Ecology of fungi in the denitrification zones of the Arabian Sea

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By

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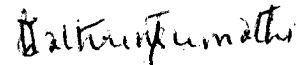
March 2010

STATEMENT

As per requirement, under the University Ordinance 0.19.8 (vi), I state that the present thesis titled **"Ecology of fungi in the denitrification zones of the Arabian Sea"** is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities or suggestions have been availed of.



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CERTIFICATE

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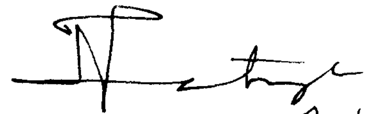

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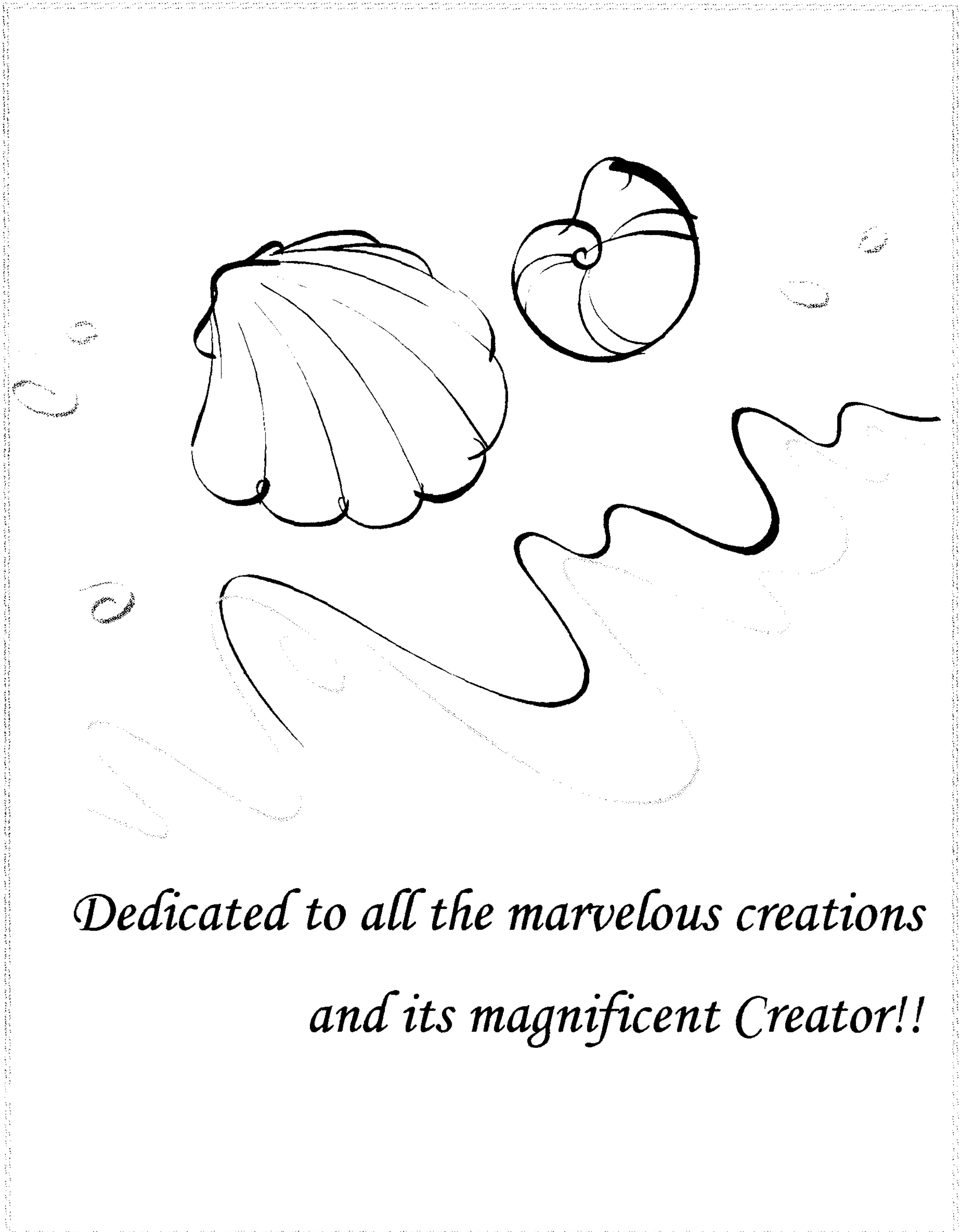

Dedicated to all the marvelous creations

and its magnificent Creator!!

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PREFACE

The microbial process, denitrification was considered to be an inherent character of a few specialized groups of bacteria. These bacteria thriving in the denitrification zones contribute to the production of nitrous oxide, a major green house gas. The pathway for this process has been studied extensively especially in the marine ecosystem, as this process leads to the loss of the fixed nitrogen and causes an imbalance in the global nitrogen budget.

Molecular ecological studies from these regions have brought to light interesting and novel microbial diversity. Environmental sequences retrieved from these regions show that fungi are one of the major components in this ecosystem. Molecular enzymological studies of a few terrestrial fungi have shown that, fungi also have the physiological adaptations to denitrify.

In this study I have attempted to estimate the abundance, diversity and activity of fungi from the denitrification zones of the Arabian Sea, that occupies more than 50% of the world's oxygen depleted marine environment.

Cathrine Sumathi Jebaraj

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Chapter 1

Fungal mediated denitrification process: an introduction.

1.1 Fungal mediated denitrification process: an introduction.

The English word "fungus" is adopted from the Latin *fungus*, meaning "mushroom", which in turn has its origin from the Greek word *sphongos* "sponge", referring to the fruiting bodies produced by mushrooms and molds. Accordingly fungi are widely popular among the common man as mushrooms, rusts, puffballs, smuts, bread moulds etc. because of the macroscopic structures they produce. Only a small fraction of fungal groups grow these macroscopic structures and are known to occur only in the terrestrial environment. The majority of the fungal forms grow embedded in the detritus' and beneath the sediment layer. Fungi along with bacteria are the major heterotrophs that play a key role as decomposers and recyclers in a large number of ecosystems such as the tropical forests (Isaac *et al.*, 1993; Hyde, 1997), marine ecosystem (Hyde *et al.*, 1998) and mangrove regions (Cheng *et al.*, 2009). They are ubiquitous and almost occupy every niche.

The heterotrophic microorganisms lack the ability to fix atmospheric carbon dioxide and use them as their carbon source and are dependent on the phototrophs *en masse* for their survival. Because of their inability to synthesize their own organic compounds, they have acquired mechanisms to degrade complex organic substances for their growth and activity. The three basic metabolic strategies they have adapted are i) aerobic glycolysis, ii) anaerobic fermentation and iii) dissimilatory anaerobic respiration / reduction.

Glycolysis is the metabolic pathway that converts glucose, into pyruvate. The free energy released in this process is used to form the high energy compounds, adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Glycolysis is a sequence of ten reactions involving ten intermediate compounds and in aerobic

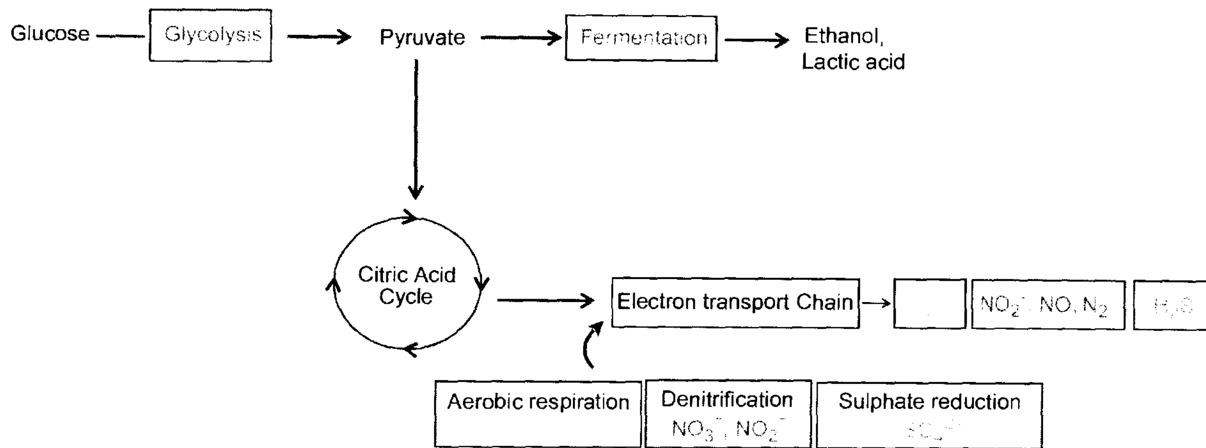


Fig.1.1 Schematic representation of the major metabolic pathways

organisms the pyruvate produced is converted to acetyl-CoA and Carbon-di-oxide (CO_2) within the mitochondria in a process called pyruvate decarboxylation. The acetyl-CoA enters the citric acid cycle, where it is fully oxidized to carbon dioxide and water, producing yet more NADH. The NADH is oxidized to NAD^+ by the electron transport chain, using oxygen as the final electron acceptor. This process creates a "hydrogen ion gradient" across the inner membrane of the mitochondria and the proton gradient generated is used to produce a large amount of ATP in a process called oxidative phosphorylation (Fig. 1.1). In situations when there is lack of oxygen, pyruvate is converted to a variety of other compounds through the fermentation process. For example pyruvate is converted to lactate in a process called lactic acid fermentation which causes the milk to curdle and yeasts convert pyruvate to acetaldehyde and carbon dioxide through ethanol fermentation. Some microbes can use a wide variety of compounds as the terminal electron acceptors in cellular respiration in the absence of oxygen. Specialised group of bacteria can utilize nitrogenous compounds such as nitrates and nitrites, sulfur compounds such as

sulfates, sulfites, sulfur dioxide and elemental sulfur, carbon dioxide, iron compounds, manganese compounds, cobalt compounds and uranium compounds (Voet & Voet, 2004; Nelson & Cox, 2005).

1.1.2 Fermentation in fungi

Role of fungi in the fermentative process was known to man since the early days of civilization, the most popularly known example is the involvement of *Saccharomyces cerevisiae* popularly known as baker's yeasts in bread-making, brewing, and wine-making. During fermentation, organic compounds usually sugars such as glucose is oxidized and byproducts such as ethanol, lactic acid and butyric acid are formed. This is a low energy yielding process and is not affected by the oxygen concentration. Fermentation products of yeasts are known for their medicinal properties even from the first century as recorded in the traditional Chinese medicinal books. One of the well-known examples is the "red yeast rice" which is fermentative product of a specific fungus *Monascus purpureus* (Went, 1895). The grain of the red rice has the outer bran which is deep reddish brown color and the rice is known to have a number of medicinal properties which is imparted during the fermentative process (Heber *et al.*, 1999). The fermentative or the secondary metabolites produced by fungi are known to have a wide range of applications as antibiotics, anti-fungal agents and pharmaceutical products (Calvo, 2002; Pointing & Hyde, 2001) and they are also known to be used as dietary supplements in the nutraceutical industry (Agahar-Murugkar & Subbulakshmi, 2005). Though in many instances, the necessity of these metabolities to the organisms producing them are not clearly defined (Challis & Hoopwood, 2003). However the biotechnological industry has

harnessed a great deal from these compounds and has found a wider application (Parekh *et al.*, 2000).

Though the ability of fungi as fermenters is well documented from the ancient times, fungi were always characterized as obligate aerobes with a requirement of oxygen for their growth. One of the salient features defining Kingdom Fungi was their requirement of oxygen environment for growth and survival. A major shift to this was brought about when a group of fungi, known as "rumen-fungi" first discovered inhabiting the rumen of cattles (Orpin, 1977) as obligate anaerobes. They are chytridiomycete fungi found in the gastrointestinal tracts of sheep, cattle and goats, as well as in many other domesticated ruminant and non-ruminant herbivores and a wide variety of wild herbivorous mammals (Trinci *et al.*, 1994). They are principally found associated with the fibrous plant particles of digesta and aid in the fermentative degradation of their cellulose rich diet (Gordon & Philip, 1998).

The fermentative ability of fungi was known from ancient times and it had found a wide variety of applications. However, the role of fungi in oxygen depleted environments was known to be restricted and they were thought to have only a minor role in the ecosystem processes of anoxic environments (Mansfield & Barlocher, 1993; Dighton, 2003). The effects of low-oxygen conditions tested on the viability of wood-decay fungi *Coniophora puteana* and *Antrodia vaillantii* grown on artificial growth medium, showed that the anoxic treatment affected the regeneration of the cultures and this increased steadily with the prolongation of anoxic treatment (Tavzes *et al.*, 2001). Studies on the survival of *Sclerotinia* species in a combination of high temperature, high soil moisture, and reduced oxygen in irrigated fields contributed to the lower survival (Wu *et al.*, 2008). The survival and growth of fungi in anoxic conditions and the groups adapted to this are not yet very clear.

1.1.3 Anaerobic respiration: Denitrification

Anaerobic respiration or the dissimilatory reduction is an alternate respiratory process where the microorganism is capable of utilizing inorganic compounds such as nitrate, nitrite or sulphate as the final electron acceptor in the absence of oxygen. Oxygen is the most preferred electron acceptor which provides the maximum energy during the metabolic process, but in oxygen depleted environments, nitrate or nitrite is the alternate compound which could be utilized by a selected group of microorganisms the nitrate reducers belonging to archaebacteria, proteobacteria and Gram-positive eubacteria (Knowles, 1982; Braker *et al.*, 2001).

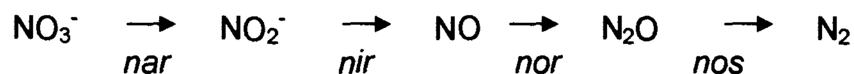


Fig. 1.2 Classsical denitrification pathway

Denitrification is a microbial process (Fig.1.2),where nitrate or nitrite is reduced under anaerobic condition to gaseous nitrogen (N_2) or its intermediary products such as nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). This is catalysed successively with the help of the enzymes dissimilatory nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*) and nitrous oxide reductase (*nos*). This process is a major sink of the fixed nitrogen in the marine nitrogen cycle (Fig. 2.1). Studies on the denitrification process have evoked the interests of microbiologists and climatologists because of its direct impact on global warming, climate change and are vulnerable to anthropogenic inputs (Conrad, 1996).

Anaerobic **amm**onium **ox**idation (ANAMMOX) is another specialized metabolic process that leads to the N_2 gas formation in the anoxic conditions. This is identified to be carried out in a few bacterial groups belonging to Planctomyces (Kuypers *et al.*, 2003). Anammox reaction was first identified from the wastewater bioreactors and subsequently in oxygen depleted marine waters and sediments (Dalsgaard *et al.*, 2003). The anammox reaction is estimated to contribute 30 – 50 % of the N_2 production from the oceans (Devol, 2003). During this process the nitrate is initially reduced by the denitrifiers to nitrite, then the anammox bacteria uses this nitrite and oxidizes ammonia to form N_2 gas (Fig. 1.3). But this process has not yet been reported in any of the fungal isolates.

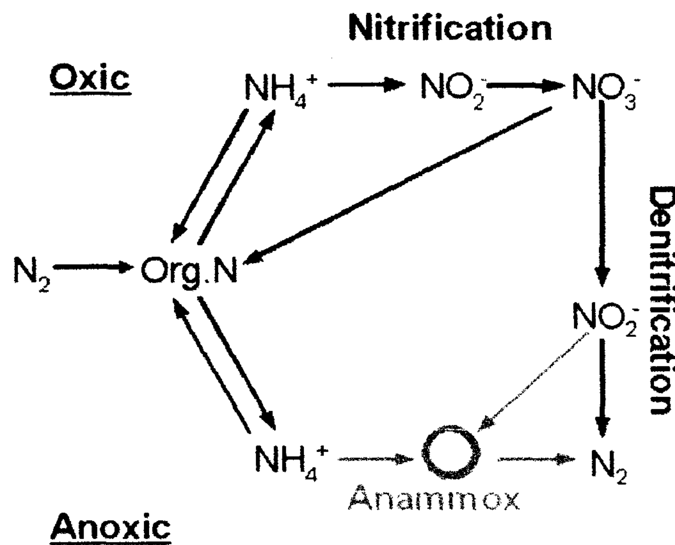


Fig. 1.3 Simplified marine nitrogen cycle including the anammox 'sink' (Kuypers *et al.*, 2003).

1.1.4 Anaerobic respiration in eukaryotes

Anaerobic or alternate respiratory process was thought to be restricted only to the specialized bacterial groups, but involvement of eukaryotes in this pathway was also reported. *Loxodes* sp. belonging to protozoa is known to be capable of living in the waters of anoxic lakes. Their capacity to participate in the denitrification pathway can be attributed to a dissimilatory nitrate reductase located within the inner mitochondrial membrane (Finlay, 2006). Recently, a benthic foraminifer *Globobulimina pseudospinenscens* has been demonstrated to show complete denitrification in marine sediments (Risgaard-Peterson *et al.*, 2006). Increased occurrence of foraminifers *Nonionella cf. stella* and a *Stainforthia* species within the oxygen minimum zone of the continental shelf off Chile and intracellular accumulation of nitrate and evolution of N_2 in these species have also been shown (Ingvardsen *et al.*, 2006). Studies conducted using cultivation-independent, 18S rDNA-based survey in suboxic waters and anoxic sediments has also shown large microeukaryotic diversity. The bulk of their sequences represent deep novel branches within green algae, fungi, cercozoa, stramenopiles, alveolates, euglenozoa, unclassified flagellate and a number of novel lineages that have no similarity with any of the known sequences (Stoeck & Epstein, 2003; Stoeck *et al.*, 2003; Stoeck *et al.*, 2006). Fungal sequences have also been retrieved from anaerobic sulfide and sulfur-rich springs of Zodletone Spring, Oklahoma (Luo *et al.*, 2005). Apart from this study Straminipiles (thraustochytrids) have also been reported from anoxic habitats (Raghukumar *et al.*, 2001; Kolodziej & Stoeck, 2007) but no studies have been attempted so far to study the denitrification pathway in the eukaryotic organisms. Though a large number of micro-eukaryotes

have been studied from the anoxic habitats, only foraminiferas and fungi have been studied and proven for their denitrification abilities.

1.1.5 Denitrification in fungi

Involvement of fungi in anaerobic dissimilatory process was completely unheard till recent times. Initial studies that reported growth of fungi in anoxic environments suggested that they required supplementation of certain inorganic salts such as nitrate, selenite, or ferric iron in the culture media (Gunner & Alexander, 1964). Bollag & Tung (1972) were the pioneers to show N_2O production by fungi when grown under anaerobic conditions. They also showed growth of fungi under these conditions when supplemented with nitrate or nitrite and concluded that nitrate respiration may be a possibility in fungi.

Isolation, purification and characterization of an intracellular P-450 enzyme from *Fusarium oxysporum* was carried out for the first time in 1983 and was shown to have lipoxygenase activity (Shoun *et al.*, 1983). Subsequent studies on this cytochrome P-450 enzyme showed that they were induced by nitrate/nitrite and not directly related to lipid metabolism. Based on this, P-450 was also called P-450_{dNIR} representing that it was related to dissimilatory nitrite reduction in the fungus *F. oxysporum* (Nakahara *et al.*, 1993). The major breakthrough came when the cDNA of the P-450_{dNIR} was sequenced by immunoscreening technique. The full length sequence had a very high homology to the P-450 of *Streptomyces* sp. and it came to be the first soluable, microsomal P-450 of eukaryote origin (Kizawa *et al.*, 1991). The novelty of the enzyme was further enhanced by its role in dissimilatory nitrate reduction activity and the gene sequence was called *CYP55*. But these studies were concentrated on a

single fungus and the property of the denitrification in other fungal cultures was not known.

Screening of terrestrial fungal cultures for their denitrification activity showed that several fungi were involved in this process (Shoun *et al.*, 1992; Usuda *et al.*, 1995). This study showed that dissimilatory nitrate reduction was seen in fungal cultures such as *Fusarium oxysporum*, *Cylindrocarpon tonkinense and Aspergillus oryzea* and were shown to release N_2O under denitrifying conditions (Shoun *et al.*, 1992). Nitrate reductase activity and denitrification process was shown in another *Fusarium oxysporum* (11dn1) culture from Moscow (Kurakov *et al.*, 2000). Screening of cultures obtained from agricultural fields showed that the cultures varied widely in their denitrification ability and N_2O production (Morozkina et al., 2005). Denitrification activity has been shown in fungi belonging to Ascomycota, selected Basidiomycota and Zygomycota (Kurakov *et al.*, 2008). This shows that the fungal denitrification activity is prevelant among all the major groups, but the molecular basis on how the members of Kingdom Fungi acquired the ability is yet to be studied

The fungal denitrification system is localized in the mitochondria and functions for anaerobic respiration, as seen in the denitrification system of bacteria (Kobayashi & Shoun, 1995). Dissimilatory *nar* partially purified from the mitochondrial fraction of the denitrifying fungus *F. oxysporum* was shown to be distinct from assimilatory *nar* (Uchimura *et al.,* 2002). The properties of this fungal *nar* were similar to those of the dissimilatory *nars* of *Escherichia coli* and other denitrifying bacteria. Analyses of mutants defective for dissimilatory *nar* or assimilatory *nar* have shown that *nar* is essential for fungal denitrification. *nir* is one of the key enzymes in the dissimilatory denitrification process. Two structurally different *nirs* are found among denitrifying bacteria: one contains copper (Cu-*nir*) and is encoded by the *nirK* gene, and the other contains heme c

and heme d1 (cd1-nir) and is encoded by the *nirS* gene (Zumft, 1997). No functionally significant differences between Cu-*nir* and cd1-*nir* have been reported. Fungal *nir* contains copper and is an ortholog of bacterial *nir*K (Kobayashi & Shoun, 1995). However, fungal n*or* is distinct from bacterial *nor.* Fungal *nor* has been classified in the cytochrome P450 superfamily on the basis of its primary and tertiary structures. All bacterial *nors* reported to date contain cytochromes *bc* in their catalytic activity.

The denitrification activities of bacteria are induced in the presence of either nitrate or nitrite under a limited O_2 supply and are suppressed by excess O_2, whereas fungal denitrification activity is induced in the presence of nitrate or nitrite and significant amounts of O_2, but not in excess O_2 (Zhou *et al.,* 2001). Two types of denitrifying fungi have been categorized according to their ability to reduce or in a dissimilatory manner. A few fungi, such as *F. oxysporum* and *Gibberella fujikuroi,* reduce both nitrate and nitrite to N_2O (Shoun & Tanimoto, 1991), whereas most other reported denitrifying fungi reduce only nitrite to N_2O (Shoun *et al.,* 1992). Thus, it appears that the minimal requirements in the fungal denitrification system are *nir* and P450*nor.* The homologies studied in the *nor* gene has shown that there are conserved regions between the bacterial and fungal *nor* gene. This shows that fungi could have acquired the denitrification ability through horizontal gene transfer from bacteria (Daiber *et al.*, 2005).

Many denitrifying fungi produce hybrid dinitrogen (N_2) or N_2O molecules by combining nitrogen atoms from nitrite and other nitrogen compounds (cosubstrates) under denitrifying conditions. This phenomenon was named "codenitrification" to indicate that nitrogen compounds (cosubstrates), such as azide and ammonia, are denitrified by the system induced by nitrite (or nitrate), but are incapable by themselves

of inducing the denitrifying system (Shoun *et al.,* 1992; Tanimoto *et al.* 1992).

Ammonia fermentation is another mode of alternate respiration observed in fungi during complete anoxic conditions (Zhou *et al.*, 2002). In ammonia fermentation, nitrate is reduced to ammonia and ethanol is simultaneously oxidized to acetate to generate ATP. In the reaction, nitrate acts as the terminal electron acceptor for fermentation, but not for anaerobic respiration. Ammonia fermentation is demonstrated to be common in many of the soil fungi. Ammonia fermentation supports fungal growth under conditions that are more anoxic than those of denitrification. This pathway is similar to the dissimilatory nitrate reduction to ammonia (DNRA) pathway known in some bacterial cultures (Tobias *et al.*, 2001). Studies on the denitrifying activities of *F. oxysporum* have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O_2 tension, ammonia fermentation under anoxic conditions, denitrification when hypoxic, and oxygen respiration under aerobic conditions, thus demonstrating that a eukaryote can use a multimodal type of respiratory system to rapidly adapt to changes in the oxygen supply (Daiber *et al.*, 2005; Hayatsu *et al.*, 2008).

1.1.6 Fungal mediated denitrification process

The involvement of fungi as denitrifiers has been evaluated based on the effect of cycloheximide on fungal denitrification activity in woodland and arable soils Castaldi & Smith (1998). Cyclohexamide is widely known anti-fungal agent and the denitrification activity reduced on treatment with this anti-fungal agent. Recent studies from the grassland ecosystem based on substrate-induced respiratory inhibition studies have also shown that they account for nearly 80% of the N_2O production (Laughlin & Stevens, 2002).

Contribution of fungi as the dominant gaseous producers has also been shown in semiarid soil (MacLain & Martens, 2006). Screening of fungal isolates has shown that all the major groups of fungi are capable of denitrification process (Shoun *et al.*, 1992; Kurakov *et al.*, 2008). It is interesting to study the ecological role of fungi as they predominantly form only nitrous oxide which directly affects the ecosystem.

As fungi have the ability to perform denitrification and O_2 respiration simultaneously in a range of O_2 stress conditions, the potential exists for fungi to produce N_2O in a wider range of soil aeration conditions than bacteria. Fungi are widely distributed in soils and water; hence the potential exists for fungi to make a significant contribution to the global N_2O budget.

An understanding of these processes can give us insights into the role of fungi in the biogeochemical cycling of nitrogen and can also be used for biotechnological applications in sewage treatment plants. Till now nitrifying and denitrifying bacteria have been used for biological treatment of sewage wastes. But filamentous fungi which can perform both nitrifying and denitrifying process even under low oxygen conditions is proving to be a better alternative (Guest & Smith, 2002). These interesting studies on the fungal denitrification show that fungi could have an important ecological role in such special niches such as the denitrification zones of the marine environment. For this study I have chosen to study the fungal diversity and contribution in the denitrification zones of the Arabian Sea which occupies more than 60 % of the world denitrification zones. The bio-geochemical significance and the dynamics of the Arabian Sea oxygen depleted zone is explained in the subsequent chapter.

Chapter 2

Quantification of fungi in the sediments of the denitrification zones of the Arabian Sea.

2.1 Introduction

Oceans play a significant role in the global budgets of key biogenic elements like Carbon, Nitrogen and Oxygen (Falkowski, 1997). The cycling of these elements is tightly linked and together forms the dynamic components of the climate and ecosystem functioning. The major pathways of the oceanic biogeochemical cycle are: Carbon fixation; during this process Carbon-di-oxide (CO_2) is converted to organic matter in the presence of light by autotrophs. Nitrogen fixation: where atmospheric nitrogen (N_2) is converted to ammonium, which in turn is used by other organisms. The fixed carbon and nitrogen are transferred to higher trophic levels in the ecosystem. This process is entirely fueled by oxygen and the delicate balance between the supply and consumption of oxygen is maintained in the ecosystem. When this dynamic steady state is altered the microbes involved in all these process respond in a variety of ways to elude the adverse circumstances.

Alternate respiration or denitrifying activity is one such adaptation well known among bacteria. In oxygen deficient regions the denitrifying bacteria utilize nitrate or nitrite as the final electron acceptor in their respiratory cycle. The nitrite is further reduced to gaseous products such as nitric oxide (NO), nitrous oxide (N_2O) and nitrogen gas (N_2). The implications of this process are manifold and they affect the entire biogeochemical cycling. (i) The liberation of N_2 gas back to the atmosphere is a direct loss of the fixed nitrogen. The rate of supply of nitrogen to the ocean is much lower than the removal, hence the oceanic nitrogen (N) budget is unbalanced (Codispoti, 1995). (ii) Denitrification decreases the amount of nitrogen available for phytoplankton thus affecting primary productivity; hence it directly and indirectly decreases carbon sequestration (Liu *et al.*, 2003). (iii) Thirdly, the intermediary

products such as NO and N_2O produced during the denitrification process are harmful green house gases. These contribute to the destruction of the ozone layer leading to global warming and climate change (Knowles, 1982). The major concern that drives intensive research on this system is due to its direct effect on the global climate and the microbial processes that catalyse these reactions vulnerable to anthropogenic inputs (Conrad, 1996; Naqvi *et al.*, 2000). The present day ecosystem is greatly influenced by human activities and the extent of denitrifying zones is increasing, especially along the continental shelf following industrialization.

The key processes in the marine nitrogen cycle are nitrogen fixation, assimilation by higher microorganisms, sinking of these as dead organic matter to the sediment bed, upward flux of the nitrates to the surface regions and denitrification (Fig. 2.1). Studies on this delicate and highly influential marine system are largely based on the measurements of transformation rates of one form of nitrogen oxide to another (Naqvi *et al.*, 1998) and by estimating the bacterial production and activity of their enzymes in these regions (Naqvi & Shailaja, 1993; Shailaja, 2001; Shailaja *et al.*, 2006). The advent of molecular biology tools to study the N cycle has helped in the study of in-situ pathways and has brought to light many new metabolic processes like the anaerobic ammonia oxidation (anammox) to form N_2 gas (Kuypers *et al.*, 2003; Devol, 2003). The rapid increase in genetic and biochemical investigations has helped us to understand the ecological significance and vast diversity of the microorganisms involved specifically in the N cycle (Braker *et al.*, 2001; Liu *et al.*, 2003). Knowledge of the gene encoding for the enzymes involved in the biogeochemical transformation provides useful tools for assaying the gene expression and for understanding the role of the microbes involved in the N cycle (Goregues *et al.*, 2005)

The Arabian Sea plays a disproportionately large role in the biogeochemical cycling of N. This region is influenced largely by the southwest and northeast monsoons, which leads to upwelling of bottom water nutrients and high productivity (Warren, 1994; Naqvi, 1994).

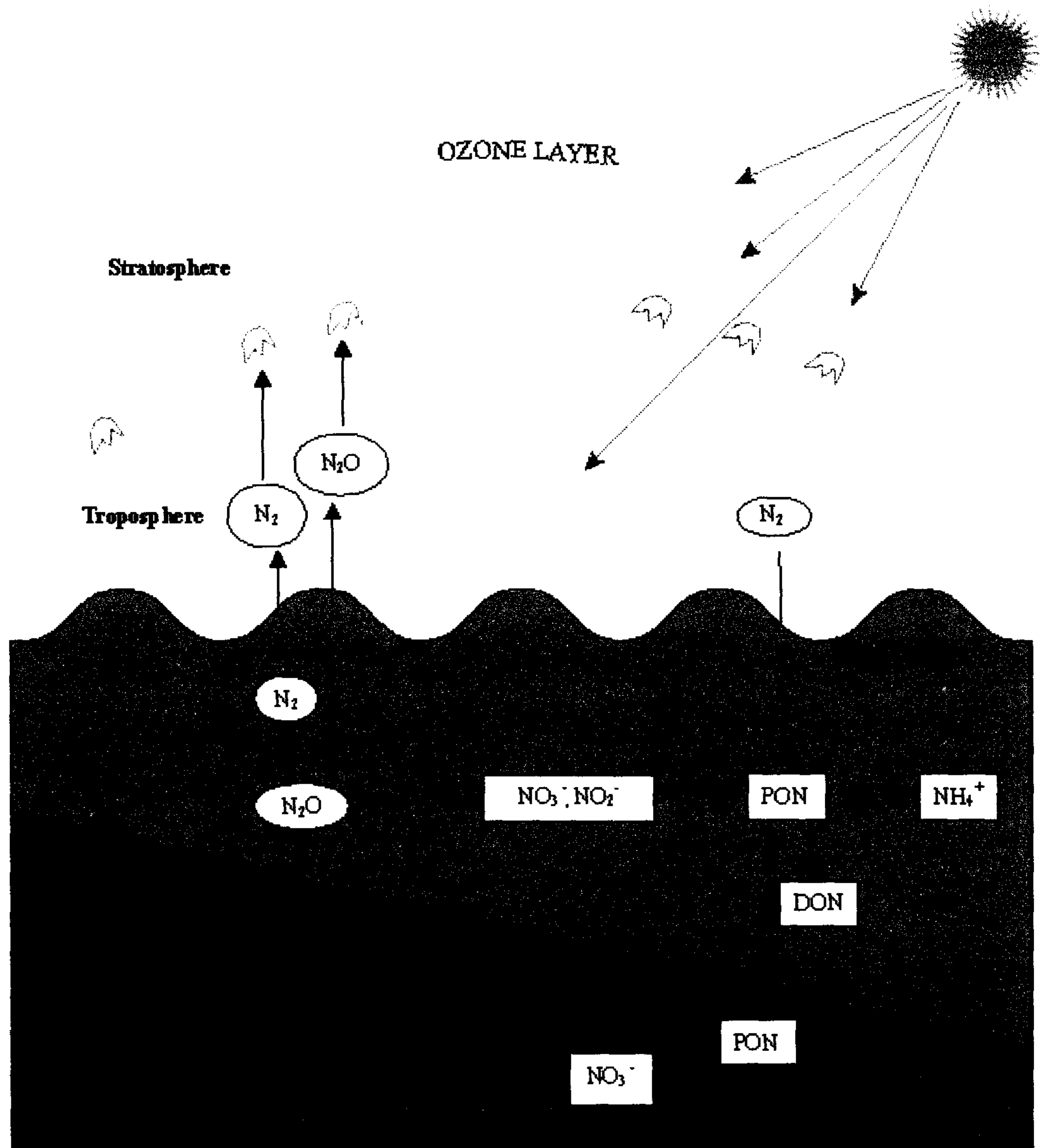


Fig. 2.1 Schematic representation of the oceanic nitrogen cycle: A: Nitrogen fixation, B: Assimilation C: Downward flux and burial into sediment, D: Advection from deep water, E: Denitrification.

A combination of high productivity and limited mixing or circulation with poorly oxygenated waters leads to the development of the world's major mid-water oxygen minimum zone (Naqvi *et al.*, 1998). These are regions where the dynamic steady state between oxygen supply and consumption is altered and the oxygen concentration is < 0.2 ml L^{-1}. Such permanent anoxic zones are seen in the eastern Pacific Ocean, Indian Ocean and in West Africa where the fixed N is transformed in the water column to dissolved N gas (Fig. 2.2). This process is called pelagic denitrification and the global denitrification rate is calculated to be 150 Tg N yr^{-1} and about 40% (60 Tg N yr^{-1}) is from the Arabian Sea (Bange *et al.*, 2005). Denitrification is a major sink for fixed N and is therefore crucial to budget the loss of oceanic nitrogen through this process. Global quantification of naturally occurring hypoxic seabed, reveals that 764,000 km^2 of shelf and bathyal sea floor is under the influence of the hypoxic overlying waters, where dissolved oxygen is 0.2 ml L^{-1}; over half (63%) occurs in the Indian Ocean (Arabian Sea and Bay of Bengal) (Helly & Levin, 2004). The extent of the palegic denitrification zone or the OMZ region of the Arabian Sea extends from between the 45°/ 73°/ 0°/ 30° (W/E/S/N) in a depth range of 200 m to 1200 m and in the Bay of Bengal 73° /100°/ 0° / 30° (W/E/S/N) between 104 m to 389 m.

Apart from the mid water pelagic denitrification, sedimentary denitrification also takes place in very high concentration along the seasonal anoxic zone. This is seen along the coastal waters on the western Indian shelf upto 200 m depth, during June to December, with the maximum intensity in September and October (Naqvi *et al.*, 2000) The perennial open-ocean system occupies two orders of magnitude larger volume than the seasonal coastal system; however, the latter offers more extreme conditions. The coastal anoxic zone extends over the continental shelf, at shallower depths and directly impinges its effect on the

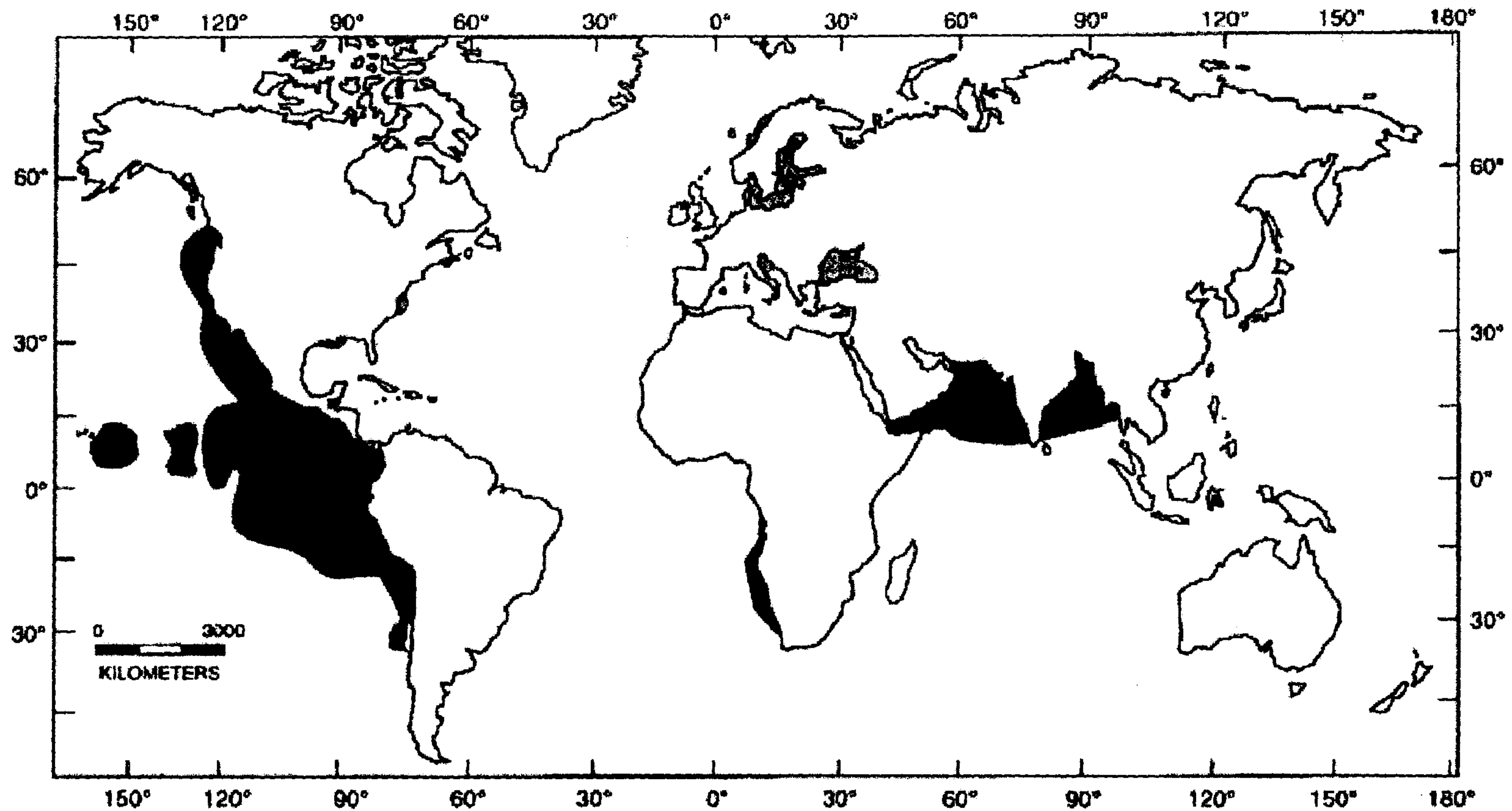


Fig 2.2 Distribution of the world oxygen minimum zones. Open water oxygen minima are shown in black, hypoxic enclosed seas and fjords are grey (Diaz & Rosenberg, 1995)

sediments. Sedimentary denitrification in the continental shelf region is estimated to be 0.4 to 3.5 Tg N y^{-1} (Naqvi *et al.*, 2006). The coastal anoxic region is a 'hot spot' for N_2O emission, a green house gas that influences the earth's climate by the destruction of the ozone in the stratosphere. Recent computations of the annual N_2O production from the Arabian Sea shows an annual production of 0.33 to 0.7 Tg N_2O excluding the N_2O production from the coastal regions which ranges from 0.06 to 0.39 Tg N_2O. If the N_2O production from the shelf region was also taken into account, then the Arabian Sea region would account for approx. 0.4 to 1 Tg N_2O y^{-1} accounting for one-fifth of the total oceanic emissions of nitrous oxide (Devol *et al.*, 2006).The anthropogenic inputs along the continental shelf are intensifying lately and are largely influencing the biogeochemical processes of the Arabian Sea, especially along the coastal regions (Naqvi *et al.*, 2000; Naqvi *et al.*, 2006).

