

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

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Running Title: anaerobic denitrification in marine fungi

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 N₂O. 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.

Key words: Oxygen minimum zone, denitrification, fungi, anoxic sediments, Goa, India

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 (N₂O) 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 N₂O emission, a green house gas that influences the 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).

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)

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 days. At the end of the 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 (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. 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).

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 $1200^{\circ}C$ 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 (*Byssoschlamys* 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 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 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 through the medium for 2 min. 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 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.

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 3A, B). Enrichment culturing was carried out with samples collected from the two stations during the 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 epifluorescence microscopy. Bacterial and fungal biomass for four depths of sediment cores at the two stations ranged between 1.2 to 500 x 10³ pg C g⁻¹ sediment and 0.01 to 0.206 x 10³ 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 & 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 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 calcofluor 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 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 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.

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 $\mu\text{M O}_2$) but excess oxygen ($> 900 \mu\text{M O}_2$) is 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 6B) 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 N₂O. Recently, a benthic foraminifer *Globobulimina pseudospinenscens* has been demonstrated to show complete denitrification 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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Figure Captions

Fig 1- Map showing the site of sampling.

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

Fig 3- Fungal CFU g^{-1} sediment at St-I (A) & St-II (B) (\triangle October 2005, \blacktriangle November 2005, \square January 2006, \blacksquare March 2006, $*$ April 2006).

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 $\times 10^4$ $pg\ C\ g^{-1}$ dry sediments whereas it is numbers $\times 10^3$ $pg\ C\ g^{-1}$ dry sediment during the other sampling periods.

Fungal C biomass in the sediment sections at St-I (C) and St-II (D). (\triangle October 2005, \blacktriangle November 2005, \square January 2006, \blacksquare March 2006, $*$ April 2006).

Fig 6- Percentage OC at St-I (A) & St-II (B). (\triangle October 2005, \blacktriangle November 2005, \square January 2006, \blacksquare March 2006, $*$ April 2006).

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	<i>Aspergillus</i> sp.	12	# 1	<i>Aspergillus</i> sp.	51
	# 2	<i>Aspergillus</i> sp.	5	# 8	<i>Aspergillus</i> sp.	2
	# 8	<i>Aspergillus</i> sp.	17	# 10	<i>Scolicobasidium</i> sp.	2
	# 11	<i>Tritirachium</i> sp.	2	# 11	<i>Tritirachium</i> sp.	6
	# 12	<i>Humicola</i> sp.	33	# 12	<i>Humicola</i> sp.	31
	# 21	<i>Aspergillus</i> sp.	2	# An-3	<i>Aspergillus</i> sp.*	ND
	# An-1	<i>Aspergillus</i> sp.*	ND		Unidentified	8
	# An-2	<i>Fusarium</i> sp.*	ND			
Nov 05	# An-4	<i>Asp.ergillus</i> sp.*	ND			
		Unidentified	17			
	# 1	<i>Asp.ergillus</i> sp.	5	# 2	<i>Aspergillus</i> sp.	3
	# 2	<i>Asp.ergillus</i> sp.	22	# T-27	Thraustochytrid	81
	# T-27	Thraustochytrid	20	# 28	Yeast	3
	# 28	Yeast	2	# 33	<i>Aspergillus</i> sp.	3
	# 30	<i>Myceliophthora</i> sp.	5		Unidentified	11
	# 31	<i>Byssochlamys</i> sp.	5			
	# 31a	<i>Paecilomyces</i> sp.	2			
# 32	<i>Cleistothecial</i> form	2				
Jan 06	# 21	<i>Aspergillus</i> sp.	2			
		Unidentified	34			
	# 42-y	Yeast	3		Unidentified	100
	# 43-y	Yeast	6			
Mar 06	# Th	Thraustochytrids	52			
		Unidentified	39			
	# 1	<i>Aspergillus</i> sp.	40	# 1	<i>Aspergillus</i> sp.	67
	# 2	<i>Aspergillus</i> sp.	12	# 52a	Non-sporulating	27
	# F0	<i>Cladosporium</i> sp.	22	# 54a	<i>Trichoderma</i> sp.	7
	# 21	<i>Aspergillus</i> sp.	1			
	# 57-aY	Yeast	1			
Apr 06	# 57-bY	Yeast	1			
		Unidentified	23			
	# 1	<i>Aspergillus</i> sp.	59	# 1	<i>Aspergillus</i> sp.	59
	# 2	<i>Aspergillus</i> sp.	6	# 2	<i>Aspergillus</i> sp.	6
			# Th	Thraustochytrid	6	
# Th	Thraustochytrid	6		Unidentified	29	
# 60-Y	Yeast	6				
# 11	<i>Tritirachium</i> sp.	6				
# 21	<i>Aspergillus</i> sp.	6				
	Unidentified	12				

