

Chapter 2

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

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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 N₂O 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 N₂ 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).

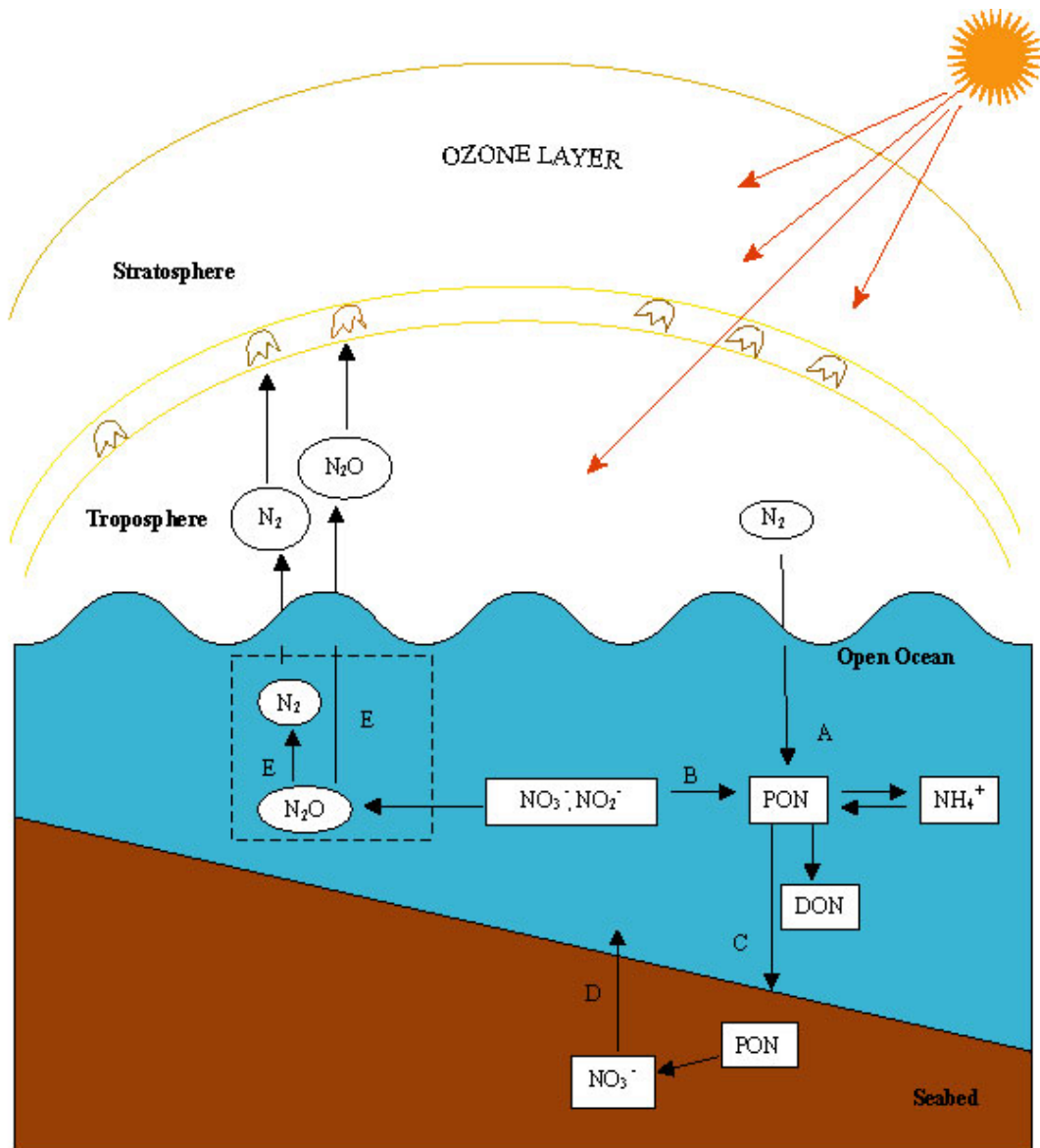


Fig. 2.1 Schematic representation of the oceanic nitrogen cycle: A: Nitrogen fixation, B: Assimilation C: Downward flux and burial into sediment, D: Advection from deep water, E: Denitrification.