Studies on the microbial process from this ecosystem has been studied based on chemical (nitrite concentrations, NO and N_2O productions), microbiological (bacterial productivity) and enzymatic parameters such as electron transport system (ETS), nitrate reductase (*nar*), nitrite reductase (*nir*) and ammonia oxygenase (*amo)* of the bacterial population (Shailaja, 2001; Shailaja *et al.*, 2006). Diversity and distribution of *nirS* genes in the water column of the Arabian Sea coastal denitrifying region shows that there is a functional relationship between the water column chemistry, especially the nitrite concentration and the distribution of nirS, nirK and 16S rDNA gene sequences (Jayakumar *et al.*, 2004; Jayakumar *et al.*, 2009). Though detailed studies on the chemical characteristics of the region has been extensively carried out, the microbial process and their community structure are least studied.

With increasing evidence showing the involvement of fungi in the denitrification process I proposed to study the ecological role of fungi in the denitrification zones of the Arabian Sea with the following objectives:

- To quantify fungal C biomass and relate it with bacterial biomass and study the influence of reduced DO levels.
- To isolate fungal cultures from sediment and water collected from the oxygen depleted environments
- To study the unculturable fungal phylotypes by constructing 18S rRNA based clone libraries from environmental samples and
- To screen a few selected cultures for their denitrification capacity.

2.2 Materials and Methods

Table 2.1 Sampling sites

Sampling stations	Depth (m)	Latitude (N)	Longitude (E)
Coastal station (CSt) within the seasonal anoxic zone off Goa			
CSt-08	8	15° 31.19'	73° 44.37'
CSt-14	14	15° 31.30'	73° 42.16'
CSt-26	26	15° 29.85'	73° 39.03'
Slope station (SSt) within the perennial oxygen minimum zone			
SSt-200	200	15° 20.10'	72° 54.11'
SSt-500	500	17° 33.50'	71° 11.35'
SSt-700	700	17° 32.28'	71° 11.09'
SSt-800	800	17° 31.50'	71° 10.31'
SSt-1100	1100	17° 31.44'	71° 04.88'
Oxic Coral reef station in Lakshwadeep (LSt-03)			
LSt-03	3	10° 34.59'	72° 38.02'

2.2.1 Sampling site

For this study, sampling was carried out from three coastal stations (CSt-08, CSt-14 and CSt-26) within the seasonal anoxic zone off Goa (Table 2.1).

Sampling to the CSt stations were carried out periodically during eight field trips (Ft-1 to Ft-8) from October 2005 to February 2007 on fishing trawlers hired for field surveys (Table 2.2).

The first field trip was carried out during the post monsoon season where the dissolved oxygen (DO) level was nearly zero, followed by the second sampling in Nov 2005 when the DO levels were returning to oxic condition. Periodic sampling was carried out in the three selected stations for a full season till anoxic condition set-in during the following post-monsoon period and during Ft-8, the oxic condition was restored (Table 2.2).

Table 2.2 List of filed trips to the coastal stations

Field trip	**Ft-1**	**Ft-2**	**Ft-3**	**Ft-4**	**Ft-5**	**Ft-6**	**Ft-7**	**Ft-8**
Date	Oct 05	Nov 05	Jan 06	Mar 06	Apr 06	Oct 06	Dec 06	Feb 07
Condition	anoxic	oxic	oxic	oxic	suboxic	anoxic	oxic	oxic

Five stations were sampled within the perennial OMZ region of the Arabian Sea, along the continental slope (SSt-200, SSt-500, SSt-700, SSt-800 and SSt-1100). Sampling from the SSt-200 station was carried out in May 2007 during the cruise SaSu-129 on board CRV Sagar Sukti. Sampling from the deeper stations (SSt-500, SSt-700, SSt-800 and SSt-1100) was carried out on the dive cruise YK08-11 on board RV Yokosuka and its manned submersible Shinkai 6000 during Nov 2008. The geographical positions of the sampling sites are plotted in the site map (Fig. 2.3).

Sampling was also carried out from a coral reef station in the Lakshwadeep islands (LSt-03) situated outside the denitrification zone (Fig. 2.3). The coral reef ecosystem is a self contained, pristine well-oxygenated system. Fungal cultures isolated and uncultured phylotypes obtained from this station were analysed and compared with those obtained from other oxygen depleted regions.

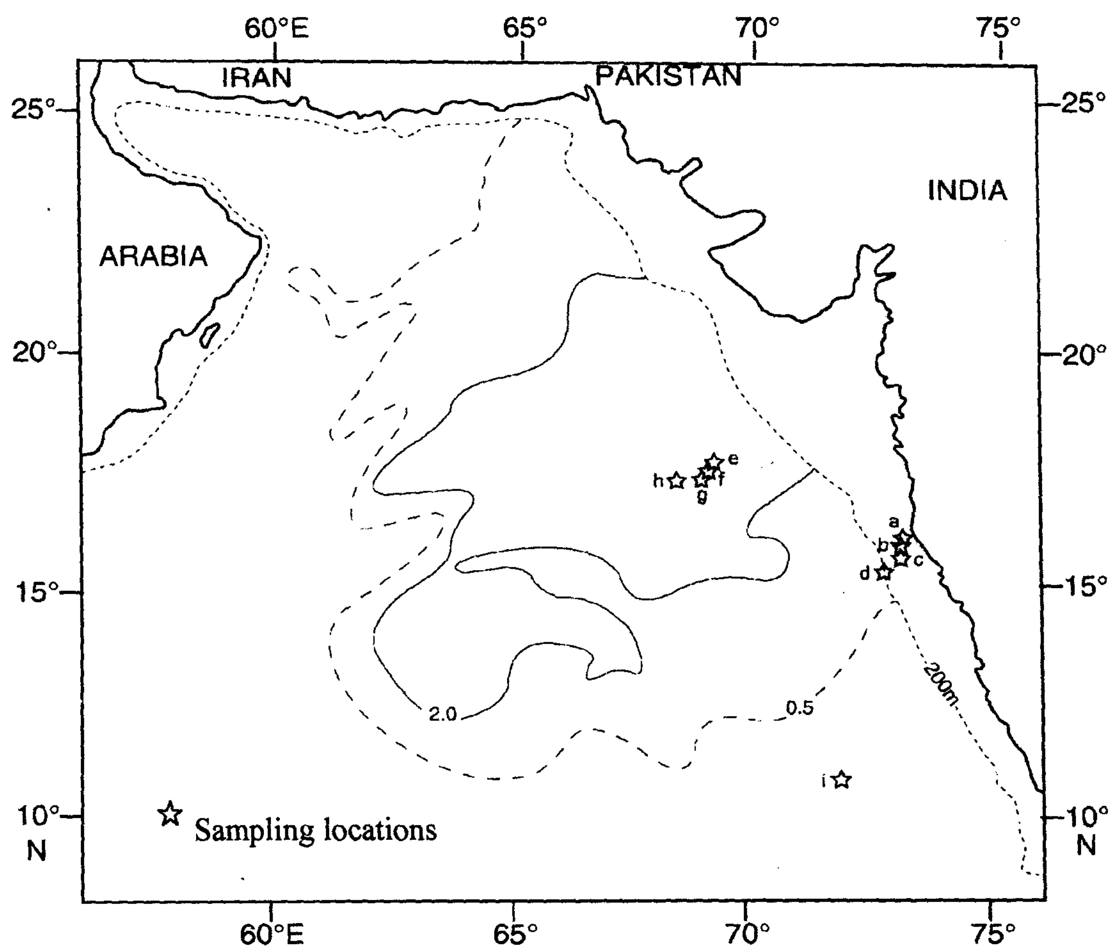


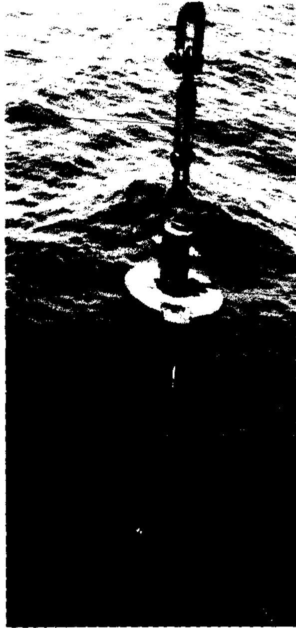
Fig. 2.3 Sampling sites in the Arabian Sea. The contours, represents the distribution of nitrite (µM) accumulation due to denitrification, the 0.5 µM contour marks the boundary of the oxygen minimum zone (Naqvi & Shailaja, 1993). Locations are represented as **a**: CSt-08, **b**: CSt-14, **c**: CSt-26, **d**: SSt-200, **e**: SSt-500, **f**: SSt-700, **g**: SSt-800, **h**: SSt-1100 and **i**: LSt-03

2.2.2 Sampling procedures

Sediment samples were collected using a ~ 60 cm long gravity corer (Fig. 2.4) and about 20- 30 cm long sediment core was obtained during each operation. The overlaying water (OLW) was collected from the top of the sediment core in clean sterile 15 mm teflon tubes (Fig. 2.4). The sediment cores were cut at 2 cm intervals down to 8 cm and extruded into alcohol sterilized clean plastic containers and the lower sections were used by co-participants in the cruise team or discarded.

Near bottom water, about 1 – 2 m above the sediment surface was collected in 5-L Niskin bottles (Fig. 2.4) and immediately sampled for dissolved oxygen (DO). 100-125 ml of water was collected into glass stoppered bottles with minimum contamination to atmospheric oxygen and was fixed immediately using Wrinkler's solution A and B. The fixed samples were allowed to precipitate and brought on ice to the laboratory. On reaching the laboratory the precipitate was dissolved in 10 N sulphuric acid and titrated against 0.01 N Thiosulphate using starch as an indicator (Strickland & Parsons, 1968). In some instances, when the DO levels were very less, titration was not possible and hence the absorbance of the dissolved precipitate was determined by spectrophotometric measurement at 456 nm (Pai *et al.*, 1993). The absorbance maxima recorded at 456 nm was converted to µM of DO using the factor 476.

Fungi are not free living and grow attached to the substratum; hence most of our studies were carried out in the sediment collected from these regions. Sediment samples obtained from the corer were divided into aliquots for the isolation of fungi and a portion was fixed in formalin for direct detection of fungi and bacteria. Samples for isolation were stored at 5° C and processed within 24 hours. Approximately 5 -10 g of sediment was frozen at -20 °C for estimation of total organic carbon.

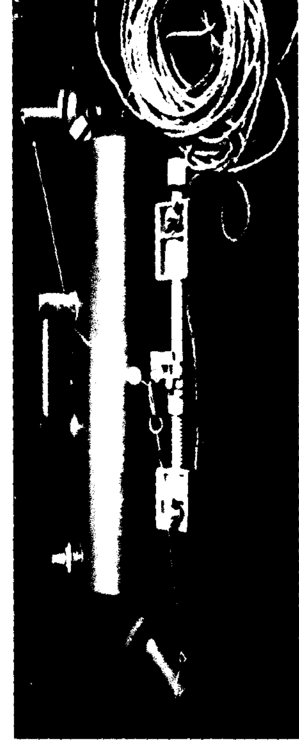


(a) deplyment of corer

(b) retreived corer on the deck

(c) sediment core

(d) core and overlaying water (OLW)



(e) Niskin sampler

Fig. 2. 4 Oceanographic instruments used for sampling

Samples from the open ocean OMZ stations were collected and processed as mentioned above for the coastal stations, except for the sediment collection, which was done using a ~ 20 cm long push corer (Fig. 2.4).

Reference samples from an oxic site were collected from a shallow 3 m site (LSt-03) in the coral reef region off Kavaratti in February 2007. Coral sand samples were collected by divers in sterile containers and processed for isolation of fungal cultures.

2.2.3 Isolation of fungi by particle plating technique

Particle plating is based on soil washing and it is a selective isolation technique minimizing the recovery of common saprobic fungi (Bills & Polishook, 1994). Particle palting technique has been used to isolate fungi from a variety of substrates such as forest litter (Polishook *et al.*, 1996), mangrove plants (Kumerasan & Suryanarayan, 2001), coral reef ecosystem (Ravindran *et al.*, 2001) and deep sea sediment (Damare *et al.*, 2006). For this, a portion of the sediment from the middle of each sub-section was removed with a flame-sterilized spatula and placed in sterile vials for isolation of fungi. Approximately 1 g of sediment slurry was sieved successively through a mesh size of 200 µm and 100 µm screens. The particles that passed through 200 µm mesh but were retained on the 100 µm mesh were spread-plated on malt extract agar (MEA), Corn meal agar (CMA) and Czapek Dox agar (CDA). All the media (HiMedia Pvt. Ltd., India) were used at 1/5 strength to discourage the growth of fast growing fungi and the plates were fortified with antibiotics. Broad range antibiotics Streptomycin (0.1 g in 100 ml medium) and Penicillin (40,000 Units in 100 ml medium) were added to inhibit bacterial growth. The plates were incubated for 15 - 20 days at 27 - 30 °C and at the end of the stipulated

time period culturable colony forming units (CFU) of fungi were expressed as numbers g^{-1} dry sediment. Duplicate plates were maintained for each sediment sample and culture media used. Media plates were exposed to air for 10 min near the sample processing site on the deck of the research vessel or the trawler and in the microbiology laboratory to check for the presence of aerial fungal contaminants and was repeated for each sampling. This did not yield any fungal colonies, thus ruling out any chance of aerial contamination. All the fungal cultures isolated were sub-cultured on MEA slants and maintained at 5 °C.

2.2.4 Direct detection of fungal mycelia in sediments

To estimate fungal biomass, about 100 – 200 mg of formalin-fixed sediment was treated with 10% EDTA (Ethylene Diamine Tetraacetic Acid), stained with 0.01% of filter sterilized calcoflour (Sigma Chemicals, USA) in sterile 2 ml tubes. EDTA is used as a mild chelating agent to release the fungal filaments associated within the sediment particles and calcaflour is an optical brightner that binds to the chitin of the fungal hyphae (Mueller & Sengbusch, 1983). This revealed the presence of hyphae in the sediments, confirming active growth of fungi therein. Occasionally fungal spores were also detected. Microscopic mounts of the sediment were then examined under ultraviolet light filter (excitation wave length 330 to 385 nm and barrier filter BA 420) of an epifluorescence microscope (Olympus BX60, Japan) to detect fluorescing fungal hyphae. The hyphal lengths were measured using an ocular micrometer. Considering the hyphae as a cylinder, length (h), the hyphal diameter as 2 μm, the length of mycelium was measured applying the formula $3.14 * r^2 * h$, the total hyphal lengths were expressed as biovolume g^{-1} dry sediment. The biovolume was converted to biomass using the conversion factor 0.2

g cm^{-3} (Newell *et al.*, 1986). The C biomass was calculated by considering that 50% of the biomass content was C (Bittman *et al.*, 2005). The results of fungal C biomass were expressed as pg C g^{-1} sediment. The values are average of 2 replicate sediment samples examined. Bacterial cells, fungal hyphae and spores were photographed with a digital camera (Olympus 4.1 Mp, Japan).

2.2.5 Direct counts of bacteria using Acidine Orange staining

For direct counts of total bacteria, 1 g wet sediment was suspended in 10 ml sterile seawater with formalin as a fixative (5 % final concentration) and was stored in the dark at 4°C. The formalin-fixed samples were sonicated (3x30 sec) in a water bath sonicator (Biosystems Ltd, India) and allowed to settle for 5 min on ice. The overlying clear water sample was filtered over 0.22 μm black polycarbonate nuclepore filters (Millipore, USA) and stained with an aqueous solution of acridine orange (0.01 %) for 3 min. Acridine orange is a nuclear stain that binds to the nucleic acids present in the cell. Bacterial cells were counted from 10-20 microscope fields with an epifluorescence microscope (Olympus BX60, Japan). The final numbers were expressed as total counts g^{-1} dry sediment. The bacterial numbers were converted to fg carbon using the conversion factor of 20 (Peduzzi & Hendle, 1991). The final values were expressed as pg C g^{-1} dry sediment.

2.2.6 Estimation of Organic Carbon (OC)

The OC content of the samples was determined by the difference between total carbon (TC) and inorganic carbon (IC). TC was analyzed by combustion of the samples at 1200°C in an oxygen atmosphere and detection of CO_2 by coulometry (Ströhlein®) following the protocol as

described in Prakash Babu *et al.* (1999). Inorganic C was analyzed in an automated CSN analyzer (NCS 2500, EAINA 1110, CE instruments, Italy). An in-house reference standard (TW-TUC) was used for testing reproducibility and accuracy. The values are expressed as % OC and are average of 2 replicates.

2.3 Results

2.3.1 Dissolved oxygen levels

Intense anoxic conditions develop along the western continental shelf region up to 200 m depth annually at the end of the monsoon. The sampling stations CSt-08, CSt-14 and CSt-26 lie within this seasonal anoxic zone, in the coastal region off Goa. Eight field trips (Table 2.1) were made to these stations to collect sediment samples and study the changing trend of the fungal abundance and C biomass with the changing DO levels (Fig. 2.5).

The first field trip (Ft-1) was carried out in the post-monsoonal season in Oct 2005. Sediment samples were collected from the three coastal stations, and the average DO levels in the overlying water was near zero. Determination of DO directly in the sediment requires special lander system and micro-electrodes as this was not possible from the trawler which was used for sampling. Hence the near bottom water about 1 m above the sediment surface was sampled for DO and other chemical parameters. Near zero DO levels and nitrite accumulation was seen (data not shown) in the coastal stations signifying typical denitrification conditions during Oct 05 (Fig. 2.5). The DO levels was 175 µM during Ft-3 in Jan 06 as the condition was restored to normalcy and remained oxic till the onset of monsoon in the following season and anoxic condition (17

μM) was recorded in Oct 2006 (Ft-6) and restored back to oxic conditions by February 2007 (Fig. 2.5). This annual cycle has been studied intensively since 2000 as a part of the Candolim Time Series (CATS) program and sulphate reducing condition have also been recorded during the anoxic season (Naqvi *et al.*, 2000; Naqvi *et al.*, 2006).

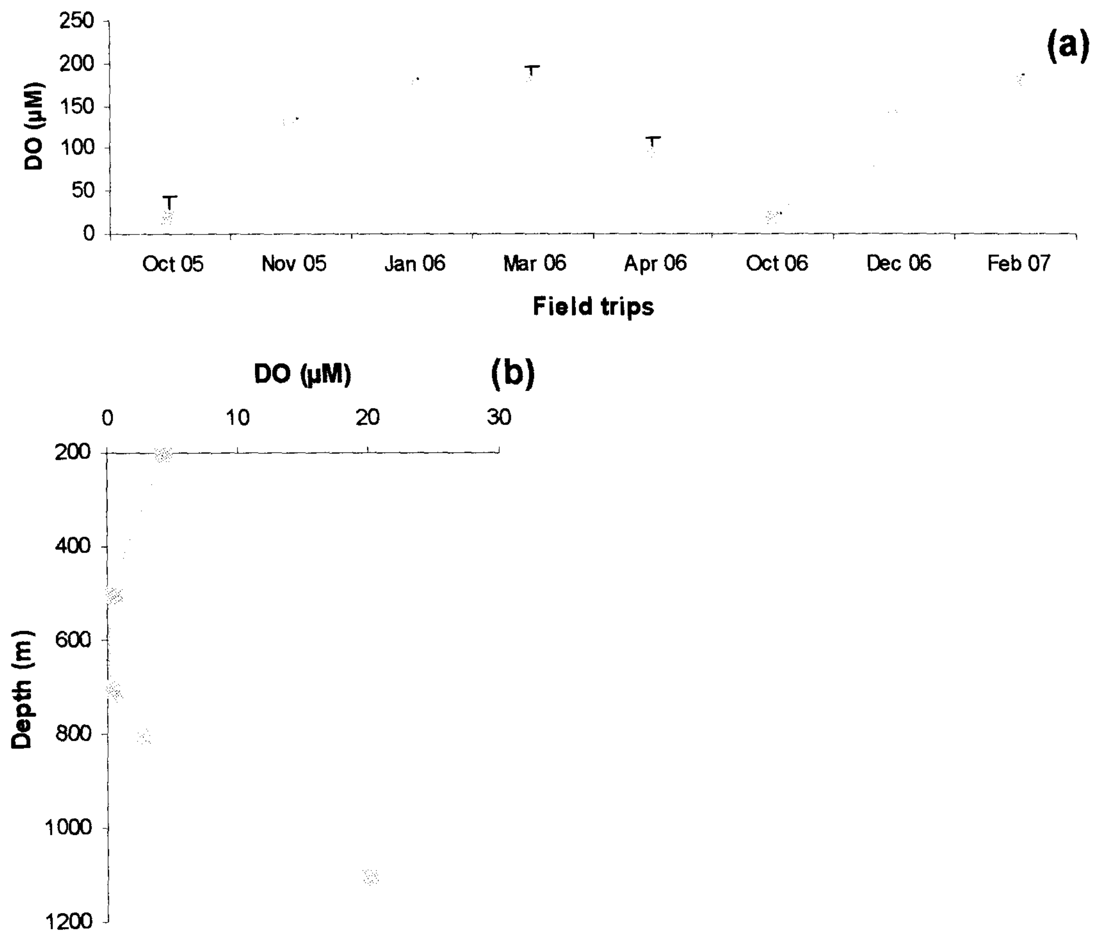


Fig. 2.5 DO levels (a) in the overlying waters of the coastal stations (Cst-08, CSt-14 and CSt-26) and (b) in the overlying waters of the slope stations (SSt-200, SSt-500, SSt-700, SSt-800 and SSt-1100) marked at their respective depths.

In the perennial open ocean OMZ region the DO level between 150 m and 1200 m water column depth is <5 µM and <1 µM within the upper half of this depth range (Bange *et al.*, 2005). Five sampling stations were selected in this region (Table 2.1) within the oxygen depleted region where the anoxic water column impinges its effect on the sediment. The DO value of the near bottom water was estimated from the water collected approximately 1 m above the sediment bed. The DO level at SSt-200 was about 4 µM, and at SSt-500 and 700 it was less than 1 µM and the DO level at SSt-800 and SSt-1200 was about 2.75 µM and 20 µM respectively (Fig. 2.5) and the DO level at the coral reef sampling point was ~ 94 µM .

2.3.2 Fungal CFU & biomass C, bacterial biomass C and organic carbon

Fungal colony-forming unit (CFU) is a measure of viable fungal numbers that can be retrieved from any natural habitat. Unlike in direct microscopic counts where all cells, dead and living, are counted, CFU measures viable cells. The number of fungal colony forming units obtained from each section was counted and expressed as no g^{-1} dry sediment. The fungal CFUs obtained from the coastal station ranged from 2000 nos g^{-1} sediment to zero colonies and an average of 1000 nos. g^{-1} sediment were obtained (Fig. 2.6a). Maximum number of fungal colonies was obtained during March 2006 from all the three CSt stations during the Ft-4, when the DO level was oxic (175 µM). The fungal CFUs obtained from the SSt stations was about 100 - 200 nos. g^{-1} sediment (Fig. 2.6e). Number of fungal CFUs from the SSt stations were an order of magnitude lesser than the CSt stations and this difference is reflected in the t-Test (Table 2.3).

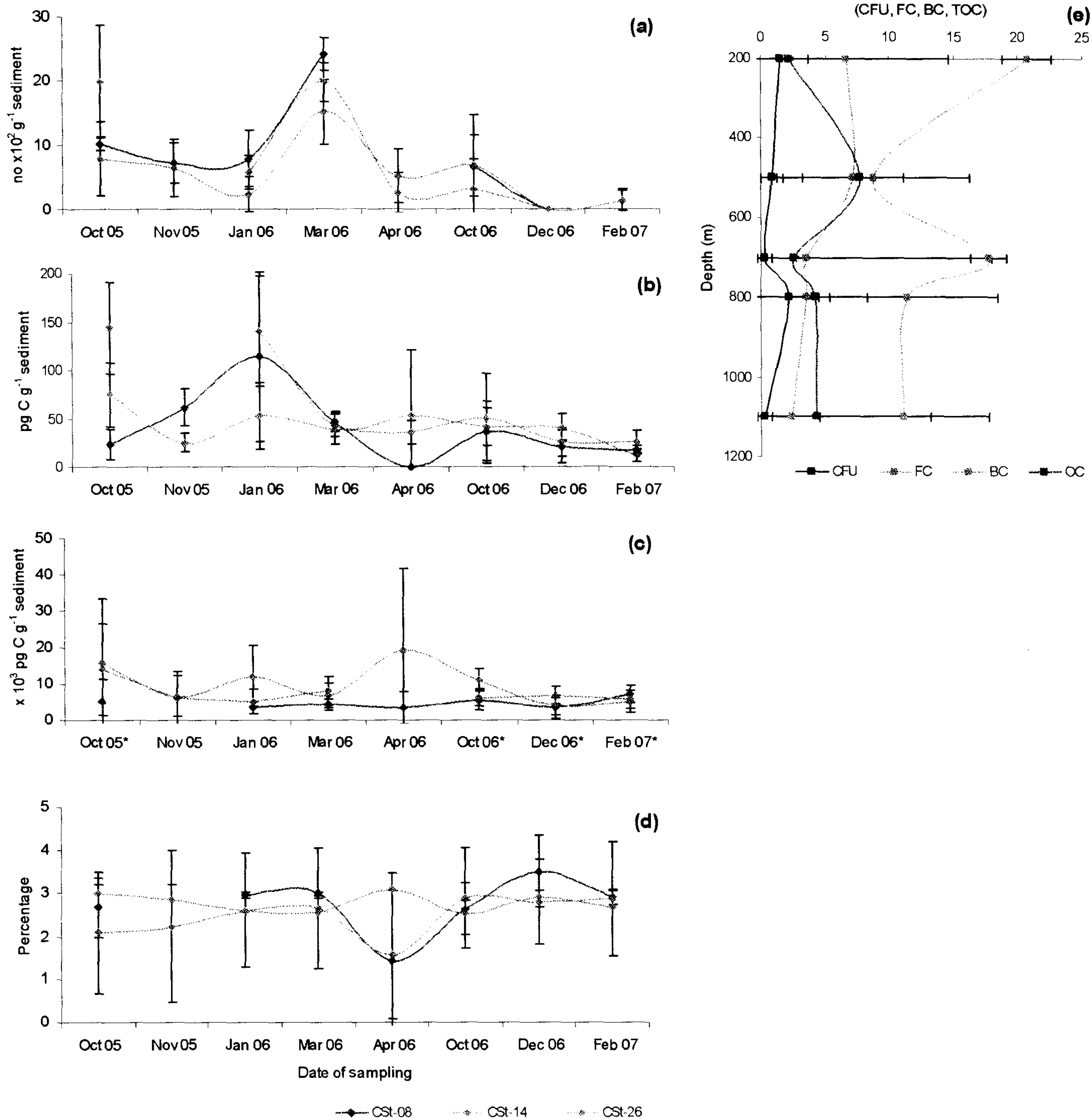


Fig. 2.6 Seasonal changes in the (a) fungal CFUs, (b) fungal biomass C, (c) bacterial biomass C and * represent x 10^4 pg C g^{-1} sediment (d) Organic Carbon in the CSt stations and (e) represents the values of CFUs (no x10^2 g^{-1} sediment), fungal biomass C (pg C g^{-1} sediment), bacterial biomass C (x 10^3 pg C g^{-1} sediment) and OC (%) from the sediments collected in the SSt stations marked at their respective depths.

Table 2.3 Results of t-Test between the coastal (CSt) and continental slope stations (SSt) of the OMZ

	df	P Value
Fungal colony forming Units (CFUs)	6	**0.001*** **
Fungal C biomass	6	**0.0005*** **
Bacterial C biomass	6	0.17
Total organic Carbon	6	**2.49×10^{-5}*** **

Df= degrees of freedom, (Df =6, where n=8 (four sections of the sediment cores from the CSt and SSt stations); F value greater than F-critical value indicates statistical significance, *** significant at 0.001 %.

Fungal biomass C and bacterial biomass C was determined by direct measurement of Calcoflour stained hyphae (Fig. 2.7a) and direct counting of acridine orange stained bacterial cells respectively (Fig. 2.7b) in the sediment. The microscopic measurements made was converted to fungal and bacterial biomass C. Fungal biomass C in the sections of the sediment cores at the three CSt stations ranged between 2.09 to 209.86 pg C g^{-1} sediment. Maximum fungal C was in 2-4 cm sediment section of the CSt-08 station during Oct 2005 (Fig. 2.6b). The fungal C biomass was significantly different between the CSt and SSt stations (Table 2.3). Bacterial biomass C was three orders of magnitude higher than the fungal biomass C and it ranged between 0.87 to 413.98 x 10^3 pg C g^{-1} sediment. Maximal bacterial biomass C was found in the 2-4 cm section of the Oct 2005 sampling at CSt-26 station (Fig. 2.6c).

Total organic carbon (OC) ranged from 2.5 to 3.5 % in the CSt stations (Fig. 2.6d). The SSt stations also recorded a similar OC % in all the stations except in SSt-500 where the OC % was about 8 % (Fig. 2.6e). The t-Test between organic carbon content in the coastal (CSt) and continental slope stations (SSt) of the OMZ in the SSt stations showed a significant difference (Table 2.3).

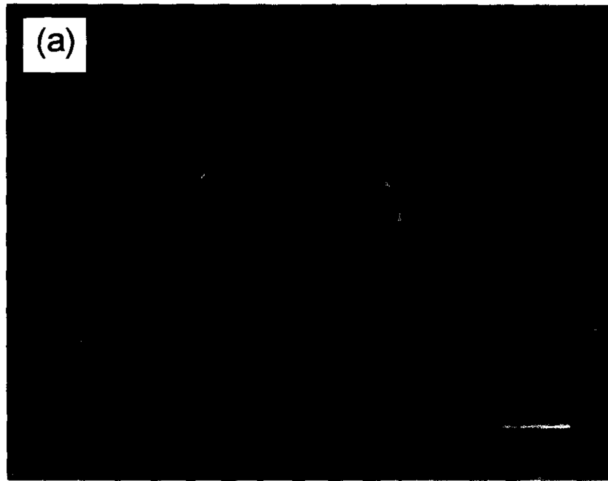


Fig. 2.7(a) Fungal hyphae in sediments stained with optical brightner, Calcoflour
bar = 10 μM

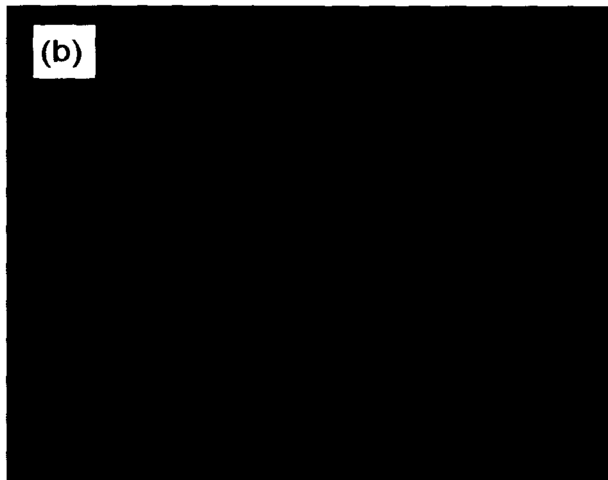


Fig. 2.7(b) Acridine Orange stained bacterial cells from sediments
bar = 10 μM

Table 2.4 Two factor Analysis of variance (ANOVA) to show the significance in distribution of different parameters at spatial and temporal levels

	Df	F value	F critical value	P value
Fungal colony forming Units (nos *10^2 g^{-1} sediment) (within coastal stations)	2	0.36	3.7	0.70
(between seasons in coastal stations)	7	10.17	2.7	**0.0001*** **
(within OMZ slope stations)	4	1.22	3.01	0.34
(between different water column depths in slope station)	4	1.68	3.01	0.20
Fungal C (pg C g^{-1} sediment) (within coastal stations)	2	0.9	3.74	0.40
(between seasons in coastal stations)	7	2.7	2.76	**0.05***
(within OMZ slope stations)	4	1.01	3.01	0.18
(between different water column depths in slope station)	4	1.76	3.01	0.43
Bacterial C (*10^3 pg C g^{-1} sediment) (within coastal stations)	2	2.1	3.7	0.2
(between seasons in coastal stations)	7	9.7	2.8	**0.0002*** **
(within OMZ slope stations)	4	3.6	3.01	**0.03***
(between different water column depths in slope station)	4	1.9	3.01	0.16
Total organic carbon (%) (within coastal stations)	2	0.74	3.74	0.49
(between seasons in coastal stations)	7	1.0	2.76	0.31
(within OMZ slope stations)	4	20.36	3.01	**4.07 *10^{-16}*** **
(between different water column depths in slope station)	4	0.67	3.01	0.62

Df= degrees of freedom (Df =2, where n=3 (three CSt stations); Df=7, where n=8 (eight field trips) Df= 4, where n= 5 (five SSt stations or five depth of the sediment core), F value greater than F-critical value indicates statistical significance, *** significant at 0.001 %, * significant at 0.05 % level.

Fungal CFU and biomass C, bacterial biomass C and the OC % were more or less uniformly distributed within the three CSt stations and showed no statistical significance (Table 2.4). Significant difference was obtained between the different sampling periods in the number of fungal

CFUs, fungal biomass C and bacterial biomass C (Table 2.4). The fungal C and CFU distribution within the SSt stations of the OMZ region was almost uniform, but there was a significant difference in the bacterial biomass C and OC % (Table 2.4). Distribution of the CFU nos, fungal and bacterial biomass C and the OC % was uniform in all the depths of the sediment sections of the SSt stations as shown by no significance in ANOVA analysis (Table 2.4).

Table 2.5 (a) Correlation coefficient (r) between the biological parameters with DO as a dependent variable within the coastal stations (CSt).

	DO	Fungal CFU	Fungal C	Bacterial C	OC
DO	1				
Fungal CFU	0.47	1			
Fungal C	**0.84**	-0.09	1		
Bacterial C	**-0.98**	-0.28	**-0.93**	1	
TOC	-0.14	**-0.94**	0.42	-0.06	1

Table 2.5 (b) Correlation coefficient (r) between the biological parameters with DO as a dependent variable within the OMZ slope stations (SSt).

	DO	Fungal CFU	Fungal C	Bacterial C	OC
DO	1				
Fungal CFU	-0.38	1			
Fungal C	-0.20	0.03	1		
Bacterial C	-0.57	0.28	0.12	1	
OC	-0.09	-0.03	**-0.87**	0.35	1

Statistical analysis of the correlation between changing DO levels in the CSt and fungal CFUs, fungal biomass C, bacterial biomass C and OC% was carried out (Table 2.5). This result indicated that DO levels

have a positive correlation with fungal biomass C and a negative correlation with bacterial biomass C. Negative correlation is also seen between fungal biomass C with bacterial biomass C and between fungal CFU and OC (Table 2.5a). In the SSt stations no correlation is seen between DO levels and the microbial abundance, but a correlation between OC and fungal biomass C was seen (Table 2.5b).

2.4 Discussion

Indian Ocean occupies a disproportionately large fraction of the open ocean hypoxic area, in spite of occupying only 21% of the sea floor in the world oceans. The microbes play an inevitable role in the marine nitrogen cycle which takes place in this oxygen depleted environments (Zehr & Ward, 2002). But studies on the microbial process in this system are very limited. The microbial processes studied from this region are restricted mostly to the bacterial denitrification process (Shailaja *et al.*, 2006; Jayakumar *et al.*, 2004) Molecular techniques have been used to quantify the bacterial denitrifying genes from many of the world anoxic zones using *nar, nir, nos* as the marker genes. Metagenomic approaches have also brought to light a number of novel process and new taxa which are involved in this denitrification process. Involvement of higher eukaryotic organisms is shown in this denitrification process from various anoxic regions. Fungal denitrification has also been reported from terrestrial ecosystem such as grassland and in cultivated and uncultivated soils of a wildlife area in Canada (Laughlin & Stevens, 2002; Ma *et al.*, 2008). The role of fungal denitrification in the marine ecosystem is largely unaddressed as of today in spite of the large occurrences of fungal sequences in the uncultured clone libraries studied from various anoxic habitats (Dawson & Pace, 2002; Stoeck & Epstein, 2003; Brad *et al.*, 2007).

In this study the microbial biomass in the sediments of the denitrification zones of the Arabian Sea was estimated from three coastal (CSt stations) and slope stations in OMZ region (SSt stations). The DO levels determined in the near bottom water of the CSt sampling sites showed the changing DO levels with the season (Fig. 2.5). Periodic sampling of sediment was carried out in this region to estimate the fungal CFUs, fungal biomass C, bacterial biomass C and OC %. Statistical analyses of these parameters show that there is a seasonal variation in these parameters with the changing DO levels.

Fungal mycelia were detected in the sediments by staining with calcoflour, an optical brightener that enhances fluorescence of cellulose and chitin, the latter being signature of the fungal cell wall (Mueller & Sengbusch, 1983). Such staining revealed the presence of hyphae in the sediments, confirming active growth of fungi therein; occasionally fungal spores were also detected. Organic material in the sediments apparently supports growth of fungi. There was almost a uniform distribution of bacterial, fungal and organic carbon in the sediment core from 0-8 cm. Water mixing, high sedimentation rates and intense activity of the benthic meiofauna could have brought about this homogeneity of the sediments. Such a phenomenon has been also reported in deep-sea sediments of the Central Indian Ocean, where the conditions are more stable (Raghukumar *et al.*, 2006).

Changing DO levels (during the different field trips) in the coastal station has its influence on the fungal biomass C abundance (Table 2.4). Fungi are well known to thrive in anaerobic condition, inside the rumen guts using fermentative process (Gordon & Phillips, 1998). But recent studies on fungi have shown that they can participate in denitrification process (Shoun *et al.,* 1992; Usuda *et al.*, 1995). Fungi are also known to alternate their mode of respiration with changing oxygen concentrations

from being aerobic in oxic conditions, to becoming nitrate/ nitrite utilisers in suboxic condition and ammonia fermenters under anoxic conditions (Daiber *et al.*, 2005). Experimental studies on the growth pattern of cultures under varying oxygen concentrations has shown that some cultures grow equally good in both the oxic and anoxic conditions, but a few cultures are affected by the reducing oxygen concentrations (Jebaraj & Raghukumar, 2009). The change in the fungal biomass C with changing DO levels shows that there could be a few cultures which are affected by the decrease in oxygen levels. The decrease in the activity of the fungal cultures in anoxic conditions becomes a favorable condition for the denitrifying bacterial population. The bacterial cultures have a well established nitrate utilizing pathway, these denitrifies revive and flourish under such conditions (Campbell *et al.*, 1998; Brettar *et al.*, 2001). Bacteria are also known to adapt to severe anoxic condition when the denitrifying condition becomes sulphate reducing (Marty, 1981). Though fungal phylotypes have been reported from super-sulphidic springs, their role and adaptations to these unique environments are not known. The negative correlation in our study (Table 2.5a) between DO levels and bacterial biomass C may be due to the ability of bacteria which can adapt to changing DO levels and also survive sulphate reducing conditions much faster than fungi. The short life span of bacteria and their faster generation time gives them an advantage over fungi. In the open ocean OMZ region there is no evident effect of DO on the fungal CFUs or on the microbial abundance (Table 2.5b). The condition in the open ocean OMZ is a perennial condition in comparison to the seasonal anoxic condition. Hence the microbes thriving in this condition could have adapted to the low oxygen levels and a mutual balance between the fungal and bacterial population adapted to this environment is a possibility. Fungal CFUs are not affected by changing DO levels in both the coastal and perennial

station. This shows that fungal cultures though are affected by the changing DO levels are able to thrive and play a role in the denitrifying environments, in contrast to the existing reports which show that fungi has no ecological role in the oxygen depleted environments (Dighton, 2003; Mansfield & Barlocher, 1993).

