ECOLOGY OF DENITRIFIERS IN MANGROVE SEDIMENTS

Thesis submitted to
Goa University
for the degree of
Doctor of Philosophy
In
Marine Sciences

By

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DECLARATION

As required under the university ordinance 0.19.8 (IV), I state that the present thesis entitled "***Ecology of denitrifiers in mangrove sediments***" 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 wherever facilities and suggestions have been availed of.

Sheryl Oliveira Fernandes

CERTIFICATE

This is to certify that the thesis entitled, "***Ecology of denitrifiers in mangrove sediments***", submitted by Ms. Sheryl Oliveira Fernandes for the award of the degree of Doctor of Philosophy in Marine Sciences is based on her original studies carried out under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any university or institute.

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Dedicated to my parents

Hipolito & Linda

"The most important role of microbes is their ability to synergistically link various elemental cycles"

P. A. Loka Bharathi

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Sheryl Oliveira Fernandes

Acronyms

ACE	Abundance-based coverage estimator
ANOVA	Analysis of variance
Anx	Anammox
ATP	Adenosine triphosphate
ATU	Allythiourea
C	Carbon
CFU	Colony forming unit
DGGE	Denaturing gradient gel electrophoresis
$^{D}N_2O$	N_2O produced by denitrifiers
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrate reduction to ammonium
DNT	Denitrification
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen
DOTUR	Distance-based OTU and richness
D_{tot}	Total denitrification
Eh	Oxidation-reduction potential
EMBL	European molecular biology laboratory
$^{I}N_2O$	Incomplete denitrification
IPM	Isotope pairing method
IPTG	Isopropyl b-D-1-thiogalactopyranoside
kPa	Kilo Pascal
LB	Luria-Bertani broth
LOM	Labile organic matter
mg	Milli gram
MIMS	Membrane inlet mass spectrometry
ml	Milli litres
mM/mmol	Milli mole
MPN	Most-probable number
MS	Mass spectrometer

mV	Milli volt
N	Nitrogen
ng	Nanno gram
Nif	Di-nitrogen fixation
nM/nmol	Nanno mole
$^{N}N_2O$	Nitrous oxide produced through nitrification
NPL	National physical laboratory
NRA	Nitrate reduction activity
NRB	Nitrate reducing bacteria
OD	Optical density
OMZ	Oxygen minimum zone
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase chain reaction
pg	Pico gram
pH	Hydrogen ion concentration
pmol	Pico mole
Ppbv	Part per billion by volume
psu	Practical salinity unit
PVC	Polyvinyl chloride
RFLP	Restriction fragment length polymorphism
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TC	Total bacterial count
TDA	Phenylalanine deamination
Tg	Terra gram
THB	Total heterotrophic bacteria
TOC	Total organic carbon
µm	Micro meter
µM/µmol	Micro mole

Chapter 1

General Introduction

CHAPTER 1. General introduction

Living organisms utilise nitrogen for the synthesis of organic molecules particularly amino acids, proteins, and nucleic acids. Though the nitrogen gas makes up almost 78% of the earth's atmosphere, it cannot be directly incorporated unless taken up by nitrogen fixing organisms. This fixed nitrogen is converted into more utilisable form (nitrate) for use by plants. Of the various processes in the nitrogen cycle, denitrification converts available NO_3^- to inert gases making it unavailable for uptake. Nitrogen gas is released into the atmosphere following the reduction of nitrate mostly under anoxic conditions. Thus, the production of gaseous nitrogen by microbial reduction of nitrogenous oxides is known as *biological denitrification* (Tiedje, 1982). Denitrification can be distinguished as either assimilative or dissimilative. In assimilative metabolism, nitrate is reduced as a source of nutrient for growth e.g. plants, fungi, bacteria. This process functions under aerobic conditions.

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_2OH \rightarrow NH_3 \rightarrow R\text{-}NH_2$$

In dissimilative metabolism, nitrate is used as an electron acceptor for energy e.g. bacteria.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

It occurs in terrestrial as well as marine environments mainly in regions where oxygen depletion results in nitrate being used as a terminal electron acceptor. Nitrate respiration is kinetically and thermodynamically favorable (Aivasidis *et al.*, 2005) and is preferred over other electron acceptors following the order: $O_2 > NO_3^- > MnO_2 > FeO(OH) > SO_4^{2-} > CO_2$ (Canfield *et al.*, 2005). The oxidation of organic matter during denitrification coupled to nitrate reduction results in a higher ATP yield (Tiedje, 1982).

Denitrification is mediated by facultative anaerobes (Tiedje, 1988; Rich and Myrold, 2004). Many heterotrophic bacteria are involved to some extent in denitrification, most of which are found to be incomplete denitrifiers capable of only reducing nitrates to nitrites with no further reduction of the nitrites produced (Drysdale *et al.*, 1999). True denitrifiers complete denitrification by the formation of byproducts such as nitric oxide, nitrous oxide and di-nitrogen. Some of the major denitrifiers are *Paracoccus denitrificans*, *Thiobacillus denitrificans* and various Pseudomonads. The

process has also been reported to be carried out by lithotrophs (Betlach, 1982; Trouve and Chazal, 1999) and phototrophs (Hiraishi *et al.*, 1995). Many fungi have denitrifying abilities (Shoun and Tanimoto 1991; Shoun *et al.*, 1992). However, fungi evolve nitrous oxide (N_2O) instead of di-nitrogen (N_2) as the final product as they lack N_2O reductase thus differing from bacterial denitrification (Kubota *et al.*, 1999). Most denitrifiers are unable to survive in the absence of nitrogenous oxides as they do not possess the ability to ferment (Tiedje, 1982). Later studies by Jørgensen and Tiedje (1993) have shown that denitrifying organisms have the capacity for long-term survival without O_2 or NO_3^- and appear to survive by carrying on a low level of fermentation. Kennedy and Lawless (1985) propose that chemotaxis may be one mechanism by which naturally occurring populations of denitrifiers survive by successfully utilising available NO_3^- and NO_2^-.

Denitrifying microbial communities have been detected in marine and terrestrial habitats. In terrestrial ecosystems, denitrifier density ranges between 10^{5-6} cells g^{-1} dry soil (Cheneby *et al.*, 2000). In coastal sediments, culture based techniques have shown up to 10^{2-4} cells/g of denitrifier abundance (Michotey *et al.*, 2000; Nogales *et al.*, 2002; Fan *et al.*, 2006) whereas molecular techniques have detected up to 10^6 cytochrome cd1 type denitrifiers in marine samples (Michotey *et al.*, 2000). Denitrification is spread among phylogenetically diverse microbial groups (Falk *et al.*, 2007) and is present in many prokaryotic families like Thermoproteaceae, Cytophagaceae, Corynebacteriaceae, Streptomycineae, Bacillaceae, Rhodospirillaceae, Rhodobacteraceae, Rhizobiaceae, Burkholderiaceae, Nitrosomonadaceae, Neisseraceae, Pseudomonaceae (Philippot and Germon, 2005). In agricultural soils, denitrifiers are more diverse belonging to the genera *Burkholderia-Ralstonia, Pseudomonas, Xanthomonas-Frateuria, Bacillus, Streptomyces* (Cheneby *et al.*, 2000). The genus *Pseudomonas* includes the most commonly isolated denitrifying bacteria from both soils and aquatic sediments (Gamble *et al.*, 1977; Okereke, 1984; Kariminiaae-Hamedaani *et al.*, 2004) and may represent the most active denitrifiers in natural environments (Knowles, 1982). Other dominant denitrifiers are representative of *Alcaligenes* (Jørgensen and Tiedje, 1993; Guynot *et al.*, 1998) and genus *Flavobacterium* (Gamble *et al.*, 1977). *Bacillus jeotgali, Bacillus sphaericus, Bacillus firmus* and *Bacillus bataviensis* related strains have been isolated by Fan *et al.* (2006)

from estuarine sediments. Phylogenetic analysis of denitrifier communities by targeting functional genes mediating the denitrification process indicate that the marine environment is dominated by diverse and novel denitrifiers that are not yet cultured (Falk *et al.*, 2007). *nirS* gene sequences from estuarine sediments have shown close relationship to *Psuedomonas stutzeri*, *Roseobacter denitrificans* (Nogales *et al.*, 2002) while majority of the *nosZ* genes have similarity to *nosZ* genes from isolates affiliated with alpha-subclass of the class Proteobacteria (Magalhães *et al.*, 2008). Sequence analysis of the *nirS* clones from continental margin sediments have been found to relate closely to the *nirS* genes of *Alcaligenes faecalis* and *Pseudomonas stutzeri* whereas *nirK* clones closely related to the *nirK* genes of *Pseudomonas* sp. strain G-179, *Bradyrhizobium japonicum*, *Blastobacter denitrificans* and *Alcaligenes xylosoxidans* (Liu *et al.*, 2003). Differences in denitrifier community composition can potentially influence *in situ* N_2O production in soils indicating that the taxonomic diversity present among denitrifiers is functionally significant (Cavigelli and Robertson, 2001).

Many denitrifiers are metabolically versatile. They are capable of degrading aromatic hydrocarbons like toluene (Evans *et al.*, 1991; Schocher *et al.*, 1991; Fries *et al.*, 1994; Zhou *et al.*, 1995), ethylbenzene (Rabus and Widdel, 1995), naphthalene, phenanthrene and biphenyl (Rockne and Stuart, 2001). Other compounds that can be degraded by denitrifiers include phenol (Tschech and Fuchs, 1987; Van Schie and Young, 1998) and dimethyl phthalate (Liang *et al.*, 2007). Bonin *et al.* (1994) have measured denitrifying activity in marine sediments heavily contaminated by petroleum hydrocarbons indicating that denitrifying activity remained unaffected. Their potential as competent bioremediators has been highlighted in a number of investigations. Rakhimova *et al.* (2004) have demonstrated the efficiency of an oil-oxidizing denitrifying community which was capable of degrading up to 60% oil on nitrate application. Ehrenreich *et al.* (2000) have revealed the capacity of denitrifying bacteria to completely oxidise alkanes and reduce nitrate under anoxic conditions. Denitrifiers have been effectively used in sewage treatment (Satoshi *et al.*, 2005) to convert organic nitrogen to nitrogen gas thus preventing nitrogenous pollutants from being released into the ambient seawater avoiding eutrophication. Some novel denitrifiers mainly relating to *Azoarcus* are able to derive energy from the oxidation of arsenite to arsenate coupled to the reduction of nitrate

whereas inorganic C is used as the carbon source under aerobic conditions (Rhine *et al.*, 2006). Denitrifying strains like *Thiobacillus denitrificans and Pseudomonas stutzeri* can oxidize ferrous iron under autotrophic conditions suggesting widespread occurrence of anaerobic ferrous iron oxidation in sub-oxic zones of aquatic sediments with active denitrification (Straub *et al.*, 1996). Some denitrifiers isolated from tannery wastewaters have been shown to possess high denitrifying potential and can tolerate toxic compounds like chromium and sulphide (Leta *et al.*, 2004).

Denitrification plays a significant role in sediment ecology. Coastal ecosystems are often subjected to eutrophication resulting from run-off from agricultural systems and sewage discharge. Studies have shown that nitrogen is the critical limiting factor to algal growth and eutrophication in coastal marine waters (Ryther and Dunstan, 1971). Denitrification helps to mitigate the excess nitrate by converting it to nitrogen gas, making it unavailable for algal uptake thus maintaining a balance in the ecosystem. In freshwater, high nitrate content is toxic. Denitrification helps to maintain potability of the water. In agricultural systems, dissimilative denitrification is regarded as the major mechanism for N loss. Over irrigated or waterlogged soils develop anoxic conditions promoting denitrification. This affects the fertility of soil and consequently agricultural productivity. Some of the factors promoting the process are high soil moisture conditions, high soil temperature, a low rate of oxygen diffusion, presence of soluble organic matter and nitrate concentration (Luo *et al.*, 1999).

Denitrification is also regarded as a major source of nitrous oxide, a potent green house gas. Though N_2O is responsible for 5–6% of the greenhouse effect (Houghton *et al.*, 1996), its lifetime of about 150 years makes the greenhouse warming potential of this biogenic gas 310 times greater than that of CO_2 (Albritton *et al.*, 1996). N_2O contributes to the destruction of the stratospheric ozone layer (Yamagishi *et al.*, 2007) which protects the earth from harmful ultraviolet radiations from the sun. Estuaries and coastal regions account for approximately 60% of the total oceanic N_2O flux (Bange *et al.*, 1996). Nitric oxide (NO) and nitrous oxide (N_2O) are assumed to be obligatory gaseous intermediates of denitrification. However, there is evidence to show that nitric oxide can be also be emitted during nitrification (Jousset *et al.*, 2001; Stüven and Eberhard, 2001; Kampschreur *et al.*, 2007). Similarly, nitrous oxide is released in high quantities under

low oxygen conditions (Bonin *et al.*, 2002) and could also be released to the atmosphere during nitrification of ammonium (Bremner and Blackmer, 1978). Experiments by Itokawa *et al.* (1996) have shown that nitrification accounted for more than 99.5% of the total emissions whereas 60-98% of N_2O was reduced under anoxic conditions. The ability of denitrifiers to evolve N_2 as a denitrification product varies as many of the predominant isolates are not able to reduce N_2O (Cheneby *et al.*, 2004).

In ecosystems with high inputs of nitrogen, such as estuaries, denitrification mediates nitrogen load reduction and therefore contributes to eutrophication control (Nogales *et al.*, 2002). One such ecosystem is mangroves which constitute nearly 75% of tidal vegetation in tropical regions (Alongi *et al.*, 1989). Mangroves play an important role in the biogeochemical cycles of coastal ecosystems (Thorsten and José, 2001). The proximity of mangroves to human inhabitation, aquaculture farms, waste discharge from industrial units, domestic sewage discharge-points, etc.. make them vulnerable to high nutrient inputs. They protect the coast from tidal erosion, storm surges and trap sediment for land accretion (Pernetta, 1993). Nitrogen is the critical limiting factor to algal growth and eutrophication in coastal waters (Ryther and Dunstan, 1971). The nitrogen cycle within mangrove forests is mediated predominantly by microbial rather than chemical processes (Alongi *et al.*, 1992). A substantial loss of N in mangrove sediment has been attributed to denitrification (Chiu *et al.*, 1996). High litter fall, it's degradation and re-mineralization is one of the factors contributing to high nitrogen concentrations in mangrove forests (Ramos E Silva *et al.*, 2007). Mangrove sediments are largely anaerobic and nitrate availability is the factor controlling denitrification rates (Seitzinger, 1990). Nitrate can either be generated through intrinsic nitrification (Krishnan *et al.*, 2008) or supplied extraneously through runoff from land (Naqvi *et al.*, 2000). Denitrification could therefore play a significant role in sediment ecology by mitigating excess nitrate in the system.

Marine ecological studies aim to understand the interactions of organisms with their surrounding environment which could be either biotic or abiotic in nature. Most of the present knowledge on mechanisms and ecological role of denitrification have been obtained from studies estimating denitrification activity (Bianchi *et al.*, 1994; Vance-Harris and Ingall, 2005; Naqvi *et al.*, 2006) and the factors affecting the process (Yoon

and Benner, 1992; Tuominen *et al.*, 1998). These studies have highlighted the importance of denitrification as a significant sink of fixed/anthropogenically derived nitrogen. At a cellular level, attempts have been made to characterize denitrifiers (Yoshie *et al.*, 2006) and to improve denitrification efficiency or to isolate (Shieh *et al.*, 2004) and assess their diversity in marine sediments (Liu *et al.*, 2003; Santoro *et al.*, 2006). Many studies have dealt with ecological aspects of denitrification in marine habitats but relatively few studies have been undertaken in potential denitrifying sites such as mangrove swamps where active denitrification has been reported to occur (Rivera-Monroy *et al.*, 1995; Chiu *et al.*, 2004; Meyer *et al.*, 2005). Further, mangrove sediments are known to harbour novel denitrifiers (Lin and Shieh, 2006). Molecular studies targeting functional genes like *nirK* and *nirS* have shown rhizosphere associated strains belonging to α-, β-, and γ-*Proteobacteria* (Flores-Mireles *et al.*, 2007). Relatively little research has been carried out to understand the factors that influence denitrifiers in mangrove swamps.

The present study represents the first attempt to examine the extent of denitrification in mangrove sediments of Goa and the bacteria that mediate the process. Their inter-relationships with other physico-chemical and biological parameters have been probed to gain deeper insights into the importance of benthic denitrification. This benthic denitrification is also compared to pertinent oxidative and reductive phases of the cycle particularly nitrification, N_2 fixation, anammox and dissimilatory nitrate reduction to ammonium (DNRA). Interestingly, the study projects the importance of DNRA as an important mechanism that minimizes nutrient loss thereby contributing to the modulation of N_2O, a green house gas to the atmosphere.

Aim

To understand the environmental factors affecting denitrification rates in mangrove ecosystems and to delineate the physiology and taxonomy of the denitrifying population.

Objectives of the present study

- To quantify the abundance and activity of denitrifying bacteria
- To understand the influence of environmental parameters on denitrification
- To identify the denitrifiers at cellular and molecular level
- To delineate the influence of bioturbating organisms on denitrification

Chapter 2

Literature review

CHAPTER 2. Literature review

2.1 The marine nitrogen cycle

Nitrogen (N_2) occurs in the oxidation states ranging from –3 to +5. It is an essential macronutrient limiting biological productivity in aquatic as well as terrestrial ecosystems. Only a few organisms are capable of utilising free N_2 and converting it into other utilizable forms. This conversion involves a series of processes mainly facilitated by a consortium of microbes e.g. nitrogen fixers, nitrifiers and denitrifiers producing a number of intermediate products like ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-) and nitrous oxide (N_2O) as by-products. Among these, NO_3^- is the most oxidized nitrogenous compound and serves as an easily utilizable substrate to primary producers in aquatic systems. Enrichment experiments have shown an increase in phytoplankton standing stock and bacterial production indicating that N is the main limiting nutrient in oceans (Dufour *et al.*, 1999). Nitrogen in the oceans is cycled (Fig. 1) through the following key processes:

Nitrogen fixation

Nitrogen enters the marine environment through deposition and solution.

$$N_2 + 8H^+ + 8e^- + 16ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

Fixed nitrogen is generally considered to limit primary production (Capone, 2001) in many parts of the oceans and serves to relieve nitrogen stress to phytoplankton assemblages (Kustka *et al.*, 2002). Gruber and Sarmiento (1997) have estimated a global nitrogen fixation rate of 28 Tg N y^{-1} (Tg = terragrams / 10^{12} g). Extensive blooms of the cyanobacteria *Trichodesmium* spp. have largely been attributed to fix bulk of nitrogen in the oceans (Capone *et al.*, 1997; Capone and Carpenter, 1982). These cyanobacteria account for annual inputs of 4.8 Tg nitrogen to the world's oceans while benthic environments such as seagrass beds or coral reef flats contribute 15 Tg (Capone and Carpenter, 1982). Other potential nitrogen fixers are populations of picocyanobacteria comprising mainly *Synechococcus* sp. (Spiller and Shanmugam, 1987; Ohlendieck *et al.*, 2000). Molecular studies have revealed a high diversity of heterotrophic nitrogen-fixing organisms in marine cyanobacterial mats represented by Firmicutes, Deltaproteobacteria,

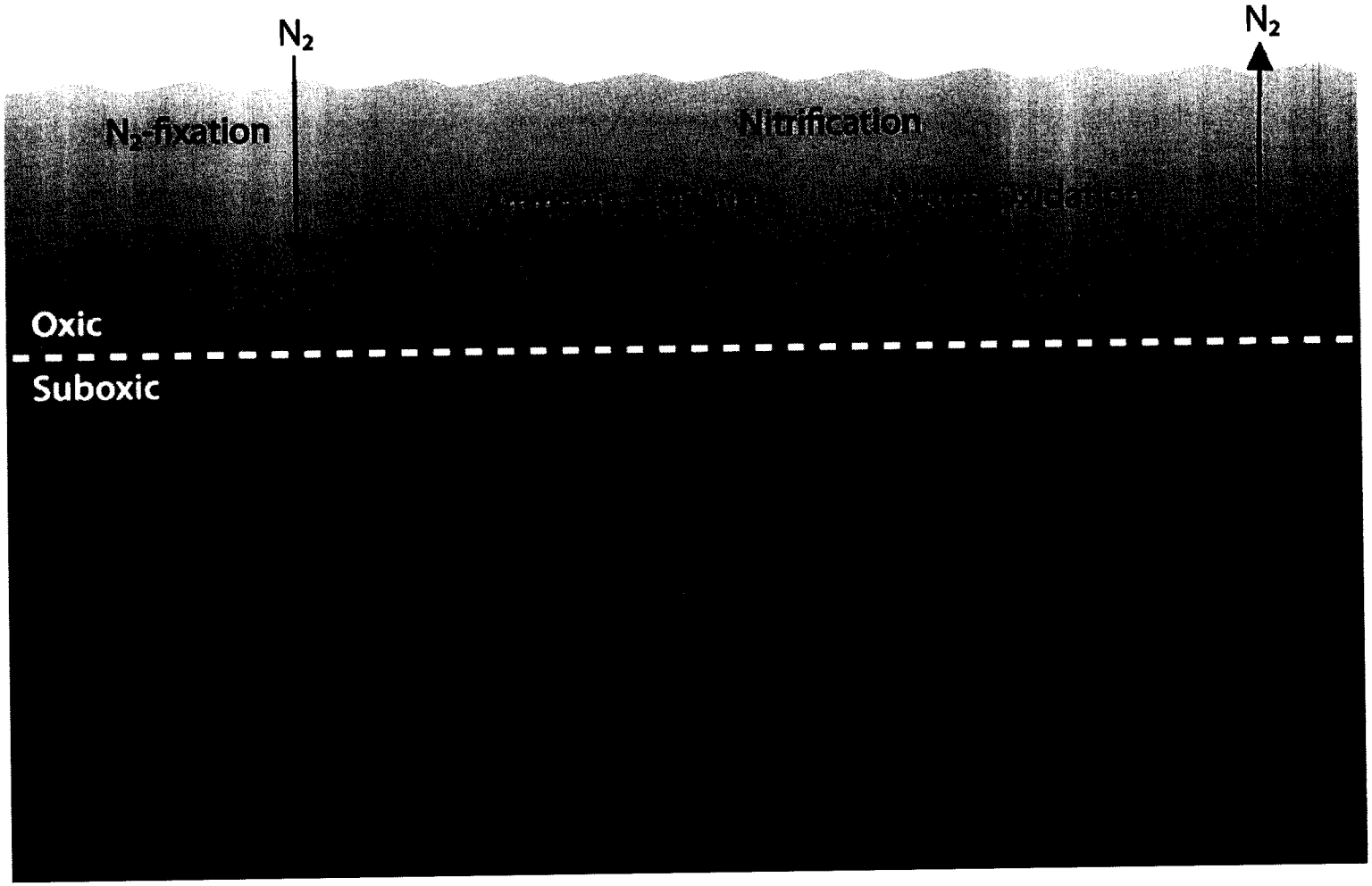


Fig. 1: Key processes in the marine microbial nitrogen cycle (Francis *et al.*, 2007).

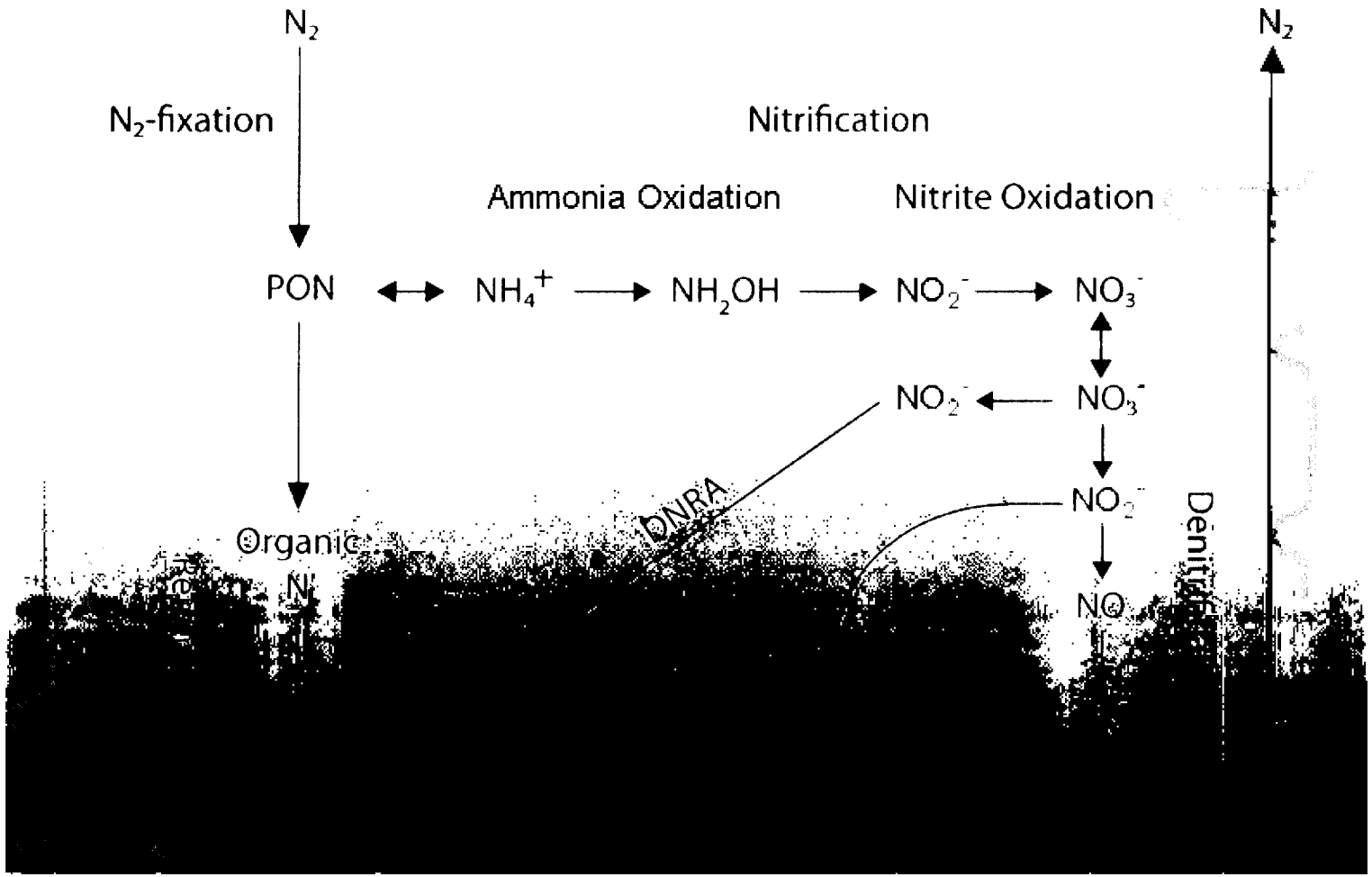


Fig. 1: Key processes in the marine microbial nitrogen cycle (Francis *et al.*, 2007).

Gammaproteobacteria and anaerobic bacteria (Zehr *et al.*, 1995). Nitrogenase, the enzyme catalyzing nitrogen fixation has also been reported in archaea (Zehr *et al.*, 2000), but is not encoded in any eukaryotic genome (Berman-Frank *et al.*, 2003). The total amount of fixed nitrogen in the ocean can be changed substantially by the denitrification processes (Altabet *et al.*, 1995, 2002; Devol, 2002).

Ammonification

Ammonification or mineralization refers to any chemical reaction that generates ammonia as an end product or its ionic form, ammonium. Ammonification occurs through various inorganic reactions or as a result of the metabolic function of microorganism, plants and animals. Nitrogen, in organic form, is converted into ammonium (NH_4^+) by microorganisms. Because it has a positive charge, ammonium can be adsorbed and fixed on to the negatively charged sediment particles or be taken by plants (Brady and Weil, 2002). Nitrates are the forms of nitrogen most commonly assimilated by plant species, which, in turn are consumed by heterotrophs for use in compounds such as amino and nucleic acids. The remains of heterotrophs are then decomposed into nutrient rich organic material. Bacteria or in some cases, fungi, will convert the nitrates within the remains back into ammonia (Smil, 2000).

In aquatic ecosystems, plankton play an important part in the transfer of organic matter to the bottom, and microbial activity in the surficial sediment leads to mineralization of a great part of the organic nitrogen quickly after its deposition (Fernex, *et al.*, 1996). In coastal estuarine surficial sediments, ammonification rates range between 5.8-220 ng N-atoms g^{-1} sediment h^{-1} (Sumi and Koike, 1990).

Nitrification

During nitrification, biological oxidation of ammonia to nitrite is followed by the oxidation of these nitrites into nitrates.

$$\text{Organic N} \rightarrow NH_4^+ \rightarrow N_2O\uparrow \rightarrow NO_2^- \rightarrow NO_3^-$$

This important step in the nitrogen cycle was discovered by the Russian microbiologist, Sergei Winogradsky (Waksman, 1946). The oxidation of ammonia into nitrite, and the

subsequent oxidation to nitrate is performed by two nitrifying bacteria. The first step is done by bacteria of the genus *Nitrosomonas* and *Nitrosococcus*.

$$NH_3 + O_2 \rightarrow NO_2^- + 3H^+ + 2e^-$$

The second step of oxidizing nitrite into nitrate is mainly done by bacteria of the genus *Nitrobacter*, with both steps producing energy to be coupled to ATP synthesis.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e$$

In most environments, both organisms are found together, yielding nitrate as the final product (Mosquera-Corral *et al.*, 2005). Together with ammonification, nitrification forms a mineralization process which refers to the complete decomposition of organic material, with the release of available nitrogen compounds. This replenishes the nitrogen cycle.

In sediments, the extent of nitrification is limited to the zone of oxygen penetration though nitrifying bacteria are found within the anoxic layer (Henriksen *et al.*, 1981). Strauss *et al.* (2002) suggest that NH_4^+ availability and pH are the most important variables regulating nitrification in aquatic sediments in addition to organic C availability which plays a significant role only under high environmental C:N conditions and if most available C is relatively labile. Koike and Hattori (1978) have demonstrated the co-occurrence of nitrification and nitrate reduction in coastal sediments indicating that the nitrate reduction rate was 11 to 17 times higher than the nitrification rate, and nitrogenous oxides derived from ammonium accounted for only 6 to 9% of the N_2 evolution by denitrification. Rysgaard *et al.* (1993) state that in anoxic environments, nitrification is generally coupled to denitrification as the NO_3 produced during nitrification is reduced to N_2 by denitrifying bacteria and lost from the environment or it can be reduced to NH_4^+ by fermentative bacteria or diffuse out of the sediment.

Autotrophic nitrification rates in estuarine sediments have been found to share a marked seasonality with highest rates of 0.92 pg N d^{-1} g^{-1} dry wt. sediment occurring during the summer (Herbert, 1986). Significant rates of nitrification have also been measured in the surficial oligotrophic oceanic waters, with rates of ammonium and nitrite oxidation generally within the range of 10–500 pmol kg^{-1} h^{-1} (Clarke *et al.*, 2007). In estuarine regions, wide variation in nitrification rates occurring between 0 to 1712 ± 666 μmol NH_4^+ m^{-2} h^{-1} have been reported (Magalhaes *et al.*, 2005).

Anammox

Anaerobic ammonium oxidation (anammox) was discovered by researchers in Netherlands while working on NH_4^+ oxidation in a denitrifying fluidized bed reactor (Mulder *et al.*, 1995). Van de Graaf *et al.* (1995) have shown it to be a biologically mediated process. It has been now considered as an important process in the marine nitrogen cycle (Devol, 2003; Dalsgaard *et al.*, 2005; Nakajima *et al.*, 2008).

$$NH_4^+ + NO_2^- \longrightarrow N_2 + 2H_2O$$

During anammox, NH_4^+ is oxidized at the expense of NO_2^- (Meyer *et al.*, 2005) produced by either heterotrophic NO_3^- reduction (Dalsgaard *et al.*, 2003) or aerobic ammonium oxidation (Francis *et al.*, 2005). Van Dongen *et al.* (2001) have proposed the following stoichiometry for anammox:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \longrightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_20$$

Dalsgaard and Thamdrup (2002) ascribe the dependence of anammox on elevated nitrite concentrations. Experiments by Trimmer *et al.* (2005) have shown that decreasing the concentration of NO_3^- but holding NO_2^- at 5 µmol decreased the significance of anammox as a sink for NO_2^- suggesting that anammox is likely to be regulated by the availability of NO_3^- and NO_2^- and the relative size or activity of the anammox population.

Kuypers *et al.* (2005) have shown massive N loss in the pelagic oxygen minimum zones (OMZs) attributing it to anaerobic ammonium oxidation. Up to 67% of the N_2 formation in continental shelf sediments has been attributed to anammox while only 33% of the N_2 formation was due to denitrification (Thamdrup and Dalsgaard, 2002). However, Dalsgaard *et al.* (2003) have shown that anammox accounts for only 19–35% of the total N_2 formation in the anoxic water column in coastal areas. The process could be responsible for up to 50% of the global removal of fixed nitrogen from the oceans (Dalsgaard *et al.*, 2005). Anammox bacteria generally live under ammonium limitation and could be mediating dissimilatory nitrate reduction to ammonium (DNRA) in natural ecosystems (Kartal *et al.*, 2007). Candidatus *Brocadia anammoxidans* (Strous *et al.*, 1999), Candidatus *Kuenenia stutgartiensis*, Candidatus *Scalindua wagneri* and

Candidatus *Scalindua brodae* (Kuypers *et al.*, 2003) are some of the *Planctomycetes* responsible for anaerobic ammonium oxidation.

Denitrification

Denitrification is largely a bacterially-mediated anaerobic process wherein the oxides of nitrogen (NO_3^-, NO_2^-, N_2O) are used as alternative electron acceptors in the absence of oxygen. Organic compounds serve as hydrogen donors. The pathway of denitrification is:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

When nitrate is used as a source of electron acceptors, there is a net loss of nitrogen from the sediment. This process is therefore called dissimilatory nitrate reduction. The oxidation of organic matter during denitrification coupled to nitrate reduction results in a higher ATP yield (Tiedje, 1982). Heterotrophic denitrification follows the stoichiometry (Vance-Harris and Ingall, 2005):

$$5CH_2O_{(\text{organic matter})} + 4NO_3^- + H^+ \longrightarrow 2N_2 + 5CO_2 + 7H_2O$$

In terrestrial ecosystems, denitrification is harmful to crops as nitrogenous fertilizers added to soil are lost resulting in low nutrient availability to the plants. The process is carried out by a variety of facultative anaerobic bacteria like *Pseudomonas, Alcaligenes, Flavobacterium, Bacillus,* etc....

Denitrifying activity is also known to release nitrous oxide, a potent greenhouse gas in the atmosphere. The trace gas plays an important role in the destruction of stratospheric ozone which protects the earth from ultraviolet radiation from the sun. This trace gas with a lifetime of approximately 150 years accounts for 2 to 4% of total greenhouse warming potential (Watson *et al.*, 1992). The atmospheric concentrations of N_2O have increased from 280–290 ppbv before industrial revolution to 350 ppbv at present (Pathak, 1999). Sediment is considered to be one of the major contributors with 65% of the total global emission. In terrestrial ecosystems, 6 to 21% of the annual N_2O flux occurs in agricultural land during thawing of top soil (Bremner *et al.*, 1980). In India, it is estimated that 0.19 and 0.27 Tg yr^{-1} of N_2O is contributed by rice and wheat fields alone (Parashar *et al.*, 1998).

A number of factors are known to influence denitrification. Some of the important factors affecting denitrification rates include variation in oxygen concentrations (Bonin

and Raymond, 1990), trace metals (Labbé *et al.*, 2003), sediment temperature (Holtan-Hartwig *et al.*, 2002; El-Sayed, 2003), sediment pH (Rust *et al.*, 2000; Simek *et al.*, 2002) and bioturbation (Gilbert *et al.*, 1995). Substrate availability limits microbial activity and may alter rates of N_2 fixation and denitrification (Lee and Joye, 2006). Experimental manipulation have shown that denitrification rates are generally limited by the supply of NO_3^-, either from nitrification or diffusion from the overlying water (Seitzinger, 1990; Morell and Corredor, 1993; Corredor and Morell, 1994; Rivera-Monroy and Twilley, 1996; Kana *et al.*, 1998). The quantity and quality of organic matter is also known to influence denitrification (McCarty and Bremner, 2003; Hill and Cardaci, 2004; Qin *et al.*, 2005). Studies have shown that N_2O production rates are affected by the type of organic carbon available as an electron donor and increased organic carbon concentration, indicating that the denitrification potential is organic carbon limited (Pfenning and McMahon, 1997). Denitrification studies in domestic wastewater indicate that whenever the carbon/nitrogen ratio is low, full denitrification is difficult to obtain (Marsili-Libelli and Manzini, 2000). In sediment, denitrifying activity is highly correlated with water-extractable organic carbon and is frequently stimulated by the addition of exogenous carbon (Knowles, 1982; Hahndel and Isermann, 1993).

Though denitrification is regarded mainly as an anaerobic process, it has also been shown to occur aerobically. Reports on aerobic denitrification have shown the denitrification enzymes to be active under aerobic conditions (Robertson and Kuenen, 1984). Robertson *et al.* (1988) have demonstrated the ability of a heterotroph *Thiosphaera pantotropha* to simultaneously carry out nitrification and denitrification under aerobic conditions.

Dissimilatory nitrate reduction to ammonium (DNRA)

Alternate respiratory pathways like DNRA lead to N conservation in the system. Rapid and direct transformation of NO_3^- to NH_4^+ via DNRA plays an important role in N conservation in the ecosystem as NH_4^+ is generally favoured for assimilation by plants and microbes (Silver *et al.*, 2001).

$$NO_3^- + 2H^+ + 4H_2 \longrightarrow NH_4^+ + 3H_2O$$

Dissimilatory nitrate reduction has been reported to occur in coastal and estuarine systems (Koike and Hattori, 1978; Buresh and Patrick, 1981, Kaspar, 1983, Gardner *et al.*, 2006). Jørgensen (1989) has shown that NH_4^+ production accounted for 4 to 21% of the total NO_3^- reduced in estuarine sediments. The significance of DNRA varies from one ecosystem to another (Tomaszek and Gruca-Rokosz, 2007). In systems having high sulfide concentrations, nitrification and denitrification is inhibited but DNRA is enhanced by providing an electron donor (An and Gardner, 2002). In freshwater sediments the importance of DNRA appears to be minor relative to denitrification (Scott *et al.*, 2008). Labile carbon is the key factor influencing the partitioning of nitrate reduction between denitrification and DNRA (Yin *et al.*, 2002). DNRA is favoured when NO_3^- is limiting, while denitrification is favoured when carbon is in limited supply (Kelso *et al.*, 1997).

2.2. Measuring denitrification in marine ecosystems

A range of methods are available for measuring denitrification in marine ecosystems. However, the accuracy, cost, scale and design of the experiments to be undertaken are some of the factors that need to be considered while selecting the most appropriate technique (Greatorex, 2000). Some different methods to measure denitrification and their limitations have been listed in Table 1.

Among the different methods available to measure denitrification, the acetylene inhibition technique is a simple, reliable, rapid and cost effective widely used method to determine near *in situ* denitrification rates (Yoshinari *et al.*, 1977; Sørensen, 1978; Chan, and Knowles, 1979; Lohse *et al.*, 1993; Bonin *et al.*, 2002). Acetylene at concentrations of 0.3 to 4 mmol (0.7 to 10 kPa) inhibits the enzyme nitrous oxide reductase which catalyzes the conversion of nitrous oxide to nitrogen leading to the accumulation of nitrous oxide (Sørensen, 1978). However, studies have shown the technique to underestimate denitrification (Mengis *et al.*, 1997) due to (i) incomplete blockage of N_2O reductase at low nitrate concentrations (Kaspar, 1982, Binnerup *et al.*, 1992) (ii) incomplete blockage of nitrous oxide by acetylene in the presence of inorganic sulphide (Tam and Knowles, 1979) (iii) diffusion of N_2O to deeper sediment layers and reduction to N_2 (Seitzinger *et al.*, 1993) (iv) catalytic oxidation of NO into NO_2 (Bollmann and Conrad, 1997) and (v) inhibition of coupled nitrification-denitrification by acetylene (Lohse *et al.*, 1996). Nitrous oxide can be produced during nitrification and the acetylene

Table 1: Some different approaches to measuring denitrification (modified from Cornwell *et al.*, 1999).

Sr. No.	Technique	Approach	Limitations	References
1	Acetylene inhibition	Blocks N_2O conversion to N_2, used to measure N_2O accumulation	Incomplete block; blocks nitrification; diffusion of N_2O to deeper sediment layers	Sørensen, 1978; Lohse *et al.*, 1993
2	Stoichiometry	Difference between C or O_2 based N remineralization and net DIN flux	Not direct, imprecision, problem with 'chemical' oxygen demand or storage of reducing species (S(II), Fe(II) when O_2 is used	Nixon, 1981 ; Joye *et al.*, 1996 ; Giblin *et al.*, 1997
3	Pore water modeling	Model rates from pore water chemistry	In shallow environments, need mmscale profiles and transport coefficients	Jahnke *et al.*, 1982
4	Nitrification-N balance	Measure all DIN fluxes and nitrification, make a mass balance	Indirect	Kemp *et al.*, 1990
5	Direct N_2	Time course change in N_2 measured by gas chromatography	Degassing step, long-term incubation, high background, gas disequilibria from changing temperature	Seitzinger, 1987; Devol, 1991; Nowicki, 1994; La Montagne and Valiela, 1995
6	Membrane inlet mass spectrometry (MIMS)	Measure time-course changes N_2/Ar ratio via mass spectrometry, continuous flow gas stripping	Gas disequilibria from changing temperatures or from CH_4 ebullition	Kana *et al.*, 1994, 1998

Table 1 contd..

Sr. No.	Technique	Approach	Limitations	References
7	Isotope pairing method (IPM)	Label NO_3^- or NH_4^+, follow incorporation in N_2	Potential enhancement by NO_3^- additions	Nielsen, 1992; Lohse *et al.*, 1996
8	Whole-system mass balance	Measure all N fluxes, calculate denitrification by difference	Imprecise, major error associated with measurement of other terms in the budget	Nielsen *et al.*, 1995; Nixon *et al.*, 1996
9	Sediment diagenetic modeling	Using labile organic matter inputs and bottom water O_2, the nitrogen cycle is modeled.	Need information on organic loading, water column chemistry. May be difficult to validate.	Soetaert *et al.*, 1996; Middelburg *et al.*, 1996
10	Molecular approach	Targeting functional genes involved in denitrification	Allows quantification of bacteria capable of performing a single step of the denitrification cascade, interference of tannins, polyphenols and polysaccharides in DNA isolation, efficiency of bacterial cell wall lysis and the non-specific adsorption of DNA to sediment influence yield, variability in clonal composition, presence of gene does not guarantee function	Goregues *et al.*, 2005

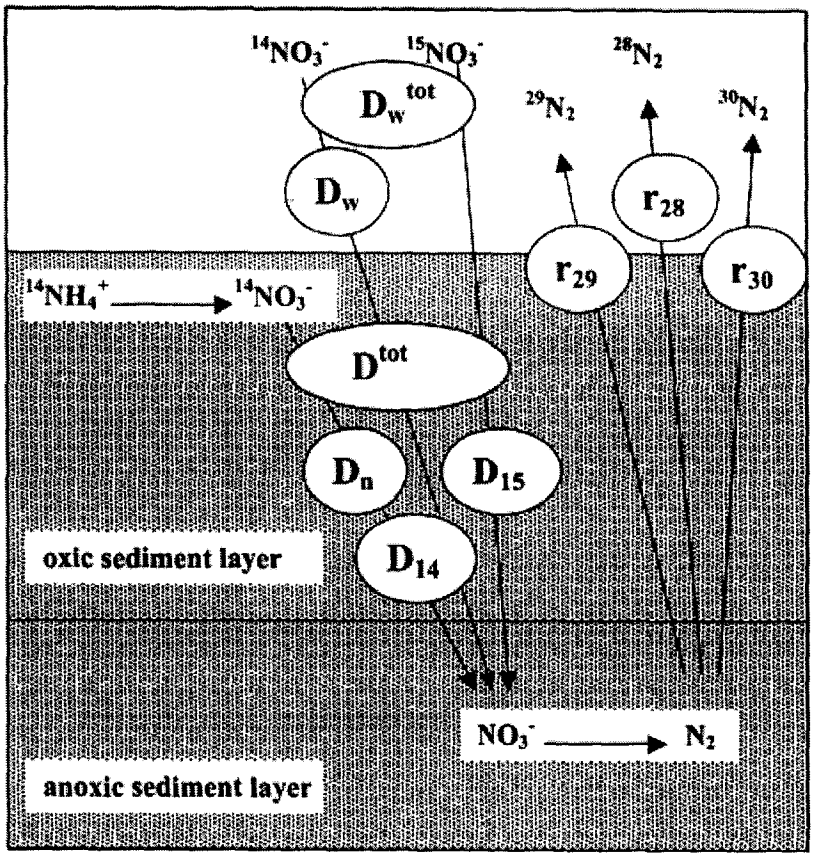


Fig. 2: Schematic representation of the transformation rates during a $^{15}NO_3^-$ tracer experiment (Steingruber *et al.*, 2001) where

D_w^{tot} = Total denitrification of nitrate from the water column

D_w = Denitrification of nitrate from the water column without tracer addition

D_n= Coupled nitrification-denitrification

D_{tot}= Total denitrification rate during the tracer experiment

D_{15} and D_{14}= Specific denitrification rates of ^{15}N and ^{14}N nitrate respectively

P_{28}, P_{29} and P_{30} = Production rates of N_2 with masses of 28, 29 and 30 respectively.

can inhibit the process as it blocks nitrification (Walter *et al.*, 1979) thereby affecting nitrate supply to denitrification.

Acetylene methods have given a large number of denitrification rate estimates which are reasonably robust, especially for systems with moderate or high NO_3^- levels (Groffman *et al.*, 2006). This method is still being used in denitrification assays in conjunction with chloramphenicol (Murray and Knowles, 1999) and allythiourea (Ginestet *et al.*, 1998) amendments to measure *in situ* profiles (Castro-González and Farías, 2004) in the marine environment. The C_2H_2 inhibition method with chloramphenicol addition, conducted over short incubation intervals, provides a cost-effective method for estimating denitrification and rate estimates are comparable to those obtained by the membrane inlet mass spectrometry (MIMS) (Bernot *et al.*, 2003).

Another method for measuring denitrification used in the present study is the ^{15}N Isotope Pairing method (IPM). This method was developed by Nielsen (1992) to quantify denitrification of both NO_3^- diffusing from the overlaying water (D_w) and NO_3^- from nitrification within the sediment (D_n). A representation of the transformation rates during a $^{15}NO_3^-$ tracer experiment (Steingruber *et al.*, 2001) is shown in Fig. 2. Reduction of $^{14}NO_3^-$ (naturally occurring) and $^{15}NO_3^-$ (tracer added) results in the formation of ^{15}N labelled N_2 molecules ($^{29}N_2=^{14}N+^{15}N$, $^{30}N_2=^{15}N+^{15}N$) which can be measured by mass spectrometry after a few hours incubation. Some of the assumptions employed in this technique are that (i) the rate of coupled nitrification-denitrification is not influenced by the addition of labelled nitrate and (ii) the $^{15}NO_3^-$ added to the overlying water mixes homogeneously with the $^{14}NO_3^-$ pool down to the zone where denitrification occurs (Lohse *et al.*, 1996).

The main shortcoming of the IPM is that the formation of N_2O is ignored resulting in an underestimation of sediment denitrification. Another limitation is the inability to account for a possible influx of nitrate through the hyporheic zone (region beneath and lateral to a stream bed, where there is mixing of shallow groundwater and surface water) into the anoxic sediment layer (Master *et al.*, 2005). Herrman and White (2008) state that artificial mixing of $^{15}NO_3^-$ tracer into the sediment disrupts the natural redox processes near the sediment-water interface resulting in yields that are not true representative of the field conditions. Further, the technique cannot distinguish anammox

from denitrification as sources of N_2 and may lead to large errors where anammox is significant (Trimmer *et al.*, 2006). Thus, the original IPM has been modified (An *et al.*, 2001; Master *et al.*, 2005) to improve the estimation of sediment denitrification capacity and account for N_2O formation mechanism(s) and fluxes.

The use of the isotope pairing technique in conjunction with other techniques like ^{15}N dilution of the NO_3^- provides a powerful tool to evaluate in the same experimental setup, the rate of denitrification and the co-occurring processes such as nitrification, mineralization and dissimilatory nitrate reduction to ammonium (Rysgaard *et al.*, 1993, Master *et al.*, 2005).The technique being sensitive and robust (Nielsen 1992, 1993, Rysgaard *et al.*, 1993, 1995, Risgaard-Petersen *et al.*, 1994) has been widely used to quantify denitrification in a variety of marine environments like streams (Mulholland *et al.*, 2004), rivers (Whalen *et al.*, 2008), estuaries (Tuominen *et al.*, 1998; Gran and Pitkänen, 1999; Cabrita and Brotas, 2000; Wang *et al.*, 2003; Dong *et al.*, 2006), mangroves (Kristensen *et al.*, 1998), coastal waters (Sundbäck *et al.*, 2000, Welsh *et al.*, 2000), fjords (Kristiansen and Schaanning, 2002) and continental shelf sediments (Lohse *et al.*, 1996).

2.3. Denitrification in the marine environment

Oxygen minimum zones (OMZ) in the oceans are known to be major centres for denitrifying activity contributing to 30-50% of the total nitrogen loss commonly attributed to heterotrophic denitrification (Gruber and Sarmiento, 1997; Codispoti *et al.*, 2001). These zones are known to occur along regions of intense upwelling. In the Atlantic and Pacific oceans, OMZs in the depth range 100 to 900 m cover the eastern tropical regions (Karstensen *et al.*, 2008).

In the Eastern Tropical Pacific, water column denitrification occurs at a rate of 48±5 Tg N yr^{-1} while sedimentary denitrification amounts to 15±3 Tg N yr^{-1} (Deutsch *et al.*, 2001). However, in the oxygen-deficient water column of the eastern tropical south Pacific off Chile, Thamdrup *et al.* (2006) have shown that anaerobic ammonium oxidation is the dominant process which is more responsible for nitrogen loss than denitrification with highest rates of up to 0.7 nmol N_2 L^{-1} h^{-1} just below the oxycline.

In the eastern tropical south Atlantic, anammox has been shown to occur in the Benguela upwelling system during *in situ* experiments (Kuypers *et al.*, 2005) indicating that nitrate is not directly converted to N_2 by heterotrophic denitrification in the sub-oxic zone. Earlier studies had suggested denitrification to occur close to the shore in the region (Tyrrell and Lucas, 2002).

In the Indian Ocean, seasonal oxygen deficient conditions are known to be prevalent within a large part of the central and north-eastern Arabian Sea. Though the Arabian Sea makes up ~2% of the oceanic regime, it is considered to be one of the most productive ecosystems and contains one of the three major open-ocean denitrification zones in the world (Naqvi *et al.*, 2006). Severe anoxia over the western Indian continental shelf develops during late summer and autumn (Naqvi *et al.*, 2000). These anoxic conditions favour alternate respiratory pathways viz., denitrification and sulfate reduction. Intense water column denitrification at a rate of 30 Tg N y^{-1} has been found to occur within the OMZ (Naqvi, 1994). Naqvi *et al.* (2000) have also reported accumulation of N_2O (nitrous oxide) along the western Indian continental shelf during the anoxic period. Bange *et al.* (2001) state that the emission of N_2O due to vigorous denitrification in the Arabian Sea ranges from 0.33 to 0.70 Tg N_2O yr^{-1}. On a global scale, it has been speculated that the Arabian Sea represents a hot spot for N_2O emissions and could contribute significantly to the global budget of atmospheric N_2O thereby adding up to the increase in the earth's climate. Naqvi *et al.* (2006) have cited differences between open-ocean and coastal denitrification in the Arabian Sea stating that the former occupies two orders of magnitude larger volume wherein an accumulation of secondary nitrite is invariably accompanied by depletion of N_2O. However, in the seasonal coastal system, greater nitrate consumption leading to complete anoxia is observed. High NO_2^- and very high N_2O have been recorded by Naqvi *et al.* (2006) within the sub-oxic zone indicative of net consumption and net production of N_2O by denitrifiers.

In coastal marine sediments, the range of denitrification rates measured is greater than that measured in lake or river sediments ranging commonly between 50 and 250 pmol N m^{-2} h^{-1} with extremes from 0 to 1.067 pmol N m^{-2} h^{-1} (Seitzinger, 1988). The author suggests that the major source of nitrate for denitrification in most rivers, lakes and coastal marine sediments underlying an aerobic water column is nitrate produced in

the sediments and not from nitrate diffusing into the sediments from the overlying water. Very often, coastal ecosystems are subject to eutrophication due to increase in nutrients (carbon, nitrogen and phosphorous) by human activities (Valiela, 1995; Richardson and Jorgenson, 1996). These induce hypoxia as a consequence of seasonal stratification of the water column combined with the decomposition of organic matter derived from accelerated rates of primary production (Childs *et al.*, 2002). Potential denitrification rates in the affected surficial sediments range between 39.8 and 108.1 pmol m^{-2} h^{-1}.

2.4. Denitrifying bacteria

A potential for denitrification exists in most habitats (Knowles, 1982). Denitrification, which is carried out solely by denitrifying bacteria, can have a direct impact on sediment nitrogen availability and is also a major source of the greenhouse gas N_2O (Firestone and Davidson, 1989) responsible for stratospheric ozone decay. The modern era of studies of denitrification began in 1886 with Gayon and Dupetit's report revealing the isolation of two strains of denitrifying bacteria (Payne, 1986). Denitrifiers have now been isolated from different areas ranging from soil to freshwater and marine environs. Denitrifiers have also been detected gut contents of earthworms averaging 10^{6-7} g^{-1} (dry weight) of gut material producing N_2O at rates exceeding that of soil homogenates (Karsten and Drake, 1997). A number of novel denitrifiers have been reported like *Bacillus thermodenitrificans* (Manachini *et al.* 2000), *Comamonas nitrativorans* (Etchebehere *et al.*, 2001), *Thioalkalivibrio denitrificans* (Sorokin *et al.*, 2001), *Pseudovibrio denitrificans* (Shieh *et al.*, 2004), *Denitratisoma oestradiolicum* (Fahrbach *et al.*, 2006), etc. Denitrifies have also been reported from extremely halophilic habitats (Hochstein and Tomlinson, 1985; Mancinelli and Hochstein, 1986).

Denitrifiers are among the most diverse groups of bacteria in terrestrial ecosystems (Zumft, 1992). Denitrification has been reported in more than 50 bacterial genera (Zumft, 1997), archaebacteria and fungi (Tiedje, 1988). In marine ecosystems, a variety of taxonomically unrelated bacterial groups are capable of denitrification. Of these, 96% of cultured denitrifiers belong to the gamma-Proteobacteria (Brettar *et al.*, 2001). Denitrifying bacteria isolated from the marine environment are heterotrophic, gram-negative, motile, facultatively anaerobic cells predominantly straight or curved rods

exhibiting optimal growth at about 30°C, pH 8 and 3% NaCl (Shieh *et al.*, 2004). They gain energy by oxygen-dependent respiration under aerobic conditions (Knowles, 1982). They are capable of anaerobic growth by carrying out denitrifying metabolism using nitrate, nitrite or nitrous oxide as terminal electron acceptors (Shieh *et al.*, 2004). Betlach (1982) suggests that denitrifiers have evolved from a common ancestor within the purple photosynthetic bacterial group but not from a nitrate-reducing organism such as those found today.

The most-probable number (MPN) technique has been widely used for the enumeration of denitrifiers (Davidson *et al.*, 1985; Mancino and Torello, 1986; Jones and Knowles, 1991; Hou *et al.*, 2000; Bigelow *et al.*, 2002; Horiba *et al.*, 2005) instead of plate counts. The technique is based the presence or absence of microorganisms in several individual aliquots of each of several consecutive dilutions of sediment. Upon incubation, change in characteristic of the nitrate medium (e.g. presence of gas bubble, NO_3^- depletion in the MPN cultures) is noted as positive for the presence of denitrifiers. Denitrifiers in sediment dilutions have also been identified by depletion of both NO_3^- and NO_2^- by a negative spot test with diphenylamine (Martin *et al.*, 1988). Allievi *et al.* (1987) devised an MPN method for the determination of denitrifiers, based on the qualitative gas-chromatographic detection of N_2O produced during incubation in the presence of acetylene. In this method, tubes with sediment extract medium containing glucose, nitrate and ammonium sulfate were sealed with rubber stoppers and fitted with taps for air extraction. Air in the tubes was replaced with helium/acetylene 90:10 v/v. The tubes were incubated at 28°C for 15 days and tubes testing positive for nitrous oxide production were scored. Allievi and Möller (1992) developed and tested a plate-count based method to evaluate sediment denitrifiers. Saitoh *et al.* (2003) have improvised the MPN method using less equipment, labor and time to give more precise results than the conventional test tube method. They used a 96-well microtiter plates to add dilutions of samples with a medium and incubated them anaerobically using the AnaeroPouch culture system.

Conventional denitrifier cultivation techniques in natural samples yield only 0.1 to 10% of the denitrifying population. Using molecular techniques, every bacterium that contains the target DNA can be counted compared to MPN culture techniques that may

give varying results due to differences in the physiological state of the bacteria, depending on the sample and the composition of the culture medium (Michotey *et al.*, 2000). Therefore, molecular techniques are being increasingly used to replace time-consuming MPN-based approaches to quantify denitrifiers. Molecular methods like real-time Polymerase Chain Reaction (PCR) targeting functional genes like *nirK* have shown their densities to range between 10^{4-6} copies per gram of sediment (Henry *et al.*, 2004). Michotey *et al.* (2000) quantified denitrifiers by targeting the cytochrome cd_1 denitrifiers by MPN-PCR and competitive PCR techniques. Their results have shown 10^{2-3} copies per millilitre of water and 10^6 copies per gram of dry sediment. For high-throughput quantification of the denitrifying community, the microarray technique is promising but the method is countered by its low-sensitivity (Philippot, 2006).

Quantification of microorganisms by cultivation based techniques are generally biased as it is generally believed that only a small fraction of environmental bacteria are recovered (Michotey *et al.*, 2000). Molecular approaches are being widely applied to identify and enumerate denitrifying bacteria in environmental samples. These methods target functional genes (Smith and Tiedje, 1992) to give an indication of the presence of the bacterial group which is capable of that function for e.g. using nitrite reductase gene as a probe for denitrifying bacteria. The use of a functional gene requires sufficient genetic homology of the structural genes and the availability of multiple sequences in order to reliably design primers (Michotey *et al.*, 2000). Specific metalloenzymes (Table 2) catalyze each step (Payne, 1983) of the denitrification process (Fig. 3) of which two contain copper.

The first step of the pathway is catalyzed by either the *nap* gene encoding periplasmic nitrate reductase synthesized during aerobic growth or the membrane-bound *nar* gene synthesized during anaerobic growth (Bell *et al.*, 1990). The second step can be catalyzed by a cytochrome cd_1-containing nitrite reductase (*nirS*) or a copper nitrite reductase (*nirK*) in some species. The third step catalyses the conversion of nitrite oxide to nitrous oxide by two different types of nitric oxide reductases (*norB, norC*) whereas the final step of converting nitrous oxide into di-nitrogen gas is carried out by the enzyme *nosZ*. Expression of nitrate reductase, nitrite reductase and N_2O reductase is controlled by discrete oxygen levels and by the nature of the nitrogenous oxide available for respiratio

Table 2: Metalloenzymes of the denitrification pathway

Sr. No.	Enzyme	Gene	Associated metal	Location	Reference
1	Nitrate reductase	*narG*	Molybdenum	Membrane-bound	Lanciano *et al.*, 2007
2	Nitrate reductase	*napA*	Molybdenum	Periplasmic	Hettmann *et al.*, 2004
3	Nitrate reductase	*narH*	Iron	Membrane-bound	Richardson *et al.*, 2001
4	Nitrate reductase	*narI*	Molybdenum	Membrane-bound	Watts *et al.*, 2005
5	Nitrate reductase	*narJ*	Molybdenum	Membrane-bound	Blasco *et al.*, 1998
6	Cytochrome cd_1 nitrite reductase	*nirS*	Iron	Periplasmic	Saunders *et al.*, 2000
7	Nitrite reductase	*nirK*	Copper	Periplasmic	Beaumont *et al.*, 2002
8	Nitric oxide reductase	*norB*	Iron	Membrane-bound	Cramm *et al.*, 1999
9	Nitric oxide reductase	*norC*	Iron	Membrane-bound	Matsuda *et al.*, 2002
10	Nitrous oxide reductase	*nosZ*	Copper	Periplasmic	Zumft *et al.*, 1990

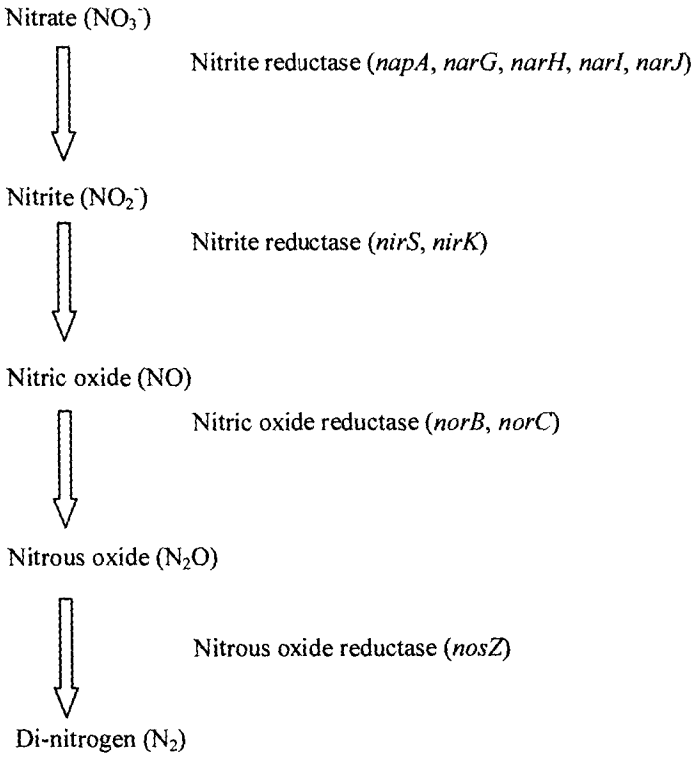


Fig. 3: Enzymes catalyzing the denitrification process and genes involved

(Körner and Zumft, 1989). Denitrification enzyme activity could be used as a useful index for comparing relative N exposure and potential denitrification activity (Wigand, 2004). Smith and Parsons (1985) have found significant increase in enzyme activity in waterlogged cores which was always greater than denitrification rates. However, denitrification enzyme assay cannot be used as a reliable estimate of actual N gas production or seasonal patterns of denitrification in sediment due to the persistence of inactive denitrification enzymes (Groffman, 1986).

Researchers have been able to probe the diversity of denitrifying genes by using culture-independent approaches (Ward, 1995, Braker *et al.*, 2000, 2001; Jayakumar *et al.*, 2004, Prieme *et al.*, 2002, Liu *et al.*, 2003). Though a variety of functional genes have been used to study denitrifier communities in the environment, the most deeply investigated at present are those involved in nitrite reduction (Ward, 2005) and encoded by *nirS* and *nirK* genes that are unique to denitrification pathway. Of these, *nirS* has so far has been reported only within Proteobacteria (Jayakumar *et al.*, 2004; Ward, 2005). A study by Jayakumar *et al.* (2004) in the water column of the coastal Arabian Sea has indicated the highest *nirS* diversity related to relatively high nitrite concentrations implying the presence of active denitrification.

The *nosZ* gene encoding for nitrous oxide reductase, an enzyme catalyzing the final step of denitrification is largely unique to denitrifying bacteria (Scala and Kerkhof, 1999). It represents the process leading to the loss of biologically available nitrogen from the sediment (Mills *et al.*, 2008) and has been used for determining the diversity of denitrifiers (Scala and kerkhof, 2000; Horn *et al.*, 2006; Yuguang *et al.*, 2006). Investigations in continental shelf sediments have shown *nosZ* genes relating closely to the *nosZ* genes of *Paracoccus denitrificans* or *Rhizobium meliloti* (Scala and kerkhof, 1998). Magalhães *et al.* (2008) suggest that denitrifiers with specific *nosZ* genotypes have competitive advantage over others when NO_3^- fluctuates in the system thereby affecting denitrification rates.