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 $< 0.2 \text{ ml L}^{-1}$. 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 $764,000 \text{ km}^2$ 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 pelagic denitrification zone or the OMZ region of the Arabian Sea extends from between the $45^\circ / 73^\circ / 0^\circ / 30^\circ$ (W/E/S/N) in a depth range of 200 m to 1200 m and in the Bay of Bengal $73^\circ / 100^\circ / 0^\circ / 30^\circ$ (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

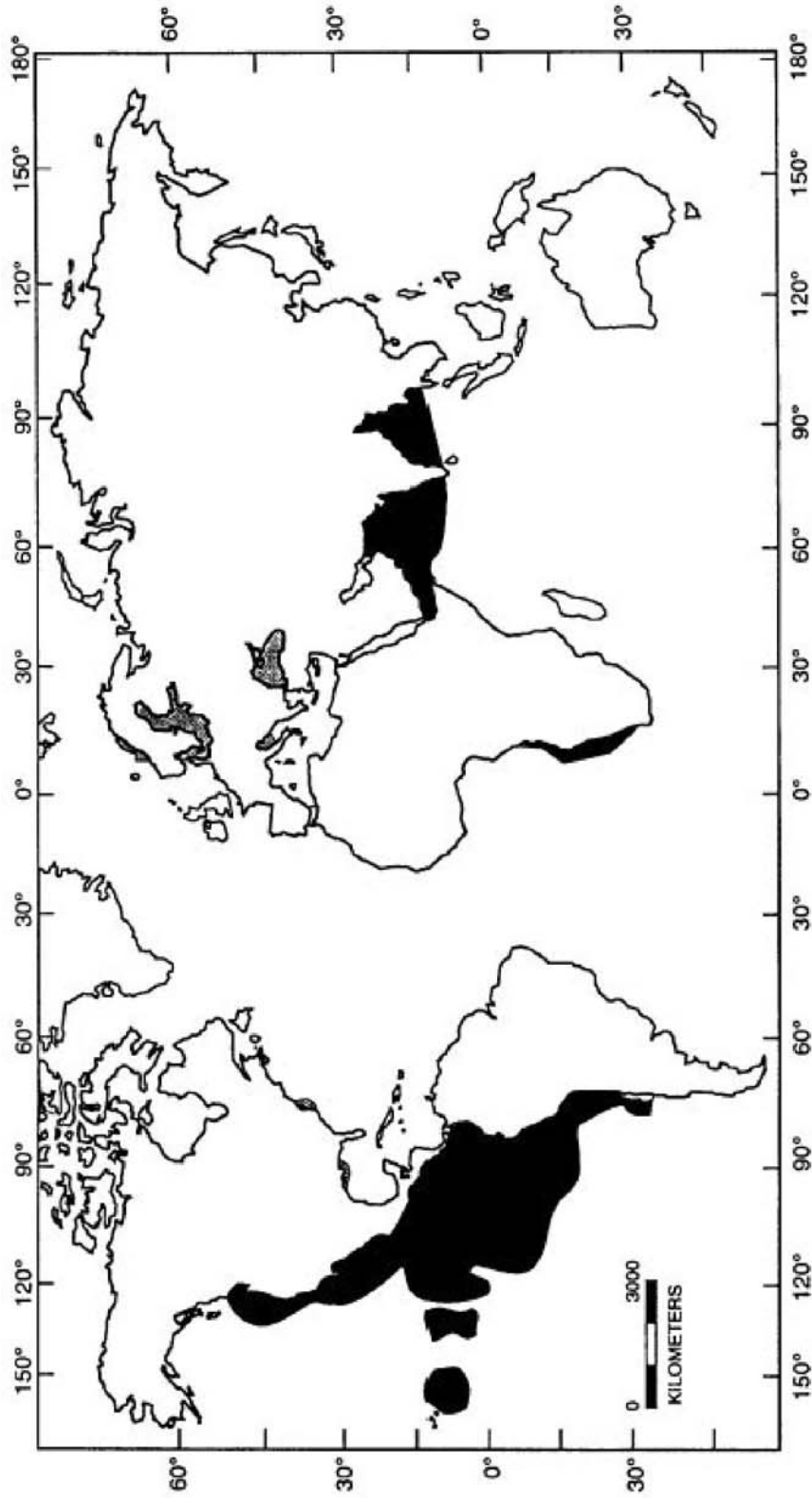


Fig 2.2 Distribution of the world oxygen minimum zones. Open water oxygen minima are shown in black, hypoxic enclosed seas and fjords are grey (Diaz & Rosenberg, 1995)

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 N₂O 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 N₂O production from the Arabian Sea shows an annual production of 0.33 to 0.7 Tg N₂O excluding the N₂O production from the coastal regions which ranges from 0.06 to 0.39 Tg N₂O. If the N₂O production from the shelf region was also taken into account, then the Arabian Sea region would account for approx. 0.4 to 1 Tg N₂O 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 N₂O 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.