Fungi are ubiquitous and can survive in arid and extremely low nutrient environments such as desserts and rocks (Gorbushina *et al.*, 2005; Grishkan *et al.*, 2006). Their presence has been detected from surfaces of optical lenses where the nutritional availability is very minimal (Marqués-Calvo, 2002). Fungi from deep sea habitats that have contrasting fast or feast conditions are also reported, where the organic load is 4 - 12 mg g^{-1} (Raghukumar *et al.*, 2001). The OC % in our study has a negative correlation with the fungal CFU formation and fungal biomass C in the coastal and OMZ region respectively (Table 2.5a & b). Isolation of cultures from natural environments was remarkably increased on low organic media (Giovannoni & Rappe, 2000) and in special media which could mimic the natural cues present in the natural environments (Kaeberlein *et al.*, 2002).

Advent of molecular ecological studies has brought to light an array of microbes and microbial process that are involved in the global biogeochemical cycles (Epstein & Lopez-Garcia, 2007; Lopez-Garcia & Moreira 2008). In this study we have for the first time quantified the fungal biomass C and have shown the presence of fungi in oxygen depleted environments (Jebaraj & Raghukumar, 2009). In the subsequent chapters I have studied the culturable and uncultivable fungal diversity, denitrification ability of a few select isolates and have also attempted to quantify the role played by fungi using *in-situ* microcosm experiments.

Chapter 3

Culturable and unculturable diversity of fungi from the denitrification zones of the Arabian Sea.

3.1 Introduction

Fungi live in very diverse habitats and display specialized adaptations in structure and biochemistry to thrive in each of these habitats (Maheshwari, 2005). Some of the unique habitats they are known to occupy include the outer stratosphere (Wainwright *et al.*, 2003; ISRO News Letter, 2009), the psychrophilic fungi inhabiting the cold artic and antarctic regions (Robinson, 2001), deep sea sediments (Raghukumar *et al.*, 2004), those living in extreme high temperatures ranging upto 62 °C (Maheshwari *et al.*, 2000) and the xerotolerent fungi living on the bark of trees and on dead, dry wood stumps (Petrovič *et al.*, 2006).

Presence of fungi in the marine environment was first studied in 1930 by Hönhk and Sparrow, followed by Barghoorn and Linder (1944). Detailed study on the diversity and physiology of fungi living in marine environment was carried out by Kohlmeyer and Kohlmeyer (1979). Following this, research on the fungal diversity in the marine habitats was carried out to understand their role in the marine ecosystems such as the detritus of salt marsh grass (Gessner, 1982), in mangrove detritus (Sathe-Pathak *et al.*, 1993) and from plant detritus in the coastal regions (Newell, 1996). Studies of fungi was also carried from deep sea environments, during which fungi was isolated from 4450 m depth (Roth *et al.*, 1964) and the barotolerance in fungi isolated from deep sea habitat was studied (Raghukumar *et al.*, 1992). These studies are based on the classical, cultivation-dependent, isolation and enumeration technique. Traditional work has been carried out to study the fungal communities colonizing the floating and submerged wood in the marine system and those associated with algae and marsh plants (Jones, 1985; Jones *et al.*, 1998). These studies of the fungal diversity are very incomplete because of the inability of many fungi to grow and sporulate in the culture media (Jones & Hyde, 2002). Inspite of the short comings in the cultivation-based diversity

assessment, about 800 species of obligate marine fungi could be isolated so far (Hyde *et al.*, 2000).

Advancements in the field of molecular ecology have revolutionized the study of fungal diversity. Molecular biological techniques, particularly the analysis of nucleotide sequences and phylogenetic approaches has greatly influenced the fungal systematics (Guarro *et al.*, 1999). Molecular tools such as RAPD and T-RFLP analysis of the amplified ITS and 18S rDNA region were used to identify the fungal cultures isolated from the marine ecosystem (Roberts *et al.*, 1996, Buchan *et al.*, 2002). These studies showed that the diversity of the marine fungi was more extensive than documented so far (Pang & Mitchell, 2005).Identification of the isolated fungal cultures using molecular markers was not without drawbacks, as all the fungal cultures could not be retrieved and maintained in laboratory conditions.

The introduction *in-situ* community profiling without the need to isolate cultures brought to light numerous hitherto undescribed organisms from various environments. Some of the groups described only based on environmental DNA analysis are presumably of high relevance for a variety of research disciplines like ecology, evolution, physiology and biogeochemistry (Epstein & Lopez-Garcia, 2007; Lopez-Garcia & Moreira, 2008). The well established phylogenetic analysis of prokaryotic SSU rRNA genes isolated from various environments has expanded our understanding of the diversity and ecological role of bacteria in the ecosystems (Giovannoni & Rappé, 2000; Freitag & Prosser, 2003; Molina *et al.*, 2007; Lehours *et al.*, 2007). In contrast to prokaryotes, molecular diversity surveys targeting microbial eukaryotes started only in recent times (Moon-van der Stay *et al.*, 2001; Lopez-Garcia *et al.*, 2001; Edgcomb *et al.*, 2002; Dawson & Pace, 2002).

A strong focus of eukaryote diversity research has been on a variety of environments such as shallow water anoxic sediments (Dawson & Pace, 2002; Stoeck *et al.*, 2003), hydrothermal vent systems (Edgcomb *et al* 2002, Lopez-Garcia *et al.,* 2003, 2007), oxygen-depleted deep basins (Stoeck *et al.*, 2003b, 2006), anoxic fjords (Behnke *et al.*, 2006, Stoeck *et al.*, 2007, Zuendorf *et al.*, 2006), saline meromictic lakes (Takishita *et al.*, 2007a), unique deep sea habitats (Takishita *et al.*, 2005, Takishita *et al.*, 2007b), hypersaline systems (Alexander *et al.*, 2009), a variety of oxygen-depleted freshwater environments (Dawson and Pace 2002, Luo *et al* . 2005, Slapeta *et al.* 2005) and terrestrial habitats (Schadt, *et al.* 2003). These studies using eukaryote specific primers has revealed a much larger diversity comprising of Green algae, Ciliates, Cercomonads, Stramenopiles, Radiolarians and Ophisthokonts (Dawson & Pace, 2002, Stoeck & Epstein, 2003; Stoeck *et al.*, 2003, Takishita *et al.*, 2005; Lou *et al.*, 2005; Bhenke *et al.*, 2006; Alexander *et al.*, 2009). Most, if not all these studies have shown that fungal representatives constitute a large proportion in most of the environmental libraries.

The fungal sequences obtained in many instances, especially during the initial studies were restricted to known and well described taxa (Dawson and Pace 2002, Stoeck and Epstein 2003, Brad et al., 2007). Community patterns from the supersulphidic anoxic Fijords of Norway (Bhenke et al., 2006), anoxic Mariager Fjord in Denmark (Zunderof *et al.*, 2006), hyper saline anoxic basin in the Mediterranean etc. (Alexander *et al.*, 2009) show that the fungal diversity is very low and a multiple PCR approach too does not improve the fungal diversity from the previously studied Cariaco Basin (Stoeck *et al.*, 2006). However there is a possibility that this could be due to the other dominant groups present in the sampling site that might have masked the presence of fungal sequences.

Recent reports of novel fungal lineages have been identified from anoxic, deep sea environments (Takishita et al., 2005, 2007a, 2007b, Bass *et al.*, 2007). Survey in the Lost City, hydrothermal vent system in the Mid-Atlantic ridge has also reported a unique clade "hydrothermal and/or anaerobic fungal group" (Lopez-Garcia *et al.*, 2007). With increasing group specific studies based on 18S rRNA and internal transcribed spacer (ITS) rRNA gene sequences, novel clusters of fungal sequences from diversified locations such as the tundra regions and temperate forest soil (Schadt, *et al.* 2003, O'Brien *et al.*, 2005) are reported.

Several of these environmental fungal sequences are highly divergent from previously deposited sequences of known fungi, indicating, that oxygen-depleted aquatic habitats may hold great promise for the discovery of novel fungi. Culture-independent techniques offer an alternative and a more detailed insight into the diversity, but it may not be an exclusive method to monitor the communities (Pang & Mitchell, 2005). Incorporation of molecular information into diversity studies and defining the fungal communities can provide a near complete picture of the ecological functioning. Hence in this study fungal specific PCR primers have been used to study the extent of fungal diversity in combination with cultivation based approaches.

3.2 Materials and methods

3.2.1 Diversity of culturable fungi

Fungal colonies were isolated from the sediments colleted from the three coastal stations and five open ocean slope stations by particle plating technique. Sampling procedure and the oceanographic instruments

used for the collection are described in Chapter 2 (Fig. 2.4). Fungal colonies were also isolated from the oxygen depleted water and the overlaying water (OLW) of the sediment core collected from four open ocean stations (SSt-500, SSt-700, SSt-800 and SSt-1100). Water was collected approximately 1 m above the sediment bed using the Niskin sampler. About 100ml of water was filtered on sterile Durapore filters (0.45 µm, Millipore (India) Pvt Ltd, Bangalore). The filter papers were plated on malt extract agar (MEA), Corn meal agar (CMA), Czapek Dox agar (CDA) and Brewer's agar (BA). All the media (HiMedia Pvt. Ltd., India) were used at 1/5 strength to discourage the growth of fast growing fungi and the plates were fortified with antibiotics as described in section 2.2.3. Fungal colonies appearing after incubation at 30°C for 15 – 20 days were picked using sterile needles and each of them was designated with a unique isolate number. OLW was collected from top of the sediment core in clean sterile 15 mm teflon tubes. Fungal colonies were isolated from OLW following the similar method which was used for isolating fungal colonies from water as described above. Fungal cultures were also isolated from the coral reef sand collected from Lakshwadeep sampling station (LSt_03) by particle plating technique.

Individual fungal colonies identified as unique colonies based on the colony morphology were catalogued as separate isolates. Fungal colonies isolated from the three coastal stations, SSt-200 station and LSt_03 are given unique isolate numbers (# 1, # 2, etc.). Fungal colonies isolated from the SSt stations (SSt-500, SSt-700, SSt-800 and SSt-1100) were designated with a suffix YK-1, YK-2, YK-3 etc., representing the research vessel RV-Yokosuka used for sampling. The distribution of the fungal cultures obtained is represented as percentage frequency.

The occurrence of the total fungal species from each sampling site and across the changing oxygen concentrations were studied based on

the species richness and Pielou's evenness values. The diversity of fungi from the coastal stations (CSt) during the field trips with the changing oxygen levels and in the oxygen depleted slope stations (SSt) was assessed based on the Shannon's and Simpson's diversity index. The statistical analysis was carried out using the software PRIMER 5 (Clarke & Gorley, 2001).

3.2.2 Isolation by enrichment culturing

Isolation of cultures by enrichment method was carried out from the sediment collected during the anoxic season (Ft-1) at the coastal station (CSt-26). Approximately, 5 g of sediment samples were incubated in airtight glass bottles in 80 ml of enrichment media that consisted only of 10 mM sodium nitrate solution prepared in artificial seawater and were supplemented with antibiotics to inhibt the growth of bacteria. The medium was then flushed with nitrogen gas and incubated for 30 days. At the end of the incubation period, fungal colonies were isolated from these sediments by particle plating as described in section 2.2.3.

3.2.3 Identification of culturable fungi

The sporulating cultures isolated from the different sampling stations were identified based on the taxonomic keys described in Domsch et al., (1980) or based on the partial (~ 1600 bp) 18S rDNA gene sequence. For the 18S rDNA sequence analysis each fungal culture was grown for 5 days at room temperature in 50 ml of malt extract broth (MEB, HiMedia Pvt. Ltd., India) in 250-ml Erlenmeyer flask. The total genomic DNA was extracted from the freeze-dried mycelial mats of each culture

using a high salt concentration extraction buffer. The components of the buffer were 100 mM Tris HCl (pH 8), 100 mM sodium phosphate buffer (pH 8), 1.5 M NaCl, 100 mM EDTA (pH 8.) and 1 % CTAB. Total genomic DNA was extracted using the buffer followed by chloroform-phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003). The 18S region of the rDNA was amplified using fungal specific primers NS1 and FR1 (Table 3.2). The 50 µl PCR reaction included 1x HotStart Taq buffer (Qiagen, Hildesheim, Germany), in a reaction buffer provided by the company containing Tris-Cl, KCl, $(NH_4)_2SO_4$], 15 mM $MgCl_2$ (pH 8.7), 200 µM concentrations of each deoxynucleoside triphosphate (dNTPs), and 0.5 µM concentrations of each oligonucleotide primer and the final volume was adjusted to 50 µl with sterile water. PCR was performed using initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 2 min, with a final extension at 72°C for 7 min with Hot Start Taq DNA polymerase (Qiagen, Valencia, CA). The PCR products were cloned separately for each fungal culture using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were isolated from positive overnight cultures using the Fast Plasmid Mini Prep kit (Eppendorf AG, Hamburg, Germany). One representative clone for each culture was sequenced bidirectionally (M13 sequencing primers) by MWG-Biotech on an Applied Biosystems (ABI) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. Sequences were included in phylogenetic analyses as described below. GenBank accession numbers of sequences deposited so far from cultured isolates are GQ120154- GQ120179.

3.2.4 Diversity of unculturable fungi

Environmental clone libraries were constructed from samples collected from five sampling sites (Table 3.1).Total genomic DNA from the sediment or water filters were extracted using a high salt concentration extraction buffer containing 100 mM Tris HCl (pH 8), 100 mM sodium phosphate buffer (pH 8), 1.5 M NaCl, 100 mM EDTA (pH 8.) and 1 % CTAB, followed by chloroform-phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003).

Table 3.1 Sampling sites for construction of five environmental clone libraries

Sampling site	Material	Quantity	DO(µM)
CSt-26 (Ft-1)	sediment	~ 500 mg	~ 0
CSt-26 (Ft-3)	sediment	~ 500 mg	118. 4
SSt-200	sediment	~ 500 mg	4.28
SSt-200	water	~ 5 l	4.28
LSt-03	sediment	~ 500 mg	93.74

DO = dissolved oxygen

Amplification of 18S rDNA was carried out using three different primer sets (Table 3.2). The primer sets were chosen based on their specificity and adequate length of amplified fragments in order to carry out robust phylogenetic analyses. The first primer set (Fung1) consisted of the fungal specific NS1 and FR1 primers resulting in ~ 1650 bp-fragments. According to homology searches this primer set has only moderate specificity and co-amplifies a range of non-fungal eukaryote genes like Metazoa, Cercozoa, Viridiplantae, Alveolata, Centroheliozoa and Bangiophyceae

(Pang & Mitchell, 2005). The second primer set (Fung2) included the fungal specific UF1 and S3 primers amplifying ~ 900 bp-fragments. This primer set has relatively high fungal specificity and previously amplified only few non-target taxa (green algae and *Pseudomonas*) from maritime Antarctic soil samples (Malosso *et al.*, 2006). We also used the universal eukaryote primer pair EukA and EukB, routinely used in environmental eukaryote diversity surveys (Euringer & Lueders, 2008; Marshall *et al.*, 2008; Stoeck *et al.*, 2006; Massana *et al.*, 2004) that amplifies nearly the full-length of the 18S rDNA (Table 3.2) of a wide range of higher eukaryote taxon groups (Medlin *et al.*, 1988).

Table 3.2 Primer sets used in this study to amplify 18S rDNA sequences from genomic environmental DNA

Primer set	Primers	Primer Sequence (5' -3')	Reference
Fung1	NS1	GTA GTC ATA TGC TTG TCT C	Vainio & Hantula,2000
	FR1	AIC CAT TCA ATC GGT AIT	Gomes *et al.*, 2003
Fung2	UF1	CGA ATC GCA TGG CCT TG	Kappe *et al.*, 1996
	S3	AGT CAA ATT AAG CCG CAG	Maiwald *et al.*, 1994
EukAB	EukA	AAC CTG GTT GAT CCT GCC AGT	Medlin *et al.*, 1988
	EukB	TGA TCC TTC TGC AGG TTC ACC TAC	Medlin *et al.*, 1988

Amplicons were ligated into pGEM-T vector and transformed into *E. coli* cells (TOP 10 strain) using Invitrogen's TA-cloning kit as described above. For each library and primer set we selected nearly 100 positively screened colonies (blue-white screening) for overnight growth and plasmid extraction using the 96 well Directprep Kit (Qiagen, Valencia, CA). The presence of 18S rDNA inserts was confirmed by the standard M13-PCR

amplification of extracted plasmids. Between 200 and 400 ng of amplification products with expected sizes were digested with restriction enzyme *Hae III* (New England Biolabs, USA) because its recognition site GGCC is reported to have a relatively high frequency of occurrence in the genomic DNA compared to many other enzymes. Digestion was carried out with 7.5 U enzymes for 60 min at 37°C, followed by an inactivation step for 20 min at 80°C. The resulting bands were separated by electrophoresis in a 2.5% low-melting point agarose gel at 80 V for 2 to 3 h. Clones with identical amplified ribosomal DNA restriction fragment analysis (ARDRA) patterns were considered members of the same operational taxonomic unit (OTU). At least one clone of each OTU was sequenced bidirectionally using M13 forward and reverse primers at MWG-Biotech as described above.

Sequences of each individual primer set were grouped separately into OTUs based on a 99.0% sequence similarity cutoff value using DOTUR (Schloss & Handelsman, 2005). ARDRA patterns were then assigned to their respective sequences. The sequences were checked for chimeras using the Bellerophon Chimera Check program and the Check_Chimera utility (Ribosomal Database Project (Cole *et al.*, 2003), as well as partial treeing analysis (Robison-Cox *et al.*, 1995). Putative chimeras and low quality reads were removed prior to subsequent sequence analyses. Similarities between two sequences were calculated using a custom program (PairAlign) provided by M. Nebel (University of Kaiserslautern), which employs IUB matrix-based pairwise alignments. These environmental sequences have been deposited in Genbank under the accession numbers GQ120105- GQ120153.

3.2.5 Phylogenetic analyses

Environmental 18S rDNA gene sequences initially were compared to those in GenBank using BLAST analysis to determine their approximate phylogenetic affiliation. Sequences of environmental clones together with their closest GenBank cultured and uncultured matches were aligned using the ARB FastAligner utility. Alignments were manually refined using phylogenetically conserved secondary structures. The conserved and unambiguously aligned positions were used in subsequent phylogenetic analyses. Evolutionary distance analyses under maximum likelihood criteria were carried out in PAUP* v4.0b8 (Swofford, 2002), with all characters equally weighted and unordered. The TBR heuristic option was used to search tree space, running ten random additions with the MulTree option on. The evolutionary model that best fit each of our aligned data sets was chosen among 56 possible models using Modeltest (Posada & Crandall, 2001). Support for the evolutionary distance analyses under Maximum Likelihood came from 1000 bootstrap replicates using heuristic searches. Bayesian analyses were carried out using MrBayes v3.2.1 (Huelsenbeck & Ronquist, 2001) with posterior probability support values calculated using four chains/two runs and running 10 million generations for each alignment. Trees were sampled every 1000th generation. The first 25% of sampled trees were considered 'burn-in' trees and were discarded. A 50% majority rule consensus of the remaining trees was used to calculate posterior probability values. The hLTR in MrModeltest v2 was used to estimate the best fitting evolutionary model and based on this GTR+I+G was used for analysis.

3.2.6 Statistical analyses

The program package SPADE (Chao & Shen, 2003-2005) was used to calculate the Jaccard index as a measure of similarity between two communities based on abundance of environmental fungal OTUs ($J_{abundance}$). An unweighted pair group mean average (UPGMA) cluster analyses based on $J_{abundance}$ data was performed using the Cluster analysis module of Statistica v. 7 (StatSoft, Tulsa OK). A Venn diagram was constructed to display the overlap in OTU composition between the different primer sets using VENNY (Oliveros, 2007).

Species accumulation curves and Abundance-based Coverage Estimator (ACE) of species richness for all the clone libraries were calculated in EstimateS (Colwell, 2005).

3.3 Results

3.3.1 Diversity of culturable fungi

Fungal cultures could be isolated from the coastal stations during all the season by particle plating technique. Maximum fungal diversity was seen in the pre-monsoon season in April 06 during the field trip 5 (Table 3. 3). During Ft-7, in Dec 06 no culturable fungal colonies could be isolated, this may be due to faulty incubator when the temperature set at 30 °C increased upto 37- 40 °C and absence of recoverable fungi may not necessarily mean absence of fungi in the natural ecosystem during this season.

About 57 different fungal isolates were recovered from the coastal stations during the eight field trips to the three coastal stations. Among the mycelial fungi that formed CFUs, *Aspergillus* species showed the highest

Table 3.3 List of cultures isolated from the sediments collected from the coastal stations (CSt) during the different field trips and their percentage frequency

Isolate number	Identification	Ft-1 (Oct 05)	Ft-2 (Nov 05)	Ft-3 (Jan 06)	Ft-4 (Mar 06)	Ft-5 (Apr 06)	Ft-6 (Oct 06)	Ft-7 (Dec 06)	Ft-8 (Feb 07)
# 1	*Aspergillus* sp.	28	3	—	37	22	—	—	—
# 2	*Aspergillus* sp.	6	13	—	9	3	—	—	—
# 4	*Isaria* sp.	0.5	—	—	—	—	—	—	—
# 6	*Aspergillus* sp.	2.5	—	—	—	—	—	—	—
# 8	*Aspergillus* sp.	5	—	—	—	—	—	—	—
# 9	*Ulospora bilgramii*	0.5	—	—	—	—	—	—	—
# 10	*Scolicobasidium* sp.	0.5	—	—	—	—	—	—	—
# 11	*Tritirachium* sp.	5	—	—	—	10	—	—	—
# 12	*Humicola* sp.	17	—	—	16	—	—	—	—
# 20	*Beauveria* sp.	0.5	—	—	—	—	—	—	—
# 21	*Aspergillus wentii*	0.5	—	—	1	2	—	—	—
# 24	*Cladosporium* sp.	22	—	—	—	—	—	—	—
# Th	Thraustochytrid colony	—	50	25	—	19	—	—	—
# 28	Yeast colony	—	3	—	—	—	—	—	—
# 30	*Microascus cirrosus*	—	3	—	—	—	—	—	—
# 31	*Myrothecium verrucaria*	—	3	—	—	—	—	—	—
# 31a	*Penicillium namyslowskii*	—	1	—	—	—	—	—	—
# 32	*Cleistothecial form*	—	1	—	—	—	—	—	—
# 33	*Aspergillus* sp.	—	1	—	—	—	—	—	—
# 35	*Aspergillus penicillioides*	—	—	9	—	—	—	—	—
# 36	*Aspergillus penicillioides*	—	—	9	—	—	—	—	—

Table 3.3 contd.

Isolate number	Identification	Ft-1 (Oct 05)	Ft-2 (Nov 05)	Ft-3 (Jan 06)	Ft-4 (Mar 06)	Ft-5 (Apr 06)	Ft-6 (Oct 06)	Ft-7 (Dec 06)	Ft-8 (Feb 07)
# 41	*Penicillium* sp.	—	—	5	—	—	—	—	—
# 46	Unidentified mycelial fungi	—	—	6	—	2	—	—	—
# 50	*Aspergillus* sp.	—	—	6	—	—	—	—	—
# 42-y	Yeast colony	—	—	1	—	—	—	—	—
# 43-y	Yeast colony	—	—	3	—	—	—	—	—
# 40	*Aspergillus candidus*	—	—	3	—	—	—	—	—
# 44	*Ermodothis angulata*	—	—	1	—	—	—	—	—
# 45	Unidentified mycelial form	—	—	14	—	—	—	—	—
# 51	Yeast colony	—	—	—	17	—	—	—	—
# 57 a	Yeast colony (orange)	—	—	—	1	—	—	—	—
# 57 b	White filamentous yeast	—	—	—	1	—	—	—	—
# 52 a	non-sporulating fungal colony	—	—	—	3	5	—	—	—
# 53	Ascomycetes with ascocarps	—	—	—	1	—	—	—	—
# 54	*Geosmiithia putterillii*	—	—	—	2	—	—	—	—
# 55	Unidentified yeast colony	—	—	—	2	—	—	—	—
# 56	*Aspergillus* sp.	—	—	—	1	—	—	—	—
# 65a	*Cladosporium* sp.	—	—	—	—	2	—	—	—
# 65b	*Aspergillus* sp.,	—	—	—	—	2	—	—	—
# 66	Unidentified mycelial form	—	—	—	—	2	—	—	—
# 68	*Aspergillus* sp.	—	—	—	—	2	—	—	—
# 67	Unidentified mycelial fungi	—	—	—	—	5	—	—	—
# 60	Unidentified yeast	—	—	—	—	2	—	—	—
# 61	*Aspergillus* sp.	—	—	—	—	2	—	—	—

Table 3.3 contd.

Isolate number	Identification	Ft-1 (Oct 05)	Ft-2 (Nov 05)	Ft-3 (Jan 06)	Ft-4 (Mar 06)	Ft-5 (Apr 06)	Ft-6 (Oct 06)	Ft-7 (Dec 06)	Ft-8 (Feb 07)
# 62	*Aspergillus* sp.	—	—	—	—	3	—	—	—
# 71-Y	Filamentous yeast colony	—	—	—	—	—	1	—	—
# 72	*Aspergillus* sp.	—	—	—	—	—	1	—	—
# 74	Unidentified mycelial colony	—	—	—	—	—	1	—	—
# 75	mycelial fungi (pink)	—	—	—	—	—	1	—	—
# 76	Unidentified mycelial colony	—	—	—	—	—	1	—	—
# 73	Unidentified mycelial colony	—	—	—	—	—	5	—	—
# 77	non-sporulating fungal colony	—	—	—	—	—	5	—	—
# 100	Filamentous yeast colony	—	—	—	—	—	—	—	4
# 104	*Aspergillus* sp.	—	—	—	—	—	—	—	4
# 103	Orange yeast	—	—	—	—	—	—	—	4
# 101	Yeast colonies (white)	—	—	—	—	—	—	—	15
# 102	Filamentous yeast	—	—	—	—	—	—	—	18
	Unidentified mycelial fungi	12	22	17	8	19	84	0	55
	Total Species (S)	13	10	12	13	16	8	0	6
	Species richness (d)	2.6	2.0	2.4	2.6	3.2	1.5	0	1.1
	Pielou's eveness (J')	0.8	0.7	0.9	0.7	0.8	0.3	0	0.7
	Shannon's diversity index (H')	1.9	1.5	2.2	1.9	2.3	0.7	0	1.3
	Simpson's diversity index (1-λ)	0.8	0.7	0.9	0.8	0.9	0.3	0	0.6

Table 3.4 List of cultures and their percentage frequency from the slope stations within the oxygen depleted region.

Isolate number	Closest named species / colony morphology	SSt-200			SSt-500			SSt-700			SSt-800			SSt-1100		
		wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed
# 90	clone LKM33			8												
# 122	*Zasmidium cellare*			8												
# 123	*Aspergillus versicolor*			8												
# 132	*Tritirachium* sp.			8												
# 129	*Cordyceps sinensis*			9												
# 133	*Tritirachium* sp.			9												
# 125	*Tritirachium* sp.			8									1			
YK-1	Mycelial fungi (pink)															
YK-2	Mycelial fungi (yellow)															
YK-3	*Acremonium* sp. KR21-2													1		
YK-4	White mycelial form												2			
YK-5	*Alternaria alternata*											1			12	
YK-6A	Orange yeasts										50					
YK-6B	Orange yeasts										50					
YK-6C	*Rhodotorula graminis*				28	5		[illegible]	[illegible]			2		[illegible]		
YK-6D	Yeast colonies (orange)													1		
YK-6E	Yeast colonies (orange)													1		
YK-6F	Yeast colonies (orange)													1		
YK-6G	Yeast colonies (orange)															
YK-6H	Yeast colonies (orange)															
YK-7	Yeast colonies (brown)							[illegible]								
YK-8B	*Pichia guilliermondii*								[illegible]			1				
YK-8C	*Melanopsichium pennsylvanium*														3	
YK-8D	Yeast colonies (white)								[illegible]							

wat : water, OLW: overlaying water, sed: sediment

Table 3.4 contd.

Isolate number	Closest named species / colony morphology	SSt-200			SSt-500			SSt-700			SSt-800			SSt-1100		
		wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed
YK- 9	*Cochliobolus* sp.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-10	Pale brown mycelia	—	—	—	—	—	—	—	—	—	—	—	—	—	26	—
YK-11	*Phialophorophoma litoralis*	—	—	—	—	—	—	—	—	—	—	—	—	—	12	—
YK-12	Cream coloured mycelia	—	—	—	—	—	—	—	—	—	—	—	—	—	6	—
YK-13	*Aspergillus unguis*	—	—	42	—	—	64	—	—	—	—	1	—	3	—	—
YK-14	Unidentified mycelial colony	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11
YK-15	Unidentified mycelial colony	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
YK-16	*Zasmidium cellare*	—	—	—	68	83	—	18	—	—	—	—	—	3	9	—
YK-17	Unidentified mycelial colony	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-18C	*Hortaea werneckii*	—	—	—	—	—	—	—	—	56	—	—	—	—	—	—
YK-19C	Yeast colony (pale orange)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-19D	*Rhodotorula nymphaeae*	—	—	—	1	5	—	9	—	—	—	—	—	—	—	—
YK-20	*Fusarium oxysporum*	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
YK-21	*Aspergillus* sp.,	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—
YK-22	*Aspergillus* sp.,	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—
YK-23	*Tilletiopsis albescens*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YK-24	*Cladosporium cladosporioides*	—	—	—	—	—	14	—	—	—	—	—	—	—	—	—
YK-25	*Cerrena unicolor*	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-26	Thraustochytrid colony (??)	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-27	*Fusarium* sp. MBS1	—	—	—	—	—	—	—	—	33	—	—	—	—	—	—
YK-28	*Chaetomium globosum*	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-29	Unidentified mycelial colony	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—
YK-30	*Zasmidium cellare*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11
YK-31	*Lepidosphaeria nicotiae*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	11
YK-32	*Coriolopsis byrsina*	—	—	—	—	—	—	—	—	11	—	—	—	—	—	—

wat : water, OLW: overlaying water, sed: sediment

Table 3.4 contd.

Isolate number	Closest named species / colony morphology	SSt-200			SSt-500			SSt-700			SSt-800			SSt-1100		
		wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed	wat	OLW	sed
YK-33	Non-sporulating fungi		—	—	—	—	—				—	—	—	—	—	—
YK-34	*Arthrinium marii*		—	—	—	1	—				—	—	—	—	—	—
YK-35	Unidentified mycelial colony		—	—	—	—	4				—	—	—	—	—	—
YK-36	Unidentified mycelial colony		—	—	—	—	4				—	—	—	—	—	—
YK-37	Unidentified mycelial colony		—	—	—	—	—				—	—	—	—	—	44
YK-38	*Aspergillus* sp. (Brown)		—	—	2	2	—				—	—	—	—	—	22
	Penicillium sp.		—	—	—	—	—				—	—	1	2	32	—
	Unidentified mycelia		—	—	—	4	—	55			—	51	92	[illegible]	—	—
	Unidentified yeast colony		—	—	—	—	—				—	43	—	—	—	—
	Total Species (S)	—	—	8	4	6	8	7	4	3	2	6	6	9	7	5
	Species richness (d)	—	—	1.5	0.7	1.1	1.5	1.3	0.7	0.4	0.2	1.1	1.1	1.7	1.3	0.9
	Pielou's eveness (J')	—	—	0.9	0.5	0	0.6	0.7	0.6	0.8	1.0	0.5	0.2	0.4	0.9	0.9
	Shannon's diversity index	—	—	1.8	0.7	0.7	1.3	1.3	0.8	0.9	0.7	0.9	0.4	0.9	1.7	1.4
	Simpson's diversity index	—	—	0.8	0.5	0.3	0.6	0.6	0.4	0.6	0.5	0.6	0.2	0.4	0.8	0.7

wat : water, OLW: overlaying water, sed: sediment

frequency of occurrence during most of the sampling period. *Cladosporium* sp. was also frequent during the anoxic period during Ft-1 in Oct 05. The straminipilan fungi, thraustochytrids were the next most abundant fungi (Table 3.3). The total species recovered from the different seasons varied from 6 to 16, the species richness (d) and Pielou's eveness (J') calculated also reflects that there was difference between the sampling seasons. The Shannon's diversity index varied between 2.3 and 1.3, and the Simpsons's diversity index was between 0.9 and 0.3 in the coastal stations during the eight field trips. During all the stations a large percentage of fungal CFUs were unidentified (Table 3.3).

Fungal cultures were isolated from five stations situated within the open ocean oxygen depleted environment along the continental slope region. Fungal cultures were isolated from water, overlaying water and the sediment collected from these stations. Yeast colonies and filamentous yeasts were the most dominant culturable fungi isolated from this region (Table 3.4). Among the culturable fungi *Zasmidium cellare* followed by *Aspergillus* sp. were the most dominant forms. A large number of mycelial colonies and yeasts colonies were unidentified and could not be retrieved during subsequent sub-culturing. The fungal diversity in the five sampling stations was uniform and the diversity was similar in the water, overlaying water and in the sediment. The species richness was very low in the slope stations when compared to the coastal stations (Table 3.4). Shannon's diversity index and Simpson's diversity index also reflected a poor diversity in the slope stations. The Values ranged between 0.4 and 1.8 for the former and between 0.2 and 0.8 for the latter (Table 3.4).

3.3.2 Isolation by enrichment culturing

Fungal cultures were isolated from the sediment incubated in anaerobic condition. Particle plating of sediment after incubation yielded four fungal

CFUs. They were identified to be one *Fusarium* sp. and three *Aspergillus* spp. (Table 3.5).

Table 3.5 List of cultures obtained by enrichment culturing

Isolate No.	Identification
# An-1	*Aspergillus* sp.
# An-2	*Fusarium* sp.
# An-4	*Aspergillus* sp.
# An-3	*Aspergillus* sp.

3.3.3 Identification of culturable fungi

Table 3.6: Selected list of cultures identified based on 18S rDNA sequence

Isolate No.	Isolated from	closest BLAST hit	Acc No	% similarity
Phylum Ascomycota				
# 9	CSt-26	*Ulospora bilgramii*	DQ384071.1	97.8
# 44	CSt-26	*Eremodothis angulata*	DQ384067.1	99.4
# 36	CSt-26	*Aspergillus penicillioides*	AB003077.1	99.7
# 35	CSt-26	*Aspergillus penicillioides*	AB003077.1	99.8
# 41	CSt-26	*Penicillium namyslowskii*	AB028190.1	99.4
# 31a	CSt-26	*Penicillium namyslowskii*	AB028190.1	99.5
# 40	CSt-26	*Aspergillus candidus*	AB008396.1	100.0
# 21	CSt-26	*Aspergillus wentii*	AB002063.1	99.6
# 50	CSt-26	*Aspergillus penicillioides*	AB003077.1	99.7
# 20	CSt-26	*Beauveria felina*	AY261369.1	99.9
# 30	CSt-26	*Microascus cirrosus*	M89994.1	97.5
# An-2	CSt-26	*Fusarium oxysporum*	AB110910.1	99.9
# 31	CSt-26	*Myrothecium verrucaria*	AJ301999.1	98.7
# 54	CSt-26	*Geosmithia putterillii*	AB031390.1	99.1
# 122	SSt-200	*Zasmidium cellare*	EF137362.1	99.9
# 123	SSt-200	*Aspergillus versicolor*	AB008411.1	99.6
# 132	SSt-200	*Tritirachium sp.*	AB003951.1	99.5
# 133	SSt-200	*Tritirachium sp.*	AB003951.1	99.5
# 129	SSt-200	*Cordyceps sinensis*	AJ301999.1	98.5

Table 3.6 contd.

Isolate No.	Isolated from	closest BLAST hit	Acc No	% similarity
Phylum Ascomycota				
# 125	SSt-200	*Tritirachium sp.* IAM 14522	AB109761.1	99.9
YK-24	SSt-500	*Cladosporium cladosporioides*	DQ678004.1	98.0
YK-28	SSt-500	*Chaetomium globosum*	AY545725.1	98.0
YK-16	SSt-500	*Zasmidium cellare*	EF137362.1	96.0
YK-18C	SSt-700	*Hortaea werneckii*	Y18700.1	99.0
YK-34	SSt-500	*Apiospora montagnei*	AB220229.1	95.8
YK-13	SSt-700	*Aspergillus unguis*	EF067336.1	99.0
YK-5b	SSt-800	*Alternaria alternata*	U05194.1	97.0
YK-8B	SSt-1100	*Pichia guilliermondii*	DQ534403.1	98.0
YK-9	SSt-1100	*Cochliobolus* sp.	FJ235087.1	97.0
YK-11	SSt-1100	*Phialophorophoma litoralis*	EU754077.1	95.0
YK-30	SSt-1100	*Zasmidium cellare* strain	EF137362.1	98.0
YK-31	SSt-1100	*Lepidosphaeria nicotiae*	DQ384068.1	96.0
# 85	LSt-03	*Penicillium namyslowskii*	AB028190.1	99.5
Phylum Basidiomycota				
# 11	CSt-26	*Rhodotorula aurantiaca*	AB030354.1	92.1
# 90	SSt-200	*Graphiola cylindrica*	D63929.1	97.1
YK-6C	SSt-500	*Rhodotorula graminis*	EU563924.1	95.0
YK-19C	SSt-500	*Aureobasidium pullulans*	EU707928.1	98.0
YK-19D	SSt-500	*Rhodotorula minuta*	FJ196163.1	98.0
YK-25	SSt-500	*Cerrena unicolor*	AY850007.1	98.0
YK-8C	SSt-700	*Melanopsichium pennsylvanicum*	DQ363314.1	99.0
YK-32	SSt-700	*Coriolopsis byrsina*	AY336773.1	99.0
# 87	LSt-03	*Sporisorium reilianum*	DQ832229.1	62.5
# 88	LSt-03	*Rhodotorula calyptogenae*	AB126648.1	99.8
# 89	LSt-03	*Rhodotorula calyptogenae*	AB126648.1	99.8

Culturable forms of fungi could be retrieved from all the sampling locations, from these 49 different cultures were sequenced for 18S rDNA region (Table 3.6).

Thirty eight of them belonged to Phylum Ascomycetes and most of them predominantly belonged to Eurotiomycetes. This is one of the biggest division and most of the commonly occurring and well studied forms belong

to this group. Eleven cultures grouped with the phylum Basidiomycetes. A majority of the sequences had more than 95 % similarity with the sequences available in the NCBI database with the different environmental sequences obtained from this study (Table 3.6). Except for two cultures # 87 and # 11 which had a greater percentage of dissimilarity compared to other cultures. # 87 has only 62.54 % similarity with *Sporosorium reilianum* and #11 belonging to Basidimycota had 92.13 % similarity with *Rhodotorula aurantica.*

3.3.4 Unculturable diversity

Five clone libraries were constructed using the total genomic DNA extracted from the environmental samples collected from a CSt and SSt station and a reference sample from the coral reef station (Table 3.1). Each of the libraries were constructed using two fungal specific primer sets (Fung1 and Fung2) for selectively amplifying fungal sequences and a universal eukaryote specific primer set EukAB that is routinely used in environmental eukaryote SSU rRNA diversity surveys (Table 3.2).

About 1440 clones obtained from five clone libraries were analyzed for their phylogenetic affiliation. Surprisingly, GenBank BLASTn analyses of these clones resulted in only 456 fungal targets, 19 of which were of low quality sequences (insufficient lengths, including Ns), and were not considered for further analyses. Of the 456 fungal targets obtained, 152 fungal sequences (grouping into 19 OTUs) were obtained with the Fung1 primer set, 172 sequences (35 OTUs) with the Fung2 primer set, and the universal eukaryotic primer set retrieved 113 fungal sequences (9 OTUs). Nearly two-third of the clones analyzed (984 clones) using BLASTn emerged as non-fungal sequences. As expected, numerous (n = 346) such non-fungal sequences were retrieved with the universal eukaryote

primer set EukAB. But also putatively fungal specific primer sets retrieved 313 (Fung1) and 281 (Fung2) non-fungal amplicons (Table 3.7).