Molecular studies to investigate denitrifying communities have extensively used the Denaturing Gradient Gel electrophoresis (DGGE) technique (Noda *et al.*, 2002; Tadashi *et al.*, 2003; Enwall *et al.*, 2005; Hallin *et al.*, 2006; Desnues *et al.*, 2007; Ma *et al.*, 2008). Denaturing gradient gel electrophoresis separates PCR generated DNA

products (200-700 bp genomic restriction fragments) when run on a low to high denaturant gradient acrylamide gel. As the products migrate, they encounter increasingly higher concentrations of chemical denaturant. Weaker melting domains of the double-stranded PCR product denature retarding the progress of the molecule in the gel. Sequence differences from different bacteria denature at different denaturant concentrations resulting in a pattern of bands wherein each band represents a different bacterial community. The fingerprints can be uploaded into databases and the similarity can be assessed to determine microbial structural differences between environments or among treatments. This method is efficient for PCR fragments shorter than 500 base pairs (Myers *et al.*, 1985) providing high resolution and allowing bands of interest to be excised from the gel for straightforward sequencing to gain insight into the identities of the pre-dominant bacteria present. The major drawback of DGGE is that sequences amplified from different organisms may have the same melting temperature and migrate to the same position on the gel meaning that a single band can contain a mixture of genotypes (Enwall, 2008). Sequence divergence in the ribosomal genes can be obtained using restriction fragment length polymorphism (RFLP) analysis (Ward, 1995; Scala and Kerkhof, 2000) to account for variations in phenotype.

2.5. Denitrification in mangrove ecosystems

Mangrove ecosystems are known to provide coastal protection from tidal erosion, storm surges and trap sediment for land accretion (Pernetta, 1993). They play a major socio-economic role to human communities in developing countries having traditionally used mangroves for wood and tannin products on a sustainable level (Tomlinson, 1994). Until recently mangrove forests had been considered wastelands. Large tracts of mangrove forest are now being 'reclaimed' for aquaculture and industrial development (Ong *et al.*, 1995). Juvenile fish and prawns use mangroves as habitats (Robertson and Duke, 1987; Robertson and Blaber, 1992). The Indian coastline extends 7516.6 km of which 5% comprises of a luxuriant mangrove cover. Mangrove swamps are located along the west coast of India along Saurashtra, Karnataka and Goa (Jagtap 1985; Jagtap *et al.*, 1993, 1994) while dense vegetation is found in the Sundarbans of West Bengal on the east coast. Extensive deforestation of mangroves for firewood, cattle grazing intense sewage and industrial pollution have threatened the mangroves along Bombay and southern

Kerala coast. According to a survey on forest by the Government of India (1997), Goa region has mangrove vegetation amounting to approximately 500 hectares. The major mangrove zones extend along swampy banks of Zuari and Mandovi estuaries. There are other sporadic mangrove patches distributed in the other 4 estuaries and along the Cumbharjua canal connecting the two estuaries.

The denitrifying ability of mangroves make them effective sewage 'filters', converting dissolved inorganic nutrients to particulate matter (Robertson, 1992) make them plausible sites for denitrifying activity. The nitrogen cycle within mangrove forests (Fig. 4) is mediated predominantly by microbial rather than chemical processes (Alongi *et al.*, 1992). The sediment N pool is composed largely of the organic N form which is not readily available for plant uptake (Clarke, 1985). Studies by Clarke (1985) and Alongi *et al.*, (1992) have revealed concentrations of dissolved inorganic forms, particularly nitrate to be low compared to concentrations in other marine sediments. Denitrification in the sediments occurs in close proximity to the oxygenated surface of sediments and is dependent on the diffusion of nitrate into the sediments (Tomaszek, 1995). Mangrove sediments are largely anaerobic and NO_3^- availability is the factor controlling denitrification rates (Seitzinger, 1990). Kristensen *et al.* (1998) have indicated that more than 90% of the NO_3^- needed by denitrifiers originates from nitrification (coupled nitrification-denitrification) while only 1 to 2% of the measured NO_3^- influx from the overlying water was consumed by the process. Denitrification results in emission of nitrous oxide, a potent green house gas. As mangroves lie in close proximity to areas prone to anthropogenic activity, there is growing concern towards their contribution to increased nitrous oxide fluxes to the atmosphere. Muñoz-Hincapié *et al.* (2002) have demonstrated enhanced nitrous oxide fluxes on nitrate enrichment to mangrove sediments resulting in maximal mean flux of 36.7 $\mu mol\ m^{-2}\ h^{-1}$. Corredor *et al.* (1999) have encountered rates ranging between 0.12 and 7.8 $\mu mol\ N_2O\ m^{-2}\ h^{-1}$ along the Southwest coast of Puerto Rico. The Muthupet mangrove system in South India emits nitrous oxide at a rate of 0.41 and 0.77 $\mu mol\ m^{-2}\ h^{-1}$ (Krithika *et al.*, 2008).

Research on denitrification in mangrove ecosystems has focussed on the measurement of direct rates of denitrification (Rivera-Monroy *et al.*, 1995; Kristensen *et al.*, 1998; Meyer *et al.*, 2005). Denitrification rates reported in mangroves are generally

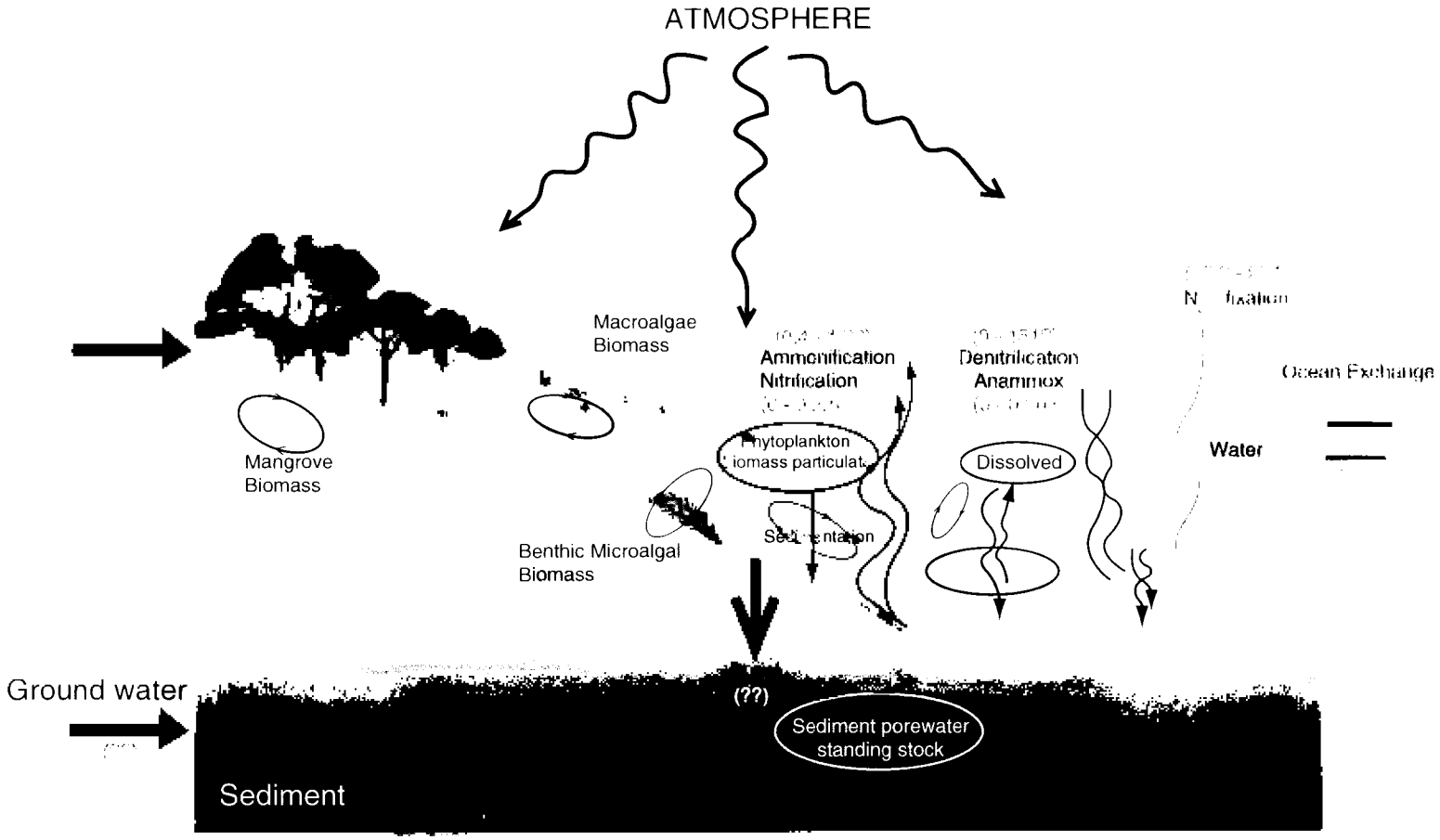


Fig. 4: Major processes and transformation rates in the N-cycle of mangrove ecosystems (Purvaja *et al.*, 2008). Units for N-processes are in $\mu g\ m^{-2}\ d^{-1}$ except for N_2 fixation which is $mg\ m^{-2}\ d^{-1}$

much lower than estuarine sediments (Rivera-Monroy and Twilley, 1996). These rates have been estimated directly by experimental procedures using different techniques like gas chromatography, N_2-gas flux technique, ^{15}N isotope techniques in intact sediment cores, etc. Chiu *et al.* (2004) state that as much as 55% of the N loss in mangrove sediments occurs through denitrification and the process is limited by carbon availability. Little has been understood on the ecology of the organisms mediating the process. So far, attempts have been made to assess the denitrifiers associated with mangrove roots at a molecular level (Flores-Mireles *et al.*, 2007) using functional marker genes (*nirS* and *nirK*) indicating that they belong to α-, β-, and γ-*Proteobacteria*. Mangrove sediments are also known to harbour novel denitrifiers (Lin and Shieh, 2006) like *Zobellella denitrificans* and *Zobellella taiwanensi*. Therefore, it would be interesting to explore the taxonomic and functional diversity of microbes capable of denitrification from other mangrove habitats. The physico-chemical parameters that influence their distribution and abundance would help gain further insights on benthic denitrification in mangrove ecosystems.

Chapter 3

Materials & methods

Chapter 3. Materials and methods

SECTION I – Field observations

3.1. Study Area

Investigations were carried out at mangrove forests located along the Mandovi and Chapora rivers in Goa, west coast of India (Fig. 5). The locations were fixed using a hand-held *Magellan* Global Positioning System.

3.1.1. Location and description of mangroves

Mangrove forests are one of the most productive and biodiverse ecosystems. The Indian coastline extends up to 7516.6 km of which 8% comprise of a luxuriant mangrove cover (Jagtap and Komarpant, 2003). Mangrove swamps are located along the west coast of India along Saurashtra, Karnataka and Goa (Jagtap, 1985) while 80% of the vegetation is found on the east coast (Untawale, 1984). Some of the major mangrove zones in Goa are located along the banks of the Mandovi and Zuari estuarine systems.

The mangroves are subjected to an annual average rainfall of up to 325 cm. The fresh water input into the riverine systems during the rainy season lowers the salinity from approximately 32 ppt during the pre-monsoon to 0 ppt at the peak of the monsoon.

3.1.2. Tuvem mangrove ecosystem

The control site was located at Tuvem (15°39'09"N and 073°47'71" E). The site is located across the picturesque village of Camurlim (approx. 7 Kms from Mapusa town, North Goa) along the river Chapora which meanders through palm-lined banks. The site is linked by ferry. This serene ecosystem is set amidst lush green hills, banana and coconut plantations and is comparatively less influenced by anthropogenic activities (Krishnan *et al.*, 2007).

The Island is fringed by lush mangroves (Plate 1). The dominant species of mangroves found here are mainly represented by *Acanthus illicifolius, Excoecaria agallocha*, *Caesalpinia* spp., *Avicennia officinalis* and *Clerodendrum inerme*.

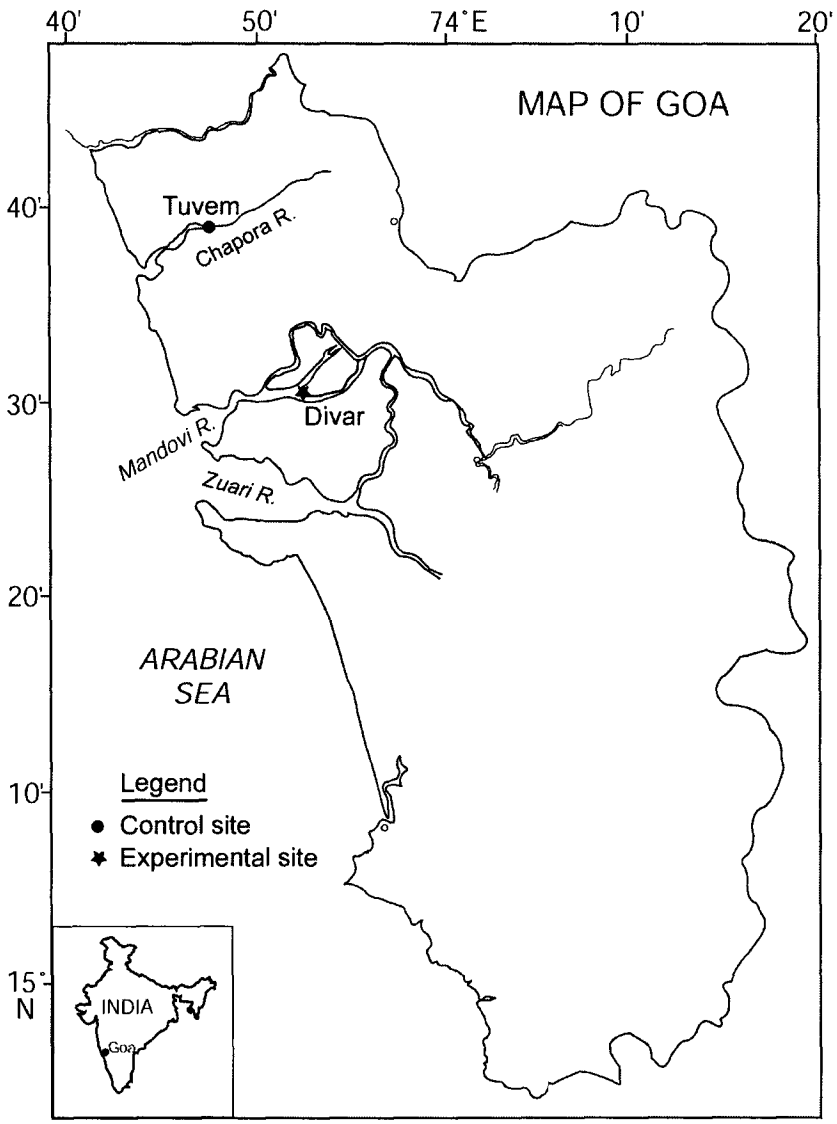


Fig. 5: Location of sampling sites along the Chapora and Mandovi estuary

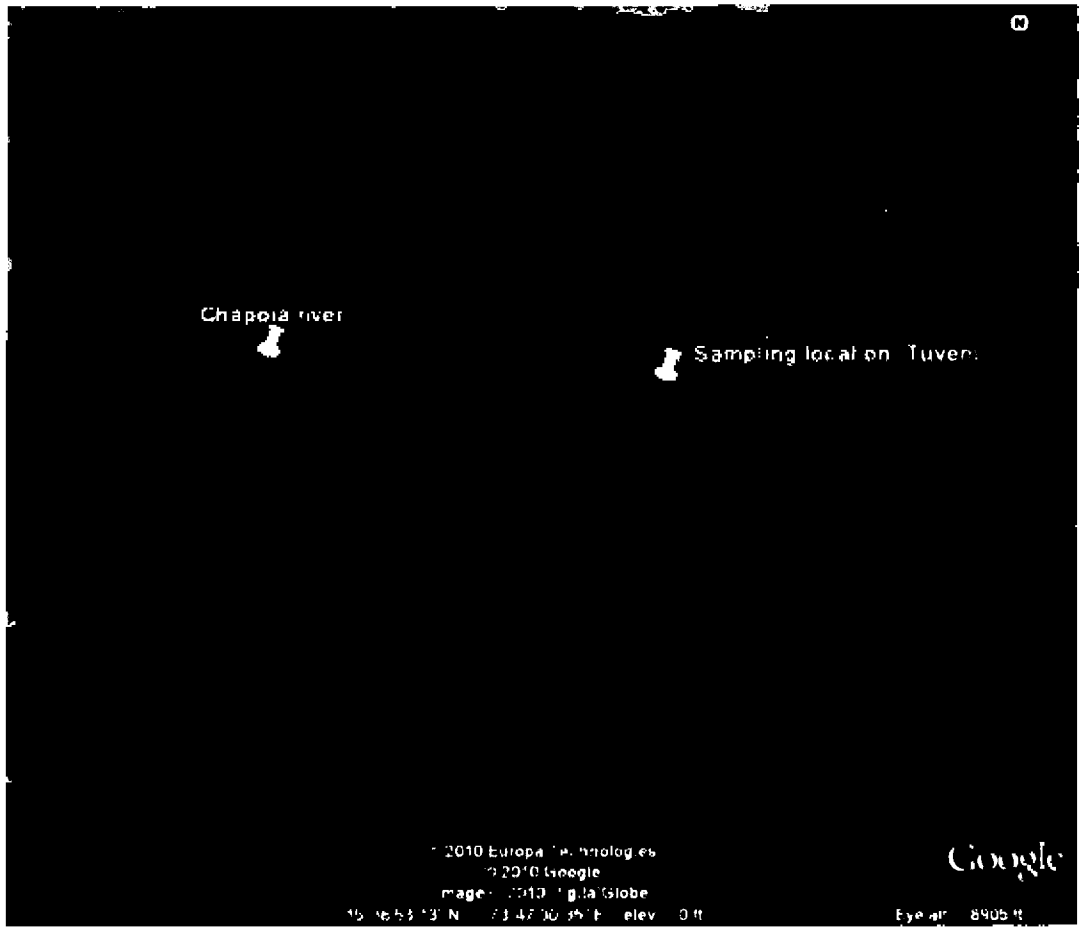


Plate 1: Mangroves at Tuvem along the Chapora river

3.1.3. Divar mangrove ecosystem

The experimental site was located at Divar island (15°30'35"N and 73°52'28"E; Plate 2) which is situated at a distance of approximately 10 km from the capital, Panjim. It is separated from the mainland by the Mandovi estuary and is accessible by ferry. The adjoining Mandovi estuary is important for the economy of the territory as it is heavily used for transportation of iron ore from mines located upstream. These iron ore beneficiation plants situated on the riverbank, discharge effluents directly into the estuary. This discharge contains high quantities of sediment rich in iron and also NH_4NO_3 used as explosive in the mining operations (De Sousa, 1999). High nutrient concentrations in the Mandovi especially during the monsoon (Divya *et al.*, 2009) have been attributed to extraneous sources (De Souza, 1983). The Divar sediments are enriched with metals due to ferromanganese mining upstream of the Mandovi (Krishnan *et al.*, 2007). These metals mainly Fe has been shown to influence N transformations in mangrove sediments (Krishnan and Loka Bharathi, 2009).

The Divar Island is also fringed by luxuriant mangroves. These mangroves consist mainly of species like *Acanthus illicifolius*, *Pongamia pinnata*, *Cyperus* spp., *Bruguiera gymnorrhiza*, *Avicennia officinalis*, *Caesalpinia* spp., *Sonneratia caseolaris* and *Rhizophora mucronata*. These mangroves support the livelihood of many islanders and are of immense ecological and economic value. A variety of birds particularly pin tailed ducks, Cormorants, Common Sandpiper, etc. have made this mangrove swamps their abode. Oysters and fish are also found. Fiddler crabs of the genus *Uca* comprise one of the dominant macro benthic communities in this ecosystem.

3.2. Sampling

3.2.1. Sampling period

Monthly sampling was carried out at low tide for a period of one year (April 2005 to March 2006) in the Divar and Tuvem mangroves swamps. The sampling covered the three seasons i.e. Pre-monsoon (February-May), Monsoon (June-September) and Post-monsoon (October-January).

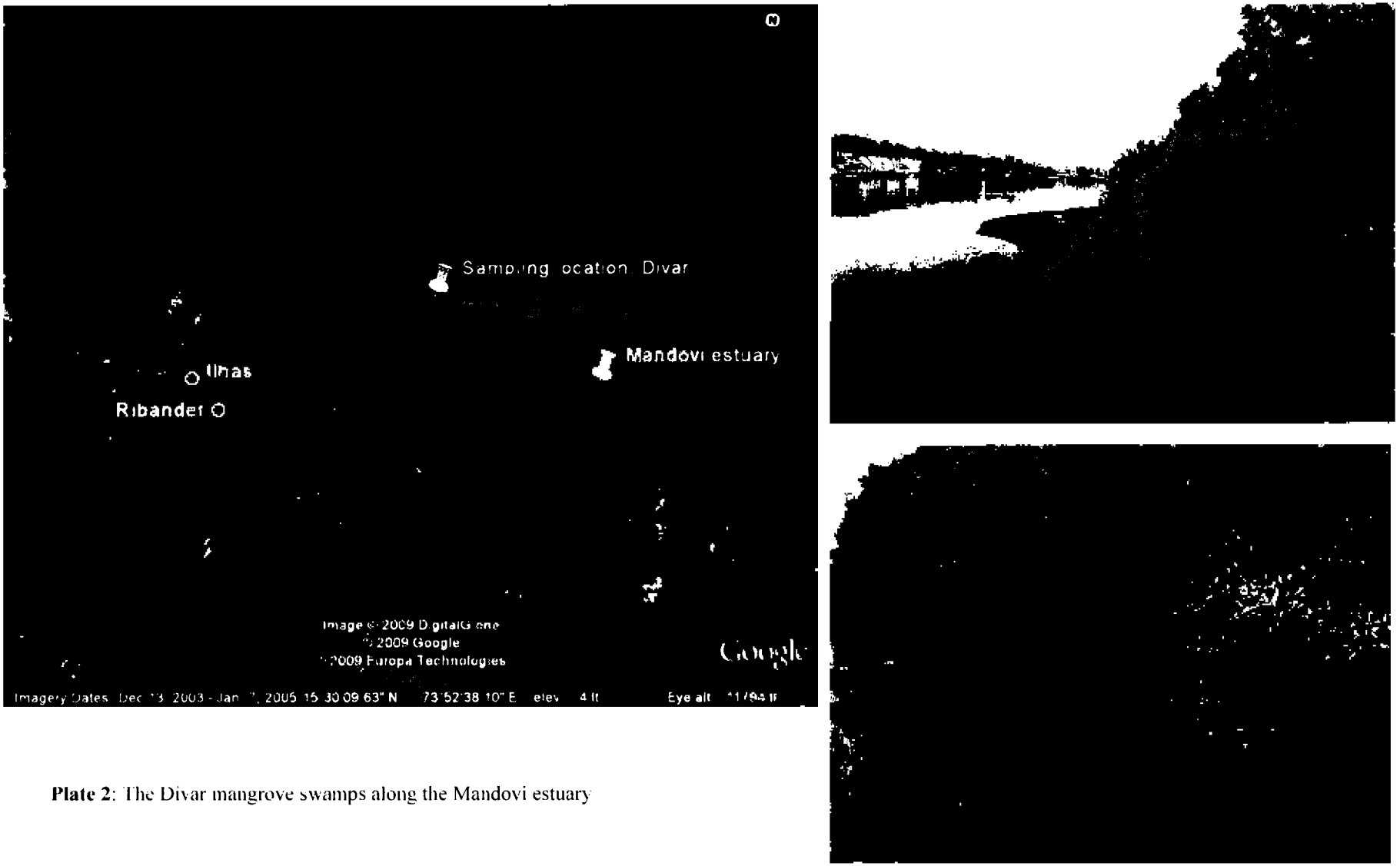


Plate 2: The Divar mangrove swamps along the Mandovi estuary

3.2.2. Sample collection

Cores up to 15 cm length were obtained, capped immediately, transferred to an insulated box and transported to the laboratory for further physico-chemical and microbiological analyses. The cores were stored at 4°C until analyses. Water from the sampling sites was collected in clean, well-rinsed carbuoys and used for media preparation during microbiological analyses.

3.3. Measurement of physico-chemical parameters

Upon arrival to the laboratory, representative cores from each site were sectioned at 0-2, 2-4, 4-6, 6-8 and 8-10 cm. The following physico-chemical parameters were immediately analyzed in the sediment: temperature, pH and redox potential. Samples for total organic carbon, grain size and metals (Fe and Mn) at each depth layer were analyzed only for the three representative seasons. Down-core variation in pore water concentration of inorganic nitrogenous species (nitrate and nitrite) was determined. Samples for the analyses of dissolved oxygen concentration in the ambient water were fixed at site. Salinity of surrounding water was also measured.

3.3.1. Temperature

Temperature at each section of the core (0-2, 2-4, 4-6, 6-8 and 8-10 cm) was measured using a digital thermometer (*Fisher Scientific*).

3.3.2. Salinity

Salinity of overlying water was measured using a hand held refractometer (model: S/Mill-E, *ATAGO*, Japan). The zero setting (calibration) was done using distilled water as per the manufacturers instructions.

3.3.3. Hydrogen ion concentration (pH)

Hydrogen ion concentration at each section was measured using an *Orion* 4-Star Plus Benchtop pH/ISE Meter after calibration with standard buffers (pH 4, 6.9 and 9; Merck).

3.3.4. Redox potential (Eh)

An intact sediment core was carefully taken out from the PVC core. Sediment oxidation-reduction potential (Eh) was measured immediately at each section of the core with the help of an *Orion* platinum redox in combination with a silver/silver chloride reference

electrode. The electrode was dipped until the reading stabilized and the values were recorded.

The redox electrode was calibrated using solutions A and B (Appendix I). The electrode was dipped in solution A, and the reading was allowed to stabilize. The potential was around 192±2 mV. The electrode was then rinsed, wiped dry with a tissue and dipped in a beaker containing solution B. The reading was allowed to stabilize until the potential was 258±5 mV (the difference potential of solutions B and A was about 66 mV).

3.3.5. Dissolved oxygen (ambient water)

Dissolved oxygen (DO) content in the ambient water was determined using the traditional Winkler's titration (Winkler, 1888) method as described by Grasshoff (1983). Water samples were collected at the sites in acid-washed (10% HCl) 300 ml glass stoppered bottles. The samples were immediately fixed using 2.4 ml of Winkler's A and B reagents (Appendix II). The DO bottles were gently shaken to mix the reagents and the precipitate formed was allowed to settle.

In the laboratory, the precipitate was acidified by adding 2.4 ml of sulphuric acid (50% v/v) and titrated against 0.01N sodium thiosulphate using starch as an indicator. End point was noted by observing colour change from pale yellow to colourless. A blank was maintained using milliQ water. Potassium iodide (0.01N) was used as a standard. The dissolved oxygen concentration of seawater was expressed as the number of millilitres of dioxygen gas (O_2) per litre of seawater (ml l^{-1}).

3.3.6. Pore water NH_4^+, NO_2^- and NO_3^-

Sub-samples were taken at every 2 cm intervals from surface to 10 cm by carefully sectioning the core. Each section (7.5 cm diameter and 2 cm thick) was transferred to 100 ml of sterile saline and homogenised using a glass rod. The slurry was centrifuged at 4°C for 10 minutes at 5000 rpm with a REMI CPR-24 centrifuge. Low spin was maintained during centrifugation to ensure minimal change in nutrient concentrations due to lysis of benthic infauna. The supernatant was filtered through a 0.2 µm filter and stored at -20°C until analysis. Ammonium (Appendix III), nitrite (Appendix IV) and nitrate (Appendix V) were measured colorimetrically (*Shimadzu* UV/VIS spectrophotometer; precision: ±

0.01 μmol l^{-1}) as described by Koroleff (1969), Bendschneider and Robinson (1952) and Wood *et al.* (1967). Weight of the sediment used in the extraction was estimated by drying in a hot air oven at 60°C for 48 hours.

3.3.7. Total organic carbon

Total organic carbon was determined by wet oxidation method (El Wakeel and Riley, 1957) with a precision of 0.01%. Dried sediment was ground using a mortar and pestle. It was sieved through a 200 μm mesh. About 0.5 grams sample from 0-2, 2-4, 4-6, 6-8 and 8-10 cm sections were taken and transferred to a conical flask. Organic matter present in sediment samples was oxidized by adding 25 ml of acid dichromate and the tubes were incubated at 60°C in a water bath for 1 hour. Then, 100 ml of distilled water was added and subsequently titrated against ferrous ammonium sulphate using diphenylamine as an indicator. A colour change from dark blue to green indicated the end point. A blank was run without sediment. The standard curve was plotted using glucose at varying concentrations.

3.3.8. Iron and manganese

Sub samples for metal analyses were dried at 60(±2)°C for 48 hours and disaggregated in an agate mortar, before chemical treatment for Fe and Mn analysis. For each sample, a known quantity (~0.2 g) of sediment was digested in a teflon vessel with a solution (10 ml) of concentrated HF (48% GR; Merck), HNO_3 (69% GR; Merck) and $HClO_4$ (35% GR; Merck) in a ratio of 7:3:1. The sediment was then dried on a hot plate in a fume hood chamber for an hour.

An aliquot of 5 ml of the above acid mixture was added and dried on the hot plate for one hour. Further, 2 ml of concentrated HCl (35% GR; Merck) was added followed by 10 ml of HNO_3 (69% GR; Merck). The residue was warmed and then transferred to a clean, dry standard flask to make up final volume to 100 ml with double distilled water. Detailed procedure of sediment digestion is given in Balaram *et al.* (1995). Trace metal concentrations (Fe and Mn) were measured using a flame atomic absorption spectrophotometer (AAS, Perkin Elmer Model 5000). The accuracy of the analytical procedures was assessed using the certified reference material MAG-1 (USGS) that yielded results within the reference value range (Flanagan, 1967; 1976).

3.4. Bacteriological studies

3.4.1. Total bacterial counts

The sediment core was thawed and sectioned at 2 cm intervals using a sterile core cutter to obtain representative samples at 0-2 cm, 2-4 cm, 4-6 cm, 6-8 cm and 8-10 cm. Sub-samples of ≈5 gm wet weight sediment was extruded using syringe cores. The sub-samples were transferred to 45 ml of filter sterilized full strength seawater (10^{-1} dilution). Tween 80 (50 µl) was added and the mixture was sonicated at 40 mHz for 15 secs. The next set of dilution (10^{-2}) for each section of the core was prepared by transferring 5 ml of 10^{-1} dilutions to 45 ml filter sterilized seawater.

The method of Hobbie *et al.* (1977) was used for the enumeration of total bacterial counts (TC) by epifluorescence microscopy. An aliquot of 5 ml sample from 10^{-2} dilution was fixed using 250 µl of buffered formalin (2% final concentration). Two millilitres of sub-sample was filtered over a 0.2 µm black Isopore polycarbonate filter paper (*Millipore*) and stained with acridine orange (final concentration 0.01% w/v). The sample was then incubated for 2 minutes and then filtered. Bacterial cells retained on the filter paper were counted using *Nikon* 50i epifluorescence microscope equipped with a 100X oil immersion objective. Cells were counted from 10-100 microscopic fields and the total counts (TC) were expressed as number of cells g^{-1} (dry sediment).

3.4.2. Heterotrophic counts

Total heterotrophic bacteria (THB) were enumerated using medium prepared in aged seawater. The seawater was amended with 0.01% nutrient broth (*HiMedia*, India) at concentrations. The pH of the medium was adjusted to 8.2 before adding agar at a final concentration of 1.5%. A 50 µl innoculum from 10^{-2} dilution was used to spread plate on to the nutrient agar plates. The plates were incubated at room temperature (28±2°C) for 7 days before and bacterial counts were taken as colony forming units (CFU) g^{-1}. Initial weight of the sediment used in the dilutions was determined by drying the filtered sediment at 60°C.

3.4.3. Nitrate reducing bacteria

Nitrate reducing bacteria (NRB) were cultured in agar shake tubes. Media for isolation (Appendix VI) was prepared using aged seawater amended with 1mmol KNO_3 and half-

strength nutrient broth (*HiMedia laboratories Ltd.*, India). About 500 µl from 10^{-2} dilution of the sediment was used. The medium was gently poured and mixed with the innoculum. The tubes were overlaid with a paraffin-paraffin oil mixture in the ration 1:2 to induce anaerobic conditions. The culture tubes were incubated at 28°C ±2 for 30 days. Gas production was observed in the tubes towards the end of the incubation period. NRB counts were taken in the form of colony forming units. Initial weight of the sediment used in the dilutions was determined by drying the filtered sediment at 60°C. The counts have been expressed as CFU g^{-1} (dry sediment).

3.4.4. Denitrifier abundance

Denitrifiers were enumerated seasonally by the N_2O-most probable number (MPN) technique as described by Michotey *et al.* (2000). The sediment core was thawed and sectioned at 2 cm intervals using a sterile core cutter to obtain representative samples at 0-2, 2-4, 4-6, 6-8 and 8-10 cm. Sediment from each depth was homogenised by mixing. Sub-samples of approximately 1 g wet weight from each depth layer were sub-sampled using sterile syringes. The sub-samples were transferred to sterile 9 ml of culture medium (Appendix VII) to give a 10^{-1} dilution. The vials were purged with high purity N_2 for 10 minutes to induce anaerobic conditions and supplemented with 20 kPa acetylene (Bonin *et al.*, 1994). Tween 80 (20 µl) was added and the mixture was sonicated at 40 MHz for 15 seconds. Dilutions up to 10^{-12} for each section of the core were prepared in triplicates. The vials were incubated at room temperature in the dark for 10 days, and the positive tubes were scored based on the accumulation of nitrous oxide. Subsequent quantification could be made using standard Mc Cready's table (Rodina, 1972). Denitrifier abundance has been expressed as MPN cells g^{-1} of dry sediment.

3.5. Denitrification rates

Denitrification rates were measured using sediment slurries by the acetylene inhibition technique based on the inhibition of the conversion of N_2O to N_2 (Sørensen, 1978). The slurry method was opted over the intact core method (Lowrance and Hubbard, 2001; Bernot *et al.*, 2003) due to low permeability of mangrove sediments (Marchand *et al.*, 2004; Schwendenmann *et al.*, 2006) as uneven penetration of acetylene in compact sediments would lead to an underestimation of denitrification rates. Sampling for

denitrification activity was carried out during April representing pre-monsoon period (February–May), September (monsoon: June–September) and January (post-monsoon: October–January).

Sediment cores were demarcated into five sections (0-2, 2-4, 4-6, 6-8 and 8-10 cm). About 1 cm^3 of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20 ml headspace vials. Three ml of sterilized ambient sea water from the sampling site was added to the sediment. This seawater was amended with chloramphenicol at a final concentration of 1g l^{-1} (Gilbert *et al.*, 1998; Simek, 2000; Bonin *et al.*, 2002; Desnues *et al.*, 2007) to inhibit *novo* synthesis of denitrifying enzymes and reflecting *in situ* activity at the time of sampling (Brooks *et al.*, 1992). No additional carbon or nitrate was amended to the seawater. The vials were capped with butyl stoppers and the slurry was vortexed for 5 seconds. The vials were purged with high purity N_2 for 10 minutes to induce anaerobic conditions. Acetylene gas at 20 kPa (Bonin *et al.*, 2002) was injected into the headspace to avoid N_2O production by nitrification and its reduction by denitrification (Castro-González and Farias, 2004). Triplicates (three cores; five depths; n=15) were maintained and the vials were incubated in the dark for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. At the end of the incubation period, bacterial activity in all incubations was terminated using 0.1 ml of 1M $HgCl_2$.

Nitrous oxide in the headspace was analyzed using a *Shimadzu* 2010 gas chromatograph fitted with a electron capture detector and Porapak Q column (Porapak Q 1/8" SS column, 3.05 m length, 80/100 mesh). The oven and detector temperatures were 40°C and 300°C, respectively. High purity nitrogen at a flow rate of 35 ml min^{-1} was used as a carrier gas. The gas chromatograph was calibrated using a secondary standard 44.16 ±0.38 nmol N_2O in nitrogen (1.11 ± 0.009 ppm_v, courtesy: National Physical Laboratory (NPL), New Delhi). Total N_2O was calculated based on the equation stated by Tiedje (1982):

$$M=C_g(V_g+V_l\alpha)$$

where, M = the total amount of N_2O in the water plus gas phases

C_g = concentration of N_2O in the gas phase

V_g = volume of gas phase

V_l = volume of liquid phase

α = Bunsen absorption coefficient

The solubility coefficients of N_2O were used to correct for dissolved N_2O in the equilibrations (Weiss and Price, 1980). As the experiment was carried out in microcosms, it is necessary to minimize error likely to be caused by variability during sediment transfer. Hence, sediment used in each microcosm was filtered through a laboratory grade filter paper and dried at 60°C for ~48 hours. Activity was considered as the linear accumulation of N_2O over time. Denitrification rates were calculated as *nmol N_2O-N g^{-1} h^{-1} of dry sediment, extrapolated to a unit area basis as described below and expressed as μmol N_2O-N m^{-2} h^{-1}.

Calculations for conversion on area basis (m^{-2}) were done as follows:

A= B x k x 10000

where,

A= denitrification rate (nmol N_2O-N m^{-2} h^{-1})

B= denitrification rate (nmol N_2O-N g^{-1} h^{-1})

k**= 1.13

** Constant k was calculated by converting volume of sample to area:

1cc wet sediment has following dimensions in syringe

Diameter = 1.2 cm

Height = 1 cm

Volume of sediment used = $\Pi r^2 h$

$=3.14x0.6x0.6x1$

$= 1.13\ cm^2$

Thus, 1.13 cm^2 represents volume of sample.

*For back conversion of DNT activity in μmol N_2O-N m^{-2} h^{-1} to nmol N_2O-N g^{-1} h^{-1}:

(DNT activity in μmol N_2O-N m^{-2} h^{-1} x 10^3) / (1.13 x 10^4)

3.6. Statistical analyses

Significant differences among physico-chemical and bacteriological parameters were analysed using two-factor analysis of variance (ANOVA) without replication in Analysis tool pack (*Microsoft Excel*). Bacterial numbers were $\log_{10}$ transformed before analyses. Pearson's correlation coefficients were used to assess inter-relationship between denitrifiers and environmental parameters. The correlation values were plotted using

Cytoscape 2.6.3 software which enabled to build an open-source network visualization. Multiple regression analysis was performed using *Statistica* 6 software to predict the combination of factors controlling denitrification and denitrifier abundance.

SECTION II – Laboratory experiments

3.7. Quantification of nitrous oxide production and its origin

3.7.1. Experimental procedure

3.7.1.1. Sampling

Sediment cores were collected at low tide and transported in an ice box. Cores for pore water nutrient and rate measurements were maintained at 4°C until analysis. Water from sampling sites was collected in carbuoys for media preparation.

In the present study, down-core variation in net N_2O production was examined at the relatively pristine site Tuvem and the Divar mangrove ecosystem which is influenced by extraneous nutrient input. Detailed studies on N_2O production mechanisms have been restricted to Tuvem.

3.7.1.2. Physico-chemical parameters

Hydrogen ion concentration (pH) was measured upon arrival using an *Orion 4-Star Plus* Benchtop pH/ISE Meter. Sediment oxidation-reduction potential (Eh) was measured with the help of an *Orion* platinum redox in combination with a silver/silver chloride reference electrode.

For nutrient analyses, triplicate sub-samples from thawed cores were taken at every 2 cm intervals from surface to 10 cm by careful sectioning of sediment. Each section (7.5 cm diameter and 2 cm thick) was transferred to 100 ml of sterile saline and gently homogenized using a glass rod. The slurry was centrifuged at 4°C for 10 minutes at 5000 rpm (x 1803 g) with a *REMI CPR-24* centrifuge. Low spin was maintained during centrifugation to ensure minimal change in nutrient concentrations due to lysis of benthic infauna. The supernatant was filtered through a 0.2 µm filter and stored at -20°C until analysis. Ammonium, nitrite and nitrate were measured colorimetrically (*Shimadzu* UV/VIS spectrophotometer; precision: ± 0.01 µmol l^{-1}) as described by Koroleff (1969),

Bendschneider and Robinson (1952) and Wood *et al.*, (1967). Weight of the sediment used in the extraction was estimated by drying in a hot air oven at 60°C for 48 hours. Total organic carbon (TOC) was determined by wet oxidation method with a precision of 0.01% (El Wakeel and Riley, 1957).

3.7.1.3. Net nitrous oxide production and its origin

Sediment cores were demarcated into five sections (0-2, 2-4, 4-6, 6-8 and 8-10 cm). About 1 cm^3 of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20 ml headspace vials. Three ml of sterilized ambient sea water from the sampling site (containing 4.5 µmol NO_3^--N l^{-1}) was added. Further, sample preparations were amended with chloramphenicol (1 g l^{-1}) to prevent de novo enzyme synthesis during the incubations (Gilbert *et al.*, 1998; Simek, 2000; Bonin *et al.*, 2002; Desnues *et al.*, 2007). No additional carbon or nitrate was added as substrate. The vials were capped with butyl stoppers and were briefly vortexed to form slurry. To determine the denitrification (DNT) rate, some of the tubes were put under anaerobic conditions by flushing with N_2 for 15 min. The headspace over these slurries was assigned with 20 kPa acetylene (Bonin *et al.*, 2002) and the tubes were vortexed. To determine net N_2O production (from nitrification plus denitrification) and for N_2O produced by incomplete denitrification ($^{D}N_2O$), aerobic conditions were maintained and the headspace over slurries was assigned respectively with 0 or 10 Pa acetylene to inhibit nitrification (Bonin *et al.*, 2002). Triplicates were maintained at each depth and the vials were incubated in the dark for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. Following incubation, each tube was treated with 0.1 ml of 1M $HgCl_2$ solution, and vigorously shaken for 2 min to stop the reaction.

Nitrous oxide in the headspace was analyzed using a *Shimadzu* 2010 gas chromatograph fitted with a electron capture detector and Porapak Q column (Porapak Q 1/8" SS column, 3.05 m length, 80/100 mesh). The oven and detector temperatures were 40°C and 300°C respectively. High purity nitrogen at a flow rate of 35 ml min^{-1} was used as a carrier gas. The gas chromatograph was calibrated using a secondary standard 44 ±0.38 nmol N_2O in nitrogen (NPL, New Delhi). The rate of N_2O production was determined based on its linear accumulation over time (Tiedje, 1982). The Bunsen

solubility coefficient for the measured salinity and temperature in the microcosms was used to correct for dissolved N_2O (Weiss and Price, 1980). As the experiment was carried out in microcosms, it is necessary to minimize error likely to be caused by variability during sediment transfer. Hence, sediment used in each microcosm was filtered through a laboratory grade filter paper and dried at 60°C for approximately 48 hours. Average N_2O production was calculated as nmol N_2O-N g^{-1} h^{-1} of dry sediment, extrapolated to a unit area basis (m^{-2}) and converted to μmol N_2O-N m^{-2} h^{-1}.

Nitrous oxide produced through nitrification ($^{N}N_2O$) was calculated as:

$$^{N}N_2O = \text{Net } N_2O \text{ production} - {}^{D}N_2O$$

Percentage of N_2O through incomplete denitrification ($^{I}N_2O$) was calculated as:

$$\%\ {}^{I}N_2O = \frac{{}^{D}N_2O}{DNT} \times 100$$

3.7.1.4. Quantification of denitrifiers

Denitrifiers were enumerated by the N_2O-most probable number (MPN) technique. The sediment core was thawed and sectioned at 2 cm intervals using a sterile core cutter to obtain representative samples at 0-2, 2-4, 4-6, 6-8 and 8-10 cm. Sediment from each depth was homogenised by mixing. Approximately 1 g wet weight sediment from each depth was sub-sampled using sterile syringes. The sub-samples were transferred to 9 ml of sterile culture medium to give a 10^{-1} dilution. The culture medium was prepared as described by Michotey *et al.*, (2000). Tween 80 (20 μl) was added and the mixture was sonicated at 40 MHz for 15 seconds. Serial dilutions for each section of the core were prepared in triplicates. The vials were purged with high purity N_2 for 10 minutes to induce anaerobic conditions and supplemented with 20 kPa acetylene (Bonin *et al.*, 1994). The vials were incubated at room temperature in the dark for 10 days and the positive tubes were scored based on the accumulation of N_2O. Subsequent quantification was made using standard McCready's table (Rodina, 1972). Denitrifier abundance has been expressed as MPN cells g^{-1} of dry sediment.

3.7.1.5. Statistical analyses

All analyses have been carried out using *Statistica* version 6. Bacterial numbers were $\log_{10}$ transformed before analysis. Nitrous oxide production rates were checked for normal distribution using the Kolmogorov-Smirnov test. As the data was normally distributed (p>0.2), t-Test was used to check for statistically significant differences in mean value of nitrous oxide production between the two sites. Pearson's correlation coefficients were used to assess inter-relationship between biotic and abiotic parameters. Principal component analysis (PCA) was used to examine the combined influence of environmental parameters on N_2O production. Depth was not included in the matrix since maximum N_2O production was restricted between 0-4 cm.

3.8. Down-core profiling of denitrification along with other co-occurring processes in the N cycle

3.8.1. Experimental procedure

3.8.1.1. Sampling

Sediments were collected from each sampling site at low tide during pre-monsoon (May, 2008). Undisturbed sediment cores (six cores per site) were sampled by hand using PVC cores (inner diameter 7.5 cm, 20 cm depth). The top 10 cm of sediment cores were sectioned into 2 cm thick segments. For each sampling site, sediment corresponding to the same depth were pooled and homogenized. Each homogenized sample was further sub-divided into (i) 3 replicates (1 ml) stored at -20°C for molecular biology experiments, (ii) duplicates (10 ml) for immediate pore water analyses and (iii) duplicate laboratory replicates to determine an average value of microbial activities in each homogenized sample were maintained at every incubation interval (n= 12). The coefficient of variation was consistently lower for laboratory replicates than for homogenized samples from any given sample location. Therefore, the level of replication reported here is for the homogenized samples obtained at each site (Rich *et al.*, 2008).

3.8.1.2. Grain size analysis

De-ionized water was used for desalination of 15g sediment sub-samples by repeated washing followed by oven-drying at 45°C. Samples were then treated overnight by adding 20 ml of 10% Na-Hexa metaphosphate solution. The sand contents were

determined after 'wet-sieving' on 63 μm sieve and subsequent weighing. The remaining mud fraction was made up to 1000 ml in a measuring cylinder. Silt (63 to 2 mm) and clay (<2 mm) content were determined by the standard pipette analysis (Folk, 1968). The separated fractions were oven dried and weighed to calculate silt and clay ratios.

3.8.1.3. Nutrient and total organic carbon analyses

For extractable ammonium analyses, 2 ml solution of 2M KCl were added to 1 ml sediment sub sample for extraction of easily exchangeable ammonium (Mackin and Aller, 1984). The tubes were vortexed and the samples were incubated at 4°C for about 2 hours with brief vortexing every 15 minutes. The samples were then centrifuged at 8000 rpm for 10 minutes using a Beckman GS-15R centrifuge. The supernatant was stored at -20°C until analysis. Ammonium was subsequently measured using the phenol/hypochlorite method (Koroleff, 1969).

For nitrate and nitrite analysis, 5 ml sediment sub-sample retrieved using syringe core was diluted with 5 ml nitrate-free distilled water, centrifuged at 8000 rpm for 10 minutes and the supernatant was transferred to a clean tube. Nutrient analysis was performed using the Technicon auto analyser as described by Tréger and Le Corre (1975). Weight of the sediment used in the nutrient analyses was estimated by drying in a hot air oven at 60°C for 48 hours.

Total organic carbon (TOC) was determined by the wet oxidation method with a precision of 0.01% (El Wakeel and Riley, 1957).

3.8.1.4. Di-nitrogen fixation

Di-nitrogen fixation rates were measured in sediment by acetylene reduction assays following the procedure of Bebout *et al.* (1987). Two millilitres of the headspace gas was replaced by acetylene in the rubber-stopper-sealed flasks containing the sediment as previously described by Bonin and Michotey (2006). The ethylene concentration was measured using a mass spectrometer (*Anagaz* 100 MKS) by monitoring the signal at m/z=27 and by taking into account the cracking pattern to avoid m/z=28, where the ethylene peak would have been masked by N_2 (m/z=28) (Lloyd and Scott 1983). Considering the salinity and the ratio of liquid/gas phases in the incubation flask, we assumed that the amount of soluble ethylene was negligible. Ethylene production rates

were transformed to N_2 production rates using a theoretical conversion factor of 3 (3 C_2H_4 per N_2) found applicable to the sediment communities (Hardy *et al.*, 1968, Joye and Paerl, 1994). Di-nitrogen was determined from the linear accumulation of N_2 over time and has been expressed on a dry weight basis as nmol g^{-1} h^{-1}.

3.8.1.5. Net nitrification rate

To measure nitrification rates, 10 mmol sodium chlorate was added to sediment slurries (4 ml sediment sample + 4 ml seawater) to inhibit oxidation of nitrite (Gilbert *et al.*, 1997). The slurries were prepared in triplicates and incubated for up to 10 hours. Incubations were terminated using $HgCl_2$ (10 mmol final concentration) and stored at room temperature. At the end of the incubation period, the vials were briefly vortexed and samples were centrifuged at 5000 rpm for 15 min. Nitrification rate was determined from the linear production of nitrite during the incubation period using the Technicon auto analyser (Tréger and Le Corre, 1975) and has been expressed on a dry weight basis as nmol g^{-1} h^{-1}.

3.8.1.6. Nitrate reduction activity

Approximately 1 g wet weight sediment obtained from each representative section was transferred to 60 ml serum bottles. Sterile ambient seawater collected from site for media preparation. This seawater contained approximately 4.5 µmol l^{-1} nitrate. The seawater was amended with allythiourea (ATU) at a pre-standardized concentration of 125 µmol l^{-1} to inhibit nitrification (Ginestet *et al.*, 1998). The sediment slurry was briefly vortexed and the bottles were then filled with seawater up to the brim. The bottles were capped with butyl stoppers and the slurry was gently mixed and incubated in triplicates under static conditions for 3 hours as the nitrification inhibitor used became ineffective beyond this period.

At the end of the sampling period, the bottles were gently swirled. The contents were transferred 100 ml centrifuge tubes and centrifuged (*REMI Compufuge* CPR-24) at 5000 rpm and 4°C for 10 minutes. Nitrate in the supernatant was measured spectrophotometrically (*SHIMADZU* UV/VIS) as described by Wood *et al.* (1967). Nitrate reduction activity (NRA) was determined from the fall in nitrate level over time and the rate has been expressed on a dry weight basis as µmol NO_3-N g^{-1} h^{-1}.

3.8.1.7. Denitrification and anammox

Experiments for anammox and denitrification activities followed the procedure of Thamdrup and Dalsgaard (2002) and Risgaard-Petersen *et al.* (2003) with some modifications as described by Minjeaud *et al.* (2008). The isotope pairing method involves the use of stable nitrogen isotope ($^{15}NO_3^-$) and the production of single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) di-nitrogen by a mass spectrometer (Nielsen 1992).

Four ml of homogenised sediment from each section was transferred into 22 ml headspace vials containing 4 ml of filter sterilized seawater amended with 10 µmol NO_3-N. The vials were sealed with butyl rubbers stoppers, purged with He and pre-incubated for about an hour before addition stock solution of $^{15}NO_3$- (97.4 atom%, *Isotech Mathesson*, USA) (Rich *et al.*, 2008) to obtain a final concentration of 50, 80 and 150 µmol $^{15}NO_3^-$. The production of single-labelled ($^{14}N^{15}N$) and double-labelled ($^{15}N^{15}N$) di-nitrogen was followed during an incubation period of up to 10 hours in the dark. Two or three vials were sacrificed by adding $HgCl_2$ (final concentration of 10 mmol) at each point of the time series (0, 2, 4, 6, 8 and 10 hours).

The amount of $^{29}N_2$ and $^{30}N_2$ produced in the incubation vials during the incubations was measured with a quadrupole mass spectrometer (*Anagaz* 100, MKS, England) (Minjeaud *et al.*, 2008). Argon was used as an internal standard. Signals at 5 m/z values were collected every 0.5 seconds and stored onto a computer for later analyses. N_2 was measured at m/z=28, 29 and 30 corresponding to $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ respectively while O_2 or Ar were measured at m/z=32 and m/z=40 respectively. The raw value collected at m/z=30 was corrected according to Minjeaud *et al.* (2008) in order to take into account interference due to NO^+ ions formation from N_2 and O^+ inside the mass spectrometer (MS). The natural abundance of nitrogen isotopes is 99.64% of ^{14}N and 0.36% of ^{15}N (Steingruber *et al.*, 2001). The measured ratio in the present study was very close to the theoretical value and calculated to account small variability between measurements. The concentration of $^{29}N_2$ and $^{30}N_2$ were plotted against time and fitted to the linear model ($A_{(t)}=A_0 \pm m*t$) using the least squares method where: t= incubation time; A_0=concentration at t=0; m= slope of linear curve. Rate uncertainties were calculated from the errors in the linear regressions.

Total denitrification (D_{tot}) and anammox were calculated from the production of N_2 molecules of different isotopic compositions upon enrichment with stable isotope tracer ($^{15}NO_3^-$). The values of $^{29}N_2$ and $^{30}N_2$ production were used in the equation described by Thamdrup and Dalsgaard (2002) with some modifications ($_{mod}$-IPM) as described by Minjeaud *et al.* (2008) to calculate anammox (Anx) and total denitrification (D_{tot}) rates. The production rates of N_2 with masses of 28, 29 ($^{29}N_2$=^{14}N+^{15}N) and 30 ($^{30}N_2$=^{15}N+^{15}N) have been represented as P_{28}, P_{29}, and P_{30} respectively. The original IPM developed by Nielsen (1992) estimates denitrification by monitoring changes in di-nitrogen gas with different isotope compositions ($^{29}N_2$, $^{30}N_2$) after enrichment with $^{15}NO_3$. However, if anammox is present, the traditional IPM (Nielsen, 1992) is erroneous and cannot be used. In the presence of $^{15}NO_3$, the production of N_2 from anammox modifies the proportion of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ productions compared to the case where denitrification alone is implied (Risgaard-Petersen *et al.*, 2003, Trimmer *et al.*, 2006, Minjeaud *et al.*, 2008). The total production of $^{28}N_2$ and $^{29}N_2$ depends on the ratio of intensities between the reactions of denitrification and anammox. The relative contribution of anammox and denitrification in the total N_2 fluxes was calculated according to the equations described by Minjeaud *et al.* (2008) as indicated below:
The percentage of anammox is represented by R_a.

$$R_a = \frac{A_{28}}{P_{14}}$$

A_{28} is the production of $^{28}N_2$ coming from anammox. P_{14} can be expressed as function of P_{15} and R_{14}.

$$P_{14} = R_{14} \times P_{15} = R_{14} \times (P_{29} + 2P_{30})$$

The calculation of R_{14} is thus essential for the calculation of P_{14}. R_{14} is the ratio between the quantities $^{14}NO_3$ and $^{15}NO_3$ in the zone of expression of the activities, this ratio is different from the initial percentage of labelling

$$R_{14} = \frac{2D_{28}}{D_{29}} = \frac{A_{28}}{A_{29}} = \frac{D_{29}}{2D_{30}}$$

where,

D_{29} and D_{30} represent the productions of the $^{29}N_2$ and $^{30}N_2$ coming from denitrification respectively.

A_{29} is the production of $^{29}N_2$ coming from anammox.

According to Risgaard Petersen *et al.* (2003) R_{14} can be experimentally determined from measurements of P_{29} and P_{30} in the presence of different $^{15}NO_3$ concentrations as it allows the calculation of R_{14} and consequently the determination of the production of N_2 formed from $^{14}NO_3$.

$$R14_V = \frac{P_{29(1)} - V \times P_{29(2)}}{2 \times (P_{30(1)} - V^2 \times P_{30(2)})}$$

where,

$$V = \frac{[^{15}NO_3^-]_{c_1}}{[^{15}NO_3^-]_{c_2}}$$

c_1= nitrate at concentration 1

c_2= nitrate at concentration 2

If R_a equals zero, no anammox occurs and denitrification rates can be determined according to the original procedure of Nielsen (1992). The total denitrification rate ($D_{tot} = D_w + D_n$) in the sediment is therefore calculated as:

$$D_{tot} = D_{14} + D_{15} \qquad \text{Eqn (1)}$$

Denitrification from $^{15}NO_3^-$ (D_{15}) and from $^{14}NO_3$ (D_{14}) can be calculated from the production rate of $^{29}N_2$ (P_{29}) and $^{30}N_2$ (P_{30}) as follows:

$$D_{15} = P_{29} + 2\,P_{30} \qquad \text{Eqn (2)}$$

$$D_{14} = P_{29} + 2\,P_{28} \qquad \text{Eqn (3)}^*$$

* Derivation of equation as described by Minjeaud *et al.*, (2008).

When denitrification is the sole process producing D_{14} and D_{15}, D_{14} can be expressed as:

$$D_{14} = \frac{P_{29}}{2P_{30}} \times (P_{29} + 2P_{30}) \qquad \text{Eqn (4)}$$

The part of D_{14} that is based on NO_3^- from the water phase (D_w) is calculated from D_{15} and the ^{14}N:^{15}N ratio of the water column NO_3^- :

$$D_w = D_{15} \frac{[^{14}NO_3^-]_w}{[^{15}NO_3^-]_w}$$

where:

$[^{14}NO_3^-]_w$ = concentration of $^{14}NO_3^-$ in the water column

$[^{15}NO_3^-]_w$ = concentration of $^{15}NO_3^-$ in the water column

In situ denitrification of NO_3^- produced by nitrification (D_n) is calculated as:

$$D_n = D_{14} - D_w$$

All rates have been expressed as nmol N_2 g^{-1} h^{-1}.

3.8.1.8. Dissimilatory nitrate reduction to ammonium (DNRA)

For each sampling site, sediment corresponding to the same depth were pooled and homogenized. Four ml of homogenised sediment from each section was transferred into 22 ml headspace vials. Four ml of filter sterilized seawater containing NO_3^--N at a final concentration of 10 µmol l^{-1} was added. The vials were sealed with butyl stoppers, purged with He and pre-incubated for about an hour before addition of stock solution of $^{15}NO_3$- (97.4 atom%, *Isotech Mathesson*, USA) (Rich *et al.*, 2008) to obtain a final concentrations of 50, 80 and 150 µmol $^{15}NO_3^-$. Nitrate ammonification was measured by monitoring the progressive increase in isotopic enrichment of $^{15}NH_4^+$ over time. NH_4^+ in pore water and sediment was extracted by microdiffusion (Gilbert *et al.*, 1997) and the nitrogen was analyzed by mass spectrometry. Unlabelled ammonium (1 µmol) was added before microdiffusion and this quantity was taken into account when calculating the DNRA activity. The samples were treated with a mild-alkali (MgO) to convert NH_4^+ to NH_3, which was trapped on acidified (50 µl, 0.5 N H_2SO_4) pre-combusted Whatman GF/C filters. To calculate the rate of flux from dissolved nitrate to dissolved ammonium, equations derived by analogy with that of Dugdale and Goering (1967) were used. DNRA

was caculated as described by Gilbert *et al.* (1997) and the rate has been expressed at μmol NH_4-N g^{-1} h^{-1}.

3.8.1.9. Net nitrous oxide production

About 1 cm^3 of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20 ml headspace vials. Three ml of sterilized ambient sea water from the sampling site was added. This seawater contained chloramphenicol at a final concentration of 1 g l^{-1} (Gilbert *et al.*, 1998; Simek, 2000; Bonin *et al.*, 2002; Desnues *et al.*, 2007) to inhibit *de novo* synthesis of denitrifying enzymes thus reflecting *in situ* activity at the time of sampling (Brooks *et al.*, 1992). The vials were capped with butyl stoppers and were briefly vortexed to form slurry. Triplicates were maintained at each depth and the vials were incubated in the dark for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. At the end of the incubation period, bacterial activity was terminated using 0.1 ml of 1M $HgCl_2$. Nitrous oxide in the headspace was analyzed using a *Shimadzu* 2010 gas chromatograph fitted with an electron capture detector. Net nitrous oxide production was calculated based on its linear accumulation over time (Tiedje, 1982). The solubility coefficients of N_2O were used to correct for dissolved N_2O in the equilibrations (Weiss and Price, 1980). Sediment used in each microcosm was filtered through a laboratory grade filter paper and dried at 60°C for approximately 48 hours. Average N_2O production rate was calculated as nmol N_2O-N g^{-1} h^{-1} of dry sediment.

3.8.1.10. Macrofaunal abundance

Macro benthic samples were sampled using a box quadrant having an area of 15x15x10 cm. Sediment was scooped and transferred to clean plastic bags. Samples were immediately fixed at site using 10% seawater formalin with Rose Bengal solution. Samples were then transferred to laboratory and sieved through a 500 μm sieve. The stained organisms were sorted and preserved in 5% buffered seawater formalin + Rose Bengal solution (10%). The sorted samples were enumerated for macrofaunal density and identified to the group level with stereo zoom microscope (4X magnification).

3.8.1.11. Quantification of *nosZ* genes

Genomic DNA was extracted in triplicates for each layer from 0.25 g (dry weight) sediment using a *MOBIO PowerSoil*™ DNA isolation kit (*MoBio Laboratories*, California) according to the manufacturer's protocol (Refer 3.12.1. for detailed procedure). Dilutions of genomic DNA (1/5, 1/10, 1/50 and 1/100) were made in sterile water and stored at -20°C.

Each PCR reaction (25 µl) reaction tube contained the following: Mg free buffer (2.5 µl) (*Promega*, USA); dNTPs (2.5 µl of 10mM *Promega*, USA); $MgCl_2$ (1.5 µl of 25 mM *Promega*, USA); *nosZ* Primer 0.5 µl of each nosZ 1211F and *nosZ* 1897R (100 pmol μl^{-1}) (Rösch *et al.*, 2002); Taq polymerase: 0.2 µl (5 units/µl; *Promega*, USA). The PCR conditions for *nosZ* were maintained as follows: an initial 3 min denaturation at 94°C followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 1 minute. The amplified products were analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel and visualized using an UV transilluminator (GelDoc 2000, gel documentation system, *Bio-Rad*). Detailed procedure for amplification of *nosZ* by PCR has been given in 3.12.2.

The competitive PCR method was used to create standard curve for the quantification of *nosZ* genes in the DNA sample. A competitor internal standard was co-amplified with the target DNA. The standard was shortened artificially to 120 bp. Standards for *nosZ* quantification ($6.8x10^{-4}$ to $6.8x10^{-2}$) were prepared as a mixture of cloned *nosZ* fragments of *Pseudomomas*, *Marinobacter* and *Achromobacter* spp. Each replicate of DNA extract for a layer (n=3) were subject to PCR reactions in 25 µl mixtures as follows:

nosZ 10^{-6} : 4 µl

nosZ 10^{-6} : 2 µl + 2 µl sterile H_2O

nosZ 10^{-6} : 1 µl + 3 µl sterile H_2O

nosZ 10^{-7} : 4 µl

nosZ 10^{-7} : 2 µl + 2 µl sterile H_2O

nosZ 10^{-7} : 1 µl + 3 µl sterile H_2O

where,

10^{-6} dilution of standard contained $6.8x10^{3}$ *nosZ*/µl

10^{-7} dilution of standard contained $6.8x10^2$ *nosZ*/µl

The extracted DNA was diluted to 1/10 concentration. PCR reactions were carried out in triplicates in 25 ml mixtures. Each reaction tube contained the following:

Mg free buffer: 2.5 µl (*Promega*, USA)

dNTPs: 2.5 µl (*Promega*, USA; 10 mM)

$MgCl_2$: 1.5 µl (*Promega*, USA; 25 mM)

nosZ Primer 1211F: 0.5 µl

nosZ Primer 1897R: 0.5 µl

Distilled water: 15.3 µl

Taq polymerase: 0.2 µl (5 units/µl; *Promega*, USA)

Standard (10^{-7}): 1 µl ($1.3x\ 10^6$ molecules of *nosZ*)

DNA: 2 µl

The pooled products were subjected to gel electrophoresis on a 1.5% agarose gel. The intensity of each band was measured using *Quantity One* software. The ratio between the two bands (one from *nosZ* and the other of the internal standard) was plotted against the number of *nosZ* in the PCR tube (Fig. 6). PCR were run with each DNA extract from mangrove sediments having the same quantity of internal standard ($3x10^3$ *nosZ*) and quantification of *nosZ* per gram dry sediment was calculated from the standard curve.

3.8.1.12. Statistical analysis

Principal component analysis was performed on the data matrix (bacterial activities and chemical parameters) using Pearson rank correlation. This method provided an ordination of sampling sites and of bacterial activities which were plotted in two dimensions based on the scores in the first two principal components. PCA test were performed using the XLSTAT software 2009 (*Addinsoft,* Paris, France). The results of the ordination analyses have been visualized as a bi-plot (Cattell 1966, Gabriel, 1971). Only significant relationships have been discussed. Depth was included in the biplot analysis as the N cycle processes in this study are depth dependent.