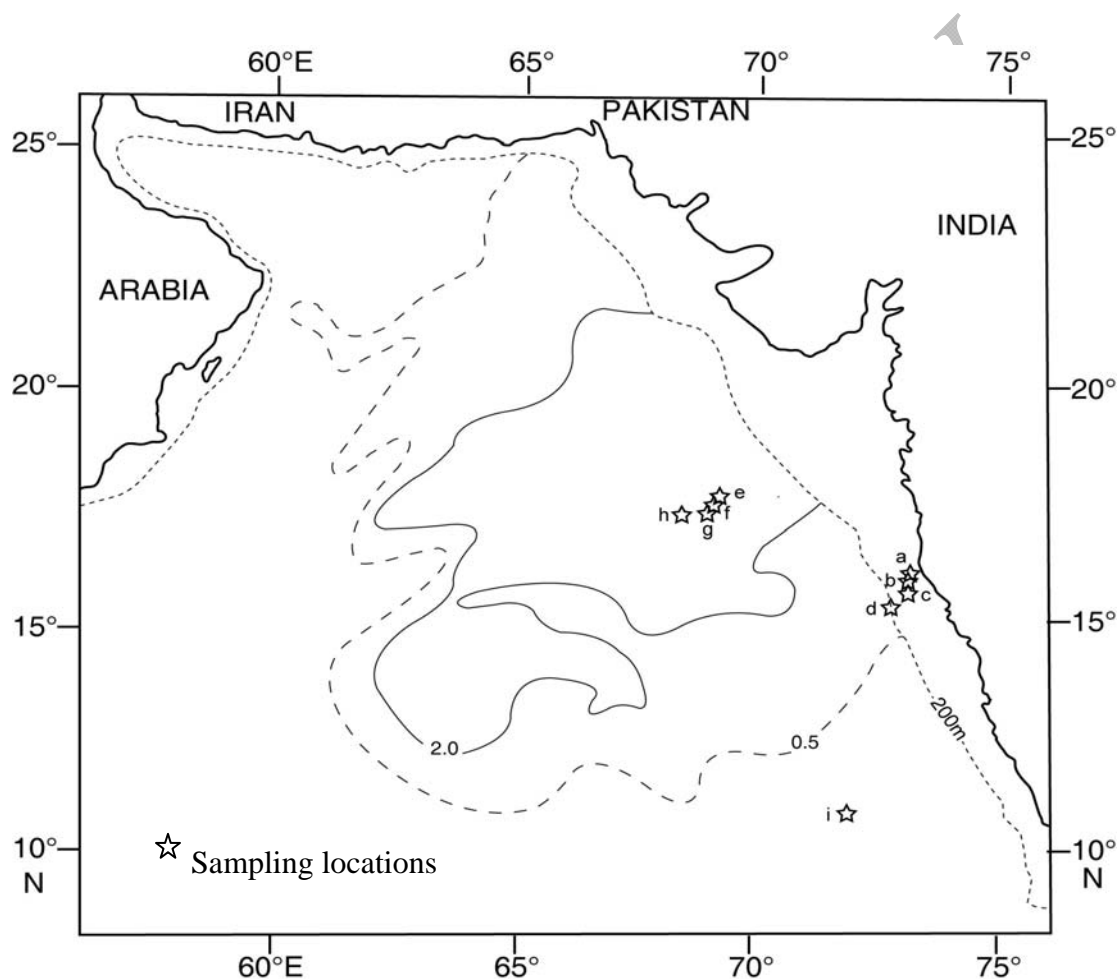


Fig. 2.3 Sampling sites in the Arabian Sea. The contours, represents the distribution of nitrite (μM) accumulation due to denitrification, the 0.5 μM contour marks the boundary of the oxygen minimum zone (Naqvi & Shailaja, 1993). Locations are represented as **a**: CSt-08, **b**: CSt-14, **c**: CSt-26, **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) deployment of corer



(b) retrieved 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 plating 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 subsection 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 $^{\circ}\text{C}$ 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 calcoflour is an optical brightener 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).