Table 3.7 Taxonomic distribution of non-fungal operational taxonomic units (OTUs)

Primer set	Non-fungal GenBank BLAST matches
Fung1	*Corallochytrium limacisporum*; Choanoflagellates; Metazoa (Gastrotricha)
Fung2	Bacteria: (Proteobacteria); Viridiplantae; Rhizaria; Corallochytrium; Metazoa (Gastrotricha)
EukAB	Bacteria (Proteobacteria, Bacteroidetes, Fimicutes); Choanoflagellates, Rhizaria, Eugleonozoa; Alveolata; Metazoa (Arthropods, Nemaotodes, Annelids)

The Venn diagram (Figure 3.1) shows that the number of OTUs that were retrieved simultaneously with both fungal-specific primer sets is only small (n=5) indicating that each of the two primer sets amplified a different subset of taxa from the fungal communities under study. Fung1 and Fung2 primer set retrieved about 9 and 28 unique OTUs, but EukAB derived OTUs were only an overlap of the OTUs obtained from the fungal specific primer sets. Though, we were able to retrieve about 37 unique OTUs by using a combination of two fungal specific primer sets, the sequences belonged primarily to either phylum ascomycota or basidiomycota and only one OTU belonging to phylum zygomycota was obtained. There were no sequences belonging to phylum chytridiomycota.

Sampling saturation analysis (Figure 3.2) of the fungal OTUs showed that sampling was not done up to saturation. This may be due to the inefficiency of the primer sets used to amplify sequences belonging to

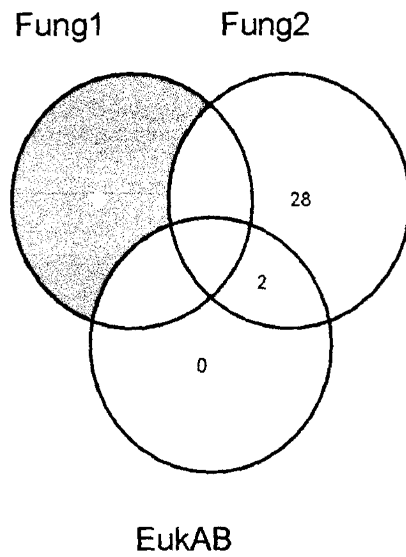


Fig.3.1 Venn diagram showing the number of OTUs that were recovered simultaneously by two or three of the primer sets used in this study (Fung1, Fung2, EukAB, see Table 3. 2) and number of OTUs recovered exclusively with a single primer set.

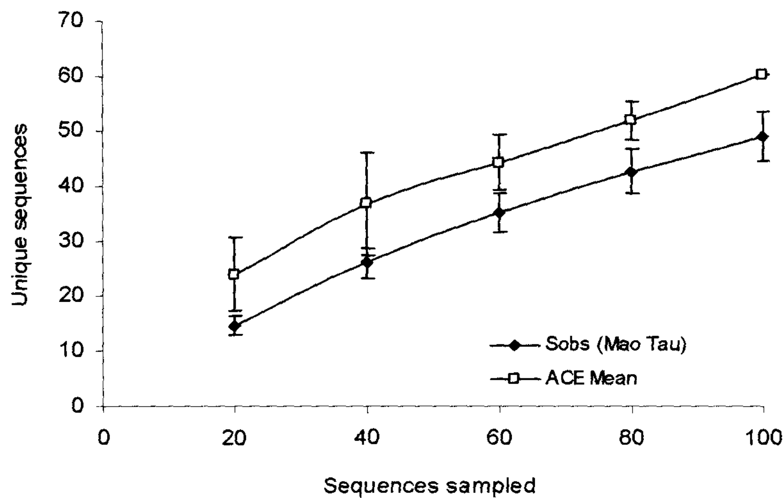


Fig. 3.2 Collectors curves of fungal OTUs. Species accumulation curve (Sobs) gives the number of species expected in the pooled samples, for the given empirical data and Abundance-based Coverage Estimator (ACE) is an estimator of species richness (mean among runs) for the clone libraries are plotted.

zygomycota and chyrtidiomycota. The primer sets also retrieved a large number of non-target OTUs, nearly two-third of the clones analysed (940 clones) on BLAST analysis matched with non-fungal data base entries and 44 sequences did not show similarity to any known sequences. Numerous (n = 346) non-fungal sequences were retrieved with the universal eukaryote primer set. Interestingly, also the putative fungal specific primer sets retrieved 313 (Fung1) and 281 (Fung2) non-fungal clones. Each primer set retrieved sequences belonging to a wide range of metazoa and unicellular eukaryotes (Table 3.7). The primer sets EukA-EukB and Fung2 even amplified SSU rRNA fragments that could be assigned to the domain Bacteria.

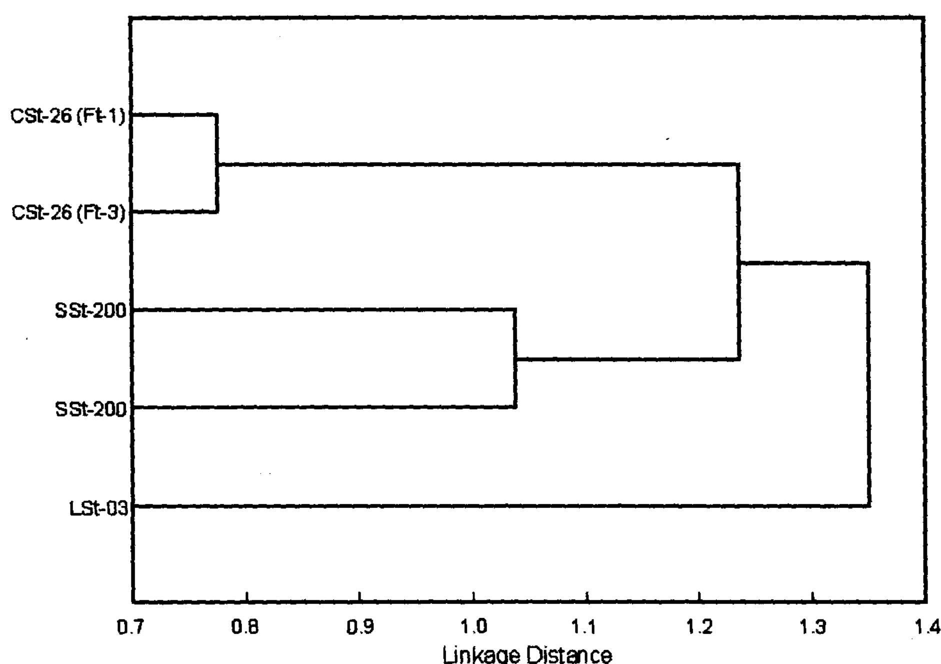


Fig. 3.3 Dendrogram resulting from calculated Jaccard index based on incidence ($J_{incidence}$) of unique operational taxonomic units (OTUs), as a measure of community similarity between the samples under study. For information on sampling sites, see Table 3. 1. Similarity values were transformed into a distance matrix and subsequent cluster analysis was performed using the unweighted pair group mean average (UPGMA) algorithm.

A UPGMA cluster analysis of J_{abund} index (Fig. 3.3) reveals that the fungal communities from the different sampling sites (Table 1) are distinctly different from each other. Of all comparisons, the two coastal sites sampled during anoxic and oxic conditions (CSt-26 during Ft-1 & Ft-3) are most similar in their fungal community membership (J_{abund} = 0.48). The sediment and water column-derived fungal communities from the open ocean (SSt-200) OMZ site cluster together with a J_{abund} of 0.35. The fungal community from the coral reef reference site (03_CRS) showed highest dissimilarity to all other samples (J_{abund} = 0.06-0.1) Our analysis suggests a clear separation of fungal communities adapted to temporal anoxic condition and those adapted to permanent anoxic zone. There is no difference in the community structure of fungi between the anoxic and oxic field trip samplings in the CSt-26 station. This may be due to the physiological adaptation of the fungal community to the changing oxygen concentrations.

3.3.5 Phylogenetic analysis:

Environmental OTUs identified in this study are designated as "Fungal OTUs from the Arabian Sea": FAS_1 to FAS_49. The OTUs from the five sample sites were assigned to the Dikarya (Ascomycota: 27 OTUs; Basidiomycota: 21 OTUs), and one OTU branched within the subphylum Mucoromycotina.

Ascomycota OTUs (Fig.3.4) belonged to the subphylum Saccharomycotina and Pezizomycotina. FAS_2 was the most frequently represented phylotype and is closely related to the yeast *Kodamaea* sp. with a similarity of 99.67%. Maximum number of environmental clones FAS_6, FAS_7, FAS_8, FAS_9, FAS_10, FAS_11 and FAS_12 belonged to Sordariomycetes and their closest known named species being *Fusarium* sp. *Fusarium oxysporum* is the most widely studied fungus of

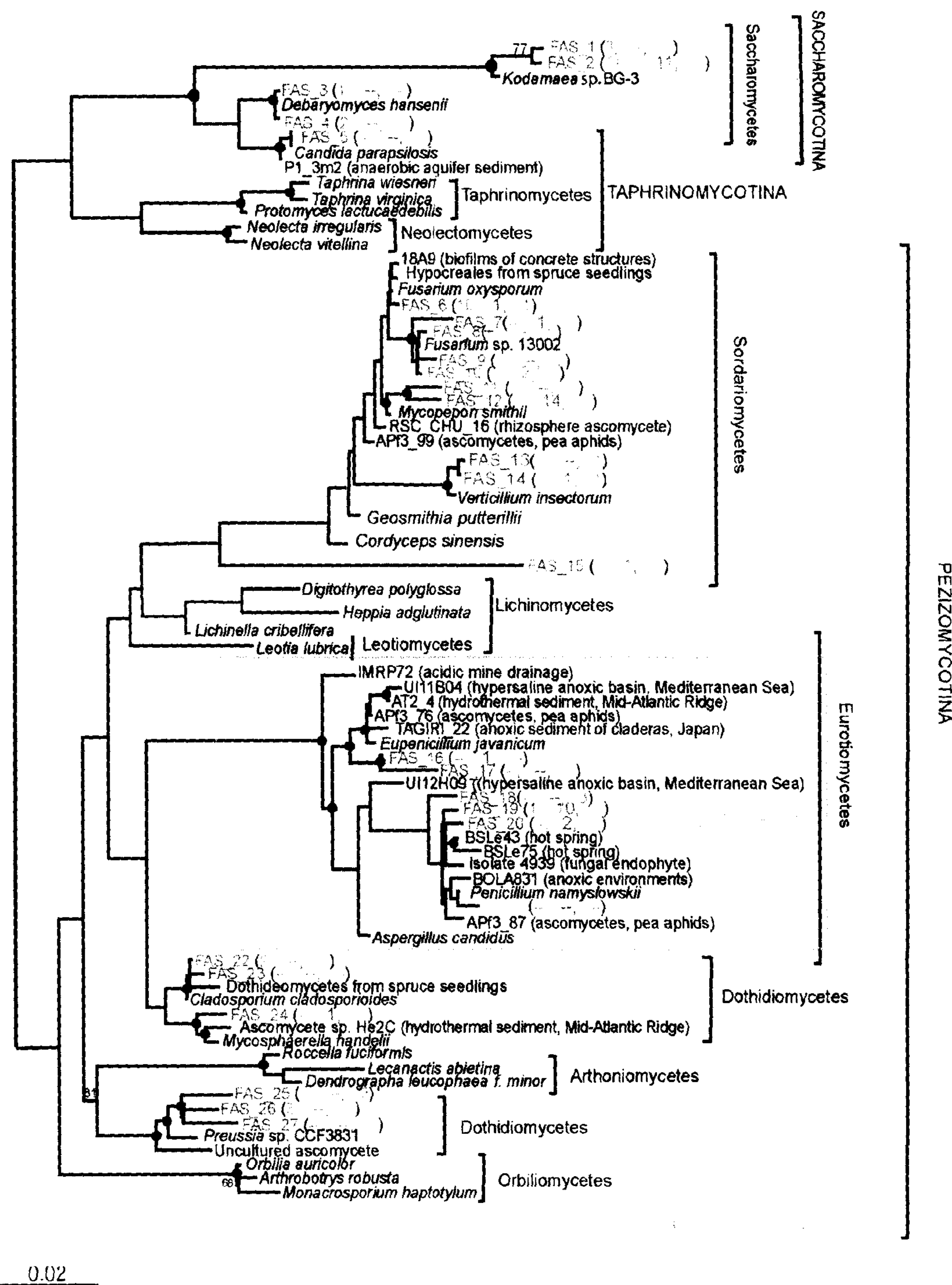


Fig. 3.4 Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Ascomycota showing the position of environmental OTUs obtained from the Arabian Sea.

Fig. 3.5 Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Basidiomycota showing the position of environmental OTUs obtained from the Arabian Sea. The tree was constructed under maximum-likelihood criteria by using a GTR+I+G evolutionary model with the variable-site gamma distribution shape parameter at 0.5470 and the proportion of invariable sites at 0.3147 as suggested by MrModeltestv2, based on 785 unambiguously aligned positions. The values given at respective nodes indicate distance posterior probabilities (PP) obtained from Bayesian MCMC analysis respectively. The numbers given within parenthesis next to the environmental OTU names are the number of representatives of them from different clone libraries. They are from coastal sampling site during anoxic and oxic season, OMZ sampling site sediment and water sample and the oxic reference site respectively.

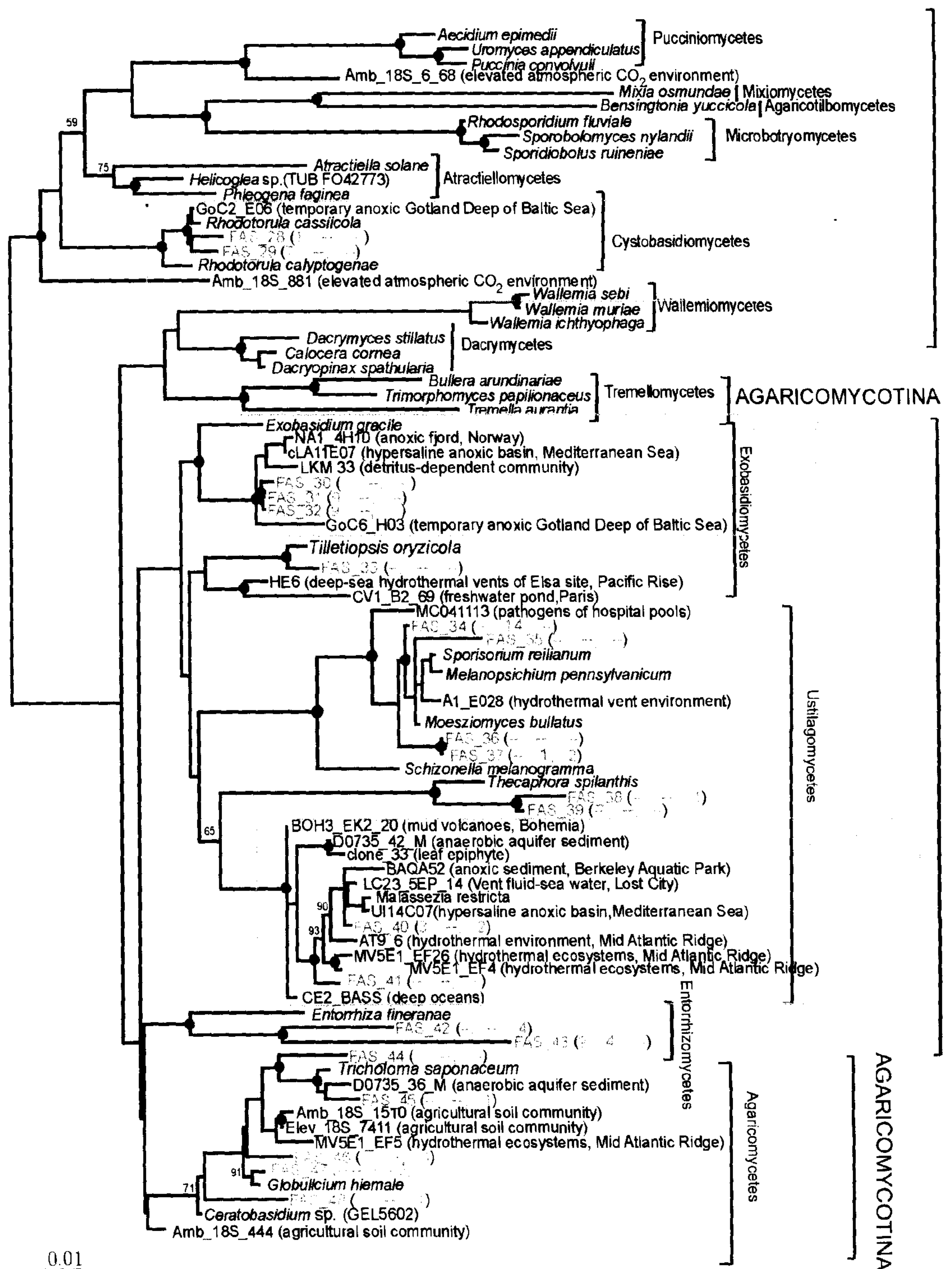


Fig. 3.5 Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Basidiomycota showing the position of environmental OTUs obtained from the Arabian Sea.

sequences were closely (> 97% sequence similarity) related to previously deposited sequences of described basidiomycetes such as FAS_47 exhibiting 99.46% similarity to the wood-decaying saprophyte *Globulicium hiemale* and FAS_28 and FAS_29 with the nitrogenous compounds scavenger *Rhodoturula cassiicola* as a closest relative with 99.83 % and 98.60 % sequence similarity respectively. Most environmental fungal sequences exhibited more than 3 % sequence divergence from sequences of described taxa of the cystobasidiomycete sequences such as the ustilagomycetes FAS_38 and FAS_39 being 93 % divergent to the plant pathogen *Thecaphora spilanthis.* Many fungal sequences from the Arabian Sea branch in clades that include other environmental fungal sequences from a variety of oxygen-depleted habitats. Examples are the three exobasidiomycete sequences FAS_30, FAS_31 and FAS_32 which were related to fungal sequences found in an anoxic Norwegian Fjord (Acc No. EF527154), an anoxic Mediterranean deep-sea basin (Acc No. EU446368) and an anoxic basin in the central Baltic Sea (Acc No FJ153746). The closest described relative of this sequence clade is the plant pathogen *Exobasidium gracile* with at least 37.3 % sequence divergence. The sequences FAS_40 and FAS_41 branch together with sequences from Mid-Atlantic Ridge hydrothermal vents (Acc Nos. AF530542, EF638640, EF638640), the Lost City hydrothermal field (Acc No DQ504335), mud volcanoes (Acc No. DQ504358), anoxic intertidal sediment (Acc No. AF372708), an anoxic Mediterranean deep-sea basin sequence (Acc No EU446358) and a few others such as epiphytes and deep ocean fungal sequences (Acc No. FJ153767, EU154971). The closest described species branching in this clade is the rare skin pathogen *Malassezia restricta*, with 0.39% and 2.36% sequence divergence to the OMZ clones FAS_40 and FAS_41. Interestingly phylotypes obtained exclusively from oxic regions of our study grouped together within the

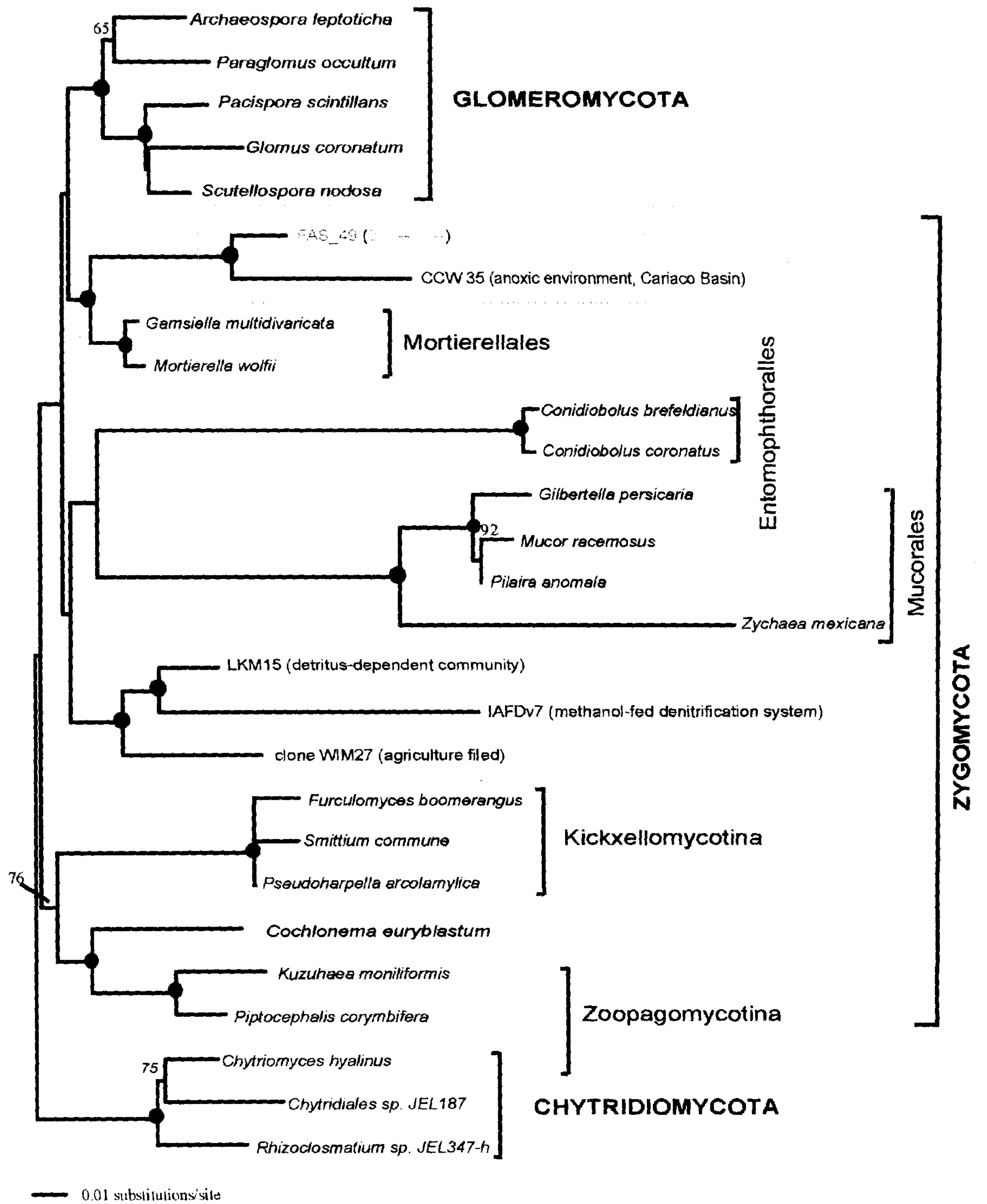


Fig. 3.6 Minimum evolution phylogenetic tree of the 18S rDNA sequences of phylum Zygomycota, Glomeromycota & Chytridiomycota showing the position of environmental OTUs obtained from the Arabian Sea.

agaricomycetes along with culture sequences and environmental clones belonging to anaerobic aquifers, hydrothermal sediment and agricultural soil (Acc Nos. EU647038, EF638641, EF024029, EF025042, EF023155).

Only one phylotype FAS_49 belonged to zygomycetes (Fig. 3.6), and it grouped with uncultured environmental clone CCW35 (Acc No: AY180022) from the anoxic Cariaco Basin with a similarity of only 90.45 %. The closest named species was *Mortierella wolfii* with 90.02 % similarity belonging to Mortierellales.

3.4 Discussion

3.4.1 Diversity of cultured fungi

Traditionally, fungal diversity studies in environmental samples are based on cultivation approaches. Limitations of this strategy are the inability to separate biomass from particulate material and lack of growth media and cultivation conditions suitable for all members of the community has lead to an underestimation of the total fungal diversity (Anderson *et al.*, 2003). Molecular phylogenetic analyses of clone libraries constructed from environmental samples have become the gold-standard in fungal diversity research (Pang & Mitchell, 2005). But this strategy is no panacea, as biases due to primer (un)specificities as discussed above. Combining cultivation-based and cultivation-independent methods may allow for a more complete picture of fungal diversity as each of the methodological strategies may compensate for the biases of the other.

Fungal cultures could be isolated using cultivation based approach from all the sampling stations (Table 3.3 and 3.4). The diversity among the cultures isolated from the coastal station based on the Shannon's index

shows that the diversity was less compared to the mangrove ecosystem (Maria & Sridhar, 2002). The Shannon's index in the mangrove ecosystem was about 5, whereas our study was only between 1.3 and 2.3. However these values are comparable to the diversity index of fungal cultures obtained from the deep sea environment (Damare *et al.*, 2006) which ranged between 1.2 and 2.0. The fungal diversity studied from the open ocean stations (Table 3.4) was very less even in comparison to the deep sea environment (0.4 to 1.8). Simpson's index also shows that the diversity from the oxygen depleted environment was lower in comparison to the mangrove ecosystem (Maria & Sridhar, 2002). It had an almost 0.9 index whereas our sampling sites showed a variation of 0.2 to 0.9 from the coastal and slope station.

The cultures identified based on the 18S rDNA sequence could be assigned to Basidiomycetes and Ascomycetes (Table 3.5). Eleven isolates belonged to Basidiomycetes, predominantly Pucciniomycotina; twenty-one of them belonged to Ascomycetes, a majority of it belonged to Eurotiomycetes. Both divisions of fungi have a high representation of cultured taxa (Hyde *et al.*, 2000), indicating the accessibility of these groups to cultivation. The 18S rDNA sequence of the cultures studied have >99% sequence similarities to previously studied, cultured and well-described fungi. This indicates that cultivation using standard techniques generally produces more isolates of already known taxa. Possibly, the development of more specific cultivation techniques and media that are adapted to the environmental conditions of the habitat under study might reveal a larger number of unknown taxa.

We obtained few isolates that show only low similarity to previously described and sequenced fungi. These are for example the isolates belonging to Basidiomycota, # 87 with only 62.5% similarity to *Sporisorium reilianum* and isolate # 11 with 92.1% sequence similarity to *Rhodotorula*

aurantiaca. These examples reveal another decisive advantage of cultivation over clone library analysis. Having access to (putatively) novel cultures in living condition paves way for an in-depth analysis of their phylogeny, morphology and ecology. A current multi-gene analysis of isolate # 11 along with ultrastructural analyses points to the discovery of a novel fungal taxon on class-level with the capability of anaerobic denitrification (Jebaraj CS, Boekhaut T, Muller W, Kauff F, Stoeck T, unpublished). Detailed analysis of the enzymes involved in the nitrate reducing pathways of cultured fungi and their expression profiles *in situ* can help us to understand their role in the OMZ of the Arabian Sea and other anoxic aquatic systems in global biogeochemical cycles.

3.4.2 Diversity of uncultured fungi

Analysis of fungal communities based on the environmental clone libraries studied across the anoxic habitats of the Indian Ocean shows that they are ubiquitous and the species diversity was not affected by the oxygen concentration (Fig 3.3). Indian Ocean has a characteristic seasonal anoxic condition that develops along the western margin during October to January (Naqvi *et al.,* 2006). Analysis of clone libraries constructed from the CSt-26, which comes under the influence of the seasonal DO changes shows that the community structure was not greatly affected by the seasonal changes of oxygen concentration and there was an overlap between the communities obtained during the anoxic and oxic season (Fig 3.3). Recent studies have shown that fungi can adapt to alternate modes of respiration (Daiber *et al.*, 2005) and the trend showed in our study is possible as the fungal community in large could adapt to the denitrification process during the oxygen depleted condition. But spatial difference was evident in the distribution of fungal communities, as seen in the difference

between the communities of the coastal, open ocean and the coral reef site. This can be seen from the J abundance index which shows that the community structure in the coral reef station was distinctly different from the coastal and slope station communities (Fig 3.3).

A number of recent studies in the anoxic habitats have shown that novel clusters and undescribed clusters are a major component in all the environmental communities studied so far. (Takishita *et al.*, 2005; Takishita *et al.*, 2007; Brad *et al.*, 2008). Exclusive studies on the fungal diversity from the cold Antarctic regions and deep sea environments has also shown the presence of a large number of uncultured sequences and novel clusters which branch out separately (Malosso *et al.*, 2006; Bass *et al.*, 2007). In this study I have attempted for the first time to use fungal specific primers to study the fungal diversity from the anoxic regions of the Arabian Sea. Phylogenetic anlaysis of the environmental sequences obtained from this study has shown that we can categorize phylotypes specific to each library and at least five clusters of environmental sequences could be identified which groups with sequences obtained from similar anoxic and extreme environments.

3.4.3 Phylogenetic analysis

Of the five unique environmental clusters identified, one cluster with six phylotypes from this study groups within the Eurotiomycetes of Ascomycota (Fig. 3.4). This cluster has sequences obtained from the anoxic Mediterranean basin, anoxic Meromictic Lake sediment and marine anoxic Bolinas Lagoon DNA and a few common culturable forms such as *Penicillium* and *Aspergillus* sp. These commonly occurring species are very versatile and are shown to participate in denitrification activity. Experimental studies with # 31a (99.45% similarity with *Penicillium*

namyslowskii) have shown that they can grow under anaerobic conditions by reducing nitrate (Jebaraj & Raghukumar, 2009).

Three clusters were seen within the basidiomycota belonging to Cystobasidiomycetes, Exobasidiomycetes, and Ustilagomycetes (Fig. 3.5). The cluster within the Cystobasidiomycetes groups with environmental sequences of uncultured clones from anoxic Gotland Deep of Baltic Sea, environments with elevated atmospheric CO_2 and with two basidiomycete yeasts *R cassiicola* and *R calyptogenae*. The second environmental cluster within the basidiomycota grouped along with environmental sequences closely related to Exobasidiomycetes. The sequences clustered with phylotypes obtained from anoxic Norwegian fjords, anoxic Mediterranean basin, anoxic Gotland Deep of Baltic Sea and a fungal clone obtained from detritus community. The closest known named species was *Exobasidium gracile* which branched out separately from this environmental cluster which is supported with a high bootstrap support. The third cluster belonging to Ustilagomycotina is within a clade that has been recognized as "hydrothermal and/or anaerobic fungal group" (Lopez-Garcia *et al.*, 2007). This cluster has representatives from mud volcanoes, hydrothermal ecosystems, anoxic Mediterranean basin and anoxic Berkeley Aquatic Park sediment, deep oceans and a leaf epiphyte (Fig. 3.5).

Only one phylotype belonging to zygomycete was obtained from this study and this OTU grouped with CCW35 from the anoxic Cariaco Basin (Fig. 3.6). Though there was a very poor representation of zygomycetes and chyrtids in the study, they are reported to be a major component of the fungal environmental community. Zygomycete representatives and a full clade of uncultured fungal sequences were recovered from the anoxic polluted aquifer sediments (Brad *et al.*, 2007). A large number of chytrid sequences have been retrieved from other

anoxic environments (Brad *et al.*, 2007) and universal microeukaryotic libraries (Stoeck *et al.*, 2007). Lack of representatives from these groups may be attributed to the primer sets used for analyses which were efficient to recover the major ascomycetes and basidiomycetes but missed out the other two groups. Similar views have been expressed by researchers studying the fungal diversity from environmental DNA analysis (Anderson *et al.*, 2003; Malosso *et al.*, 2006). It is probable that some fungal sequences present could not be retrieved due to problems associated with primer selection and DNA quality. Increasing studies from across the globe in the anoxic environments has strengthened the database available on the fungal community structure. Hence further studies with this basis should be carried out with more refined primer sets that can amplify fungal taxa belonging to all the phylums. Studies to specifically study individual groups and subgroups may also bring to light many new and interesting dimensions to our understanding of the ecosystem and their functioning.

Aid of molecular tools has definitely broadened the understanding of several ecosystems. In spite of the increasing efficiency of techniques to study the in-situ process at molecular level, an in-depth understanding of the role played by the microorganisms can be achieved only by studying the culturable forms in-vitro conditions. Screening studies of a few cultures isolated from the oxygen depleted environment has been studied for their denitrification ability (Chapter 4).

The major inferences that can be drawn from the study based on the culture dependent and independent approach are: this study represents the first fungal specific SSU rRNA based survey of the oxygen depleted regions of the Arabian Sea and representative environmental sequences from the major fungal groups could be retrieved. Five clusters of environmental sequences that group with sequences from metagenomic libraries constructed from similar anoxic regions could be

identified. Unique fungal phylotypes were obtained from each of the libraries constructed from seasonally and spatially distinct locations. Isolation of cultures from anoxic regions which are divergent from well described taxa gives an opportunity to study them in detail for their phylogenetic and ecological significance. Detailed analysis of the genes that code for the enzymes involved in the nitrate reducing pathways and their expression profiles will help to quantify their role in the OMZ of the Arabian Sea.

Chapter 4

Denitrification ability of fungi isolated from the marine denitrification zones.

4.1 Introduction

Fungi are primarily aerobic heterotrophs that play an essential role as decomposers of organic matter in a variety of environments. In marine ecosystems fungi are the major decomposers of woody and herbaceous substrates and their importance lies in their ability to aggressively degrade lignocellulose (Newell, 1996). Marine fungi also contribute to the degradation of dead animals and animal parts (Kushwaha & Gupta, 2008), and are important pathogens of animals (Lecampion-Alsumard *et al.*, 1995) and plants (Raghukumar, 1986), or partners in mutualistic symbioses (Mishra & Lichtwardt, 2000; Pivkin, 2000). Fungi have been isolated from diverse marine substrata such as marine alage (Raghukumar *et al.*, 1992a), mangrove plants (Kumaresan *et al.*, 2002), calcareous shells of marine organisms (Raghukumar *et al.*, 1992b), corals (Ravindran *et al.*, 2001, Golubic *et al.*, 2005) and deep sea sediments (Damare *et al.*, 2006). There was a strong conviction that all fungi required oxygen for growth and it was considered as one of the major metabolic differences between bacteria and fungi (Foster, 1949). This was disproved by the discovery of obligate anerobic chytrid fungi from the gut of the herbivores (Orpin, 1977). However the ability of fungi to live in anaerobic conditions was restricted to fermentative rumen-fungi and studies have shown that colonization of fungi on wood in marine environment is slower or almost nil in oxygen minimum zones (Kohlmeyer & Kohlmeyer, 1979). Role of fungi in natural oxygen depleted environments was considered to be insignificant (Mansfield & Barlocher, 1993; Dighton, 2003) and there have been no attempts to study the diversity of fungi from oxygen-depleted marine environments.

Pioneering work on soil fungi capable of denitrification was shown in early 70s (Bollag & Tung, 1972). Denitrifying activity in fungi has been

studied in detail after 1991 when the involvement of cytochrome P450 in the fungal denitrification pathway was shown (Shoun & Tanimoto, 1991). Now several fungi like *Fusarium oxysporum*, *Aspergillus oryzea* and yeasts *Trichosporon cutaneum, Cylindrocarpen tonkinesea* are shown to be capable of denitrification (Shoun *et al.,* 1992). The denitrification process in *Fusarium oxysporum* has been studied extensively and they have shown that fungi have an incomplete denitrification process. In bacterial denitrification process, nitric oxide (NO) is reduced to nitrous oxide (N_2O) by nitric oxic reductase, which is further reduced to the final product nitrogen gas (N_2). In fungi the denitrification process largely stops with the reduction of NO to N_2O. The enzyme involved in the NO to N_2O reduction is nitric oxide reductase (p450 nor), which is specific for fungi (Kobayashi *et al* 1995, Daiber *et al.*, 2005). The role of fungi in the denitrification process of the grassland ecosystem has shown that the fungal denitrification accounts for nearly 80% of the nitrous oxide production based on substrate induced respiratory inhibition studies (Laughlin & Stevens 2002). Fungi are also known to be involved in co-denitrification and chemo-denitrification to produce N_2 gas (Laughlin & Stevens 2002).

Ammonia fermentation is another mode of alternate respiration observed in fungi during complete anoxic conditions. Studies on the denitrifying activities of *F. oxysporum* have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O_2 tension, ammonia fermentation under anoxic conditions, denitrification when hypoxic, and oxygen respiration under aerobic conditions, thus demonstrating that a eukaryote can use a multimodal type of respiration (or ATP-producing) system to rapidly adapt to changes in the oxygen supply (Zhou *et al.*, 2002).

Ammonia oxidation to N_2 by bacteria, through annamox reaction has been identified in the marine environment, both in the water column and in the sediments. The annmaox reaction is estimated to contribute 30-50% of the N_2 production from the oceans (Devol, 2003). But much of the molecular biology, physiology and ecology of anammox are yet to be studied. Though the involvement of fungi in ammonia oxidation is not reported till now, an investigation on their ability to involve in anammox reaction and other alternate respiration modes are worth attempting.

Microeukaryotic diversity studied from the anoxic regions based on culture independent methods have always included a great number of sequences belonging to mycelial fungi and other lower fungal forms such as Stramenopîles (Dawson & Pace, 2002; Stoeck & Epstein 2003; Massana *et al.*, 2004). The thraustochytrid dynamics studied in the water column of the Arabian Sea OMZ shows that the total numbers were as high as that of bacterial numbers in the order of 10^6 and 10^9 respectively (Raghukumar *et al.*, 2001). Thraustochytrids have also been found associated in large numbers in the sediments of the continental shelf region. The preliminary studies in the sediments off Goa also showed large number of thraustochytids, especially Labyrinthulomycetes during the post monsoon season (Jebaraj & Raghukumar, 2009). Studying fungi in the denitrifying environment can give us insights about their involvement in the biogeochemical cycling of nitrogen. An understanding of this process can be used for biotechnological applications in sewage treatment plants. Till now nitrifying and denitrifying bacteria are used for biological treatment of sewage wastes. But filamentous fungi can perform both nitrifying and denitrifying process even under low oxygen conditions. Hence the use of fungi can be beneficial in many ways (Guest & Smith, 2002).

Microbes involved in the denitrification pathway are studied by their ability to utilize nitrate or nitrite as an alternate electron acceptor in the absence of oxygen by studying the most probable numbers (especially of bacteria) from denitrifying zones (Michotey *et al.*, 2000) and studying the electron transport system activity and the enzymes involved in the denitrifying pathway (Devol *et al.*, 1976; Shailaja *et al.*, 2006). Functional markers coding for the key enzymes such as *nir*K, *nir*S, *nor*B, *nos* of the denitrification process are used to study the community structure of the denitrifiers (Scala *et al.*, 1999; Braker *et al.*, 2000). Real time quantitative analysis of the genes involved such as nitrite reductase (*nir*K and *nir*S) provides us with the in-situ analysis to quantify bacterial denitrifiers in the natural ecosystems (Henry *et al.*, 2004). Apart from studying the natural oxygen depleted environments, isolation of cultures from these regions are also carried out and their utilisation of nitrate in the medium under anoxic condition has been used as the common and quick method to test the denitrifiers (Simbert & Kreig, 1981). Though this step is a basic for the denitrification process, identification of other complex microbial process involved in the nitrogen cycle has been brought to light (Zher & Ward, 2002), hence more detailed analysis of the denitrification process is required. Mahne and Tiedje (1995) has suggested that denitrifiers should satisfy two criteria to be called so which includes the production of nitrogen gases principally N_2O and N_2, coupled with growth yield which can be quantified by the increase in biomass.

A selected set of cultures from our study isolated from the coastal station (CSt-26) during the anoxic and oxic season (Table 4.1) were screened for their ability to grow under anaerobic conditions to reduce nitrate. Measurement of nitrite accumulation, ammonia formation and respiratory electron-transport activity was carried out in these cultures.

Table 4.1 List of cultures used for experimental studies

Isolate	MTCC Acc No.	Identification	Isolated from
# An-2	MTCC 9376	*Fusarium* sp	CSt-26, Ft-1 (Oct 05)
#11	MTCC 9381	*Tritirachium* sp	CSt-26, Ft-1 (Oct 05)
# 31	MTCC 9375	*Myrothecium verrucaria*	CSt-26 FT-3 (Jan 06)
# 31a	MTCC 9388	*Penicillium namyslowskii*	CSt-26 FT-3 (Jan 06)
# MT-811	---	*Fusarium oxysporum*	Gift from Dr Shoun, Univ of Tokyo, Japan.

MTCC: Microbial Type Culture Collection, IMTECH, Chandigarh, Inida.

Activity of fungi and bacteria individually and in combination under *in-situ* condition was studied by setting up various microcosms. The different microcosm set-ups included incubation of fresh sediment collected from the coastal anoxic region, sediment supplemented with nitrate, sediment with antifungal or antibacterial agents and with fungal inoculum. Nitrate and nitrite formation, ETS activity and the Eh were measured from the microcosm after 7 days of incubation.