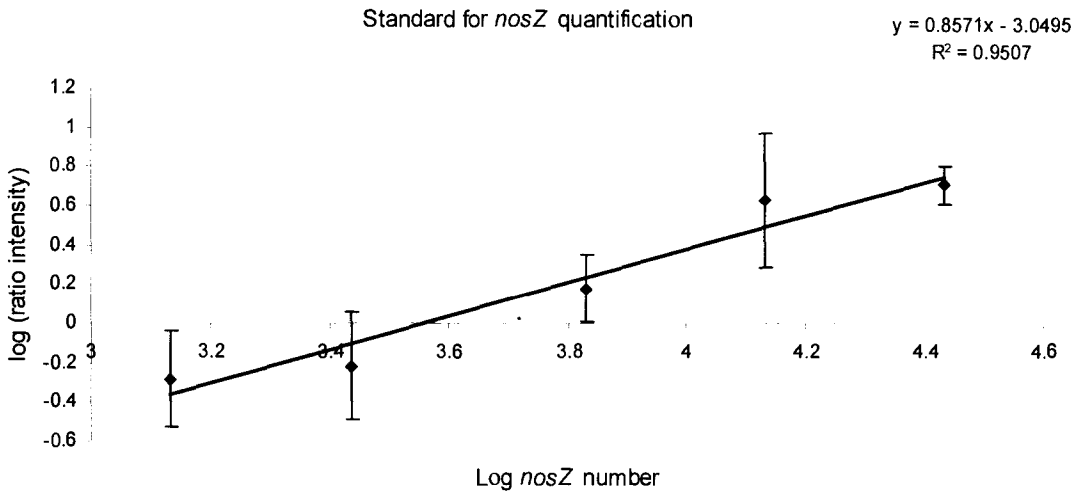


Fig. 6: Standard graph for *nosZ* quantification

where log (ratio intensity)= corrected *nosZ* volume/corrected volume of *nosZ* standard

3.9. Influence of nitrate and organic C amendments on denitrification

3.9.1. Experimental procedure

3.9.1. 1. Sampling

The anthropogenically influenced site Divar was selected for detailed investigations. Sediment cores were collected at low tide and transported in an insulated box. Cores for activity measurements were maintained at 4°C. For measurement of labile organic matter, cores were immediately sectioned at 0-2, 2-4, 4-6, 6-8 and 8-10 cm interval. The sections were dried at 60°C ±2, powdered and sieved through a 200 µm sieve and stored in clean PVC vials until analysis.

3.9.1. 2. Pore water nitrate

For the estimation of pore water nitrate concentration, sub-samples were taken at every 2 cm intervals from surface to 10 cm by carefully sectioning the core. Each section (7.5 cm diameter and 2 cm thick) was transferred to 100 ml of sterile saline and homogenized using a glass rod. The slurry was centrifuged at 5000 rpm for 10 mins and 4°C. Low spin was maintained during centrifugation to ensure minimal change in nutrient concentration due to lysis of benthic infauna. The supernatant was filtered through a 0.2 µm filter and stored at -20°C until analysis. Nitrate was measured colorimetrically (*Shimadzu* UV/VIS spectrophotometer; precision: ± 0.01 µmol l^{-1}) as described by Wood *et al.* (1967). Weight of the sediment used in the extraction was estimated by drying in a hot air oven at 60°C for 48 hours.

3.9.1. 3. Total organic carbon

Total organic carbon (TOC) was determined by wet oxidation method with a precision of 0.01% (El Wakeel and Riley 1957).

3.9.1. 4. Labile organic matter

The labile organic matter (LOM) was measured as a sum of proteins, carbohydrate and lipid content in the sediments. Proteins were estimated as described by Lowry *et al.* (1951). Carbohydrate was estimated by phenol-sulphuric acid method (Dubois *et al.*, 1956) using glucose as standard. Lipid content was extracted from sediment as described by Bligh et al. (1959) and estimated by using the method outlined by (Parsons *et al.*, 1984).

3.9.1. 5. Effect of NO_3^- addition on denitrification

Sediment cores were demarcated into five sections (0-2, 2-4, 4-6, 6-8 and 8-10 cm). About 1 cm^3 of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20 ml headspace vials. Three ml of sterilized ambient seawater from the sampling site was added to the sediment. This seawater used for slurry preparation was spiked with a KNO_3 solution to give final concentrations of 0 (unamended to reflect *in situ* denitrification activity), 5, 10, 20, 40 and 60 µmol NO_3-N l^{-1}. This seawater was also (Gilbert *et al.*, 1998; Simek, 2000; Bonin *et al.*, 2002; Desnues *et al.*, 2007) to inhibit *de novo* synthesis of denitrifying enzymes thus reflecting *in situ* activity at the time of sampling (Brooks *et al.*, 1992). No additional carbon substrates were added. The vials were capped with butyl stoppers and were briefly vortexed to form slurry. The vials were purged with high purity N_2 for 10 minutes to induce anaerobic conditions. Acetylene gas at 20 kPa (Bonin *et al.*, 2002) was injected into the headspace to inhibit N_2O production by nitrification and its reduction by denitrification (Castro-González and Farias, 2004). Triplicates were maintained at each depth and the vials were incubated in the dark for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. At the end of the incubation period, bacterial activity was terminated using 0.1 ml of 1M $HgCl_2$.

Nitrous oxide in the headspace was analyzed using a *Shimadzu* 2010 gas chromatograph fitted with a electron capture detector and Porapak Q column (1/8" SS column, 3.05 m length, 80/100 mesh). The oven and detector temperatures were 40°C and 300°C respectively. High purity nitrogen at a flow rate of 35 ml min^{-1} was used as a carrier gas. The gas chromatograph was calibrated using a secondary standard 44 ±0.38 nmol N_2O in nitrogen (NPL, New Delhi). Denitrification activity was calculated based on the linear accumulation of N_2O over time. The solubility coefficients of N_2O were used to correct for dissolved N_2O in the equilibrations (Weiss and Price, 1980). Sediment used in each microcosm was filtered through a laboratory grade filter paper and dried at 60°C for approximately 48 hours and weighed. Denitrification activity was calculated as nmol N_2O-N g^{-1} h^{-1} of dry sediment, extrapolated to a unit area basis (m^{-2}) and expressed as µmol N_2O-N m^{-2} h^{-1}.

3.9.1. 6. Effect of organic C addition on denitrification

Sterilized seawater used for slurry preparation was amended with 1 g l^{-1} chloramphenicol and glucose solution at a final concentration of 0, 0.1, 0.3, 0.5, 0.75 and 1%. Samples were prepared and analyzed as described above. No additional nitrate was added.

3.9.1. 7. Potential denitrification rates

Samples were prepared and analyzed as described above except that the seawater used for slurry preparation was amended with KNO_3 and glucose at a final concentration of 1 mmol l^{-1} in addition to chloramphenicol.

3.9.1. 8. Statistical analyses

Statistical analyses have been carried out using analysis tool pack in *Microsoft Excel.* Significant differences in denitrification rates have been determined using analysis of variance (ANOVA).

3.10. Influence of bioturbation on denitrification

3.10.1. Experimental procedure

3.10.1.1. Study area and sampling

The Divar mangrove ecosystem was selected to examine the influence of bioturbation on denitrification. The Divar sediments are sandy in nature and organically rich with organic carbon levels varying between 0.1 to 6.5% (Krishnan and Loka Bharathi, 2009). During high tide most of the mangrove area is inundated with seawater while at low tide, a large area of the mangroves get exposed due to the receding tide. A large number of crabs of the *Uca* spp. can be seen surfacing out from burrows during low tide (Plate 3).

Sediment cores for de-faunation were collected at low tide and transported to the laboratory (May, 2009). De-faunation was achieved by freezing (Gerino, 1990) the cores at -70°C. The de-faunated cores were thawed and covered at both ends using a 200 μm mesh to prevent entry of macrofauna. The cores were then transplanted back to the experimental site and left undisturbed for 7 days. After a week, the de-faunated cores were retrieved. Additional cores of natural sediment containing autochthonous infauna (faunated cores) were also collected for measuring physico-chemical and biological parameters. All cores were transferred to an insulated box and transported to the

Plate 3: Crabs of *Uca* spp. seen burrowing/emerging out from the sediment at Divar

laboratory. Upon arrival, faunated and de-faunated cores were maintained at 4°C for activity measurements. Down-core nutrient profiles, denitrification activity and abundance of macrofauna were estimated and compared between faunated and de-faunated cores. All analysis was completed within 36 hours of sample collection.

3.10.1.2. Physico-chemical analyses

Hydrogen ion concentration at each section was measured using an *Orion* 4-Star Plus Benchtop pH/ISE Meter after calibration with standard buffers (pH 4, 6.9 and 9; Merck). Sediment oxidation-reduction potential (Eh) was measured at each section of the core with the help of an *Orion* platinum redox in combination with a silver/silver chloride reference electrode.

For the estimation of N compounds (nitrate and nitrite) in pore water, sub-samples were taken at every 2 cm intervals from surface to 10 cm by carefully sectioning the core after thawing. Each section (7.5 cm diameter and 2 cm thick) was transferred to 100 ml of sterile saline and homogenized using a glass rod. Low spin was maintained during centrifugation to ensure minimal change in nutrient concentrations due to lysis of benthic infauna. The supernatant was filtered through a 0.2 µm filter and stored at -20°C until analysis. Nitrate was measured colorimetrically as described by Wood *et al.* (1967) using a *Shimadzu* UV/VIS spectrophotometer. Nitrite was measured as described by Bendschneider and Robinson (1952). Weight of the sediment used in the extraction was estimated by drying in a hot air oven at 60°C for 48 hours.

For measuring denitrification in faunated and de-faunated cores, thawed cores were demarcated into five sections (0-2, 2-4, 4-6, 6-8 and 8-10 cm). About 1 cm^3 of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20 ml headspace vials. Sterilized ambient sea water from the sampling site was added to the sediment. This seawater was also amended with chloramphenicol at a final concentration of 1 g l^{-1} (Gilbert *et al.*, 1998; Simek, 2000; Bonin *et al.*, 2002; Desnues *et al.*, 2007) to inhibit *de novo* synthesis of denitrifying enzymes thus reflecting *in situ* activity at the time of sampling (Brooks *et al.*, 1992). The vials were capped with butyl stoppers and were briefly vortexed to form slurry. The vials were purged with high purity N_2 for 10 minutes to induce anaerobic conditions. Acetylene gas at 20 kPa (Bonin *et al.*, 2002) was injected into the headspace to inhibit N_2O production by nitrification and its

reduction by denitrification (Castro-González and Farias, 2004). Triplicates were maintained at each depth and the vials were incubated in the dark for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. At the end of the incubation period, bacterial activity was terminated using 0.1 ml of 1M $HgCl_2$.

Nitrous oxide in the headspace was analyzed using a *Shimadzu* 2010 gas chromatograph fitted with a electron capture detector and Porapak Q column (Porapak Q 1/8" SS column, 3.05 m length, 80/100 mesh). The oven and detector temperatures were 40°C and 300°C respectively. High purity nitrogen at a flow rate of 35 ml min^{-1} was used as a carrier gas. The gas chromatograph was calibrated using a secondary standard 44 ±0.38 nM N_2O in nitrogen (NPL, New Delhi). Denitrification (DNT) activity was calculated based on the linear accumulation of N_2O over time. The solubility coefficients of N_2O were used to correct for dissolved N_2O in the equilibrations (Weiss and Price, 1980). Sediment used in each microcosm was filtered through a laboratory grade filter paper and dried at 60°C for approximately 48 hours. DNT was calculated as nmol N_2O-N g^{-1} h^{-1} of dry sediment, extrapolated to a unit area basis (m^{-2}) and expressed as µmol N_2O-N m^{-2} h^{-1}.

3.10.1.3. Denitrifier abundance

Denitrifiers were enumerated by the N_2O- most probable number (MPN) technique. The sediment core was thawed and sectioned at 2 cm intervals using a sterile core cutter to obtain representative samples at 0-2, 2-4, 4-6, 6-8 and 8-10 cm. Sediment from each depth was homogenised by mixing. Approximately 1 g wet weight sediment from each depth was sub-sampled using sterile syringes. The sub-samples were transferred to 9 ml of sterile culture medium to give a 10^{-1} dilution. The culture medium was prepared as described by Michotey *et al.* (2000). Tween 80 (20 µl) was added and the mixture was sonicated at 40 MHz for 15 seconds. Serial dilutions for each section of the core were prepared in triplicates. The vials were purged with high purity N_2 for 10 minutes to induce anaerobic conditions and supplemented with 20 kPa acetylene (Bonin *et al.*, 1994). The vials were incubated at room temperature in the dark for 10 days and the positive tubes were scored based on the accumulation of N_2O. Subsequent quantification was made using standard McCready's table (Rodina, 1972). Denitrifier abundance has been expressed as MPN cells g^{-1} of dry sediment.

3.10.1.4. Macrofaunal abundance

Cores containing natural sediment were sectioned at every 2 cm intervals from surface to 10 cm. The sections were transferred to clean plastic bags. The samples were preserved by adding 10% seawater formalin Rose Bengal solution. Samples were then sieved using a 500 μm sieve, sorted, identified and enumerated identified to the lowest taxa possible with stereo zoom microscope (4X magnification). The density of organisms was calculated as:

Area of section= Πr^2

where radius=0.0375 m

Therefore, area of section=0.0044 m^2

$$\text{Area } (m^{-2}) = \frac{1}{0.0044}$$

$$= 226$$

Organisms (m^{-2}) = Count * 226

3.10.1.5. Statistical analysis

Data analysis was carried out using *Statistica* version 6. Differences in denitrification activity between faunated and de-faunated cores were analyzed using analysis of variance (ANOVA).

SECTION III –Diversity of denitrifying bacteria

The diversity of denitrifiers in mangrove sediments was assessed using three different approaches. At a cellular level, the culturable denitrifiers isolated from MPN tubes were subjected to biochemical characterization. The isolates were then identified up to the generic level using pertinent taxonomic keys. At the community level, whole DNA from sediments was analyzed to understand both taxonomic and functional diversity of denitrifiers. For functional diversity, the *nosZ* gene catalyzing the reduction of nitrous oxide to di-nitrogen was probed. A more recent technique viz., the 454 pyrosequencing technology involving sequencing of the V6 region of 16S rRNA gene was used to delineate the community diversity up to the species level.

3.11. Taxonomic diversity of denitrifiers: a biochemical approach

3.11.1. Isolation

The most-probable number method has been widely used for the culture and enumeration of denitrifiers (Shieh *et al.*, 2004; Fan *et al.*, 2006). Pure isolates of facultatively anaerobic denitrifiers from mangrove sediments were obtained from MPN culture tubes which were amended with nitrate. A 100 µl innòculum from every dilution was surface plated on solid media prepared using the MPN denitrifier culture medium (Appendix VII) supplemented with 1.5% agar. The plates were incubated at room temperature in aerobic conditions. Colonies of 1-3 pre-dominant types appearing on each plate were isolated, transferred to liquid MPN culture medium tubes having medium filled up to the brim. A total of 126 denitrifier strains (Divar n=76; Tuvem n=50) were isolated.

3.11.2. Characterization and identification

Pure denitrifier isolates were characterized and identified up to the generic level (Oliver, 1982; Holt *et al.*, 1994). Identification was done based on morphological and physiological (Appendix VIII) tests. Biochemical characterization was done using the following tests:

1. Citrate utilization
2. Lysine utilization
3. Ornithine decarboxylation
4. Urease detection
5. Phenylalanine deamination (TDA)
6. Nitrate reduction
7. H_2S production
8. Glucose utilization
9. Adonitol utilization
10. Lactose utilization
11. Arabinose utilization
12. Sorbitol utilization

Test cultures were grown overnight in liquid denitrifier medium. The cell suspension was washed with sterile saline. Each test vials from the biochemical test kit (*Microxpress*,

India) was inoculated with 100 µl of culture suspension (OD_{600}:1.0) and incubated at room temperature for approximately 48 hours. Qualitative results were recorded based on the presence of by-products. Results were interpreted according to the specifications provided by the manufacturer.

3.12. Functional diversity: probing the nosZ gene

3.12.1. DNA extraction

Sediment samples were lyophilized overnight and genomic DNA was extracted using a *MO BIO PowerSoil* DNA isolation kit (CA, USA). The kit eliminates humic substances/brown colour permitting high level of purity for successful amplification by the PCR. The DNA was isolated as follows:

1. Freezed sediment samples from respective depth layer were thawed and homogenised using a sterile rod.
2. A sub-sample weighing 0.25 gm each was weighed and added to the PowerBead tubes provided.
3. The tubes were briefly vortexed and 60 µl of **Solution C1** containing sodium dodecyl sulfate (SDS) was added. The tubes were inverted several times and then fastened onto a horizontal vortexer. They were vortexed at maximum speed for 10 minutes. This step helps to disperse the soil particles, dissolves humic acids and protects nucleic acids from degradation.
4. The bead tubes were centrifuged on *Thermo Heraus Fresco 17* centrifuge at 13,000 x *g* for 1 minute at room temperature.
5. The supernatant was transferred to a clean 2 ml collection tube and 250 µl of **Solution C2** was added. The tubes were vortexed for 5 seconds and incubated at 4°C for 5 minutes. Solution C2 contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins.
6. The tubes were centrifuged at room temperature for 1 minute at 13,000 x g.
7. Avoiding the pellet, 600 µl of supernatant was transferred to a clean 2 ml collection tube.
8. To the supernatant, 200 µl of **Solution C3** was added. The solution precipitates additional non-DNA organic and inorganic material including humic acid, cell

debris and proteins. The tubes were vortexed briefly and incubated at 4°C for 5 minutes.

9. The tubes were centrifuged at room temperature for 1 minute at 13,000 x *g*.
10. Avoiding the pellet, 750 µl of supernatant was transferred into a clean 2 ml collection tube.
11. To the supernatant collected, 1200 µl of **Solution C4** was added and vortexed for 5 seconds. Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solutions salt concentration to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the spin filters.
12. Approximately 675 µl was loaded onto a spin filter and centrifuge at 13,000 x *g* for 1 minute at room temperature. The flow through was discarded, an additional 675 µl of supernatant was added to the spin filter and centrifuge at 13,000 x *g* for 1 minute at room temperature. A total of three loads for each sample were processed.
13. To the spin filters, 500 µl of **Solution C5** was added and centrifuged at room temperature for 1 minute at 13,000 x *g*. Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the spin filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.
14. The flow through was discarded and the tubes were centrifuged again at room temperature for 1 minute at 13,000 x *g*.
15. The spin filters were carefully placed in a clean 2 ml collection tube avoiding splashing of any Solution C5 onto the spin filter.
16. A 100 µl volume of **Solution C6** was added to the centre of the white filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mmol Tris) which lacks salt.
17. The tubes were centrifuged at room temperature for 1 minute at 13,000 x *g*.
18. The spin filter was discarded and the DNA in the tube was used for PCR amplification.

19. Samples were checked for purity (OD_{260}/OD_{280} ~1.8).

3.12.2. Amplification of *nosZ* genes by PCR

The extracted DNA was diluted to 1/10 concentration. PCR reactions were carried out in 25 ml mixtures. Each reaction tube contained the following:

Mg free buffer: 2.5 µl (*Promega*, USA)

dNTPs: 2.5 µl (10 mM; *Promega*, USA)

$MgCl_2$: 1.5 µl (25 mM; *Promega*, USA)

nosZ Primer 1211F: 0.5 µl (Rösch *et al.*, 2002)

nosZ Primer 1897R: 0.5 µl

Sterile distilled water: 15.3 µl

Taq polymerase: 0.2 µl (5 units/µl; Promega, USA)

DNA: 2 µl

The PCR runs were made as follows:

An initial 3 min denaturation at 94°C

Denaturation at 94°C for 30 seconds
Annealing at 55°C for 30 seconds
Elongation at 72°C during for 1 minute
} 30 cycles

Final elongation at 72°C during for 3 minutes

A negative control tube (2 µl of sterile distilled water + PCR mix) and a positive tube containing (2 µl DNA from *Marinobacter hydrocarbonoclasticus* + PCR mix) were also prepared. About 10 µl of PCR product was mixed with 2 µl of loading dye, mixed, loaded onto a 1% (wt/vol) agarose gel (Appendix IX) containing 0.5 mg ml^{-1} ethidium bromide and subjected to electrophoresis in TBE 1X buffer (Appendix X). A 2 µl volume of DNA molecular weight marker III (0.25 µg/µl; *Roche Diagnostics*, Germany) was loaded along with the samples. The separated products were visualized using an UV transilluminator (GelDoc 2000, gel documentation system, *Bio-Rad*). Each PCR was performed thrice and the PCR products were pooled prior to Denaturing Gradient Gel Electrophoresis (DGGE) analysis.

3.12.3. Denaturing gradient gel electrophoresis

Denaturing Gradient Gel electrophoresis (DGGE) was performed using a D-code Universal Mutation Detection System (Bio-Rad Laboratories Inc.). Samples containing approximately equal amounts of PCR products (600 ng) were loaded onto 1 mm thick, 6% (wt/vol) polyacrylamide gel (Appendix XI) with a denaturation gradient from 20% to 80% (100% of denaturation corresponds to 7M urea and 40% formamide). Electrophoresis was run at 60°C for 280 minutes at 150V in 1X TAE (Appendix XII). Following electrophoresis, the gel was incubated for 30 minutes in 1X TAE buffer containing ethidium bromide (0.5 mg ml^{-1}) and photographed on a UV transilluminator (GelDoc 2000, gel documentation system, *Bio-Rad*). A 10 µl volume of the following mixture of markers were used-

- *nosZ* of *Pseudomonas* sp NBP39
- *nosZ* of *Marinobacter* sp BC38
- *nosZ* of *Achromobacter cycloclastes* ATCC 21921
- 16S *Micrococcus* sp SR283
- 16S *Clostridium* sp.

Representative bands were excised from the polyacrylamide gel using sterile toothpicks and transferred into clean eppendorf tubes. The gel fragments were rinsed with 50 µl of sterile distilled water. Another 50 µl volume of sterile distilled water was added and the tubes were stored at 4°C overnight for allowing diffusion of the DNA into the water. The following day, the gel pieces were agitated using sterile tips and centrifuges at 13,000 x for 1 minute. The supernatant was transferred to a clean 1.5 ml eppendorf tube. A 5 µl volume of these samples were used for amplification of *nosZ* by PCR. The products were observed by electrophoresis. Amplified *nosZ* bands were cut and transferred to clean 1.5 ml eppendorf tubes. The DNA in the agarose gel (approx. 100 mg or 100 µl vol) was purified using the *QIAGEN MinElute Gel Extraction Kit* as follows:

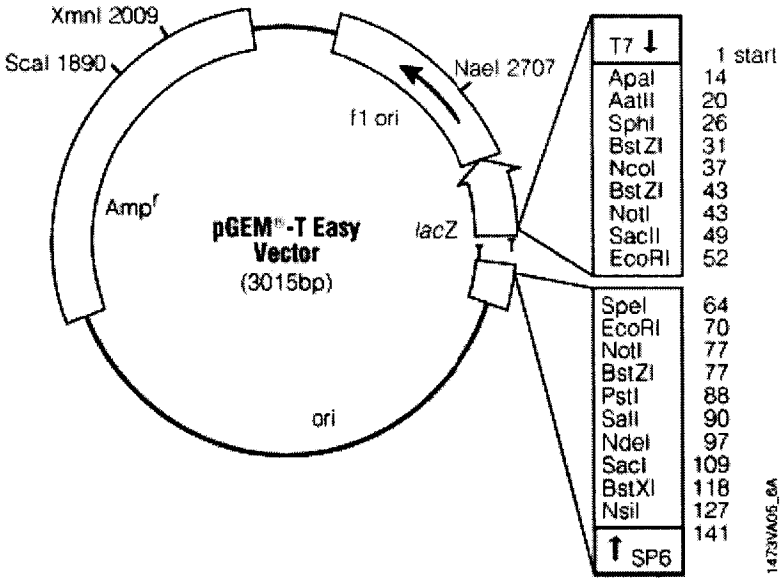
1. A 300 µl volume of buffer QG was added to the gel.
2. The tubes were incubated at 50C for 10 minutes until the gel slice had completely dissolved. To help the dissolution, the tubes were vortexed briefly for a few seconds every 3 minutes.

3. After the gel had dissolved completely, 100 µl of isopropanol was added to the samples and the contents were mixed by inverting the tubes several times.
4. The *MinElute* column was placed in a clean 2 ml collection tube. The samples were transferred to the column and allowed to stand for a minute.
5. The samples were centrifuged at 13,000 x for 1 minute.
6. The flow-through was discarded from the collection tube and the *MinElute* column was placed back into the collection tube.
7. A 500 µl volume of buffer QG was added to the spin column and centrifuged at 13,000 x for 1 minute.
8. The flow-through was discarded and the column was placed back into the collection tube.
9. To wash, 750 µl of buffer PE was added to the *MinElute* column and centrifuged for 1 minute.
10. The flow-through was discarded and the column was centrifuged for an additional 1 minute.
11. The *MinElute* column was placed in a clean 1.5 ml collection tube.
12. To elute the DNA, 10 µl of buffer EB (10mM Tris Cl; pH 8.5) was added to the centre of the membrane. The column was allowed to stand for a minute and then centrifuged for 1 minute.
13. The purified DNA was used for downstream application i.e. cloning.

3.12.4. Cloning of PCR fragments

Cloning of PCR products was performed using the pGEM-T and pGEM-T Easy Vector Systems. The vectors are prepared by cutting *Promega's* pGEM-5Zf(+)[(b)] and pGEM-T Easy vectors with EcoR V and adding a 3′ terminal thymidine to both ends. These single 3′ - T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing re-circularization of the vector and providing a compatible overhang for PCR products (Fig. 7).

The high copy number pGEM-T and pGEM-T Easy Vectors contain T7 and SP3 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Successful cloning of an insert into the



Pic: www.promega.com

Fig. 7: pGEM®-T Vector circle map and sequence reference points (3015bp)

vectors interrupts the coding sequence of β-galactosidase. Insertional inactivation of the α-peptide and allows recombinant clones to be directly identified by colour screening on indicator plates. Clones that contain PCR products produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lacZ* gene.

3.12.5. Ligation of PCR products

Ligation of PCR products was performed using *Promega's* pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer. The pGEM®-T or pGEM®-T Easy Vector and control insert DNA tubes were briefly centrifuged to collect contents at the bottom of the tubes.

Ligation reactions were set up as described below:

2X Rapid Ligation Buffer, T4 DNA Ligase:	5 µl / reaction
pGEM®-T or pGEM®-T Easy Vector (50 ng):	1 µl / reaction
T4 DNA Ligase (3 Weiss units/µl):	1 µl / reaction
PCR product:	1.5 µl / reaction

The reactions were mixed by pipetting and incubated overnight at 4°C for obtaining maximum number of transformants.

3.12.6. Transformation of ligation reactions into competent cells

Transformations was performed using *Promega's* pGEM®-T and pGEM®-T Easy Vector Ligation Reactions

1. Two LB/ampicillin/IPTG/X-Gal plates (Appendix XIII) were prepared for each ligation reaction. The plates were equilibrated to room temperature prior to plating.

- Ampicillin sodium salt: A b-lactam antibiotic with an amino group side chain attached to the penicillin structure. Penicillin derivative that inhibits bacterial cell wall synthesis (peptidoglycan cross-linking) by inactivating transpeptidases on the inner surface of the bacterial cell membrane. Bactericidal only to growing *E. coli.*
- Isopropyl b-D-1-thiogalactopyranoside (IPTG) functions by binding to the *lacI* repressor and altering its conformation, which prevents the repression of the b-galactosidase coding gene *lacZ.*
- X-Gal (Bromo-4-chloro-3-indolyl b-D-galactoside) is a chromogenic substrate for b-galactosidase that produces a rich blue color that can easily be detected visually

over background. X-Gal is a substrate for blue-white selection of recombinant bacterial colonies with the *lac*+ genotype.

2. Tubes containing the ligation reactions were centrifuged at 10,000× *g* for 10 minutes to collect contents at the bottom of the tube. About 3 µl of each ligation reaction was transferred to a sterile 1.5 ml micro centrifuge tube on ice.

3. Tubes containing frozen JM109 High Efficiency Competent Cells were removed from storage (liquid nitrogen) and placed in an ice bath until just thawed (about 5 minutes). The cells were mixed by gently flicking the tube.

4. 50 µl of cells were carefully transferred into each tube prepared in Step 2.

5. The contents were mixed by gently flicking the tubes and placed on ice for 20 minutes.

6. The cells were subjected to heat-shock for 45–50 seconds in a water bath at exactly 42°C.

7. The tubes were immediately returned to ice for 2 minutes.

8. A 950 µl innoculum of SOC medium at room temperature was added to the tubes containing cells transformed with ligation reactions.

9. The tubes were incubated for 1.5 hours at 37°C.

10. A 100 µl innoculum of each transformation culture was plated onto LB/ampicillin/IPTG/X-Gal plates. For a higher number of colonies, the cells were pelleted by centrifugation at 10,000 × *g* for 10 minutes, re-suspended in 200 µl of SOC medium, and 100 µl plated on each of two plates.

11. The plates were incubated overnight (16–24 hours) at 37°C.

3.12.7. Analysis of clones

Whitish colonies (containing insert) from LB/ampicillin/IPTG/X-Gal plates (Plate 4) were transferred in 2 ml liquid LB medium amended with ampicillin (10 mg/ml). The culture tubes were incubated for 24 hours on a shaker at 22°C until the cells (plasmid culture) were grown to a density of A_{600}=2.0 or higher.

3.12.8. Purification of plasmid culture

Plasmid culture was purified using the *MO BIO* UltraClean™ 6 Minute Mini Plasmid Prep Kit. In this method cells are lysed using alkaline lysis reagents. Plasmid DNA is bound to a silica spin filter, washed once, and recovered in Tris buffer or water. The

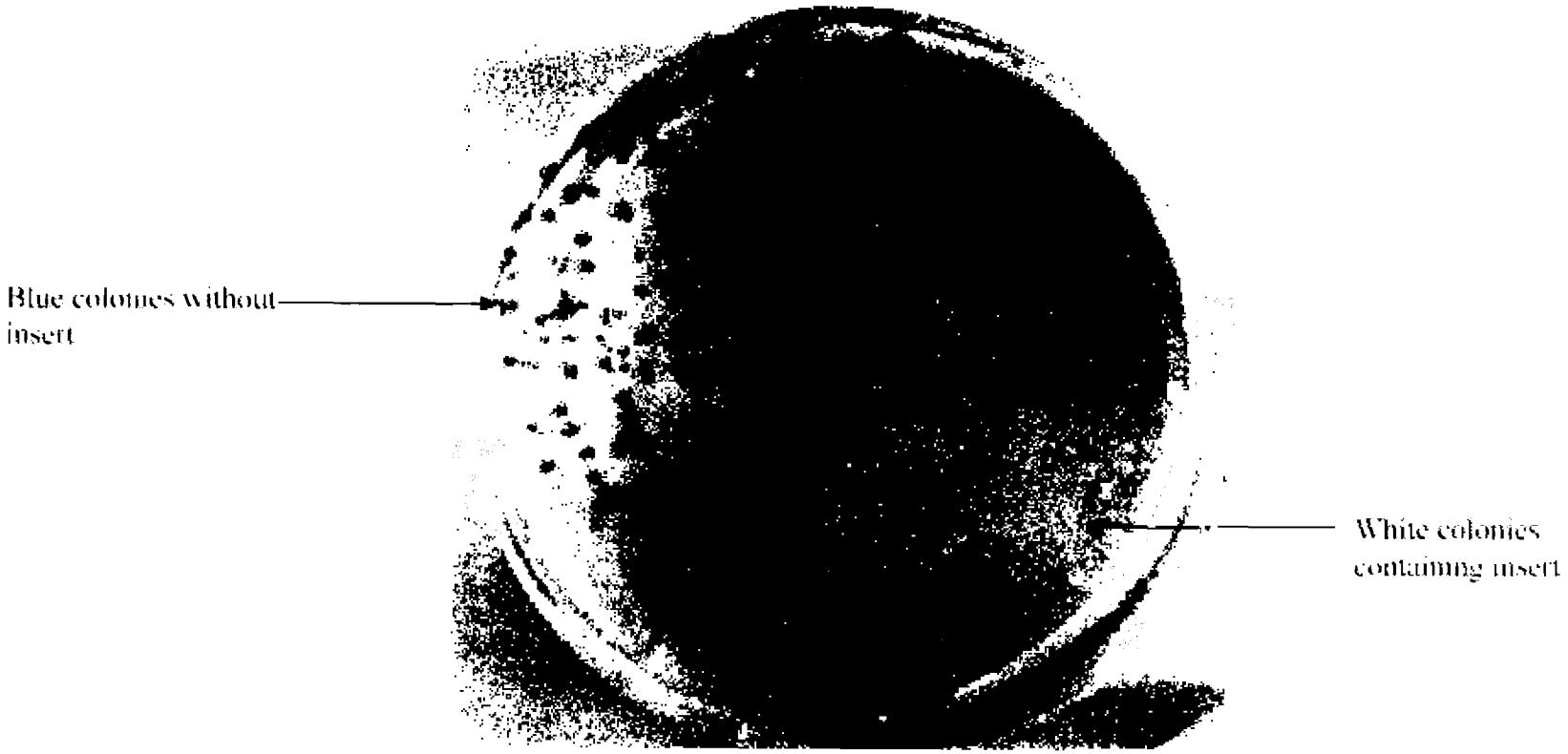


Plate 4: Cloned PCR products

DNA retrieved can be directly used for automated sequencing or other downstream applications. The plasmid culture was purified as per the method described by the manufacturer as follows:

1. Overnight grown cells were transferred into sterile labelled micro centrifuge tubes and centrifuged for 1 minute at 13,000 x g.

2. The supernatant was discarded. Any remaining liquid from tube was removed with a pipette tip.

5. The cell pellet was re-suspended cell in 50 µl of **Solution 1** by bump vortexing for 1 minute to yield a homogenous suspension. The buffer in the solution prevents the bacterial cells from lysing.

8. A 100 µl volume of **Solution 2** was added. Solution 2 contains a detergent SDS the addition of which causes the bacterial cells to lyse due to denaturation of proteins in the cell membrane. All DNA becomes denatured to its single stranded form at this point. The bacterial chromosomal DNA is long and is attached to broken pieces of the cell membrane. Plasmid DNA is linked so it forms two attached circles like two links of a chain. All RNA is digested during this very short step because RNase A is active even in very alkaline conditions.

10. The tubes were inverted once to mix.

11. A 325 µl volume of **Solution 3** was added and the tubes were inverted just once to mix. Solution 3 contains potassium acetate and salt. The potassium acetate forms a precipitate when it interacts with SDS. At the same time denatured proteins co-precipitate with the SDS. Solution 3 neutralizes the alkaline pH to a more neutral pH 7. All DNA tries to re-nature. Plasmid can easily re-form to its double stranded form. Bacterial chromosomal DNA finds it difficult to re-nature because it has no reference point and homologous pieces of DNA may be blocked from finding each other by the cell debris present.

12. The tubes were centrifuged for 1 minute at 13,000 x g.

14. The supernatant was transferred into a spin filter by decanting.

15. The spin filters were centrifuged for 1 minute.

16. The liquid in the collection tube was discarded.

17. A volume of 300 µl of **Solution 4** was added. Solution 4 washes the DNA that is bound to the spin filter. Solution 4 is about 50% ethanol. The ethanol keeps the plasmid DNA bound to the filter as impurities are washed away.

18. The tubes were centrifuged for 1 minute.

19. The flow through was discarded and the tubes were centrifuged again for another 1 minute.

20. The spin filter baskets were carefully transferred to new micro centrifuge tubes.

21. A 50 µl volume of **Solution 5** was added to the middle of spin filter membrane. Solution 5 is 10 mM Tris. As it passes through the spin filter, the plasmid DNA is released (eluted) off the filter and it passes into the collection tube. The plasmid DNA is released because it will not stay bound to the spin filter when there is no salt present.

22. The tubes were centrifuged for 1 minute.

23. The spin filter baskets were removed. The Plasmid DNA in the micro centrifuge tube was stored at -20°C until sequencing.

3.12.9. Sequencing of cloned products and analysis of *nosZ* sequences

Sequencing was performed by *GATC Biotech SARL* (Germany). The partial sequences were aligned with the same region of the closest relative strains available in the GenBank database by using the BLAST facility (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was achieved using ClustalX (Thompson *et al.*, 1997). A phylogenetic tree was constructed in *TreeView* using the neighbor-joining method (Saitou and Nei, 1987). Sequences obtained in this study (Table 3) are available from European Molecular Biology Laboratory (EMBL) nucleotide sequence database under the accession numbers FN356193, FN356194, FN356195 and FN356196.

3.13. Bacterial diversity in mangrove sediments: A 454 pyrosequencing approach

Massively parallel tag sequencing of the V6 region of 16S rRNA gene is a cost-effective (Huse *et al.*, 2008) and more superior alternative to examine the phylogenetic diversity of microbial populations (Galand *et al.*, 2009). It is fast replacing conventional 16S rRNA gene sequencing which underestimates the full extent of microbial diversity (Huber *et al.*,

2007). It generates hundreds of thousands of short (100-200 nucleotide) DNA sequence reads in a short time period (few hours) eliminating the need for preparing sequence templates by conventional cloning (Huse *et al.*, 2007).

Low abundance populations accounting for most of the observed phylogenetic diversity in marine samples (Sogin *et al.*, 2006) demand high resolution surveys like the pyrosequencing technology which is based upon sequencing-by-synthesis protocol (Ronaghi *et al.*, 1996). Parallel processing of large numbers of samples can be easily carried out by using high-density PicoTiterPlate™ (Huse *et al.*, 2007) and microinjector technology (Ronaghi *et al.*, 1998).

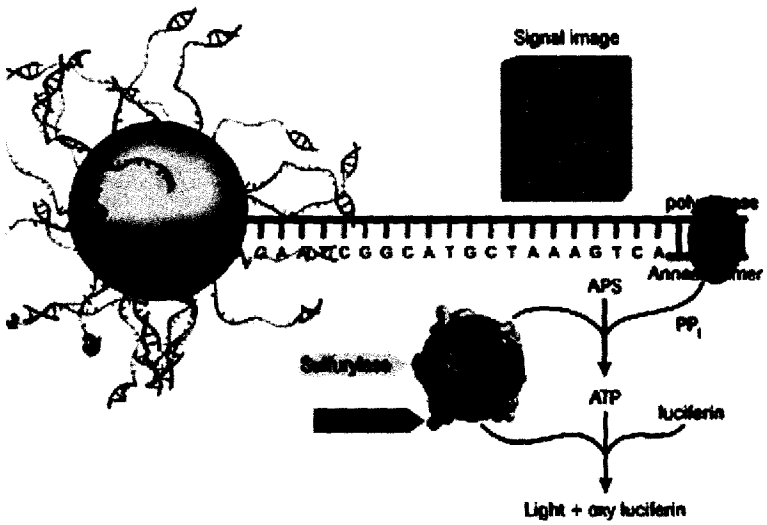


Fig. 8: Sequencing reaction of the Genome Sequencer System wherein millions of copies of a single clonal fragment are contained on each DNA Capture Bead.

In this method, nucleotides are flowed sequentially in a fixed order across the PicoTiterPlate™ device during a sequencing run. During the nucleotide flow, hundreds of thousands of beads each carrying millions of copies of a unique single-stranded DNA molecule are sequenced in parallel. If a nucleotide complementary to the template strand is flowed into a well, the polymerase extends the existing DNA strand by adding nucelotide(s). Addition of one (or more) nucleotide(s) results in a reaction that generates a light signal that is recorded by the CCD camera in the Genome Sequencer System (Fig.

Table 3: *nosZ* sequence identification

Accession number	Base pairs (bp)	Sequence	Description
FN356193	589	agtatcagcc cggccacaat catacctcca tgggggagac caaggatgcc gacggcaagt ggctggtgtc actgaacaag ttctccaagg acagatttat caatgtcggt ccactcaagc ctgaaaacga gcagctcatc gacatcaccg gtgataagat gaagctcctg catgatgggc ctaccttcgc ggagccccat gactgcatca ttgtgcgtgc ggacatcgtc aatcccaact ctgtatggac gcggggcgat ccgatgtggg cagacgtgcg tgagtgggcc agcaaggacg gcatcaacct ggactctgac agcaaggtgg tccgcgacgg taatgaggtg cgcgtctaca tggtatccat cgcacccaac ttcagcatgc agaagtttac agtgaaggag ggcgatgaag tcacggtgat tgtgaccaac atggatcgca ttgatgacct gacccatggc ttcaccctcg ccaactatgg tatcgccgta gagatcggac cgcaggcgac ctcctctgtg acctttgtcg ctgatcgacc gggcgtacac tggttttact gtcagtggtt ctgccacgc	Uncultured marine bacterium partial *nosZ* gene for nitrous oxide reductase, isolate DGGE band BD1
FN356194	666	tgttcatcga cagccagatg gtgaagtgga acatccagaa ggcgatcgac ctctacgcga accccgtcga gggagagacg cccgtcctcg atcgtctcga catccactat caagtaggac acaccatggc ctcgatggcg gagacgaagg aggcggacgg gaaatacctc atttccctca acaagatctc caaggatcgc ttcctcaacg tagaacccct caaaccggag aacgatcagc tcatcgacat ctccggagaa gagatggtcc tgctcgaaga cgagcccgcg tatatcgagc ctcatgactg catcatcgtc cgcagggaca tcatcgagga caaggttcag catcgtgcgg ttctcgaaga gcatcccgac gcggtgaccc agagctcagt ggagcgaaac ggacgaagag tcacggcccg gatcaccgct tccgcgcccg tctacggcct acaggaggtc gtggtcaatc agggtgacga ggtgaccttc atcgtgacca ataccgatga gatccccgat ctcgcgcacg ggttcgcgat ctcgaactac ggcatccagt tcgtcgtggg accgtttcag acgaagtcgg tcacgttcgt cgcggacaag ccgggggtcc actggatcta ctgcacgaac ttctgccacg cactgc	Uncultured marine bacterium partial *nosZ* gene for nitrous oxide reductase, isolate DGGE band BD2

Accession number	Base pairs (bp)	Sequence	Description
FN356195	698	tgttcctcga cagccaggtc gccaagtgga acatcgatct ggcgaagaag aaattcgccg gtgaggacgt cgacccgatc ctcgacaagg tggatgtgca gtaccagccg gggcacaacc acacctcaat gggcgagacg tccaaggcgg atggcaagtg gctggtgtca ttgaacaagt tctccaagga ccggttcatc aacgtcggcc cgctgaaacc ggaaaacgag cagctcatcg atatctccgg cgacaagatg caggtggcgc acgacggccc gacattcgcc gaaccgcatg actgcatcat cgtcgatgca tcggtcatca acccgcgtca gacctatgac cgggccgatc cgatgttcga cgacatcaag gcgatggcca aggcggacgg tgtcgacctg gaggcggccg aacacgtcat ccgcgacggc aacaaggtgc gggtctacat gcactcggtg gcgccgaact tctcgctcga gaagttcacc gtcaagcagg gagacgaggt cacgatcatc atcaccaaca tcgatgacgt cgacgatctc acgcatgggt tcacgctcgc caaccacggc atcgcgatgg agattggccc gcaggcgacc gcctcggtga cgttcacggc cgacgttccg ggcgtacact ggttctactg ccagtggttc tgccacgccc tgcacatg	Uncultured marine bacterium partial *nosZ* gene for nitrous oxide reductase, isolate DGGE band BD3
FN356196	653	aagaggcgat acaggcntat cagggcaaga aggtcaacta cctgcgccag aagctggacg tacantntca gccaggccac aatcacacct ctcaggncga aacccgcgac gccgatggca agtggctagt gtctctgtgt aagttntcca aggacagatt cctgcccgtt ggcccgctgc gccccgagaa cgatcagttg atcgacatct caggcgatga gatgaagctg gtgcacgacg gcccaacctt cgccgagcct cacgactgca tcatagtgca ccgtagcaag ctcaagccga agaagctgtg gacccgtgac gatccgctgt tttccgatac tgtggccatg gccgagaaag atggcgtgcg cctgatgatg gacaacaagg tgatccgcga cggcaacaag gtccgcgtct acatgacctc ggtcgcgccc aactacggca tgagcgagtt taaggtcaag ctaggcgacg aggtaaccgt tgtagtcacc aacctggatc aggtggaaga cgtgacccac ggcttctgta tgaccaacca tggcgtacag atggagattg gcccacaggc cacggcatct gtcaccttca tcgccgacaa gccaggggta cagtggtact actgcaactg gttctgccac gca	Uncultured marine bacterium partial *nosZ* gene for nitrous oxide reductase, isolate DGGE band BD4

8). The signal strength is proportional to the number of nucleotides incorporated in a single nucelotide flow (www.454.com).

3.13.1. DNA extraction

Genomic DNA was extracted from the surface sediment (0-2 cm) using a *MO BIO PowerSoil* DNA isolation kit as described above (Refer 3.12.1).

3.13.2. Concentration of DNA

The final volume of eluted DNA (100 µl) was concentrated by adding 4 µl of 5M NaCl and inverting 3-5 times to mix. Next, 200 µl of 100% cold ethanol was added and the tubes were inverted 3-5 times to mix. Samples were centrifuged at 10,000 x g for 5 minutes at room temperature. All liquid was decanted. Residual ethanol was removed by drying overnight in a dessicator.

3.13.3. High-throughput pyrosequencing

Precipitated DNA was re-suspended in sterile water. The hypervariable region of rRNA genes (BV6-rRNA tags) were amplified and subjected to high-throughput pyrosequencing using the 454 technology as described by Sogin *et al.* (2006) and Huber *et al.* (2007). The following sequence adaptors and primers were used (vamps.mbl.edu):

Roche amplicon sequencing adaptors:
A-adaptor 5'-GCCTCCCTCGCGCCATCAG-3'
B-adaptor 5'-GCCTTGCCAGCCCGCTCAG-3'

Forward Primers (967F)
CNACGCGAAGAACCTTANC
CAACGCGAAAAACCTTACC
CAACGCGCAGAACCTTACC
ATACGCGARGAACCTTACC
CTAACCGANGAACCTYACC

Reverse Primers (1046R)
CGACAGCCATGCANCACCT
CGACAACCATGCANCACCT
CGACGGCCATGCANCACCT
CGACGACCATGCANCACCT

Thus, to amplify the V6 hypervariable region of bacterial 16S rRNAs (*Escherichia coli* positions 967-1046) (Sogin *et al.*, 2006) and sequence it in the forward direction (relative to the 5'–>3' orientation of the gene) using the Roche A primer, the forward primer consisted of the A–adaptor, 5–base key, and sequence designed to bind to the 967F region of the SSU:

5'-GCCTCCCTCGCGCCATCAG**gatct**CNACGCGAAGAACCTTANC-3'

The reverse primer would consist of the B-adaptor and a sequence designed to bind to 1046R:

5'-GCCTTGCCAGCCCGCTCAG CGACAGCCATGCANCACCT-3'

3.13.4. Data analysis

The sequence reads (tags) were trimmed as described by Sogin *et al.* (2006) and Huber *et al.* (2007). Sequences likely to be of low-quality were identified based on previous assessment of pyrosequencing error rates (Huse *et al*, 2007) and were removed. The 454 tags served as query to identify its closest match in a reference database (V6RefDB) containing ≈40,000 unique V6 sequences (Sogin *et al.*, 2006). Taxonomic counts from the VAMPS database (vamps.mbl.edu) were then downloaded and imported into *Microsoft Excel*. Sequence characteristics like average length and tag aggregates were estimated using *R* package (R Development Core Team, 2007). Multiple sequence alignment was done using *ClustalX* 1.83. Distance matrices were calculated using DNAdist from *PHYLIP* 3.69 (Felsenstein, 2005). These pairwise distances served as input to DOTUR (Schloss and Handelsman, 2005) for clustering tags that ranged from unique sequences (no variation) to 20% dissimilarity. These clusters served as operational taxonomic units (OTUs) for generating rarefaction curves, calculating two indices of diversity- Chao1 (Chao, 1987) and ACE (abundance-based coverage estimator (Chao and Lee 1992)) and the Simpson evenness index.

Chapter 4

Results

Chapter 4. Results

SECTION I – Field observations

4.1. Physical parameters

4.1.1. Temperature

Sediment temperature was found to decrease with increasing depth at both the sites. At the control site Tuvem, temperature varied from 25.9°C in January to 31.6°C in March. Average temperature values (n=4) varied from 29.60 (±1.82) to 30.00 (±1.31), 27.58 (±0.84) to 27.60 (±0.74) and 26.68 (±0.80) to 26.93 (±0.75) during the pre-monsoon (Feb-May), monsoon (June-Sept) and post-monsoon (Oct-Jan) seasons respectively (Fig. 9a).

At the experimental site Divar, sediment temperature fluctuated from 23.6°C in December to 31.1°C in March. Average temperature during pre-monsoon varied between 28.65 (±2.07) to 29.30 (±1.98) whereas in the monsoon it fluctuated from 28.00 (±0.74) to 28.18 (±0.85). In the post-monsoon season, lower average values varying from 25.95 (±1.64) to 26.20 (±1.49) were recorded (Fig. 9b). Seasonal fluctuation of temperature was highly significant ($p > 0.001$) at both the sites.

4.1.2. Salinity

Annual salinity variations of overlying seawater varied from 0-30 psu and 1-32 psu at Tuvem and Divar respectively. The lowest salinity values were recorded during the monsoon with an average value of 1.25 (±0.96) psu at the control site and 3.25 (±3.20) psu at the experimental site (Fig. 10). During the non-monsoon seasons, salinity values at the control site were lower with a pre-monsoon average of 23.75 (±6.13) psu and 18.50 (±8.50) at post-monsoon. Average values of 28.75 (±2.22) psu and 26.75 (±9.18) psu were recorded at pre and post-monsoon at the experimental site.

4.1.3. pH

Hydrogen ion concentration in Tuvem sediments showed a wider variation from 5.57-7.32 in comparison to the Divar sediments where the fluctuation was relatively less varying between 6.08-7.32 within the 0-10 cm depth range. The control site exhibited a decrease in pH with depth. Average values (Fig. 11a) of 6.52 (±0.65) to 6.71 (±0.35),

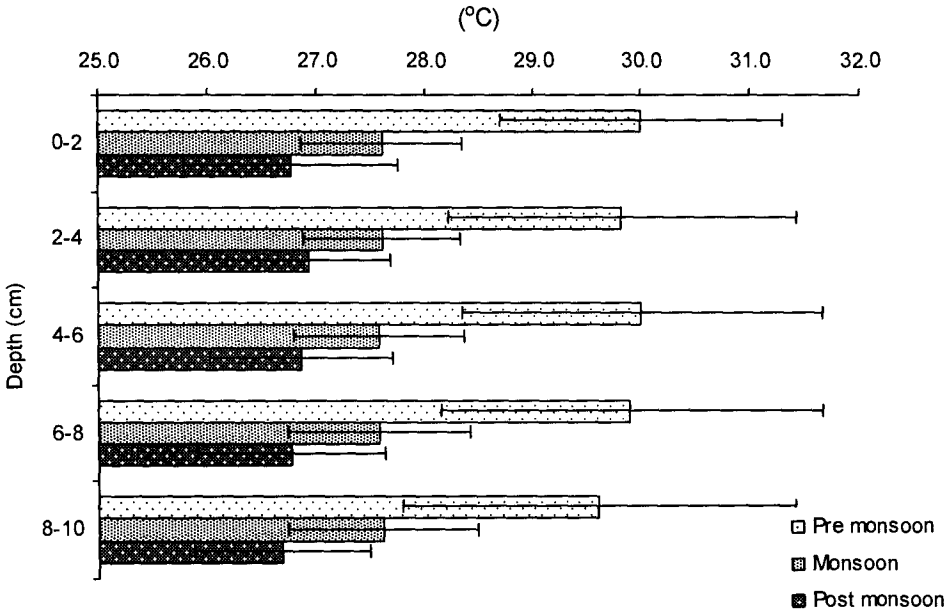


Fig. 9a: Seasonal down-core variation in sediment temperature (±SD) at Tuvem where n=4 at each season

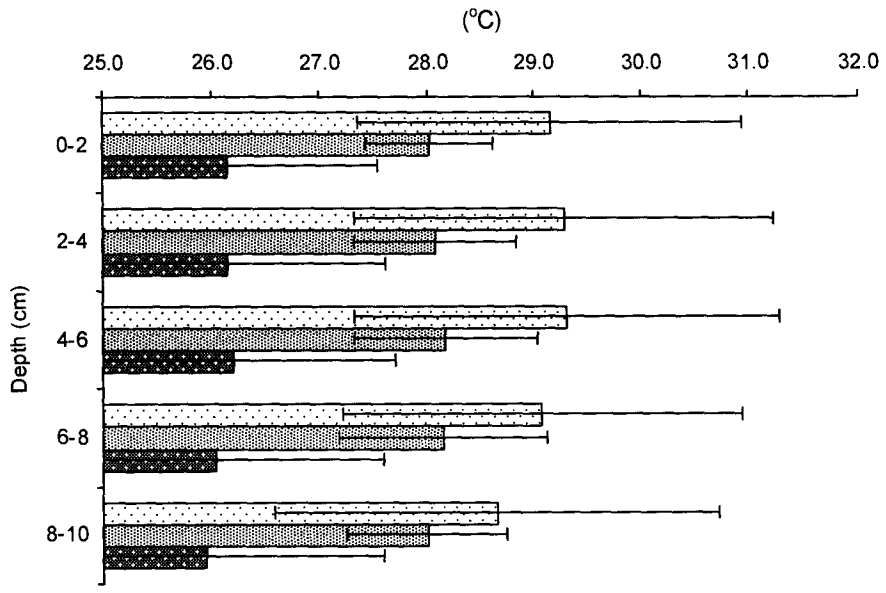


Fig. 9b: Seasonal down-core variation in sediment temperature (±SD) at Divar where n=4 at each season

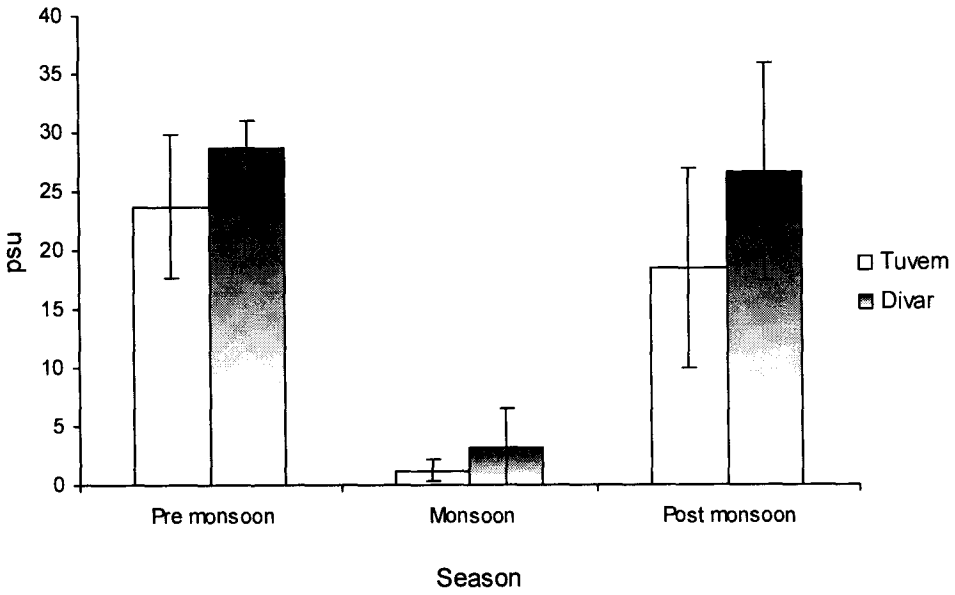


Fig. 10: Seasonal variation in salinity (±SD) of overlying seawater at Tuvem and Divar where n=4 at each season

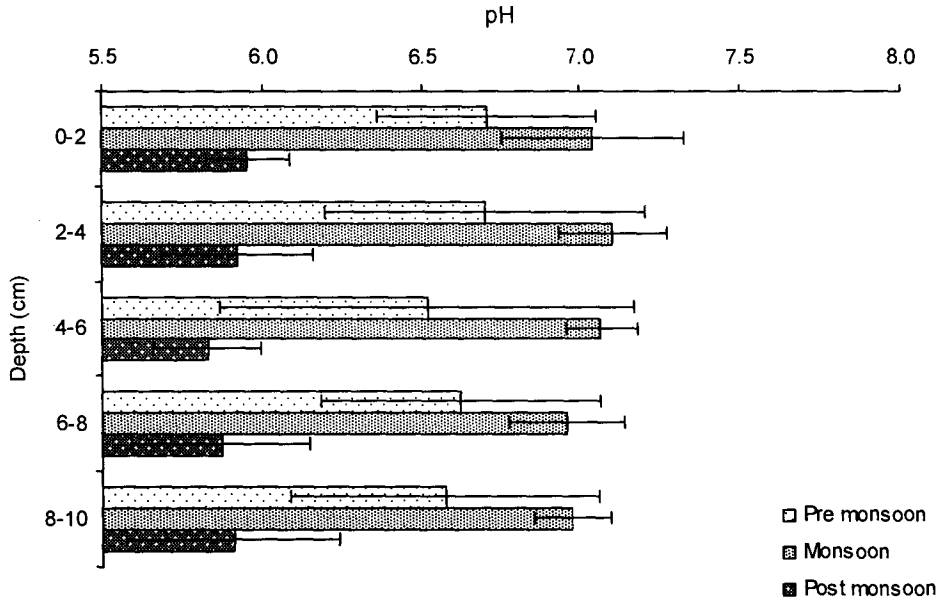


Fig. 11a: Seasonal down-core variation in sediment pH (±SD) at Tuvem where n=4 at each season

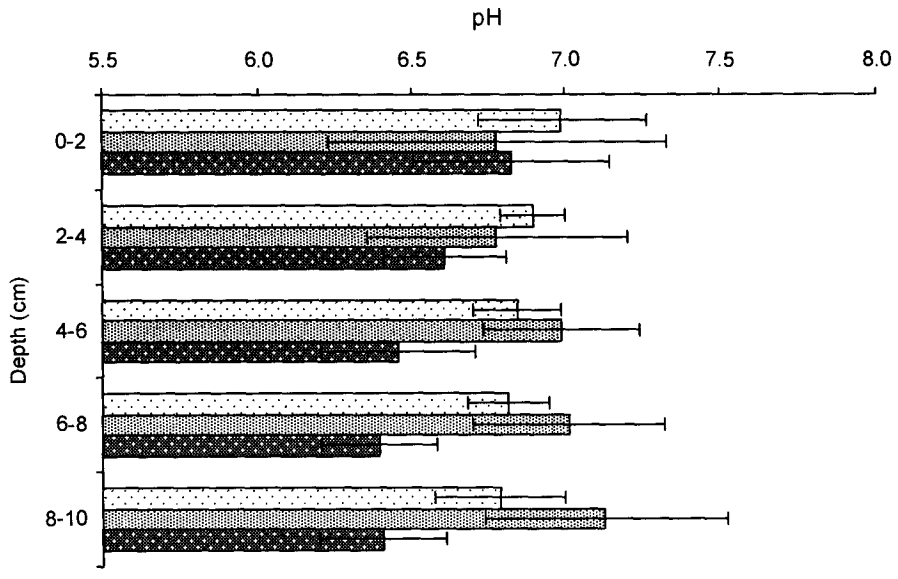


Fig. 11b: Seasonal down-core variation in sediment pH (±SD) at Divar where n=4 at each season

6.95 (±0.18) to 7.11 (±0.17) and 5.83 (±0.17) to 5.95 (±0.13) were recorded during the pre-monsoon, monsoon and post-monsoon respectively.

At the experimental site (Fig. 11b), average pH values of 6.79 (±2.07) to 6.99 (±0.27), 6.78 (±0.43) to 7.13 (±0.43) and 6.39 (±0.19) to 6.83 (±0.32) were recorded during the pre-monsoon, monsoon and post-monsoon respectively.

4.1.4. Redox potential

The redox potential differed markedly during the different seasons (Fig. 12a). The values were lowest in July at the control site with comparatively less reducing conditions prevalent during pre and post monsoon.

The Divar sediments were (Fig. 12b) relatively more oxidizing in nature at depth <6 cm prior to the commencement of rainfall in the region. Reducing conditions prevailed through the monsoon with the lowest value of -248.4 mV at 2-4 cm depth in the month of June.

4.2. Chemical parameters

4.2.1. Dissolved oxygen

At the control site, dissolved oxygen in the ambient water varied from 0.12-4.79 ml l^{-1} through the year. The seasonal pattern was distinct with the monsoon season showing the highest average value of 3.63 (±1.24) ml l^{-1}(Fig. 13).The post-monsoon dissolved oxygen content was lower at 0.87 (±1.36) ml l^{-1} compared to the pre-monsoon where the average value for this season was 1.36 (±1.59) ml l^{-1}.

At the experimental site Divar, similar annual variation in oxygen concentrations were observed with values ranging between 0.19-4.75 ml l^{-1}. The highest average value of 3.85 (±1.32) ml l^{-1} was also recorded during the monsoon. Between the non-monsoon periods, the pre-monsoon value was lower at 1.11 (±1.18) ml l^{-1} while the post-monsoon value was marginally higher at 1.29 (±1.97) ml l^{-1}.