* fungi obtained by enrichment culturing; ND: no data

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)

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 (<i>Fusarium oxysporum</i>)	130.2	29.3
# An-2 (<i>Fusarium</i> sp)	86.7	64.0
# 31 (<i>Byssochlamys</i> sp)	70.6	11.0
# 11 (<i>Tritirachium</i> sp)	60.2	61.0
# 31(a) (<i>Paecilomyces</i> sp)	250.3	46.0

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 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 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 6A- Ammonia formation by different fungi (*10³ 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 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 %)

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%)

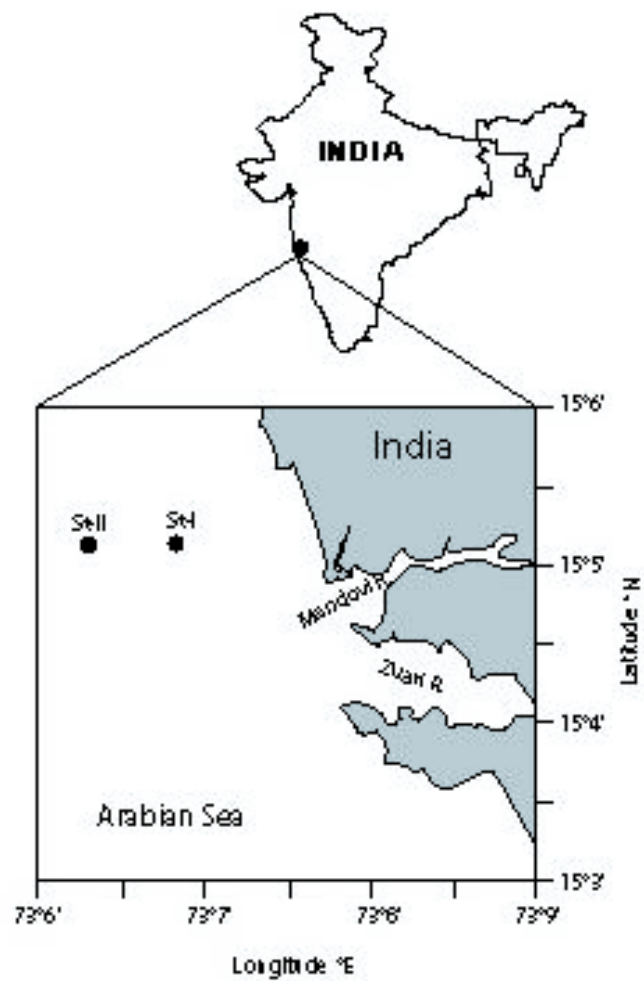


Fig 1- Map showing the site of sampling.