4.2 Materials and methods

4.2.1 Screening of fungi for their nitrate utilization capacity under aerobic and anaerobic conditions

For screening studies the growth of four different fungi was studied in an inorganic mineral medium (Appendix) supplemented with sodium nitrate. The final concentration of the nitrate supplement in the medium was 10 mM and the fungal cultures were studied for their ability to reduce nitrate by estimating the nitrite formed in the culture medium and their biomass formed. These were were # An-2 (*Fusarium* sp.) isolated after anaerobic incubation of the sediment, # 11 (*Tritirachium* sp.) which was isolated from the sediment during anoxic condition and # 31 (*Byssochlamys* sp.) and #

31a (*Paecilomyces* sp.) isolated from the sediments when the conditions were oxic (Table 4.1). These cultures were compared with a well studied denitrifier of terrestrial origin, *Fusarium oxysporum* # MT-811 (Shoun & Tanimoto 1991), a gift from Dr. Shoun, Tokyo University, Japan.

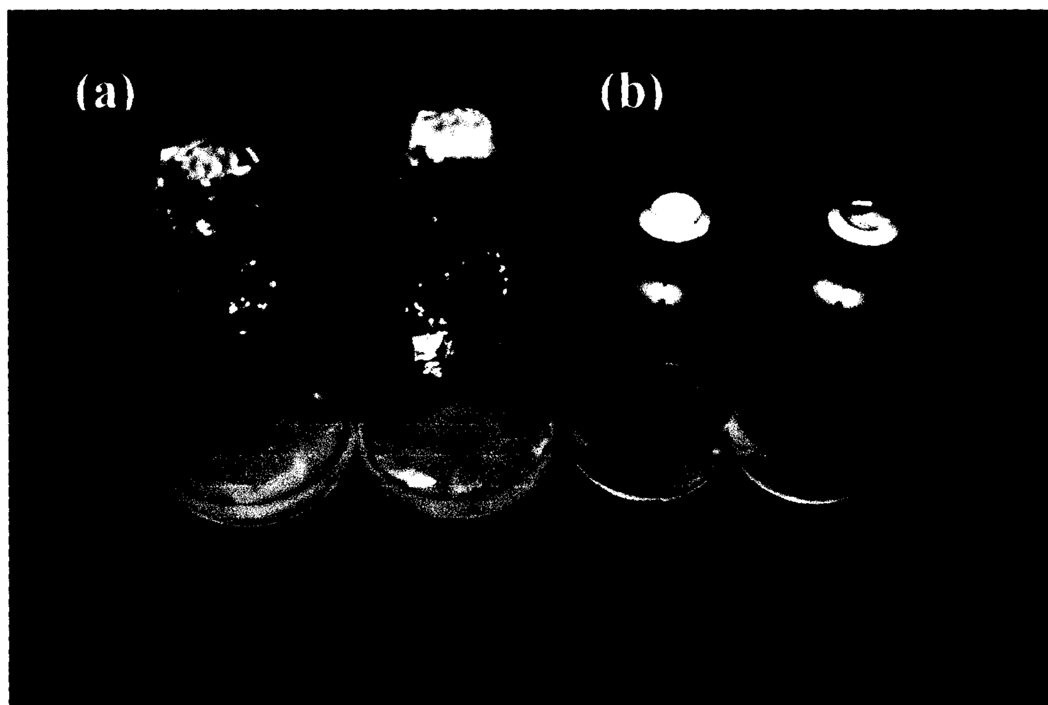


Fig. 4.1 Experimental set-up of fungal cultures grown in (a) aerobic and (b) anaerobic condition

Starter cultures of these fungi were grown in mineral medium supplemented with 10 mM of sodium nitrate for 3 to 5 days. Approximately 10-15 mg (dry weight) of the mycelial suspension was used as an inoculum. The cultures were maintained under aerobic conditions in 100 ml conical flasks (Fig 4.1a) plugged with cotton containing 20 ml of medium and under anaerobic conditions in 100 ml serum bottles (Fig 4.1b) sealed air tight with butyl rubber stoppers and steel crimps. The gas present in the medium was replaced with the inert nitrogen gas by flushing

nitrogen gas through the butyl septum for 2 min with a help of a 5 mm hypodermic needle.

The dissolved oxygen (DO) was determined by spectrophotometric method (Pai *et al.* 1993) at 0 hour and at the end of the experiment on the day 10 and on days when there was significant nitrite formation. Replicate bottles were used exclusively for DO measurement. The cultures were harvested every 48 hours up to 10 days and nitrite and ammonia formed were determined by spectrophotometric measurement (Strickland & Parsons, 1968).

The growth of the cultures was also measured on day 10 and biomass in mg dry weight was determined. The cultures grown in aerobic and anaerobic conditions were filtered on a pre-weighed filter paper to harvest the mycelia. The harvested mycelia on the filter paper was washed several times with distilled water and dried in an oven at 50 °C till it attained constant weight.

Ammonia was estimated in the culture filtrate, by treating it in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside which acts as a catalyzer. The blue indophenol color formed with ammonia was measured spectrophotometrically at 640 nm (Strickland & Parsons, 1968).

Nitrite in the culture filtrate was allowed to react with sulfanilamide in an acid solution. The resulting diazo compound is reacted with N-(1-naphthyl)-ethylenediamine which forms a highly colored azo dye that was measured at 543 nm (Strickland & Parsons, 1968). All chemicals used were of analytical grade.

Statistical analyses were carried out using Excel (Microsoft) programme. The data were transformed and tested for normality before analysis by Cochran Q test (Sokal & Rohlf 1981).

4.2.2 Determination of Electron transport system (ETS) enzyme activity in fungal cultures grown under aerobic and anaerobic conditions

Estimation of ETS activity in the aerobic and denitrifiying condition is proportional to the respiratory activity and denitrification rates respectively (Packard *et al.*, 1983). ETS activity was determined for the four cultures ie., # AN- 2 and # 11, # 31, # 31a, # and compared with the terrestrial control culture (# MT-811).

The biomass of the fungal cultures grown under aerobic and anaerobic conditions was harvested and freeze dried. The freeze dried biomass (approximately 100 mg) was homogenized in 3 ml of 0.05 M phosphate buffer (pH 7.7) containing 9 mg of polyvinyl pyrrolidone and 0.5 mg of dithiothreitol. Precautions were taken to minimize bacterial contamination throughout the experimental procedure by preparing buffers in filter sterilized water and using sterilized glass wares. The crude homogenized mixture was centrifuged at 6000 g for 5 min at 4°C. The supernatant was immediately transferred to clean tubes and the volume obtained was recorded. One ml of the crude enzyme extract was incubated in dark for 20 minutes at room temperature with 1 ml of 4 mM 2(p-Iodophenyl)-3(p-nitrophenyl)-5phenyl tetrazolium chloride (INT) and 3 ml of substrate solution containing 0.6 g NADH, 0.2 g NADPH, 36 g sodium succinate hexahydrate and 2 ml Triton X 100 per liter of 0.05 M phosphate buffer (pH 8). During the incubation process INT is reduced to its formazan and this gives a pink coloration to the reaction mixture. The reaction was terminated by addition of 1 ml of 1:1 (V/V) solution of 1 M H_3PO_4 and concentrated formalin at the end of the 30 min incubation time. The quenched reaction mixture was centrifuged at 6000 g for 5 min at 4°C. The absorbance was recorded at 490 nm and 760 nm within 3 hours at

room temperature in a spectrophotometer. The peak at 490 nm is proportional to INT-formazan produced and the 760 nm reading serves as turbidity blank. Pigment blank consisting of an assay run without pyridine nucleotides or succinate on the clarified homogenate was run with each assay; in addition a reagent blank was also run.

The ETS activity was calculated from the equation:

$$ETS\ (nq\ h^{-1}\ \mu g^{-1}\ protein) = 60*S*(COD\text{-}RB)\ /1.42*f*t * 5.6$$

where S: volume of quenched reaction mixture, COD: corrected absorbance, RB: reagent blank, f: conc. of protein in reaction mixture (µg), t: incubation time, the constants 60 and 1.42 conversion factor is based on the molar extinction coefficient of the INT formazan and the stoichiometry of oxygen consumption by the respiratory electron transport system. The constant 5.6 is used to convert the ETS activity obtained in µl O_2 h^{-1} μg^{-1} protein to neq h^{-1} μg^{-1} protein. The total protein concentration in the crude homogenate was determined by Bradford method using the commercially available Bradford reagent (Sigma-Aldrich, Bangalore, India).

4.2.3 Determination of Electron transport system (ETS) enzyme activity under simulated *in-situ* condition (microcosm):

For this study, fresh sediment and near bottom water were collected from CSt-26 during the pre-monsoon season in April 09 (Table 2.2). Sediment samples were collected with the help of a Van-veen grab and a Niskin water sampler (5 L) was used to collect water samples. The samples were brought to the lab on ice and five different microcosms were set-up as described below.

For all the microcosms, sediment slurry was prepared with 15 - 20 g of sediment and ~ 100 ml of near bottom water. The slurry was added to

100 ml serum bottles and flushed with N_2 gas to remove the dissolved oxygen present in the sediment slurry. These bottles were closed with a butyl rubber stopper and sealed immediately with an aluminum crimp.

Table 4.2 List of microcosm set-ups studied

Microcosm	components	Purpose of the set-up
Mic-1a	Plain sediment slurry	To study the total microbial activity
Mic-2a	Sediment slurry with anti-bacterial agents	To study the fungal activity by suppressing the bacterial growth
Mic-3a	Sediment slurry with anti-fungal agents	To study the bacterial activity by suppressing the fungal growth
Mic-4a	Sediment slurry with culture suspension of # An-2	To study the activity of fungal culture # An-2 in the *in-situ* condition
Mic-5a	Sediment slurry with culture suspension of # MT-811	To study the activity of fungal culture # MT-811 in the *in-situ* condition

Note: Similar microcosms Mic-1b to Mic-5b were set-up with nitrate supplement.

In the first microcosm (Mic-1a) plain sediment was incubated in anaerobic condition without any supplementation. To study the activity of fungi, we fortified the sediment slurry with streptomycin (0.1 g in 100 ml medium) and penicillin (40,000 Units in 100 ml medium) to inhibit bacterial growth in microcosm-2 (Mic-2a). Likewise, the fungal activity was specifically suppressed with an anti-fungal compound Bavistin to a final concentration of 0.5% in microcosm-3 (Mic-3a). Bavistin is a commonly used benzimidazole fungicide containing methyl benzimidazol-2-yl carbamate (MBC). The concentration of the anti-bacterial and anti-fungal agent used was standardized by growing three isolates of bacteria and fungi in varying concentrations of both the inhibitors. The concentration

that did not show cross-inhibition (ie. fungicide not inhibiting bacteria and vice versa) were used in the study. In microcosm-4 and 5 (Mic-4a & Mic-5a) culture suspension with approximately 30 mg (dry weight) of two cultures # An-2 and # MT-811 was added to the sediment slurry respectively (Table 4.2).

As the initial nutrient concentration was very low in the fresh sediment collected, a set of experiments with nitrate (Sodium nitrate) supplementation to a final concentration of 10 mM was also carried out. The five microcosms with nitrate (Sodium nitrate) supplementation are Microcosm-1b to 5b (Mic-1b to Mic-5b). The experimental bottles were incubated for 7 days under controlled conditions at 30°C with mild agitation to prevent the slurry from settling to the bottom.

Concentrations of nitrate, nitrite, DO levels and Eh of the sediment in the slurry were determined at the start and at the end of the experiment. The DO levels were determined by spectrophotometric methods (Pai *et al.* 1993). Eh was recorded in mV using pH analyzer (PHAN, LabIndia, Thane, India). Nitrate in the sea water used in the experimental set-up was estimated by quantitatively reducing it to nitrite by passing the sample through the column containing cadmium fillings coated with metallic copper. The nitrate reduced to nitrite was estimated by measuring the azo dye formed and any nitrite present in the medium was corrected for (Strickland & Parsons 1968). Nitrite present in the sea water was estimated as described in section 4.2.1.

At the end of the experiment the incubated sediment was freeze dried in clean plastic containers. ETS activity in the freeze dried sediment was determined as mentioned in section 4.2.2 with slight modifications. Approximately 600 mg of freeze dried sediment was homogenized in 6 ml of 0.05 M phosphate buffer (pH 7.7) and incubated for 60 minutes at room temperature and all the experiments were carried out in triplicates.

4.3 Results

4.3.1 Screening of a few fungal cultures for their nitrate utilization capacity under aerobic and anaerobic conditions

The cultures # An-2, isolated after anaerobic incubation of sediments obtained from CSt-26 during the anoxic season in Oct 05 (Ft-1), # 11 isolated from CSt-26, during the anoxic season in Oct 05 (Ft-1). # 31 and # 31a isolated from CSt-26 during the oxic season in Jan 06 (Ft-3) were screened for their ability to reduce nitrate. The culture # MT-811 *Fusarium oxysporum*, a terrestrial isolate studied extensively for its denitrification abilities was included as a positive control (Table 4.1).

These cultures were grown in an inorganic medium supplemented with sodium nitrate to a final concentration of 10 mM. The cultures grew under aerobic and anaerobic conditions; their biomass was estimated after 10 days of incubation. Two cultures, # An-2 and # 11 showed equally good growth under aerobic and anaerobic conditions, the cultures # 31 and # 31a showed seven and fivefold less growth respectively under anaerobic condition and # MT-811 showed a tenfold decrease in biomass (Table 4.3).

Table 4.3 Fungal biomass mg (dry weight) 20 ml^{-1} under aerobic and anaerobic culture conditions.

Isolate number	Biomass under aerobic culture condition	Biomass under ànaerobic culture condition
# An-2	86.7	64.0
# 11	60.2	61.0
# 31	70.6	11.0
# 31(a)	250.3	46.0
# MT-811	130.2	29.3

Nitrate utilization by the cultures was studied based on their ability to accumulate nitrite by reducing the available nitrate. During denitrification process nitrate is reduced into nitrite and further reduced to produce gaseous products. To screen the cultures isolated from the marine habitats for their denitrification ability we estimated the nitrite accumulation under varying oxygen concentrations for 10 days. The initial dissolved oxygen DO level in the flasks was 115.2 µM signifying an oxic condition and as the cultures grew DO was utilized and suboxic conditions developed (18 - 97 µM) on day 4 or 6 depending on the increase in biomass. The DO was estimated every alternate day till the end of the incubation on the 10th day. The initial DO recorded in the anaerobic bottles was 9.5 µM on the first day and the amount of DO on the 10th day was almost undetectable in both the aerobic flasks and anaerobic bottles.

Fungal denitrification differs from bacterial denitrification because the former requires suboxic condition, whereas the latter takes places largely under strict anoxic conditions. Nitrite formation in fungi due to denitrification takes place during suboxic condition and they require about 690 and 250 µM O_2 h^{-1} g^{-1} dry cell weight for reduction of nitrate and nitrite respectively (Zhou *et al.*, 2001). Accordingly nitrite accumulation could be recorded intermittently in cultures grown in flasks. As the conditions became suboxic the cultures started to utilize nitrate for respiration and nitrite accumulation was seen in the cultures maintained under aerobic conditions. The cultures widely varied in their nitrite accumulation capacity and in the time taken to produce nitrite (Fig.4.2). About 400 nM of nitrite was produced on the 8th day by #An-2 under aerobic condition and almost a similar amount of nitrite in the anaerobic condition. The culture # 11 produced a maximum of 605 nM on the 4th day and maximum nitrite formation in anaerobic condition was 356.17 nM. Among all the cultures, # 31 produced the maximum nitrite of about 904 nM on the 6th day and only

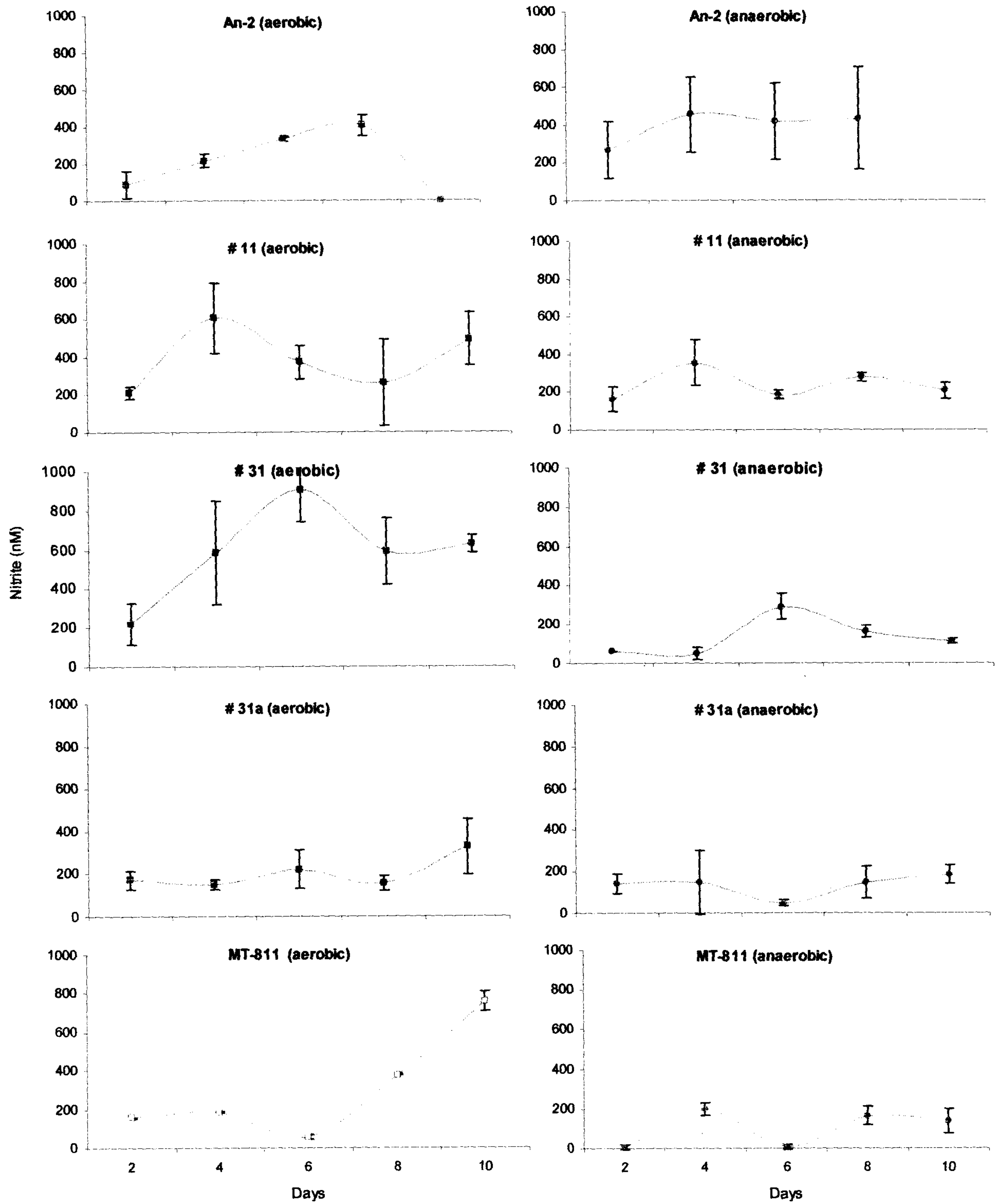


Fig. 4.2. Estimation of nitrite in the culture supernatant of fungi screened for their denitrification ability.

Table 4.4 Two factor Analysis of variance (ANOVA) to show the significance of nitrite accumulation by different cultures on different days

Variables	Df	F value	F critical value	P value
Different days				
(aerobic)	4	1.4	3	0.27
(anaerobic)	4	1.3	3	0.31
Different cultures				
(aerobic)	4	1.7	3	0.19
(anaerobic)	4	**3.3**	3	**0.04***

Df= degrees of freedom, Df=4, n=5 (five sampling days or five cultures);
F -critical value indicates statistical significance, * significant at 5 % level

Table 4.5 Two factor Analysis of variance (ANOVA) to show the significance of nitrite accumulation by different cultures in aerobic vs anaerobic conditions

Isolate number	Df	F value	F critical value	P value
# An-2	9	0.9	5.3	0.37
# 11	9	3.4	5.3	0.1
# 31	9	**14.6**	5.3	**0.005****
# 31(a)	9	3	5.3	0.12
# MT-811	9	2.7	5.3	0.14

Df= degrees of freedom, Df=9, n=10 (five sampling of each culture in aerobic and anaerobic conditions); F value greater than F-critical value indicates statistical significance, ** significant at 1 %

about 292 nM was produced under anaerobic condition. Isolate # 31a produced a maximum of 327 nM of nitrite on the 10th day in aerobic condition and under anaerobic condition it was about 185 nM. Statistical analysis showed significant variation between cultures grown under anaerobic conditions (Table 4.4). Maximum nitrite accumulation was seen in # 31 and the nitrite production varied with a high significance (P= 0.005) between aerobic and anaerobic conditions (Table 4.5).

Fungi under complete anoxic condition are known to perform an ammonia fermentation process (Zhou *et al.*, 2002). To study this process

Table 4.6 Two factor Analysis of variance (ANOVA) to show the significance of ammonia accumulation by different cultures on different days

Variables	Df	F value	F critical value	P value
Different days				
(aerobic)	4	0.8	3	0.53
(anaerobic)	4	1.0	3	0.42
Different cultures				
(aerobic)	4	1.8	3	0.17
(anaerobic)	4	**11.5**	3	**0.00014*** **

Df= degrees of freedom, Df=4, n=5 (five sampling days or five cultures); F value greater than F- critical value indicates statistical significance, *** significant at 0.1 %

Table 4.7 Two factor Analysis of variance (ANOVA) to show the significance of ammonia accumulation by different cultures in aerobic vs anaerobic conditions

Isolate number	Df	F value	F critical value	P value
# An-2	9	**37**	5.3	**0.0003*** **
# 11	9	1.4	5.3	0.3
# 31	9	0.9	5.3	0.4
# 31(a)	9	0.1	5.3	0.7
# MT-811	9	3.5	5.3	0.09

Df= degrees of freedom, Df=9, n=10 (five sampling of each culture in aerobic and anaerobic conditions); F value greater than F-critical value indicates statistical significance, *** significant at 0.1 %

in the fungal cultures, ammonia accumulation in the culture supernatant maintained under aerobic and anaerobic condition was also determined. The four cultures studied and the positive control # MT-811 showed varying degree of ammonia formation during the incubation (Fig. 4.3). # An-2 produced about 26 µM of ammonia under anaerobic condition and only 6 µM under aerobic condition. The cultures # 11 and # 31 produced almost similar levels of ammonia in both the conditions, which were about 6 and 9 µM respectively. Ammonia formation in the positive control MT-

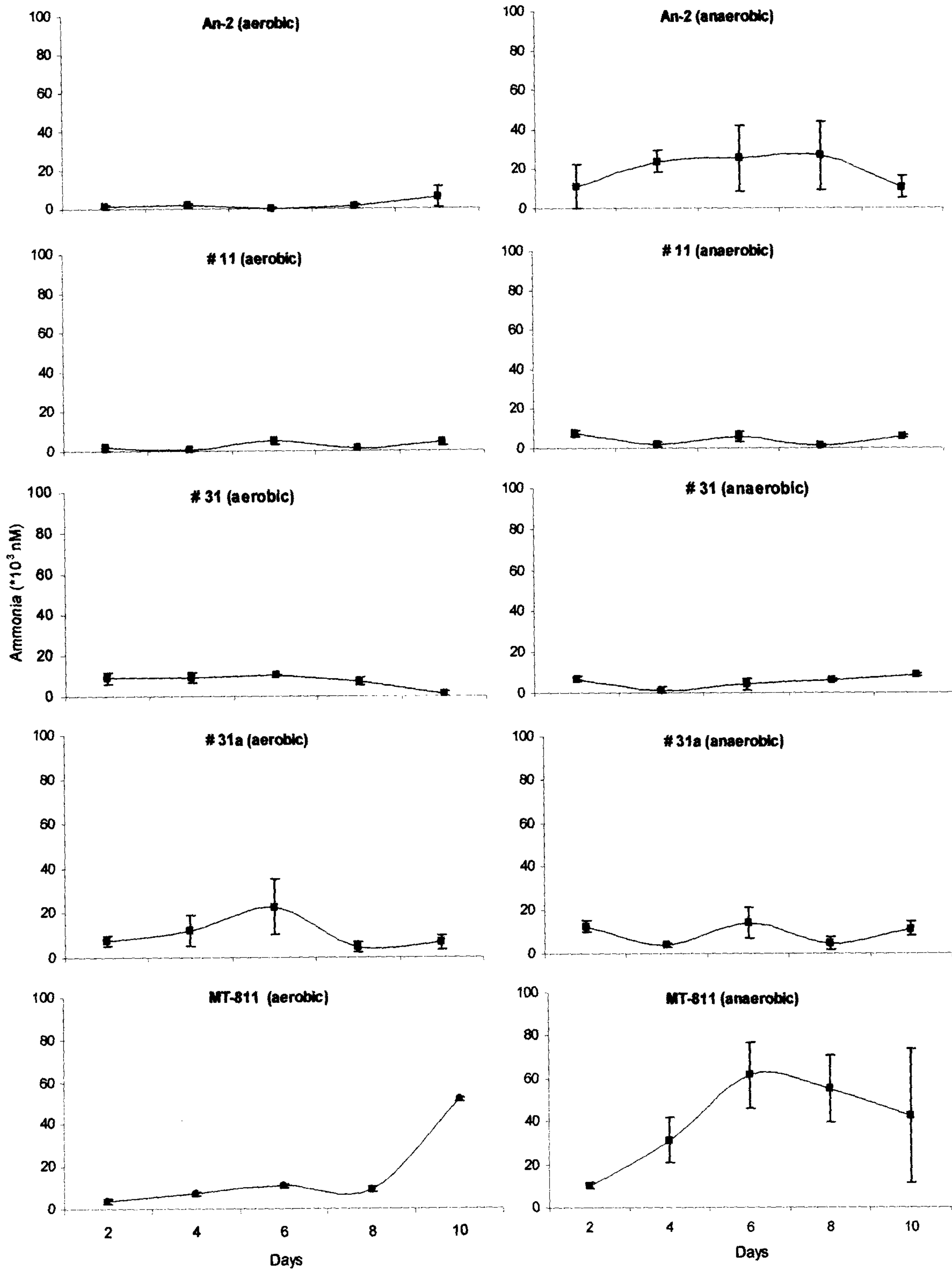


Fig. 4.3. Estimation of ammonia in the culture supernatant of fungi screened for their denitrification ability.

811 was about 50 and 51 μM under anaerobic and aerobic condition. Isolate # 31a produced a maximum of 22 μM under aerobic condition but was able to produce only 14 μM in anaerobic condition. A 2-way analysis of variance indicated significant difference between cultures in their capacity to accumulate ammonia (Table 4.6). Ammonia accumulation between aerobic and anaerobic cultures was significantly different in the culture # An-2 (Table 4.7).

4.3.2 Determination of Electron transport system (ETS) enzyme activity in fungal cultures grown under aerobic and anaerobic conditions

Electron transport system (ETS) activity was measured as an estimate of the respiratory activity under aerobic and anaerobic conditions. The ETS activity measured in the anaerobic condition show that the cultures were viable and active under anaerobic denitrifying conditions (Fig 4.4). The ETS activity measured in the aerobic cultures reached a maximum of about 719 neq h^{-1} μg^{-1} protein and in the anaerobic condition it reached a maximum of about 350 neq h^{-1} μg^{-1} protein only. ETS activity varied widely in the aerobic and anaerobic conditions in the cultures screened (Table 4.8) with a high significance. The cultures # MT-811, # An-2 and # 31 were significantly different in their ETS activity in the aerobic and anaerobic conditions (Table 4.9). On the other hand # 11 and 31a did not show any significant difference and the activity was uniform under both the conditions.

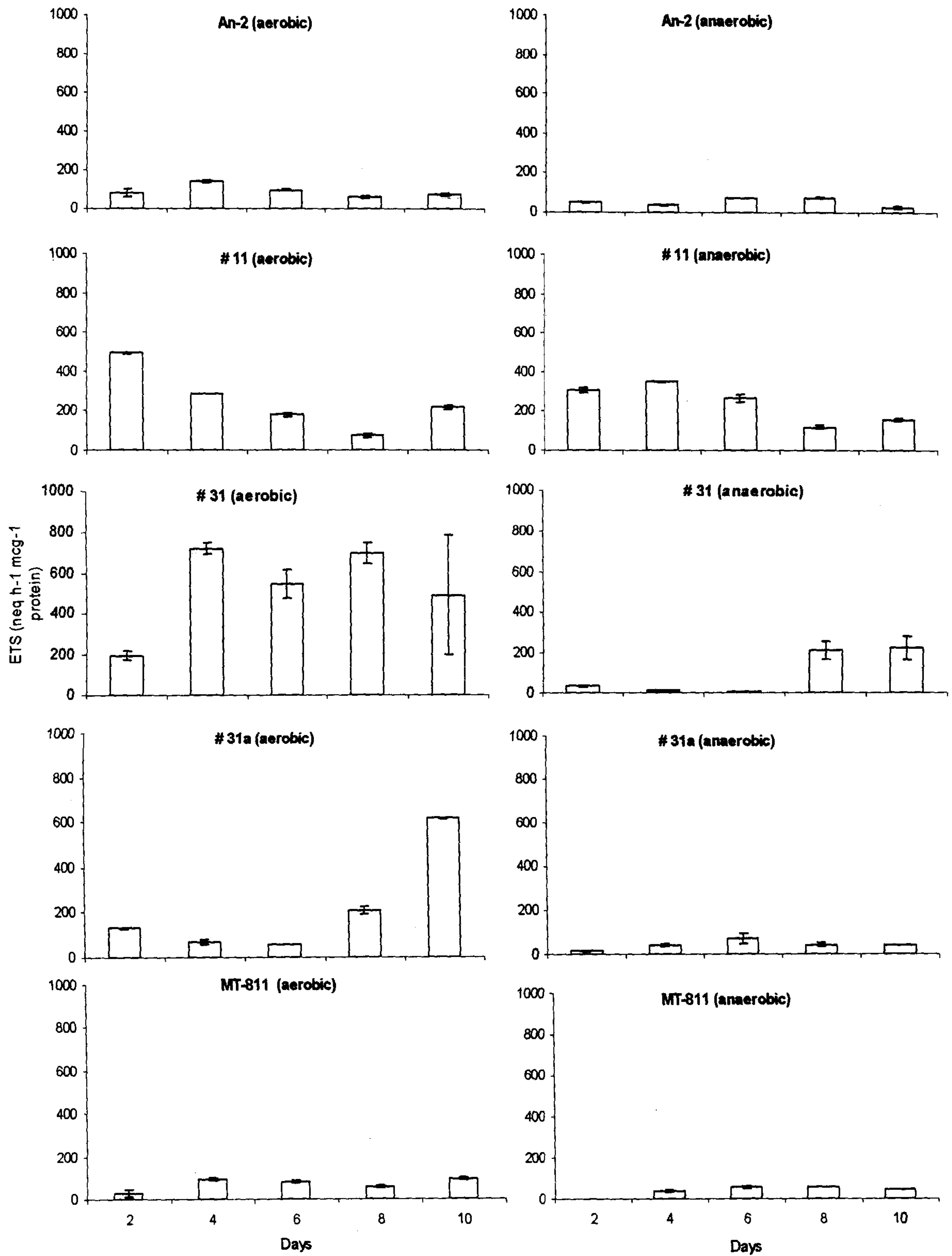


Fig. 4.4. Estimation of electron transport system (ETS) activity in cultures screened for their denitrification ability.

Table 4.8 Two factor Analysis of variance (ANOVA) to show the significance of ETS activity by different cultures on different days

Variables	Df	F value	F critical value	P value
Different days				
(aerobic)	4	0.4	3	0.8
(anaerobic)	4	0.05	3	0.99
Different cultures				
(aerobic)	4	**6.0**	3	**0.003****
(anaerobic)	4	**6.2**	3	**0.003****

Df= degrees of freedom, Df=4, n=5 (five sampling days or five cultures); F value greater than F- critical value indicates statistical significance, ** significant at 1 %

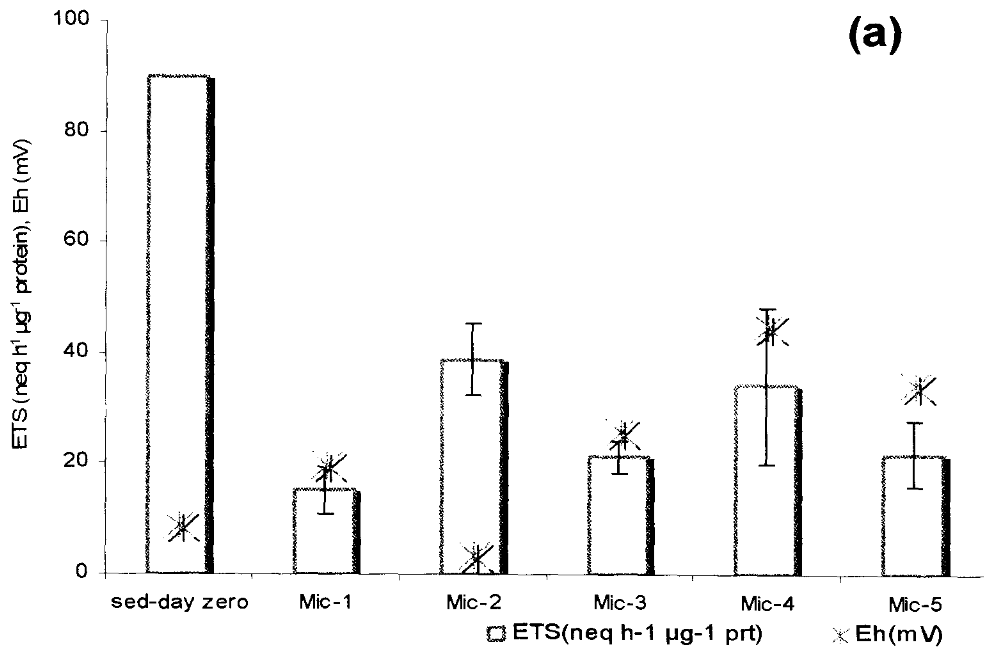
Table 4.9 Two factor Analysis of variance (ANOVA) to show the significance of ETS activity by different cultures in aerobic vs anaerobic conditions

Isolate number	Df	F value	F critical value	P value
# An-2	9	**4.9**	5.3	**0.05***
# 11	9	0.01	5.3	0.9
# 31	9	**16.5**	5.3	**0.004****
# 31(a)	9	2.8	5.3	0.13
# MT-811	9	**4.5**	5.3	**0.07***

Df= degrees of freedom, Df=9, n=10 (five sampling of each culture in aerobic and anaerobic conditions); F value greater than F-critical value indicates statistical significance, ** significant at 1 % * significant at 5 %

4.3.3 Determination of Electron transport system (ETS) enzyme activity under simulated *in-situ* condition (microcosm)

The sediment samples were collected during the suboxic condition during the pre-monsoon season and the DO level in the near bottom water was 112 μM. The Eh of the sediment was -9 mV, the nitrate and nitrite value was less than 1 μM and ETS activity on day zero was about 90 neq h^{-1} μg^{-1} protein. The sediment was incubated in anaerobic microcosms as described (in Table 4.2) for 7 days. At the end of the incubation period the DO level was 3.81 μM, and the Eh recorded was -19.4 mV in Mic-1a. In a



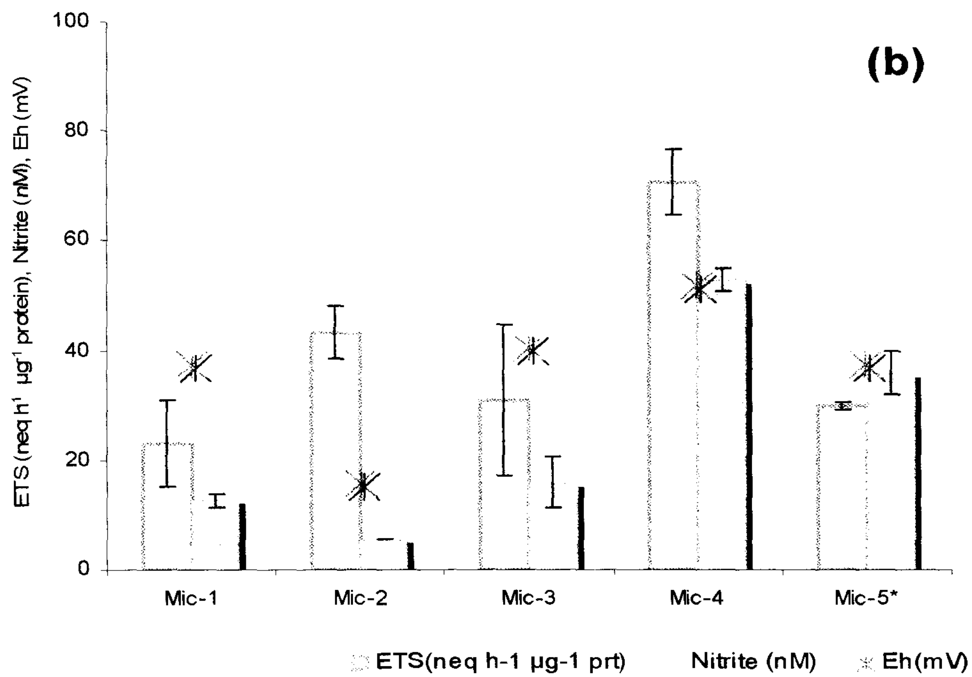


Fig. 4.5 ETS activity and nitrite formation in the (a) anaerobic microcosms without nitrate supplement and (b) with nitrate supplement (Table 4.2). indicates the nitrite concentration was 10 times higher than shown in the graph.

similar set-up which was supplemented with antibiotics and anti-fungal compounds (Mic-2a and 3a), the Eh value was -2.5 mV and -25.8 mV respectively. In the microcosm set-up with addition of fungal inoculums (Mic-4a & 5a) the ETS activity was 34. 3 and 21.9 neq h^{-1} μg^{-1} protein respectively. Nitrate or nitrite could not be detected on the initial day and in the experimental set-ups which was not supplemented with ntirate. Because the initial nitrate and nitrite levels were very low, sediments supplemented with 10 mM of sodium nitrate were also set-up (Mic-1b to 5b).

Nitrite accumulation could be seen in all the microcosm set-ups with nitrate supplement (Mic-1b to 5b) and ETS activity proportionately increased with the nitrate supplementation (Fig. 4.5). Increased activity was detected in the microcosm with external fungal inoculation and the ETS activity in Mic-4b and 5b was 70.9 and 29.8 neq h^{-1} μg^{-1} protein respectively. Nitrite accumulation recorded in the microcosms with nitrate supplementation was about 53 µM and 358 µM in Mic-4b and 5b respectively (Fig 4.5).

4.4 Discussion

4.4.1 Screening of a few fungal cultures for their nitrate utilization capacity and ETS activity under aerobic and anaerobic conditions

Besides bacteria, fungi are the major agents of decomposition and nutrient cycling in the ecosystem. Role of fungi in the marine ecosystem has been increasingly recognized over the past 50 years since the initial studies by Barghoorn and Linder (1944). Studies on fungi in the marine ecosystem are largely based on the isolation of fungi from various marine substrata

(Maria & Sridhar, 2002) and by studying the quantitative fungal biomass distribution from various habitats (Damare *et al.*, 2006). The role of fungi in the natural ecosystem was largely based on the various studies on the extracellular and intracellular enzymes produced by the fungal isolates. The physiology of fungi and their activity in the marine ecosystem has been studied based on their growth and enzyme production (Molitoris & Schaumann, 1986). The ability of fungal cultures to utilize nitrate in the aerobic conditions has been studied based on the the nitrate reductase activity (Bresinsky & Schnieder, 1975). A number of terrestrial isolates have also been tested for their ability to denitrify in anaerobic conditions (Shoun *et al.*, 1992). Screening of various isolates and studying their capacity under various oxygen concentrations has shown the ability of fungi to respond variously to the oxygen concentrations.