4.2.2. Pore water NH_4^+, NO_2^- and NO_3^-

Monthly variation in pore water ammonium at both the control and experimental sites showed generally higher values at depths >4 cm. At the control site, the highest seasonal average of 28.43 (±5.03) NH_4^+-N l^{-1} (Fig. 14a) was recorded at 4-6 cm depth during the

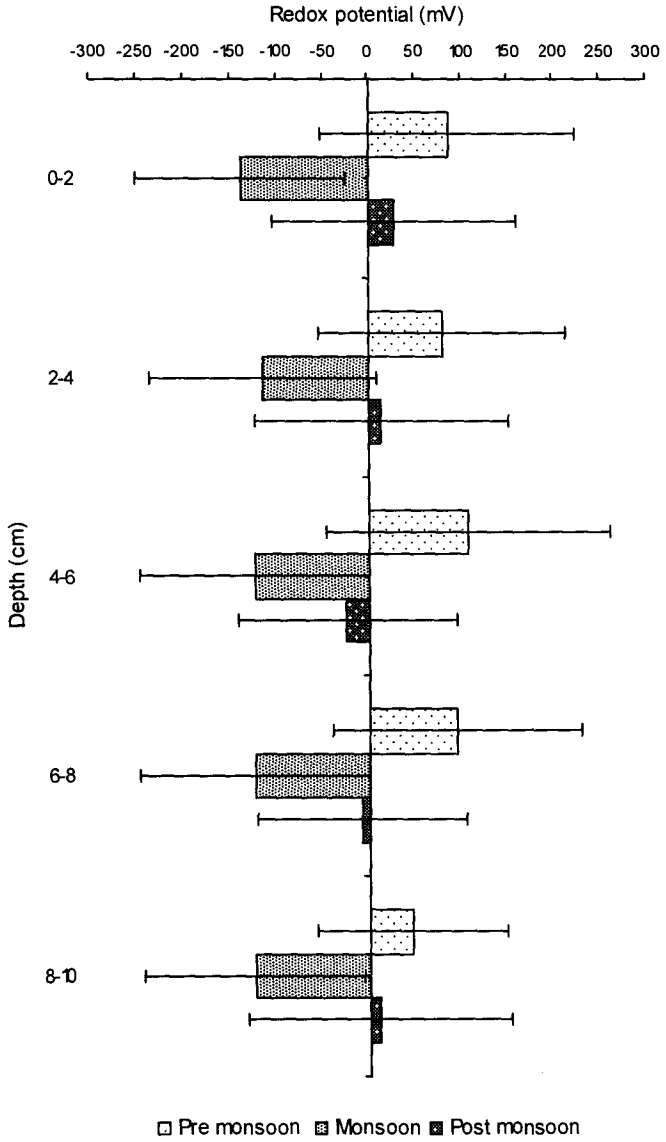


Fig. 12a: Seasonal down-core Eh (±SD) profile at Tuvem where n=4 at each season

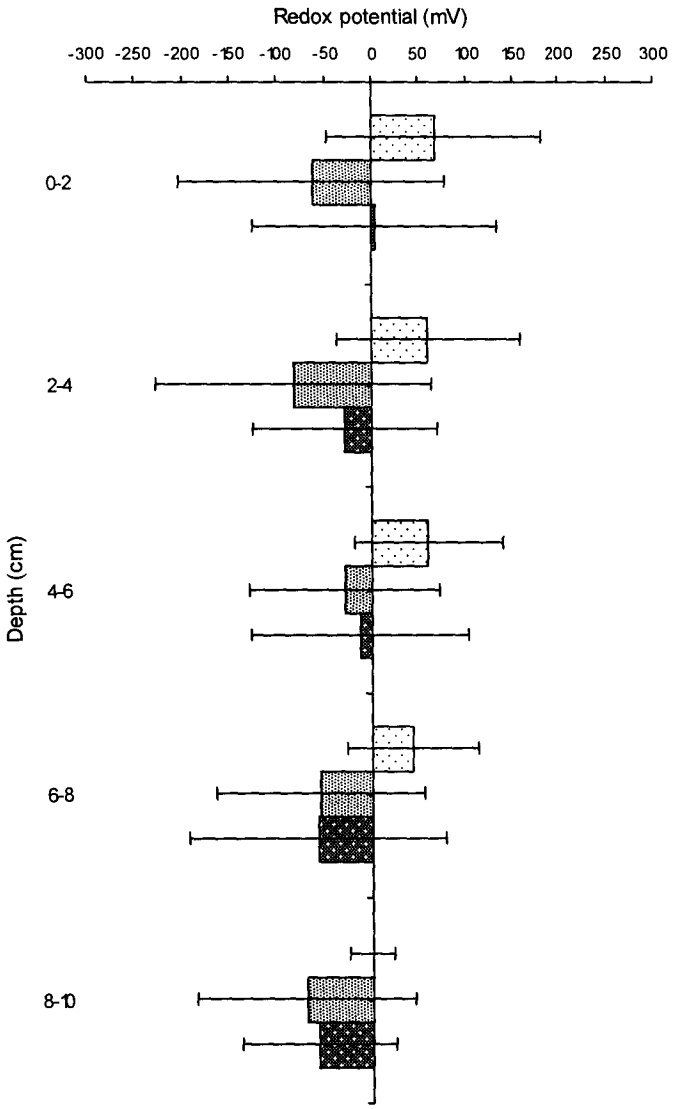


Fig. 12b: Seasonal down-core Eh (±SD) profile at Divar where n=4 at each season

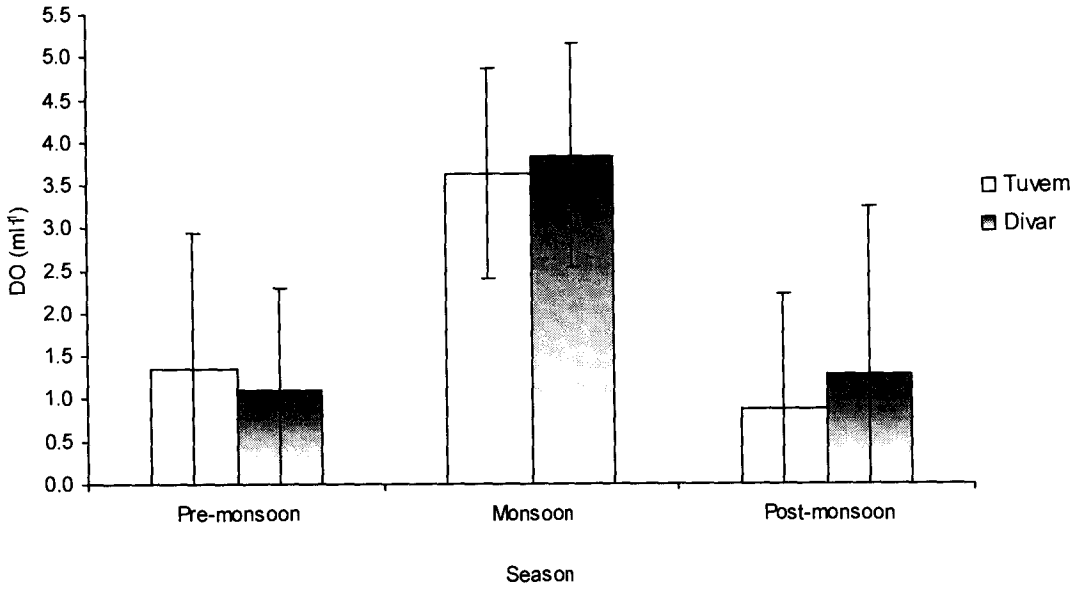


Fig. 13: Seasonal variation in dissolved oxygen (±SD) in the overlying water at Tuvem and Divar where n=4 at each season

post-monsoon. Similarly at the experimental site, higher concentrations were recorded during the post-monsoon season with average values ranging between 20.56 (±3.07) at 6-8 cm to 32.05 (±4.63) at 8-10 cm (Fig. 14b). NH_4^+ showed significant seasonal variability at the experimental site ($p<0.001$, df=2) while there was no significant variability at the control site. Between the two sites, a significant variation ($p<0.05$, df=1) was observed only during the monsoon and could be attributed to the variability observed at 0-2 cm ($p<0.01$, df=1).

Monthly variation in nitrite concentrations at both the sites varied widely with depth (Figs. 14c-d). At the control site the lowest concentration of 0.41 µmol NO_2-N l^{-1} was recorded at the surface during the post-monsoon while the highest concentration of 5.35 µmol NO_2-N l^{-1} was recorded at 4-6 cm during the same season. At the experimental site, similar nitrite concentrations varying between 0.41-6.42 µmol NO_2-N l^{-1} within the 0-10 cm depth range were observed. Seasonal averages have shown relatively higher nitrite concentration during the monsoon at the experimental site with up to 3.77 (±1.80) µmol NO_2-N l^{-1} at 2-4 cm. Average nitrite concentrations in the surficial sediments at the control site were relatively lower through the seasons as compared to the deeper layers. Inter-seasonal variability in NO_2^- concentration was observed only at the experimental site ($p<0.001$, df=2). Between the two sites, significant variation was observed only during the post-monsoon ($p<0.04$, df=1) caused due to variation in the nutrient concentration at 4-6 cm ($p<0.01$, df=4).

Nitrate concentration in mangrove sediments also varied with depth (Figs. 14e-f). Average values at the control site showed low nitrate concentration at all depths. At pre-monsoon, the concentration decreased with depth with an average value of 15.32 (±12.65) µmol NO_3-N l^{-1} at 0-2 cm to 7.66 (±4.85) µmol NO_3-N l^{-1} at 8-10 cm. The highest average pre-monsoon value of 13.35 (±6.07) µmol NO_3-N l^{-1} was recorded at 4-6 cm. The monsoon season significantly increased nitrate levels at the experimental site as compared to the non-monsoon seasons. Seasonal variability in nitrate concentrations were observed at the control ($p<0.05$, df=2) and experimental sites ($p<0.001$, df=2) sites. The monsoon season showed significant variation ($p<0.001$, df=1) between both the sampling locations.

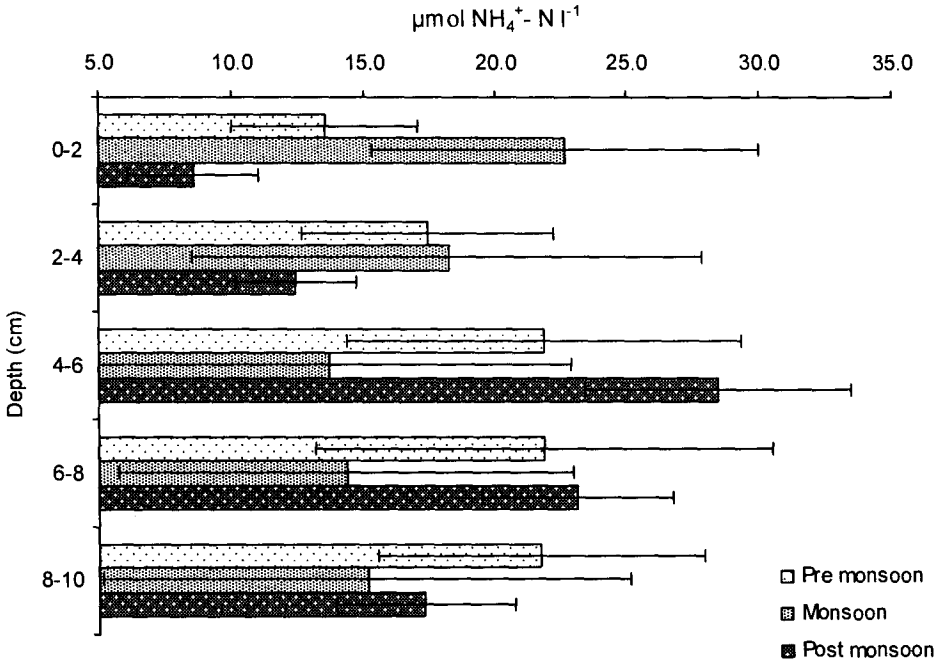


Fig. 14a: Seasonal down-core variation of pore water ammonium (±SD) at Tuvem where n=4 at each season

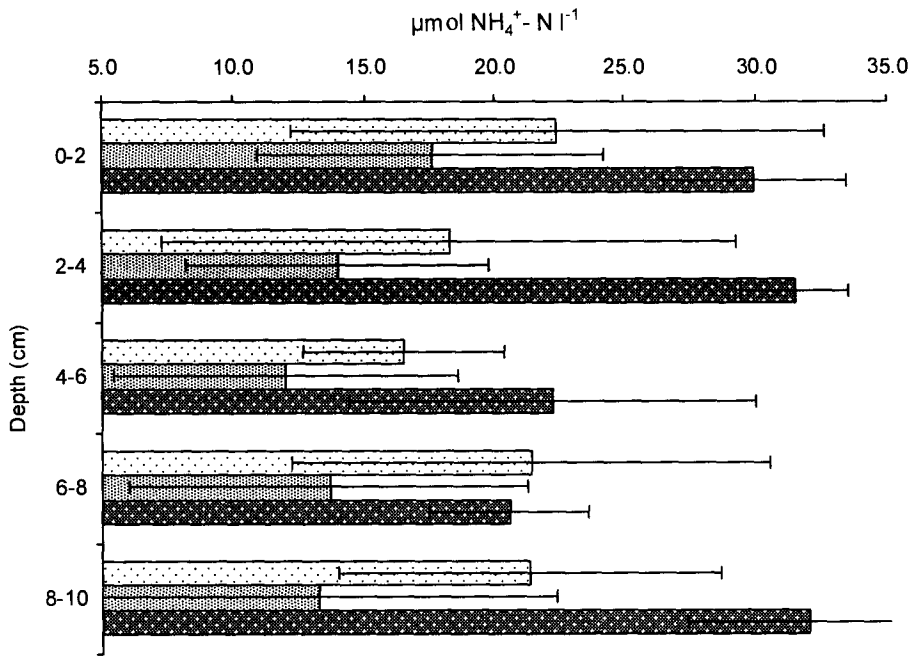


Fig. 14b: Seasonal down-core variation of pore water ammonium (±SD) at Divar where n=4 at each season

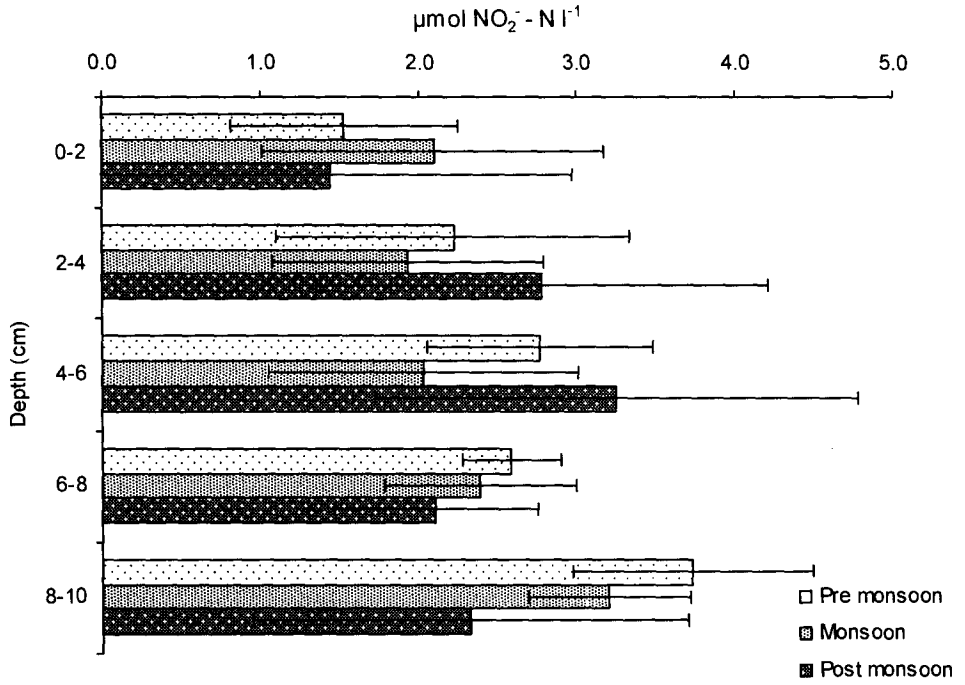


Fig. 14c: Seasonal down-core variation of pore water nitrite (±SD) at Tuvem where n=4 at each season

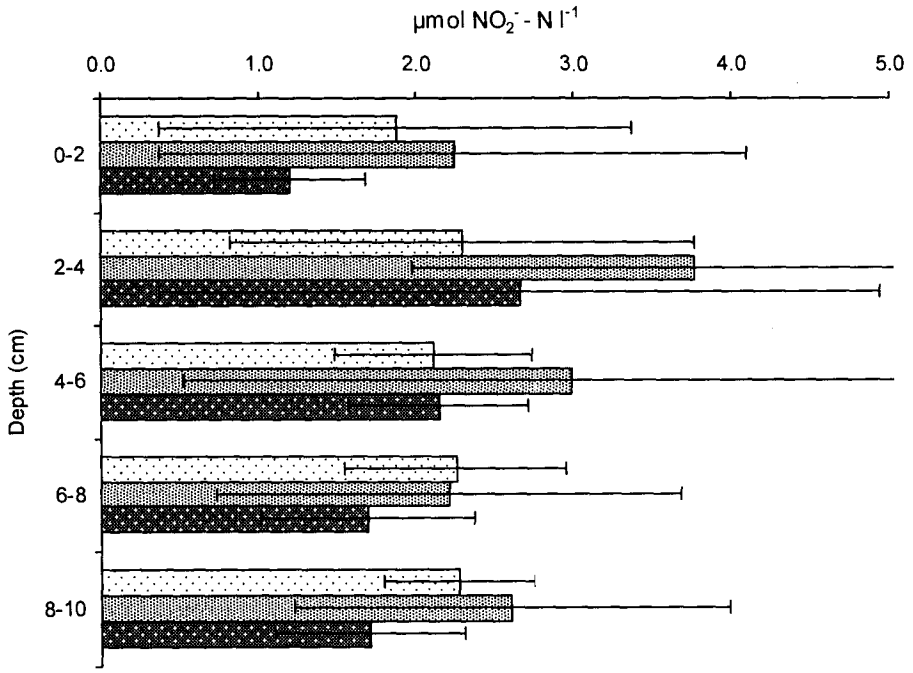


Fig. 14d: Seasonal down-core variation of pore water nitrite (±SD) at Divar where n=4 at each season

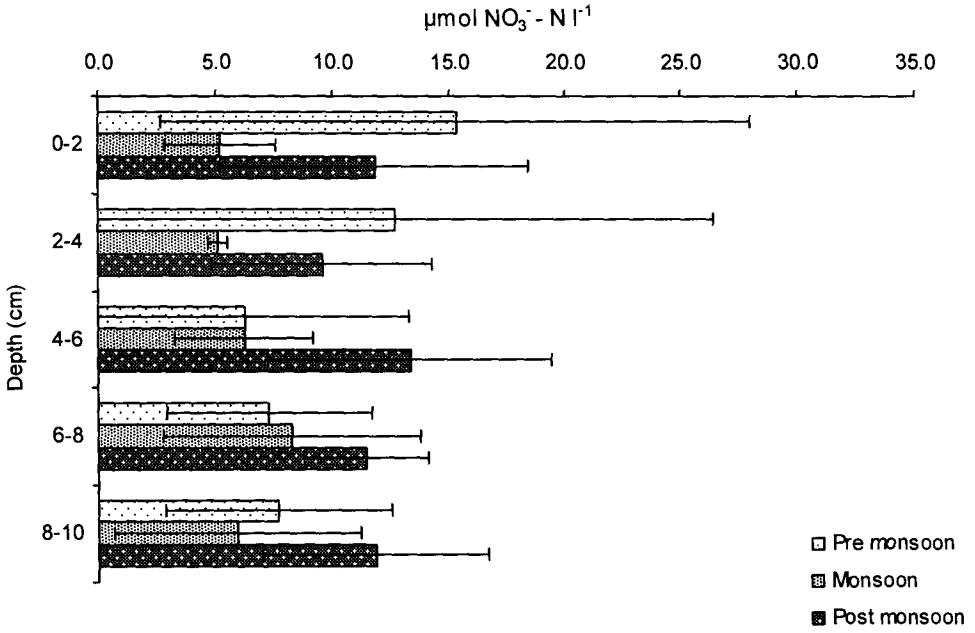


Fig. 14e: Seasonal down-core variation of pore water nitrate (±SD) at Tuvem where n=4 at each season

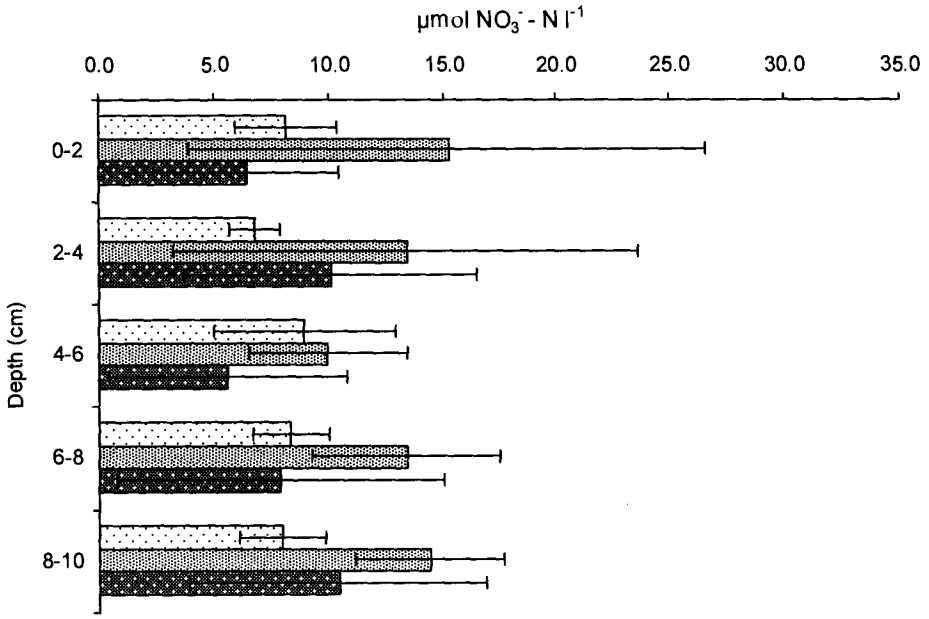


Fig. 14f: Seasonal down-core variation of pore water nitrate (±SD) at Divar where n=4 at each season

4.2.3. Total organic carbon

Down-core variability in TOC for the different seasons are illustrated in figures 15a and b. TOC at the control site varied from 1(±0.05) % during the monsoon to 4.52 (±3.13) % at post-monsoon. These values were recorded at 4-6 cm depth. Similar trend was observed at the experimental site where TOC varied from 1.65(±1.37) at 4-6 cm during monsoon to 4.88 (±2.76) % at 2-4 cm during the post-monsoon. Seasonal variation in TOC was observed at the control ($p<0.01$, $df=2$) and experimental ($p<0.01$, $df=2$) sites. The monsoon season showed variability in TOC ($p<0.01$, $df=1$) content between both sites.

4.2.4. Iron and manganese concentration

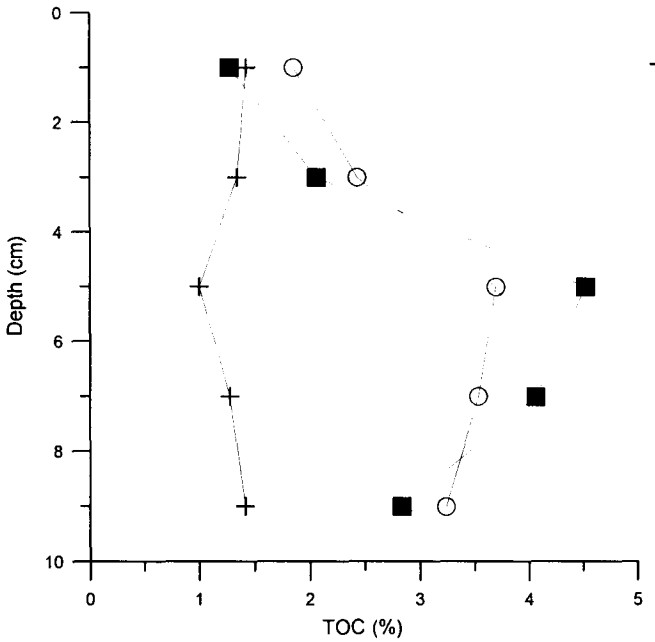
The down-core variation of Fe and Mn during pre-monsoon, monsoon and post-monsoon seasons have been shown in Figs. 16a-d. Sediment Fe values at the control site ranged from 4.5% at 0-2 cm to 9.3% at 4-6 cm during the post-monsoon. Seasonal variation in Fe was observed only at the experimental site ($p<0.001$, $df=2$) with highest Fe content of up to 30% at 8-10 cm during the pre-monsoon. Variation in Fe between both sampling locations was observed during all the three seasons i.e. pre-monsoon ($p<0.001$, $df=1$), monsoon ($p<0.001$, $df=1$) and post-monsoon ($p<0.001$, $df=1$).

Highest Mn content of 0.9 (±1.0) % was recorded at 4-6 cm at the control site during the post-monsoon. At the experimental site, a maximum of 2.5 (±1.9) % was recorded at 6-8 cm during the monsoon. Seasonal variation in Mn content was observed at only the experimental site ($p<0.01$, $df=2$). Between the two sites, variation in Mn was observed only during the pre-monsoon ($p<0.0001$, $df=1$) and monsoon ($p<0.01$, $df=1$).

4.3. Bacteriological parameters

4.3.1. Total bacterial counts

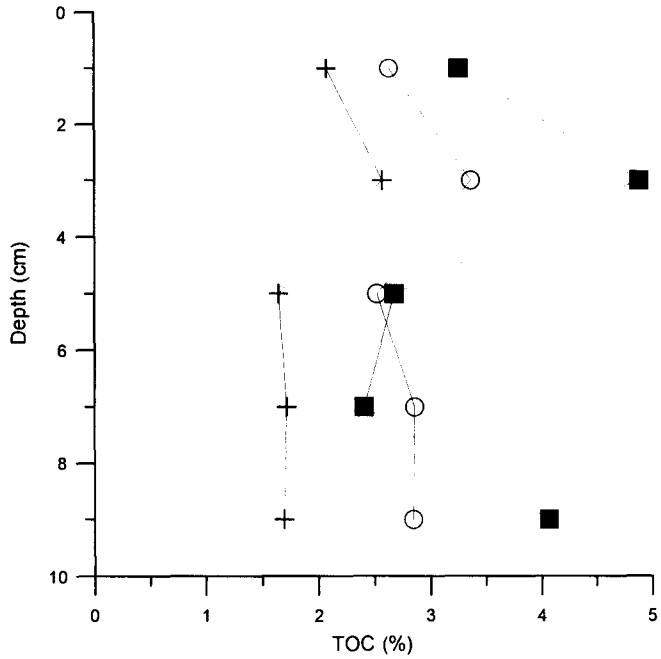
Total bacterial cells (TC) in the Tuvem sediments ranged from 3.35×10^9 to 3.37×10^{10} cells g^{-1} while at Divar it varied from 3.16×10^9 to 5.82×10^{10} cells g^{-1}. Generally, the cell number was marginally higher at 4-6 cm as compared to the surface. Irrespective of the depth, the abundance pattern showed considerable monthly variation. At Tuvem, cells number was high during April, June, July and September. However at Divar, high cell count was recorded during the months of May, June and July. Inter-seasonal variation in



Control site: Tuvem

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.80	0.28	0.85
2-4	1.43	0.46	1.43
4-6	2.88	0.05	3.13
6-8	2.91	0.41	2.75
8-10	2.66	0.40	2.02

Fig.15a

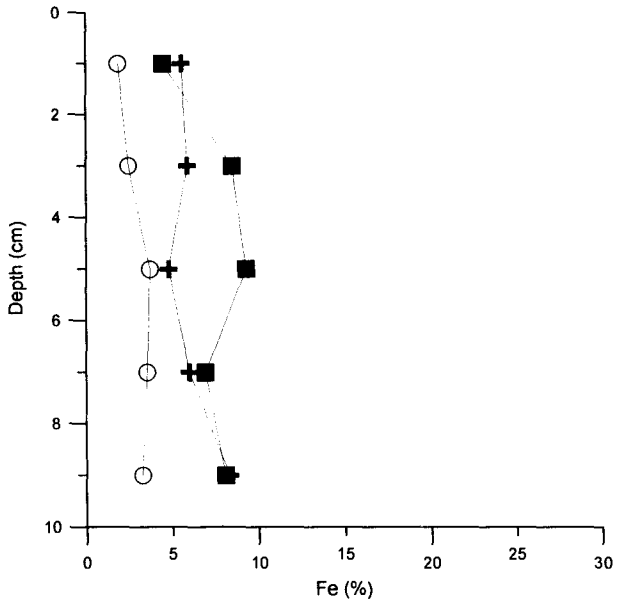


Experimental site: Divar

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	1.42	1.14	1.83
2-4	2.16	1.33	2.76
4-6	0.86	1.37	1.21
6-8	1.63	1.51	1.23
8-10	1.51	1.65	2.64

Fig.15b

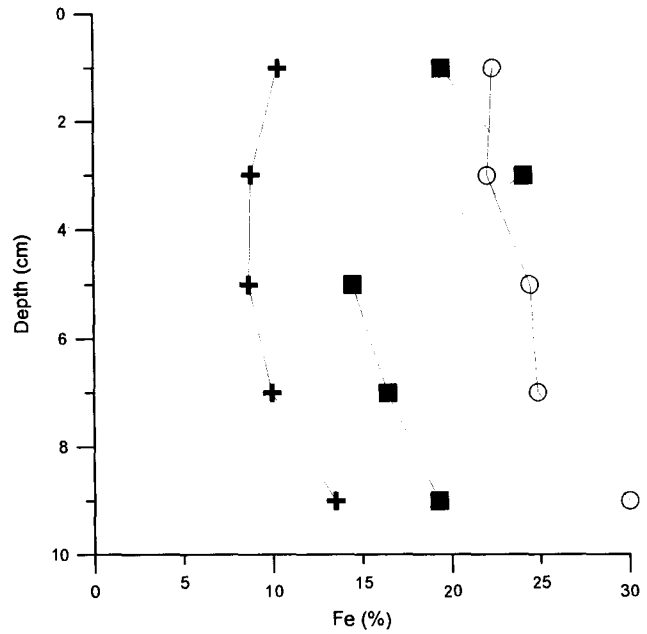
Figs. 15a & b: Down-core variation in Total Organic Carbon (TOC) the Control (15a) and experimental site (15b) where o Pre Monsoon, + Monsoon and ■ Post Monsoon. SD values have been shown in adjacent tables



Control site: Tuvem

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	1.75	2.96	3.70
2-4	2.97	2.36	1.87
4-6	2.23	0.92	4.33
6-8	1.23	1.03	2.26
8-10	2.27	1.72	3.25

Fig.16a



Experimental site: Divar

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	7.96	4.87	8.66
2-4	7.14	6.10	10.36
4-6	8.43	3.51	4.40
6-8	8.57	2.38	6.00
8-10	13.37	11.35	8.47

Fig.16b

Figs. 16a & b: Down-core variation in Fe at the control (16a) and experimental site (16b) where **o** Pre monsoon, + Monsoon and ■ Post monsoon. SD values have been shown in adjacent table

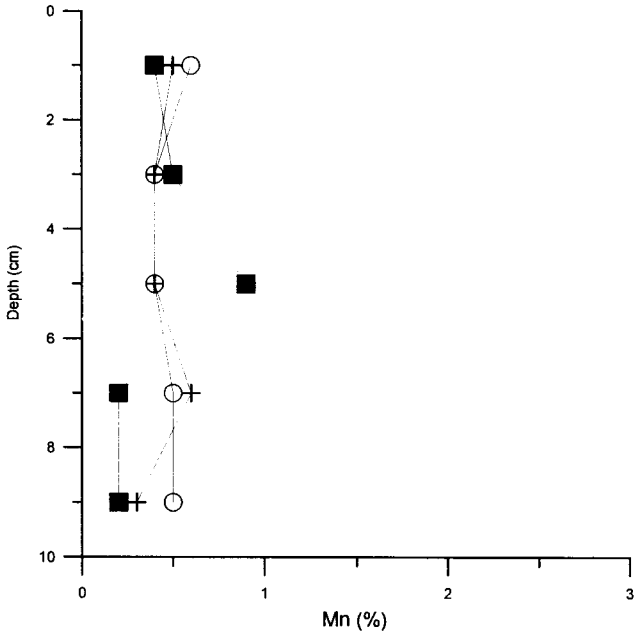


Fig.16c

Control site: Tuvem

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.30	0.20	0.20
2-4	0.30	0.10	0.30
4-6	0.30	0.10	1.00
6-8	0.30	0.20	0.10
8-10	0.30	0.10	0.00

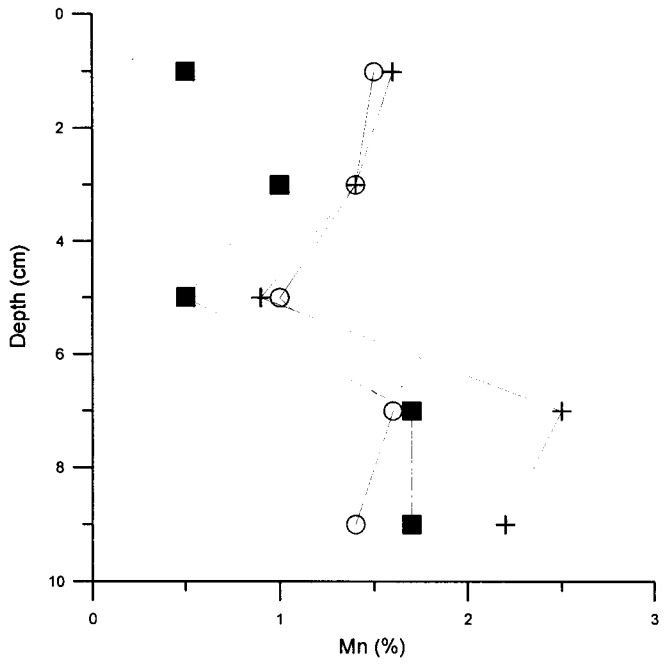


Fig.16d

Experimental site: Divar

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	1.20	1.50	0.40
2-4	1.10	1.40	0.80
4-6	0.40	0.60	0.20
6-8	1.40	1.90	1.90
8-10	0.70	1.50	1.40

Figs. 16c & d: Down-core variation in Mn at the control (16c) and experimental site (16d) where **o** Pre monsoon, + Monsoon and ■ Post monsoon. SD values have been shown in adjacent table

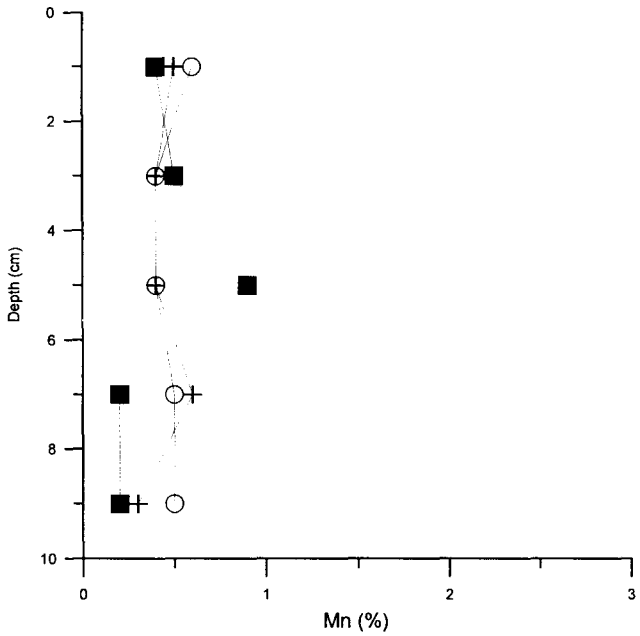


Fig.16c

Control site: Tuvem

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.30	0.20	0.20
2-4	0.30	0.10	0.30
4-6	0.30	0.10	1.00
6-8	0.30	0.20	0.10
8-10	0.30	0.10	0.00

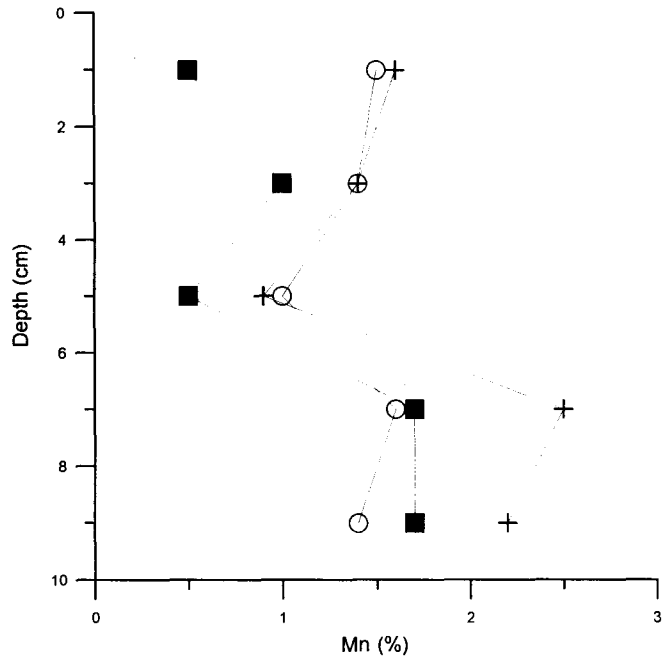


Fig.16d

Experimental site: Divar

Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	1.20	1.50	0.40
2-4	1.10	1.40	0.80
4-6	0.40	0.60	0.20
6-8	1.40	1.90	1.90
8-10	0.70	1.50	1.40

Figs. 16c & d: Down-core variation in Mn at the control (16c) and experimental site (16d) where o Pre monsoon, + Monsoon and ■ Post monsoon. SD values have been shown in adjacent table

bacterial cells was observed at the control ($p<0.001$, df=2) and experimental site ($p<0.05$, df=2). Cell number was maximum during the monsoon at both the sites with higher density at 4-6 cm depth (Figs. 17a-b). No significant seasonal variation in cell abundance was observed between the two sites.

4.3.2. Heterotrophic counts

The abundance of total heterotrophic bacteria (THB) in mangrove sediments varied from 10^{2-5} CFU g^{-1}. At the control site, their abundance averaged between 10^{4-5} CFU g^{-1} with a maximum at 2-4 cm. Their abundance was non-detectable at depth ≥4 cm during December. A maximum abundance of $8.48x10^5$ CFU g^{-1} was recorded at 2-4 cm depth during February. Though THB abundance was higher during the post-monsoon (Fig. 18a), no inter-seasonal variability in their abundance was observed at either location.

At the experimental site, heterotrophic abundance averaged between 10^{3-5} CFU g^{-1} decreasing steadily with depth. On a monthly basis, their abundance varied from $3.77x10^2$ CFU g^{-1} at 6-8 cm in November to $3.83x10^5$ CFU g^{-1} at 2-4 cm in January. Lower abundance was observed during the monsoon as compared to the non-monsoon period (Fig. 18b). Between the two sites, variation in THB abundance was observed during the monsoon ($p<0.03$, df=1).

4.3.3. Nitrate reducing bacteria

The nitrate reducers (NRB) were one order lower than the heterotrophs averaging between 10^{2-4} CFU g^{-1}. Their abundance was higher between 2-6 cm at both the study sites. Average NRB abundance at the control site varied from $1.37x10^3$ CFU g^{-1} at 8-10 cm during the post-monsoon to $1.38x10^4$ CFU g^{-1} at 0-2 cm during the pre-monsoon. Inter-seasonal variation in NRB abundance was observed at the control ($p<0.001$, df=2) and experimental ($p<0.001$, df=2) site. Their abundance at the experimental site Divar was one order higher during the pre-monsoon as compared to the monsoon and post-monsoon seasons. NRB showed a sub-surface maxima during the pre-monsoon season at both study sites (Figs. 19a-b). Between the sites, NRB abundance varied significantly during the monsoon ($p<0.01$, df=1).

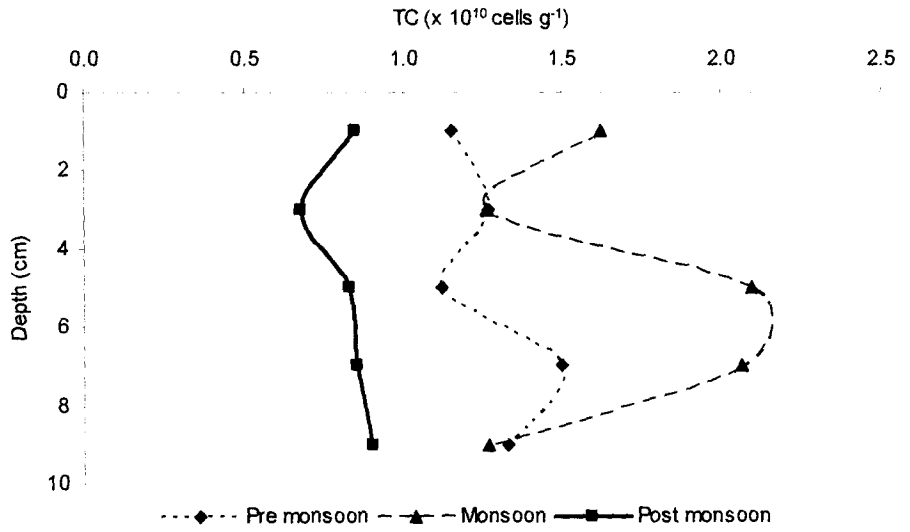


Fig. 17a: Seasonal variation in total bacterial counts at Tuvem. Standard deviation values have been shown in table below.

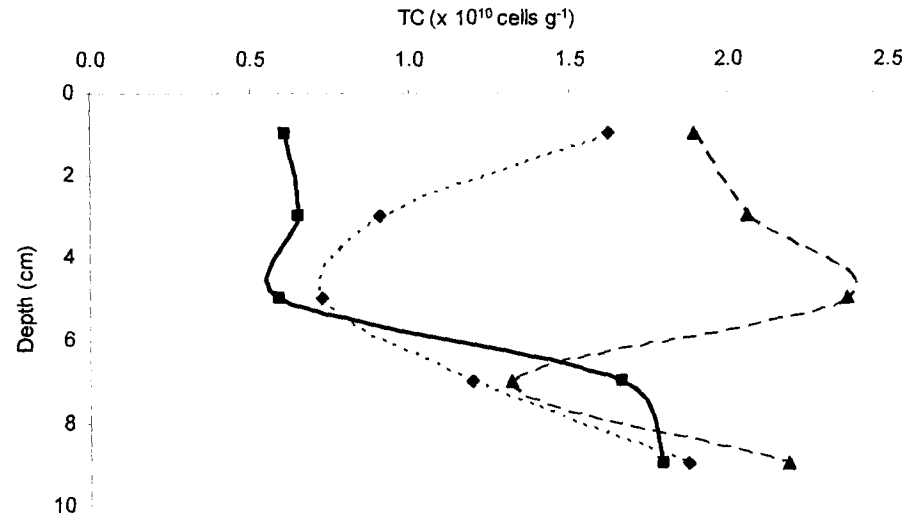


Fig. 17b: Seasonal variation in total bacterial counts at Divar. SD values have been shown below.

	Tuvem			Divar		
Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.65	0.76	0.40	1.41	1.16	0.22
2-4	0.92	0.57	0.28	0.43	0.65	0.30
4-6	0.77	0.77	0.46	0.34	2.33	0.19
6-8	1.27	0.96	0.69	0.50	1.23	2.00
8-10	0.51	0.40	0.59	1.58	1.51	2.32

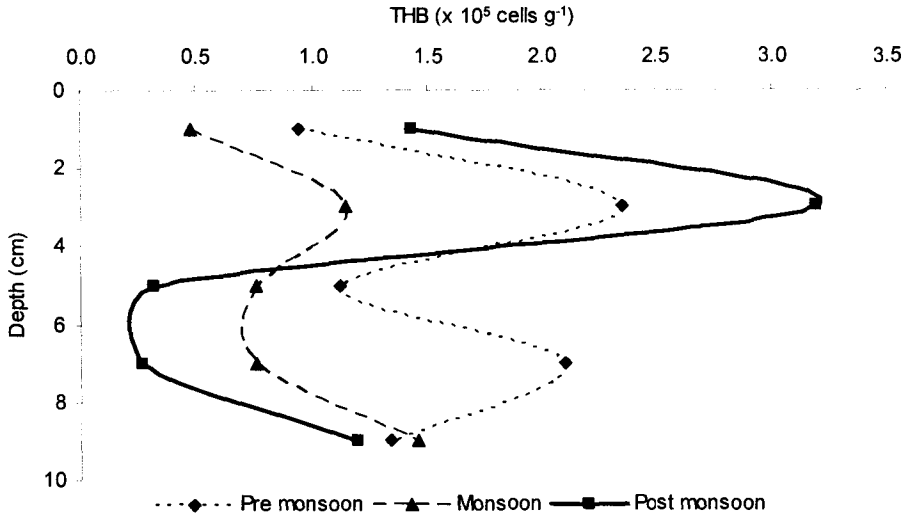


Fig. 18a: Seasonal variation in heterotrophic abundance at Tuvem. SD values have been shown below.

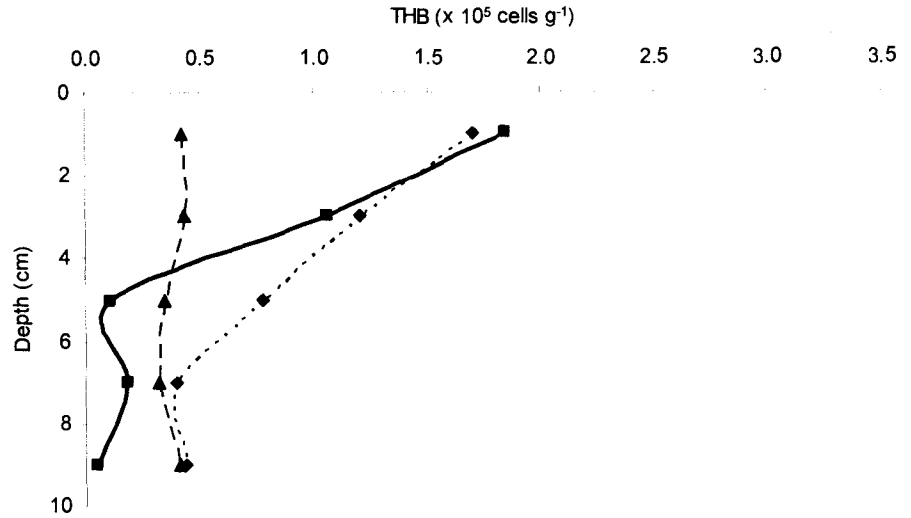


Fig. 18b: Seasonal variation in heterotrophic abundance at Divar. SD values have been shown below.

	Tuvem			Divar		
Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.94	0.34	0.89	0.66	0.30	1.20
2-4	4.09	0.58	2.99	0.92	0.35	1.85
4-6	1.04	0.72	0.30	0.96	0.39	0.13
6-8	2.25	0.57	0.34	0.15	0.23	0.31
8-10	2.33	1.07	2.18	0.54	0.38	0.03

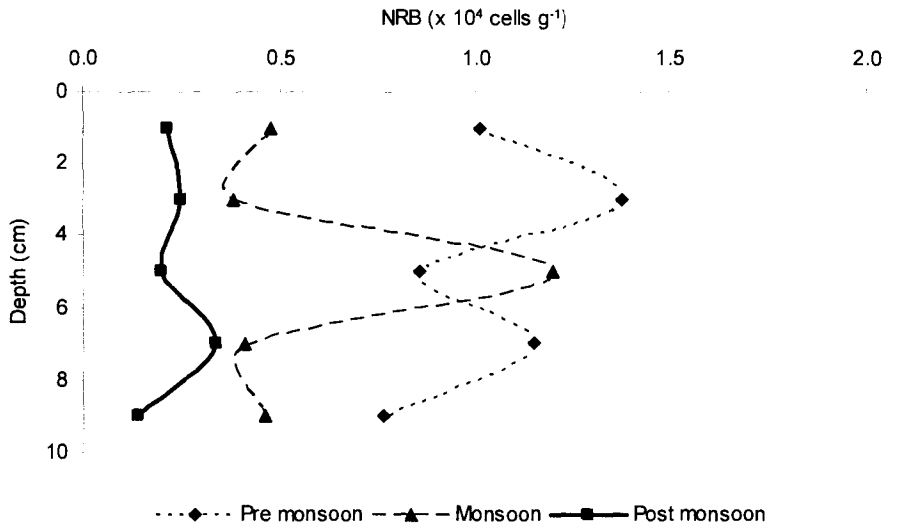


Fig. 19a: Seasonal variation in abundance of heterotrophic nitrate reducing bacteria (NRB) at Tuvem. SD values have been shown below.

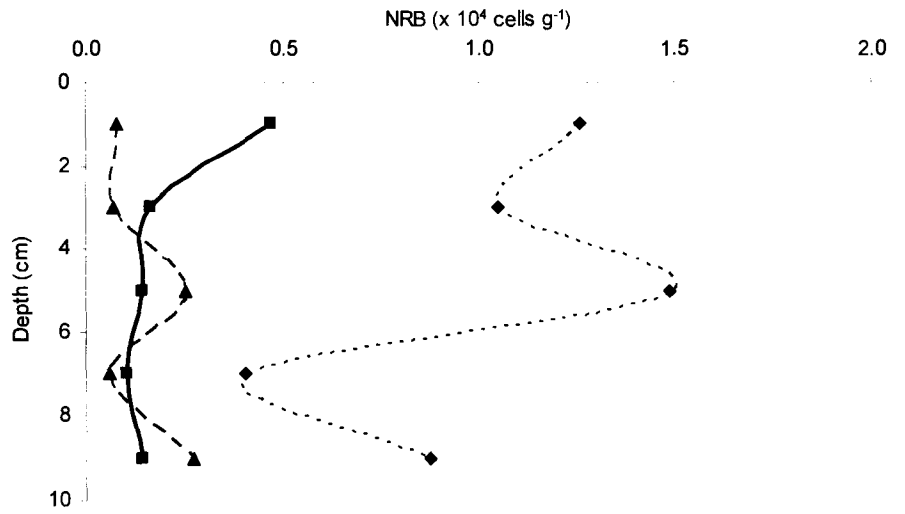


Fig. 19b: Seasonal variation in abundance of heterotrophic nitrate reducing bacteria (NRB) at Divar. SD values have been shown below.

	Tuvem			Divar		
Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.95	0.43	0.27	1.51	0.03	0.41
2-4	2.37	0.26	0.25	1.20	0.04	0.18
4-6	1.27	1.73	0.23	1.30	0.31	0.12
6-8	1.82	0.29	0.43	0.66	0.03	0.07
8-10	1.02	0.41	0.10	0.97	0.22	0.11

4.3.4. Denitrifier abundance

The denitrifiers were most abundant at 4-6 cm at both the sites investigated. Their abundance at the control site varied from $6.17x10^4$ cells g^{-1} at 2-4 cm to $1.87x10^8$ cells g^{-1} at 4-6 cm during the monsoon. Their abundance was stable during the pre and post-monsoon varying from 10^{6-7} cells g^{-1} while it increased to 10^8 cells g^{-1} (at 4-8 cm) during the monsoon (Fig. 20a). However, no significant inter-seasonal variation in denitrifier abundance was observed.

At the experimental site too, no seasonal variation in denitrifier abundance was observed. Their abundance varied from $2.34 x10^5$ cells g^{-1} at 6-8 cm during the monsoon to $8.96 x10^8$ cells g^{-1} at 4-6 cm during the post-monsoon. Maximum denitrifier counts were recorded at 4-6 cm at all seasons (Fig. 20b). Comparative analysis between the two study sites showed no significant variation in denitrifier abundance during any of the 3 seasons.

4.4. Seasonal denitrification rates

DNT was higher at depths ≤4 cm at both the study sites (Figs. 21a-b). The activity was the highest at pre-monsoon at both the study sites. Between seasons, denitrification activity was significantly different at the control ($p<0.001$, df=2) and experimental sites ($p<0.001$, df=2). However, the maximum denitrification rate of 237.29 (±145.07) μmol N_2O-N m^{-2} h^{-1} at the control site was recorded at the surface while at the experimental site, a maximum of 235.21 (±87.57) μmol N_2O-N m^{-2} h^{-1} was recorded at the sub-surface (2-4 cm) during the pre-monsoon season. On the onset of the monsoon, DNT at the control and experimental site decreased to a maximum of 22.01 (±9.55) μmol N_2O-N m^{-2} h^{-1} and 23.86 (±1.45) μmol N_2O-N m^{-2} h^{-1} respectively. At post-monsoon, DNT at the experimental site showed increased to 130.35 (±7.62) μmol N_2O-N m^{-2} h^{-1} compared to the control site where the rate remained steady at a maximum of 25.23 (±3.80) μmol N_2O-N m^{-2} h^{-1}. DNT at the control and experimental sites differed in the monsoon ($p<0.001$, df=1) and post-monsoon ($p<0.001$, df=1). The difference observed during the monsoon between the two sites could be attributed to the variability in activity observed at 4-6 ($p<0.05$, df=1), 6-8 ($p<0.001$, df=1) and 8-10 ($p<0.05$, df=1) cm. The difference observed during the post-monsoon between the two sites could be attributed to the

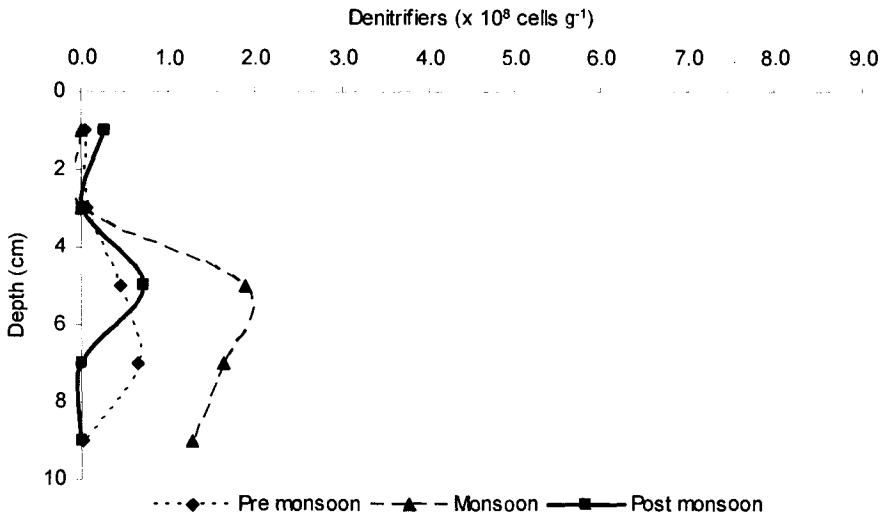


Fig. 20a: Seasonal variation in abundance of denitrifiers at Tuvem. SD values have been shown below.

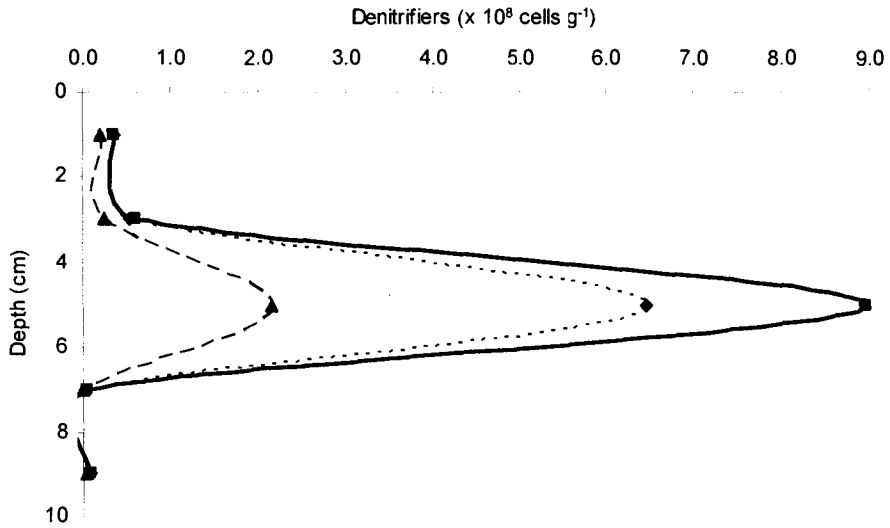


Fig. 20b: Seasonal variation in abundance of denitrifiers at Divar. SD values have been shown below.

	Tuvem			Divar		
Depth (cm)	Pre-monsoon SD	Monsoon SD	Post-monsoon SD	Pre-monsoon SD	Monsoon SD	Post-monsoon SD
0-2	0.34	0.59	0.72	0.11	0.28	0.34
2-4	0.23	0.25	1.73	1.00	0.09	0.14
4-6	1.20	1.37	0.33	0.95	0.23	0.10
6-8	1.08	0.21	0.37	0.77	0.03	0.05
8-10	0.98	0.44	0.10	0.81	0.32	0.17

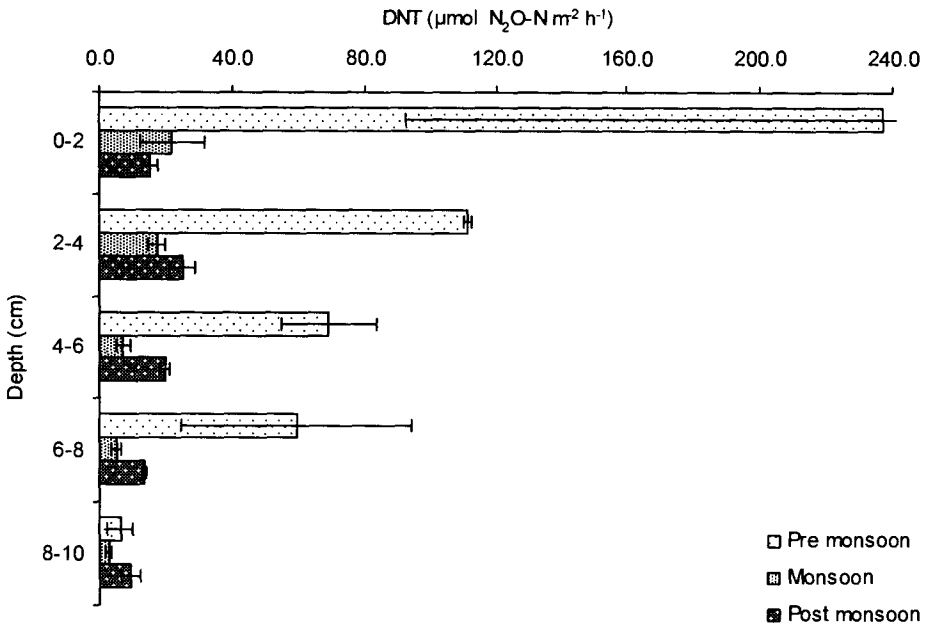


Fig. 21a: Seasonal down-core variation in denitrification activity (±SD) at Tuvem where n=4 at each season. Only negative SD bar for pre-monsoon value at 0-2 cm has been shown due to large variability.

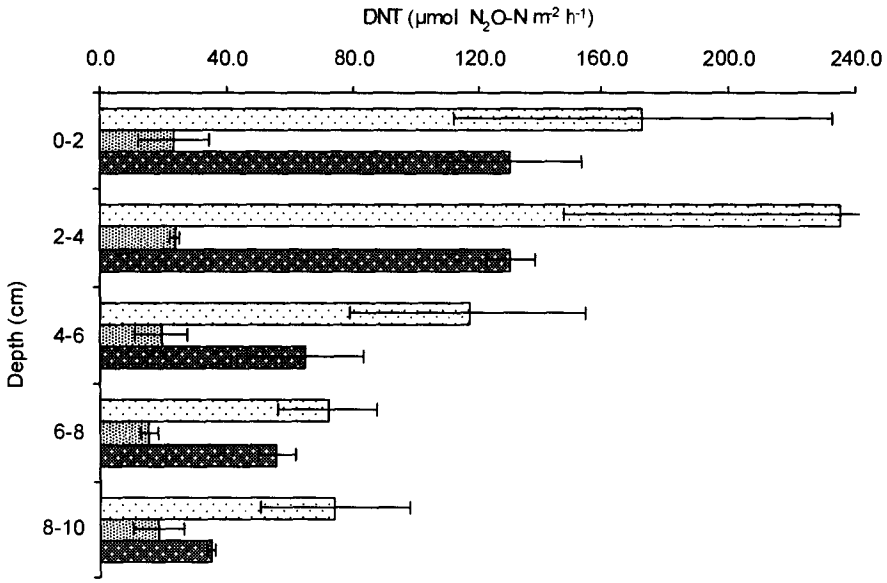


Fig. 21b: Seasonal down-core variation in denitrification activity (±SD) at Divar where n=4 at each season. Only negative SD bar for pre-monsoon value at 2-4 cm has been shown due to large variability.

variability in activity observed at 0-2 cm ($p<0.01$, df=1), 2-4 ($p<0.001$, df=1), 4-6 ($p<0.01$, df=1) and 8-10 ($p<0.05$, df=1) cm.

4.5. Statistical analysis

4.5.1. Factors controlling denitrification

The collective effect of different environmental factors on denitrification at the control (Table 4a) and experimental sites (Table 4b) were evaluated by multiple linear regression analyses. A predictive model to determine denitrification rates was done using 13 essential parameters which gave the best regression equation (Table 5). The regression equation has been derived on the assumption that the observed values would repeat for two annual cycles without variation for purpose of multiple regression analysis.

4.5.2. Factors controlling denitrifier abundance

Pearson's correlation coefficients were used to examine the relationships of environmental variables on denitrifiers in mangrove sediments. Generally, denitrifier abundance decreased with depth mainly at the experimental site as evident from an inverse relationship with depth. At the control site, their abundance was found to increase with depth only during the monsoon (Figs. 22a-c).

At the experimental site, metals (Fe and Mn) were negatively correlated to denitrifiers especially during the monsoon season (Figs. 23a-c). However, this relationship with Fe was not evident during the pre-monsoon and post-monsoon. About 22% of the variation in denitrifiers were brought about due to the variation in NRB ($n=15$; $r=0.46$; $p<0.05$) during the pre-monsoon.

Multiple regression analysis was also used to assess the combined effect of environmental variables on denitrifier abundance in mangrove sediments. Metals mainly Mn was an important parameter influencing denitrifiers at the both the locations (Tables 6a-b). The influence of manganese on denitrifier abundance was persistent throughout the year except during the monsoon where NO_2^- was seen to influence the bacterial forms at Tuvem.

Table 4a: Results of multiple regression analysis of selected parameters which influence denitrification at the control site Tuvem

Pre-monsoon

R= .96085115 R^2= .92323494, F(8,21)=31.570, p<.00000, Std. Error of estimate: 44.261

	Coefficient B	Std. Err. of B	p-level	Semi part. Cor.	Order of influence
Intercept	1847.487	471.386	7.88E-04		
Fe	-126.500	11.719	5.00E-10	-0.653	
NO_2^-	72.023	15.614	1.50E-04	0.279	
Eh	-1.114	0.190	7.96E-06	-0.355	
NH_4^+	13.142	2.208	6.59E-06	0.360	Fe> NH_4^+>Eh> NO_3^-> NO_2^->Mn>TC
NO_3^-	19.744	3.655	2.33E-05	0.327	
Mn	-455.996	132.941	2.51E-03	-0.207	
TC	-113.697	46.829	2.43E-02	-0.147	
TOC	-16.342	12.126	1.92E-01	-0.081	

Monsoon

R= .99623967, R^2= .99249347, F(11,18)=216.36, p<.00000, Std. Error of estimate: .93926

	Coefficient B	Std. Err. of B	p-level	Semi part. Cor.	Order of influence
Intercept	-53.995	19.162	1.14E-02		
Denitrifiers	-1.270	0.290	3.63E-04	-0.089	
NH_4^+	1.106	0.050	1.90E-14	0.449	
THB	-19.393	1.208	4.10E-12	-0.328	
NO_2^-	-8.719	0.696	2.53E-10	-0.256	NH_4^+>THB> NO_2^->TC>TOC> NO_3^- >Mn>Eh>Denitrifiers>NRB>pH
NO_3^-	1.014	0.129	3.13E-07	0.161	
TC	15.288	1.700	4.46E-08	0.184	
TOC	13.134	1.635	2.31E-07	0.164	
Mn	-23.602	3.205	7.80E-07	-0.150	
Eh	-0.025	0.005	5.18E-05	-0.108	

Post-monsoon

R= .83669268, R^2= .70005463, F(5,24)=11.203, p<.00001, Std. Error of estimate: 4.9938

	Coefficient B	Std. Err. of B	p-level	Semi part Cor.	Order of influence
Intercept	24.785	74.969	7.44E-01		
NO_3^-	-0.806	0.250	3.65E-03	-0.360	
pH	-35.157	6.803	2.72E-05	-0.578	pH>THB> NO_3^->TC >NRB
THB	2.644	0.697	8.83E-04	0.424	
TC	18.232	6.959	1.50E-02	0.293	
NRB	5.439	2.369	3.07E-02	0.257	

Table 4b: Results of multiple regression analysis of selected parameters which influence denitrification at the experimental site Divar

Pre-monsoon

R= .93130013, R^2= .86731993, F(11,18)=10.697, p<.00001, Std. Error of estimate: 35.464

	B	Std. Err. of B	p-level	Semi part Cor.	Order of influence
Intercept	423.273	1053.619	6.93E-01		
Fe	8.763	7.198	2.39E-01	0.105	
TOC	142.330	21.445	3.14E-06	0.570	
NRB	186.567	38.600	1.33E-04	0.415	
Mn	766.597	249.908	6.63E-03	0.263	
Denitrifiers	96.937	69.154	1.78E-01	0.120	TOC>NRB>pH>TC>Mn> NO_2^-
pH	-221.613	62.063	2.18E-03	-0.307	
Eh	0.026	0.153	8.68E-01	0.014	
TC	-193.410	60.675	5.10E-03	-0.274	
NO_2^-	-53.917	20.712	1.80E-02	-0.223	
NH_4^+	4.383	2.706	1.23E-01	0.139	

Monsoon

R= .95596427, R^2= .91386769, F(10,19)=20.159, p<.00000, Std. Error of estimate: 2.4820

	B	Std. Err. of B	p-level	Semi part Cor.	Order of influence
Intercept	-356.771	182.682	6.57E-02		
NO_3^-	1.407	0.256	2.64E-05	0.370	
Eh	0.073	0.067	2.92E-01	0.073	
Mn	-11.599	4.324	1.47E-02	-0.181	
THB	-10.583	2.153	9.61E-05	-0.331	
NRB	30.941	7.053	3.17E-04	0.295	NO_3^->THB>NRB>TC>Mn>Denitrifiers> NO_2^-
TC	23.191	8.413	1.26E-02	0.186	
NO_2^-	-1.566	0.724	4.36E-02	-0.146	
Denitrifiers	-8.399	3.537	2.82E-02	-0.160	
pH	24.485	14.569	1.09E-01	0.113	
TOC	-3.157	3.043	3.13E-01	-0.070	

Post-monsoon

R= .99339908, R^2= .98684173, F(12,17)=106.25, p<.00000, Std. Error of estimate: 6.7716

	B	Std. Err. of B	p-level	Semi part Cor.	Order of influence
Intercept	-173.057	155.370	2.81E-01		
Mn	-122.250	13.707	8.06E-08	-0.248	
Fe	15.066	2.415	9.01E-06	0.174	
TOC	-2.630	8.816	7.69E-01	-0.008	
THB	-12.518	6.194	5.93E-02	-0.056	
TC	35.736	5.238	2.97E-06	0.190	
Eh	-0.377	0.045	2.21E-07	-0.231	Mn>Eh>TC>Fe>NRB> NH_4^+> NO_3^- >Denitrifiers>THB
NRB	55.079	9.037	1.19E-05	0.170	
NO_2^-	3.562	2.205	1.25E-01	0.045	
NH_4^+	-3.666	0.962	1.40E-03	-0.106	
NO_3^-	1.338	0.452	8.78E-03	0.082	
Denitrifiers	-24.570	9.428	1.85E-02	-0.073	
pH	-12.603	12.024	3.09E-01	-0.029	

Table 5: The best multiple regression models obtained were:

Location	Season	Multiple regression model
Control site: Tuvem	Pre-monsoon	DNT = 1847.487 - 126.50 * Fe + 72.02 * NO_2^--1.114 * Eh + 13.142 * NH_4^+ + 19.744 * NO_3^- - 455.996 * Mn - 113.697 * TC - 16.342 * TOC
	Monsoon	DNT = -53.994 - 1.27 * Denitrifiers + 1.106 * NH_4^+ - 19.393 * THB - 8.719 * NO_2^- + 1.014 * NO_3^- + 15.287 * TC + 13.133 * TOC - 23.601 * Mn - 0.025 * Eh + 8.594 * NRB - 4.621 * pH
	Post-monsoon	DNT = 24.785 - 0.805 * NO_3^- - 35.156 * pH + 2.644 * THB + 18.232 * TC + 5.439 * NRB
Experimental site: Divar	Pre-monsoon	DNT = 423.27 + 8.763 * Fe + 142.330 * TOC + 186.567 * NRB + 766.597 * Mn + 96.937 * Denitrifiers - 221.613 * pH + 0.026 * Eh - 193.410 * TC - 53.917 * NO_2^- + 4.383 * NH_4^+ + 45.427 * THB
	Monsoon	DNT = -356.771 + 1.407 * NO_3^- + 0.073 * Eh -11.599 * Mn - 10.583 * THB + 30.941 * NRB + 23.191 * TC - 1.566 * NO_2^- - 8.399 * Denitrifiers + 24.458 * pH - 3.157 * TOC
	Post-monsoon	DNT = -173.057 - 122.250 * Mn + 15.066 * Fe - 2.630 * TOC -12.518 * THB + 35.736 * TC - 0.377 * Eh + 55.079 * NRB + 3.562 * NO_2^- - 3.666 * NH_4^+ + 1.338 * NO_3^- - 24.570 * Denitrifiers - 12.603 * pH

where DNT = denitrification rate (µmol N_2O-N m^{-2} h^{-1}); NH_4^+-N, NO_2^-N & NO_3^-N= concentration in µmol L^{-1}; Fe & Mn = concentration in %; TC= total bacterial cells; THB = total heterotrophic bacteria; NRB = nitrate reducing bacteria. All bacterial parameters have been $\log_{10}$ transformed.