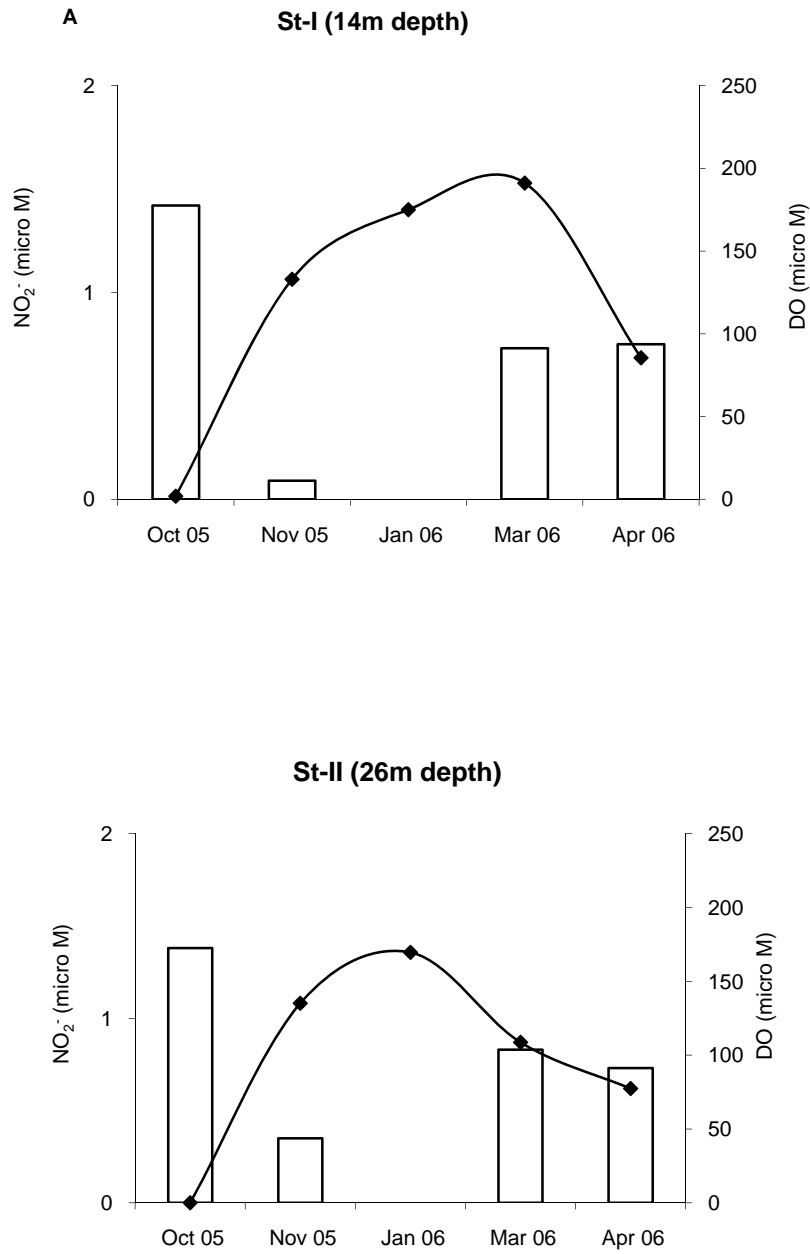


Fig 2- Nitrite levels in micromole (bar) and dissolved oxygen concentration (line) of the near bottom water at St-I (A) & St-II (B).

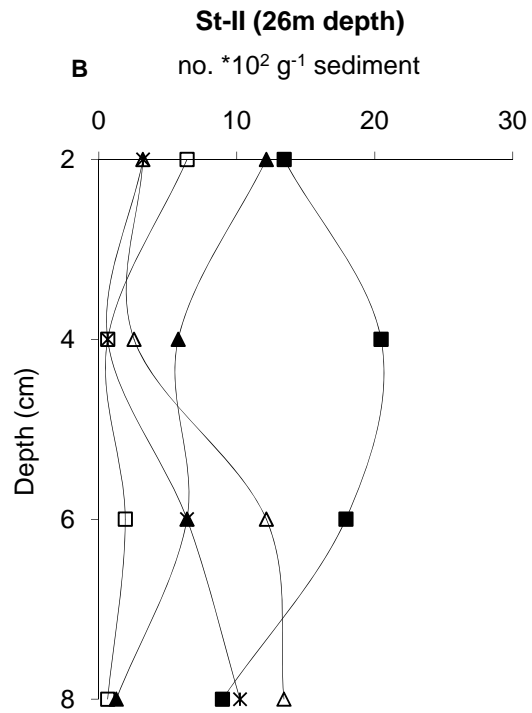
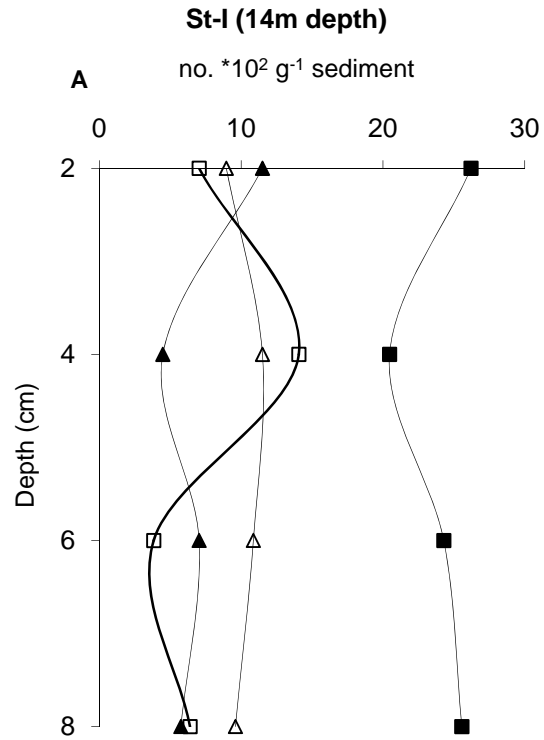


Fig 3- Fungal CFU g⁻¹ sediment at St-I (A) & St-II (B) (△ October 2005, ▲ November 2005, □ January 2006, ■ March 2006, * April 2006).