Here we have tested the ability of four cultures isolated from the anoxic coastal marine sediments for their growth under anaerobic conditions. While all the cultures grew much better under aerobic conditions, one culture (#11) produced almost the same amount of biomass under both aerobic and anaerobic conditions (see Table 4.2). These results indicate that fungi isolated from the anoxic sediments might have adapted to a facultative anaerobic mode of life. Shoun and Tanimoto (1991) have shown that # MT-811 can grow under anaerobic conditions and utilize nitrate and nitrite for dissimilatory purpose. They have also shown that there is a substantial amount of cell growth during this process, which shows that the dissimilatory nitrate or nitrite reduction is an energy yielding reaction.

Fungal denitrification process differs significantly from classical bacterial denitrification. Bacterial denitrification takes place only in the complete absence of oxygen and even a trace of oxygen could be toxic to the obligate anaerobes and inhibit denitrification. In fungi, reduction of

nitrate takes place under suboxic conditions, but excess oxygen (> 900 µM O_2) is shown to inhibit the process (Zhou *et al.* 2001). My study on the denitrifying ability of the cultures under varying oxygen concentrations has shown that all the cultures were able to grow and reduce nitrate to nitrite. The isolate # 31 showed the maximum nitrite accumulation on day 6 when suboxic conditions set in (Fig. 4.2). The cultures showed marked difference in their ability to reduce nitrate under the denitrifying conditions. The ability of the cultures was however much less in comparison with the positive control culture # MT-811. This may be due to the inability of the cultures to utilize nitrate, as all fungi are not capable of nitrate reduction, but can use nitrite more efficiently as an electron acceptor (Takaya, 2002). Hence experiments to screen isolates for their nitrite reducing capacity are to be carried out. Further fungi are also known to require supplements to aid their growth under anaerobic conditions (Bartnicki-Garcia & Nickerson, 1961). There was no organic nitrogen source in the medium during the present study, which may be the one of the reasons for the low efficiency of the cultures to utilize nitrate.

Fungi also follow another pathway to reduce nitrate under complete anoxic conditions, which is referred to as ammonia fermentation. This process in fungi appears to be widespread as 15 of 17 fungi tested by Zhou *et al.* (2002) showed ammonia formation under anaerobic condition. This process was studied in the same four cultures under both aerobic and anaerobic conditions. There was ammonia formation by all the cultures under anaerobic conditions and # An-2 produced the maximum ammonia under anaerobic culture condition (Fig 4.3). The fungal cultures screened varied widely in their capability to produce ammonia under anaerobic condition (Table 4.5). Ammonia formation in An-2 was distinctly different between aerobic and anaerobic condition (Table 4.6). The cultures were isolated from the seasonal anoxic zone from the coastal

waters, their ability to utilize nitrate and grow under suboxic and anoxic condition shows that the fungal cultures are adapted to a facultative mode of denitrification activity with varying oxygen concentration. Studies on the denitrifying activities of *Fusarium oxysporum* # MT-811 have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O_2 tension (Takaya, 2002). Fungi show a multimodal type of respiration to rapidly adapt to changes in the oxygen supply, in anoxic conditions ammonia formation takes place, while denitrification process in suboxic and oxygen respiration under aerobic conditions (Takaya, 2002). This may be a survival strategy for mycelial fungi to thrive in extreme and dynamic environments.

Electron transport system (ETS) activity is a measure of the respiratory activity and is used as a measure of the denitrification rates under anoxic condition in the presence of nitrate as final electron acceptor. The ETS was active in the fungi under aerobic and anaerobic conditions and the potency of the cultures highly varied. Measuring the ETS activity directly shows that the respiratory system was active in fungal cultures in the anaerobic conditions also. The accumulation of nitrite, ammonia and the active ETS in the anerobic condition shows that the fungal cultures utilizes the alternate respiratory process or denitrification in the absence of oxygen.

4.4.2 Determination of Electron transport system (ETS) enzyme activity under simulated *in-situ* condition (microcosm)

Initial ETS activity in the natural sediment sample collected during suboxic condition in April 09 was about 90 neq h^{-1} μg^{-1} protein and the activity reduced approximately four times at the end of the incubation period in Mic-1a on the 7th day (Fig 4.5a). When the bacterial growth was

suppressed the ETS activity was higher in comparison to the plain sediment anaerobic microcosm. Microcosm study shows that the ETS activity due to fungi was higher in comparison to the bacterial activity (Fig 4.5a). Though ETS activity could be recorded, the nitrite formation was not seen, this may be due to the unavailability of nitrate in the natural condition. Nitrate or nitrite levels were undetectable on the day zero (data not shown).

Microcosms with nitrate supplementation (Mic-1b to Mic-5b) were set-up to study the denitrifying ability of the in-situ microbial population. Nitrite formation was recorded in all these microcosms and the ETS activity also proportionally increased with nitrate supplementation (Fig 4.5b). In natural system the fungal biomass C observed was 3 orders of magnitude lesser in comparison with the bacterial biomass C (Fig. 2.1). Enumeration of fungi from the forest soil capable of anaerobic growth accounts for only 0.01 to 0.1% in conifer and deciduous forest soil (Reith *et al.*, 2002). Though the abundance of fungal biomass in the leaf litter has shown to be high in comparison with the bacterial biomass, the denitrifying bacterial community may be dominating the natural oxygen depleted environment. A mix culturing of *Fusarium oxysporum* and *Pseudomonas stutzeri* in anaerobic condition was shown to be capable of faster nitrate and nitrite removal, than individual cultures (Liu *et al.*, 2006). Moreover the N_2O production recorded also reduced from the mix-culture because *P stutzeri* were able to utilize the N_2O produced by *F oxysporum* This study is a preliminary attempt to screen a few marine fungal isolates and estimate the role of fungi in marine denitrification process. Detailed studies using labeled nitrate supplement and estimation of gaseous denitrifying products can improve our understanding of the fungal role in the oxygen depleted environments.

Chapter 5

Summary

5.1 Summary

Denitrification is a microbial process, where nitrate or nitrite is reduced under anaerobic condition to gaseous nitrogen or its intermediary products such as nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). Bacteria were considered to be the major players in this pathway, but recently the involvement of micro-eukaryotes such as fungi and foraminiferans are also reported (Takaya *et al.*, 2002; Risgaard-Peterson *et al.*, 2006). This is an important pathway in the marine nitrogen cycle as the fixed nitrogen is lost leading to an imbalance in the global nitrogen budget (Codispoti *et al.*, 2003). NO and N_2O produced as intermediates are among the harmful green house gases that influence the earth's climate by the destruction of the ozone in the stratosphere. Denitrification process takes place in enclosed, stagnant water bodies, open ocean oxygen depleted zones and in the coastal waters affected by eutrophication.

Denitrification activity in the Arabian Sea has been reported from the perennial open ocean oxygen minimum zone (OMZ) that contributes to near 50% of the world's oxygen depleted environment and from the coastal regions along the western continental shelf of India (Naqvi et al., 2000). Biological diversity, especially macro organisms are greatly affected by depleting oxygen levels (Levin 2003), but a vast diversity of prokaryotes and micro-eukaryotes have been reported in OMZs from across the globe (Zhou *et al.*, 1997; Giovannoni & Rappé 2000; Dawson & Pace, 2002; Stoeck *et al.*, 2003; Takishita *et al.*, 2005; 2007). Some of the groups described based on environmental DNA analysis are presumably of high relevance for a variety of research disciplines like ecology, evolution, physiology and biogeochemistry (Epstein & Lopez-Garcia, 2007; Lopez-Garcia & Moreira 2008). But there is a lack of knowledge on the role of the specific groups, like fungi present in oxygen-depleted

environments. In this study I have attempted to quantify the abundance of fungi (Chapter-2), estimate the culturable and unculturable diversity (Chapter-3) of fungi and measure the growth and activity of a few selected fungi and in simulated oxygen depleted conditions (microcosm) (Chapter-4).

Abundance of fungi in the sediments of the denitrification zones of the Arabian Sea was estimated from three coastal (CSt stations) and five slope stations (SSt stations) within the OMZ region (Table 2.1).

The CSt stations were located within the coastal, seasonal anoxic region off Goa (Fig 2.). This condition is seen to occur along the coastal waters on the western Indian shelf upto 200 m depth, during June to December, with the maximum intensity during the post-monsoon season in September and October (Naqvi *et al.*, 2000). Periodic sampling was carried out to study the changes in the fungal abundance with the changing dissolved oxygen (DO) levels. Sampling was also carried out in the open ocean, perennial oxygen minimum zone to study the fungal abundance in this region. Fungal mycelia were detected in the sediments by staining with calcoflour, an optical brightener that enhances fluorescence of cellulose and chitin, the latter being signature of the fungal cell wall (Mueller & Sengbusch, 1983). Such staining revealed the presence of hyphae in the sediments, confirming active growth of fungi therein. Occasionally fungal spores were also detected.

Fungal colony forming units (CFUs), fungal biomass C, bacterial biomass C and organic carbon (OC) % was estimated in the sediments collected from the different sampling sites. Statistical analyses of these parameters show that there is a difference in the biological parameters between seasons (Table 2.4). A negative correlation (Table 2.5a) between DO levels and bacterial biomass C in the CSt was observed. This may be due to the ability of bacteria which can adapt to changing DO levels. But in

the open ocean OMZ region there is no evident effect of DO on the microbial abundance or on the fungal CFUs between the different sampling stations (Table 2.5b). The condition in the open ocean OMZ is a perennial condition in comparison to the seasonal anoxic condition. Hence the microbes thriving in this condition could have adapted to the low oxygen levels and a mutual balance between the fungal and bacterial population adapted to this environment is a possibility.

Fungal CFUs are not affected by changing DO levels in both the coastal, seasonal and perennial, oceanic station. This shows that fungal cultures though are affected by the changing DO levels are able to thrive and play a role in the denitrifying environments, in contrast to the existing reports which show that fungi have no ecological role in the oxygen depleted environments (Dighton, 2003; Mansfield & Barlocher, 1993). In this study I have for the first time quantified the fungal biomass C and have shown that fungi thrive in oxygen depleted environments (Jebaraj & Raghukumar, 2009).

Diversity of fungal cultures was studied from the CSt, SSt and LSt stations (Table 2.1) using cultivation based and cultivation-independent approach. Shannon's diversity index and Simpson's diversity index shows that the diversity was less compared to the mangrove ecosystem and deep sea sediments (Table 3.3 & 3.4). The cultures identified based on the 18S rDNA sequence could be assigned to Basidiomycetes and Ascomycetes (Table 3.5). Eleven isolates belonged to Basidiomycetes, predominantly Pucciniomycotina; twenty-one of them belonged to Ascomycetes, a majority of it belonged to Eurotiomycetes. The 18S rDNA sequence analysis of the isolates shows that two of the cultures isolated # 11 and # 87 are very divergent from previously described and sequenced fungi (Table 3.5).

Uncultured fungal diversity was studied from the five clone libraries constructed from a CSt, SSt and LSt station (Table 3.1). UPGMA analysis of the fungal communities obtained from each of the libraries shows that are no difference in the community of fungi between the anoxic and oxic seasons (Fig. 3.3). This may be due to the physiological adaptation of the fungal community to the changing oxygen concentrations. But there was a marked spatial difference between the CSt, SSt and LSt station (Fig. 3.3).

Phylogenetic analysis of the environmental sequences obtained from this study shows that the phylotypes can be categorized to atleast five unique clusters of environmental sequences. These clusters have sequences obtained from this study locations and groups with sequences obtained from similar anoxic and extreme environments. Of the five unique environmental clusters identified, one cluster with six phylotypes from this study groups within the Eurotiomycetes of Ascomycota (Fig.3.4). This cluster has sequences obtained from the anoxic Mediterranean basin, anoxic Meromictic Lake sediment and marine anoxic Bolinas Lagoon DNA and a few common culturable forms such as *Penicillium* and *Aspergillus* sp. Three clusters were seen within the basidiomycota belonging to Cystobasidiomycetes, Exobasidiomycetes, and Ustilagomycetes (Fig.3.5). The cluster within the Cystobasidiomycetes groups with environmental sequences of uncultured clones from anoxic Gotland Deep of Baltic Sea. The second environmental cluster within the basidiomycota grouped along with environmental sequences closely related to Exobasidiomycetes. The sequences clustered with phylotypes obtained from anoxic Norwegian fjords, anoxic Mediterranean basin, anoxic Gotland Deep of Baltic Sea and a fungal clone obtained from detritus community. The only known named species was *Exobasidium gracile* which branched out separately from this environmental cluster which is supported with a high bootstrap support. The third cluster belonging to Ustilagomycotina is within a clade

that has been recognized as "hydrothermal and/or anaerobic fungal group" (Lopez *et al.*, 2007). This cluster has representatives from mud volcanoes, hydrothermal ecosystems, anoxic Mediterranean basin and anoxic Berkeley Aquatic Park sediment, deep oceans and a leaf epiphyte (Fig. 3.5). One phylotype belonging to zygomycete was obtained from this study and this OTU grouped with CCW35 from the anoxic Cariaco Basin (Fig. 3.6). These unique clusters of uncultured environmental sequences show that there is a vast diversity of fungi which are yet to be isolated and studied.

The ability of four cultures (# An-2, # 11, # 31, # 31(a)) isolated from the anoxic coastal marine sediments was studied for their growth under anaerobic conditions. While all the cultures grew much better under aerobic conditions, one culture (# 11) produced almost the same amount of biomass under both aerobic and anaerobic conditions (Table 4.2). The denitrification ability of the cultures was studied by growing them in medium containing nitrate and estimating the nitrite and ammonia accumulation in the culture supernatant (Fig 4.2 & 4.3). Their activity in anaerobic condition was shown by studying the ETS activity in the presence of the nitrate in the medium (Fig. 4.4). The cultures screened varied widely in their denitrification ability (Table 4.3, 4.5 & 4.6). The activity of fungi under simulated *in-situ* condition (microcosm) was studied based on their Electron transport system (ETS) enzyme activity (Fig. 4.5). The screening studies were a preliminary attempt to study the denitrification activity of marine fungal isolates. Detailed analysis of all the denitrification products and their enzymes or the genes coding of the enzymes could give us better insights in to the contribution by fungi to the marine denitrification process.

5.2 Conclusions

Major inferences that could be drawn from this study are:

- The microbial abundance as fungal CFUs, fungal biomass C and bacterial biomass C were quantified from the oxygen depleted sediments, collected from the denitrification zones of the Arabian Sea (Jebaraj & Raghukumar, 2009).
- Fungal CFUs, fungal biomass C and bacterial biomass C significantly differ between seasons in the coastal station sediments, showing that the changing DO levels has an impact on the total microbial abundance.
- The fungal CFU numbers, fungal biomass C and total organic carbon estimated were significantly different between the coastal and open ocean sampling stations.
- Culturable fungi could be isolated from all the sampling locations irrespective of the DO levels, but the diversity index shows that the overall diversity was low in comparison with the mangrove or deep sea regions. The indices from the slope stations within the open ocean OMZ region is also lower in comparison with the coastal station.
- Fungal cultures isolated belonged to the two major phylums Ascomycota or Basidiomycota. Analysis based on the 18S rDNA sequence shows that two of the cultures (# 11 and # 87) were very divergent from any of the cultures described so far.
- Phylogenetic analysis of the environmental fungal phylotypes groups many of the uncultured sequences obtained from this study into clusters with environmental sequences obtained from similar anoxic regions from across the globe (Jebaraj CS, Raghukumar C, Behnke A, Stoeck T, MS in review process. Targeted environmental sequencing

combined with cultivation reveals high fungal diversity in oxygen-depleted regions of the Arabian Sea).

- Screening of a few fungal cultures isolated from the denitrification zones shows that they are able to grow under anaerobic condition. Their ability to denitrify was also demonstrated under suboxic and anoxic conditions. The contribution by fungi in simulated *in-situ* conditions (microcosms) shows that fungi could be playing an important role as denitrifiers in the oxygen depleted regions.

Though the abundance and diversity of fungi has been detailed from this study, the actual contribution of fungi to the denitrification activity in the marine ecosystem is yet to be estimated. Some of the studies that are being carried out as a continuation of the present study are as follows:

a) Using *nor* gene sequence as a marker to study the fungal denitrification in cultures and in the environmental samples.
b) Description of the fungus # 11 using multi-gene phylogeny

a) Using *nor* gene sequence as a marker to study the fungal denitrification in cultures and in the environmental samples.

In fungal denitrification system nitric oxide (NO) is reduced to nitrous oxide (N_2O) by an enzyme cytochrome P450*nor*. The catalytic properties of nor in fungal system is very distinct in their physiological properties and differ significantly from the bacterial denitrification system (Takaya & Shoun, 2000). The cDNA of the $P\text{-}450_{dNIR}$ was sequenced by immunoscreening technique and it came to be the first soluable P-450 of eukaryote origin (Kizawa *et al.*, 1991). Deeper insight into the evolutionary and physiological aspects of P450nor came from studying the gene sequence. A cDNA sequence revealed that the polypeptide was about 403 amino

acid residues, corresponding to a molecular weight of 44,371 Daltons (Kizawa *et al.*, 1991). The full length sequence of the p450nor was designated p450norA and the isolated protein was shown to contain an additional threonine at the N-terminus, but a second isoform was also detected with threonine and the adjacent methionine lacking (Nakahara & Shoun, 1996). This P450norB contained the N-terminus blocked by an acetylated alanine, which usually is seen after post- or co-translational elimination of a methionine residue. This latter isoform was mainly found in the cytosol, whereas the longer form (P450norA) was located to the particulate and mainly mitochondrial fraction. It was assumed that P450norA was synthesized with a presequence of 26 amino acids that targets it to mitochondria. This localization of the two isoforms was shown in the fungus *Cylindrocarpon tonkinense*, but in this fungus two separate genes (*CYP55A2* and *CYP55A3*) were found to code for the mitochondrial and cytosolic enzymes, respectively (Kudo et al., 1996). A fourth gene of the P450nor family was isolated from the yeast *Trichosporon cutaneum,* a basidiomycete (*CYP55A4*) and this showed only 65% similarity to the other three genes (zhang et al., 2001). This gene has been isolated from *Aspergillus oryzae* (*CYP55A5*) and the sequence has striking difference with previously described *nor* gene sequences (Kaya *et al.*, 2004). The sequence similarity with the other sequences is only 60%, and the fungal whole genome project of many candidate isolates such as *Neurospora crassa* has increased the database of the fungal *nor* gene sequence available for study. The fungal whole genome project has added to the available database of the p450 *nor* gene sequence obtained from fungi.

In my study, I tried to design degenerate primers based on the sequence data available on the NCBI genebank deposits. The list of primers designed is (Table 5.1) based on the Clustal W alignment of the protein and DNA sequence and identifying a conserved region. As the

homology between the sequence is less than 60%, PCR amplification using the primers designed was very difficult. With the preliminary results obtained and increasing whole genome sequencing of the fungal cultures, refined primers are being designed. Designing of primers to amplify the *nor* gene from fungal cultures and environmental samples could prove to be a power tool for studying the fungal denitrification from various oxygen depleted environments.

Table 5.1 List of primers for the *nor* gene amplification

Primer [a]	Position[b] (nt)	Primer sequence (5'-3')
nor F1	307- 323	ACD TTY GTB GAY ATG GA
nor R1	769-789	CGA ATC ATG YTB ACS AYR GT
nor F2	559-575	GCS AYB CGV ASN AAY GG
nor R2	1087-1103	TCN GCR ATR CAD CKR TG

[a] forward and reverse primers are indicated by F and R as the last letter respectively,
[b] Positions correspond to the *nor* gene of *Trichosporon cutaneum* (gi:14456619;dbj|AB052733.1 CYP55A4 gene) and nt, nucleotide.

b) Description of the fungus # 11 using multi-gene phylogeny

The fungus # 11 was isolated from the CSt-26 station during the anoxic season (Table 3.). The 18S rDNA sequence analysis of this culture showed only a 92% similarity with the known sequences and the culture grouped closely to Pucciniomycotina of phylum Basidiomycota. Based on the morphological identification, this culture resembled an Ascomycete *Tritirachium* sp. The identification was also confirmed by experts from the Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherland. Hence to resolve the taxonomic position of this culture we are presently carrying out a multi-gene phylogeny. For this sequences of the (internal transcribed spacer) ITS region, the large subunit nuclear rDNA gene

sequence (nLSU), largest subunit (RPB1) and the second largest subunit (RPB2) of RNA polymerase II gene sequence and the elongation factor (EF-a) gene sequence was amplified and aligned with the sequences available in the NCBI database (Table 5.2). Results obtained from this study will not only give us the taxonomic position of this culture, but may also give many insights on fungal phylogeny. More detailed description of the morphological features based on SEM and TEM images and physiological analysis of the culture is also being carried out.

Table 5.2 List of primers used for the multi-gene phylogeny.

Primer	Sequence (5' to 3')	Gene amplified	Reference
ITS1	TCCGTAGGTGAACCTGCGG	ITS	Mitchell *et al.*, 1994
ITS4	TCCTCCGCTTATTGATATGC	ITS	Mitchell *et al.*, 1994
983F	GCYCCYGGHCAYCGTGAYTTYAT	EF1-a	Rehner & Buckley, 2005
2218R	ATGACACCRACRGCRACRGTYTG	EF1-a	Rehner & Buckley, 2005
RPB1-Ac	GARTGYCCDGGDCAYTTYGG	RPB1	Stiller & Hall, 1997
RPB1-Df	TACAATGCYGAYTTYGAYGG	RPB1	Stiller & Hall, 1997
RPB1-G2R	GTCATYTGDGTDGCDGGYTCDCC	RPB1	Stiller & Hall, 1997
fRPB2-5F	GAYGAYMGWGATCAYTTYGG	RPB2	Liu *et al.*, 1999
RPB2-11bR	CAATCWCGYTCCATYTCWCC	RPB2	Liu *et al.*, 1999
LR0R	GTACCCGCTGAACTTAAGC	LSU	Vilgalys *et al.*, 1990
LR7	TACTACCACCAAGATCT	LSU	Vilgalys *et al.*, 1990

5.3 Future directions

- To develop probes to specifically study the unique fungal clusters obtained from the environmental clone libraries.
- Improvise isolation technique to isolate fungal cultures from oxygen depleted environments in anaerobic condition with the use of anaerobic chamber.
- Screen an increased number of cultures obtained from the anoxic regions for their denitrification ability.
- Use mRNA approach to quantify the denitrification ability in the cultures.
- *In-situ* experiments using labeled nitrates should be carried out to precisely study the fungal contribution in the marine denitrification zones.
- Substrate induced respiratory inhibition (SIRIN) studies to quantify the role of fungi in marine denitrification zones.

APPENDIX

1. Inorganic mineral medium

KH_2PO_4	1.36 g
Glucose	30 g
Peptone	2 g
$MgSO_4$	0.2 g
Trace element solution	1 ml
$NaNO_3$	10 mM
Artificial Sea Water	1000 ml

2. Trace element solution

$FeSO_4 . 7H_2O$	0.2 g
$CoCl_2 . 6H_2O$	1.0 g
$CuSO_4 . 6H_2O$	0.38 g
$Na_2MoO_4 . 7H_2O$	8.6 mg
$CaCl_2$	0.2 g
De-ionized water	1000 ml

3. Artificial sea water (pH 7.5)

NaCl	24 g
KCl	0.7 g
$MgCl_2$	7 g
De-ionized water	1000 ml

4. Magnesium Chloride 3 M solution for dissolved oxygen estimation

$MgCl_2$	6 g
De-ionized water	100 ml

5. Sodium hydroxide solution for dissolved oxygen estimation

Sodium hydroxide pellets	3.2 g
Sodium Idodide	6 g
De-ionized water	100 ml

6. Sulphuric acid 10 N for dissolved oxygen estimation

7. Standard thiosulphate solution (0.01 N) for dissolved oxygen estimation

Sodium thiosulphate	2.9 g
Na_2CO_3	0.1 g
De-ionized water	1000 ml

8. Sulfanilamide (SFN) solution for nitrate and nitrite estimation

SFN	5 g
HCl (conc.)	50 ml
De-ionized water	500 ml

9. N- (1-naphthyl) – ethylenediamine dihydrochloride (NED) solution for nitrate and nitrite estimation

NED	0.5 g
De-ionized water	500 ml

10. Concentrate ammonium chloride solution for nitrate estimation

NH_4Cl	125 g
De-ionized water	500 ml

11. Dilute ammonium chloride solution for nitrate estimation

NH_4Cl (conc)	50 ml
De-ionized water	2000 ml

12. Phenol solution for nitrate estimation

Phenol crystals	20 g
95 % Ethanol	200 ml

13. Sodium nitroprusside solution for ammonia estimation

Sodium nitroprusside	1 g
95 % Ethanol	200 ml

14. Alkaline reagent for ammonia estimation

Sodium citrate	100 g
Sodium hydroxide	5 g
De-ionized water	500 ml

15. Sodium hypochlorite solution for ammonia estimation

Commercially available Hypochlorite (Chlorox) about 1.5 N

16. Oxidizing solution for ammonia estimation

Mix 100 ml of alkaline reagent (14) and 25 ml of Sodium hypochlorite solution (15)

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ANNEXURE

List of publications from this study:

Cathrine Sumathi Jebaraj and Chandralata Raghukumar (2009) Anaerobic denitrification in fungi from the coastal marine sediments off Goa, India. Mycological Research **113**: 100 - 109.

Cathrine Sumathi Jebaraj, Chandralata Raghukumar, Anke Behnke and Thorsten Stoeck. (2010) Fungal diversity in oxygen-depleted regions of the Arabian Sea revealed by targeted environmental sequencing combined with cultivation. FEMS Microbiology and Ecology **71**, 399–412.

Cathrine Sumathi Jebaraj and Chandralata Raghukumar. Fungal activity in a marine oxygen-depleted laboratory microcosm. (MS submitted)

Cathrine Sumathi Jebaraj, Teun Boekhaut, Wally H Muller, Frank Kauff, and Thorsten Stoeck. Ultrastructural and multi-gene analysis of a novel fungus, # 11 isolated from the denitrification zone of the Arabian Sea. (MS under preparation).

Presentation in workshop:

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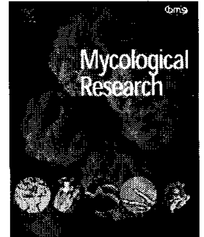

Anaerobic denitrification in fungi from the coastal marine sediments off Goa, India

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ABSTRACT

Denitrification is a microbial process during which nitrate or nitrite is reduced under anaerobic condition to gaseous nitrogen. The Arabian Sea contains one of the major pelagic denitrification zones and in addition to this, denitrification also takes places along the continental shelf. Prokaryotic microorganisms were considered to be the only players in this process. However recent studies have shown that higher microeukaryotes such as fungi can also adapt to anaerobic mode of respiration and reduce nitrate to harmful green house gases such as NO and N_2O. In this study we examined the distribution and biomass of fungi in the sediments of the seasonal anoxic region off Goa from two stations. The sampling was carried out in five different periods from October 2005, when dissolved oxygen levels were near zero in bottom waters to March 2006. We isolated mycelial fungi, thraustochytrids and yeasts. Species of *Aspergillus* and thraustochytrids were dominant. Fungi were isolated under aerobic, as well as anaerobic conditions from different seasons. Four isolates were examined for their denitrification activity. Two cultures obtained from the anoxic sediments showed better growth under anaerobic condition than the other two cultures that were isolated from oxic sediments. Our preliminary results suggest that several species of fungi can grow under oxygen deficient conditions and participate in denitrification processes.

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Introduction

Anaerobic denitrification is an alternate respiratory process in prokaryotes that enables them to thrive under oxygen-depleted conditions. Denitrifying bacteria utilize nitrate and (or) nitrite as the final electron acceptor in their respiratory cycle and release nitrogen gas to the atmosphere (Zumft 1997). During this process, they successively reduce nitrate to nitrite, nitric oxide, nitrous oxide and nitrogen with the help of the enzymes dissimilatory nitrate reductase (nar), nitrite reductase (nir), nitric oxide reductase (nor) and nitrous oxide reductase (nos). In a marine nitrogen cycle this is an important pathway through which the fixed nitrogen is lost and leads to an imbalance in the total nitrogen budget (Naqvi *et al.* 2006). Nitric oxide (NO) and nitrous oxide (N_2O) are produced as intermediates during the denitrification process. These are among the harmful green house gases that influence the earth's climate by the destruction of the ozone in the stratosphere.

The Arabian Sea is characterized by a perennial, open ocean oxygen minimum zone (OMZ) and a seasonal, coastal anoxic region along the western continental shelf of India. The anoxic condition develops during the southwest monsoon, following the upwelling and intensifies during September and October each year. The coastal anoxic region is a hot spot for N_2O emission, a green house gas that influences the

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earth's climate by the destruction of the ozone in the stratosphere (Naqvi *et al.* 2000). Microbial communities of the oxygen-depleted environment have often been assumed to have low species richness (Levin 2003). Culture independent studies in the oxygen-depleted environments have shown that these regions harbor a vast microbial diversity (Behnke *et al.* 2006; Dawson & Pace 2002; Massana *et al.* 2004; Stoeck & Epstein 2003). These microbes have unique physiological adaptations to survive in the adverse conditions. Recently reported group of anaerobic ammonia oxidizing bacteria is one of the examples (Dalsgaard *et al.* 2003). Molecular ecological studies have also shown a vast diversity of microeukaryotes in the anoxic regions of Cariaco Basin off the Venezuelan coast in the Caribbean (Stoeck *et al.* 2006) and in anaerobic sulfide and sulfur-rich spring in Oklahoma (Qingwei *et al.* 2005). The sequences of small subunit rDNA have revealed presence of deep novel branches within green algae, fungi, cercozoa, stramenopiles, alveolates, euglenozoa, unclassified flagellate and a number of novel lineages that has no similarity with any of the known sequences (Massana *et al.* 2004; Zuendorf *et al.* 2006). This suggests that oxygen-depleted environments harbor diverse communities of novel organisms, each of which might have an interesting role in the ecosystem.

The involvement of fungi as denitrifiers has been recently shown in the grassland ecosystem based on substrate-induced respiratory inhibition studies, which showed that they account for nearly 80 % of the nitrous oxide production (Ronald & Laughlin 2002). Screening of fungal isolates has shown that all the major groups of fungi are capable of denitrification process, though they predominantly form only nitrous oxide (Shoun *et al.* 1992).

The presence, diversity and role of fungi in denitrification processes in the marine nitrogen cycle have not been studied. We have attempted in this study to survey the presence of fungi in the seasonal oxygen minimum zone off Goa. We also screened a few fungi for nitrate reduction and ammonia formation under aerobic and anaerobic conditions.

Materials and methods

Sampling site and collection of sediments

Two field stations Station-I (St-I), 15° 31′ 080″ N, 73° 42′ 060″ E and Station-II (St-II), 15° 30′ 522′ N, 73° 39′ 00″ E within the coastal anoxic zone off Goa (Fig 1) were sampled from October 2005 to March 2006. Sediment samples were collected with a gravity corer (66 cm length and 7 cm diam.) from these two locations. The overlying water was siphoned out and the cores were cut at 2 cm intervals down to 8 cm and extruded into alcohol sterilized clean plastic containers. They were processed in the laboratory on the same day for isolation of fungi and fixed in formalin for direct detection of fungi and bacteria. The remaining sediments were stored at −20 °C for estimation of total organic carbon. Samples for dissolved oxygen in the near bottom water were fixed in Winkler's reagents on board and stored in an icebox. Nitrite and DO were estimated in the laboratory immediately on return (Strickland & Parsons 1968).

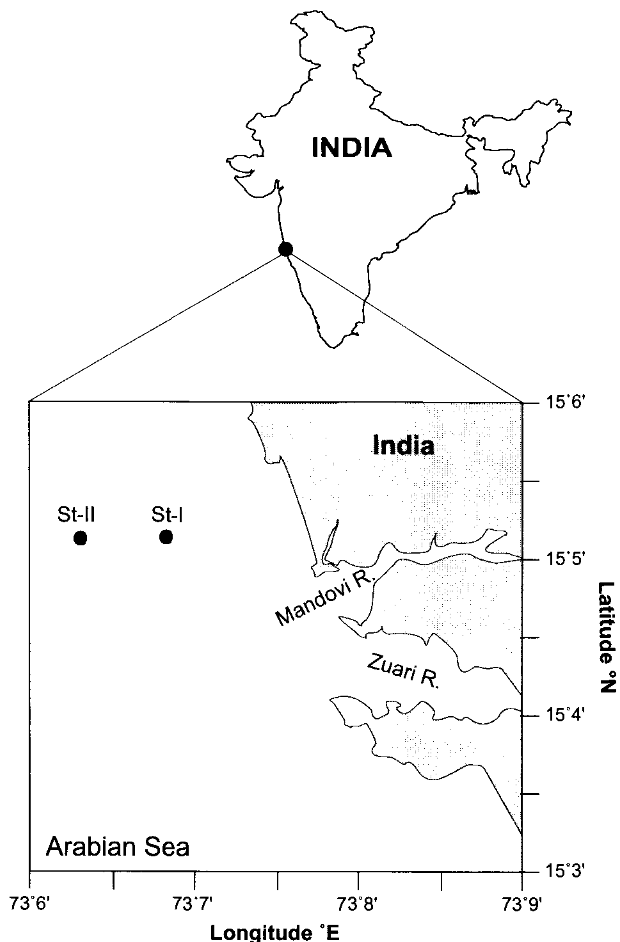

Fig 1 – Map showing the site of sampling.

Distribution of fungi

Isolation by particle plating technique

A portion of the sediment from the middle of each sub-section was removed with a flame-sterilized spatula and placed in sterile vials for isolation of fungi (Raghukumar *et al.* 2004). The media used for isolations were malt extract agar (MEA), malt extract broth (MEB), corn meal agar (CMA) and Czapek Dox agar (CDA). All the media (HiMedia Pvt. Ltd., India) were used at 1/5 strength to discourage the growth of fast growing fungi. They were prepared in seawater and fortified with streptomycin (0.1 g in 100 ml medium) and penicillin (40,000 Units in 100 ml medium) to inhibit bacterial growth. Fungi were isolated by modified particle plating technique (Bills & Polishook 1994). For this approximately 1 g of sediment slurry was sieved successively through a mesh size of 200 μm and 100 μm screens. The particles that passed through 200 μm mesh but were retained on the 100 μm mesh were spread-plated (Damare *et al.* 2006). Culturable colony forming units (CFU) of fungi were expressed as numbers g^{-1} dry sediment of 100–200 μm size particles. Fungi isolated from the sediments were sub cultured and maintained on MEA slants at 5 °C. Sporulating cultures were identified using the morphological keys (Domsch *et al.* 1980).

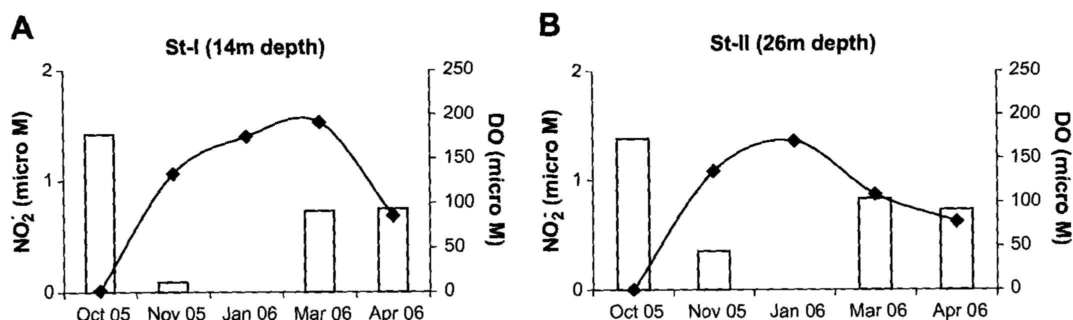

Fig 2 – Nitrite levels in micromole (bar) and dissolved oxygen concentration (line) ofthe near bottom water at St-I (A) & St-II (B).

Isolation by enrichment culturing

Isolation of cultures by enrichment method was carried out as follows. Approximately, 5 g of sediment samples were incubated in airtight glass bottles in 80 ml of enrichment media that consisted only of 10 mM sodium nitrate solution prepared in artificial seawater and were supplemented with antibiotics to inhibit growth of bacteria. The medium was then flushed with nitrogen gas and incubated for 30 d. At the end of the

Table 1 – Fungi isolated by particle plating of sediments from St-I & II in different seasons

Field Trip	St-I			St-II		
	Culture #	Identification	Percentage frequency	Isolate No.	Identification	Percentage frequency
Oct 05	# 1	*Aspergillus* sp.	12	# 1	*Aspergillus* sp.	51
	# 2	*Aspergillus* sp.	5	# 8	*Aspergillus* sp.	2
	# 8	*Aspergillus* sp.	17	# 10	*Scolicobasidium* sp.	2
	# 11	*Tritirachium* sp.	2	# 11	*Tritirachium* sp.	6
	# 12	*Humicola* sp.	33	# 12	*Humicola* sp.	31
	# 21	*Aspergillus* sp.	2	# An-3	*Aspergillus* sp.[a]	ND
	# An-1	*Aspergillus* sp.[a]	ND		Unidentified	8
	# An-2	*Fusarium* sp.[a]	ND			
	# An-4	*Asp.ergillus* sp.[a]	ND			
		Unidentified	17			
Nov 05	# 1	*Asp.ergillus* sp.	5	# 2	*Aspergillus* sp.	3
	# 2	*Asp.ergillus* sp.	22	# T-27	Thraustochytrid	81
	# T-27	Thraustochytrid	20	# 28	Yeast	3
	# 28	Yeast	2	# 33	*Aspergillus* sp.	3
	# 30	*Myceliopthora* sp.	5		Unidentfied	11
	# 31	*Byssochlamys* sp.	5			
	# 31a	*Paecilomyces* sp.	2			
	# 32	*Cleistothecial form*	2			
	# 21	*Aspergillus* sp.	2			
		Unidentified	34			
Jan 06	# 42-y	Yeast	3		Unidentified	100
	# 43-y	Yeast	6			
	# Th	Thraustochytrids	52			
		Unidentified	39			
Mar 06	# 1	*Aspergillus* sp.	40	# 1	*Aspergillus* sp.	67
	# 2	*Aspergillus* sp.	12	# 52a	Non-sporulating	27
	# F0	*Cladosporium* sp.	22	# 54a	*Trichoderma* sp.	7
	# 21	*Aspergillus* sp.	1			
	# 57-aY	Yeast	1			
	# 57-bY	Yeast	1			
		Unidentified	23			
Apr 06	# 1	*Aspergillus* sp.	59	# 1	*Aspergillus* sp.	59
	# 2	*Aspergillus* sp.	6	# 2	*Aspergillus* sp.	6
	# Th	Thraustochytrid	6	# Th	Thraustochytrid	6
	# 60-Y	Yeast	6		Unidentified	29
	# 11	*Tritirachium* sp.	6			
	# 21	*Aspergillus* sp.	6			
		Unidentified	12			

a Fungi obtained by enrichment culturing; ND: no data.

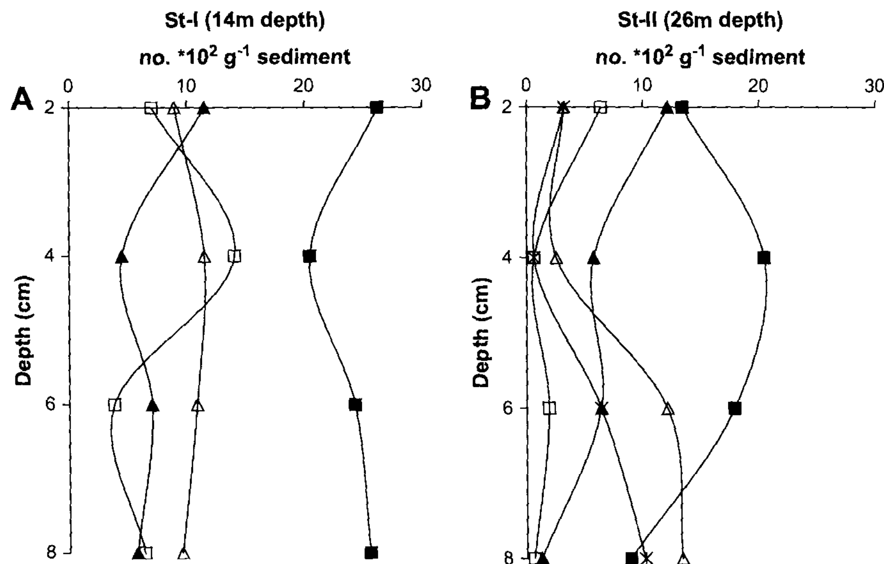

Fig 3 – Fungal CFU g^{-1} sediment at St-I (A) & St-II (B) (△ October 2005, ▲ November 2005, □ January 2006, ■ March 2006, * April 2006).

incubation period, fungal colonies were isolated from these sediments by particle plating as described above.