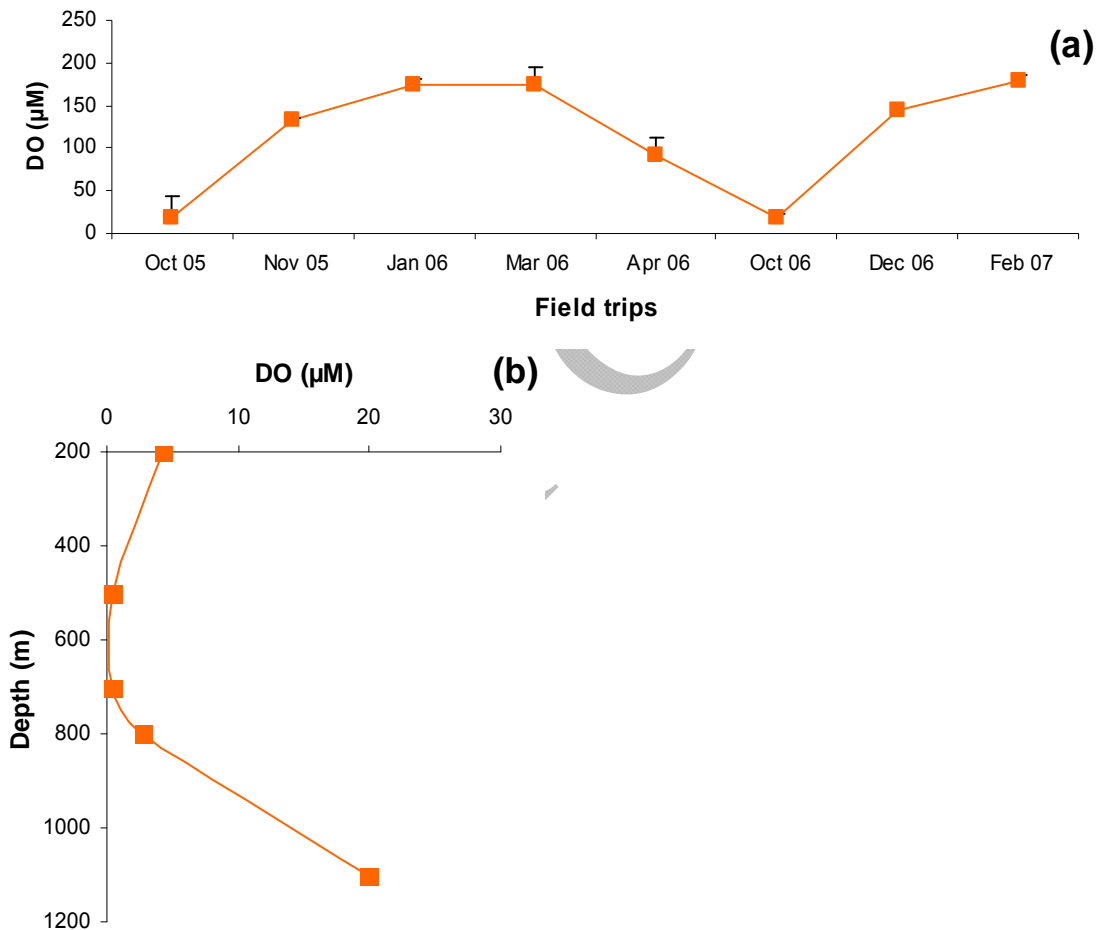


Fig. 2.5 DO levels (a) in the overlying waters of the coastal stations (Cst-08, CSt-14 and CSt-26) and (b) in the overlying waters of the slope stations (SSt-200, SSt-500, SSt-700, SSt-800 and SSt-1100) marked at their respective depths.

In the perennial open ocean OMZ region the DO level between 150 m and 1200 m water column depth is $<5 \mu\text{M}$ and $<1 \mu\text{M}$ 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 $4 \mu\text{M}$, and at SSt-500 and 700 it was less than $1 \mu\text{M}$ and the DO level at SSt-800 and SSt-1200 was about $2.75 \mu\text{M}$ and $20 \mu\text{M}$ respectively (Fig. 2.5) and the DO level at the coral reef sampling point was $\sim 94 \mu\text{M}$.

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 ($175 \mu\text{M}$). 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).