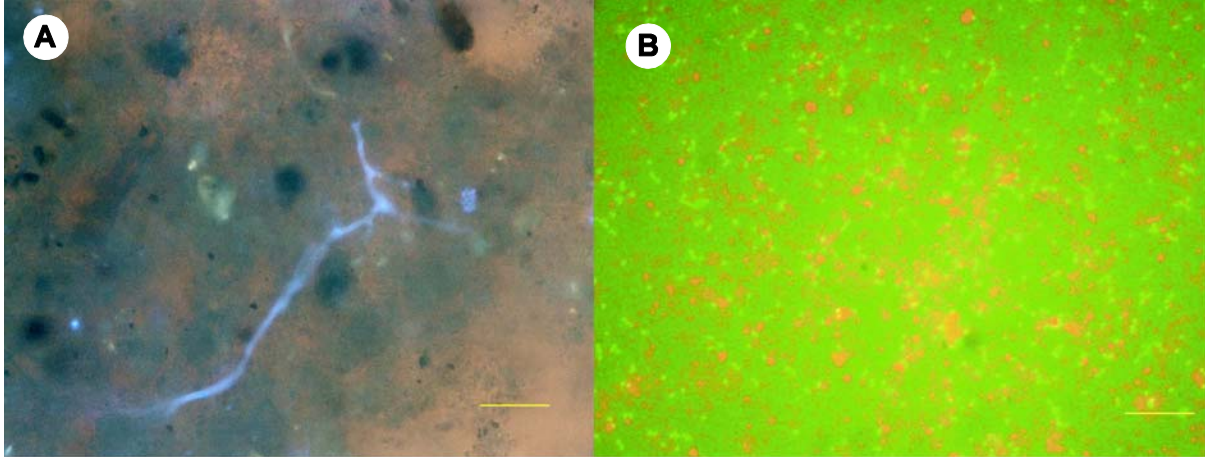


Fig 4- (A) fungal hyphae in sediments stained with the fluorescent brightner, Calcofluor. Bar= 10 μ m, (B) Acridine orange-stained bacteria from the sediments, Bar=10 μ m.