Bacterial and fungal abundance

For direct counts of total bacteria, 1 g wet sediment was suspended in 10 ml sterile seawater with formalin as a fixative (5 % final concentration) and was stored in the dark at 4 °C. The formalin-fixed samples were sonicated (3 × 30 sec) in a water bath sonicator (Biosystems Ltd, India) and allowed to settle for 5 min on ice. The overlying clear water sample was filtered over 0.22 μm black polycarbonate nuclepore filters (Millipore, USA) and stained with an aqueous solution of acridine orange (0.01 %) for 3 min. Bacterial cells were counted from 10–20 microscope fields with an epifluorescence microscope (Olympus BX60, Japan). The final numbers were expressed as total counts g^{-1} dry sediment. The bacterial numbers were converted to fg carbon using the conversion factor of 20 (Peduzzi & Hendle 1991). The final values were expressed as pgC g^{-1} dry sediment.

To estimate fungal biomass, formalin-fixed sediment was treated with 10 % EDTA, stained with 0.01 % of filter sterilized calcoflour (Sigma Chemicals, USA). Microscopic mounts of the sediment were then examined under ultraviolet light filter (excitation wave length 330 to 385 nm and barrier filter BA 420) of an epifluorescence microscope (Olympus BX60, Japan) to detect fluorescing fungal hyphae. The hyphal lengths were measured using an ocular micrometer. Considering the hyphae as a cylinder, length (h), the hyphal diameter as 2 μm and applying the formula $3.14 * r^2 * h$, the total hyphal lengths were expressed as biovolume g^{-1} dry sediment. The biovolume was converted to biomass using the conversion factor 0.2 g cm^{-3} (Newell *et al.* 1986). The C biomass was calculated by considering that 50 % of the biomass content was C (Bittman *et al.* 2005). The results of fungal C biomass were expressed as pg C g^{-1} sediment. The values are average of 2 replicate sediment samples examined. Bacterial cells, fungal hyphae and spores were photographed with a digital camera (Olympus 4.1 Mp, Japan).

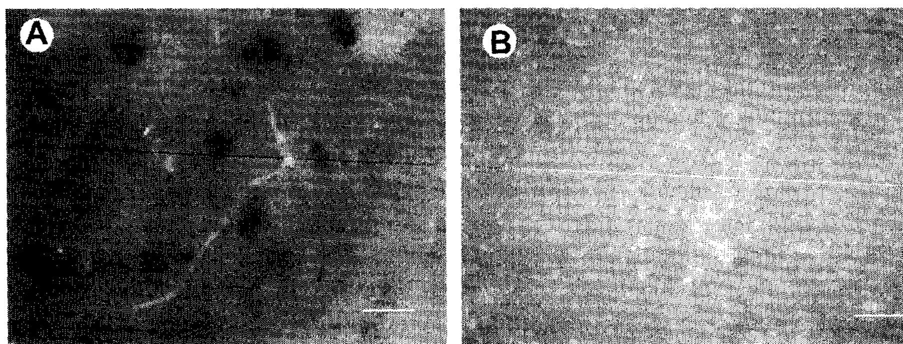

Fig 4 – (A) fungal hyphae in sediments stained with the fluorescent brightner, Calcofluor. Bar = 10 μm, (B) Acridine orange-stained bacteria from the sediments, Bar = 10 μm.

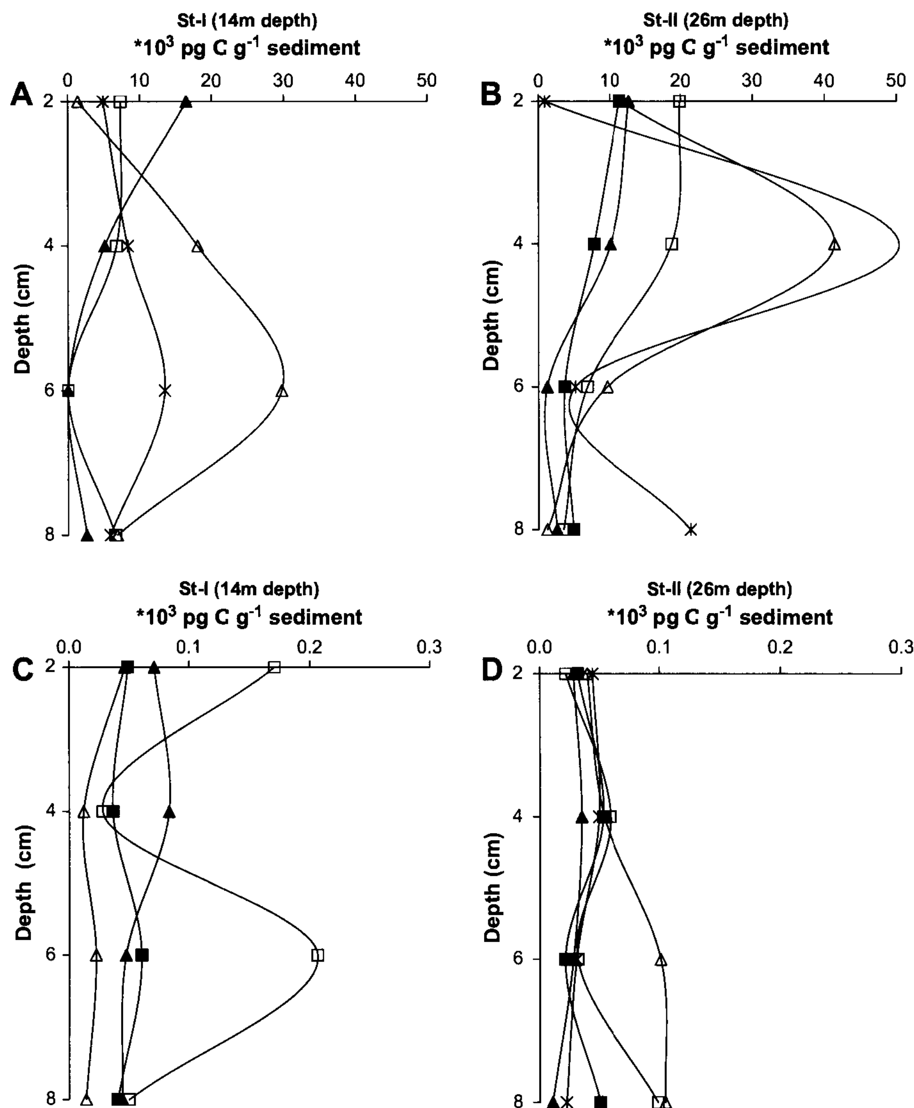

Fig 5 – Bacterial C biomass in the sediment sections at St-I (A) & St-II (B). Bacterial C data during Oct 2005 and April 2006 is depicted as numbers *10^4 pg C g^{-1} dry sediments whereas it is numbers *10^3 pg C g^{-1} dry sediment during the other sampling periods. Fungal C biomass in the sediment sections at St-I (C) and St-II (D). (△ October 2005, ▲ November 2005, □ January 2006, ■ March 2006, • April 2006).

Estimation of organic carbon (OC)

The OC content of the samples was determined by the difference between total carbon (TC) and inorganic carbon (IC). TC was analyzed by combustion of the samples at 1200 °C in an oxygen atmosphere and detection of CO_2 by coulometry (Prakash Babu et al. 1999). Inorganic C was analyzed by coulometry (UIC Coulometrics®), after liberation of CO_2 in an acidification module (Engleman *et al.* 1985). An in-house reference standard (TW-TUC) was used for testing reproducibility and accuracy. The values are expressed as % OC and are average of 2 replicates.

Screening of fungi for their nitrate utilization capacity under aerobic and anaerobic conditions

Four different fungi were studied for their growth and denitrifying capacity. They were, # An-2 (*Fusarium* sp.) isolated after anaerobic incubation of the sediment, # 11 (*Tritirachium* sp.) which was isolated from the sediment during anoxic condition and # 31 (*Byssochlamys* sp.) and # 31a (*Paecilomyces* sp.) were isolated from the sediments when the conditions were oxic. These cultures were compared with a well studied denitrifier of terrestrial origin, *Fusarium oxysporum* # MT-811 (Shoun & Tanimoto 1991), a gift from Dr. Shoun, Tokyo University, Japan. Starter cultures of these fungi were grown in mineral medium supplemented with 10 mM of sodium nitrate for 3 to 5 d. Approximately 10–15 mg (dry weight) of the mycelial suspension was used as an inoculum. The cultures were maintained under aerobic conditions in 100 ml conical flasks plugged with cotton containing 20 ml of medium and under anaerobic conditions in 100 ml serum bottles sealed air tight with butyl rubber stoppers and steel crimps after flushing with nitrogen gas

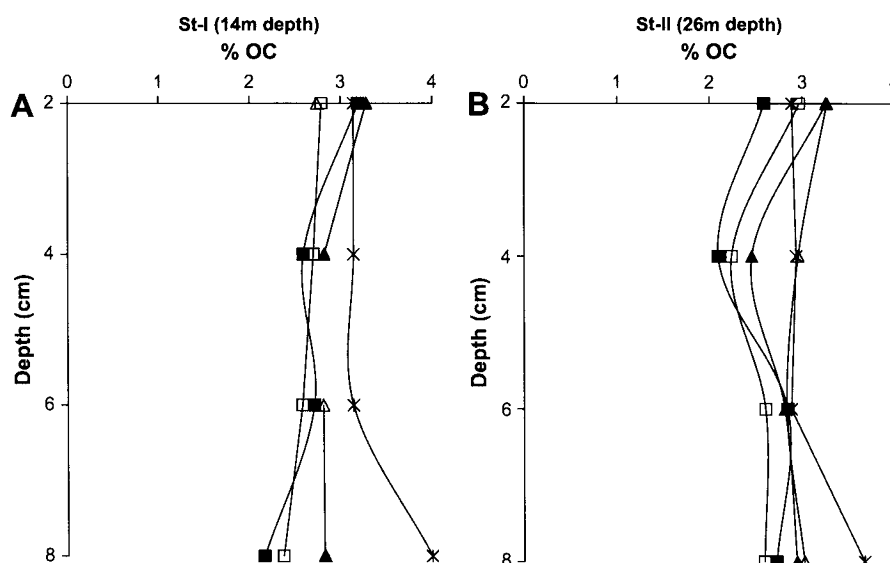

Fig 6 – Percentage OC at St-I (A) & St-II (B). (△ October 2005, ▲ November 2005, □ January 2006, ■ March 2006, • April 2006).

through the medium for 2 min. The dissolved oxygen (DO) was determined by spectrophotometric method (Pai *et al.* 1993) at 0 h and at the end of the experiment on the day 10 and on days when there was significant nitrite formation. Replicate bottles were used exclusively for DO measurement. The cultures were harvested every 48 h up to 10 d and nitrite and ammonia formed were determined by spectrophotometrically (Strickland & Parsons 1968). The growth of the cultures was also measured on day 10 and biomass in mg dry weight was determined. All chemicals used were of analytical grade.

Statistical analyses were carried out using Excel (Microsoft) programme. The data were transformed and tested for normality before analysis by Cochran Q test.

Table 2 – ANOVA: two factor to show the significance of distribution between different parameters at spatial and temporal levels

Variables	Df	F value	F-critical value	P value
Bacterial C				
(between depths)	7	1.1	2.5	0.41
(between seasons)	3	**8.2**	3.1	0.00*
Fungal C				
(between depths)	7	1.0	2.5	0.44
(between seasons)	3	1.7	3.1	0.19
Fungal CFU				
(between depths)	7	1.2	2.5	0.3
(between seasons)	3	**17.5**	3.1	<0.001***
TOC				
(between depths)	5	**2.9**	2.7	0.04*
(between seasons)	4	**3.9**	2.9	0.02*

(Df = degrees of freedom, F value greater than F-critical value indicates statistical significance, ***significant at 0.1 %, *significant at 5 % level).

Results

The physico-chemical characteristics of the near bottom water at the two stations showed typical denitrifying conditions during October 2005, when the levels of DO were near zero and nitrite accumulation was seen and oxic conditions were restored in the same site by January 2006 (Fig 2).

Distribution of fungi

Isolations using both aerobic and anaerobic incubations yielded a total of 54 fungi from sediments of both the stations during the 5 sampling periods between October 2005 and April 2006 by the particle plating technique (Table 1). Among the mycelial fungi that formed CFUs, *Aspergillus* species showed the highest frequency of occurrence during most of the sampling period at both the stations. *Humicola* sp. was also frequent during the anoxic period of October 2005. The straminipilan fungi, thraustochytrids were the next most abundant fungi. The number of CFUs obtained by particle plating technique from each section of the sediment core ranged between 64 to 2622 g^{-1} dry sediment of 100–200 μm size particles (Fig 3Aand B). Enrichment culturing was carried out with samples collected from the two stations during the

Table 3 – Correlation coefficient (r) between the biological parameters with DO as a dependent variable

	DO	Bacterial C	Fungal C	Fungal CFU	OC
DO	1				
Bacterial C	0.34	1			
Fungal C	0.30	−0.19	1		
Fungal CFU	0.52	−0.08	−0.10	1	
OC	0.10	0.36	−0.19	0.17	1

Table 4 – Fungal biomass mg (dry weight) 20 ml^{-1} under aerobic and anaerobic culture conditions

Cultures	Biomass under aerobic culture condition	Biomass under anaerobic culture condition
# MT-811 (*Fusarium oxysporum*)	130.2	29.3
# An-2 (*Fusarium* sp)	86.7	64.0
# 31 (*Byssochlamys* sp)	70.6	11.0
# 11 (*Tritirachium* sp)	60.2	61.0
# 31(a) (*Paecilomyces* sp)	250.3	46.0

Oct 2005 sampling trip, when the levels of DO were near zero. There was visible growth of mycelia at the end of the incubation period and the CFUs were predominantly *Aspergillus* sp., but an isolate (# An-2) identified as *Fusarium* sp. was also obtained (Table 1).

Bacterial, fungal and organic carbon

Fungal hyphae stained with Calcoflour, an optical brightener (Fig 4A) and bacterial cells (Fig 4B) from the sediments stained with acridine orange were measured by epiflourescence microscopy. Bacterial and fungal biomass for four depths of sediment cores at the two stations ranged between 1.2 to 500×10^3 pg C g^{-1} sediment and 0.01 to 0.206×10^3 pg C g^{-1} sediment respectively (Fig 5A–D). The maximal bacterial C biomass was found in the 2–4 cm section of the April 2006 sampling at St-II (Fig 5B) and that of fungi at St-I in 4–6 cm sediment section during January 2006 (Fig 5C). Total organic carbon ranged from 2.5 to 3.5 % at the two stations (Fig 6A and B). The bacterial, fungal biomass C and the organic C were more or less uniformly distributed in the sediment core from 0–8 cm during most of the sampling periods and showed no statistically significant difference, (Table 2). On the other hand, fungal CFUs and OC were significantly different between the various sampling periods as analyzed by 2-way ANOVA (Table 2). Bacterial and fungal biomass was not statistically related to dissolved oxygen content of the near bottom water and OC (Table 3).

Nitrate utilization capacity of fungi under aerobic and anaerobic conditions

The culture # An-2 (isolated after anaerobic incubation of sediments), # 11, from anoxic sediments and # 31 and # 31a from oxic sediments were screened for their ability to reduce nitrate. The culture # MT-811 was included as positive culture. The cultures grew under anaerobic conditions as was evident from the increase in biomass measured on the last day of the experiment (Table 4). Two cultures, # An-2 and # 11 showed equally good growth under aerobic and anaerobic conditions, the cultures # 31 and # 31a showed seven and fivefold less growth respectively under anaerobic condition and # MT-811 showed a tenfold decrease in biomass.

A distinct increase in nitrite accumulation was noticed intermittently in cultures grown in flasks (Table 5). The initial DO value in the flasks was 115.2 μM, as the cultures grew, DO was utilized and suboxic conditions developed (18–97 μM) on day 4 or 6 depending on the increase in biomass. As the conditions became suboxic the cultures started to utilize nitrate for respiration and nitrite accumulation was seen in the flasks of the cultures (Table 5A). In the anaerobic cultures (after flushing with N_2 gas) the initial DO was 9.5 μM and a significant difference in nitrite accumulation was observed under anaerobic conditions between cultures (Table 5B). Maximum nitrite accumulation was noticed in # 31 and this was statistically significant ($P = 0.005$) between aerobic and anaerobic cultures (Table 5C).

Ammonia formation was seen in cultures maintained under anaerobic condition. The positive control # MT-811 as well as all the other cultures showed ammonia formation to a varying degree during anaerobic incubation (Table 6A). A 2-way analysis of variance indicated significant difference between cultures in their capacity to accumulate ammonia

Table 5B – ANOVA: two factor to show the significance of nitrite accumulation by different cultures on different days

Variables	Df	F value	F-critical value	P value
Different days				
(aerobic)	4	1.4	3	0.27
(anaerobic)	4	1.3	3	0.31
Different cultures				
(aerobic)	4	1.7	3	0.19
(anaerobic)	4	3.3	3	**0.04***

(DF = degrees of freedom, F value greater than F-critical value indicates statistical significance, *significant at 5 % level).

Table 5A – Nitrite formation by different fungi (nM)

Culture #	Day 2		Day 4		Day 6		Day 8		Day 10	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
# MT-811	156.7 (96.4)	7.1 (12.3)	1852.1 (327.1)	199.4 (32.6)	56.9 (61.7)	7.1 (12.3)	377.5 (121.5)	163.8 (44.4)	758.6 (407.9)	135.3 (61.6)
# An-2	85.5 (74.0)	270.7 (150.1)	213.7 (37.0)	455.9 (198.6)	334.8 (12.3)	420.3 (203.1)	406.0 (56.5)	434.5 (271.4)	0.0	0.0
# 31	220.8 (105.4)	64.1 (0)	584.1 (263.8)	49.9 (32.6)	904.7 (163.2)	292.1 (267.2)	591.2 (172.7)	163.8 (32.6)	630.4 (45.3)	114.0 (12.3)
#11	206.6 (32.6)	163.8 (65.3)	605.5 (184.2)	356.2 (117.7)	370.4 (89)	185.2 (24.7)	263.6 (228.5)	277.8 (21.4)	491.5 (140.1)	206.6 (44.5)
# 31a	171.0 (42.7)	142.5 (49.4)	149.6 (21.4)	149.6 (154.1)	220.8 (89)	49.9 (12.3)	156.7 (32.6)	149.6 (77.1)	327.7 (128.8)	185.2 (44.5)

Values within brackets denote standard deviation.

Table 5C – ANOVA: single factor to show the significance of nitrite accumulation by different cultures in aerobic vs anaerobic conditions

Culture #	Df	F value	F-critical value	P value
MT8-11	9	2.7	5.3	0.14
An-2	9	0.9	5.3	0.37
# 31	9	**14.6**	5.3	**0.005****
# 11	9	3.4	5.3	0.1
# 31(a)	9	3	5.3	0.12

(Df = degrees of freedom, F value greater than F-critical value indicates statistical significance, **significant at 1 %).

(Table 6B). Ammonia accumulation between aerobic and anaerobic cultures was significantly different in the culture # An-2 (Table 6C).

Discussion

During this study except for thraustochytrids, all the other fungi obtained were common terrestrial fungi. Terrestrial runoff during monsoon might be the major source of input for such terrestrial fungi. Alternatively, many terrestrial species of fungi might have become adapted to marine conditions. This is evident from several recent reports of terrestrial species of fungi (geofungi) from the marine environment. The occurrence of endolithic fungi associated with molluscan shells as microborers (Golubic *et al.* 2005); *Aspergillus sydowii* as a pathogen of seafan in the Caribbean (Shinn *et al.* 2000); terrestrial fungi as putative pathogens in scleractinian corals (Le Campion-Alsumard *et al.* 1995) are some of the examples of active involvement of geofungi in the marine environment. Presence of *Aspergillus sydowii* in 0.42 million year old deep-sea sediments of the Chagos Trench in the Indian Ocean (Raghukumar *et al.* 2004), a *Penicillium* sp. in the sediments of the Mariana Trench at 11,500 m depth (Takami 1999) and several terrestrial fungi in the deep-sea sediments at ~5000 m depth in Central Indian Basin (Damare *et al.* 2006) add further credence to this view.

Besides culturing, direct detection of mycelia in the sediments was shown by the calcoflour staining technique. Calcofluor, is an optical brightener that enhances fluorescence of cellulose and chitin, the latter being signature of the fungal cell wall (Mueller & Sengbusch 1983). Such staining revealed the presence of hyphae in the sediments, confirming active

Table 6B – ANOVA: two factor to show the significance of ammonia accumulation by different cultures on different days

Variables	Df	F value	F-critical value	P value
Different days				
(aerobic)	4	0.8	3	0.53
(anaerobic)	4	1.0	3	0.42
Different cultures				
(aerobic)	4	1.8	3	0.17
(anaerobic)	4	**11.5**	3	**0.00014*****

(Df = degrees of freedom, F value greater than F-critical value indicates statistical significance, ***significant at 0.1 %).

growth of fungi therein. Occasionally fungal spores were also detected. Organic material in the sediments apparently supports growth of fungi. There was almost a uniform distribution of bacterial, fungal and organic carbon in the sediment core from 0–8 cm. Water mixing, high sedimentation rates and intense activity of the benthic meiofauna could have brought about this homogeneity of the sediments. Such a phenomenon has been also reported in deep-sea sediments of the Central Indian Ocean, where the conditions are more stable (Raghukumar *et al.* 2006).

Four cultures studied in the present study were capable of growth under anaerobic conditions. While all the cultures grew much better under aerobic conditions, one culture (#11) produced almost the same amount of biomass under both aerobic and anaerobic conditions (Table 4). These results indicate that fungi isolated from the anoxic sediments might have adapted to a facultative anaerobic mode of life. Shoun & Tanimoto (1991) have shown that # MT-811 can grow under anaerobic conditions and utilize nitrate and nitrite for dissimilatory purpose. They have also shown that there is a substantial amount of cell growth during this process, which shows that the dissimilatory nitrate or nitrite reduction is an energy yielding reaction.

We observed denitrification activity by fungi under varying oxygen concentrations in our present study. Fungal denitrification process differs significantly from classical bacterial denitrification. Bacterial denitrification takes place only in the complete absence of oxygen and even a trace of oxygen could be toxic to the obligate anaerobes and inhibit denitrification. In fungi, reduction of nitrate takes place under suboxic conditions (300–900 μM O_2) but excess oxygen (>900 μM O_2) is

Table 6A – Ammonia formation by different fungi (*10^3 nM)

Culture #	Day 2		Day 4		Day 6		Day 8		Day 10	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
# MT-811	3.6 (1.9)	10.5 (1.4)	7.1 (3.4)	31.3 (10.5)	10.8 (12.9)	61.4 (15.2)	9.2 (4.3)	55.0 (15.6)	51.5 (49.6)	42.6 (31.1)
# An-2	1.1 (1.0)	11.2 (11.0)	1.9 (1.0)	23.8 (5.9)	0.1 (0.3)	25.3 (16.7)	1.2 (1.0)	26.6 (17.2)	6.3 (5.3)	10.7 (5.4)
# 31	9.3 (3.0)	7.0 (2.4)	9.3 (2.5)	1.6 (1.8)	10.6 (0.3)	4.2 (1.7)	7.6 (2.1)	6.2 (5.5)	1.2 (1.4)	8.9 (1.4)
# 11	1.7 (1.5)	7.6 (1.4)	0.9 (0.8)	1.9 (1.4)	5.1 (2.1)	6.0 (2.7)	1.3 (0.3)	1.1 (0.6)	4.3 (1.6)	5.8 (0.5)
# 31a	7.5 (2.2)	12.7 (2.9)	12.3 (6.8)	4.0 (1.0)	22.6 (12.4)	14.0 (7.0)	4.8 (2.2)	4.7 (2.9)	6.7 (3.4)	11.5 (3.3)

Values within brackets denote standard deviation.

Table 6C – ANOVA: single factor to show the significance of ammonia accumulation by different cultures in aerobic vs anaerobic conditions

Culture #	Df	F value	F-critical value	P value
# MT8-11	9	3.5	5.3	0.09
# An-2	9	37	5.3	0.0003**
# 31	9	0.9	5.3	0.4
# 11	9	1.4	5.3	0.3
# 31(a)	9	0.1	5.3	0.7

(Df = degrees of freedom, F value greater than F-critical value indicates statistical significance, **significant at 1 %).

shown to inhibit the process (Zhou *et al.* 2001). It was observed in our studies that in the culture # 31, maximum nitrite accumulation occurred on day 6 when suboxic conditions set in (Table 5). As all fungi are not capable of nitrate reduction, but can use nitrite as an electron acceptor (Takaya 2002) experiments to screen isolates for their nitrite reducing capacity are to be carried out. Further, fungal denitrification is an incomplete process in comparison with the classical pathway. Fungi are known to stop with the formation of N_2O and fungal denitrifiers are not reported to produce N_2 as the final product (Bleakley & Tiedje 1982; Shoun *et al.* 1998). Because of this incomplete system, denitrification by fungi causes an increase in the green house gases and leads to detrimental effects on the global climate.

Fungi also follow another pathway to reduce nitrate under complete anoxic conditions, which is referred as ammonia fermentation. This process was studied in the same four isolates under both aerobic and anaerobic conditions. There was ammonia formation by all the cultures under anaerobic conditions (Table 6A and B) and # An-2 showed a significant difference between aerobic and anaerobic culture conditions (Table 6C). This process in fungi appears to be widespread as 15 of 17 fungi tested by Zhou *et al.* (2002) showed ammonia formation under anaerobic condition.

Studies on the denitrifying activities of *Fusarium oxysporum* # MT-811 have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O_2 tension (Takaya 2002). Fungi show a multimodal type of respiration to rapidly adapt to changes in the oxygen supply, in anoxic conditions ammonia formation takes place, while denitrification process in suboxic and oxygen respiration under aerobic conditions (Takaya 2002). This may be a survival strategy for mycelial fungi to thrive in extreme and dynamic environments.

Advancements in the area of molecular ecology have seen an advent of discoveries of new microorganisms that partake in biogeochemical process. New groups are being added to the list of microorganisms that have an active role in the marine nitrogen cycle, especially in their ability to produce harmful green house gases like NO and N_2O. Recently, a benthic foraminifer *Globobulimina pseudospinenscens* has been demonstrated to show complete denirification in marine sediments (Risgaard-Peterson *et al.* 2006). Apart from this study Straminipiles (thraustochytrids) have also been reported from anoxic habitats (Kolodziej & Stoeck 2007) but no studies have been attempted so far to understand their role in these habitats.

Our present study is the first report showing involvement of mycelial fungi in denitrification process in the marine anoxic sediments. Further studies on the presence of various enzymes that are responsible in denitrification and the genes responsible for them will shed more light on fungal processes in sedimentary denitrification in oxygen minimum zone of the Arabian Sea off Goa.

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RESEARCH ARTICLE

Fungal diversity in oxygen-depleted regions of the Arabian Sea revealed by targeted environmental sequencing combined with cultivation

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fungal diversity; Arabian Sea; cultivation; SSU rDNA; oxygen minimum zone; PCR primers.

Abstract

In order to study fungal diversity in oxygen minimum zones of the Arabian Sea, we analyzed 1440 cloned small subunit rRNA gene (18S rRNA gene) sequences obtained from environmental samples using three different PCR primer sets. Restriction fragment length polymorphism (RFLP) analyses yielded 549 distinct RFLP patterns, 268 of which could be assigned to fungi (Dikarya and zygomycetes) after sequence analyses. The remaining 281 RFLP patterns represented a variety of nonfungal taxa, even when using putatively fungal-specific primers. A substantial number of fungal sequences were closely related to environmental sequences from a range of other anoxic marine habitats, but distantly related to known sequences of described fungi. Community similarity analyses suggested distinctively different structures of fungal communities from normoxic sites, seasonally anoxic sites and permanently anoxic sites, suggesting different adaptation strategies of fungal communities to prevailing oxygen conditions. Additionally, we obtained 26 fungal cultures from the study sites, most of which were closely related (> 97% sequence similarity) to well-described Dikarya. This indicates that standard cultivation mainly produces more of what is already known. However, two of these cultures were highly divergent to known sequences and seem to represent novel fungal groups on high taxonomic levels. Interestingly, none of the cultured isolates is identical to any of the environmental sequences obtained. Our study demonstrates the importance of a multiple-primer approach combined with cultivation to obtain deeper insights into the true fungal diversity in environmental samples and to enable adequate intersample comparisons of fungal communities.

Introduction

Fungi are primarily aerobic heterotrophs that play an essential role as decomposers of organic matter in a variety of environments. In marine ecosystems, fungi are the major decomposers of woody and herbaceous substrates and their importance lies in their ability to aggressively degrade lignocellulose (Newell, 1996). Marine fungi also contribute to the degradation of dead animals and animal parts (Kohlmeyer & Kohlmeyer, 1979), and are important pathogens of animals and plants or partners in mutualistic symbioses (Raghukumar, 1986; Alsumard *et al.*, 1995; Pivkin, 2000).

Fungi were long thought to play only a minor role in the ecosystem processes of anoxic environments (Dighton, 2003). However, many fungal taxa were recently shown to possess metabolic adaptations to utilize nitrate and (or) nitrite as an alternative for oxygen (Shoun *et al.*, 1992). This testifies to their potential to participate in anaerobic denitrification processes in biogeochemically highly active ecosystems such as the oxygen minimum zones (OMZs) of the Arabian Sea, which are characterized by high microbially mediated denitrification rates (Naqvi *et al.*, 2006). Indeed, in a recent study, Jebaraj & Raghukumar (2009) showed that fungi isolated from anoxic marine waters of the Arabian Sea are capable of growth under

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oxygen-deficient conditions while performing anaerobic denitrification.

Such findings suggest that the abundance and ecological role of fungi in anoxic marine systems is probably underestimated. Support for this assumption comes from analyses of the small subunit rRNA locus (18S rRNA gene) amplified from genomic DNA isolated from environmental samples. While usually rare in open ocean surface waters, the majority of environmental 18S rRNA gene diversity surveys conducted in oxygen-depleted aquatic environments report large proportions of fungal 18S rRNA gene sequences (reviewed in Epstein & López-García, 2007). Many of these environmental sequences appear as unique phylogenetic branches that are highly divergent from previously described 18S rRNA gene fungal sequences (e.g. Takishita *et al.*, 2005, 2007a, b; López-García *et al.*, 2007; Stoeck *et al.*, 2007; Laurin *et al.*, 2008). As a rule, such molecular diversity surveys targeting microbial eukaryotes have used domain-specific PCR primers to amplify 18S rRNA gene fragments from environmental samples. As such primers target most eukaryotic organisms across all major clades and only sample a small fraction of amplicon diversity present in a sample (Epstein & López-García, 2007), it is reasonable to assume that many fungi may have escaped these surveys. Thus, the full extent of fungal diversity may be orders of magnitude higher than these domain-targeted environmental diversity studies have shown. Indeed, PCR primers with specificity for fungal DNA, while reducing coamplification of DNA from nonfungal sources, were applied successfully for estimating the extent of fungal diversity in soil (e.g. Borneman & Hartin, 2000; Anderson *et al.*, 2003; Malosso *et al.*, 2006). To date, this targeted strategy that applies fungal-specific primers to analyze fungal diversity has gone untested for anoxic aquatic habitats.

Using previously published primers designed for the specific amplification of fungal 18S rRNA gene from mixed-origin genomic DNA (Maiwald *et al.*, 1994; Kappe *et al.*, 1996; Vainio & Hantula, 2000; Gomes *et al.*, 2003), we analyzed fungal diversity in samples from the OMZ of the Arabian Sea. Therefore, we constructed clone libraries with two fungal-specific PCR primer sets and one domain-specific PCR primer set, routinely used in environmental diversity surveys. This strategy not only allowed an insight into fungal diversity in OMZs of the Arabian Sea but also revealed the substantial proportion of fungal diversity that is missed in a domain-specific PCR primer approach. Additionally, we used standard cultivation techniques to complement the molecular diversity surveys, unearthing a different subset of the fungal communities under study than the molecular approach. Finally, community structure analyses suggest that fungal assemblages thriving under different oxygen regimes are significantly distinct from each other, probably reflecting different adaptations to geochemically distinct environments.

Materials and methods

Sampling sites

Samples were collected from three sites in the Arabian Sea (Table 1). (1) Sampling of sediments at the coastal station (15°29.951′N, 73°40.827′E) off Goa at 25 m depth was carried out in October 2005 during the anoxic season (25_ANS), with no dissolved oxygen (DO) detectable, and in January 2006 during the oxic season (25_OXS), when oxygen saturation was restored. (2) Sampling in the perennial OMZ off Goa (15°20.10′N, 72°54.11′E) at a depth of 200 m was carried out during May 2007, when we collected anoxic sediment (200_ANS) and anoxic near-bottom water (200_ANW). (3) As a comparison, we sampled the permanently oxygenated sediment from a shallow 3-m-depth site (03_CRS) in the coral reef region off Kavaratti (10°34.588′N, 72°38.019′E) in February 2007. Coral sand samples were collected by divers in sterile containers. All other sediment samples were collected using an ~60-cm-long gravity corer while water was collected in 5-L Niskin bottles. All samples were divided into aliquots for the isolation of fungi for cultivation and for total genomic DNA extraction. Samples for isolation were stored at 5 °C and processed within 24 h. Water samples for molecular analyses (5 L of water) were drawn on Durapore filters [0.45 μm; Millipore (India) Pvt Ltd, Bangalore] under the

Table 1. Sampling sites and characteristics

Sampling site	Designation (color coding in Figs 1–3)	Sampling date	DO (μM)	Temp (°C)	Salinity (PSU)	Sampling material	Sample volume
Near a shore off Goa (25 m)	25_ANS (brown)	October 2005	ND	22.5	35.4	Sediment	~500 mg
Near a shore off Goa (25 m)	25_OXS (orange)	January 2006	118. 4	26.9	34.7	Sediment	~500 mg
OMZ off Goa (200 m)	200_ANS (blue)	May 2007	ND	14.8	35.6	Sediment	~500 mg
OMZ off Goa (200 m)	200_ANW (turquoise)	May 2007	ND	14.8	35.6	Water	~5 L
Coral reef off Kavaratti (3 m)	03_CRS (green)	February 2007	93.74	28.7	34.0	Sediment	~500 mg

ND, below detection limit; Temp, temperature.

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exclusion of oxygen (Stoeck *et al.*, 2003), and ~5–10 g of sediment was frozen immediately in liquid nitrogen before storage at −80 °C until further processing. The DO of the near-bottom water was determined spectrophotometrically (Pai *et al.*, 1993).

Isolation and identification of culturable fungi

Culturable fungi were isolated using the particle-plating technique of Bills & Polishook (1994), with slight modifications. In brief, approximately 1 g of sediment slurry was serially sieved through a 200-μm and a 100-μm mesh. The particles that passed through the 200-μm mesh, but were retained on the 100-μm mesh were spread-plated on different fungal media such as malt extract agar, corn meal agar and Czapek Dox agar (HiMedia Pvt Ltd, India) prepared in seawater and fortified with streptomycin (0.1 g in 100 mL medium) and penicillin (40 000 U in 100 mL medium) to inhibit bacterial growth. Incubation was carried out under oxic conditions. The cultures isolated from the different sampling stations were identified based on their morphology and partial (~1600 bp) 18S rRNA gene sequence. Genomic DNA was extracted from the freeze-dried mycelial mats of each culture grown aerobically for 5 days at room temperature in 50 mL of malt extract broth (HiMedia Pvt Ltd) in 250-mL Erlenmeyer flasks. Total genomic DNA was extracted from the freeze-dried mycelial mats of each culture using a high salt concentration extraction buffer (100 mM Tris-HCl (pH 8), 100 mM sodium phosphate buffer (pH 8), 1.5 M NaCl, 100 mM EDTA (pH 8) and 1% CTAB). One milliliter of buffer was added to approximately 500 mg of mycelia and the total genomic DNA was extracted using chloroform–phenol extraction and isopropanol precipitation as described previously (Stoeck & Epstein, 2003). The 18S region of the rDNA was amplified using fungal-specific primers NS1 and FR1 (Table 2). The 50-μL PCR reactions included 0.5 U HotStart Taq DNA polymerase (Qiagen, Hildesheim, Germany) in 1 × HotStart Taq buffer, 200 μM of each dNTP and 0.5 μM of each oligonucleotide primer. PCR was performed using initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 2 min, with a final extension at 72 °C for 7 min. The PCR products were cloned separately for each fungal culture using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmids were isolated from positive overnight cultures using the Fast Plasmid Mini Prep kit (Eppendorf, Hamburg, Germany). One representative clone of each culture was sequenced bidirectionally (M13 sequencing primers) by MWG-Biotech on an Applied Biosystems (ABI) 3730 DNA Stretch Sequencer, with the XL Upgrade and the ABI Prism BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit. In order to evaluate the intrastrain variability of the 18S rRNA gene, for five strains (FCAS35, FCAS36, FCAS41, FCAS89 and FCAS125), we have sequenced four amplicons. Sequences were included in phylogenetic analyses as described below. GenBank accession numbers of sequences from cultured isolates are GQ120154–GQ120179 and GU072534–GU072548 for duplicate amplicons.

PCR primer selection, environmental 18S rRNA gene clone libraries construction and operational taxonomic unit (OTU) calling

Total DNA from the five environmental samples (25_ANS, 25_OXS, 200_ANS, 200_ANW and 03_CRS) was extracted using a high salt concentration extraction buffer, followed by chloroform–phenol extraction and precipitation with isopropanol as described previously (Stoeck & Epstein, 2003). Amplification of 18S rRNA gene was carried out using three different primer sets (Table 2). The primer sets were chosen based on their specificity and adequate length of amplified fragments in order to carry out robust phylogenetic analyses.

The first primer set (Fung1) consisted of the fungal-specific NS1 and FR1 primers resulting in ~1650-bp fragments. According to homology searches, this primer set has only moderate specificity and coamplifies a range of non-fungal eukaryote genes such as Metazoa, *Cercozoa*, *Viridiplantae*, Alveolata, Centroheliozoa and *Bangiophyceae* (Pang & Mitchell, 2005). The second primer set (Fung2) included the fungal-specific UF1 and S3 primers amplifying ~900-bp fragments. This primer set has a relatively high fungal specificity and previously amplified only a few nontarget taxa (green algae and *Pseudomonas*) from Antarctic soil samples (Malosso *et al.*, 2006). We also applied the universal

Table 2. Primer sets used in this study to amplify 18S rRNA gene sequences from genomic environmental DNA

Primer set	Primers	Primer sequence (5′–3′)	Reference
Fung1	NS1	GTA GTC ATA TGC TTG TCT C	Vainio & Hantula (2000)
	FR1	AIC CAT TCA ATC GGT AIT	Gomes *et al.* (2003)
Fung2	UF1	CGA ATC GCA TGG CCT TG	Kappe *et al.* (1996)
	S3	AGT CAA ATT AAG CCG CAG	Maiwald *et al.* (1994)
EukAB	EukA	AAC CTG GTT GAT CCT GCC AGT	Medlin *et al.* (1988)
	EukB	TGA TCC TTC TGC AGG TTC ACC TAC	Medlin *et al.* (1988)

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eukaryote primer pair EukA and EukB, routinely used in environmental eukaryote diversity surveys (Massana *et al.*, 2004; Stoeck *et al.*, 2006; Euringer & Lueders, 2008), which amplifies nearly the full length of the 18S rRNA gene (Table 2) of a wide range of higher eukaryote taxon groups (Medlin *et al.*, 1988).