Significance level:

p<0.001 p<0.01 p<0.05; n=15

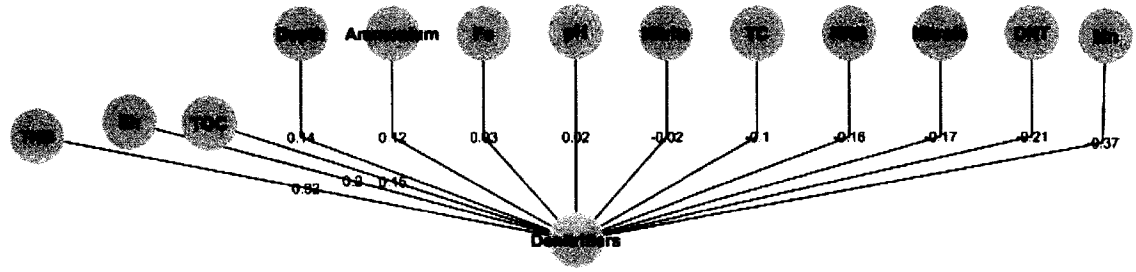


Fig. 22a

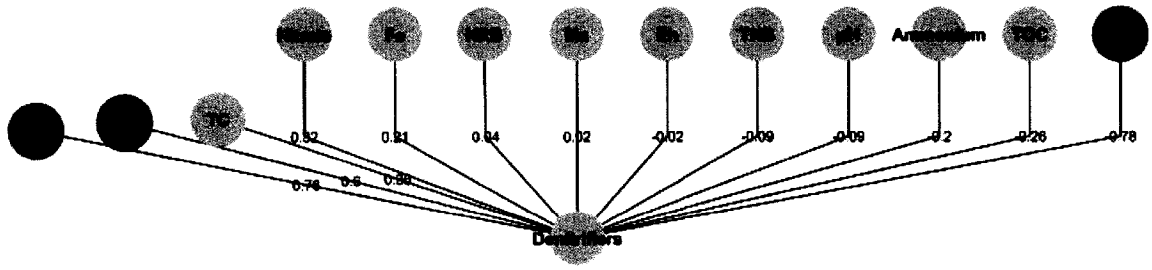


Fig. 22b

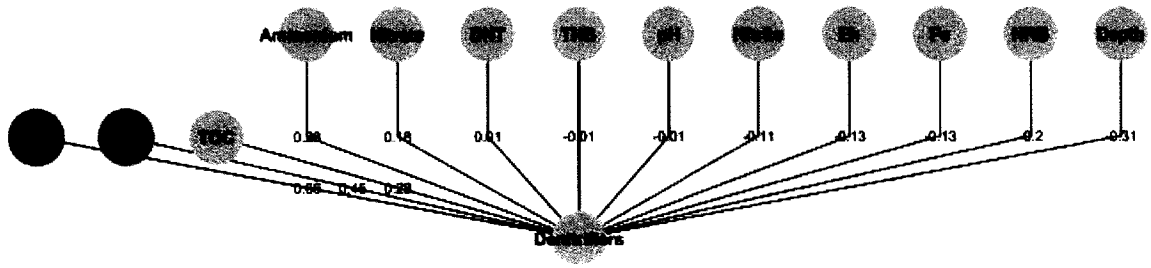


Fig. 22c

Figures 22a-c: Factors influencing denitrifiers during pre-monsoon (Fig. 22a), monsoon (Fig. 22b) and post-monsoon (Fig. 22c) at the control site. Corresponding r values have been shown at each node. Significant correlations have been highlighted in the key denoted above. Variables DNT = denitrification activity; TC= total bacterial cells; THB = total heterotrophic bacteria; NRB = nitrate reducing bacteria. All bacterial parameters have been $\log_{10}$ transformed before analysis.

Significance level:

p<0.001 p<0.01 p<0.05; n=15

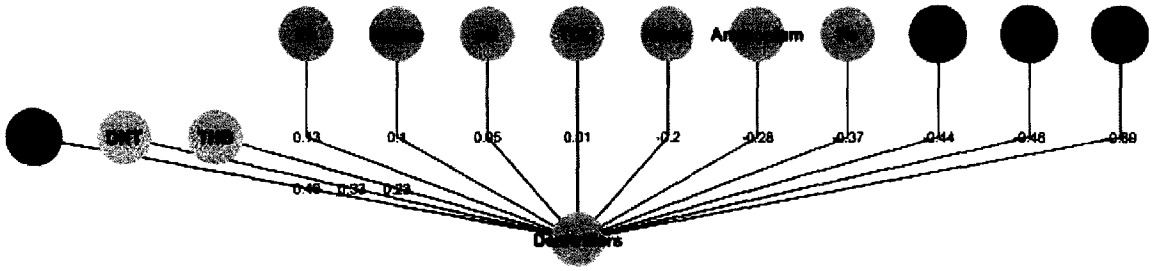


Fig. 23a

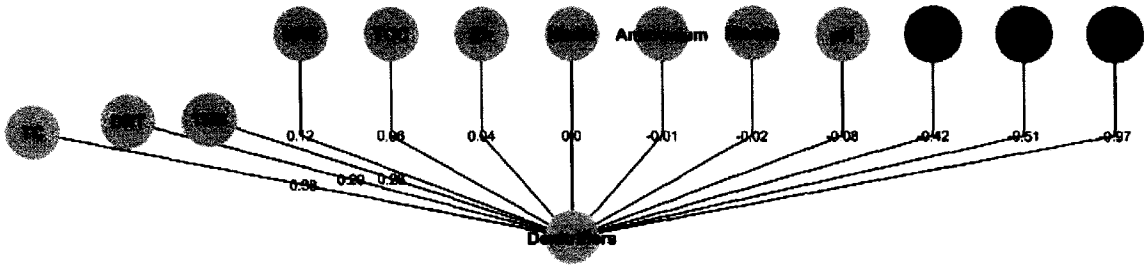


Fig. 23b

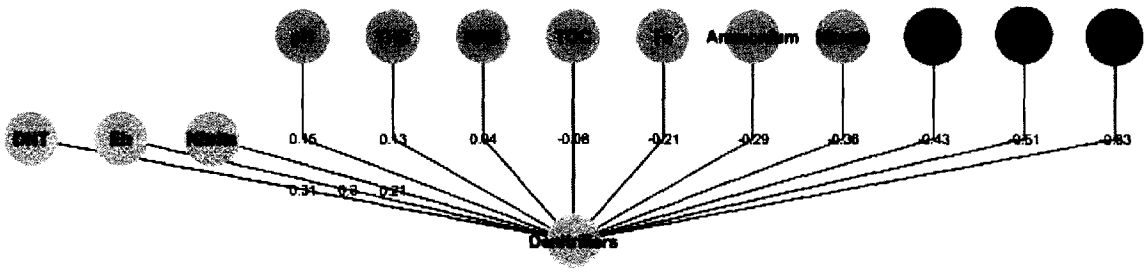


Fig. 23c

Figures 23a-c: Factors influencing denitrifiers during pre-monsoon (Fig. 23a), monsoon (Fig. 23b) and post-monsoon (Fig. 23c) at the experimental site.

Table 6a: Results of multiple regression analysis of selected parameters which influence denitrifier abundance at the control site Tuvem:

Pre-monsoon: Tuvem

R= .37305279, R^2= .13916839, F(1,28)=4.5267, p<.04231, Std. Error of estimate: .57084

	Coefficient B	Std. Err. Of B	Semi part. Cor.	p-level	Order of influence
Intercept	8.451	0.677	5.75E-13		Mn
Mn	-2.963	1.393	4.23E-02	-0.373	

Monsoon: Tuvem

R= .87966533, R^2= .77381109, F(6,23)=13.114, p<.00000, Std. Error of estimate: .77607

	Coefficient B	Std. Err. Of B	Semi part. Cor.	p-level	Order of influence
Intercept	47.474	8.772	1.69E-05		
NO_2^-	2.099	0.277	1.09E-07	0.751	
TOC	-2.420	0.590	4.33E-04	-0.407	
pH	-5.338	1.163	1.29E-04	-0.455	NO_2^->pH>TOC>Eh> NO_3^->Fe
Fe	-0.452	0.176	1.71E-02	-0.255	
Eh	0.005	0.002	3.49E-03	0.323	
NO_3^-	-0.159	0.060	1.47E-02	-0.261	

Post-monsoon: Tuvem

R= .94692978, R^2= .89667600, F(6,23)=33.267, p<.00000, Std. Error of estimate: .22288

	Coefficient B	Std. Err. Of B	Semi part. Cor.	p-level	Order of influence
Intercept	7.077	0.321	5.58E-17		
Mn	2.076	0.181	5.37E-11	0.769	
Fe	-0.214	0.049	2.11E-04	-0.295	
NH_4^+	0.029	0.008	1.10E-03	0.250	Mn>Fe> NH_4^+> NO_2^-> NO_3^->Eh
NO_2^-	-0.171	0.061	9.85E-03	-0.189	
NO_3^-	0.047	0.020	2.49E-02	0.161	
Eh	-0.002	0.001	3.68E-02	-0.149	

Table 6b: Results of multiple regression analysis of selected parameters which influence denitrifier abundance at the experimental site Divar:

Pre-monsoon: Divar

R= .98741413, R^2= .97498666, F(6,23)=149.42, p<.00000, Std. Error of estimate: .14357

	Coefficient B	Std. Err. Of B	Semi part. Cor.	p-level	Order of influence
Intercept	11.171	1.344	2.21E-08		
Mn	-3.633	0.136	8.51E-19	-0.879	
Fe	-0.106	0.010	4.22E-10	-0.340	
pH	0.583	0.186	4.68E-03	0.103	Mn>Fe>pH>Eh> NO_2^-> NH_4^+
Eh	-0.001	0.000	2.97E-02	-0.076	
NO_2^-	-0.084	0.043	6.16E-02	-0.065	
NH_4^+	0.010	0.007	1.89E-01	0.045	

Monsoon: Divar

R= .99983274, R^2= .99966550, F(7,22)=9392.7, p<0.0000, Std. Error of estimate: .02114

	Coefficient B	Std. Err. Of B	Semi part. Cor.	p-level	Order of influence
Intercept	8.149	0.274	2.94E-19		
Mn	-2.005	0.009	4.64E-38	-0.839	
Fe	0.162	0.003	1.60E-24	0.203	
NO_3^-	0.011	0.002	3.45E-05	0.020	
NO_2^-	-0.046	0.006	1.93E-07	-0.029	Mn>Fe> NO_2^->Eh> NO_3^->pH
Eh	-0.001	0.000	1.60E-06	-0.025	
pH	0.088	0.039	3.64E-02	0.009	
NH_4^+	-0.003	0.002	1.15E-01	-0.006	

Post-monsoon: Divar

R= .98449229, R^2= .96922507, F(7,22)=98.981, p<.00000, Std. Error of estimate: .16884

	Coefficient B	Std. Err. Of B	Semi part. Cor.	p-level	Order of influence
Intercept	12.080	0.981	2.43E-11		
Mn	-1.475	0.083	1.48E-14	-0.666	
pH	-0.124	0.161	4.46E-01	-0.029	
NH_4^+	-0.089	0.009	1.46E-09	-0.370	
TOC	0.855	0.080	3.30E-10	0.401	Mn>TOC> NH_4^+>Fe> NO_3^-> NO_2^-
Fe	-0.187	0.025	2.13E-07	-0.277	
NO_3^-	0.021	0.007	7.39E-03	0.110	
NO_2^-	-0.083	0.037	3.57E-02	-0.084	

SECTION II – Laboratory experiments

4.6. Nitrous oxide production

4.6. 1. Physico-chemical characteristics

The mangrove sediments were acidic in nature with pH ranging from 5.80-6.16 (Table 7). Sediment redox potentials were consistently low, in the range of -27.8(±0.9) to -6(±16.45) mV at Tuvem while the Divar sediments exhibited the lowest redox potential of -5.7(±25.10) at a depth of 8-10 cm.

Pore water had a measurable nutrient content which varied widely with depth. Up to 15.15(±3.43) μmol NH_4^+-N l^{-1} was recorded at 8-10 cm at Tuvem while 31.34(±1.83) was recorded at Divar at a depth of 6-8 cm. Nitrite and nitrate concentration generally increased with depth with up to 11.69(±0.83) μmol NO_2^--N l^{-1} and 14.17(±0.43) μmol NO_3^--N l^{-1} at Tuvem. Low nitrate levels and increase in nitrite concentration were observed at Divar. Up to 14.20(±0.08) μmol NO_2^--N l^{-1} was recorded at a depth of 6-8 cm. Total organic carbon at Tuvem varied from 2.13-4.54% while at Divar it ranged between 2.45-3.99%.

4.6. 2. Net nitrous oxide production, denitrification rate and N_2O production by denitrifiers

Nitrous oxide production was significantly different (two-tailed t-test, P = 0.003, n = 15) at both the locations and varied with depth. At Tuvem, a steady decrease in N_2O production with depth was observed. Highest production of 7.98 (±1.23) μmol N_2O-N m^{-2} h^{-1} was recorded at 0-2 cm (Fig. 24). At the deepest layer investigated (8-10 cm), net N_2O production decreased to a minimum of 1.39 (±0.23) μmol N_2O-N m^{-2} h^{-1}. At Divar, the 2-4 cm layer showed maximum N_2O production at a rate of 22.00 (±13.53) μmol N_2O-N m^{-2} h^{-1}.

A denitrification maxima of 25.23 (±3.80) μmol N_2O-N m^{-2} h^{-1} was observed at 2-4 cm (Fig. 25). DNT was otherwise relatively homogenous up to a depth of 8 cm with a rate of 15.27(±2.15) μmol N_2O-N m^{-2} h^{-1} at the surface to 13.61(±0.41) μmol N_2O-N m^{-2} h^{-1} at 6-8 cm. At the deepest layer, DNT decreased to 9.61(±2.85) μmol N_2O-N m^{-2} h^{-1}.

In presence of a nitrification inhibitor, denitrifiers produced a maximum of 14.25(±8.34) μmol N_2O-N m^{-2} h^{-1} at 0-2 cm (Fig. 26). Incomplete denitrification (^IN_2O)

Table 7: Variation in the average values of physico-chemical parameters (n=15) at Tuvem and Divar

Location: Tuvem						
Depth (cm)	pH	Eh (mV)	NH_4^+-N ($\mu mol\ l^{-1}$)	NO_2^--N ($\mu mol\ l^{-1}$)	NO_3^--N ($\mu mol\ l^{-1}$)	TOC (%)
0-2	5.84(±0.2)	-27.8(±0.9)	14.73(±4.42)	4.53(±0.13)	10.44(±0.42)	2.13(±0.71)
2-4	5.97(±0.2)	-23.2(±7.39)	15.02(±1.36)	8.59(±0.33)	9.86(±0.37)	2.85(±1.44)
4-6	6.06(±0.6)	-9.5(±15.06)	7.82(±1.45)	8.12(±0.12)	10.94(±0.61)	4.54(±2.86)
6-8	6.04(±0.6)	-6(±16.45)	12.03(±0.82)	10.89(±0.64)	14.17(±0.43)	4.43(±2.82)
8-10	6.16(±0.5)	-6.3(±14.54)	15.15(±3.43)	11.69(±0.83)	11.45(±0.01)	3.97(±2.72)
Location: Divar						
0-2	5.88(±0.3)	115(±64.7)	15.69 (±3.40)	7.18(±0.01)	4.62(±0.67)	2.94(±1.57)
2-4	5.80(±0.1)	94.3(±62.6)	15.96 (±2.01)	4.08(±0.19)	4.25(±0.20)	3.99(±2.15)
4-6	5.99(±0.1)	93.5(±45.18)	23.82(±2.27)	6.63(±0.65)	9.31(±0.16)	2.45(±1.04)
6-8	6.03(±0.1)	50.8(±61.38)	31.34(±1.83)	14.20(±0.08)	10.08(±0.16)	3.29(±1.70)
8-10	6.05(±0.04)	-5.7(±25.10)	22.10(±3.97)	8.07(±0.28)	10.01(±0.26)	3.07(±1.77)

was responsible for 43-93% (Fig. 27) of the N_2O production of which about 13-52% (net N_2O/DNT*100) of the N_2O produced through denitrification. N_2O production through nitrification ($^{N}N_2O$) was below detection.

4.6. 3. Denitrifier abundance

The cultivation based technique yielded up to 10^7 denitrifiers g^{-1} sediment. Their abundance was maximum at 4-6 cm ($7.14x10^7$ cells g^{-1}) as compared to the other sections of the core where their number was relatively stable. The denitrifiers significantly influenced (Table 8) nitrous oxide production in these sediments ($r= 0.55$; $p<0.05$). They also showed an inverse relationship with pore water ammonium ($r= -0.57$; $p<0.05$) and nitrite ($r= -0.55$; $p<0.05$) concentrations.

4.6. 4. Environmental factors influencing N_2O production

Inter-relationships of nitrous oxide production rates with physico-chemical parameters at Tuvem showed an inverse relationship between N_2O production and pore water nitrite concentration ($n=15$; $r= -0.47$; $p<0.05$). Up to 32% variation in gas production was negatively influenced by sediment pH ($r= -0.57$; $p<0.05$). Even though the relationship between nitrous oxide concentration and total organic carbon was poor, a positive relationship existed between these parameters.

Principal component analysis (PCA) on sediment variables resulted in four main components explaining nearly 80% of the total variance (Table 9). The first component in the correlation plot (Fig. 28) explained 29% of the observed variance. A forward regression of the reductive processes in the N cycle (DNT and N_2O production) was observed in the first component. Sediment pH, denitrifier abundance and nitrite significantly influenced this component (Table 10). The second component explained about 22% of the variance with pore water nitrate and organic carbon concentrations correlating strongly with this component. The third component correlated with pore water ammonium and denitrification activity and explained 16% of the variation. The only most significant variable in the fourth component was sediment redox potential and explained 12% of the variation.

Table 8: Correlation between sediment variables at Tuvem

Variable 1	Variable 2	*r* value*
N_2O production	pH	-0.57
N_2O production	Nitrite	-0.47
N_2O production	Denitrifiers	0.55

*Correlation significant at 0.05 probability level

Table 9: Eigenvalues of correlation matrix and related statistics

Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
2.64	29.31	2.64	29.31
2.02	22.48	4.66	51.79
1.46	16.27	6.12	68.05
1.05	11.71	7.18	79.76

Table 10: Results of principal component analysis of sediment variables and nitrous oxide production

Variable	Component 1	Component 2	Component 3	Component 4
N_2O production	0.856*	0.124	0.206	0.266
pH	-0.597*	-0.391	-0.541	0.134
Eh	-0.215	0.438	0.106	0.828*
Ammonium	-0.453	-0.420	0.640*	0.164
Nitrite	-0.687*	0.649	0.093	-0.004
Nitrate	-0.395	0.665*	-0.367	0.027
Denitrifiers	0.730*	-0.023	-0.496	0.326
TOC	0.369	0.779*	0.286	-0.330
DNT	0.021	0.133	-0.487*	-0.189

*Correlation significant at 0.05 probability level; TOC= Total organic carbon, DNT=Denitrification rate

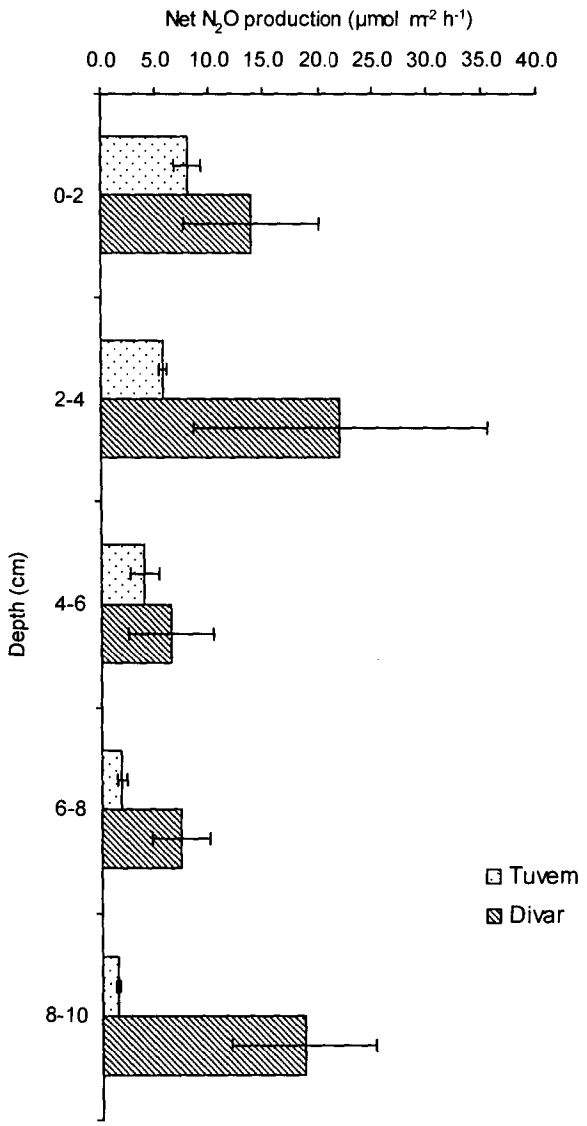


Fig. 24: Net nitrous oxide production (±SD) in the Tuvem and Divar mangrove sediments

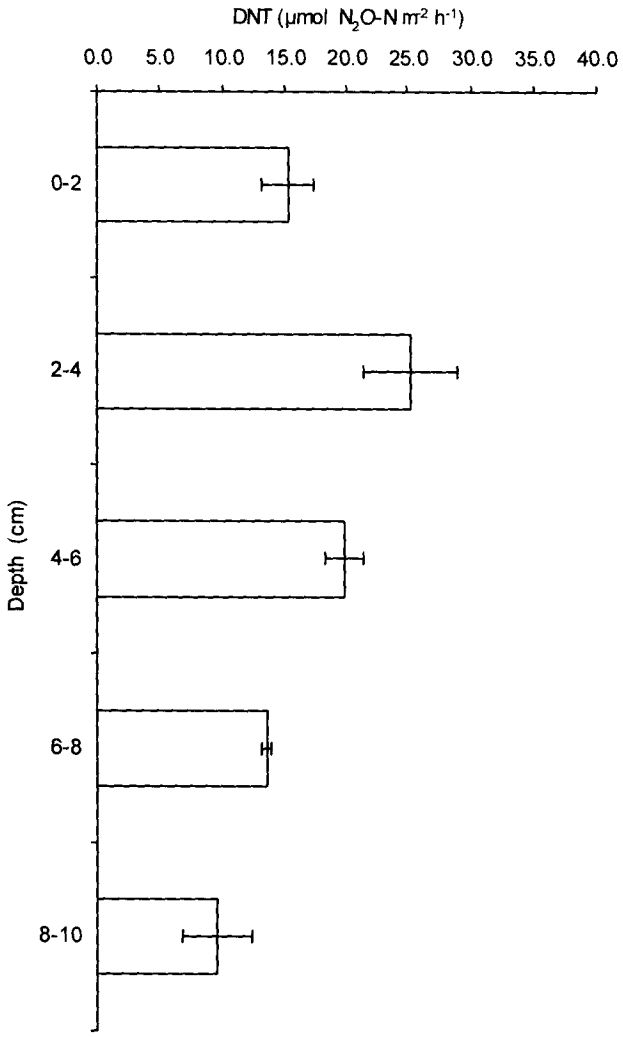


Fig. 25: Denitrification rate (±SD) at Tuvem

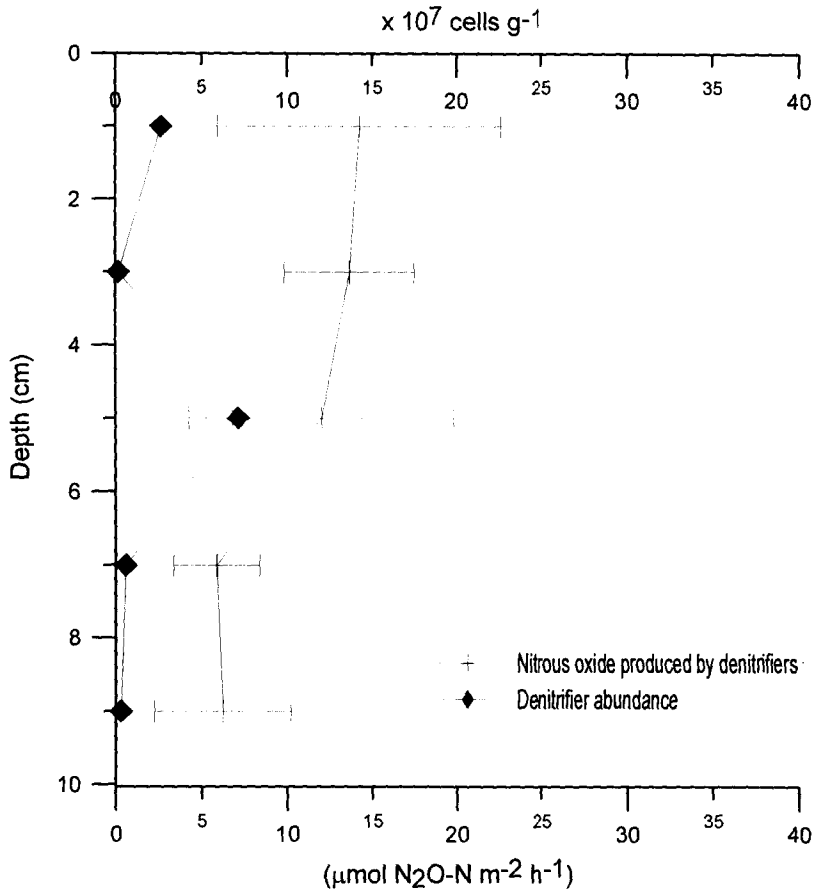


Fig.26: Nitrous oxide production (±SD) by denitrifiers under nitrification inhibited conditions at Tuvem

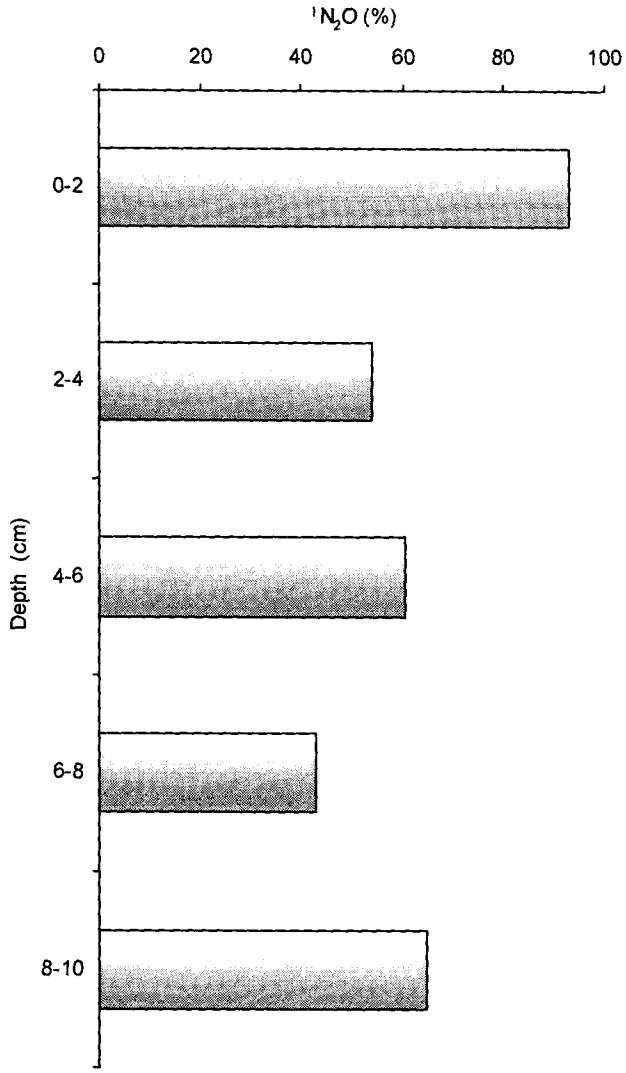


Fig.27: Percentage of nitrous oxide produced through incomplete denitrification

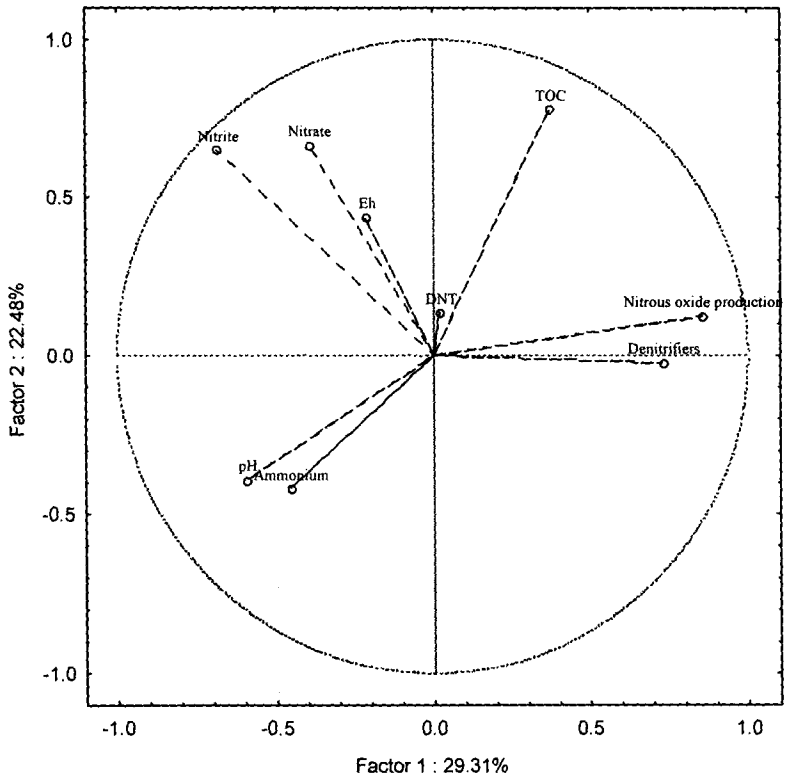


Fig. 28: Correlation plot from standardized principal component analysis (PCA) on sediment variables. Variables DNT= denitrification rate measured by C_2H_2 inhibition technique, N_2O production= Net N_2O produced from nitrification + denitrification, TOC=total organic carbon. Eigenvalues of correlation matrix and related statistics are as follows:

Eigenvalue	% Total variance	Cumulative Eigenvalue	Cumulative %
2.64	29.31	2.64	29.31
2.02	22.48	4.66	51.79
1.46	16.27	6.12	68.05
1.05	11.71	7.18	79.76

4.7. Denitrification and other co-occurring processes

4.7.1. Grain size

Analysis of sand, silt and clay percentage in mangrove sediments (0-10 cm depth) depicts sediment texture dominated by sand which contributes to the extent of 43-79%. A comparison between the two mangrove ecosystems indicates that at Divar (Table 11) sand forms a major component (≥70%) of the sediment with percentage of silt and clay composing only up to 28%. At Tuvem, the percentage of silt and clay was higher at 32% and 22% respectively.

4.7.2. Total organic carbon and nutrient analyses

Down-core total organic carbon (TOC) profiles at Divar and Tuvem showed 2 patterns corresponding to the 6 first centimeters (0-6 cm) and the remaining 4 cm (6-10 cm). For Divar, a general increase of TOC with depth appeared for the first 6 cm whereas in Tuvem TOC content was relatively stable (1.19-1.23%) for these layers at Tuvem. TOC content at 6-10 cm was similar at both sites, i.e. a decrease with depth (Fig. 29).

Down-core nutrient profiles at Divar exhibited an overall decreasing trend with depth. Sediment extractable ammonium concentration at the surface was about 2.22 mmol which steadily decreased to 1.21 mmol at 8-10 cm depth (Fig. 30). Ammonium concentrations at Tuvem however showed an opposite trend, starting with 1.1 mmol and increasing with depth. A maximum of 1.60 mmol was observed at 8-10 cm.

At Tuvem, nitrite and nitrate concentrations decreased with depth and a maxima was observed at 2-4 cm with 7.55 µmol and 36.62 µmol (Fig. 31a) respectively. Similarly at Divar, nitrite concentrations in the pore water decreased from 14.45 at the surface to 4.96 µmol at the deeper layer while nitrate varied from 19.90 at 0-2 cm to 2.63 µmol at 8-10 cm (Fig. 31b).

4.7.3. Di-nitrogen fixation

Di-nitrogen fixing activity (Fig. 32) was detected only at Tuvem with maximum activity of 12.47 nmol N_2 g^{-1} h^{-1} occurring at 6-8 cm depth. At Divar, it was below detection limits.

Table 11: Fractions of sand, silt and clay at various depths in mangrove sediment

	Location: **Tuvem**			Location: **Divar**		
Depth (cm)	Sand (%)	Silt (%)	Clay (%)	Sand (%)	Silt (%)	Clay (%)
0-2	48.42	32.02	16.31	70.14	16.69	11.59
2-4	44.56	32.36	20.05	69.88	14.32	13.60
4-6	43.07	32.45	20.10	72.88	12.37	12.71
6-8	47.93	30.71	19.62	70.03	13.16	15.62
8-10	44.16	31.86	22.54	79.31	8.68	10.50
Texture	Silty sand			Sandy		

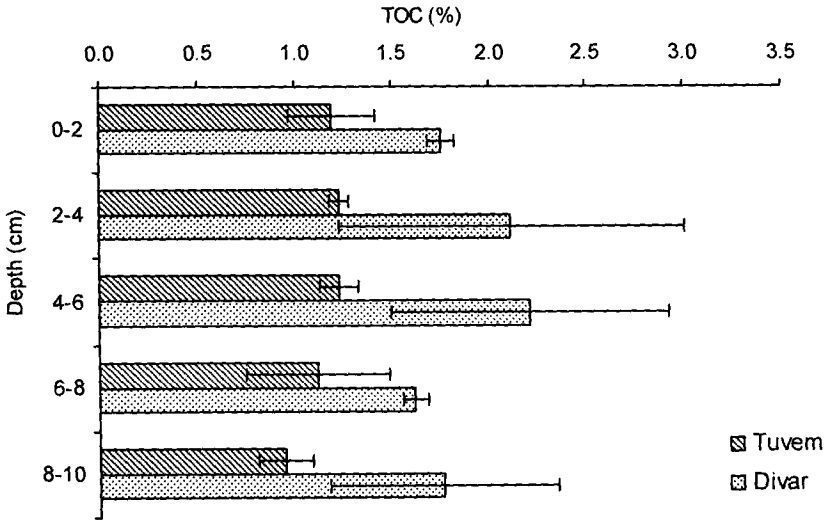


Fig. 29: Down-core total organic carbon (±SD) profile at Tuvem and Divar.

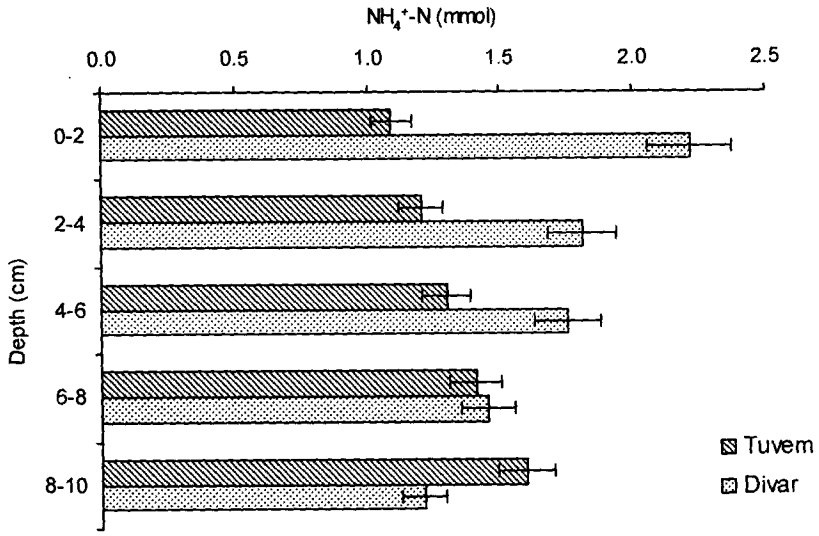


Fig. 30: Down-core variation in sediment extractable ammonium (±SD) at Tuvem and Divar.

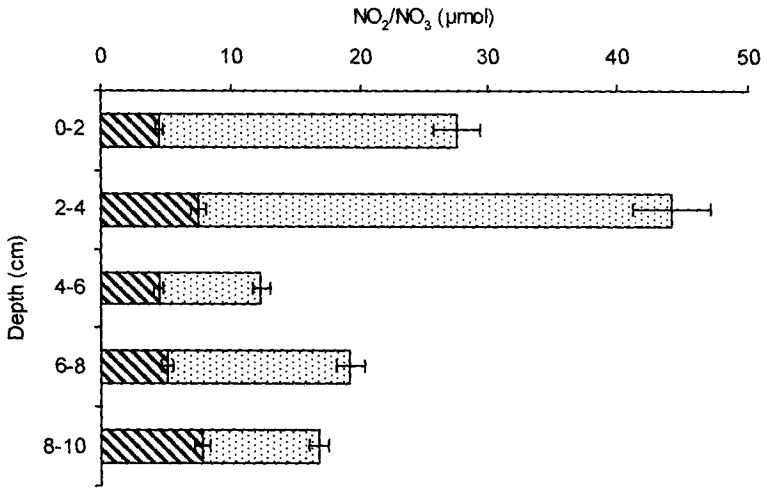


Fig. 31a

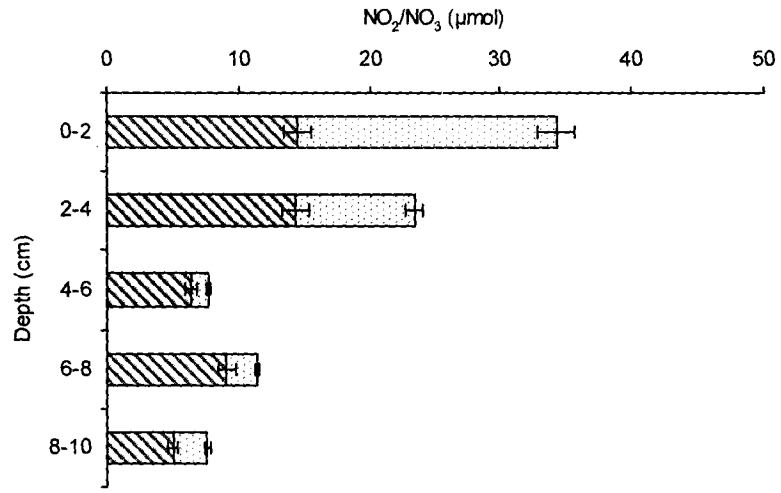


Fig. 31b

Figs. 31a-b: Down-core nitrate and nitrite concentration (±SD) profile for pore water in Tuvem (Fig. 31a) and Divar (Fig. 31b). Hatched bars represent NO_2^- while NO_3^- is represented by the dotted bar.

4.7.4. Net nitrification

Though nitrification is assumed to be maximal under aerobic conditions, it was interesting to note that the deeper layers of mangrove sediments exhibited higher nitrification potential (Fig. 33). At Tuvem, nitrification rates were relatively higher at most depth layers tested varying between 4.05 nmol g^{-1} h^{-1} at 6-8 cm to 13.78 nmol g^{-1} h^{-1} at 8-10 cm. Net potential nitrification rates at Divar were homogenous from the surface up to 8 cm depth varying between 4.62-6.06 nmol g^{-1} h^{-1}. The activity at the deepest layer (8-10 cm) doubled to 13.43 nmol g^{-1} h^{-1}.

4.7.5. Nitrate reduction

Nitrate reducing activity (NRA) in mangrove sediments determined in sediment slurry incubation under micro aerophilic conditions revealed maximum NRA of 3.52 µmol g^{-1} h^{-1} at 2-4 cm depth at Divar which consequently decreased with increasing depth (Fig. 34). At Tuvem, the trend was opposite with the deeper layers showing maximum activity. A maximum of 2.01 µmol g^{-1} h^{-1} was observed in Tuvem sediment at 8-10 cm depth. This rate however was comparatively lower than that observed at Divar.

4.7.6. N_2 production through anammox and denitrification

Di-nitrogen production through anammox and denitrification was concomitantly determined. There was wide and statistically significant variation in denitrification activity between sampling stations (α=0.05, $p<0.01$, n= 30) and sampling depth (α=0.05, $p<0.01$, n= 30). The total N_2 production rates including denitrification and anammox were higher within the first 4 cm, and then decreased with depth except at Divar where the activity was slightly higher in the deeper layer. At Tuvem, the observed rate (Fig. 35a) in the upper layer was three fold lower (81 nmol N g^{-1} h^{-1}) than that found for the same layer at Divar where N_2 production occurred at a rate of 224 nmol N g^{-1} h^{-1} (Fig. 35b). For both sampling stations denitrification was the main process leading to N_2 production and highest denitrification rates were observed in the two first layers.

The contribution of anammox was calculated according to $_{mod}$.IPM. Anammox (Anx) activity was detected in mangrove sediments. Though the process was negligible in the upper layers (up to 8 cm) at Divar, it was found to be the major source of N_2 in the deeper layer (8-10 cm) accounting for up to 101.15 nmol N_2 g^{-1} h^{-1} (67% of total N_2

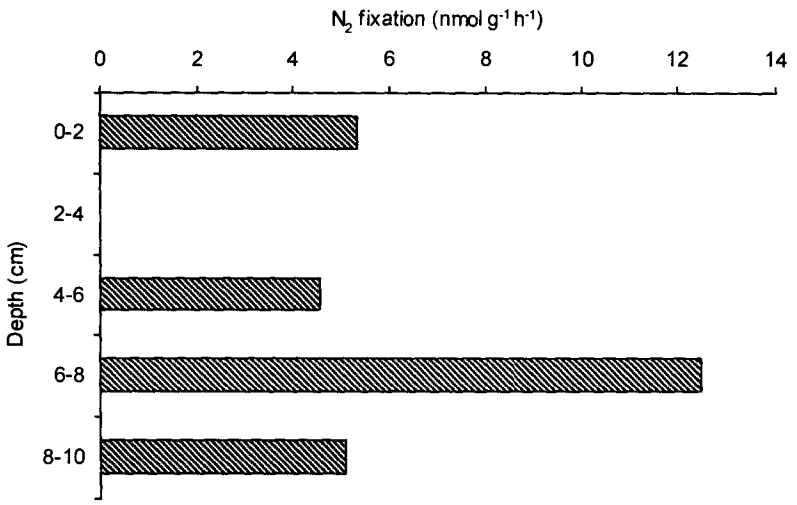


Fig. 32: Down-core di-nitrogen fixation (±SD) profile at Tuvem. No activity was detectable at Divar.

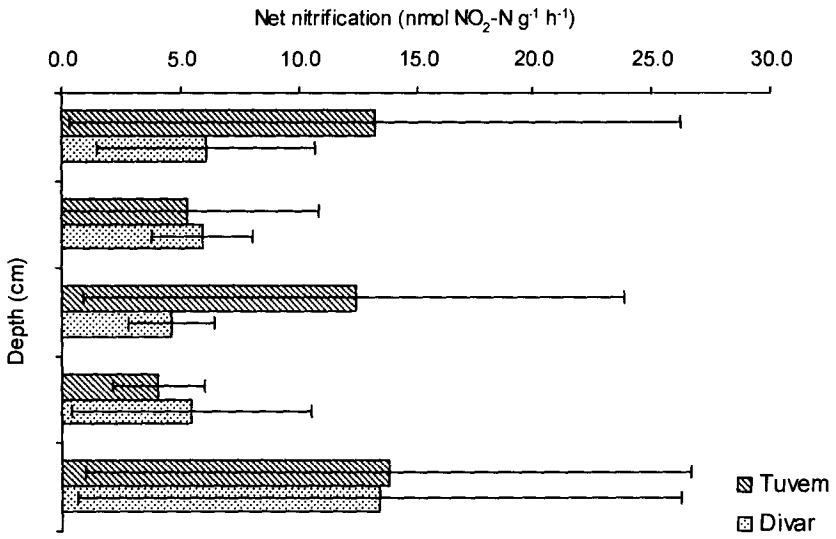


Fig. 33: Down-core profile of net nitrification activity (±SD) at Tuvem and Divar

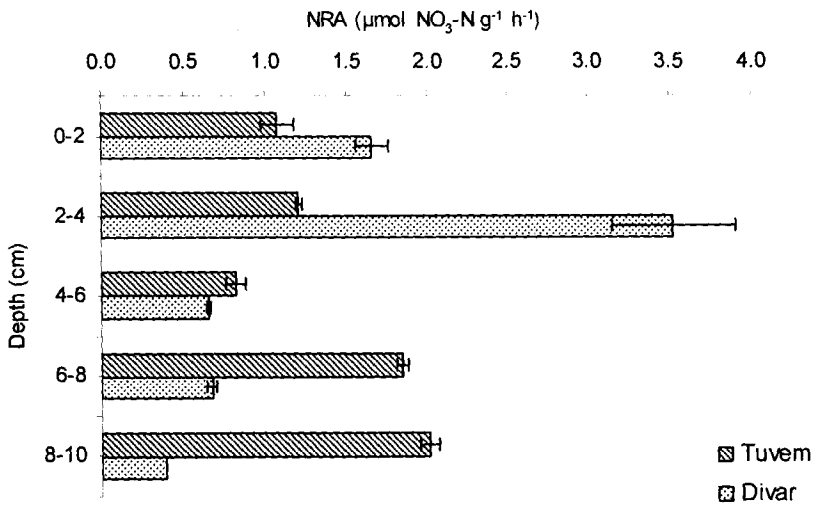


Fig. 34: Down-core variation in nitrate reduction activity (±SD) at Tuvem and Divar

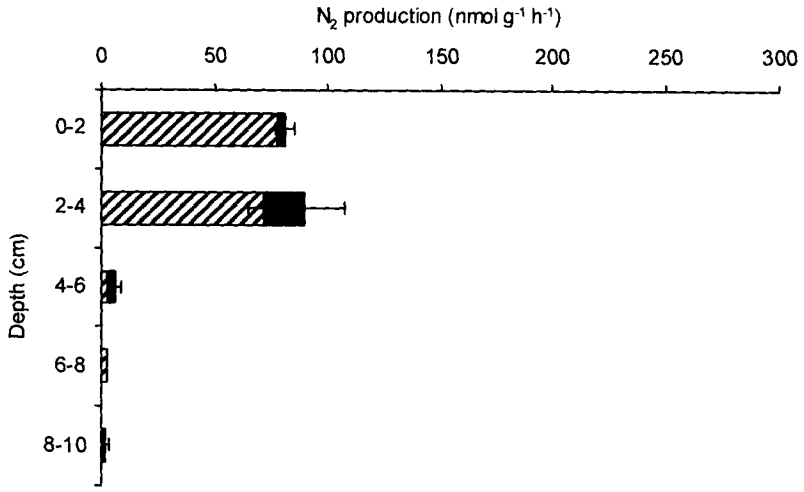


Fig. 35a

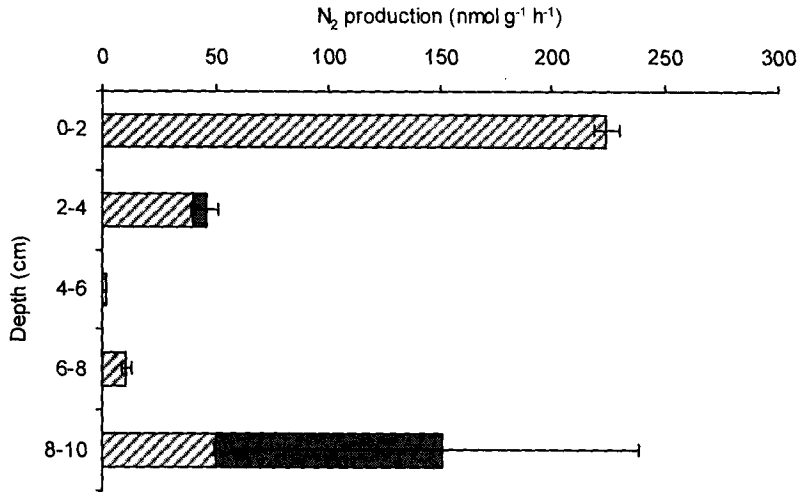


Fig. 35b

Figs. 35a-b: Di-nitrogen production (±SD) profile from denitrification (hatched bar) and by anammox (full bar) at Tuvem (Fig. 35a) and Divar (Fig. 35b).

production at this layer). At Tuvem, although the process was detected at all depths investigated, it was relatively weaker (1.48-18.31 nmol N_2 g^{-1} h^{-1}). Denitrification was the dominant process responsible for N-loss in mangrove sediments and correlated negatively with depth ($n=9$; $r=-0.65$; $p<0.05$).

We calculated a net nitrogen balance by differences between N_2-production via denitrification plus anammox and N_2-fixation (Fig. 36). Thus, there was a net N_2 production from the sediments, leading to the loss of nitrogen from the mangrove ecosystem which is not compensated by the process of N-fixation.

4.7.7. Dissimilatory nitrate reduction to ammonium (DNRA)

^{15}N labelling to measure DNRA showed a steady increase in $^{15}NH_4^+$ over time at all depths investigated (Fig. 37) at both the sites. This process was responsible for 39% nitrate removal (100% NRA= 6.91 µmol) at Divar with values varying from 0.38-0.67 µmol g^{-1} h^{-1}. At Tuvem, DNRA accounted for up to 65% NO_3^- removal (100% NRA=6.97 µmol) with a maximum rate of 1.25 µmol g^{-1} h^{-1} at 2-4 cm.

4.7.8. Net nitrous oxide production

N_2O production in the Divar sediments decreased with depth from a maximum of 2.71 nmol N_2O-N g^{-1} h^{-1} at the surface to 0.33 nmol N_2O-N g^{-1} h^{-1} at 8-10 cm (Fig. 38). At the control site Tuvem, maximum N_2O production occurred at 2-4 cm at a rate of 4.18 nmol N_2O-N g^{-1} h^{-1}.

4.7.9. Macrofaunal abundance

The mangrove sediments at Divar harbored a substantial macrofaunal population (Table 12) with a dominance of polychaetes (94%; 1376 no. m^{-2}). The gastropods, oligochaetes and crustaceans formed the minor fraction of the community. Though less in abundance, the polychaetes were the dominant macrofauna (87%; 224 no. m^{-2}) at Tuvem. Crustaceans were absent while the minor fraction of the community were represented by oligochaetes and crustaceans.

4.7.10. Abundance of denitrifying genes (*nosZ*)

Abundance of *nosZ* genes ranged from 0.1 to 2 x 10^7 target copies g^{-1} of dry sediment. At Divar, the *nosZ* genes were well dispersed within the 0-10 cm core as compared to Tuvem where their abundance was maximum at 6-8 cm (Fig. 39).

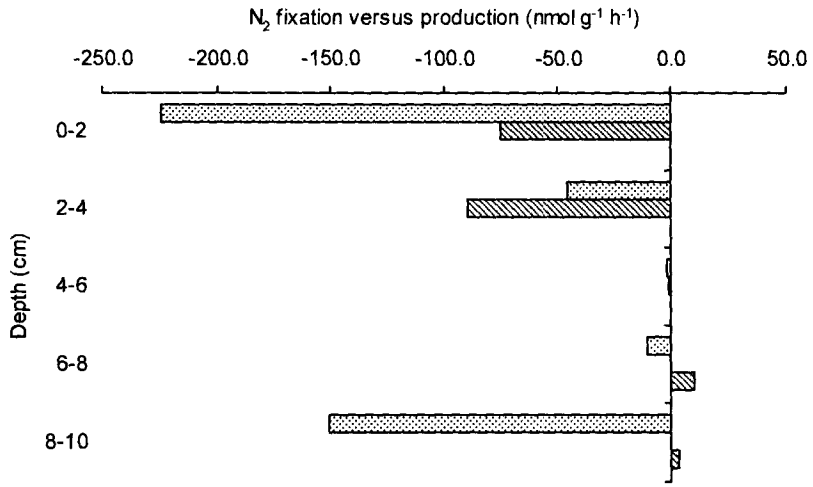


Fig. 36: Down-core profile of di-nitrogen budget (di-nitrogen fixation minus di-nitrogen production (denitrification + anammox)) in mangrove sediments. Hatched bar= Tuvem and dotted bar= Divar sample.

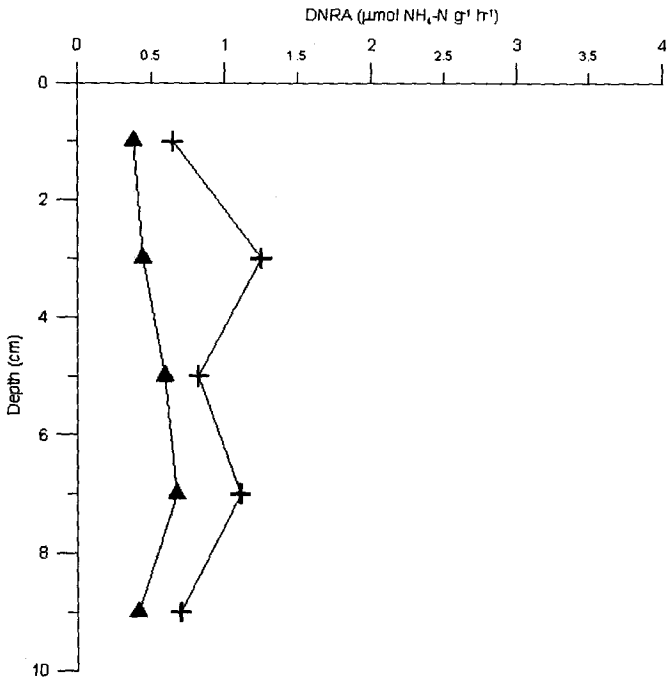


Fig. 37: Down-core variation in dissimilatory reduction of nitrate to ammonium (┼ Control site: Tuvem; ▲Experimental site: Divar).

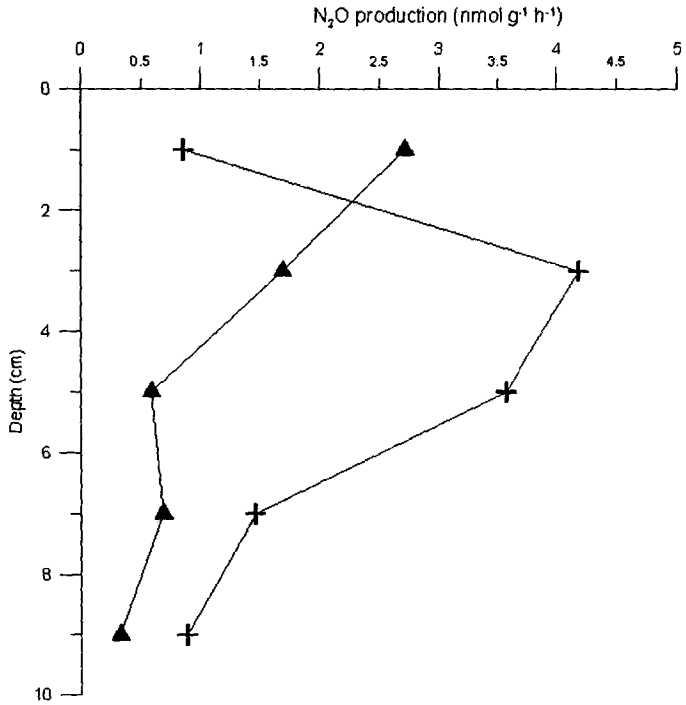


Fig. 38: Down-core variation in nitrous oxide production (┼ Control site: Tuvem; ▲Experimental site: Divar)

Table 12: Abundance of major macrofaunal groups in mangrove sediments

Macrofaunal group	Density at Tuvem (nos. m^{-2})	Density at Divar (nos. m^{-2})
Polychaeta	224	1376
Oligochaeta	16	32
Crustacea	16	32
Gastropoda	-	16

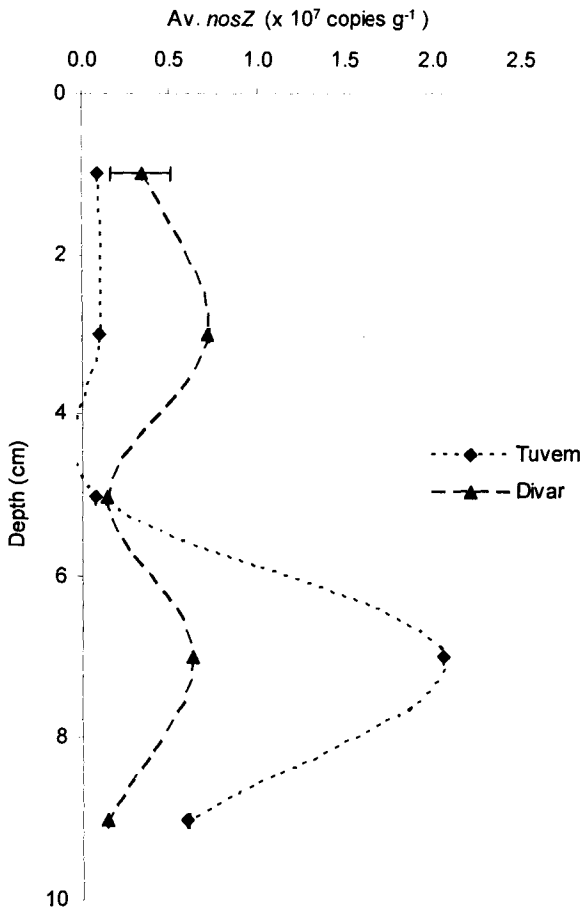


Fig. 39: Down-core variation in the abundance of *nosZ* genes (±SD) at Tuvem and Divar

4.7.11. Environmental controls on denitrification

The Principal Component Analysis (PCA) was applied to the matrix of correlation coefficient between biotic and abiotic parameters (Fig. 40). The first three factors were taken into account which corresponded to 82.2% of total data variability. Factor 1 (PC 1) explains 38.2% of total data variability. D_{tot}, N_2 production as well as nitrite were positively correlated to the axis while N_2 fixation was negatively correlated. The differences between sampling depths were clearly discriminated within the axis. Factor 2 (PC 2) explained 22.6% of data variability. The 4 top centimeters of Divar seems characterized by higher content in nitrate and higher N_2 production that was mainly explained by denitrification rates. Anammox was negatively correlated to this axis. The deeper sampling depths (D3= 2-4 cm, D5=4-6 cm, D7=6-8 cm, D9=8-10 cm) at Divar were seen to cluster along with anammox activity.

4.8. Influence of nitrate and organic C amendments on denitrification

4.8.1. Chemical analyses

The Divar sediments harbored a measurable pore water nitrate content which increased with depth (Fig. 41) ranging from 4.15 (±0.21) to 18.71 (±0.28) µmol. Down-core profiling of denitrification showed a sub-surface maxima at 2-4 cm with a rate of 20.08 µmol N_2O-N m^{-2} h^{-1} (1.43±0.66 nmol g^{-1} h^{-1}). High denitrification activity at 2-4 cm coincided with increased organic carbon availability at this depth which varied from 2.88% at 4-6 cm to 4.95% at 2-4 cm (Fig. 42). Labile organic matter also showed a similar distribution like TOC with maximum concentration at the sub-surface (2-4 cm) containing 0.68% LOM (Fig. 43).

Denitrification activity in mangrove sediments was monitored when subjected to a wide range of nitrate amendments (three times higher than ambient). Nitrate addition stimulated denitrification activity in all microcosms. Higher rates were observed especially at depth ≤4 cm. Highest activity of 129.22 (±31.94) µmol N_2O-N m^{-2} h^{-1} at 0-2 cm was observed at nitrate amendment of 40 µmol (Fig. 44). At depths >4 cm, the activity was less pronounced. Up to 46.54 (±4.77) µmol N_2O-N m^{-2} h^{-1} was recorded at a nitrate amendment of 20 µmol NO_3-N l^{-1} at 4-6 cm.