Microbial abundance in the denitrification zones of the Arabian Sea

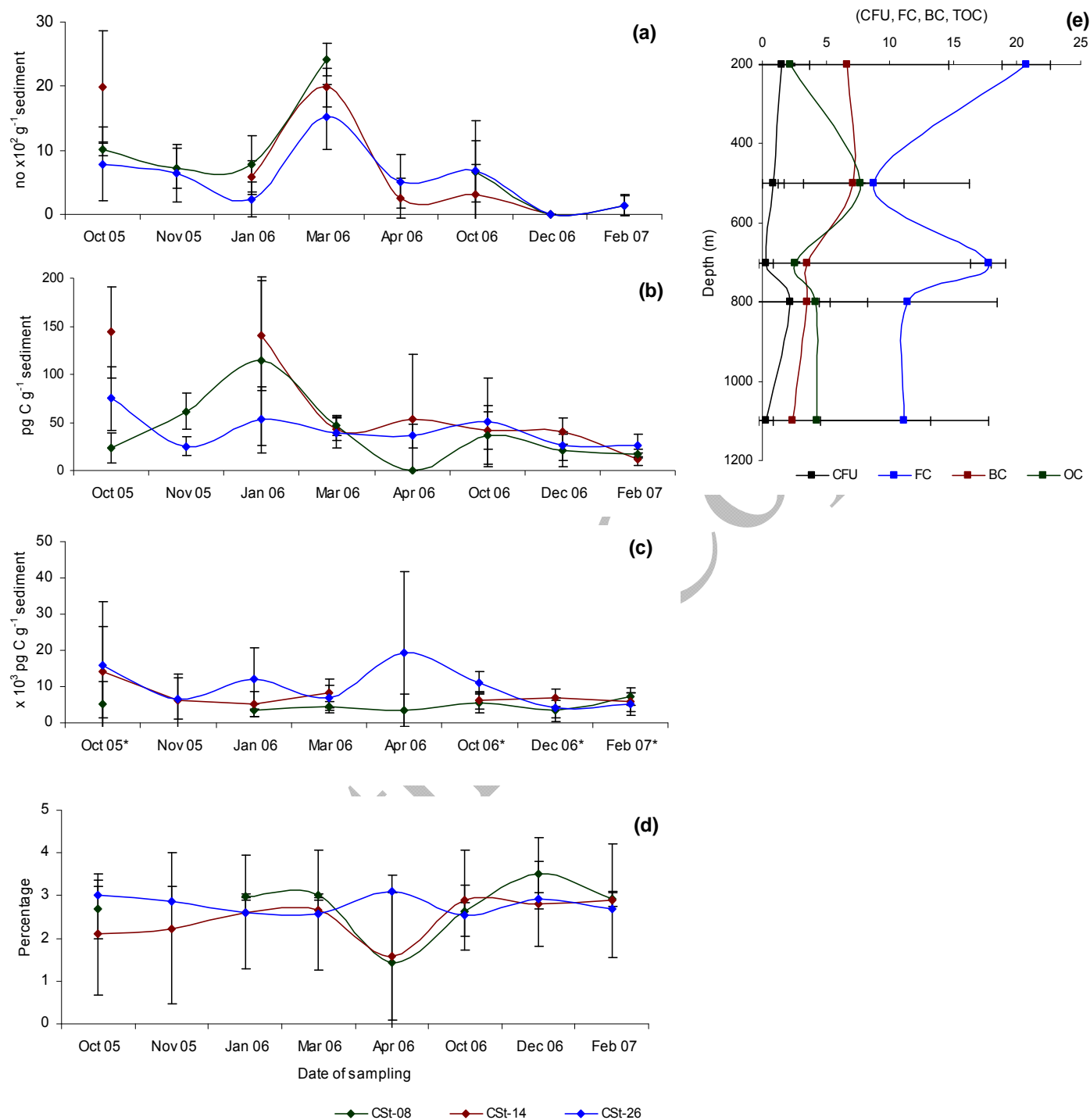


Fig. 2.6 Seasonal changes in the (a) fungal CFUs, (b) fungal biomass C, (c) bacterial biomass C and * represent $\times 10^4$ pg C g⁻¹ sediment (d) Organic Carbon in the CSt stations and (e) represents the values of CFUs (no $\times 10^2$ g⁻¹ sediment), fungal biomass C (pg C g⁻¹ sediment), bacterial biomass C ($\times 10^3$ 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	2.49x10⁻⁵***