Amplicons were ligated into pGEM-T vector and transformed into *Escherichia coli* cells (TOP 10 strain) using Invitrogen's TA-cloning kit as described above. For each library and primer set, we selected nearly 100 positively screened colonies (blue–white screening) for overnight growth and plasmid extraction using Qiagen's 96-well Directprep Kit. The presence of 18S rRNA gene inserts was confirmed by a standard M13-PCR amplification of extracted plasmids. In sum, 1440 positively screened plasmids were subjected to restriction fragment length polymorphism (RFLP) analyses. Therefore, between 200 and 400 ng of amplification products with expected sizes were digested with 7.5 U of the restriction endonuclease HaeIII (New England Biolabs, Beverly, MA) for 60 min at 37 °C, followed by an inactivation step for 20 min at 80 °C. The resulting bands were separated by electrophoresis in a 2.5% low-melting-point agarose gel at 80 V for 2–3 h. At least one representative clone of each RFLP pattern ($n = 549$) was partially sequenced (*c.* 600 nucleotides) at MWG-Biotech as described above.

Because partial fragments could only be adequately aligned within each primer set (the sequence overlap was too small for partial fragments to be aligned between sequences obtained by different primer sets), sequences of each individual primer set were grouped separately into OTUs based on 99.0% sequence similarity using three independent (one for each primer pair-derived sequence set) DOTUR analyses (Schloss & Handelsman, 2005). One representative of each OTU that was identified as a fungal sequence using a BLASTN search in the GenBank nr-database (Altschul *et al.*, 1997) was chosen for full-fragment sequencing ($n = 100$). The obtained sequences were checked for chimeras using the BELLEROPHON CHIMERA CHECK program and the CHECK_CHIMERA utility [Ribosomal Database Project (Cole *et al.*, 2003), as well as partial treeing analysis (Robison-Cox *et al.*, 1995)]. Nine potentially chimeric sequences were identified and removed before subsequent sequence analyses. This left us with 91 full-fragment sequences that were analyzed together in a final DOTUR analysis. Similarities between two sequences were calculated using a custom program (PAIRALIGN) provided by M. Nebel (University of Kaiserslautern), which uses IUB matrix-based pairwise alignments. In total, 549 partial and 91 full-fragment environmental sequences have been deposited in the GenBank database under accession numbers GU071985–GU072533, GU072549–GU072590 and GQ120105–GQ120153.

Phylogenetic analyses

Environmental 18S rRNA gene sequences were compared initially with those in GenBank using BLAST analysis to determine their approximate phylogenetic affiliation. Sequences of environmental clones, together with their closest GenBank cultured and uncultured matches, were aligned using the ARB FASTALIGNER utility. Alignments were manually refined using phylogenetically conserved secondary structures. The conserved and unambiguously aligned positions were used in subsequent phylogenetic analyses. Maximum-likelihood analyses were conducted using RAXML (Stamatakis *et al.*, 2008) on the CIPRES Portal v. 1.15 (http://www.phylo.org). The relative stability of tree topologies was assessed using 1000 bootstrap replicates. Heuristic searches for bootstrap analyses used stepwise addition, starting trees with simple addition of sequences and tree bisection and reconnection branch swapping. Maximum-likelihood bootstrapping analyses were carried out using RAXML with all free model parameters estimated by RAXML as described in Stamatakis *et al.* (2008). Details on model parameters for the individual alignments are given in the legends of Figs 1–3.

Community comparisons

The program package SPADE (Chao & Shen, 2003–2005) was used to calculate the Jaccard index as a measure of similarity between two communities based on the abundance of environmental fungal OTUs ($J_{abundance}$). An unweighted pair group mean average (UPGMA) cluster analysis based on $J_{abundance}$ data was performed using the Cluster analysis module of STATISTICA v. 7 (StatSoft, Tulsa, OK). A Venn diagram was constructed to display the overlap in OTU composition between the different primer sets using VENNY (Oliveros, 2007). Species accumulation curves and abundance-based coverage estimator of species richness for all clone libraries were calculated in ESTIMATES (Colwell, 2005).

Results and discussion

We subjected 1440 environmental clones obtained from five individual sampling events in the Arabian Sea (Table 1) to RFLP analyses. The clones grouped into 549 distinct RFLP patterns, which were subjected to 18S rRNA gene sequence analyses. Surprisingly, GenBank BLASTN analyses of one representative sequence from each RFLP pattern identified only 268 unique RFLP patterns as members of the kingdom Fungi. Considering the replicates of these unique patterns, in total, we could putatively identify 455 out of the initial 1440 clones as taxonomically affiliated to the kingdom Fungi. The remaining RFLP patterns ($n = 281$) represented sequences falling into a number of different taxonomic

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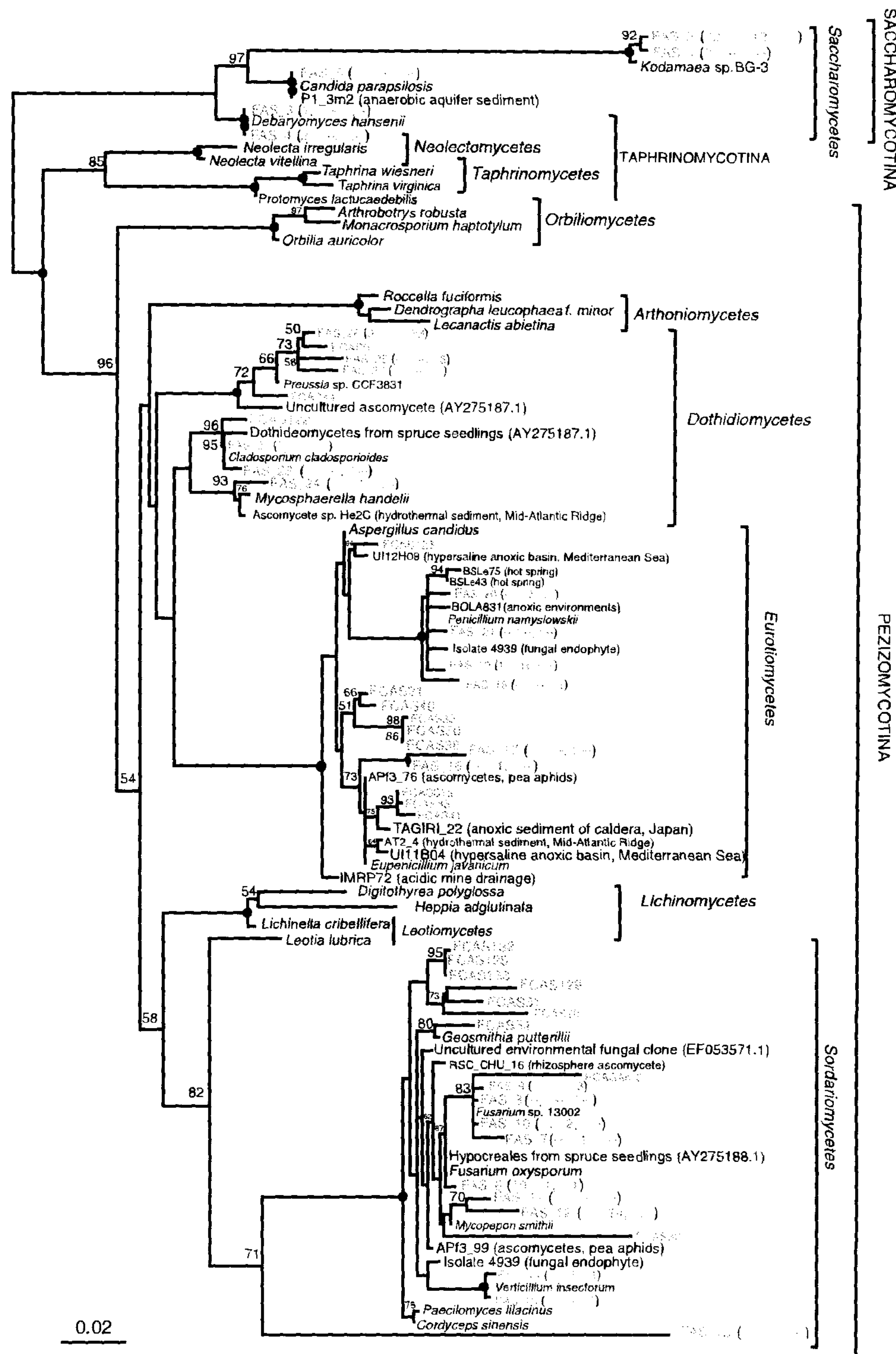

Fig. 1. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phylum *Ascomycota* showing the positions of environmental OTUs and from cultured isolates obtained from the Arabian Sea. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.645 and the proportion of invariable sites at 0.312, based on 925 unambiguously aligned positions. Full support from 1000 bootstrap replicates is indicated by a black-filled dot at the respective node. Other support values are only displayed when > 50. Numbers in parentheses following environmental OTU names indicate the number of sequences of this OTU that were found in the individual libraries. Sequences from cultured isolates are in green and designated as FCAS followed by the culture identification number. Color coding for environmental libraries is given in Table 1.

groups (Table 3, Supporting Information, Table S1). The partial sequences ($n = 268$) obtained from representatives of each unique RFLP pattern grouped into 91 distinct OTUs called at 99% sequence similarity: 25 OTUs for primer set Fung1, 54 OTUs for primer set Fung2 and 12 OTUs for primer set EukAB. After we sequenced one complete fragment from at least one representative of each individual OTU, we were able to analyze all sequences in one individual

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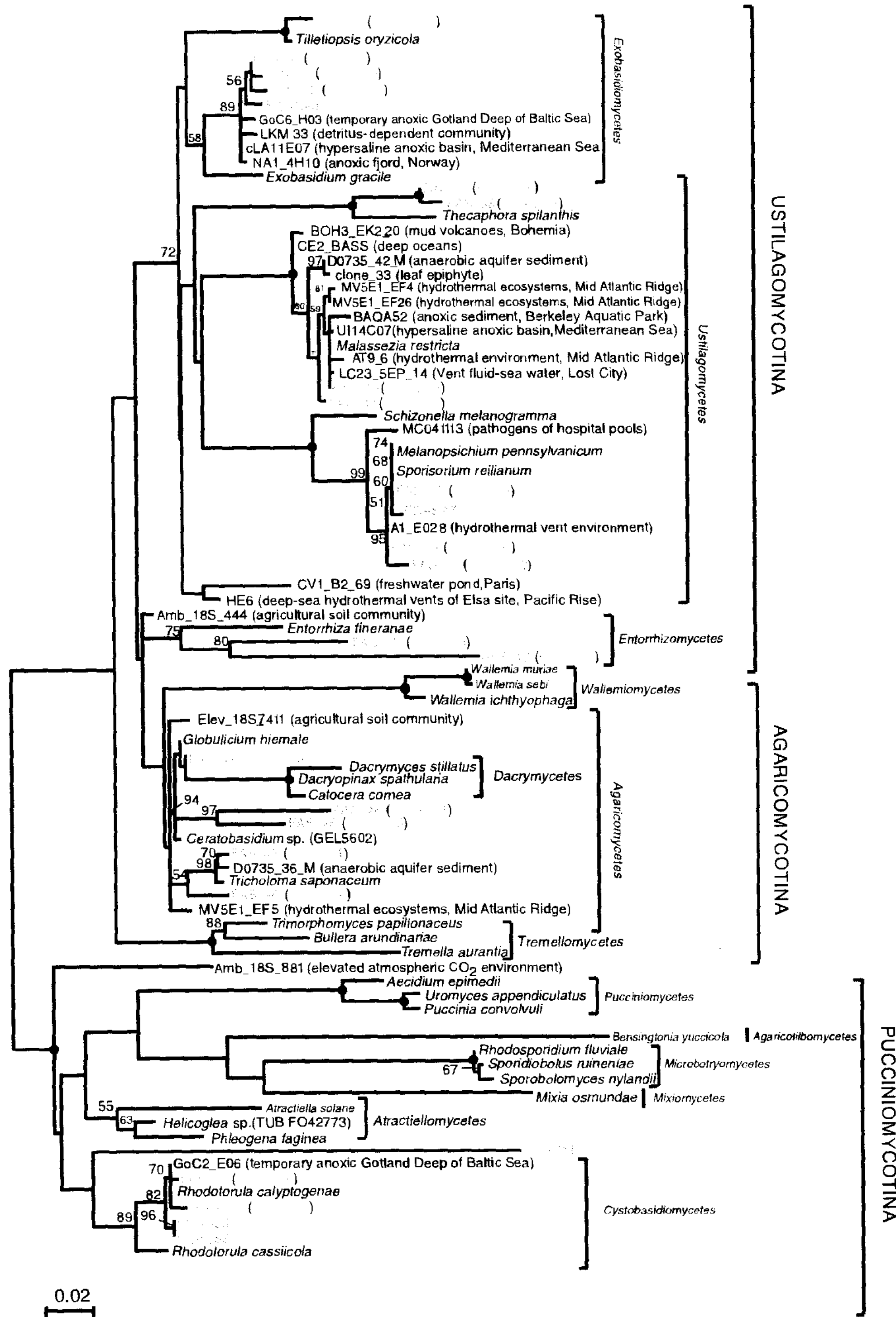

Fig. 2. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phylum *Basidiomycota* showing the positions of environmental OTUs and from cultured isolates obtained from the Arabian Sea. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.737 and the proportion of invariable sites at 0.336, based on 785 unambiguously aligned positions. Further legend as in Fig. 1.

DOTUR run, which resulted in 48 distinct OTUs overall, indicating that a number of OTUs obtained by the individual primer-set analyses were shared among two or three primer sets. Table 4 presents an overview of the numbers of clones, RFLP patterns, sequences and OTUs analyzed in this study.

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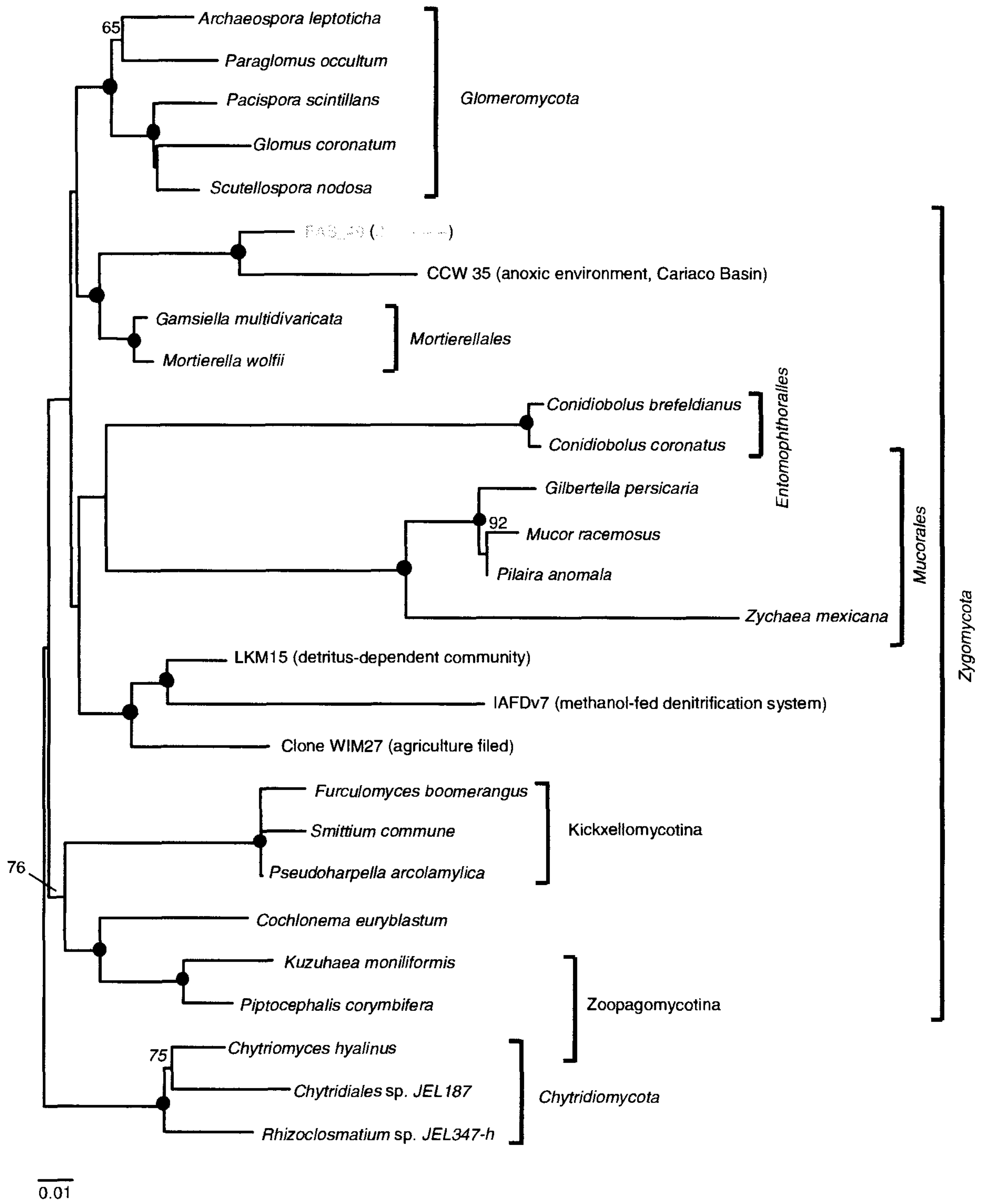

Fig. 3. Maximum-likelihood phylogenetic tree of the 18S rRNA gene sequences of phyla *Zygomycota*, *Glomeromycota* and *Chytridiomycota* showing the position of the environmental OTU obtained from the Arabian Sea branching as sister to *Mortierellales*. The tree was constructed using a GTR+I+G evolutionary model with the variable-site γ distribution shape parameter at 0.571 and the proportion of invariable sites at 0.39 based on 1436 unambiguously aligned positions. Further legend as in Fig. 1.

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Selectivity of fungal-specific primers

More than half of the unique RFLP patterns obtained and analyzed by sequencing and BLASTN ($n=549$) turned out to be of nonfungal origin ($n=281$) (Table 4). As expected, the largest proportion (74.07%) of such nonfungal sequences was retrieved within the RFLPs obtained with the universal eukaryote primer set EukAB, but also putatively fungal-specific primer sets retrieved 48.18% (Fung1) and 35.05% (Fung2) unique nonfungal taxa (Table 3). These findings met our expectations, considering that primer set Fung1 has a lower specificity to fungi compared with primer set Fung2 (see Materials and methods).

Specific PCR primers for environmental studies aim to amplify all members of the fungal community without bias, while excluding the coamplificaton of other eukaryote sequences. As noted earlier (Anderson *et al.*, 2003), this is particularly difficult using the highly conserved 18S rRNA gene region (Page & Holmes, 1998). Several authors claim that they have designed or applied truly specific fungal 18S rRNA gene primer pairs (Smit *et al.*, 1999; Borneman & Hartin, 2000). However, there is conflicting evidence for this specificity as other authors reported amplification of nonfungal templates using the same primer pairs (Borneman & Hartin, 2000; Anderson *et al.*, 2003). It seems that most, if not all, fungal 18S rRNA gene primers are prone to observable cross-kingdom amplifications. Further evidence for this comes from BLAST homology analyses for fungal 18S rRNA gene primers indicating a relatively low success rate for most primer pairs in recovering fungal sequences, but coamplification of nonfungal targets (Pang & Mitchell, 2005). The degree to which cross-kingdom amplification occurs largely depends on the choice of specific primers, PCR conditions but also the abundance of specific fungal taxon groups in relation to the co-occurrence of eukaryote nontarget organisms in the environmental samples under study, i.e. when nontarget DNA is much more abundant in an environmental sample than target DNA, it is reasonable to assume that there is a greater than usual chance of nontarget DNA being amplified. Such systematic biases can distort diversity assessments. Furthermore, different fungal-specific primer sets may amplify different subsets of the fungal community (Fig. 4; Anderson *et al.*, 2003). This severely affects the comparability of different fungal 18S rRNA gene data sets obtained from PCR amplification with different primers. A solution may arise from the application of a multiple-primer approach as suggested for general microbial eukaryote diversity surveys (Stoeck *et al.*, 2006).

Table 3. Taxonomic assignment of unique nonfungal RFLPs ($n=281$) after sequencing and BLASTN analysis of one representative sequence from each RFLP pattern ($n=549$), obtained after restriction digest of 1440 clones

Primer set	Taxonomic assignment of nonfungal RFLPs	Proportion of unique nonfungal RFLPs obtained with each primer set (%)
Fung1	*Corallochytrium*; Choanoflagellates; Metazoa (*Gastrotricha*)	48.18
Fung2	Bacteria (*Proteobacteria*); *Viridiplantae*; Rhizaria; *Corallochytrium*; Metazoa (*Gastrotricha*)	35.05
EukAB	Bacteria (*Proteobacteria*, *Bacteroidetes*, *Firmicutes*); Choanoflagellates; Rhizaria, *Eugleonozoa*, Alveolata; Metazoa (Arthropods, Nematodes, Annelids)	74.07

The three primer sets used to generate the individual clone libraries are described in Table 2. Values given in the last column depict the proportion of unique nonfungal RFLP patterns (identified after BLASTN analyses of obtained sequences from each unique RFLP) relative to the number of total distinct RFLP patterns obtained with the respective primer set (193 for Fung1; 194 for Fung2; and 162 for EukAB). A table detailing the taxonomic affiliation of each individual nontarget sequence (RFLP pattern) is given as Supporting Information.

Diversity patterns of fungal communities in the Arabian Sea

A UPGMA cluster analysis of the $J_{abundance}$ index (Fig. 5) reveals that the fungal communities from the different sampling sites (Table 1) are distinctly different from each other. Of all the comparisons, the two coastal sites sampled under anoxic and oxic conditions (25_ANS, 25_OXS) are most similar in their fungal community membership ($J_{abundance}=0.48$). The sediment (200_ANS)- and water column (200_ANW)-derived fungal communities from the OMZ offshore site cluster together with a $J_{abundance}$ of 0.35. The fungal community from the coral reef reference site (03_CRS) showed the highest dissimilarity to all other samples ($J_{abundance}=0.06$–0.1)

The Indian Ocean has a characteristic seasonal anoxic condition that develops along the western margin during October to January (Naqvi *et al.*, 2006). In this process, the coastal sites are subjected to seasonal oxygen fluctuations. Our analysis suggests a clear separation of fungal communities adapted to permanently oxic conditions, temporal anoxia and the ones adapted to permanent anoxia. Recent studies have shown that numerous fungi can adapt to alternate modes of respiration depending on oxygen availability (Daiber *et al.*, 2005; Jebaraj & Raghukumar, 2009). It is then reasonable to assume that the fungal community of the coastal site (25_ANS, 25_OXS), considered as an entity, may be capable of physiological adaptation when the oxygen

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Table 4. Overview of the total number of clones analyzed per library and primer set, total number of distinct RFLP patterns obtained, number of unique RFLP patterns that could be assigned to fungi and nonfungal taxon groups (for each distinct RFLP, we obtained a partial sequence that was analyzed by BLASTN) and number of distinct OTUs (called at 99% sequence similarity) obtained after DOTUR analyses of full-length fragments for each primer set

Clone library	Total number of clones analyzed by RFLP	Total number of distinct RFLP patterns	Number of unique RFLP patterns assigned to fungi after sequencing	Number of unique fungal OTUs	Number of unique RFLP patterns assigned to nonfungal taxon groups after sequencing
Primer set: Fung1					
25_ANS	96	38	35	10	3
25_OXS	96	24	23	6	1
200_ANS	96	36	4	3	32
200_ANW	96	62	18	5	44
03_CRS	96	33	20	3	13
Total	480	193	100	25*	93
Primer set: Fung2					
25_ANS	96	46	29	10	17
25_OXS	96	30	27	11	3
200_ANS	96	37	26	9	11
200_ANW	96	55	20	17	35
03_CRS	96	26	24	11	2
Total	480	194	126	54*	68
Primer set: EukAB					
25_ANS	96	42	17	6	25
25_OXS	96	17	7	2	10
200_ANS	96	36	17	6	19
200_ANW	96	28	0	0	28
03_CRS	96	39	1	1	38
Total	480	162	42	12*	120
Overall	1440	549	268	48†	281

The total number of OTUs does not equal the sum of OTUs of the individual libraries, but reflects the number of unique OTUs for all five individual libraries (obtained with the same primer set) together. For details, see Materials and methods.

†The overall number of OTUs does not equal the sum of OTUs of the three individual primer sets (=91), but is a result from a separate DOTUR analysis that determined the number of unique OTUs for all three individual primer sets together. For details, see the Materials and methods.

concentration decreases. This adaptation is neither a prerequisite for survival in oxygenated coral reef sediment (03_CRS) nor for life under permanently anoxic conditions (200_ANS, 200_ANW).

Apart from a few exceptions (e.g. some *Chytridiomycetes*), no strictly anaerobic fungi have been described. This, however, contrasts with molecular diversity surveys in anaerobic aquatic environments, where fungal sequences sometimes account for a significant fraction of the clones (Dawson & Pace, 2002; Edgcomb *et al.*, 2002; Stoeck & Epstein, 2003; Luo *et al.*, 2005; Stoeck *et al.*, 2006, 2007). Some recent studies in permanently anoxic habitats have found novel fungal clades to be restricted to these environments (Luo *et al.*, 2005; Brad *et al.*, 2008; Laurin *et al.*, 2008). Support for the adaptation of fungi to anoxia comes from reports of some ascomycetes (Dumitru *et al.*, 2004; Sonderegger *et al.*, 2004) and some basidiomycetes (Fell *et al.*, 2001) that are capable of fermentation and anaerobic growth. Some of these fungi have even been isolated from anaerobic deep-sea environments (Nagahama *et al.*, 2003).

Considering these wide ecological and physiological adaptations of different fungi, it is not unexpected that fungal communities thriving under oxygenated conditions (25_OXS) are distinctively different from fungal communities living under anoxic conditions (25_ANS). The same applies to those from anoxic sediments (200_ANS) and anoxic water samples (200_ANW). However, the actual degree of dissimilarity is difficult to assess with our data due to severe undersampling (Fig. 6).

Phylogeny of environmental fungal rDNA sequences

The analyses of the intrastrain variability of the 18S rRNA gene of five strains obtained in this study revealed a maximum divergence of 0.77% (FCAS35, 0.18–0.36%; FCAS36, 0.17–0.5%; FCAS41, 0.25–0.77%; FCAS89, 0–0.14%; and FCAS125, 0%). Thus, we are confident that a 1% divergence between two different OTUs is sufficient to represent distinct genotypes. The environmental OTUs identified in this study are designated as FAS (fungal OTUs

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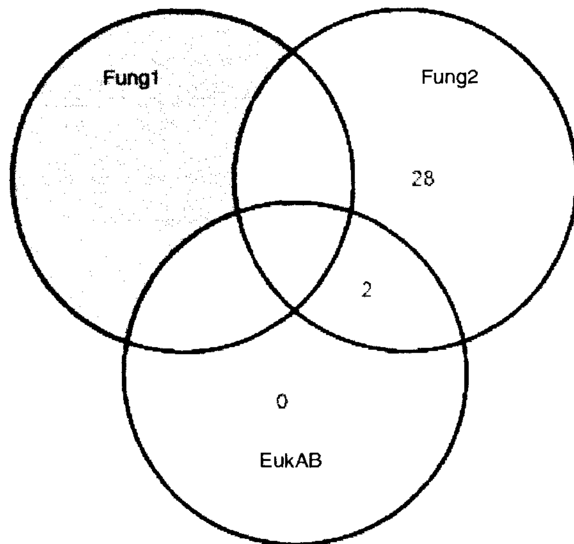

Fig. 4. Venn diagram showing the number of OTUs that were recovered simultaneously by two or three of the primer sets used in this study (Fung1, Fung2 and EukAB; see Table 2) and number of OTUs recovered exclusively with a single primer set. All OTUs, which were retrieved by the universal eukaryote primer set (EukAB), were also detected with the two fungal-specific primer sets (Fung1 and Fung2).

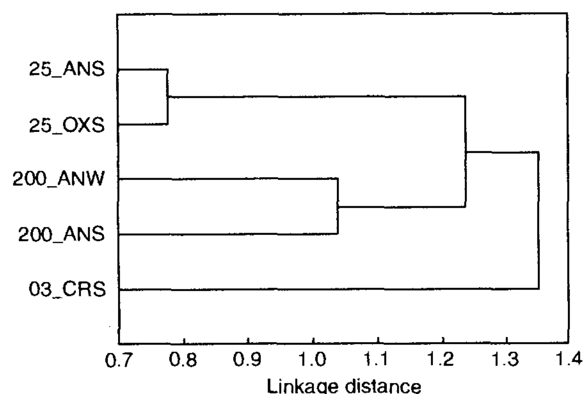

Fig. 5. Dendrogram resulting from calculated Jaccard index based on incidence ($J_{incidence}$) of unique OTUs, as a measure of community similarity between the samples under study. For information on sampling sites, see Table 1. Similarity values were transformed into a distance matrix and subsequent cluster analysis was performed using the UPGMA algorithm.

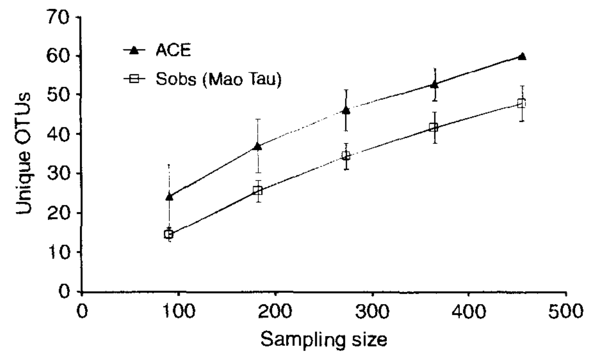

Fig. 6. Sampling saturation analyses of our fungal molecular diversity survey in the Arabian Sea. Species accumulation curve and cumulative abundance-based coverage estimator (ACE) total diversity estimate for all libraries (see Table 1) are plotted.

from the Arabian Sea). The OTUs from the five sample sites were assigned to the Dikarya (*Ascomycota*, 27 OTUs; *Basidiomycota*, 20 OTUs), and one OTU branched within the subphylum Mucoromycotina (phylum *Zygomycota*).

OTUs belonging to the *Ascomycota* (Fig. 1) grouped within the subphyla Saccharomycotina and Pezizomycotina. FAS_2 is the most frequently represented OTU with a close similarity to the yeast *Kodamaea* sp. (99.67%) belonging to *Saccharomycetes*. *Saccharomycete* yeasts are able to cope with anoxia while producing ethanol (Prior *et al.*, 1989). A large number of OTUs (FAS_6, FAS_7, FAS_8, FAS_9, FAS_10, FAS_11 and FAS_12) belong to *Sordariomycetes*. Their closest known relatives belong to the genus *Fusarium*, which serves as a model to study fungal denitrification under low-oxygen conditions (Takaya *et al.*, 1999; Daiber *et al.*, 2005). Six OTUs from the Arabian Sea (Fig. 1) branch within the *Eurotiomycetes*. These sequences are affiliated with a number of environmental sequences obtained from a hypersaline anoxic Mediterranean deep-sea basin (Alexander *et al.*, 2009), anoxic meromictic lake sediments (Takishita *et al.*, 2007a), anoxic coastal sediments (Dawson & Pace, 2002), acidic mine drainages (Baker *et al.*, 2004), hydrothermal vent habitats (López-García *et al.*, 2007) and boiling springs' lake sediment (Wilson *et al.*, 2008). This strongly indicates that the respective phyloclade includes extremophile fungi that are capable of thriving in the most extreme aquatic environments known to date. Described representatives in this clade are *Penicillium* and *Aspergillus*, versatile ubiquitously distributed species that are capable of anaerobic denitrification (Takasaki *et al.*, 2004). This agrees with our observation that under laboratory conditions, isolate FCA-S31a (99.45% similarity to *Penicillium namyslowskii*) grows anaerobically while reducing nitrate (Jebaraj & Raghukumar, 2009). The widespread distribution of ascomycete fungi in oxygen-depleted and extreme habitats is also indicated by the close affiliation of a number of further OTUs from the Arabian Sea to environmental clones obtained from other anoxic habitats and extreme environments. For example, FAS_5 is 99.88% similar to P1_3m2 obtained from anoxic aquifer sediment (Brad *et al.*, 2008), and the dothideomycete OTU FAS_24 is 98.92% similar to an environmental clone from the Mid-Atlantic hydrothermal vent sediment (López-García *et al.*, 2007).

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Within the phylum *Basidiomycota* (Fig. 2), we identified sequences branching in the subphyla Pucciniomycotina, Agaricomycotina and Ustilagomycotina. Only a few are closely (> 97% sequence similarity) related to previously deposited sequences of described basidiomycetes. For example FAS_47 is 99.46% similar to the wood-decaying saprophyte *Globulicium hiemale*, and FAS_28 and FAS_29 are similar to the nitrogenous compound scavenger *Rhodotorula cassiicola* (99.83% and 98.60% sequence similarity, respectively). Most OTUs are more divergent from sequences of described taxa such as the *Ustilagomycetes* FAS_38 and FAS_39, exhibiting only 93% sequence similarity to the plant pathogen *Thecaphora spilanthis*. Many basidiomycete OTUs from the Arabian Sea branch in clades that include other environmental fungal sequences from a variety of oxygen-depleted habitats. Examples are three exobasidiomycete OTUs (FAS_30, FAS_31 and FAS_32), which are related to sequences from an anoxic Norwegian Fjord (A. Behnke, K. Barger, J. Bunge & T. Stoeck, unpublished data), an anoxic Mediterranean deep-sea basin (Alexander *et al.*, 2009) and an anoxic basin in the central Baltic Sea (Stock *et al.*, 2009). The closest described relative of this sequence clade is the plant pathogen *Exobasidium gracile*, with at least 37.3% sequence divergence. The OTUs FAS_40 and FAS_41 branch together with sequences from hydrothermal vent ecosystems (López-García *et al.*, 2007; T. Le Calvez, S. Mahe & P. Vandenkoomhuyse, unpublished data), anoxic intertidal sediment (Dawson & Pace, 2002), an anoxic Mediterranean deep-sea basin (Alexander *et al.*, 2009) and other deep-sea sites (Bass *et al.*, 2007). This cluster, belonging to Ustilagomycotina, is within a clade that has been recognized as a 'hydrothermal and/or anaerobic fungal group' (López-García *et al.*, 2007). The closest described species branching in this clade is the rare skin pathogen *Malassezia restricta*, with 0.39% and 2.36% sequence divergence to the OMZ OTUs FAS_40 and FAS_41. Finding a sequence related to a human skin pathogen in anoxic marine environments seems unusual at first glance. However, sequences closely related to *M. restricta* were also reported from hydrate-rich deep-sea sediments of South China Sea (Lai *et al.*, 2007), indicating a wide ecological diversity of taxa falling in this sequence clade. Interestingly, OTUs obtained exclusively from oxic regions of our study grouped together within the agaricomycetes, along with culture sequences and environmental clones obtained from anaerobic aquifers, hydrothermal sediment and agricultural soil (Euringer & Lueders, 2008; Le Calvez *et al.*, submitted; Lesaulnier *et al.*, 2008).

One OTU (FAS_49) obtained from our study (Fig. 3) originates from a fungus other than Dikarya. This clone, together with clone CCW35 from anoxic salt marsh water (Stoeck & Epstein, 2003, sequence similarity 90.45%), branches among zygomycetes. The closest named species was *Mortierella wolfii*, subphylum Mucoromycotina, with only 90.02% sequence similarity. There are contrasting reports on the capability of zygomycetes to grow anaerobically (Kurakov *et al.*, 2008; Schmidt *et al.*, 2008). Even though we have a poor representation of zygomycetes in our study, they are reported to be a major component of fungal communities in oxygen-depleted environments (Slapeta *et al.*, 2005; Takishita *et al.*, 2007b; Brad *et al.*, 2008). Similarly, we did not discover *Chytridiomycetes* in our clone libraries. This was unexpected, because the latter taxon group includes fermentative anaerobes (Orpin, 1977) and was found in previous 18S rRNA gene diversity surveys in anoxic habitats (Stoeck *et al.*, 2007; Takishita *et al.*, 2007a, b). This poor representation of fungal groups other than ascomycetes and basidiomycetes may be due to undersampling (Fig. 6), combined with a higher efficiency of the primers to amplify genes from *Ascomycota* and *Basidiomycota* (Anderson *et al.*, 2003; Malosso *et al.*, 2006). Indeed, previous studies also failed to obtain chytridiomycete sequences using Fung1 (Gomes *et al.*, 2003) and Fung2 primers (Malosso *et al.*, 2006).

Even though we are not able to distinguish native from transitory fungi (both spores and mycelium contain rDNA; Osherov *et al.*, 2002), it is reasonable to assume that the majority of fungi detected in this study are indigenous. The reasoning for this assumption is their close relation to a number of fungal sequences detected (some of them exclusively) in oxygen-deficient environments. Furthermore, we conducted a laboratory experiment to largely exclude the possibility of PCR amplification of DNA from nonindigenous fungi. Wind or water currents are vectors to disperse fungal spores. Such transitory material could theoretically be sources of genomic DNA, although the corresponding organisms are not active members of the fungal communities under study. Therefore, we harvested spores from two cultured isolates originating from the sampling sites (the basidiomycete isolate FCAS11 and the ascomycete isolate FCAS21). The protocol that we used to extract nucleic acids from the environmental samples under study failed to extract DNA from these spores. This largely (even though certainly not entirely) excludes the possibility that the clone libraries constructed from the Arabian Sea samples include significant proportions of nonindigenous transitory fungi (spores) (results not shown, but available from the authors upon request).

Cultured fungi

Traditionally, fungal diversity studies in environmental samples are based on cultivation approaches. Limitations of this strategy – such as the inability to separate biomass from particulate material and lack of growth media and cultivation conditions suitable for all members of the community – are

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held responsible for our underestimation of the total fungal diversity (Anderson *et al.*, 2003). Molecular phylogenetic analyses of clone libraries constructed from environmental samples have become the gold standard in fungal diversity research (Pang & Mitchell, 2005). However, this strategy is no panacea, as it has biases such as PCR-primer (un)specificities as discussed above. Combining cultivation-based and cultivation-independent methods may allow for a more complete picture of fungal diversity as each of the methodological strategies may compensate for the biases of the other.

We obtained 26 cultures from all the sampling locations. The taxonomic breadth of the isolated strains is restricted: five isolates (FCAS11, FCAS87, FCAS88, FCAS89 and FCAS90) could be assigned to *Basidiomycota*, predominantly Pucciniomycotina (Fig. 2), and 21 of them belonged to Pezizomycotina of *Ascomycota* (Fig. 1). Both divisions of fungi have a high representation of cultured taxa, indicating the accessibility of these groups to cultivation. With the exception of two ascomycetes (FCAS31 and FCAS129) and three basidiomycetes (FCAS87, FCAS90 and FCAS11), all cultures have > 99% sequence similarities to previously cultured and well-described fungi. This indicates that cultivation using standard techniques generally produces more isolates of already known taxa. We note that cultivation under anoxic conditions may have produced a different set of fungi being more divergent to known taxa. Current efforts are in order to stimulate growth under anoxic conditions using a variety of different media. Thus far, we were able to grow one of the isolates (isolate FCAS11) discussed in this study under anoxic cultivation conditions. This supports the hypotheses that at least some fungi from the OMZ region, subjected to changing oxygen conditions, can readily adapt to anoxia.

We also obtained a few isolates that show only a low similarity to previously described and sequenced fungi. These are, for example the basidiomycete isolates FCAS90 exhibiting 97.10% sequence similarity to *Graphiola cylindrical*, FCAS87 with only 62.5% similarity to *Sporisorium reilianum* and FCAS11 with 92.1% sequence similarity to *Rhodotorula aurantiaca*. These examples reveal another decisive advantage of cultivation over clone library analysis. Having access to (putatively) novel cultures in a living condition paves the way for an in-depth analysis of their phylogeny, morphology and ecology. A current multigene analysis of isolate FCAS11, along with ultrastructural analyses, points to the discovery of a novel fungal taxon on class level with the capability of anaerobic denitrification (C.S. Jebaraj, T. Boekhout, W. Muller, F. Kauff & T. Stoeck, unpublished data). Detailed analysis of the enzymes involved in the nitrate-reducing pathways of cultured fungi and their expression profiles *in situ* can help us to understand their role in the OMZ of the Arabian Sea and other anoxic aquatic systems.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Closest BLASTN match of nonfungal sequences.

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