Organic carbon addition stimulated denitrification activity mostly at depths ≥4 cm (Fig. 45). However, maximum activity of only 35.24 (±9.93) µmol N_2O-N m^{-2} h^{-1} was

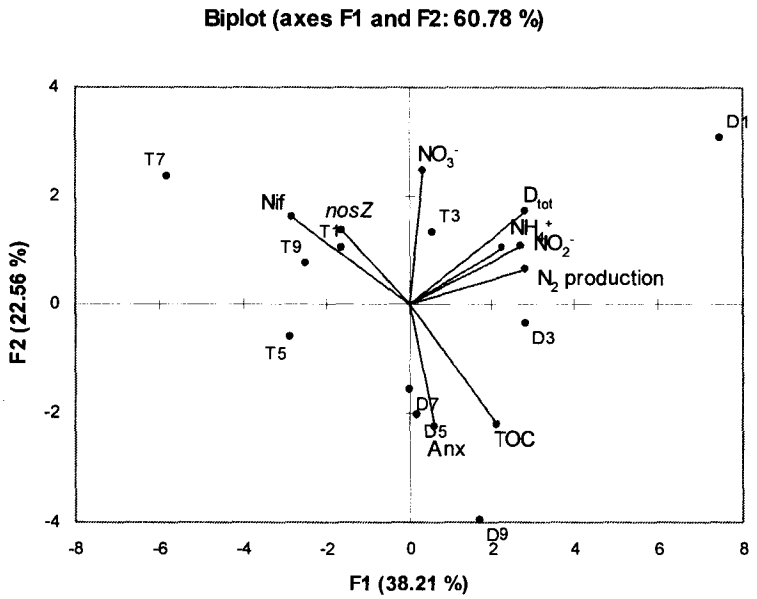


Fig. 40: Principle component analysis (PCA) bi-plot of two sites (Divar (D) and Tuvem (T); sampled at 5 sampling levels- D1/T1=0-2 cm, D3/T3=2-4 cm, D5/T5=4-6 cm, D7/T7=6-8 cm, D9/T9= 8-10 cm) based on the biogeochemical parameters (nitrate, nitrite, ammonium, total organic carbon (TOC) and bacterial activities associated with nitrogen cycle: di-nitrogen fixation (Nif), denitrification (D_{tot}), anammox (Anx). Eigenvalues for PCA are as follows:

	Factor 1	**Factor 2**	**Factor 3**
Eigenvalue	3.439	2.031	1.931
Variability (%)	38.2	22.6	21.5
Cumulative %	38.215	60.777	82.236

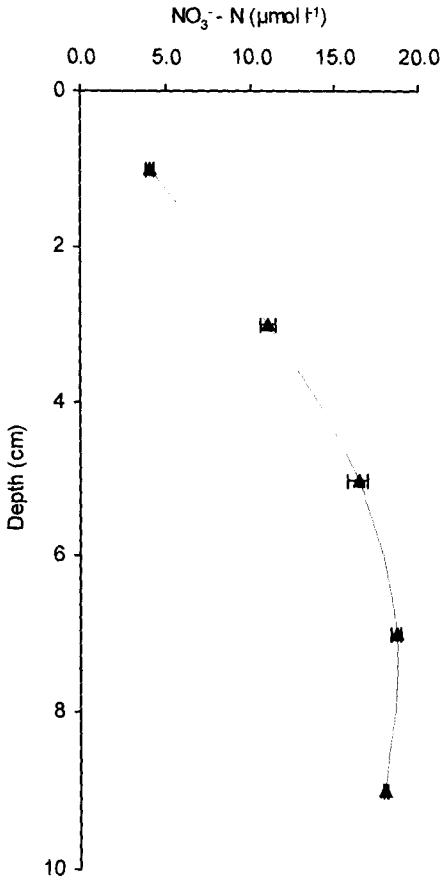


Fig. 41: Down-core variation in pore water nitrate (±SD) at Divar

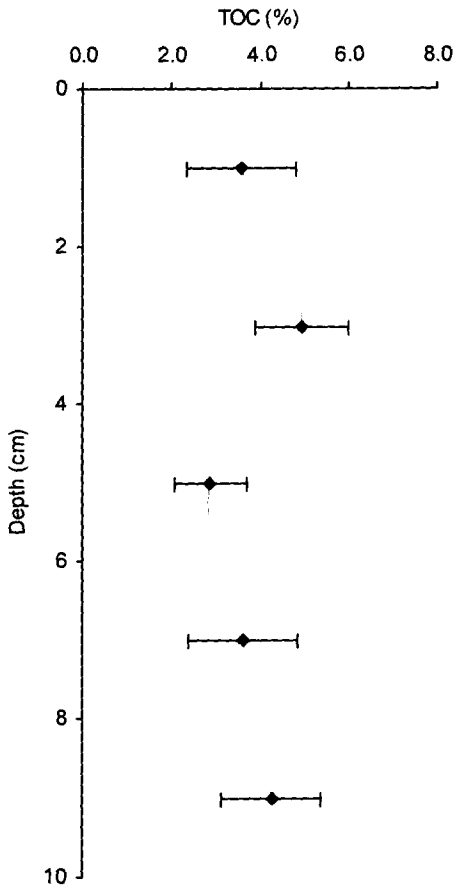


Fig. 42: Down-core variation in total organic carbon (TOC) (±SD) at Divar

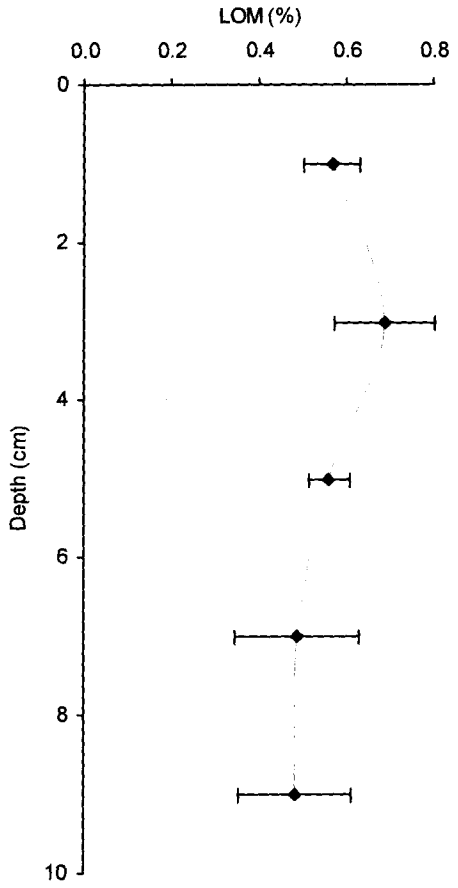


Fig. 43: Down-core variation in labile organic matter (LOM) (±SD) at Divar

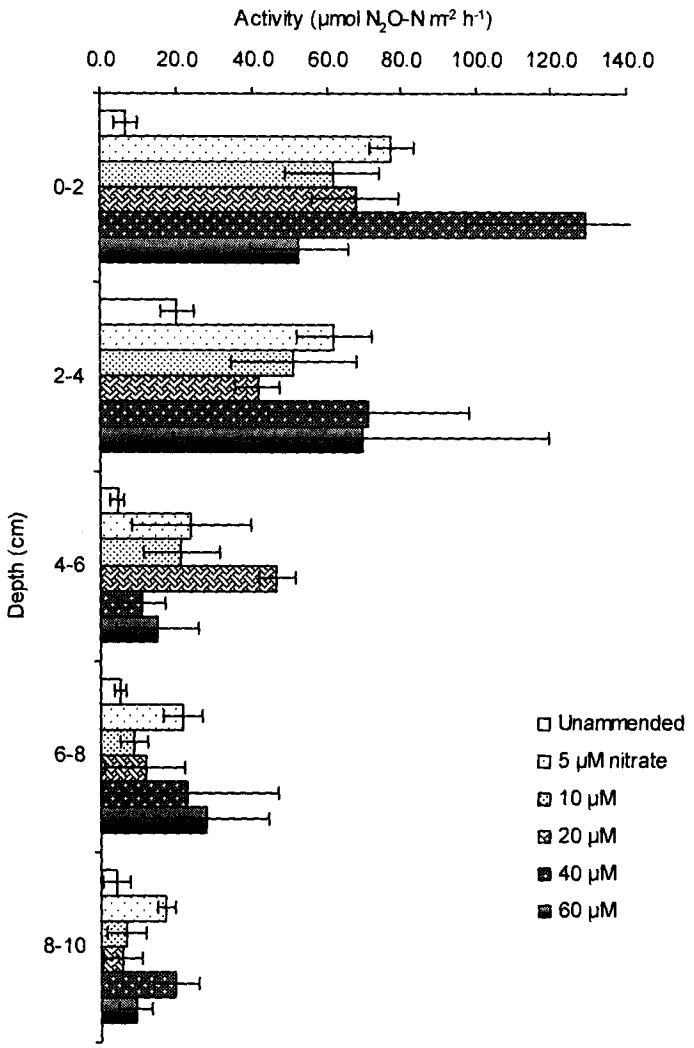


Fig. 44: Effect of nitrate addition on denitrification activity (±SD) in the Divar mangrove sediments.

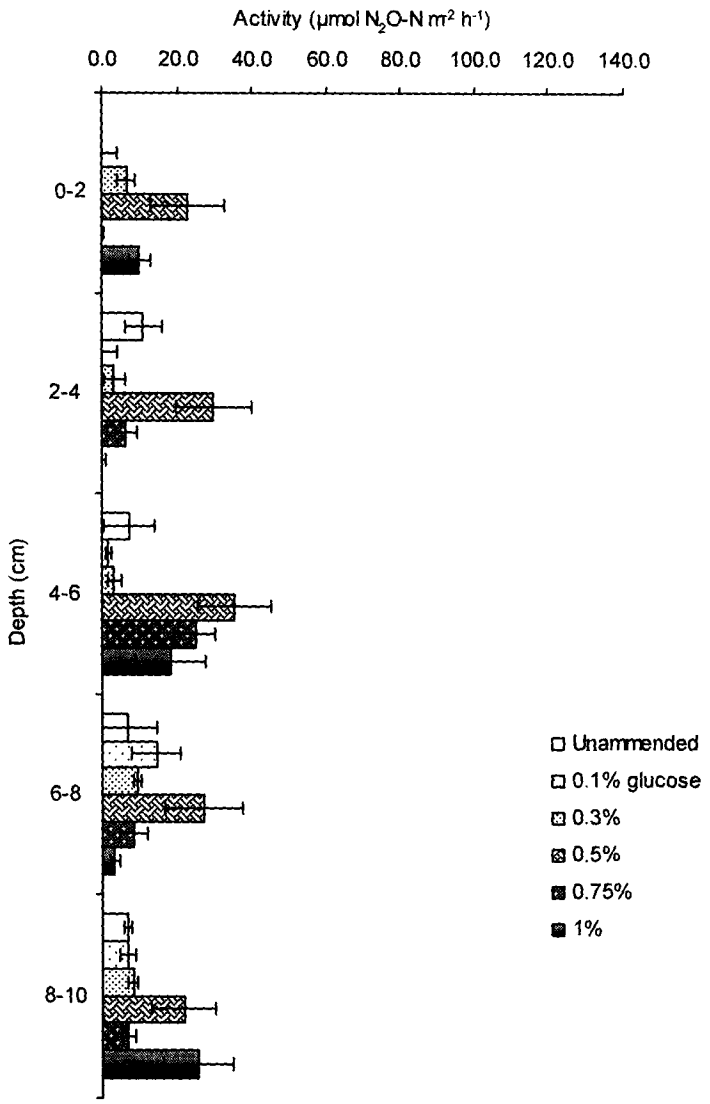


Fig. 45: Effect of varying organic carbon ammendments on denitrification (±SD) in the Divar mangrove sediments

recorded at 4-6 cm depth. A 0.5% amendment of labile organic carbon (glucose) was found to effectively stimulate denitrification activity at all depths. Statistical analyses did not show significant increase in denitrification activity on organic carbon addition as compared to amendments with nitrate which showed significant increase (one way ANOVA; n=15; $p<0.001$) at all depths.

Potential denitrification rates decreased with depth and were 15-38 times higher (within 0-10 cm core) than the ambient when both nitrate and organic carbon were in excess. Highest activity of up to 304.09 (±47.6) μmol N_2O-N m^{-2} h^{-1} (Fig. 46) was recorded at 2-4 cm. At 8-10 cm the activity decreased to 81.25 (±22.58) μmol N_2O-N m^{-2} h^{-1}.

4.9. Influence of bioturbation on denitrification

4.9.1. Physico-chemical characteristics

The pH of the non-bioturbated core varied from 7.19 to 7.5 and decreased with depth (Fig. 47). The pH in bioturbated sediments was relatively lower varying from 6.54 at 4-6 cm to 7.02 at the surface. Though the redox potential in both the cores was similar at the surface (-58.6 mV), the non-bioturbated core was comparatively more reducing in nature than the faunated core at depth >6 cm (Fig. 48). A redox potential of -120.9 mV was recorded at 8-10 cm in the de-faunated core.

Nitrate content in the faunated core was generally higher as compared to the de-faunated core and its concentration was found to increase with depth (Fig. 49) from 9.41(±0.17) μmol at the surface to 22.85(±1.28) μmol at 8-10 cm. In the de-faunated core, surficial nitrate content was 4 times lower than the faunated core with a maximum nitrate concentration of 17.41 (±4.81) μmol at 8-10 cm. Nitrite accumulation was observed in non-bioturbated conditions with up to 15.55 (±3.66) μmol at 2-4 cm (Fig 50). Nitrite concentration in the bioturbated core decreased with depth with a maximum of 4.49 (±0.54) μmol at the surface.

Denitrification activity was generally higher in the top few centimeters of the de-faunated sediment reaching to a maximum of 0.37 (±0.01) μmol N_2O-N m^{-2} h^{-1} at 2-4 cm (Fig. 51). In the bioturbated core, high DNT was observed to occur from 2-6 cm with a similar rate as observed at 2-4 cm in the de-faunated core. Statistical analysis showed that

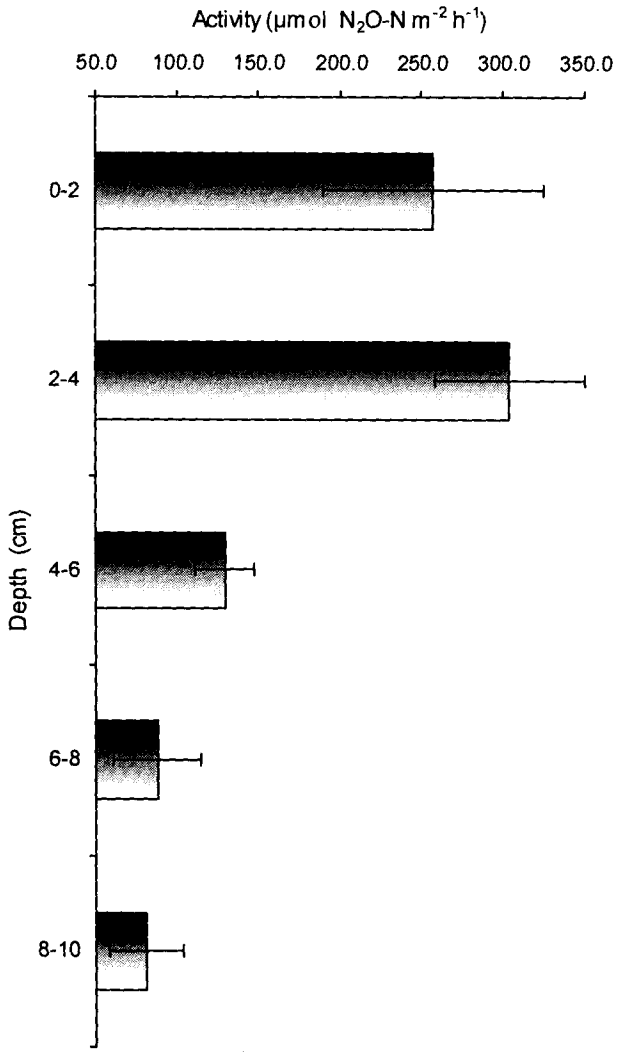


Fig. 46: Down-core variation in potential denitrification activity (±SD) in the Divar mangrove sediments

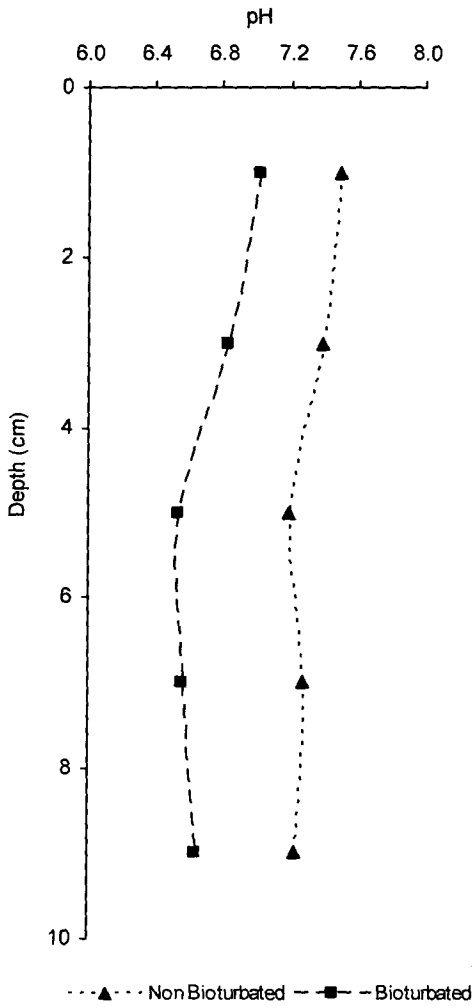


Fig. 47: Down-core variation in sediment pH

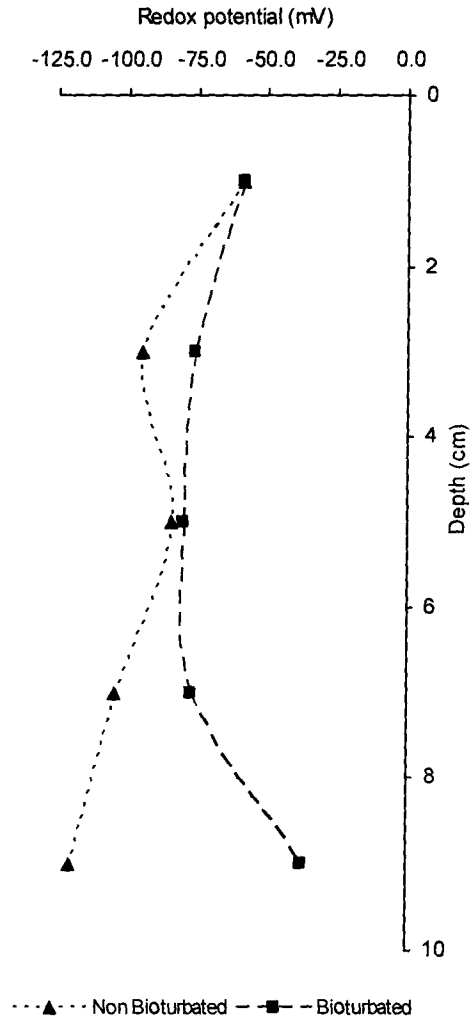


Fig. 48: Down-core variation in sediment redox potential

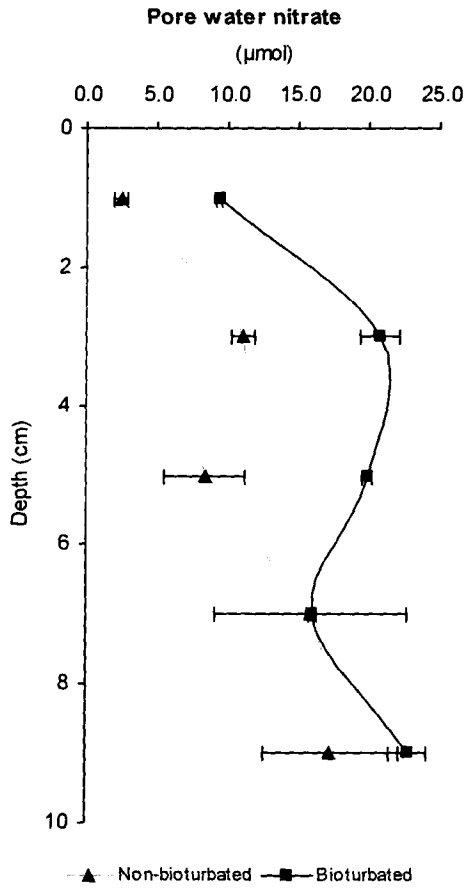


Fig. 49: Down-core variation in pore water nitrate (±SD) at Divar

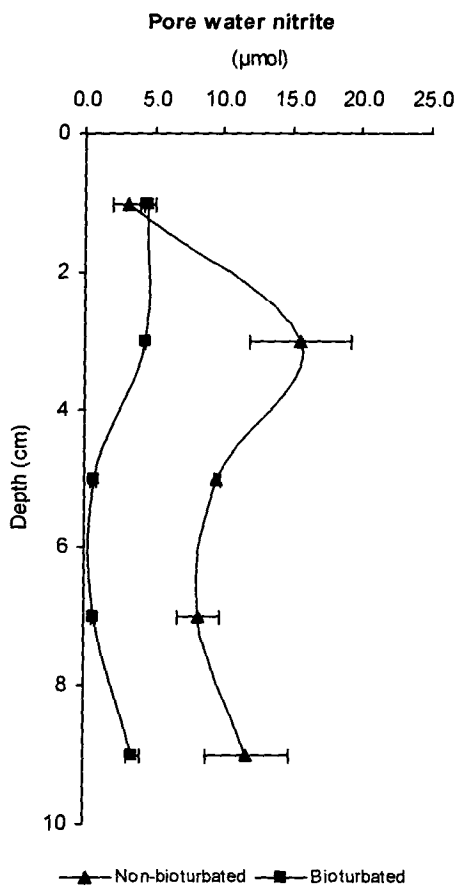


Fig. 50: Down-core variation in pore water nitrite (±SD) at Divar

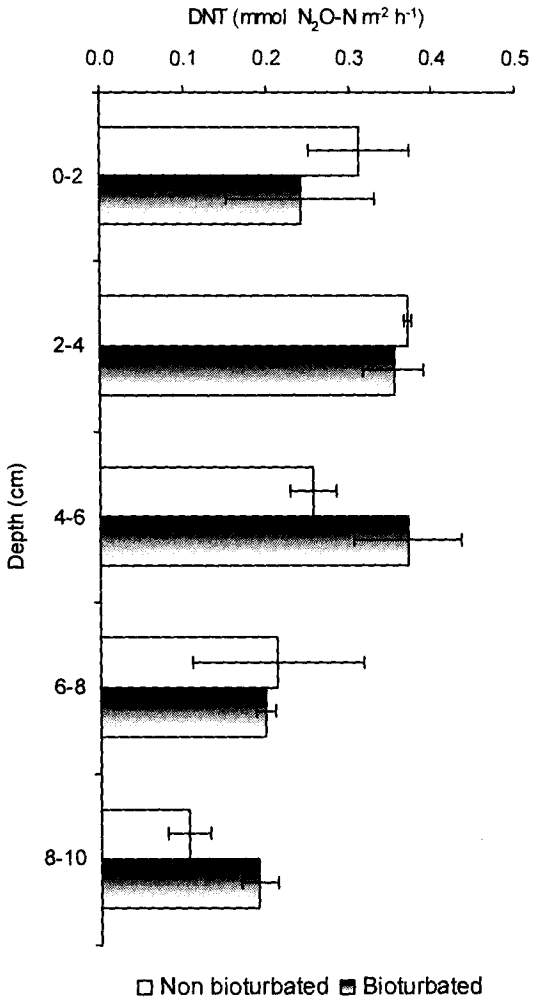


Fig. 51: Down-core variation in denitrification activity (±SD) in non-bioturbated and bioturbated conditions at Divar

bioturbation influenced DNT only at depth >4 cm (ANOVA, $p<0.05$, df=1). However, a linear regression showed that macrofauna accounted for only 18% of the variation in the denitrification activity ($R^2=0.18$).

4.9.2. Biological analyses

In natural sediment, denitrifier abundance ranged between 10^{6-8} MPN cells g^{-1} with maximum numbers at 4-6 cm (Fig. 52) which also exhibited elevated DNT activity at this depth. In de-faunated conditions, their abundance at the surface (0-2 cm) was same as that of the faunated core and decreased rapidly by up to two orders with increasing depth.

Down-core profile of macrofaunal abundance showed that they varied from 375 ind. m^{-2} at the surface to non-detectable levels at 8-10 cm. About 88% of the infauna were present up to a depth of 6 cm beyond which their density declined (Table 13). Polychaetes (*Neries* spp.) and oligochaetes represented the dominant macrofauna in the Divar sediments. The density of polychaetes was highest at the surface (0-2 cm) while the oligochaetes were most abundant at 4-6 cm with a density of 300 ind. m^{-2}. Crabs of *Uca* spp. were retrieved from a depth of 6-8 cm. Some of the macrofaunal forms have been shown in Figs. 53a-c.

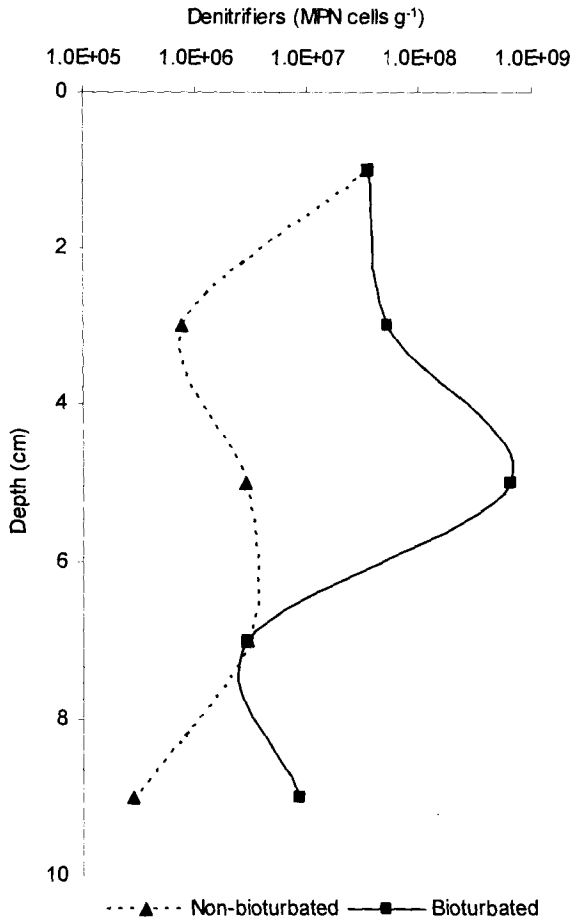


Fig. 52: Down-core variation in denitrifier abundance in non-bioturbated and bioturbated conditions at Divar

Table 13: Down-core variation in macrofaunal abundance at Divar

Depth (cm)	Species	Density (nos. m^{-2})
0-2	*Nereis*	150
	Oligochaeta	150
	Uca spp.	75
2-4	*Nereis*	75
	Jasminera spp.	75
	Nemertina spp.	75
	Uca spp.	75
4-6	Oligochaeta	300
	Polychaeta (Family:Euricidae)	75
6-8	*Nemertina* spp.	75
	Uca spp.	75
8-10		Not detected

Fig. 53a

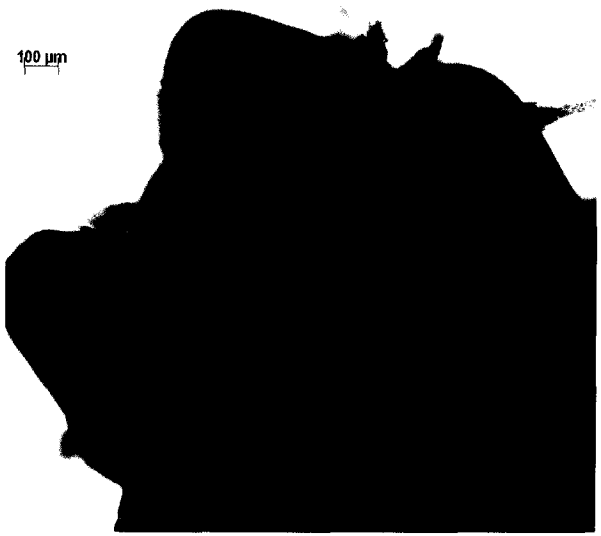


Fig. 53b

Fig. 53c

Figs. 53a-c: Anterior portion of *Jasminera* spp. (a), *Neries* spp. (b) (5X magnification on *Zeiss* Axioskop 2 Plus microscope) and *Uca* spp. (c) from the Divar sediments.

SECTION III – Denitrifier diversity

4.10.1. Taxonomic identification of culturable denitrifiers

At the control site Tuvem, gram negative denitrifiers comprised majority (68%) of the denitrifying bacteria. Of the 50 denitrifier strains isolated, only 32% were gram positive (Table 14a). All the isolates were opaque, had smooth surfaces and were generally 0.5-1 mm in size. All strains tested positive for nitrate reduction and N_2O production while H_2S production was not detected. About 88% of the isolates were catalase positive while only 26% tested positive for oxidase. The cell morphologically varied from short to curved rods. A number of cocci were also recorded. A total of 21 different genera were identified based on physiological and biochemical tests. Culturable denitrifiers isolated from the control site showed close taxonomic affinities to *Acinetobacter*, *Paracoccus* belonging to Alphaproteobacteria, *Bordetella* of Betaproteobacteria, *E. coli*, *Serratia*, *Alteromonas*, *Shigella*, *Pseudomonas*, *Aeromonas*, *Vibrio*, *Halomonas*, *Klebsiella*, *Pantoea*, *Enterogenes*, *Ewingella* all belonging to class Gammaproteobacteria, *Micrococcus* and *Corynebacterium* of Actinobacteria, *Salinicoccus*, *Staphylococcus*, *Bacillus* and *Marinococcus* of class Bacilli (Table 14b).

At the experimental site Divar, colonies of all 76 isolates had smooth surfaces (Table 15a) and were about 0.5-2.5 mm in size. Except for 3 translucent strains, the rest were opaque. N_2O production was detected in all isolates while only 3 isolates tested positive for H_2S production. A total of 19 different genera were identified (Table 15b). Denitrifiers at the experimental site were represented by the genera *Acinetobacter* of class Alphaproteobacteria, *Alcaligenes* of Betaproteobacteria, *Aeromonas*, *Alteromonas*, *Enterobacter*, *Halomonas*, *Kluyvera*, *Proteus*, *Serratia*, *Vibrio*, *Yersinia* of class Gammaproteobacteria, *Bacillus*, *Marinococcus*, *Planococcus*, *Salinococcus*, *Staphylococcus*, *Streptococcus belonging to* class Bacilli *and Micrococcus* belonging to class Actinobacteria. Up to 43% of culturable denitrifiers belonged to Gammaproteobacteria.

Table 14a: Tests for identification of denitrifiers at the control site Tuvem

Depth	ID no.	Size	Colour	Gram character	Cell shape	Catalase	Oxidase	OF test	Motility	N_2O production
0-2	Tuv_1	Medium	Cream	-	Rods	+	+	+	+	+
0-2	Tuv_2	Small	Cream	-	Rods	+	+	+	+	+
0-2	Tuv_3	Small	White	-	Cocci	+	-	-	+	+
0-2	Tuv_4	Medium	White	-	Rods	+	+	+	+	+
0-2	Tuv_5	Small	White	-	Rods	+	+	+	+	+
0-2	Tuv_6	Small	Cream	-	Rods	+	+	+	+	+
0-2	Tuv_7	Medium	Cream	-	Rods	+	-	+	-	+
0-2	Tuv_8	Medium	Cream	+	Cocci	+	-	+	-	+
0-2	Tuv_9	Medium	White	-	Short rods	+	-	-	+	+
0-2	Tuv_10	Small	White	-	Cocci	+	+	-	-	+
0-2	Tuv_11	Small	White	+	Cocci	+	-	+	+	+
0-2	Tuv_12	Medium	Yellow	+	Cocci	+	-	+	-	+
0-2	Tuv_13	Small	Yellow	+	Cocci	+	-	+	+	+
0-2	Tuv_14	Small	Yellow	+	Cocci	+	-	-	-	+
2-4	Tuv_15	Large	Cream	+	Rods	+	-	-	-	+
2-4	Tuv_16	Small	Cream	-	Rods	-	-	+	+	+
2-4	Tuv_17	Small	Cream	+	Rods	+	-	+	-	+
2-4	Tuv_18	Medium	Yellow	-	Short rods	+	-	+	+	+
2-4	Tuv_19	Small	Yellow	-	Curved rod	+	-	+	+	+
2-4	Tuv_20	Large	Orange	-	Rods	+	-	+	+	+
2-4	Tuv_21	Medium	Orange	-	Rods	+	-	+	+	+
2-4	Tuv_22	Medium	Yellow	+	Rods	+	-	+	+	+
2-4	Tuv_23	Small	Yellow	-	Rods	+	+	+	+	+
4-6	Tuv_24	Large	Cream	-	Curved rod	+	-	+	+	+
4-6	Tuv_25	Medium	Cream	-	Cocci	+	+	+	+	+
4-6	Tuv_26	Small	Cream	-	Rods	+	+	-	+	+
4-6	Tuv_27	Large	Cream	-	Rods	+	+	+	+	+
4-6	Tuv_28	Large	Cream	-	Rods	+	-	+	-	+
4-6	Tuv_29	Medium	Cream	-	Short rods	+	+	+	+	+
4-6	Tuv_30	Small	Cream	+	Cocci	+	+	-	-	+

Table 14a contd...

Depth	ID no.	Size	Colour	Gram character	Cell shape	Catalase	Oxidase	OF test	Motility	N_2O production
4-6	Tuv_31	Small	Cream	-	Cocci	+	+	-	+	+
4-6	Tuv_32	Small	Cream	+	Cocci	+	-	-	-	+
8-10	Tuv_33	Medium	White	-	Rods	-	-	+	+	+
8-10	Tuv_34	Small	White	-	Cocci	-	-	+	+	+
8-10	Tuv_35	Small	White	-	Cocci	-	-	+	+	+
8-10	Tuv_36	Small	White	+	Cocci	+	-	+	+	+
8-10	Tuv_37	Small	White	+	Cocci	+	-	+	+	+
8-10	Tuv_38	Medium	Yellow	-	Rods	+	-	-	+	+
8-10	Tuv_39	Small	Yellow	-	Rods	+	-	-	+	+
8-10	Tuv_40	Small	Cream	-	Rods	+	-	+	+	+
8-10	Tuv_41	Small	White	+	Cocci	+	-	+	+	+
8-10	Tuv_42	Small	White	+	Cocci	+	-	+	+	+
8-10	Tuv_43	Small	White	-	Rods	+	-	+	+	+
8-10	Tuv_44	Small	Cream	-	Rods	-	-	+	+	+
8-10	Tuv_45	Small	Cream	-	Rods	-	-	+	+	+
8-10	Tuv_46	Medium	Pink	-	Cocci	+	-	+	+	+
8-10	Tuv_47	Small	Pink	+	Cocci	+	-	+	+	+
8-10	Tuv_48	Small	Pink	+	Cocci	+	-	+	+	+
8-10	Tuv_49	Medium	Yellow	-	Rods	+	-	+	+	+
8-10	Tuv_50	Small	White	-	Curved rod	+	-	+	+	+

Note: Size classification of colonies: 0.5-1 mm= small; 1-1.5 mm= medium and 1.5-2 mm= large.
Surface of all isolates smooth and the colonies were opaque.

Table 14b: Biochemical tests for identification of denitrifiers at the control site Tuvem

ID no.	Citrate utilization	Lysine	Orninthine	Urease	TDA	Glucose	Adonitol	Lactose	Arabinose	Sorbitol	NO_3^- reduction	H_2S production	Genus	Class
Tuv_1	-	+	-	-	-	+	+	+	+	+	+	-	*E. coli*	γ - Proteobacteria
Tuv_2	-	+	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_3	-	+	+	-	-	+	+	+	+	+	+	-	*Alteromonas*	γ - Proteobacteria
Tuv_4	+	+	+	+	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_5	-	+	+	+	-	+	-	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_6	+	+	+	+	-	-	-	-	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_7	-	+	+	-	-	+	+	+	+	+	+	-	*Shigella*	γ - Proteobacteria
Tuv_8	-	+	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_9	+	+	+	+	-	+	-	-	+	+	+	-	*Acinetobacter*	α - Proteobacteria
Tuv_10	+	+	+	+	-	+	+	+	+	+	+	-	*Paracoccus*	α - Proteobacteria
Tuv_11	+	+	+	+	-	+	+	+	+	+	+	-	*Marinococcus*	Bacilli
Tuv_12	+	-	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_13	+	+	+	-	-	+	-	+	+	+	+	-	*Marinococcus*	Bacilli
Tuv_14	+	+	+	-	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
Tuv_15	-	-	-	-	-	-	-	-	-	-	+	-	*Corynebacterium*	Actinobacteria
Tuv_16	+	+	+	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_17	-	-	-	-	-	-	-	-	-	-	+	-	*Corynebacterium*	Actinobacteria
Tuv_18	-	-	-	-	-	+	+	+	+	+	+	-	*Pseudomonas*	γ - Proteobacteria
Tuv_19	-	-	-	-	-	+	+	+	+	+	+	-	*Pseudomonas*	γ - Proteobacteria
Tuv_20	+	+	+	+	-	+	+	-	+	+	+	-	*Aeromonas*	γ - Proteobacteria
Tuv_21	+	+	+	+	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_22	+	-	-	-	-	+	-	-	-	-	+	-	*Bacillus*	Bacilli
Tuv_23	-	+	+	-	-	-	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
Tuv_24	+	+	+	+	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_25	-	-	-	+	-	+	+	+	+	+	+	-	*Halomonas*	γ - Proteobacteria
Tuv_26	-	-	-	-	-	-	+	+	+	+	+	-	*Acinetobacter*	α - Proteobacteria
Tuv_27	-	-	-	-	-	+	+	+	+	+	+	-	*Enterogenes*	γ - Proteobacteria
Tuv_28	-	-	-	-	-	+	+	+	+	+	+	-	*Klebsiella*	γ - Proteobacteria
Tuv_29	-	-	-	+	-	+	+	+	+	+	+	-	*Halomonas*	γ - Proteobacteria
Tuv_30	-	+	-	+	-	+	+	+	-	+	+	-	*Salinicoccus*	Bacilli

Table 14b contd...

ID no.	Citrate utilization	Lysine	Ornithine	Urease	TDA	Glucose	Adonitol	Lactose	Arabinose	Sorbitol	NO_3^- reduction	H_2S production	Genus	Class
Tuv_31	-	-	-	+	-	+	+	+	+	+	+	-	*Bordetella*	β- Proteobacteria
Tuv_32	-	+	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_33	+	-	-	-	-	+	-	-	+	-	+	-	*Pantoea*	γ - Proteobacteria
Tuv_34	+	-	-	-	-	+	-	-	+	-	+	-	*Pantoea*	γ - Proteobacteria
Tuv_35	+	-	-	-	-	+	-	-	+	-	+	-	*Pantoea*	γ - Proteobacteria
Tuv_36	-	+	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_37	-	+	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_38	-	-	-	-	-	+	+	+	+	+	+	-	*Bordetella*	β- Proteobacteria
Tuv_39	-	-	-	-	-	+	+	+	+	+	+	-	*Bordetella*	β- Proteobacteria
Tuv_40	-	-	-	-	-	-	+	+	+	+	-	-	*Acinetobacter*	α - Proteobacteria
Tuv_41	-	+	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_42	-	+	-	+	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_43	-	-	-	+	-	+	+	+	+	+	+	-	*E. coli*	γ - Proteobacteria
Tuv_44	+	+	-	-	-	+	-	+	-	-	+	-	*Ewingella*	γ - Proteobacteria
Tuv_45	+	+	-	-	-	+	-	+	-	-	+	-	*Ewingella*	γ - Proteobacteria
Tuv_46	+	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_47	+	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_48	-	-	-	-	-	+	+	-	+	+	+	-	*Staphylococcus*	Bacilli
Tuv_49	+	+	+	+	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
Tuv_50	-	+	+	-	-	+	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria

Table 15a contd..

ID no.	Depth	Size	Colour	Gram character	Cell shape	Oxidase	Catalase	OF test	Motility	N_2O production
DIV_35	4-6	Small	Cream	-	Short rods	+	+	+	+	+
DIV_36	4-6	Small	Cream	-	Rods	+	-	+	+	+
DIV_37	4-6	Small	Cream	-	Cocci	-	+	+	+	+
DIV_38	4-6	Small	Cream	-	Cocci	+	+	+	+	+
DIV_39	4-6	Small	White	-	Rods	+	+	+	+	+
DIV_40	4-6	Small	White	-	Rods	+	+	+	+	+
DIV_41	4-6	Small	White	-	Rods	-	+	+	+	+
DIV_42	4-6	Small	White	-	Rods	-	+	+	+	+
DIV_43	4-6	Small	White	+	Cocci	-	+	+	+	+
DIV_44	4-6	Medium	White	-	Rods	+	+	-	+	+
DIV_45	4-6	Small	White	-	Rods	+	+	-	+	+
DIV_46	4-6	Small	White	-	Cocci	+	+	+	+	+
DIV_47	4-6	Small	White	+	Cocci	+	+	+	+	+
DIV_48	4-6	Small	Cream	+	Cocci	+	+	+	+	+
DIV_49	4-6	Small	Cream	+	Cocci	+	+	+	+	+
DIV_50	4-6	Small	Yellow	+	Cocci	+	+	+	+	+
DIV_51	4-6	Small	Cream	+	Cocci	+	+	+	+	+
DIV_52	4-6	Small	Cream	-	Cocci	+	+	+	+	+
DIV_53	6-8	Large	Yellow	-	Curved rods	+	+	+	+	+
DIV_54	6-8	Small	Cream	-	Cocci	-	+	+	+	+
DIV_55	6-8	Small	Cream	-	Cocci	+	+	+	+	+
DIV_56	6-8	Small	Cream	-	Rods	-	+	+	+	+
DIV_57	6-8	Medium	Cream	-	Rods	+	+	+	+	+
DIV_58	6-8	Small	Yellow	-	Rods	+	+	+	+	+
DIV_59	6-8	Small	Yellow	-	Rods	+	+	+	+	+
DIV_60	6-8	Small	Cream	+	Cocci	-	+	+	+	+
DIV_61	6-8	Small	White	+	Cocci	-	+	-	-	+
DIV_62	6-8	Small	White	+	Cocci	-	-	-	-	+
DIV_63	8-10	Small	Cream	-	Cocci	+	+	+	+	+
DIV_64	8-10	Medium	Cream	-	Rods	+	+	+	+	+
DIV_65	8-10	Small	Cream	-	Cocci	-	-	-	-	+
DIV_66	8-10	Small	Cream	-	Cocci	-	-	+	+	+
DIV_67	8-10	Small	Cream	+	Cocci	+	+	+	-	+
DIV_68	8-10	Small	White	+	Cocci	-	+	+	+	+

Table 15a contd..

ID no.	Depth	Size	Colour	Gram character	Cell shape	Oxidase	Catalase	OF test	Motility	N_2O production
DIV_69	8-10	Small	White	+	Cocci	-	+	+	+	+
DIV_70	8-10	Small	White	+	Cocci	-	+	+	+	+
DIV_71	8-10	Small	White	+	Cocci	-	+	+	+	+
DIV_72	8-10	Small	White	+	Cocci	-	+	+	+	+
DIV_73	8-10	Small	White	+	Cocci	-	+	+	+	+
DIV_74	8-10	Medium	White	-	Cocci	-	+	-	+	+
DIV_75	8-10	Small	Yellow	-	Cocci	-	+	+	+	+
DIV_76	8-10	Small	Yellow	-	Rods	-	+	+	+	+

Note: Size classification of colonies: 0.5-1 mm= small; 1-1.5 mm= medium and 1.5-2 mm= large.
Surface of all isolates were smooth. Except for isolate numbers DIV_10, 11 and 12 all the rest were opaque.

Table 15b: Biochemical tests for identification of denitrifiers at the experimental site Divar

ID no.	Citrate utilization	Lysine	Ornithine	Urease	TDA	Glucose	Adonitol	Lactose	Arabinose	Sorbitol	NO_3^- reduction	H_2S production	Genus	Class
DIV_1	+	-	-	-	-	+	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_2	+	+	+	V	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_3	V	V	-	-	-	+	-	V	+	-	+	+	*Aeromonas*	γ - Proteobacteria
DIV_4	V	+	V	-	V	+	-	V	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_5	+	+	+	V	-	+	V	-	-	+	+	-	*Serratia*	γ - Proteobacteria
DIV_6	+	+	+	-	-	+	+	+	+	+	+	-	*Enterobacter*	γ - Proteobacteria
DIV_7	+	+	+	-	-	+	-	+	+	+	+	-	*Marinococcus*	Bacilli
DIV_8	+	+	+	V	-	+	V	-	-	+	+	-	*Serratia*	γ - Proteobacteria
DIV_9	+	-	-	-	-	+	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_10	-	+	+	-	-	+	+	+	+	+	+	-	*Marinococcus*	Bacilli
DIV_11	+	+	+	+	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
DIV_12	+	+	+	-	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
DIV_13	-	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_14	-	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_15	-	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_16	+	+	+	-	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
DIV_17	+	+	+	-	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
DIV_18	+	-	-	-	-	+	-	-	-	-	+	-	*Bacillus*	Bacilli
DIV_19	+	-	+	+	+	+	-	-	-	-	+	-	*Proteus*	γ - Proteobacteria
DIV_20	+	+	+	-	V	+	-	-	-	-	+	-	*Aeromonas*	γ - Proteobacteria
DIV_21	+	+	+	-	-	+	-	V	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_22	-	+	-	-	ND	V	V	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_23	-	+	V	-	-	+	-	-	+	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_24	-	+	+	-	-	-	-	-	-	-	+	-	*Planococcus*	Bacilli
DIV_25	+	-	-	-	-	+	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_26	+	+	+	+	+	+	+	+	+	+	+	+	*Serratia*	γ - Proteobacteria
DIV_27	+	+	+	-	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
DIV_28	+	+	+	+	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_29	+	+	+	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_30	+	+	+	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_31	+	+	+	-	-	+	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_32	+	+	+	V	-	+	V	-	-	+	+	-	*Serratia*	γ - Proteobacteria
DIV_33	-	+	V	-	-	+	-	-	-	-	+	+	*Vibrio*	γ - Proteobacteria
DIV_34	-	+	+	-	-	-	-	-	-	-	+	-	*Aeromonas*	γ - Proteobacteria

Table 15b contd..

ID no.	Citrate utilization	Lysine	Ornithine	Urease	TDA	Glucose	Adonitol	Lactose	Arabinose	Sorbitol	NO_3^- reduction	H_2S production	Genus	Class
DIV_35	-	+	+	-	-	-	-	-	-	-	+	-	*Aeromonas*	γ - Proteobacteria
DIV_36	+	+	+	-	-	+	-	+	+	V	+	-	*Kluyvera*	γ - Proteobacteria
DIV_37	+	+	+	-	-	+	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_38	-	-	-	-	-	+	+	+	+	+	+	-	*Halomonas*	γ - Proteobacteria
DIV_39	-	-	+	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_40	-	+	-	-	-	+	+	-	+	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_41	+	-	-	+	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_42	-	-	-	-	-	+	-	-	+	-	+	-	*Aeromonas*	γ - Proteobacteria
DIV_43	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_44	-	-	-	+	-	-	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_45	+	+	+	-	-	+	+	+	+	+	+	-	*Vibrio*	γ - Proteobacteria
DIV_46	+	+	+	+	-	-	-	-	-	-	+	-	*Halomonas*	γ - Proteobacteria
DIV_47	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_48	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_49	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_50	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_51	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_52	+	-	+	+	-	+	+	+	-	+	+	-	*Halomonas*	γ - Proteobacteria
DIV_53	-	+	-	-	ND	V	-	-	-	-	+	-	*Vibrio*	γ - Proteobacteria
DIV_54	+	-	+	+	-	-	+	+	-	+	+	-	*Acinetobacter*	α - Proteobacteria
DIV_55	+	-	-		-	+	-	+	+	-	+	-	*Alcaligenes*	β - Proteobacteria
DIV_56	-	+	-	-	-	+	+	+	+	+	+	-	*Aeromonas*	γ - Proteobacteria
DIV_57	-	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_58	-	-	-	-	-	-	-	-	-	-	-	-	*Vibrio*	γ - Proteobacteria
DIV_59	+	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria
DIV_60	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_61	+	+	+	-	-	+	+	+	+	+	+	-	*Micrococcus*	Actinobacteria
DIV_62	+	+	+	-	ND	+	+	+	+	+	+	-	*Streptococcus*	Bacilli
DIV_63	+	-	-	ND	-	+	-	+	+	-	+	-	*Alcaligenes*	β - Proteobacteria
DIV_64	+	+	+	-	-	-	-	+	-	-	+	-	*Aeromonas*	γ - Proteobacteria
DIV_65	-	+	-	-	-	+	-	-	-	-	+	-	*Acinetobacter Moraxella grp I*	α - Proteobacteria
DIV_66	-	+	+	-	-	-	-	-	-	-	+	-	*Alteromonas*	γ - Proteobacteria
DIV_67	-	-	-	-	-	+	+	+	+	+	+	-	*Salinicoccus*	Bacilli

Table 15b contd..

ID no.	Citrate utilization	Lysine	Ornithine	Urease	TDA	Glucose	Adonitol	Lactose	Arabinose	Sorbitol	NO_3^- reduction	H_2S production	Genus	Class
DIV_68	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_69	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_70	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_71	-	-	-	-	-	+	+	+	+	+	+	-	*Staphylococcus*	Bacilli
DIV_72	-	+	-	-	-	+	-	-	-	-	+	-	*Staphylococcus*	Bacilli
DIV_73	-	+	+	-	-	+	-	-	-	+	+	-	*Staphylococcus*	Bacilli
DIV_74	-	+	-	-	-	+	+	+	+	+	+	-	*Alteromonas*	γ - Proteobacteria
DIV_75	-	-	+	-	-	+		+	+	+	+	-	*Yersinia*	γ - Proteobacteria
DIV_76	-	-	-	-	-	+	+	+	+	+	+	-	*Serratia*	γ - Proteobacteria

Note: V= variable; ND= No data

4.10.2. Functional diversity of denitrifiers

The main component of the *nosZ* community was analysed by DGGE. Clear difference in DGGE banding pattern was visible between sediment retrieved from Divar and Tuvem (Fig. 54). Partial *nosZ* gene sequences for nitrous oxide reductase from the present study belonged to uncultured marine bacteria. Although some of the bands have been observed at both the sites, a prominent band BT1 was observed at Tuvem at all depths investigated. This band was also found to be in the Divar sediment mainly within 0-6 cm. Its sequence fell within the Gammaproteobacteria *nosZ* cluster and showed 96.5% similarity with *Shewanella loihica nosZ* (Fig. 55). Three more major bands were observed at Divar, BD1, BD2 and BD3. Band BD1 was more prominent until 6 cm depth while the other two bands BD2 and BD3 were observed at all depths. BD2 and BD3 could be seen at Tuvem. From comparison with *nosZ* of cultured strains, BD2 was clustered within the Betaproteobacterial *nosZ* and showed 62% similarity to *Ralstonia eutropha nosZ*. BD3 and BD1 were grouped within Alphaproteobacterial *nosZ* cluster and showed 77% and 74% similarity to *Silicibacter pomeroyi nosZ* and *Azospirillum brasilense nosZ* respectively.

4.10.3. Bacterial diversity in mangrove sediments

More than 23,000 bacterial V6 amplicons were sequenced from the two sites-Tuvem and Divar. The average read length of the sequences were 60.73 (±2.63) bp. Taxonomic analysis revealed differences in community composition between the two sites. Among the 32 phyla identified, phylum Proteobacteria was the most dominant contributing ≈46% of the total V6 tags. Up to 22 and 18% of the bacterial community at Tuvem and Divar respectively remained unidentified at a class level. Deltaproteobacteria was the next most dominant class in the Tuvem sediments forming 21% of the total tags. At Divar the Gammaproteobacteria were the dominant forms at 18% followed by Deltaproteobacteria and Actinobacteria at 15%. The order Desulfobacterales dominated (Fig. 56a) the Tuvem sediments at 10% whereas at Divar they formed only 8% (Fig. 56b). Percentage wise Phylum_Class_Order distribution for taxonomically assigned tags that occurred ≤100 times (minor orders) has been tabulated in Tables (16a-b).

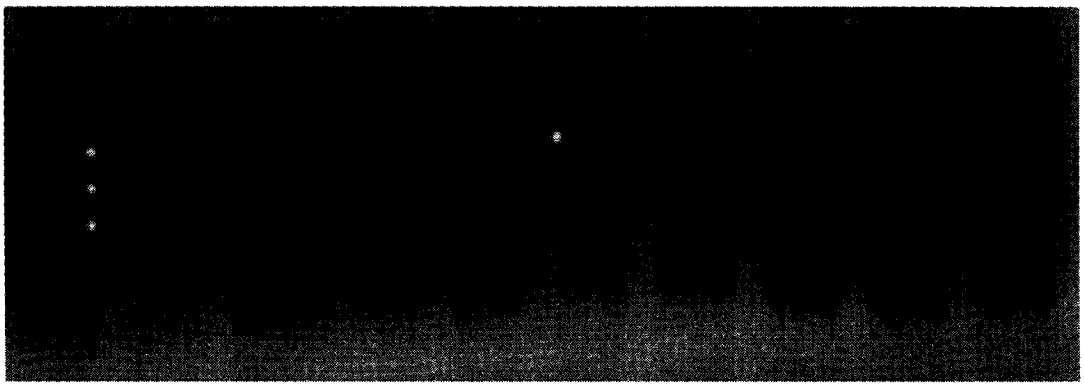


Fig. 54: *nosZ* DGGE profiles for mangrove sediments. Lanes 1-5 represent samples retrieved at every 2 cm interval within the 0-10 cm depth range at Divar while lane 6-10 represent samples from Tuvem. a= band BD1, b=BD2; c=BD3; d=BT1.

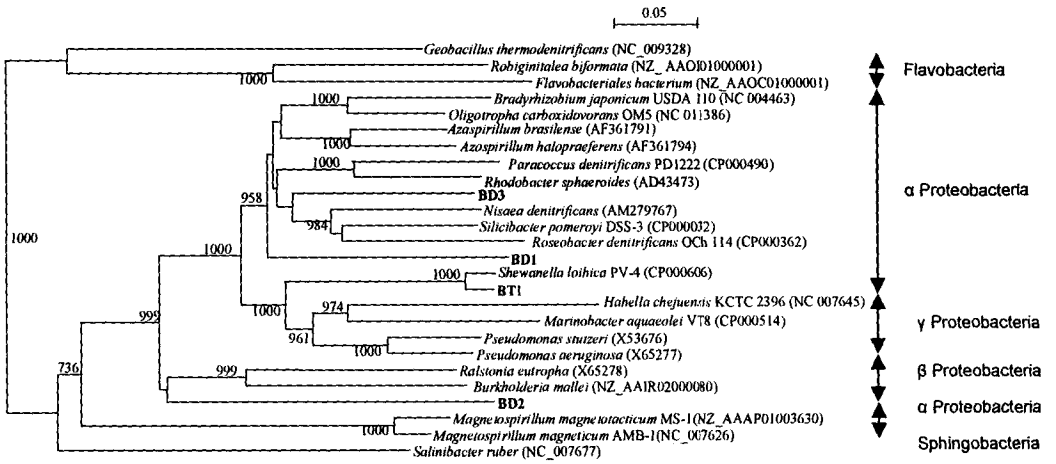


Fig. 55: Neighbor-joining phylogenetic tree incorporating *nosZ* genes from BD1, BD2, BD3 & BT1. Bootstrap analyses (1000 replications) were conducted and values greater than 700 are indicated in the nodes. Bar, 0.05 change per nucleotide position.

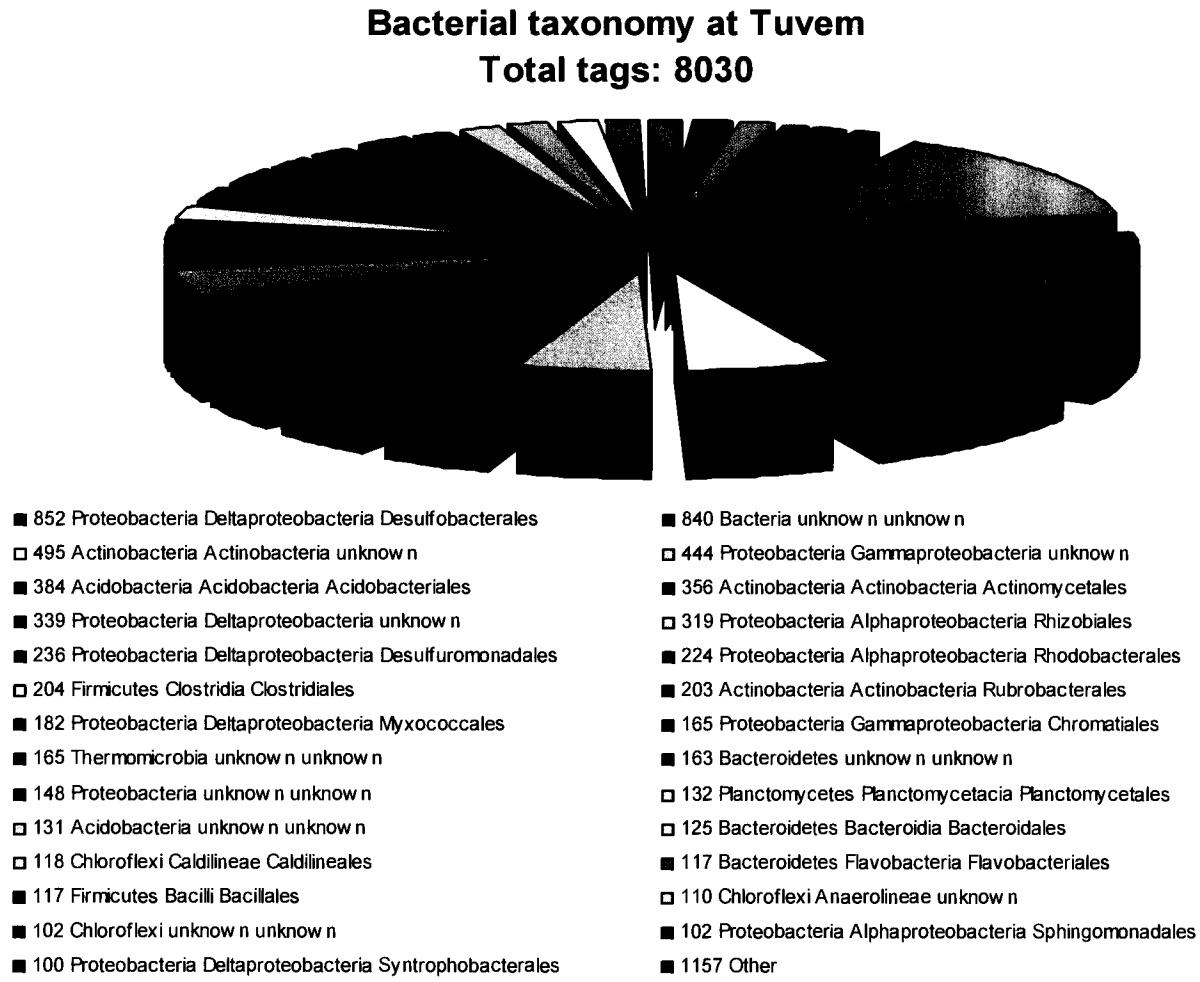


Fig. 56a: Taxonomic breakdown of bacterial V6 tags at Tuvem. Pie charts show the Phylum_Class_Order distribution for taxonomically assigned tags that occurred more than 100 times; the remaining tag sequences are grouped into "Other." Numbers denote total tags recorded.

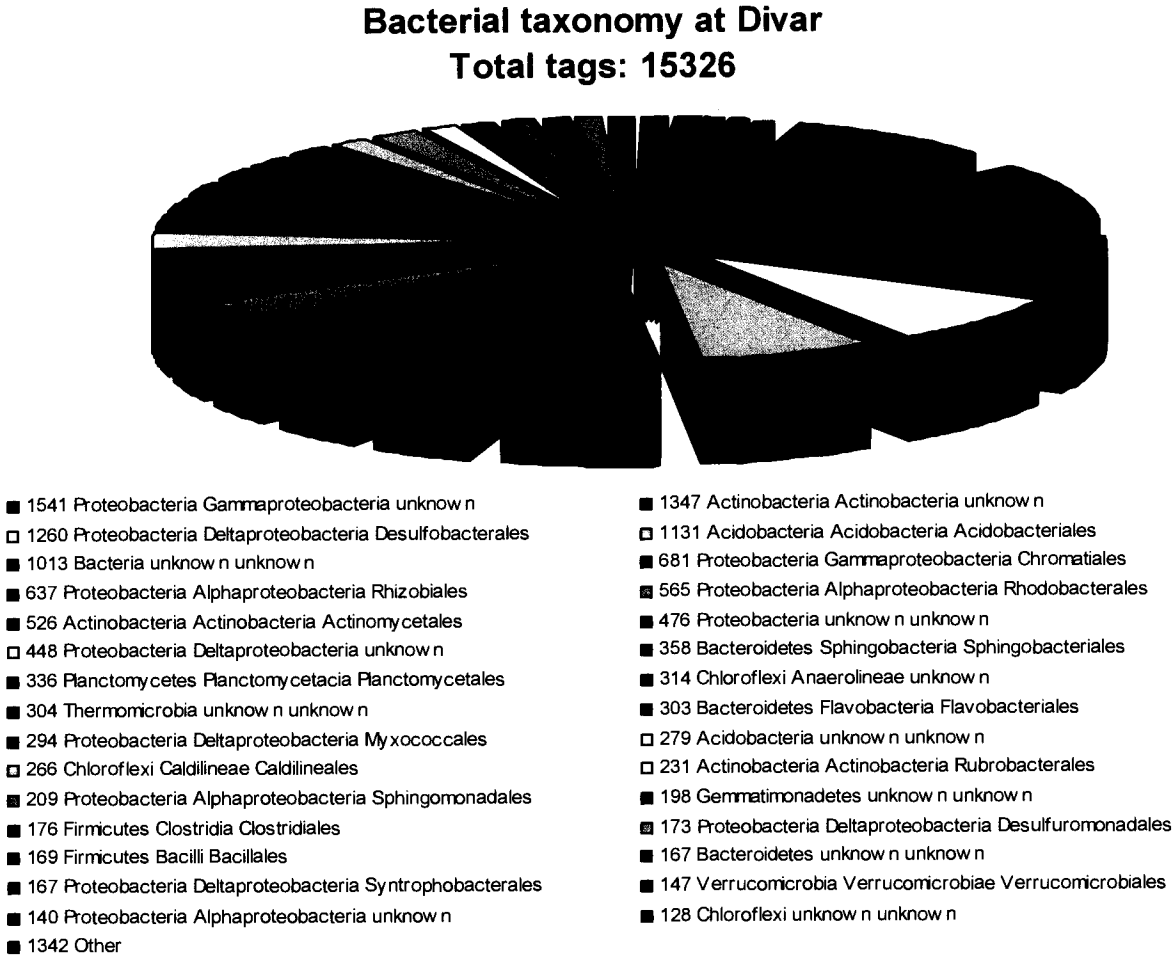


Fig. 56b: Taxonomic breakdown of bacterial V6 tags at Divar. Pie charts show the Phylum_Class_Order distribution for taxonomically assigned tags that occurred more than 100 times; the remaining tag sequences are grouped into "Other." Numbers denote total tags recorded.

Table 16a: Taxonomic breakdown of bacterial V6 tags at Tuvem showing the Phylum_Class_Order percentage wise distribution (100%= 8030) for taxonomically assigned tags that occurred ≤100 times.

Phylum_Class_Order	Tag occurrence (%)
Deferribacteres Deferribacteres Deferribacterales	1.00
Verrucomicrobia Verrucomicrobiae Verrucomicrobiales	0.98
Bacteroidetes Sphingobacteria Sphingobacteriales	0.90
Chloroflexi Anaerolineae Anaerolineales	0.83
Proteobacteria Alphaproteobacteria unknown	0.78
Gemmatimonadetes unknown unknown	0.76
Proteobacteria Gammaproteobacteria Alteromonadales	0.75
Proteobacteria Betaproteobacteria Burkholderiales	0.61
Actinobacteria Actinobacteria Acidimicrobiales	0.61
Proteobacteria Betaproteobacteria unknown	0.56
Chlorobi unknown unknown	0.54
WS3 unknown unknown	0.45
Proteobacteria Epsilonproteobacteria Campylobacterales	0.44
Nitrospira Nitrospira Nitrospirales	0.37
Proteobacteria Alphaproteobacteria Rhodospirillales	0.35
Proteobacteria Gammaproteobacteria Oceanospirillales	0.31
Actinobacteria Actinobacteria Coriobacteriales	0.27
Proteobacteria Gammaproteobacteria Legionellales	0.26
Gemmatimonadetes Gemmatimonadetes Gemmatimonadales	0.24
Planctomycetes unknown unknown	0.24
Spirochaetes Spirochaetes Spirochaetales	0.24
Actinobacteria Actinobacteria Rubrobacterales	0.22
Proteobacteria Gammaproteobacteria Methylococcales	0.20
Chlamydiae Chlamydiae Chlamydiales	0.19
Verrucomicrobia Spartobacteria Chthoniobacterales	0.17
Proteobacteria Betaproteobacteria Rhodocyclales	0.14
Proteobacteria Gammaproteobacteria Pseudomonadales	0.14
OD1 unknown unknown	0.12
Chloroflexi Anaerolineae Caldilineaceae	0.11
Cyanobacteria True Cyanobacteria Unassigned	0.11
Cyanobacteria True Cyanobacteria unknown	0.10
Lentisphaerae unknown unknown	0.10
OP8 unknown unknown	0.10
Proteobacteria Gammaproteobacteria Enterobacteriales	<0.1
Nitrospira unknown unknown	<0.1
Lentisphaerae Lentisphaeria Victivallales	<0.1
Proteobacteria Betaproteobacteria Hydrogenophilales	<0.1
Proteobacteria Gammaproteobacteria Xanthomonadales	<0.1
Fusobacteria Fusobacteria Fusobacteriales	<0.1
Proteobacteria Gammaproteobacteria Thiotrichales	<0.1
Spirochaetes unknown unknown	<0.1
Acidobacteria TM1 unknown	<0.1
Chloroflexi Dehalococcoidetes unknown	<0.1
Cyanobacteria unknown unknown	<0.1

Table 16a contd...