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 $\mu\text{g C g}^{-1}$ 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 $\times 10^3 \mu\text{g C g}^{-1}$ 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).

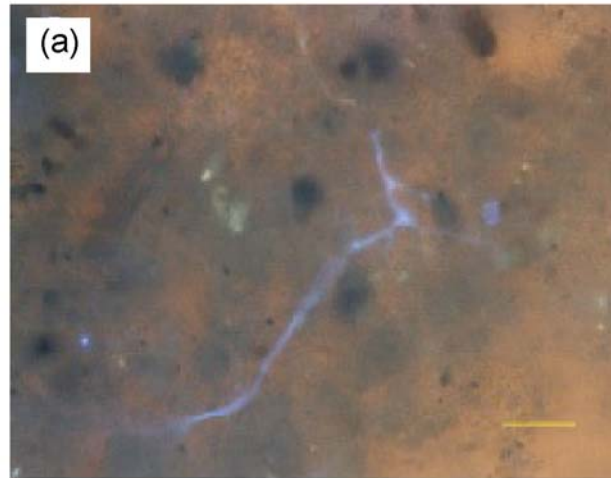


Fig. 2.7(a) Fungal hyphae in sediments stained with optical brightner, Calcofluor
bar = 10 μ M

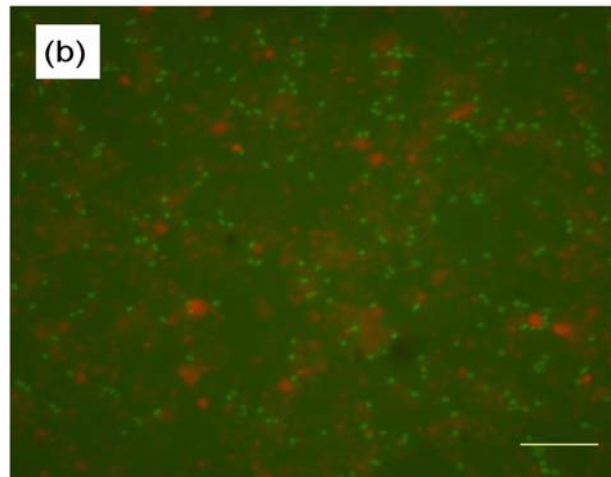


Fig. 2.7(b) Acridine Orange stained bacterial cells from sediments
bar = 10 μ M

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 (*10³ 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 deserts 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.