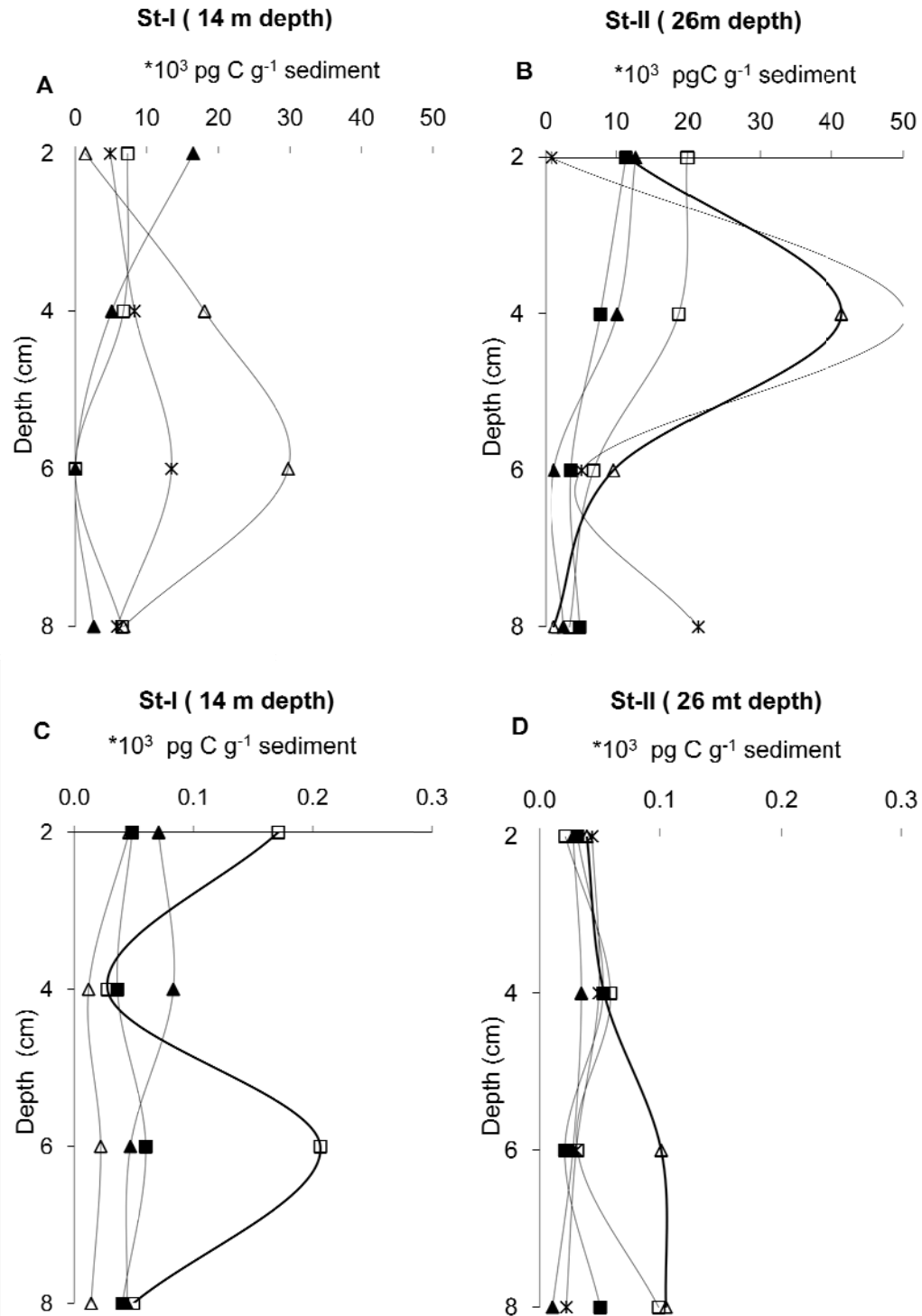


Fig 5- Bacterial C biomass in the sediment sections at St-I (A) & St-II (B). Bacterial C data during Oct 2005 and April 2006 is depicted as numbers *10⁴ pg C g⁻¹ dry sediments whereas it is numbers * 10³ pg C g⁻¹ dry sediment during the other sampling periods. Fungal C biomass in the sediment sections at St-I (C) and St-II (D). (△ October 2005, ▲ November 2005, □ January 2006, ■ March 2006, * April 2006).

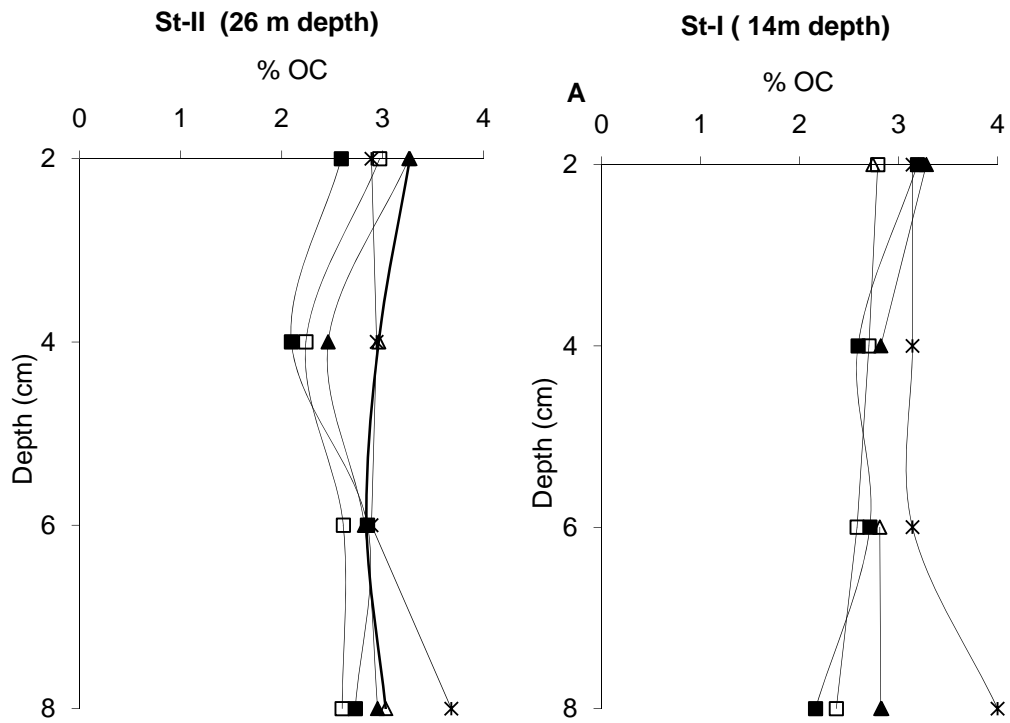


Fig 6- Percentage OC at St-I (A) & St-II (B). (\triangle October 2005, \blacktriangle November 2005, \square January 2006, \blacksquare March 2006, $*$ April 2006).