Phylum_Class_Order	Tag occurrence (%)
OP11 unknown unknown	<0.1
Proteobacteria Betaproteobacteria Neisseriales	<0.1
TM7 unknown unknown	<0.1
Unassigned Ktedonobacteria unknown	<0.1
Verrucomicrobia Opitutae Opitutales	<0.1
BRC1 unknown unknown	<0.1
Firmicutes Bacilli Lactobacillales	<0.1
Firmicutes unknown unknown	<0.1
OP3 unknown unknown	<0.1
Proteobacteria Betaproteobacteria Nitrosomonadales	<0.1
Proteobacteria Deltaproteobacteria Desulfarculales	<0.1
Acidobacteria Acidobacteria unknown	<0.1
Actinobacteria Actinobacteria Bifidobacteriales	<0.1
Deinococcus-Thermus Deinococci Deinococcales	<0.1
Fibrobacteres unknown unknown	<0.1
Firmicutes Bacilli unknown	<0.1
Firmicutes Erysipelotrichi Erysipelotrichales	<0.1
OP10 unknown unknown	<0.1
Proteobacteria Deltaproteobacteria Bdellovibrionales	<0.1
Proteobacteria Gammaproteobacteria Pasteurellales	<0.1
Proteobacteria Gammaproteobacteria Vibrionales	<0.1

Table 16b: Taxonomic breakdown of bacterial V6 tags at Divar showing the Phylum_Class_Order percentage wise distribution (100%= 15326) for taxonomically assigned tags that occurred ≤100 times.

Phylum_Class_Order	Tag occurrence (%)
WS3 unknown unknown	0.59
Proteobacteria Gammaproteobacteria Oceanospirillales	0.57
Deferribacteres Deferribacteres Deferribacterales	0.54
Proteobacteria Gammaproteobacteria Alteromonadales	0.51
Chloroflexi Anaerolineae Anaerolineales	0.49
Bacteroidetes Bacteroidia Bacteroidales	0.45
Actinobacteria Actinobacteria Acidimicrobiales	0.40
Chlamydiae Chlamydiae Chlamydiales	0.40
Lentisphaerae unknown unknown	0.39
Proteobacteria Gammaproteobacteria Methylococcales	0.38
Proteobacteria Epsilonproteobacteria Campylobacterales	0.37
Nitrospira Nitrospira Nitrospirales	0.29
Proteobacteria Betaproteobacteria Burkholderiales	0.27
Chlorobi unknown unknown	0.26
OD1 unknown unknown	0.25
Proteobacteria Betaproteobacteria unknown	0.25
Gemmatimonadetes Gemmatimonadetes Gemmatimonadales	0.23
Proteobacteria Gammaproteobacteria Legionellales	0.20
Spirochaetes Spirochaetes Spirochaetales	0.20
Cyanobacteria True Cyanobacteria unknown	0.16
Proteobacteria Gammaproteobacteria Enterobacteriales	0.14
Actinobacteria Actinobacteria Coriobacteriales	0.11
Proteobacteria Gammaproteobacteria Vibrionales	0.11
OP11 unknown unknown	<0.1
Chloroflexi Anaerolineae Caldilineaceae	<0.1
Cyanobacteria True Cyanobacteria Unassigned	<0.1
Proteobacteria Gammaproteobacteria Thiotrichales	<0.1
Planctomycetes unknown unknown	<0.1
Proteobacteria Alphaproteobacteria Rhodospirillales	<0.1
Cyanobacteria unknown unknown	<0.1
Fusobacteria Fusobacteria Fusobacteriales	<0.1
Proteobacteria Gammaproteobacteria Pseudomonadales	<0.1
Verrucomicrobia Spartobacteria Chthoniobacterales	<0.1
Firmicutes unknown unknown	<0.1
Proteobacteria Deltaproteobacteria Bdellovibrionales	<0.1
TM7 unknown unknown	<0.1
Verrucomicrobia Opitutae Opitutales	<0.1
Acidobacteria Acidobacteria unknown	<0.1
Cyanobacteria True Cyanobacteria Pleurocapsales	<0.1
Firmicutes Bacilli Lactobacillales	<0.1
OP8 unknown unknown	<0.1
Proteobacteria Betaproteobacteria Neisseriales	<0.1
Proteobacteria Betaproteobacteria Rhodocyclales	<0.1
Proteobacteria Deltaproteobacteria Desulfovibrionales	<0.1

Phylum_Class_Order	Tag occurrence (%)
Actinobacteria Actinobacteria Bifidobacteriales	<0.1
Lentisphaerae Lentisphaeria Victivallales	<0.1
Proteobacteria Gammaproteobacteria Pasteurellales	<0.1
Proteobacteria Gammaproteobacteria Salinisphaerales	<0.1
Cyanobacteria True Cyanobacteria Chroococcales	<0.1
Deinococcus-Thermus Deinococci Deinococcales	<0.1
Lentisphaerae Lentisphaeria unknown	<0.1
Proteobacteria Alphaproteobacteria Caulobacterales	<0.1
Thermomicrobia Thermomicrobia unknown	<0.1
Actinobacteria Actinobacteria Rubrobacterales	<0.1
Nitrospira unknown unknown	<0.1

Using the furthest neighbor assignment algorithm implemented in DOTUR, more than 2600 unique V6 tag sequences were recorded forming 1561 phylotypes at a distance of 3% (Table 17) at the control site Tuvem. Non-parametric statistical analysis estimates predicted the presence of 2166 phylotypes at Tuvem. At Divar, 24% of the total trimmed sequences were unique with 2198 operational taxonomic units (OTUs) while the predicted phylotypes were estimated to be ~3300. Similarly, species richness estimated by the Chao1 estimator was higher at Divar as compared to Tuvem. Phylogenetic classification of twenty most abundant clusters (≥97% similarity) at both the locations has been tabulated in (Table 18a-b). Rarefaction curves generated did not reach a curvilinear phase (Fig. 57).

Table 17: Sequencing information and diversity estimates for all bacteria at the two sites

	Tuvem	Divar
DNA recovered from 0.25g dry sediment (ng/μl)	10.8	15.2
Total number of bacterial V6 tag sequences	12954	21433
Total number of trimmed bacterial V6 tag sequences	8030	15326
Total number of unique sequences	2661	3707
Total OTUs at 3% difference	1561	2198
Chao1 estimator of richness at 3% difference, min, max	1988, 1914, 2076	3092, 2968, 3237
ACE estimator of richness at 3% difference, min, max	2166, 2136, 2196	3342, 3261, 3429
Simpson evenness index	0.0004	0.0004

Note: Value under trimmed tags are the numbers of reads remaining after the removal of low-quality data. Values under unique sequences are the numbers of discrete sequences. Average read length of sequences was 60.73 (±2.63) bp.

Table 18a: Phylogenetic classification of twenty most abundant clusters (≥97% similarity) at Tuvem where total number of OTUs = 1561.

Domain	Phylum	Class	Order	Family	Occurrence per cluster (%)
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.96
	Bacteroidetes	Bacteroidia	Bacteroidales		0.51
	Proteobacteria				0.45
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.38
	Proteobacteria				0.38
Bacteria					0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.32
Bacteria					0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.32
	Proteobacteria				0.32
Bacteria					0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.32
	Bacteroidetes	Sphingobacteria	Sphingobacteriales		0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.32
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.26
	Bacteroidetes				0.26
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.26
	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae	0.26

Table 18b: Phylogenetic classification of twenty most abundant clusters (≥97% similarity) at Divar where total number of OTUs = 2198.

Phylum	Class	Order	Family	Occurrence per cluster (%)
Proteobacteria	Gammaproteobacteria			0.68
Proteobacteria				0.50
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	0.36
Proteobacteria	Gammaproteobacteria			0.36
Proteobacteria				0.36
Proteobacteria	Gammaproteobacteria			0.36
Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	0.32
Proteobacteria	Gammaproteobacteria			0.32
Bacteroidetes				0.32
Proteobacteria	Gammaproteobacteria			0.32
Bacteroidetes				0.32
Proteobacteria	Gammaproteobacteria			0.32
Bacteroidetes				0.27
Proteobacteria	Gammaproteobacteria			0.27
Bacteroidetes	Bacteroidia	Bacteroidales		0.27
Proteobacteria	Gammaproteobacteria			0.27
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	0.27
Proteobacteria	Gammaproteobacteria			0.27
Bacteroidetes				0.27
Proteobacteria	Gammaproteobacteria			0.27

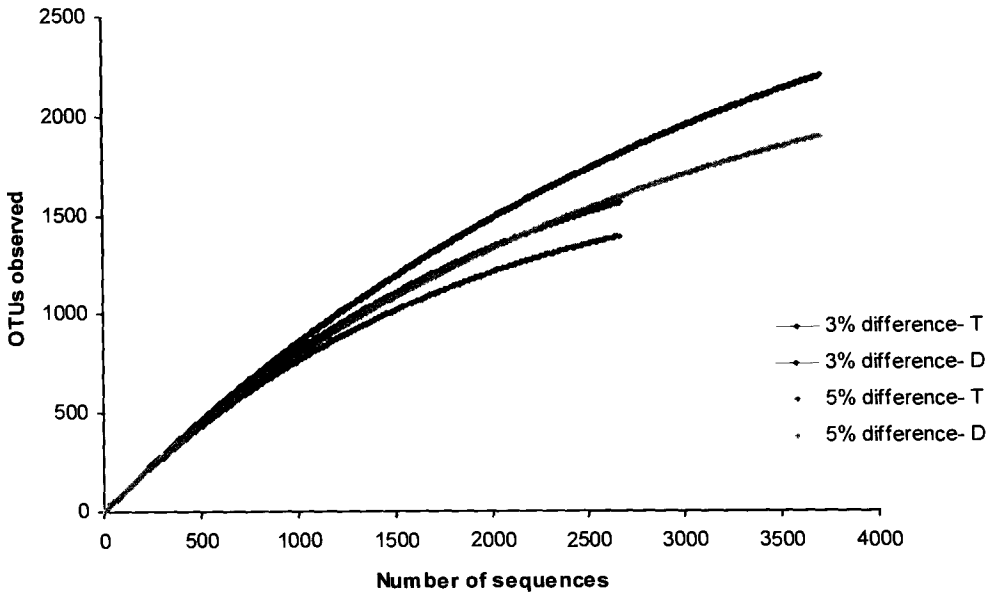


Fig. 57: Rarefaction curves based on 95 and 97% sequence similarity of sediment bacterial community at Tuvem (T) and Divar (D).

Chapter 5
Discussion

Chapter 5. Discussion

5.1. Denitrification: process and controls

Estuarine sediments are locations for bacterially-mediated removal of anthropogenically derived inorganic nitrogen from the aquatic environment (Dong *et al.*, 2009). Nitrate in particular serves as an electron acceptor for anaerobic oxidation of organic matter and is reduced to gaseous compounds like N_2O or N_2 through the denitrification pathway. Denitrification is favorable under oxygen deficient conditions and is mediated by facultatively anaerobic bacteria. However, denitrification rates vary in different ecosystems and could be affected by the prevailing environmental conditions. There is a general lack of information on the environmental parameters affecting benthic denitrification in near-shore coastal ecosystems such as mangroves. Currently, data available on denitrification dynamics and the factors influencing the process in mangrove ecosystems are relatively few. Most of the studies in mangroves have focused on quantifying benthic denitrification rates (Rivera-Monroy *et al.*, 1995; Meyer *et al.*, 2005) to understand the potential use of mangroves in nitrate depuration (Corredor and Morell, 1994) or wastewater treatment (Nedwell, 1975). Chiu *et al.* (2004) have quantified denitrification and assessed the factors affecting the process in temperate mangrove sediment. In the present study, the seasonal down-core variation of denitrification (DNT) in sediments was investigated in two tropical mangrove ecosystems of Goa. Benthic DNT in these habitats was pre-dominant within 0-4 cm at all seasons and decreased further with depth at both the control site Tuvem and the experimental site Divar. This observation is in accordance to observations made by Chiu *et al.* (2004) who state that the N loss occurs primarily in the surface rather than in the rhizosphere sediment. Gas chromatographic measurements of DNT rates showed distinct seasonality with highest activity during the pre-monsoon season at both the study sites. Highest DNT activity of 237 μmol N_2O-N m^{-2} h^{-1} at 0-2 cm was recorded at the control site Tuvem. A similar rate was recorded at 2-4 cm during the same season at Divar. Denitrification rates assayed by the acetylene block technique in other mangrove sediments have yielded average rates of 75 μmol N_2O-N m^{-2} h^{-1} (Corredor and Morell, 1994) which are 3 times lower than observed in the present study.

The progressive increase in DNT activity from monsoon to pre-monsoon could be caused due to the variation in associated environmental parameters. In this investigation, multiple regression analysis was used to examine the biotic and abiotic parameters influencing DNT. The complexity of the process increased during the monsoon with a larger number of variables affecting DNT at the control site. The multiple regression model obtained is capable of describing about 99% of the overall variation in DNT rates ($R^2 = 0.99$) during the monsoon at the control site. The model was obtained on a seasonal basis for both the sites. The advent of the south-west monsoon results in a large input of fresh water into the estuaries causing considerable changes in the chemical characteristics of the aquatic system. A marked decrease in ambient salinity has been observed during this season. Runoff also results in addition of land-derived nutrients into the estuarine system as evident from the elevated pore water ammonium concentration in the surficial (0-4 cm) mangrove sediments. Among the variables positively influencing DNT during the monsoon, NH_4^+ availability highly influenced DNT ($p<0.0000$, $n=30$) at the control site. As nitrate availability is relatively low at the control site during monsoon, DNT could be more closely linked to nitrification which oxidizes NH_4^+ and thus serves as a major source of NO_3^- (Wang *et al.*, 2003). At the experimental site Divar, DNT was influenced by the concentration of pore water NO_3^- ($p<0.001$, $n=30$) and NRB ($p<0.0001$, $n=30$). In anaerobic environments, availability of electron acceptor often limits DNT (Seitzinger, 1990). The experimental site, receives high extraneous nitrate input during the monsoon. Recently Divya *et al.*, (2009) have shown that limno-tolerant bacteria are more actively involved reducing nitrate concentrations entering the Mandovi estuary particularly during the monsoon. In the present study, the heterotrophs and nitrate reducers influenced DNT during the monsoon suggesting that they could be important in reducing elevated nutrient levels in the system thereby counteracting eutrophication.

The post-monsoon marks a transition phase from highly dynamic conditions towards a period of stability. At the control site, a significant increase in pore water NO_3^- concentration is observed during the post-monsoon. A shift in DNT regulation from NH_4^+ modulated to NO_3^- dependent is observed during this season. Sediment pH negatively influenced DNT while bacterial parameters like heterotrophic bacteria and NRB were some of the prominent variables which positively govern DNT. During the post-

monsoon, organic carbon content was higher at depth >4 cm. Its degradation could result in acidic pH at these depths. Generally, the optimum pH for DNT is 7.0–7.5 (Thomas *et al.*, 1994). In mangrove sediments, maximum DNT occurs towards more neutral pH which explains the inverse relationship of DNT with pH. As heterotrophs and NRB influence DNT, a heterotrophic mode of NO_3^- respiration could be prevailing at Tuvem during the post-monsoon. At the experimental site, DNT activity increased approximately 5 times during the post-monsoon as compared to the monsoon season. Though NO_3^-, NRB and denitrifiers influenced DNT, the process is largely controlled by metals i.e. Mn and Fe. Up to 24% Fe has been recorded at 2-4 cm during this season. The Divar ecosystem lies along the Mandovi estuary which has ferro-manganese mines located upstream. Runoff during the monsoon and subsequent mining activities (input of rejects, movement of Fe ore transporting barges, etc..) on the onset of the post-monsoon could be responsible for high Fe content in the sediments. Earlier studies by Krishnan *et al.* (2007) at the same location have shown that the 0-4 cm remains moderately contaminated by iron during the post-monsoon while it falls in the 'uncontaminated to moderately contaminated by iron' category during the pre-monsoon and monsoon season. Addition of Ferric ion (Fe_3^+) has been shown to significantly accelerate nitrate utilization in the denitrifying strain *Paracoccus pantotrophus* P16 (Pintathong *et al.*, 2009). Another denitrifying strain *Pseudomonas denitrificans,* can reduce nitrate and grow in the presence of Fe(0) (Till *et al.*, 1998). Further, denitrification-based remediation studies on contaminated marine sediment samples have shown that autotrophic denitrification results in an increase of reducible fractions of metals (Shao *et al.*, 2009). Mn^{2+} oxidation by NO_3^- has also been reported to occur by Luther *et al.* (1997). Thus, nitrate respiration by denitrifiers using various potentials electron donors like metal ions could be important in reducing environmental pollution as the mangrove ecosystem shifts from heterotrophic to autotrophic based respiration.

More stable conditions were observed during the pre-monsoon with a fewer factors influencing DNT. The influence of metals (mainly Fe) on DNT was persistent during this season at both the study sites albeit negatively correlated. Iron concentration was found to increase with depth while DNT was maximum within 0-4 cm during this season. Bioturbating organisms viz., *Uca* crabs, polychaetes, etc.. are abundant in the

Divar region. Their burrowing action could be responsible for the downward advection of metals. Co-occurring processes like anoxic nitrification result in metal reduction (Hulth *et al.*, 1999). The lower metal content in the surficial mangrove sediments could be attributed to reduction of Fe and Mn oxides and oxidation of NH_4^+. Krishnan and Loka Bharathi (2009) suggest that autotrophic nitrification could be important in mangrove sediments with higher metal content. As denitrification and nitrification operate in tandem (Rysgaard *et al.*, 1993; Nielsen *et al.*, 1996), metal immobilization could occur through the reductive phase of the N cycle while autotrophic nitrification could be responsible for an increase in reduced metal ions. Thus, a close coupling between metals and nitrogen redox cycle in mangrove sediments could be prevalent. The pre-monsoon is also marked by highest DNT activity compared to other seasons. The activity is positively influenced by the availability of inorganic nitrogenous compounds at the control site. At the experimental site, heterotrophic denitrification could be pre-dominant as organic carbon ($p<0.05$) and NRB ($p<0.05$) were some of the factors influencing the process. Organic C has significant effect in regulating DNT (Dodla *et al.*, 2008) as denitrifying organisms oxidize organic matter under sub-oxic or anoxic conditions (Vance-Harris and Ingall, 2005). The mangrove sediments investigated have considerable organic carbon loading throughout the year. Thus, availability of substrates like organic C, NO_3^-, oxygen-deficiency, etc. provide ideal conditions for heterotrophic denitrification to occur.

The present study reveals distinct seasonal DNT variability in the mangrove sediments of Goa with highest activity during the pre-monsoon. This variation is brought about by changes in the sediment chemistry and physiological groups of bacteria capable of denitrification. A large number of parameters influence DNT during the monsoon season especially at Tuvem. The complexity decreases progressively towards the non-monsoon period. The availability of inorganic nitrogenous substrates were also seen to limit DNT in these sediments. Generally, heterotrophic DNT prevails at the sites examined. However, during the post-monsoon, DNT at the experimental site is largely influenced by Mn and autotrophic mode of nitrate respiration could possibly exceed heterotrophy.

5.2. Factors influencing denitrifier abundance

Though maximum denitrification activity in mangrove sediments occurred within 0-4 cm, bacteria mediating the process were maximum at 4-6 cm indicating that activity is not a function of bacterial biomass. A similar trend in denitrifier distribution has been reported by Fan *et al.* (2006) in estuarine sediments with higher denitrifier aggregation of up to 3.64 x 10^4 cells g^{-1} at 5-7 cm whereas the activity was maximum at the surface. In the present study, up to 10^8 denitrifiers g^{-1} have been recorded. The culturable fraction of this physiological group thus form up to 1% total bacterial population (10^{10} cells g^{-1}) indicating that a large number of non-culturable forms from mangrove sediments could be actively involved in N transformations.

Some of the factors influencing the abundance and activity of denitrifiers are sediment nitrification (Stockenberg and Johnstone, 1997), nitrate availability (Seitzinger, 1990; Corredor and Morell, 1994; Kana *et al.*, 1998), organic carbon (Pfenning and Mc Mahon 1997; Mc Carty and Bremner, 1993; Ward *et al.*, 2008), sediment pH (Rust *et al.*, 2000; Simek *et al.*, 2002), oxygen concentration (Bonin and Raymond, 1990) and redox conditions (Lee and Joye, 2006). In the present study, statistical analysis revealed the influence of metals especially Mn on denitrifier abundance at both the sites investigated. Manganese and nitrate transformations appear to be linked in mangrove sediments. The correlation between denitrifiers and Mn was strongest during the post-monsoon at the control site and was responsible for bringing about 43% variation ($r=0.66$, $p<0.01$, $n=15$) in denitrifier population. This could be indicative of the beneficial effect of denitrifiers on Mn removal. Nitrate or nitrite can couple their reduction to oxidants like Mn (II) (Oguz *et al.*, 2001) as described by Luther *et al.* (1997):

$$5Mn_2^- + 2NO_3^- + 8OH^- \longrightarrow 5MnO_2 + N_2 + 4H_2O$$

Experiments using denitrifying cultures by Vandenabeele *et al.* (1995) have shown that Mn removal rate was 38% higher in the presence of nitrate and was accompanied by nitrite accumulation. In anaerobic sediments containing Mn or Fe oxides denitrifying organism (e.g. *Shewanella putrefaciens* MR-1) would will have a distinct advantage over other organisms by gaining energy from the use of Mn (IV) or Fe (III) as an electron acceptor (Myers and Nealson, 1990). Total bacterial counts also showed a positive

relationship with denitrifiers ($r=0.45$; $p<0.05$; $n=15$) indicating that a large fraction of the bacterial population could be involved in DNT and consequently play and important role in altering the sediment chemistry.

At Divar, a strong negative relationship ($r=-0.97$, $p<0.001$, $n=15$) was observed between Mn and denitrifiers during the monsoon. During monsoon, Mn accumulation is observed at depths >6 cm whereas higher denitrifier abundance is seen at depths ≤6 cm. The adjoining Mandovi estuary receives higher extraneously derived nitrate concentrations (Divya *et al.*, 2009) during this season. Thus, nitrate could be more important in governing denitrification during this period. Iron also showed a negative influence on denitrifiers during the monsoon. Though multiple regression analysis has indicated TOC as an important factor for denitrification (heterotrophic denitrification), the negative influence of metals on denitrifiers is suggestive of autotrophic denitrification to co-occur. Among the microbiological parameters, denitrifiers were significantly related to NRB ($r=0.46$; $p<0.05$; $n=15$) suggesting that heterotrophic nitrate reducers have the potential to completely denitrify NO_3^- at Divar.

Among the various factors governing denitrifier abundance during the monsoon, nitrite ($r=0.60$; $p<0.01$; $n=15$) is the most important factor influencing the group at the control site whereas metals (Fe and Mn) were more important at the experimental site. During the monsoon, terrestrially-derived nutrient input increases in the estuaries and denitrifiers could be important in reducing elevated nitrate levels in the aquatic system. Nitrate reduction results in a formation of nitrite which is further reduced to N_2 through the denitrification pathway. Thus, Pearson's correlation as well as multiple regression analyses employed in the present study indicate that denitrifier abundance in mangrove sediments is largely influenced by metals mainly Mn at both the study sites. Nitrate reduction at the expense of metal ions could thus be a principal mode of respiration during this period.

5.3. Nitrous oxide production and it's origin

The marine environment is recognized as a net source of nitrous oxide to the atmosphere (Corredor *et al.*, 1999). In the Indian Ocean region, high nitrous oxide emission has so far been reported within the oxygen minimum zones in the Arabian Sea (Naqvi *et al.*, 2000; Bange *et al.*, 2001). Estuarine sediments also play a significant role in the transformation of nitrogenous compounds leading to N_2O production. In coastal ecosystems like mangroves, a substantial flux of nitrous oxide has been observed (Corredor *et al.*, 1999; Muñoz-Hincapié *et al.* 2002; Kreuzwieser *et al.*, 2003). Although Krithika *et al.*, 2008 have reported benthic N_2O flux to vary between 0.41 and 0.77 μmol m^{-2} h^{-1} in a South Indian mangrove system, not much is known on the net production or origin of the gas. Natural N_2O production rates in estuarine sediments have been found to range from 0.1 to 8.5 μmol m^{-2} h^{-1} (Wang *et al.*, 2007). N_2O production rates estimated from sediment slurry experiments in the present study revealed higher production of the gas especially in sediments prone to elevated nutrient supply. Benthic N_2O production rates in the Divar sediments was up to 3 times higher than at the relatively pristine Tuvem varying between 6.41-22 μmol m^{-2} h^{-1}. At nitrate concentration of ~10-15 μmol (*in situ* + ambient seawater used in medium), N_2O production values observed in this study are in close range to those reported by Dong *et al.*, (2002) from the anthropogenically influenced Colne estuary at similar NO_3^- concentration. Intertidal rocky biofilms have also shown N_2O production to occur at rate of up to 17±6 μmol m^{-2} h^{-1} (Magalhaes *et al.*, 2005) which is close to values recorded in the present study. In mangrove sediments, microbial communities are capable of taking up large amounts of nitrate added to water system by sewage effluents (Corredor and Morell, 1994). Nutrient depuration occurs through the denitrification pathway and could possibly lead to evolution of greater amounts of nitrous oxide to the atmosphere. Pore water profiles have shown that the organically-rich Divar sediments are characterized by higher ammonium and nitrite content. Nitrite is an intermediate of both nitrification and denitrification and its concentration could be high when both the processes co-occur. Low nitrate concentration within 0-4 cm layer in the Divar sediments coincided with higher production of nitrous oxide and is indicative of elevated denitrification activity. Jørgensen (1989) has also shown that the denitrification capacity in estuarine sediments was always highest at the surface and declined with

depth. Denitrification depends on the supply of nitrate by nitrification and the two processes are coupled (Klingensmith and Alexander, 1983). Denitrification activity could also be dependent on nitrate supply from the ambient water. The Divar mangrove ecosystem fringes the Mandovi estuary which receives high nutrient input through anthropogenic activities. This ecosystem could act as a buffer zone by reducing nutrient levels and maintaining the water quality of the estuary through the denitrification process. The percentage of nitrous oxide produced in the Divar sediments is much higher than at Tuvem highlighting that in ecosystems prone to extraneous nutrient input, denitrification could be an important process to counteract eutrophication. However, higher N loading could also have a detrimental effect on the environment through increased N_2O production and its consequent flux to the atmosphere.

Experiments to examine the major pathway for N_2O production in the present study indicates that reducing habitats like mangroves are potential sites for denitrifying activity and incomplete denitrification (up to 93% at the surface) could contribute substantially to an increase in atmospheric N_2O. Studies by Robinson *et al.*, (1998) in hypernutrified estuarine sediments have also shown higher N_2O concentrations in the surface layer ($\leq$2 cm) attributing it to denitrification fuelled by NO_3^- availability. Similarly, Koike and Terauchi (1996) have also reported highest concentration of nitrous oxide at the top 1 cm in marine sediments. In this study, stratified sampling showed that benthic N_2O production was found to generally decrease with depth. The flux of the radiative gas to the atmosphere would however be dependent on the diffusion coefficient and N_2O consumption rates in the sediment layers it passes. A microsensor approach by Meyer *et al.*, (2008) has shown that in sub-tropical mangrove sediments, N_2O is produced through nitrification close to the surface while denitrification is responsible for its production in the deeper layers. In this study, measurements were carried out at every 2 cm intervals and not on a sub-millimeter scale unlike Meyer *et al.*, (2008). Perhaps close grid sampling could have provided evidence on the contribution of nitrification to N_2O production very close to the surface.

A number of factors are known to influence the production of nitrous oxide in marine sediments. Physical, chemical, biological and environmental factors like temperature, pH, sediment redox potential (Van Cleemput and Samater, 1996), organic C

availability (Rosswell *et al.*, 1989), nitrite concentration (Dong *et al.*, 2002) and denitrifying communities play an important role in N_2O production. Statistical analysis showed that pore water nutrient concentrations, organic carbon availability and denitrifiers were some of the important factors influencing the production of nitrous oxide in mangrove sediments. Degradation of sediment organic matter results in acidic conditions (pH=4.7–7.2). Sediments at Tuvem contain organic carbon varying between 2.13-4.54%. Though the relationship between nitrous oxide production and total organic carbon content in these sediments was not highly significant, a positive relationship existed and is indicative of its influence on the gas. N_2O production rates are by affected by levels of organic carbon which can be used as electron donor during denitrification (Pfenning and McMahon, 1997). Though the water soluble fraction of organic C was not estimated during the study, it is possible that it stimulated denitrifier activity and consequently N_2O production. Both denitrification rate and N_2O production followed a decreasing trend with depth, however no significant relationship was observed between the two parameters. The production of N_2O could be thus be governed by the availability of electron donors and acceptors like NH_4^+, NO_3^-, organic C rather than the rate of denitrification (Usui *et al.*, 2001; Mathieu *et al.*, 2006). The denitrifier community was also found influence nitrous oxide production in mangrove sediments. They numbered up to 10^7 cells g^{-1} and their abundance could be regulated by the amount of organic matter available for their growth. This can be easily explained by the fact that the denitrifiers are facultative aerobic microorganisms and their denitrifying activity is limited by the amount of nitrate available.

Estuaries are generally heterotrophic systems, with bacterial respiration exceeding primary production (Heip *et al.*, 1995, Gattuso *et al.*, 1998). Consequently, removal of dissolved inorganic nitrogen from estuaries occurs through sedimentary denitrification and/or burial in the sediment (Middelburg and Nieuwenhuize, 2000). An inverse relationship observed between denitrifiers and pore water nutrient concentrations in the present study suggests that denitrification could play an important role in mitigating excess nutrients within the system preventing eutrophication. In sulfidic sediments, the denitrification end product is known to shift from N_2 to partially reduced ones such as nitrite and N_2O (Ebrahimipour *et al.*, 2000). This could explain the high nitrite pool in the

largely anaerobic mangrove sediments. In this study, about 22% variation in nitrous oxide production was caused by the variation in nitrite concentrations suggesting that it was one of the important and statistically significant parameters regulating the production of the gas in mangrove sediments. Many studies have shown a correlation to exist between N_2O production and nitrite concentration (He *et al.*, 2001; Dong *et al.*, 2004; Alinsafi *et al.*, 2008). Denitrification activity in estuarine sediments is dependent on nitrate availability (Kana *et al.*, 1998) consequently leading to NO_2^- and N_2O production. Dong *et al.*, (2002) state that formation of N_2O from nitrite is thermodynamically favourable compared to nitrate and may be a critical factor regulating N_2O formation. Bauza *et al.*, (2002) have reported N_2O production mainly through nitrification in red mangrove forests which are characterized by oxic conditions and higher ammonium concentration. In the present study, ammonium concentration did not appear to assert a strong influence on N_2O production suggesting that nitrification was not a significant source of N_2O. Low redox potentials (<115 mV) at the sampling sites are indicative of anaerobic conditions in the sediment which are conducive for alternate respiratory pathways like denitrification, sulphate reduction, etc. to occur. When nitrate and nitrite pre-dominate, nitrous oxide arises from microbial denitrification (Corredor *et al.*, 1999). Experimental results also reveal that denitrification was the major pathway for N_2O production in the mangrove ecosystems of Goa, India. Nitrous oxide production through nitrification could be more prominent when the oxidative process is more pronounced. However, N_2O production through nitrification was not detected indicating that the reductive phase of the N cycle was pre-dominant (at post-monsoon). Recently, Krishnan and Loka Bharathi (2009) have reported highest rates of benthic nitrification during the pre-monsoon at the same sampling locations. Seasonal trends in N_2O flux and production mechanisms would provide further insights on contribution of nitrification if any.

Observations in the present study demonstrate that estuarine ecosystems like mangroves are potential sites for denitrifying activity. Incomplete denitrification leads to nitrous oxide production which could be responsible for flux of the radiative gas to the atmosphere. Though mangroves have the ability to efficiently moderate elevated nutrient concentrations in the estuarine system through the denitrification pathway, they also pose a threat by increasing green house gas production. Thus, adequate measures could be

Table 19: Denitrification rates in mangrove sediments

Sr. No.	Area	Denitrification rates	Method	Reference
1	Terminos Lagoon, Mexico	Fringe mangrove: 9.4 μmol m^{-2} h^{-1} Basin mangrove: 1.9 to 4.5 μmol m^{-2} h^{-1}	^{15}N isotope technique	Rivera-Monroy *et al.*, 1995
2	Mangrove forest, Phuket, Thailand	Vegetated sediments: 1.91μmol m^{-2} h^{-1} Unvegetated sediments: 0.54 μmol m^{-2} h^{-1}	^{15}N isotope technique	Kristensen *et al.*, 1998
3	Logan/Albert River, Queensland, Australia	85 nmol N cm^{-3} h^{-1} or 38 nmol N g^{-1} h^{-1}	^{15}N isotope technique	Meyer *et al.*, 2005
4	Matang Mangrove Forest Reserve, Malaysia	16–458 μmol N_2 m^{-2} h^{-1} 46.08 to 157.5 μmol N_2 m^{-2} h^{-1}	N_2-gas flux technique	Alongi *et al.*, 2004
5	Jiulongjiang Estuary, China		N_2-gas flux technique	Alongi *et al.*, 2005
6	Southwest coast of Puerto Rico	0.12 and 7.8 μmol N_2O m^{-2} h^{-1} Tuvem: 0.03-74.88 nmol g^{-1} h^{-1} or 0.339-846.14 μmol N_2 m^{-2} h^{-1} *	Gas chromatography	Corredor *et al.*, 1999
7	Goa, India	Divar: 1.47-222.59 nmol g^{-1} h^{-1} or 16.61-2515.26 μmol N_2 m^{-2} h^{-1} *	^{15}N isotope technique	Present study

Note: *For conversion of DNT activity in nmol N_2 g^{-1} h^{-1} to μmol N_2 m^{-2} h^{-1}:

(DNT activity in nmol N_2 g^{-1} h^{-1} x 1.13×10^4) / 10^3

initiated to minimize N loading in adjoining estuarine systems in order to lower environmental pollution but also simultaneously result in decreased N_2O emission to the atmosphere.

5.4. Denitrification and other co-occurring processes

Coastal marine ecosystems are susceptible to high nitrogen inputs through anthropogenic activities. In these regions, coupled nitrification/denitrification serves as an important mechanism for nitrogen depuration (Corredor *et al.*, 1999). Investigations carried out in the present study affirm the potential of nearshore tropical mangrove ecosystems in mitigating inorganic nitrogenous compounds mainly through denitrification. Denitrification served as a major mechanism for N loss in mangrove sediments accounting for 75-85% of the total N_2 production. Highest denitrification activity was observed at the surface at both the sites- Divar and Tuvem. Total denitrification rates of up to 222.59 nmol N_2 g^{-1} h^{-1} (≈2.51 mmol N_2 m^{-2} h^{-1}) have been measured in the Divar sediments. In sub-tropical mangrove sediments, denitrification has been reported to occur at a maximum rate of approximately 85 nmol N cm^{-3} h^{-1} (≈ 38 nmol N g^{-1} h^{-1}) (Meyer *et al.*, 2005) which is about five times less than observed in the present measurements. Benthic denitrification rates recorded in other mangrove areas (Table 19) are relatively less than those observed in the present study.

The Divar mangrove ecosystem lies along the Mandovi estuary. This estuary receives about 10 tonnes/month of ammonium nitrate used in explosives for mining activities upstream which eventually acts as a source of nitrate (De Souza, 1999). Experiments have shown that nitrate availability is one of the major factors controlling benthic denitrification (Seitzinger, 1990; Morell and Corredor, 1993; Rivera-Monroy and Twilley, 1996; Kana *et al.*, 1998). In the present study, the higher nitrate concentration recorded in the superficial sediments could be due to the continuous replenishment of the nutrient from the ambient water in addition to its *in situ* production through nitrification. Consequently, nitrate availability could be accountable for the high denitrification rates observed especially at depth ≤4 cm. Principal component analysis also revealed that nitrate and nitrite availability were important factors limiting denitrification in mangrove ecosystems. A positive relationship between nitrite, nitrate concentration and

denitrification (D_{tot}) confirms that this process could be a major sink for nitrate in mangrove sediments. The PCA bi-plot also shows that ammonium, nitrite and nitrate clustered together suggesting a link between ammonium, nitrate and nitrite probably through nitrification.

Molecular investigations carried out in the present study reveal that the abundance of denitrifier genes in mangrove sediments numbered up to 10^7 target copies g^{-1} of dry sediment. The result was comparable to those recorded in other marine environments. Michotey *et al.* (2000) have reported up to 10^6 cytochrome cd1 type denitrifiers in marine samples. No statistical correlation between *nosZ* gene abundance and denitrifying activity could be observed indicating that denitrification activity is not limited by the presence of the population able to denitrify but by the availability of nitrate as substrate. Though denitrification at Tuvem was maximum within the 0-4 cm depth range, the *nosZ* were most abundant at 6-8 cm. Experiments using the bacterial strain *Pseudomonas mandelii* has shown that *nosZ* gene expression did not respond to increasing NO_3^- concentration indicating that there is no relationship between gene expression and denitrification activity (Saleh-Lakha *et al.*, 2009). Boetius (1995) examined the potential hydrolysis rates of five different hydrolytic enzymes in deep-sea sediments and found that the activity of the enzymes most likely reflects the availability of their respective substrates and is not a function of bacterial biomass. It is possible that the expression of *nosZ* genes in mangrove sediments could be linked to the prevailing environmental conditions. Recently, Sumathi and Raghukumar (2009) have demonstrated the denitrification potential in several species of fungi isolated from the Arabian Sea sediments. Fungi commonly occur in coastal and offshore marine environments (Raghukumar, 1989). Mangrove ecosystems are known to harbour about 10^4 colonies g^{-1} of fungal flora (Prabhakaran *et al.*, 1987). Fungi are actively involved in the decomposition of mangrove leaves/wood and very little is known on their involvement in mangrove nutrient cycling (Hyde and Lee, 1995). It is uncertain whether denitrifying activity of fungi in mangrove sediments if any, is partially responsible for N loss.

At the experimental site, the denitrifying genes were found from the surface till a depth of 10 cm indicating that the organisms were well dispersed. It is possible that sandy nature of the mangrove sediment and the bioturbating activity of the infauna (crabs,

polychaetes, etc..) could aid in the dispersion of the microbes. The mangrove sediments are seen to harbour a substantially high macrofaunal assemblage. Macrofauna are known to modify nutrient fluxes in marine sediments (Mortimer *et al.*, 1999). Burrowing activities by macrofaunal communities enhances oxygen availability and creates non-local mixing between nutrient rich pore water and overlying water (Graf and Rosenberg, 1997). Studies by Pennifold and Davis (2001) have also shown that nutrient fluxes were significantly correlated with increased faunal biomass. Further, the authors state that macrofauna increased the release of ammonium into the water column but decreased the release or resulted in uptake of nitrate by the sediments. Macrofauna stimulate denitrification by providing nitrate to bacteria from the overlying water and also through *in situ* nitrification strengthening the proximity and exchanges between the two processes (Gilbert *et al.*, 1998). In contrast to the control site, the experimental site is dominated mainly by sand. The sandy nature of the sediments could be responsible for facilitating percolation of nutrients to deeper depths. In this study, we can envisage that the physical perturbation of sediments ascribed to the high density of macrofauna especially in the Divar sediments may have resulted in a stronger nitrification-denitrification coupling by enhancing the exchange of solutes between water and sediment.

Anammox is known to co-occur along with denitrification in marine sediments (Rysgaard *et al.*, 2004). In some marine environments, anammox is a major pathway for the removal of fixed inorganic nitrogen (Kuypers *et al.*, 2003; Engström *et al.*, 2005). The co-occurrence of anammox in mangrove sediments has been detected in the current study. Slurry incubations, as employed in this study, can be useful for quantifying mechanisms of N_2 production, but this approach perturbs the natural gradient and spatial arrangements of organisms carrying out N cycling process *in situ*. Whether slurry measurements yield high activity at low ra% (percentage of anammox) values is thus a concern. Trimmer *et al.* (2006) compared rates of anammox and denitrification in slurries and intact cores. In sediments with low anammox activity (ra<1%), slurries and intact core yielded similar results, but when anammox was more significant (ra>5%), ra% was about 10-15% higher in intact core than in slurries (Trimmer *et al.*, 2006). Consequently the slurry measurement used in this study probably accurately assessed the presence or absence of anammox activity, but the actual ra% in some layers with high activity may

have been underestimated. Maximum anammox rates of up to 101.15 nmol N_2 g^{-1} h^{-1} (≈1.14 mmol N_2 m^{-2} h^{-1}) have been observed in the present study. In sub-tropical mangrove sediment, Meyer *et al.* (2005) have reported anammox rates varying between 0.5 to 8 nmol N cm^{-3} h^{-1} (0.22 to 3.6 nmol N g^{-1} h^{-1}) which is approximately 25 times lower than those encountered in the present study. Reports by Rysgaard *et al.* (2004) in arctic marine sediments indicate anammox to occur at about 3 orders lower while in the north sea sediments, anammox rates were also relatively lower (Dalsgaard *et al.*, 2005). The present observations show that anammox served as an important mechanism for N_2 production mainly at deeper depths (8-10 cm) where it accounted for 67-96% of the total N_2 production. High rates of anammox occur in estuarine sediments with permanently high concentrations of NO_2^- (Risgaard-Petersen *et al.*, 2005). Dalsgaard and Thamdrup (2002) have attributed the 1:1 stoichiometry for the reaction between nitrite and ammonium to the anammox process. At pre-monsoon, nitrification occurs at a rate of up to ~18 nmol g^{-1} h^{-1} at a depth of 8-10 cm at Divar (Krishnan and Loka Bharathi, 2009) which co-incidentally shows the highest anammox activity in this study. Thus, nitrite production through nitrification at this depth could fuel the anammox process. High NO_3^- concentrations (Rich *et al.*, 2008) are also known to favour anammox rates. The mangrove sediments harbored fairly high concentrations of nitrate which could be favorable for anaerobic oxidation of ammonium to occur. At the control site, anammox was detected at all depths investigated in comparison to the experimental site where it was more restricted. It interesting to observe that anammox activity is more prominent at deeper depths and occurs at a rather low rate in ecosystems where denitrification is much higher. Dalsgaard *et al.* (2005) explain that in organic-rich sediments, denitrification is more responsive to organic carbon loading than anammox. Dalsgaard *et al.* (2005) also suggest that high organic matter content creates a higher demand for electron acceptor (i.e., NO_2^- and NO_3^-) and a smaller fraction of the reduced NO_3^- is liberated as NO_2^-. In such circumstances, anammox may not be able to keep up with denitrification when electron donor availability is high. Such a phenomenon might be occurring in mangrove sediments which have considerable organic carbon content. Dalsgaard *et al.* (2005) also state that denitrifiers have a much higher growth rate which gives them a competitive advantage over anammox bacteria in fluctuating environments. Reports from the

Benguela upwelling system indicate that anammox bacteria are metabolically versatile and can function as nitrate reducers (Kartal *et al.*, 2007). Thus, it is possible that these microbes could be mediating dissimilatory nitrate reduction to ammonium in mangrove ecosystems.

In mangrove sediments, denitrification and anammox operate concurrently leading to N_2 production. However, the anammox process is restrained in organically rich mangrove sediments and denitrification becomes the major mechanism of N loss. Most of the N loss in these sediments occurs in the superficial layers and a coupling between nitrification and denitrification is suggested. Thus, in coastal ecosystems like mangroves which are prone to high input of inorganic nitrogenous compounds through anthropogenic activities, denitrification helps to counteract eutrophication. Consequently, di-nitrogen fixation in these ecosystems is minimal as compared to the N_2 production from denitrification and anammox together.

Though denitrification is a major mechanism for NO_3^- removal in coastal sediments (Tuerk and Aelion, 2005), N_2 produced through complete denitrification of nitrate accounted for ≤1% of the total pore water nitrate reduced. The overall low contribution of denitrification in NO_3^- depuration suggests that removal of the macro nutrient could proceed through other significant pathways. Nutrient re-generation could be important in N limited ecosystems like mangroves (Lovelock *et al.*, 2006) wherein the microbial community could be competing with the vegetation for inorganic N requirements. Internal re-generation could therefore act as an efficient mechanism to meet the nitrogen demand from both the microbial and plant communities. Investigations carried out in the present study show that the anthropogenically influenced Divar sediments are characterized by higher nitrate reduction activity, nitrous oxide and N_2 production within 0-4 cm which are fuelled by nitrate availability. Nitrate can be produced intrinsically through nitrification (Krishnan *et al.*, 2008) or supplied through extraneous input (Naqvi *et al.*, 2000). The Divar ecosystem fringes the Mandovi estuary which is prone to NH_4NO_3 input from Fe-Mn mining rejects (De Sousa, 1999). Colorimetric measurements revealed that nitrate reduction in Divar sediments occurs at a rate of up to 3.52 μmol g^{-1} h^{-1} (≈1.07 μmol cm^{-3} h^{-1}) which is in range to reports from

other coastal sediments where NRA is found to range from 0.662-2.4 μmol cm^{-3} h^{-1} (Laverman *et al.*, 2006).

DNRA showed a steady increase in $^{15}NH_4^+$ at all depths investigated at both the sites. This process was responsible for 39% nitrate removal (100% NRA= 6.91 μmol; integrated for whole core) at Divar. In anoxic estuarine sediments, degradation of organic matter results in sulfide enrichment (Burton *et al.*, 2006; Laurent *et al.*, 2009). Chemolithoautotrophic DNRA couples the reduction of NO_3^- to H_2S/S^{2-} to generate ammonium which is a more readily utilisable form than nitrate. High concentrations of hydrogen sulfide in marine sediments can be lethal to marine organisms (Phillips *et al.*, 1997). As DNRA provides an electron donor (An and Gardner, 2002) the process could be linked to lowering levels of reduced sulfur forms in the system. Sulfide is also known to inhibit the last two steps of the denitrification pathway (Burgin and Hamilton, 2007). It is observed that N loss through the denitrification pathway (N_2O and N_2) in mangrove sediments was almost 3 times lower than DNRA (Fig. 58). Estuaries and coastal regions account for approximately 60% of the total oceanic N_2O flux (Bange *et al.*, 1996). The present observations reveal that N_2O production in mangrove sediments is relatively small highlighting the capacity of mangroves to buffer the climate against the green house gas.

Though the control site Tuvem is relatively free from extraneous nutrient input, it is characterized by nitrate accumulation at depth ≥2 cm which could be attributed to anoxic nitrification. As a result, NRA is more pronounced at depths ≥6 cm. The relatively higher nitrous oxide production at 2-4 cm in Tuvem could be attributed to nitrate accumulation at this depth. In this ecosystem, DNRA accounts for up to 65% NO_3^- removal (100% NRA=6.97 μmol). N retention is about 15 times higher as compared to N loss through the denitrification pathway. DNRA is an important mechanism that adds or retains available N in the system (Gardner *et al.*, 2006). Studies by Scott *et al.* (2008) have shown that in some areas, DNRA can remove more nitrate than denitrification. The present observations show that DNRA is important in reducing ecosystems and probably responsible for not only nitrite accumulation and removal but also for a non-neglectable part of ammonium production. Ammonium is known to adsorb easily onto clay particles (Laima *et al.*, 1999). In organically rich mangrove sediments (Krishnan and Loka

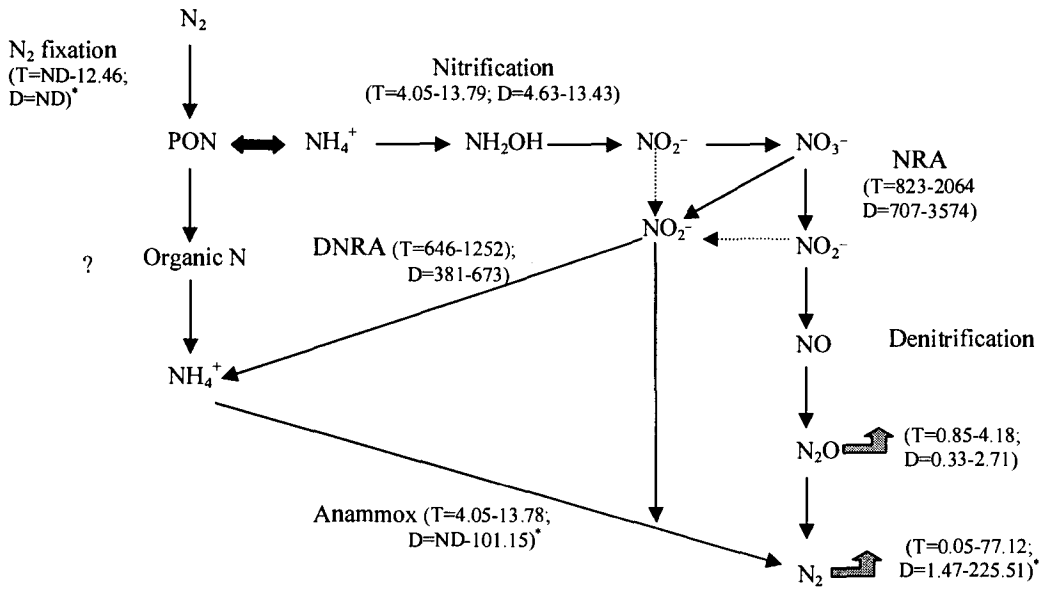


Fig. 58: Nitrogen cycling in mangrove sediments (T=Tuvem; D=Divar; ND= Not detected; activity has been expressed as nmol g^{-1} h^{-1}). Pore water nutrient profiles during above activity measurements have been given in Figs. 30 and 31a-b.

Bharathi, 2009), ammonium released through degradation of organic compounds could easily get bound to clay particles making it unavailable for biological uptake. The larger contribution of DNRA in pristine habitats like Tuvem indicates that this ecosystem efficiently re-circulates available N and conserves it to overcome limitation. A similar scenario could be expected at Divar. However, this ecosystem receives additional nutrients from external sources. As a result, the contribution of DNRA is relatively less as compared to ecosystems that need to conserve N.

Until now, mangroves have been known to function as efficient buffer zones mitigating large amounts of intrinsically produced nutrients as well as extraneously derived anthropogenic inputs (Corredor and Morell, 1994). This buffering capacity could be mainly attributed to the efficient functioning of the autochthonous microbial flora especially bacteria. However, mangroves have also been shown to emit a substantial flux of the green house gas N_2O to the atmosphere (Krithika *et al.*, 2008). On the contrary, the present study has shown that the microbial population in the mangrove swamps could contribute to considerably decreasing the N_2O emission for the first time. This is achieved in exchange for ammonium that gets retained in the system perhaps within biologically acceptable limits.

5.5. Influence of nitrate and organic C amendments on denitrification

Estuarine sediments are known to have considerable nutrient loading mostly derived from extraneous inputs like sewage outfall (King and Nedwell, 1987). The present study showed that the Divar sediments harbored a measurable pore water nitrate content which increased with depth. Down-core profiling of denitrification showed a sub-surface maxima at 2-4 cm with a rate of 20.08 μmol N_2O-N m^{-2} h^{-1} (1.43±0.66 nmol g^{-1} h^{-1}). Isotopic measurements by Chiu et al. (2004) have shown much higher denitrification activity in surficial mangrove sediments as compared to the deeper non-rhizosphere soil with rates of up to 120 nmol g^{-1} h^{-1}. Thomas and Lloyd (1994) have also reported maximum denitrification to occur at the surface in estuarine sediment. The process is known to be dependent on nitrate availability (Seitzinger, 1990). Low nitrate values encountered at the surface could thus be attributed to higher nitrate removal in the upper few centimeters of the sediment.

High denitrification activity at 2-4 cm coincided with increased organic carbon availability at this depth. Naturally occurring organic carbon in sediments is critical because it can influence nutrient availability (Moore, 1989), enhance biological activity and can increase acidity of ambient waters through organic acids (Eshleman and Hemond, 1985; Kerekes *et al.*, 1986). The primary source of TOC in mangrove swamps is plant material which is decomposed by sediment organisms and converted to organic compounds viz., carbohydrates, proteins and lipids. A sizeable fraction of organic matter could be transported to deeper depths by bioturbating infauna and retained within the system. Natural processes and human activities can also result in elevated content of TOC in the area. Sardessai (1993) have shown that decomposition of mangrove litter and influx of fresh water during monsoons contributes to organic matter derived from humic acids. In the present investigation, labile organic matter showed a similar depth-wise distribution like TOC with a maximum concentration of 0.68% LOM at 2-4 cm. Labile organic matter is known to limit denitrification (McCutchan and Lewis, 2008). In the reductive phase of the N cycle, NO_3^- ions are reduced to N_2O or N_2 whereas organic C gets oxidized to CO_2 and H_2O. In the Divar sediments, about 11-19% of the TOC is present in readily utilizable form (LOM) and could be important for heterotrophic metabolism.

Denitrification activity was stimulated in microcosms containing seawater with all combinations of nitrate amendments i.e. 5, 10, 20, 40 and 60 μmol NO_3-N l^{-1}. In estuarine sediments prone to high nitrate inputs, the bacterial communities adapt to changes in the concentration exhibiting higher rates of nitrate reduction and also increasing the proportion of nitrate reduced to gaseous products (King and Nedwell, 1987). Laverman *et al.* (2007) have shown that denitrification in estuarine sediments is nitrate limited and the resident denitrifying community rapidly adjusts its level of activity to increased nitrate availability. Denitrification accounts for 27 to 57% of the nitrate consumption in estuarine and coastal sediments (Nishio *et al.*, 1982). The Divar mangrove ecosystem is prone to high nutrient input from mining rejects, land runoff and domestic sewage discharge. The increase in denitrification activity especially in the first few centimeters is indicative of the high nitrate removal capacity of these sediments. Corredor and Morell (1994) have confirmed that mangrove sediment-microbial communities are capable of depurating up

to 10-15 times the nitrate added. Recently, Krishnan and Loka Bharathi (2009) have shown that nitrification rates in the Divar sediments vary between 2.7 to 18.2 nmol g^{-1} h^{-1}. A strong coupling between redox processes of the N cycle could exist in these sediments wherein nitrate supplied continuously through the nitrification process could be fuelling denitrification especially in the upper few centimeters.

Organic carbon addition stimulated denitrification activity in mangrove sediments mostly at depths ≥4 cm. A 0.5% amendment of labile organic carbon (glucose) was found to effectively stimulate denitrification activity at all depths suggesting that the process is optimal at this concentration. Statistical analyses did not show significant increase in denitrification activity on organic carbon addition as compared to amendments with nitrate (one way ANOVA; n=15; $p<0.001$) at all depths indicating that organic carbon was not a limiting factor for denitrification in mangrove sediments. Denitrification in oxygen minimum zones is known to be fuelled almost entirely by organic matter supplied by particles sinking vertically from the euphotic zone (Anderson *et al.*, 2007; Ward *et al.*, 2008). In these environs, organic carbon is the main limiting factor controlling denitrification. In contrast, estuarine systems have considerable organic C loading and labile organic matter is readily available for metabolic activity. In such circumstances, denitrification is more dependent on nitrate availability. Similar observations have been made by Davidsson and Leonardson (1996) using peaty and sandy sediment in which NO_3^- has been shown to be a stronger regulator of denitrification than organic carbon.

Potential benthic denitrification rates at Divar were 15-38 times higher (within 0-10 cm core) than the *in situ* denitrification activity when both nitrate and organic carbon were in excess with highest activity of up to 304.09 (±47.6) μmol N_2O-N m^{-2} h^{-1} at the surface. Flemer *et al.* (1998) have reported relatively high potential denitrification rates in estuarine sediments ranging between 500 to 1000 μmol N m^{-2} h^{-1} which were also limited by nitrate availability. Laverman *et al.* (2007) have encountered maximum *in situ* rates two fold lower than the maximum potential rate in surficial sediment indicating that *in situ* denitrification was nitrate limited. These observations indicate that the surficial sediments have a comparatively higher denitrifying potential. This could be possible since the denitrifying communities at depths ≤4 cm get a continuous availability of electron acceptors through lateral supply from the ambient waters in addition to relatively

intense nitrification in the surficial sediments. Thus, these microbes are able to eliminate elevated nitrate concentration through the denitrification pathway.

The Divar mangrove sediments harbored sufficient amount of labile organic matter to support denitrification activity. Microcosm experiments have indicated that nitrate addition enhanced denitrification at *in situ* organic carbon concentration. The benthic denitrifying community in these habitats respond rapidly to episodic events of elevated nutrient supply by increasing the rate of nitrate removal through the denitrification pathway. Thus, these sediments could act as a sink for nitrate and this nutrient is more crucial in controlling denitrification activity. Potential denitrification rates were up to 38 times higher than *in situ* denitrification activity indicating that denitrification was an important process maintaining low concentration of nitrate and helping to maintain the water quality of the adjoining aquatic system.

5.6. Influence of bioturbation on denitrification

Nutrient-rich reducing habitats like mangroves are excellent locations for denitrification to occur. Though a few studies have quantified denitrification rates in mangrove sediments (Meyer *et al.*, 2005; Alongi *et al.*, 2005) and identified some of the factors limiting the process (Chiu *et al.*, 2004), the influence of bioturbation on denitrification in these regions is poorly understood. Benthic denitrification in coastal habitats is largely dependent on nitrate availability (Laverman *et al.*, 2007). The exchange of nutrients and oxygen is known to be facilitated by the feeding and burrowing activities of benthic macrofauna (Aller, 1980, Huttel 1990, Binnerup *et al.*, 1992; Sasaki *et al.*, 2003). In the present study, differences in sediment physical, chemical and biological parameters have been compared between faunated and de-faunated cores to ascertain the effect of bioturbation at different strata. Marked differences in the physico-chemical and biological characteristics of bioturbated and non-bioturbated sediment were observed. Firstly, the de-faunated core showed relatively lower redox potential as compared to natural sediments indicating reduced oxygenation. Recently, Pischedda *et al.* (2008) have shown increased sediment oxygen heterogeneity particularly in the presence of polychaetes which construct complex burrows. Stratified enumeration of macrofaunal abundance in the present study has shown a well dispersed macrofaunal community

represented by oligochaetes forming the dominant forms followed by polychaetes. Further, crabs belonging to *Uca* spp. were retrieved from 6-8 cm depth indicating that their burrowing activity creates channels that could facilitate the exchange of solutes between overlying water and sediments. The bioturbated sediments were also characterized by decrease in pH with depth. The Divar sediments have a considerable organic and metal content (Krishnan *et al.*, 2007; Krishnan and Loka Bharathi, 2009). In the absence of oxygen, alternate electron acceptors like Fe(III) and Mn(IV) support degradation of organic compounds (Lovley *et al.*, 2004) consequently leading to acidic condition (Ogner *et al.*, 2001) in the sediment. This explains the lower pH encountered in the bioturbated core.

Denitrification in particular is mainly affected by nitrate concentration (Morell and Corredor 1993; Corredor and Morell 1994; Rivera-Monroy and Twilley 1996; Kana *et al.* 1998). Earlier studies have shown that bioturbation enhances nitrate supply (Kristensen *et al.* 1991) and stimulates coupling of nitrification-denitrification (Aller *et al.*, 1983, Pelegri *et al.*, 1994). In the present investigation, pore water nitrate concentration in the bioturbated core was relatively higher as compared to the de-faunated core and is indicative of elevated nutrient supply in irrigated sediments. Nitrate can be supplied either from the ambient seawater or result from intrinsic nitrification (Krishnan *et al.*, 2008). Experiments by Svensson (1997) have shown that bioturbation mobilizes the ammonium to the water and stimulates denitrification by reducing the diffusive barrier blocking nitrate from reaching anoxic zones in the sediment. Downward movement of crabs and polychaetes in the sediments could induce sediment re-working facilitating the transfer of NO_3^- from the water column and also penetration of O_2, which in turn stimulates nitrification (Gilbert *et al.*, 1997). Unlike the de-faunated core, overall low nitrite levels in the bioturbated core especially at 4-6 cm coincided with high DNT activity. In the denitrification pathway, nitrite is further reduced to gaseous products like N_2O and N_2. Thus, high DNT enhances NO_2^- uptake preventing its accumulation to toxic levels. Autochthonous infauna can stimulate denitrification activity significantly by up to 160% in coastal sediments (Gilbert *et al.*, 1998) indicating that denitrification is directly dependent on macrofaunal activity. In this study, significant influence of macrofaunal abundance on DNT was evident at depth $\geq$4 cm ($p<0.05$) and was responsible for about

18% of the variation in the activity. Gilbert *et al.*, (1998) have reported benthic macrofaunal density varying from 2580 to 3160 ind. m^{-2} in the Gulf of Fos sediments. In this investigation, higher faunal density of organisms were observed at 0-2 and 4-6 cm (375 ind. m^{-2}). Lower contribution of the mangrove macrofauna in enhancing denitrification could be attributed to their overall low density and patchy distribution pattern in the sediment.

The exclusion of macrofauna in mangrove sediment was also seen to influence the distribution and abundance of the denitrifying community. Though denitrifier abundance in both the cores was same at the surface, their number decreased rapidly by up to two orders in de-faunated conditions especially between 2-6 cm. The 2-6 cm layers has a sizable fraction of polychaetes and *Uca* crabs. Bioturbating activity of these forms could be responsible for enhancing nitrate concentration at these depths which in turn stimulates denitrifier abundance as they are dependent on substrate availability. The presence of the marine polychaetes favour the development of bacteria which may play an active role in natural bioremediation processes (Cuny *et al.*, 2007). Further, sediment re-working of macrofauna also aids in the dispersion of denitrifiers to deeper depths as evident from higher number of cells at 8-10 cm in the bioturbated core. It can be concluded that bioturbating activity of autochthonous infauna in the Divar sediments could be one of the important factors influencing denitrification activity. Relatively higher redox potential, elevated nitrate supply, low nitrite accumulation and higher denitrifier abundance in natural sediments further substantiate the findings in the present study.

5.7. Taxonomic and functional diversity of denitrifiers

Very little is known about benthic bacterial diversity in productive coastal habitats like mangroves. So far, 16S rRNA gene analysis based studies have been used to assess their diversity in these regions (Sjöling *et al.*, 2005; Liang *et al.*, 2007; Gomes *et al.*, 2008) providing some knowledge of the bacteria therein. The technologically advanced 454 pyrosequencing approach was used to examine bacterial diversity in two mangrove ecosystems of Goa- the anthropogenically influenced Divar and the relatively pristine Tuvem. The present study showed that the surficial mangrove sediments (0-2 cm)

harbour all major sediment bacterial groups affiliated with the phyla Proteobacteria (classes Alpha, Beta, Gamma, Delta and Epsilonproteobacteria), Bacteroidetes, Firmicutes, Cyanobacteria, Planctomycetes, Actinobacteria, etc. Early studies in mangrove swamps have also shown predominant bacterial phylotypes to cluster within Proteobacteria, Bacteroidetes, Gemmatimonadetes, Actinobacteria and Firmicutes (Zhang *et al.*, 2009). Interestingly, this study reveals the existence of bacteria belonging to the newly devised phylum like Acidobacteria and Gemmatimonadetes and members of the candidate divisions OD1, OP3, OP8, OP10 and OP11 in mangrove sediments. According to the frequency of V6 tag occurrence, the Proteobacterial community at Tuvem was dominated by members of the class Deltaproteobacteria (21% of total V6 tags; up to 0.96% total OTUs), in particular Desulfobacterales and to a lesser extent by Actinobacteria (14% of total V6 tags). The identities of organisms provide clues on the bacterially-mediated processes likely to occur in these habitats. At Tuvem redox-potential of <-150 mV have been observed at the surface implying that the sediments are largely anaerobic as compared to Divar. Anoxic environments are known to be dominated by Deltaproteobacteria (Schwarz *et al.*, 2007). This class of bacteria have been reported to occur in coastal (Paisse *et al.*, 2008; Zhang *et al.*, 2008), continental shelf (Hunter *et al.*, 2006) as well as cold-seep sediments (Reed *et al.*, 2009). The Desulfobacterales have implications in sulfur cycling (Vrionis *et al.*, 2005; Borin *et al.*, 2009) as they are primarily involved in sulfate-reduction (Reed *et al.*, 2009) which is an important electron-accepting process for mineralization of carbon (Asami *et al.*, 2005; Scholten *et al.*, 2005). Earlier studies by Loka Bharathi *et al.*, (1991) have also shown the occurrence of SRB representing the orders Desulfovibrionales, Desulfobacterales, Desulfococcus and Clostridiales in mangrove swamps of the Zuari estuarine system in Goa. Other SRB sequences within the class Deltaproteobacteria found at Tuvem belonged to the orders Desulfuromonadales, Myxococcales and Synthrophobacterales. Some Desulfuromonadales can obtained energy from the reduction of Fe(III), Mn(IV), nitrate, elemental sulfur using a variety of electron donors like organic acids, alcohols, biological extracts and hydrogen (Greene *et al.*, 2009). Investigations in the present study have shown that the Tuvem sediments have a considerable organic carbon, Fe, Mn and nutrient (N compounds) content. It is possible that Deltaproteobacteria in this anoxic

habitat could not only participate in sulfur cycling but also prevent accumulation of metal and inorganic nitrogenous compounds. Members of the phylum Actinobacteria are ubiquitously found in estuarine and oceanic environments (Crump *et al.*, 2004; Piza *et al.*, 2004; Stach and Bull, 2005). Most Actinobacteria are of economic importance (Ward and Bora, 2006) as they are as a source of antibiotics (Ellaiah and Zeeck, 2006; Adinarayana *et al.*, 2006; Kim *et al.*, 2006; Manivasagan *et al.*, 2009). Like Deltaproteobacteria, they play multiple roles in the environment that includes degradation of cellulose (Pankratov *et al.*, 2006) and hydrocarbons (Harwati *et al.*, 2007; Kim *et al.*, 2008), metal oxidation (Bryan and Johnson, 2008; Johnson *et al.*, 2009), nitrate reduction (Van Keulen *et al.*, 2005), etc.. Their presence and activity in mangrove sediments could be vital in altering the benthic chemistry.

Rarefaction curves at both locations did not reach an asymptotic stage as the bacterial richness was not fully covered. Diversity estimates suggest that the bacterial community at the anthropogenically influenced site Divar is relatively more diverse than at Tuvem with ~3300 phylotypes which are an order of magnitude higher than previously reported (Zhang *et al.*, 2008) in marine sediments. A large number of low-abundance OTUs of the so called "rare biosphere" (Sogin *et al.*, 2006) were responsible for the high diversity observed in mangrove sediments and indicate that they have the potential to become dominant when favorable environmental conditions arise. The Divar ecosystem receives comparatively higher extraneously derived organic and inorganic compounds of different types. Consequently, availability of a wide variety of substrates could result in a higher taxonomic and metabolic bacterial diversity. The frequency of Proteobacterial tags at Divar formed >40% of total V6 tag sequences as in Tuvem. However, the Gammaproteobacteria dominated (2% higher tag occurrence than Deltaproteobacteria) at Divar as compared to Deltaproteobacteria at Tuvem. Molecular investigations by Liang *et al.*, (2007) in a Chinese mangrove ecosystem have also shown that the Gammaproteobacteria affiliated sequences constituted the largest portion in their clone library. The Gammaproteobacteria are active mediators of the N and S cycles. Gammaproteobacteria are also involved in methane oxidation (Sorokin *et al.*, 2000). Some of the Gammaproteobacteria recorded at Divar are classified under the order Alteromonadales, Chromatiales, Pseudomonadales, Vibrionales, Enterobacteriales,

Salinisphaerales etc.. Species belonging to the genus *Marinobacter*, *Shewanella*, *Alteromonas* of order Alteromonadales have been commonly found in the marine environment (Rontani *et al.*, 1999; Zhuang *et al.*, 2009). *Marinobacter spp.* use a variety of hydrocarbons as the sole source of carbon and energy (Gauthier *et al.*, 1992). These halophilic bacteria also have a high capacity for denitrification (Yoshie *et al.*, 2006). Similarly, *Shewanella* (Brettar *et al.*, 2002; Zhao *et al.*, 2006) and *Alteromonas* (Haijun, 2002) are known to mediate reductive phase of the N cycle. Facultatively anaerobic species of the order Pseudomonadales (Carlson and Ingraham, 1983; Castignetti and Hollocher, 1984; Rezaee *et al.*, 2008), Vibrionales (Yoshinari, 1980; Bianchi *et al.*, 1992; Kim *et al.*, 2000), Enterobacteriales (Bezbaruah 1983, Calmels *et al.*, 1996; Herbert, 1999) etc.. have been reported to participate in both oxidative and reductive phases of the N cycle. The Divar ecosystem is prone to high nutrient input from mining rejects (De Souza, 1999) predominantly during the monsoon (Divya *et al.*, 2009). Pore water analysis in the present study have shown measurable concentrations of inorganic nitrogenous compounds. However, pore water ammonium was <50 μmol l^{-1} throughout the year at Tuvem and Divar indicating close coupling between synthetic and degradative processes. The existence of metabolically versatile bacteria could indicate their contribution to the C and N cycle. The presence of a large number of enteric Gammaproteobacteria reflect their terrestrial origin in the Divar mangrove ecosystem. The micro-aerophilic purple sulfur bacteria (order Chromatiales) capable of photosynthesis (Antony and Philip, 2006) have also been recorded at Divar suggesting their importance in the microbial food web. A significant fraction of bacteria in this habitat have not been identified up to the class level indicating that the mangrove sediments harbour novel bacterial species of scientific value that might play a more dominant role than believed hitherto.

Denitrification is mediated by heterotrophic anaerobic facultative bacteria which can use nitrate or nitrite as a terminal electron acceptor for respiration and reduce it to nitrous oxide or nitrogen (Desnues *et al.*, 2007). In marine ecosystems, a variety of taxonomically unrelated bacterial groups are capable of denitrification. Of these, 96% of cultured denitrifiers belong to the Gammaproteobacteria (Brettar *et al.*, 2001). Biochemical characterization of denitrifier strains carried out in the present study have shown that in addition to Firmicutes (class Bacilli) and Actinobacteria, the denitrifies

belonged to the classes Alpha, Beta and Gammaproteobacteria establishing that denitrifiers in mangrove systems are polyphyletic. Bacteria belonging to these classes are known to catalyze carbon and nitrogen cycling as discussed above. The Gammaproteobacteria constituted nearly 50% of all the denitrifying strains at Divar. The pyrosequencing approach too has shown the occurrence of high Gammaproteobacteria tags in this region suggesting that they could be primarily contributing to the reductive phase of the N cycle. Undoubtedly, the pyrosequencing approach reveals that a vast majority of the denitrifiers from mangrove sediments remain unculturable. Only a few viable phylotypes make their appearance in enriched medium and can be cultured under laboratory conditions.

The functional gene *nosZ* encoding for nitrous oxide reductase, an enzyme catalyzing the final step of denitrification (Scala and Kerkhof, 1999) was used for determining the diversity of denitrifiers (Horn *et al.*, 2006). The *nosZ* derived clones in marine sediments are affiliated to the class Alphaproteobacteria (Hunter *et al.*, 2006; Magalhaes *et al.*, 2008). At the two sites investigated, the *nosZ* community was found to be resilient and was represented by members belonging to class Alpha, Beta and Gammaproteobacteria. The dominant denitrifiers at the control site were represented by strains showing close similarity to *Shewanella* spp. which are known to show considerable respiratory versatility. *Shewanella* spp. can use a wide range of compounds as electron acceptors (Tiedje, 2002, Munn, 2004). Oxygen fluctuation during tidal cycles and diffusion of nutrients from overlying water could favor proliferation of versatile bacteria capable of using alternate electron acceptors.

The present study shows that both biochemical characterization of denitrifiers and the functional gene phylogeny give a fair understanding of the major bacterial groups involved in N transformations in mangrove sediments as they have also been encountered in the 454 pyrosequencing approach. For the first time, this study provides insights on the yet uncultured and ecologically significant bacterial communities in mangrove sediments. Overall, diversity in anthropogenically influenced estuarine zones is higher than in relatively pristine locations. Prevailing environmental conditions could be crucial in influencing the composition of sediment bacterial communities. Large physico-chemical gradients in these systems can lead to the formation of complex microbial communities

which play a vital role in ecosystem functioning. Both culturable and molecular approaches adopted to reveal the taxonomy of dominant denitrifying communities in tropical mangrove sediments indicates that the process is mainly carried out by members belonging to different classes of the phylum Proteobacteria.

Chapter 6

Summary & conclusion

Chapter 6. Summary and conclusion

The study on the "*Ecology of denitrifiers in mangrove sediments*" elucidates the role of environmental parameters in governing the reductive phase of the N cycle in two mangrove ecosystems of Goa, India- the anthropogenically influenced Divar and the relatively pristine site Tuvem. Field and lab based experiments were carried out to meet the following objectives:

- To quantify the abundance and activity of denitrifying bacteria
- To understand the influence of environmental parameters on denitrification
- To identify the denitrifiers at cellular and molecular level
- To delineate the influence of bioturbating organisms on denitrification

Down-core investigations were carried out at 2 cm intervals within 0-10 cm depth range at both the locations. Some of the salient findings from this study are as follows:

1. Denitrifier abundance in mangrove sediments varied from 10^{5-8} cells g^{-1} and constituted an important fraction of the total bacterial community (10^{9-10} cells g^{-1}) in mangrove sediments. Maximum denitrifier abundance by both culturable and molecular methods showed higher abundance at deeper depths (within 4-8 cm) at both the sites.
2. Denitrification activity (DNT) was found to be maximum within 0-4 cm at both the sites suggesting that the activity depends on the prevailing environmental conditions and is not a function of bacterial biomass.
3. DNT at Divar was nearly 3 times the value at Tuvem with maximum activity of 224.51 nmol g^{-1} h^{-1} observed at 0-2 cm. Other co-occurring processes in the N cycle were also measured in conjunction with denitrification. Highest anammox activity of 101.15 nmol N_2 g^{-1} h^{-1} at Divar was recorded at 8-10 cm and was 5 times higher than at Tuvem. Di-nitrogen fixation was minimal in estuarine habitats prone to high nitrate inputs and denitrification rather than anammox served as an important mechanism for counteracting N loading.
4. Alternate respiratory pathways like dissimilatory nitrate reduction to ammonium (DNRA) removes up to 3 times more nitrate than DNT resulting in N retention.

5. DNT results in a significant flux of nitrous oxide (N_2O), a potent green house gas. Microcosm studies showed that net nitrous oxide production at Divar occurred at a maximum rate of 22 μmol N_2O-N m^{-2} h^{-1} which was 3 times higher than at Tuvem and is indicative of higher emission of the radiative gas in anthropogenically influenced regions.
6. Among the environmental factors influencing DNT, nitrate had a larger influence ($n=15$; $p<0.001$) than organic carbon suggesting that mangrove sediments are NO_3^- limited and these regions could act as a sink for nitrate. Multiple regression analysis showed that Fe and Mn also influenced DNT which is indicative of DNT coupled to metal oxidation.
7. Bioturbating infauna were responsible for only 18% of the variation in DNT. The little influence on the process is attributed to the low and patchy distribution of macrofauna in the sediments.
8. Culturable methods have shown that up to 43% of culturable denitrifiers belonged to Gammaproteobacteria.
9. The dominant denitrifier community probed based on the functional gene (*nosZ*) phylogeny showed that they belonged to the sequences of uncultured organisms and were clustered within phylum Proteobacteria. However, it is possible that some of these genes belong to the culturable counterparts.
10. Analysis of bacterial diversity using the 454 pyrosequencing technology revealed a complex and rich bacterial community in mangrove sediments with ~3300 phylotypes recorded at Divar. The phylum Proteobacteria was the most dominant phylum at both the locations. The class Deltaproteobacteria dominated the Tuvem sediments while the Gammaproteobacteria were more dominant at Divar. Deltaproteobacteria include most of the sulfur cycle bacteria whereas the Gamma and Alphaproteobacteria are involved in N cycling. The existence of these bacteria in mangrove sediments reflects their ability to thrive on reduced substrates and could therefore play an important role in altering the chemistry of inorganic N compounds in coastal ecosystems.

11. Though benthic DNT in mangrove ecosystems serves as an important mechanism for counteracting N loading, it can be concluded that these habitats effectively conserve N through the DNRA pathway thereby minimizing nutrient loss that would otherwise occur through DNT. Most importantly DNRA contributes to minimizing the flux of green house gas N_2O to the atmosphere.

Chapter 7

Implication, application

&

Future scope

Chapter 7. Implication, application and future scope

Implication and application

- Mangrove systems overcome N limitation by effectively conserving N through the dissimilatory nitrate reduction to ammonium (DNRA) pathway thereby minimizing nutrient loss that would otherwise occur through denitrification (DNT). As the reduction of nitrate through the DNRA pathway in anoxic sediments could be coupled to the oxidation of reduced forms of sulfur, the process contributes to lowering levels of toxic ions in the system. Most importantly this process contributes to minimizing the emission of the potent radiative gas N_2O to the atmosphere.

- In estuarine habitats prone to high nitrate inputs, DNT overrides other co-occurring processes like anammox. Consequently, the process serves as an important mechanism for counteracting N loading.

Future scope

1. Examination of oxidizing and reducing processes of N cycle could be carried out in tandem to elucidate spatial/temporal coupling at the genetic, cellular and community level.

2. Mn/Fe coupled denitrification in mangrove sediments could be examined to gain deeper understanding of their contribution to the reductive phase of the N cycle.

3. Nitrous oxide flux across the sediment - atmosphere interface could be quantified to enhance the contribution of mangrove ecosystem to N_2O inventory from the Indian Ocean region.

4. Contrary to our understanding that pristine mangrove habitats have a higher bacterial diversity, taxonomic investigations in the present study have shown

anthropogenically influenced mangrove sediments to contain more complex and diverse bacterial communities. It would also be interesting to examine if the metabolic diversity also follows a similar trend.

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APPENDIX

Appendix

APPENDIX I

The redox electrode was calibrated using solutions A and B.

Solution A:

Potassium ferrocyanide $K_4Fe(CN)_6.3H_2O$; 4.22 g: 0.1M

Potassium ferricyanide $K_3Fe(CN)_6$; 1.65 g: 0.05M

Add 50 ml d/w in volumetric flask. Transfer weighed chemicals and stir to dissolve. Dilute to volume with d/w.

Solution B:

Potassium ferrocyanide $K_4Fe(CN)_6.3H_2O$; 0.42 g: 0.01M

Potassium ferricyanide $K_3Fe(CN)_6$; 1.65 g: 0.05M

Potassium fluoride $KF.2H_2O$; 3.39g 0.36M

Add 50 ml d/w in volumetric flask. Transfer weighed chemicals and stir to dissolve. Dilute to volume with d/w.

Transfer solutions to beaker. Place the electrode in the solution and wait until reading stabilizes. The potential should be about 192 mV (solution A) while for solution B it is about 256 mV. Thus, B-A=66 mV.

APPENDIX II

1. **Winkler's A:** Manganese (II) chloride (3M: reagent grade): Dissolve 100 g of $MnCl_2 4H_2O$ in 100 ml distilled water. After complete dissolution, make the solution up to a final volume of 1 litre with distilled water and then filtered into an amber plastic bottle for storage.

2. **Winkler's B:** Sodium iodide (4M: reagent grade) and sodium hydroxide (8M: reagent grade): Dissolve 600 g NaI in 600 ml of distilled water. If the color of solution becomes yellowish brown, discard and repeat preparation with fresh reagent. While cooling the mixture, add 320 g NaOH to the solution, and make up

the volume to 1 liter with distilled water. The solution is then filtered and stored in an amber glass bottle.

3. **Sulfuric Acid (50% v/v)**: Slowly add 500 ml of reagent grade concentrated H_2SO_4 to 500 ml distilled water. Cool the mixture during addition of acid.

4. **Starch indicator solution:** Place 1.0 g of soluble starch in a 100 ml beaker, and add a little distilled water to make a thick paste. Pour this paste into 1000 ml of boiling distilled water and stir for 1 minute. The indicator should be stored in a refrigerator.

5. **Sodium Thiosulfate Stock solution (0.18 M: reagent grade)**: Dissolve 45 g $Na_2S_2O_3*5H_2O$ and 2.5g sodium borate, $Na_2B_4O_7$ (reagent grade) for a preservative, in 1 liter of distilled water. This solution is stored in a refrigerator and used to make the working thiosulfate solution. Some variation on the method call for a 0.1N solution, which can be purchased ready-made.

6. **Sodium Thiosulfate working solution (0.018 M: reagent grade)**: Bring 100 ml of the sodium thiosulfate stock solution to 1000 ml with distilled water in a 1 liter volumetric flask. This solution is stored in a refrigerator and used for titrations. If a ready-made 0.1 N solution was used for the stock, a working solution of 0.01 N will be fine.

7. **Potassium Iodate Standard (0.00167 M = 0.01 N: analytical grade)**: Dry the reagent in a desiccator under vacuum. Weigh out exactly 0.3567 g KIO_3 and make up to 1.0 liter with distilled water. It is important to note the temperature of the solution so that a precise molarity can be calculated.

APPENDIX III

Reagents for ammonium estimation:

Phenol nitroprusside:

Solution A: 10 gm of phenol in 100 ml of 95% ethanol.

Solution B: 0.6 gm of sodium nitroprusside dehydrate in 100 ml of distilled water.

Mix both 100 ml of Solution A and 100 ml of Solution B. (light sensitive, should be freshly prepared)

Sodium hypochlorite:

4 ml of hypochlorite in 100 ml of 0.5 N NaOH.

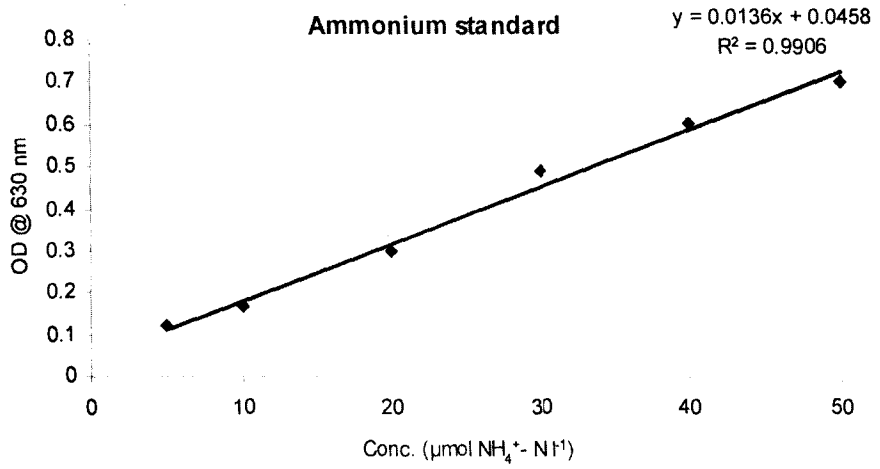
Trisodium citrate:

Dissolve 120 gm of trisodium citrate in 250 ml of distilled water. Add 5 ml of 0.8 N NaOH (stable indefinitely).

Ammonium standard:

NH_4Cl sock solution (1 mM): 0.05349 g of NH_4Cl dissolved in 1000 ml of distilled water.

Working standard: prepare the standard up to 50 ml of different concentration.



Determination of ammonium in sample:

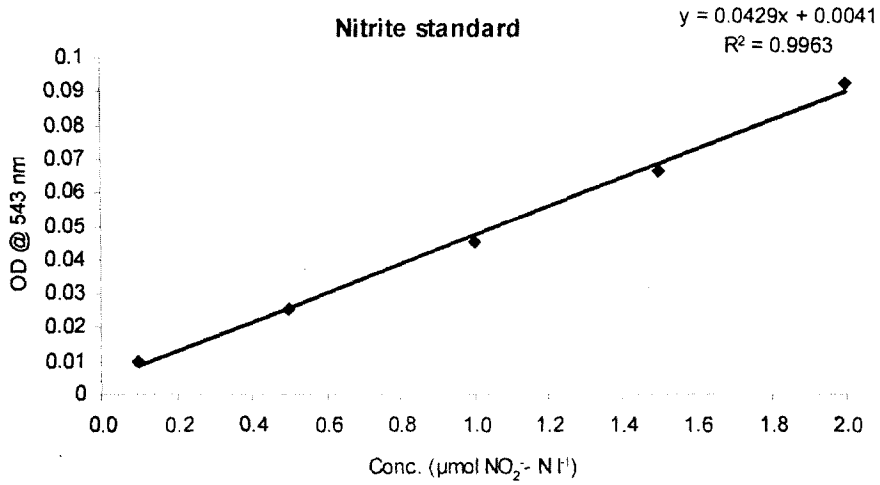
For 5 ml of sample, add 200 µl of phenol nitroprusside solution and mix it well, then add 100 µl of trisodium citrate, mix it and 200 µl of hypochlorite reagent. Incubate in dark for 6 hr and measure the Optical density (OD) at 630 nm.

APPENDIX IV

Nitrite standards

Sodium nitrite stock solution: 0.0345 gm of sodium nitrite is dissolve in 100 ml of distilled water.

Working standard: from the stock solution 1 ml is taken and the volume made up to 100 ml with distilled water. Prepare standard solutions containing 0, 0.1, 0.5, 1, 1.5 and 2 µg at. NO_2- N l^{-1}.



Determination of nitrite

For 5 ml of nitrite sample, add 100 µl of sulphanilamide solution. Incubate for 2 – 8 min then add 100 µl of N – (1 - Napthyl) – ethylene diamine dihydrochloride. Appearance of pink colour is conformation of nitrite in sample. Calculate the nitrite concentration by checking the OD at 543 nm by spectrophotometer.

APPENDIX V

Reagents for determination of nitrate

1% sulphanilamide:

1g sulphanilamide in 10 ml concentrated HCl, made upto 100 ml with distilled water.

N(1-naphthyl)ethylenediamine dihydrochloride:

100mg N(1-naphthyl)ethylenediamine dihydrochloride in 100 ml distilled water.

Preparation of cadmium column

Mercuric chloride solution:

Add 2.5 gm of mercuric chloride in 250 ml of distilled water.

Cadmium fillings:

Cadmium fillings cut into 3 mm size.

Preparation:

Weigh about 50 fillings and stir with mercuric chloride solution until clear solution changes black. Wash fillings with distilled water. Push glass wool at bottom of the column. Fill column with distilled water, add filling by tapping gently, pack without air bubble and spaces should be minimum. Put glass wool plug at the top. Wash column with distilled water. Adjust flow rate such that 100 ml passes through 8 – 12 min. column should be left completely covered with distilled water.

Nitrate standard

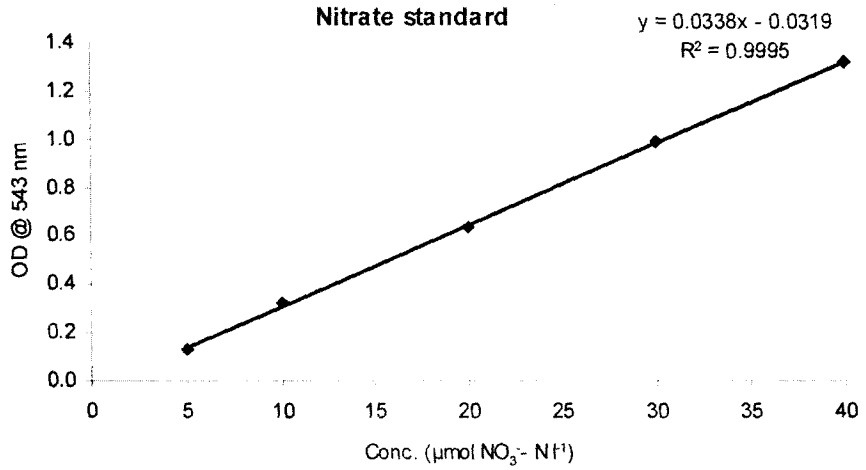
Potassium nitrate stock solution: (1mM NO_3^- N l^{-1}): 5.05 gm of potassium nitrate in 50 ml of distilled water (1M KNO_3). Pipette 50 µl of 1M solution and make up volume with distilled water to 50 ml.

Working standard: From the stock solution 0 µl, 250 µl, 500 µl, 1 ml, 1.5 ml and 2 ml is taken and volume is made up to 50 ml of distilled water to prepare standard solutions containing 0, 5, 10, 20, 30, 40 and 50 µg at. NO_3^- N l^{-1}.

Reduction:

Pass 10 ml through the cadmium column twice. Then pour the rest 30 ml. Discard the initial 15 ml and collect approx. 15 ml sample required for nitrate analysis. Add 200 µl of

sulphanilamide in 5 ml of sample, wait for 5 min, and then add 200 µl of N – (1 - napthyl)- ethylene diamine dihydrochloride. Take OD between 10 min to 2 hrs at 543 nm.



Determination of nitrate in sample

Pass 10 ml through the cadmium column twice. Then pour the rest 30 ml. Discard the initial 15 ml and collect ~ 15 ml sample required for nitrate analysis. Add 200 µl of sulphanilamide in 5 ml of sample wait for 5min and then add 200 µl of N- (1-napthyl)-ethylene diamine dihydrochloride. Take OD between 10 min to 2 hrs at 543 nm.

APPENDIX VI:

Culture medium for nitrate reducers:

KNO_3	0.101 g (1 mmol)
Nutrient Broth (*HiMedia laboratories*)	6.5 g
Phenol red	0.01 g
Agar	5 g
Aged seawater	1000 ml

APPENDIX VII:

Culture medium for denitrifiers:

KNO_3	0.101 g
NH_4Cl	0.05 g
Sodium acetate	0.1 g
Sodium succinate	0.1 g
Bacto Tryptone	1 g
Aged seawater	1000 ml

Adjust pH to 8.

APPENDIX VIII:

Morphological tests:

Gram staining (Gram, 1884)

- Smear of the isolates was prepared on clean dry grease free slides.
- The smears were air-dried and heat fixed.
- They were then treated with crystal violet for 1 min followed by Gram's iodine for 1 min.
- The slide was then washed with decolorizing solution (ethyl alcohol) till the blue color disappears.
- Counter stained with safranine for 30 sec. The slide was then washed with water.
- Dried and observed under oil-immersion

Oxidase test (Kovacs, 1956)

Oxidase discs (*HiMedia*, India) were used to detect the presence of cytochrome oxidase in the isolates.

Observation	Report
1. Deep violet color developed Immediately after smearing.	Oxidase positive
2. Deep violet color developed after 30 sec.	Delayed positiveness

3. No color change. Oxidase negative

Catalase test

- This test was performed using 3 % hydrogen peroxide on a glass slide.
- Scrape the growth from a slant or plate with a non- metallic instrument.
- Suspend it in 3 % hydrogen peroxide on a slide.
- Examine for effervescence, presence of effervescence denotes catalase positive and absence denotes negative reaction.

Marine Oxidation /Fermentation (MOF) test: -

Test medium was prepared as follows:

Sea water- 100 ml

Dextrose- 1 g

Peptone- 0.2 g

Agar- 1.5 g

K_2HPO_4- 0.03 g

Bromothymol blue- 0.002 g

Combine Peptone, K_2HPO_4, Bromothymol blue. Adjust pH of medium to 7.2. Then add agar and boil to melt. Add Dextrose, pour into tubes, autoclave and allow to cool and solidify. Inoculate the tubes with test culture using a nichrome stab and incubate.

Observe for color change of the medium and interpret results as follows:

1. Bottom to top yellow/bottom yellow — Fermentative (with or w/o gas)
2. Yellow only on top — Oxidative
3. Blue colour — Alkaline
4. Growth, no colour change — Growth only
5. No growth — Inert

Motility test

Prepare wet smear of the culture on slide and observe under microscope (100X).

APPENDIX IX:

Agarose gel (1%):

Agarose	0.5 g
1X TBE	49.5 ml
Ethidium bromide	1µl

APPENDIX X:

Preparation of 1X TBE buffer:

Add 10.8 g Tris-(hydroxymethyl) aminomethane in 500 ml distilled water. Add 5.5 g boric acid, 4 ml sodium ethylenediamine tetraacetic acid (Na_2EDTA; 0.5 M). Make up volume to 1000 ml. The solution is stable for a maximum of 3 months.

APPENDIX XI:

PREPARATION of 0% and 80% acrylamide solutions

0% solution (100 ml):

-	40% acrylamide/bis 37.5 solution	15 ml
-	TAE 50 X	2 ml
-	Distilled water	83 ml

Blend the above with a magnetic stirrer for 5 - 10 minutes. Using a syringe, filter contents through a 0.2 µm filter. Refrigerate filtrate at (4°C) in a plastic bottle protected from light (covered with aluminium foil). The product is stable for a maximum of 3 months.

80% solution (100 ml):

-	40% acrylamide/bis 37.5 solution	15 ml
-	TAE 50 X	2 ml
-	Formamide de-ionized	32 ml
-	Urea	33.6 g
-	Distilled water	18 ml

Blend the above with a magnetic stirrer for 5 - 10 minutes. Using a syringe, filter contents through a 0.2 µm filter. Refrigerate filtrate at (4°C) in a plastic bottle protected from light (covered with aluminium foil). The product is stable for a maximum of 3 months.

Preparation of gel for Denaturing Gel Gradient Electrophoresis (DGGE):

Bottom gel:

- 1 ml acrylamide 0%
- 30 µl Ammonium Per Sulfate (APS) 10%
- 2.5 µl TEMED(N,N,N',N' -tetramethylenediamine)

} 1 hour polymerisation

Introduce the gel between the glass plates with the help of a 1 ml pipette. See that the gel spreads evenly and overlay it immediately with 2 - 3 ml of distilled water so that the gel is set uniformly. Before casting the gradient gel, invert the DGGE gel unit and soak the water onto a tissue paper.

Gradient migration gel 20-80%:

- 20% Solution
  - 8.2 ml acrylamide 0%
  - 2.8 ml acrylamide 80%
  - 30 µl Ammonium Per Sulfate (APS) 10%
  - 7 µl de TEMED
- 80% Solution
  - 11 ml acrylamide 80%
  - 30 µl Ammonium Per Sulfate (APS) 10%
  - 7 µl de TEMED

} 3 hours polymerisation

Mix the contents of the 20% solution in a beaker using a magnetic stirrer. Similarly, mix the contents of the 80% solution in another separate beaker. Transfer the contents from the two beakers into the gel unit using a mechanical pump. Overlay the gel with distilled

water for uniform alignment. Before adding the topmost gel, remove the water by soaking it onto a tissue paper.

Top gel:

- 5 ml acrylamide 0%
- 30 µl Ammonium Per Sulfate (APS) 10%
- 5 µl de TEMED

} 1 hour polymerisation

Introduce the gel with the aid of a 5 ml pipette. Before arriving absolutely in the top of the glass, introduce the comb and add the remaining gel. Restrict the formation of air bubbles.

NOTE:

- Store TEMED (*Bio-Rad*) solution at 4°C.
- Store sub-samples of 10% APS in eppendorf tubes at -20°C.

APPENDIX XII:

Preparation of 50X TAE buffer (stock solution):

Add 242 g Tris base and dissolve in approximately 750 ml deionised water. Carefully add 57.1 ml glacial acid and 100 ml of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 litre. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.

Preparation of 1X TAE buffer (working solution):

The working solution of 1x TAE buffer is made by diluting the stock solution by 50x in de-ionized water. Final solute concentrations are 40 mM Tris acetate and 1 mM EDTA. The solution is stable for a maximum of 3 months.

APPENDIX XIII:

Composition of Buffers and Solutions for cloning

Isopropyl b-D-1-thiogalactopyranoside (IPTG) stock solution (0.1M)

1.2 g IPTG

Add water to 50 ml final volume. Filter sterilize and store at 4°C.

X-Gal (2 ml)

100 mg 5-bromo-4-chloro-3-

Indolyl-β-D-galactoside

Dissolve in 2 ml N, N′-dimethylformamide.

Cover with aluminum foil and store at –20°C.

LB medium (per liter)

10 g Bacto®-tryptone

5 g Bacto®-yeast extract

5 g NaCl

Adjust pH to 7.0 with NaOH

LB plates with ampicillin

Add 15 g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100 μg ml^{-1}. Pour 30–35 ml of medium into 85 mm Petri dishes. Let the agar harden. The plates can be stored at 4°C for up to 1 month or at room temperature for up to 1 week.

LB plates with ampicillin/IPTG/X-Gal

LB plates with ampicillin were made as above; then supplement with 0.5 mM IPTG and 80 μg ml^{-1} X-Gal and the plates were poured. Alternatively, 100 μl of 100 mM IPTG and 20 μl of 50 mg ml^{-1} X-Gal may be spread over the surface of an LB ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

SOC medium (100 ml)

2.0 g Bacto®-tryptone

0.5 g Bacto®-yeast extract

1 ml 1M NaCl

0.25 ml 1M KCl

1 ml 2M Mg^{2+} stock, filter sterilized

1 ml 2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile, distilled water. The final pH should be 7.0.

2M Mg^{2+} stock

20.33 g $MgCl_2 \cdot 6H_2O$

24.65 g $MgSO_4 \cdot 7H_2O$

Add distilled water to 100 ml. Filter sterilize.

2X Rapid Ligation Buffer, T4 DNA

Ligase (provided)

60 mM Tris-HCl (pH 7.8)

20 mM $MgCl_2$

20 mM DTT

2 mM ATP

10% polyethylene glycol

(MW8000, ACS Grade)

Store in single-use aliquots at –20°C. Avoid multiple freeze-thaw cycles.

List of publications

List of publications

(*Manuscripts from thesis)

1. **Fernandes S.O.**, Krishnan K.P., Khedekar V. D., Loka Bharathi P.A. 2005. Manganese oxidation by bacterial isolates from the Indian Ridge System. *Biometals* 18:483–492. **(IF 1.704)**

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4. Krishnan K.P., **Fernandes S.O.**, Loka Bharathi P.A., Krishna Kumari L., Nair S, Pratihary A.K., Ramalingeswara Rao B. 2008. Anoxia over the western continental shelf of India: Bacterial indications of intrinsic nitrification feeding denitrification. *Marine Environmental Research* 65: 445-455. **(IF 2.032)**

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7. **Fernandes S.O.**, Bonin P.C., Michotey V.D., Loka Bharathi P.A. 2010. Denitrification: An important pathway for nitrous oxide production in tropical

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8. **Fernandes S.O.,** Loka Bharathi P. A. 2010. Nitrate levels modulate denitrification activity in tropical mangrove sediments (Goa, India). *Environmental Monitoring and Assessment* (DOI: 10.1007/s10661-010-1375-x; **IF 1.356**). *

9. Antony R., Sujith P.P., **Fernandes S.O.**, Verma P., Khedekar V.D., Loka Bharathi P.A. 2010. Cobalt immobilization by manganese oxidizing bacteria from Indian Ridge system. *Current Microbiology* (In press; **IF 1.33**).

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2. **Fernandes S.O.,** Michotey V.D., Guasco S., Bonin P.C., Loka Bharathi P.A. Denitrification prevails over anammox in tropical mangrove sediments (Goa, India): indications from preliminary insights.*

3. **Fernandes S.O.**, Kirchman D.L., Loka Bharathi P.A. Gammaproteobacteria dominate Deltaproteobacteria in anthropogenically influenced tropical mangrove sediments (Goa, India).*

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Publications from thesis

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Bacterial contribution to mitigation of iron and manganese in mangrove sediments

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Abstract

The Mandovi and Chapora are two tropical estuaries lying in close geographic proximity on the west coast of India. Seasonal changes in down core variation of Fe, Mn and Total Organic Carbon (TOC) in the mangrove sediments adjoining these estuaries were studied to assess their influence on some of the representative benthic bacteria belonging to heterotrophic and autotrophic groups. Heterotrophic bacteria (HB) cultured on different nutrient concentrations (0.01%, 0.1% and 25%) together with nitrifiers (NtB; representating autotroph) were chosen to assess the influence of the above-mentioned abiotic parameters on the former. The experimental site located along the Mandovi is under the influence of extensive ferromanganese ore mining, while the control site at Chapora is relatively free from such influences. Geoaccumulation index computed for Mandovi showed that sediments (0–10 cm) were 'uncontaminated to moderately contaminated' by Fe during the pre monsoon and monsoon seasons, while in the post monsoon season the 4–10 cm fraction was almost completely restored from contamination. Similar computations for Mn showed that in pre monsoon, sediments fell in the 'moderately contaminated' and 'moderately to strongly contaminated' categories, while in the monsoon and post monsoon seasons all the sections were 'Uncontaminated'. The difference observed in correlation between Fe and Mn with the various fractions of heterotrophs and nitrifiers indicated that though these two elements shared a similar chemistry in the environment, microbes involved in biogeochemical processes might prefer them differentially. The relationship between TOC and HB enumerated on 0.01% dilute nutrient agar remained at $r = 0.50$, $p < 0.05$ throughout the year. Hence, it could be apparently linked to their preferred concentration of organic carbon requirement. A relationship of $r = 0.61$, $p < 0.01$ between manganese concentration and heterotrophs recovered on different strengths of nutrient agar is suggestive of their response to the metal enrichment. They could thus contribute towards maintaining the level of Mn at par with reference levels at Chapora. A positive correlation between Mn with NtB ($n = 10$, $p < 0.05$, $r = 0.58$) at the experimental site during the non-monsoon months is suggestive of the latter's contribution to regulation of the metal concentration in the sediment probably through anaerobic nitrification at the expense of manganese. The study therefore supports our hypothesis that both autochthonous autotrophs and heterotrophs work in tandem to mitigate concentration of Mn and related metals in mangrove sediments.

Keywords: Iron; Manganese; Mangroves; Heterotrophs; Nitrifiers; Geoaccumulation

1. Introduction

The Mandovi and Chapora are two tropical estuaries lying in close geographic proximity to each other on the west coast of India, but the latter is relatively distanced from pollution. The hydrological characteristics of these two estuarine systems are governed by the monsoon regime. The physical characteristics of the Mandovi and Chapora estuaries have been described earlier (Varma and Rao, 1975; Varma and Cherian, 1975; Murthy et al., 1976). The estuarine channel of the Mandovi is used to transport large quantities of ferromanganese ores from mines located upstream to the Marmagao harbour (Arabian Sea), while the Chapora is free from movement

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of ferromanganese ore bearing barges. Lush mangrove vegetation fringes both of the estuarine systems. Though the impacts of iron ore processing on the surface sediments of the Mandovi estuary have been documented earlier by Alagarsamy (2006), its influence on the benthic bacterial flora is sparsely understood.

In this study, an attempt has been made to understand the downcore distributory patterns of heterotrophic and autotrophic bacteria together with total Fe and Mn during monsoon and non-monsoon periods. Retrievable heterotrophic bacteria were enumerated on various nutrient strengths to estimate the various fractions requiring different carbon and nitrogen requirements. Nitrifiers were enumerated as a representative autotrophic community as they are one of the most important physiological groups in this ecosystem responsible for converting ammonia to its oxidized forms.

Geoaccumulation index (I_{geo}, Müller, 1979) was used to assess the impact of mining on the mangrove sediments. Although I_{geo} was originally devised for use with global standard shale values as background metal levels, Rubio et al. (2000) have shown the use of regional background values to give more appropriate results. In this study, I_{geo} has been calculated using regional metal concentrations in Chapora mangrove sediments. A comparison between the Mandovi and Chapora mangrove sediments was therefore made to assess the probable influence of mining on bacterial groups in this region. We hypothesize that bacterial populations in the mangrove sediments could play an important role in preventing the concentration of the metal species from accumulating.

2. Materials and methods

2.1. Site description and sampling procedure

Sediment cores were collected from fringing mangrove forests along the Chapora and Mandovi estuaries. The control site in the Chapora estuary was located at Tuvem, whereas the Divar mangrove ecosystem was selected as the experimental site along the Mandovi estuary (Fig. 1). The experimental site is under the influence of ferromanganese ore mining. Samples were collected during the months of April (Pre Monsoon), July (Monsoon) and October (Post Monsoon) of 2005 using a PVC hand-held sediment corer. Sub samples were taken at 2 cm intervals from the surface to 10 cm by carefully sectioning the core in the lab.

2.2. Chemical analyses

Organic carbon was determined by the wet oxidation method with a precision of 0.01% (El Wakeel and Riley, 1957). Sub samples for metal analyses were dried at 60(±2) °C for 48 h and disaggregated in an agate mortar before chemical treatment for Fe and Mn analysis. For each sample, a known quantity (~0.2 g) of sediment was digested in a teflon vessel with a solution (10 ml) of concentrated HF (48% GR; Merck), HNO_3 (69% GR; Merck) & $HClO_4$ (35% GR; Merck) in a ratio of 7:3:1. The sediment was then dried on a hot plate in a fume hood chamber.

An aliquot of 5 ml of the above acid mixture was added and dried on the hot plate for 1 h. Further, 2 ml of concentrated HCl (35% GR; Merck) was added, followed by 10 ml of HNO_3 (69% GR; Merck). The residue was warmed and then transferred to a clean, dry standard flask to make a final volume of 100 ml with double distilled water. The detailed procedure of sediment digestion is given in Balaram et al. (1995). Trace metal concentrations (Fe and Mn) were measured using a flame atomic absorption spectrophotometer (AAS, PerkineElmer Model 5000). The accuracy of the analytical procedures was assessed using the certified reference material MAG-1 (USGS) that yielded results within the reference value range (Flanagan, 1967, 1976).

2.3. Microbial analyses

Sediment core was sectioned at 2 cm intervals in sterile conditions to obtain representative samples at 0–2, 2–4, 4–6, 6–8 and 8–10 cm depths. Sub samples of approximately 5 g wet weight sediment were sampled using sterile syringe cores. The sub samples were transferred to 45 ml of full strength sterile seawater (10^{-1} dilution). Tween80 (50 μL) was added and the mixture was sonicated at 40 mHz for 10 s. Serial dilutions of the sediment samples were made in autoclaved seawater to yield dilutions from 10^{-1} to $10^{-6/7}$.

Medium for the isolation of heterotrophic bacteria (HB) was prepared using various nutrient strengths of 0.01, 0.1 and 25%, which correspond to 0.01%, 0.1% and 25% nutrient broth + 2% agar, respectively. A concentration of 100% corresponds to 8 g nutrient broth (HiMedia Laboratories Pvt. Ltd., Bombay, India) per 1000 ml seawater. About 100 μl from 10^{-2} dilution was plated onto the medium. The plates were incubated for 15 days at room temperature. Bacterial counts in the form of colony forming units (CFU) formed on the medium were recorded after a 15-day incubation period at 28(±1) °C. Dry weight of the sediment used for the dilutions was determined by drying the filtered sediment in an oven at 60 °C for 48 h.

Nitrifiers (NtB) were enumerated by the most probable number (MPN) method of Alexander and Clark (1965). Nitrifying media (seawater amended with NH_4Cl of 2 mM final concentration) was distributed in 5 ml quantities in 15 ml screw capped tubes. From each dilution ranging from 10^{-1} to $10^{-6/7}$, 500 μl was inoculated in triplicate in the nitrifying media until the inoculation from the highest dilution into the culture tubes yielded negative results. The culture tubes were incubated in the dark for a period ~60 days at 28(±1) °C. After incubation, the tubes were tested for the presence of NO_2^- and/or NO_3^-. The combinations of positive and negative tubes were scored and MPN was assessed from McCready's table (Rodina, 1972).

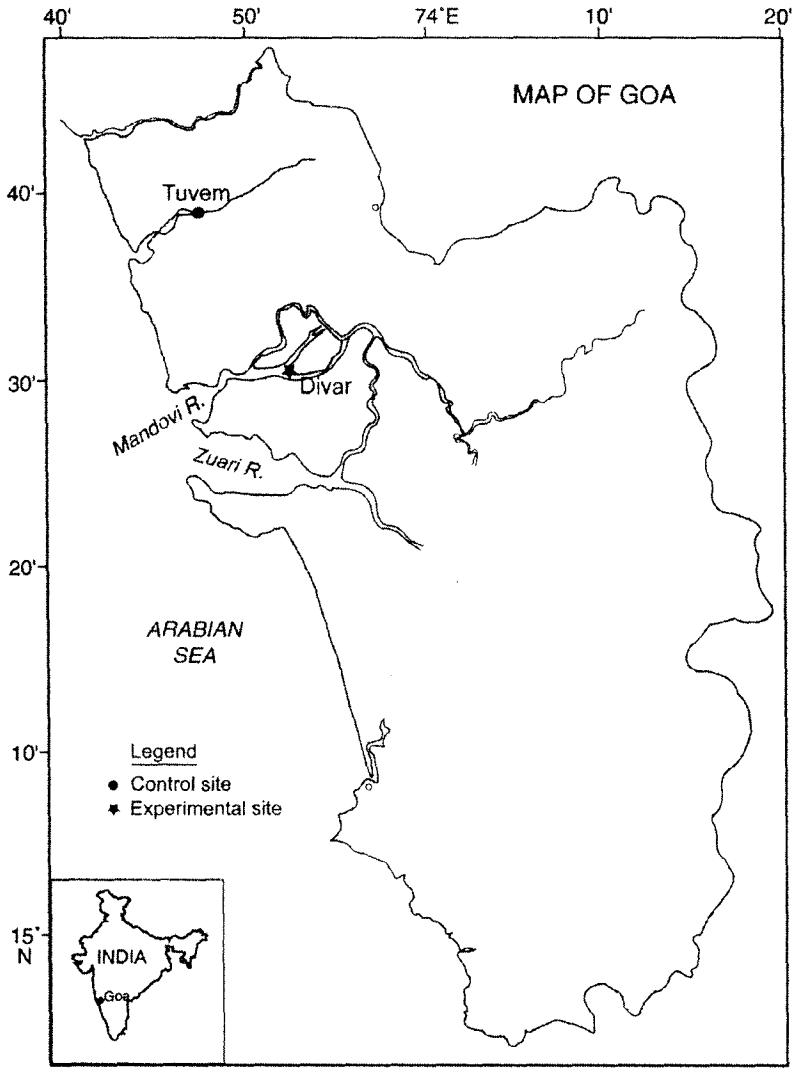

Fig. 1. Location of sampling sites in the Chapora and Mandovi estuary.

Statistical analysis was carried out using Pearson's Correlation Coefficient in order to explore the possible associations existing between different variables. The analysis was done using Microsoft Excel 2000. The bacterial parameters were normalized by log transformation before analyses.

3. Results and discussion

3.1. Down core variation and index of geoaccumulation for iron and manganese

Figs. 2a, b and 3a, b show the down core variation of Fe and Mn during pre monsoon, monsoon and post monsoon seasons. The overall range in sedimentary Fe values ranged between 8% and 12.6% at the experimental site, while it ranged between 4.7% and 9.3% at the control site. The Mn values varied from 0.06% to 0.52% at the experimental site and 0.05–0.48% at the control site. The values reported

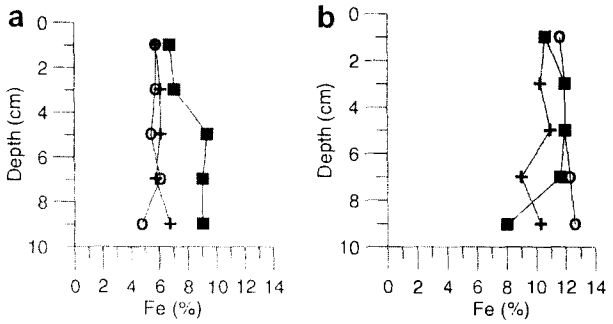

Fig. 2. Down core variation in Fe. (a) refers to the control site while (b) refers to experimental site (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

in the present study are comparatively higher to those reported by Ray et al. (2006) from the Godavari estuarine mangrove ecosystem on the eastern coastline of India. These authors reported that the average sedimentary Fe

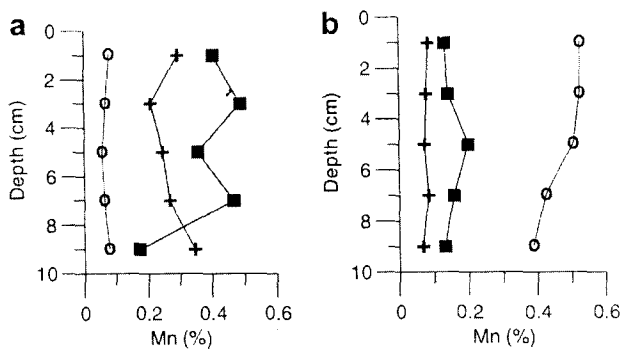

Fig. 3. Down core variation in Mn. (a) refers to the control site while (b) refers to experimental site (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

and Mn values were 0.004% and 0.001%, respectively. Studies by Alagarsamy (2006) showed that the concentrations of Fe varied from 2.2% to 49.7% on the surface sediments of the Mandovi estuary, while the concentration of Mn ranged below detection limit to 1.61%. Though reports from the Mandovi estuary (Alagarsamy, 2006) showed that metal concentrations were generally low during monsoon, compared to the pre and post monsoon seasons, at the adjoining mangrove sediments, they were found to vary. In the present study, the highest accumulation of Fe was observed during pre monsoon, while the values in the monsoon and post monsoon seasons were lower and comparable. The variation of Mn values was similar to observations made by Alagarsamy (2006) in the surface sediments of the adjoining Mandovi estuary.

Irrespective of the depth, the experimental site showed an enrichment of Fe > 100% of the control site during pre monsoon. Percentage of enrichment has been calculated using the formula:

If $a > b$:

Percentage of enrichment $= (a - b)/b * 100$

If $b > a$ under washout conditions:

Percentage of enrichment $= (b - a)/a * 100$

where a is the concentration of Fe/Mn at experimental site and b is the concentration of Fe/Mn at control site.

In general, enrichment increases with depth to reach values >165% at an 8–10 cm interval during pre monsoon. During monsoon, the general enrichment pattern is reversed with the highest enrichment (85.6%) at 0–2 cm. Moreover, observations from statistical analysis indicate that there is no significant correlation between Fe and Mn in the monsoon months, neither at the control nor at the experimental site. However, a direction is suggested in the relationship. It is negative at the experimental site and positive at the control site, perhaps suggesting that under a lower concentration of iron, manganese concentration tends to increase. With a higher concentration of iron up to 12–13%, this trend changes, suggesting that the increase of both of the elements do not get coupled after a threshold.

During post monsoon, it could be observed that, though the trend in down core variation fairly resembled the monsoon season, the magnitude fell sharply to show negative enrichment. It was interesting to note that, although the control site was free from the influence of mining, the experimental site showed negative Mn enrichment in all sections during monsoon and post monsoon. This could be due to enhanced fresh water supply to the Mandovi compared to the Chapora. These observations are contrasted by very high enrichment (393–773%) of Mn at the experimental site during the pre monsoon season, especially in the depth range of 4–6 cm. A positive relation between Fe and Mn during the non-monsoon months ($r = 0.64$, $p < 0.02$, $n = 10$) at the control site and the absence of such a relation at the experimental site showed that, though the chemistry of Fe and Mn are closely related, they could be differentially preferred by organisms, which in turn is influenced by the prevailing environment. Alternatively, the concentration of iron is too high to warrant any bacterial dependance.

The geoaccumulation index (I_{geo}) was originally defined by Müller (1979) for metal concentrations in the <2 μ fraction and developed for global standard shale values, which is expressed as follows:

$$I_{geo} = \log_2(C_n/1.5B_n)$$

where C_n is the measured concentration of metal 'n' in the sediment and B_n is the background value for metal 'n'.

The factor 1.5 is used for the possible variations of the background data due to lithological variations.

The choice of the background value plays an important role in the interpretation of geological data. I_{geo} has been widely utilized as a measure of pollution in freshwater (e.g. Müller, 1980; Singh et al., 1997; Kralik, 1999) and marine sediments (e.g. Stoffers et al., 1986; Bryan and Langston, 1992; Dickinson et al., 1996). Geoaccumulation index (I_{geo}) of Fe and Mn in the experimental site (control site metal values were taken as reference values) was computed based on Müller (1979) for all the depths and seasons. The results are plotted in Fig. 4a and b.

According to the I_{geo} classification (Table 1), it could be inferred that the sediments in the depth range 0–10 cm fall in the 'uncontaminated to moderately contaminated by iron' category during the pre monsoon and monsoon season. While, in the post monsoon season, though the 0–4 cm still remains 'uncontaminated to moderately contaminated by iron', the 4–10 cm layer has recovered from Fe contamination and could be termed as 'Uncontaminated'. The contamination due to Mn is more acute than Fe during the pre monsoon season. During the pre monsoon, the 0–8 cm section falls under the 'Moderately to strongly contaminated' category, while the 8–10 cm section falls under the 'Moderately contaminated category'. All the depths fall in the 'Uncontaminated' group during the monsoon and post monsoon and hence could be assessed as free

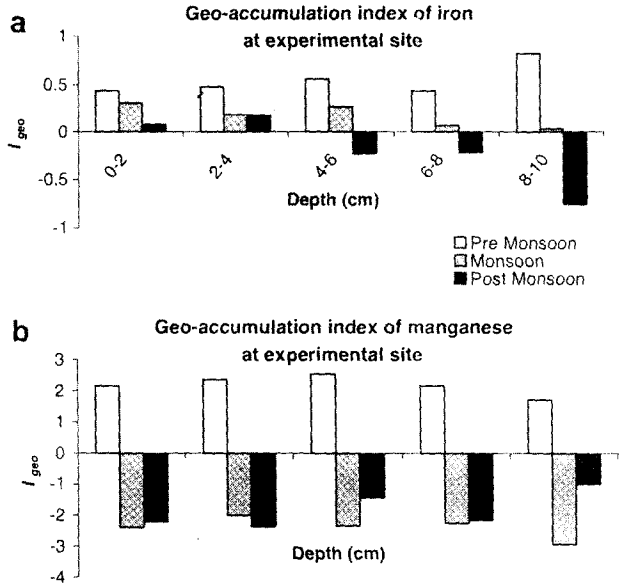

Fig. 4. (a) and (b) I_{geo} values of Fe and Mn, respectively, for the experimental site at Divar. The *y*-axis scale and legend to (b) is the same as that of (a).

Table 1
Description of sediment quality by geoaccumulation index (Müller, 1979)

I_{geo} Value	I_{geo} Class	Description of sediment quality
>5	6	Extremely contaminated
4–5	5	Strongly to extremely strongly contaminated
3–4	4	Strongly contaminated
2–3	3	Moderately to strongly contaminated
1–2	2	Moderately contaminated
0–1	1	Uncontaminated to moderately contaminated
<0	0	Uncontaminated

from the Mn pollution. These observations could again suggest that, though the elements are closely related, the biogeochemical cycling of Mn could be more efficient and rapid when compared to Fe in the mangrove sediments. Moreover, the mangrove ecosystems play a buffering role by reducing the enrichment levels of Fe and Mn in the sediments. The overall assessment could be that, though the Mandovi estuary is under the influence of ferromanganese ore mining with significant impact on the estuarine sediments, the sediments of the adjoining mangroves are comparatively less contaminated, but when strongly contaminated, could be self-regulatory and recover in the time scale tested.

3.2. Total organic carbon (TOC) and bacterial trophic structure

Down core variability in TOC for the different seasons are illustrated in Fig. 5a and b. TOC varied from 0.02 to 1.8% in the control site, whereas at the experimental site, it varied from 0.12 to 2.74%. Lowest TOC values were recorded during the post monsoon season at the control

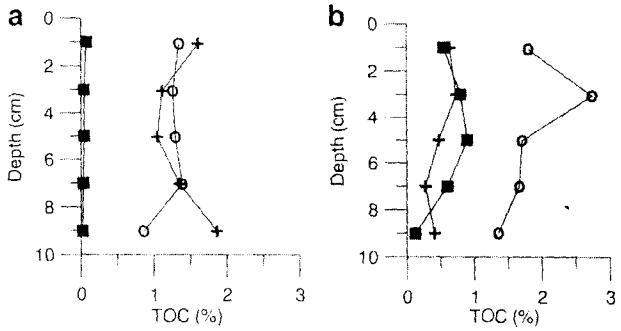

Fig. 5. (a) Control site and (b) Experimental site show the down core variation in Total Organic Carbon (TOC) in sediments. (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

site and the highest values were recorded during the pre monsoon at the experimental site.

TOC varied with different fractions of retrievable heterotrophic bacteria (Figs. 6 and 7). In the pre monsoon season, TOC and HB retrieved on all three strengths of nutrient agar showed a positive relationship at the control site. However, only on 0.01% HB was the correlation significant at $r = 0.80$, $p < 0.05$. Despite the season, it could be observed that at the control site the abundance of HB retrieved on 0.01% dNA is limited ($r = 0.50$, $p < 0.05$, $n = 5$) by the availability of organic carbon. The extent of limitation due to carbon availability for HB retrieved on 0.01% ($r = 0.89$, $p < 0.01$, $n = 5$) and 0.1% dNA ($r = 0.96$, $p < 0.001$, $n = 5$) is maximum during the monsoon. Though there is a heavy organic flux into the estuary during the monsoon, the higher dependency of HB on TOC could be due to the lower lability of the organic carbon. Reduced residence time during monsoon could be responsible for the delayed degradation of complex organic matter into simpler labile forms. Short residence time could also be due to the large seasonal influx of freshwater into the estuary, with unchanged tidal amplitude over large distances (Unnikrishnan et al., 1997). As in the control site, as well as at the experimental site, TOC showed a positive relation with 0.01% HB ($r = 0.72$, $p < 0.1$) during pre monsoon. There does not exist any significant relationship between the 25 and 0.1% fraction of HB with TOC at the experimental site during the pre monsoon and monsoon seasons.

The scenario in post monsoon is very different from the preceding seasons at both the control and experimental sites. It was observed that the 0.01% dNA fraction of HB had a positive relationship of $r = 0.889$ ($p < 0.02$) and $r = 0.79$ ($p < 0.05$), respectively, with TOC. The other fractions bore an insignificant, negative relationship. However, it is suggestive of enhanced utilization compared to production of organic carbon. Though the estuarine environment is eutrophic with considerable organic loading, a persistent relationship between TOC and the 0.01% dNA fraction of HB could be due to a dominant fraction of heterotrophs, which constantly require an optimal organic carbon concentration of 0.01%. Similar observations have

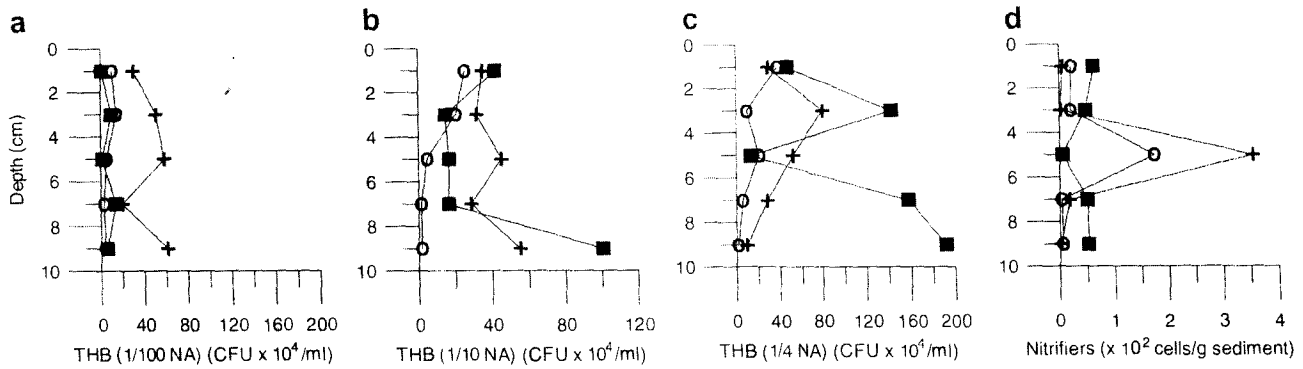

Fig. 6. Control site shows the down core variation of bacterial groups with different nutritional requirements (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

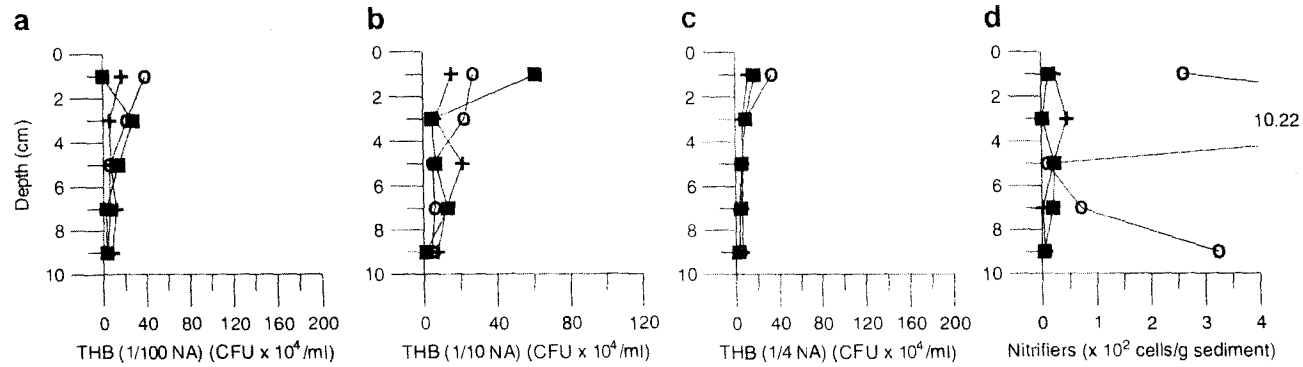

Fig. 7. Experimental site shows the down core variation of bacterial groups with different nutritional requirements (○ – Pre Monsoon, + – Monsoon and ■ – Post Monsoon).

been made earlier by Goltekar et al. (2006) in the Mandovi estuary, which indicate that bacteria from relatively more dynamic estuarine systems seemed less resilient compared to the coastal and offshore populations, as they were best retrieved only on 1% nutrient strength.

3.3. Relation of sediment geochemistry with bacterial trophic status

It was interesting to note that TOC related positively to Fe ($r = 0.67$, $p < 0.01$, $n = 15$) and Mn ($r = 0.92$, $p < 0.001$, $n = 15$) at the experimental site, whereas the relationships were negative at the control site ($r = -0.72$, $p < 0.001$, $n = 15$ for Fe and $r = -0.51$, $p < 0.05$, $n = 15$ for Mn). These relationships could imply that at the experimental site there is considerable extraneous input of TOC favoring the accumulation of Fe and Mn. Wangersky (1986) has reported that coatings of organic matter prevalent in fine-grained sediments bind a variety of trace elements. Enrichment of Mn in the pre monsoon and its subsequent removal in the monsoon indicate that Mn turnover time could be much less, as it is actively removed both by physical processes and biogeochemical sequestration. It could be observed that at the experimental site NtB are higher at the depth ranges of 2–4 cm and 8–10 cm. Irrespective of the season, a positive correlation between Mn and NtB ($r = 0.61$, $p < 0.01$, $n = 15$) at the experimental site suggests that Mn could be used actively as a co-factor for the ammonia monoxygenase enzyme. More importantly, it could also serve as an alternate terminal electron acceptor in anaerobic respiration (Hulth et al., 1999). This relationship disappeared when the Mn levels decreased during the monsoon season and was re-established during the post monsoon season ($r = 0.62$, $p < 0.02$, $n = 10$) when the Mn levels increased. Absence of a significant relationship between Fe with various fractions of heterotrophs and nitrifiers at both the control and experimental sites suggested that Fe is present in excess and perhaps non-limiting. Moreover, Mn(IV) reduction preceeds that of Fe(III) because of reduction energetics of the solid phases (Burdige et al., 1992).

The relationship between Mn and various fractions of HB indicate that the latter has a considerable influence in regulating the levels of Mn. At the experimental site, HB cultured on 0.01% dNA related to Mn during pre monsoon ($r = 0.826$, $p < 0.05$, $n = 5$) and further strengthened during the monsoon ($r = 0.968$, $p < 0.001$, $n = 5$). HB on 0.1% dNA also showed a relationship to Mn concentrations ($r = 0.951$, $p < 0.001$, $n = 5$) during the monsoon. However, during post monsoon, the variation in HB on a higher

strength of 25% nutrient agar affected the variation in Mn concentration up to 90% ($r = 0.949$, $p < 0.001$, $n = 5$). All these observations show that the different fractions of HB could respond in sequence to the Mn enrichment taking place in the pre monsoon season. These relationships demonstrate that HB are actively involved in maintaining the level of Mn on par with reference levels at the Chapora estuary.

A very strong positive correlation between NtB and TOC in the pre monsoon season ($r = 0.987$, $p < 0.001$, $n = 5$) showed that heterotrophic nitrification could be a dominant process controlling the cycling of ammonia in sediments. The potential of heterotrophic nitrification in sediments has been previously reported by Schimel et al. (1984). Furthermore, the absence of a relationship in monsoon and a negative relationship in post monsoon ($r = -0.673$, $p < 0.1$, $n = 5$) could indicate a shift from a relatively heterotrophic to relatively autotrophic mode of nitrification.

4. Conclusion

The present study shows that the heterotrophs respond to the Mn enrichment and could be active in maintaining its level and the other associated metal concentrations like that of Fe on par with the reference site. A positive correlation between Mn with NtB at the experimental site during the non-monsoon months is indicative of the latter's contribution to regulation of the metal concentration, especially of Mn in the sediment, probably through its use as a terminal electron acceptor in respiration at the expense of manganese oxide. The study therefore lends support to our hypothesis that both autochthonous autotrophs and heterotrophs work in tandem to mitigate manganese and perhaps related metals like iron in mangrove swamps. Though the Mandovi estuary is under the influence of ferromanganese ore mining with relatively higher sediment metal concentrations, the adjoining mangroves are comparatively less contaminated due to the self-regulation facilitated by the native bacterial flora.

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Limno-tolerant bacteria govern nitrate concentration in Mandovi Estuary, India

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ABSTRACT

The spatial and temporal abundances of limno-tolerant and halo-tolerant bacteria were investigated in the tide-dominated Mandovi estuary along the west coast of India. These investigations were carried out in relation to various environmental parameters on a monthly basis at three fixed stations for a year. On an annual basis, the estuary showed an average salinity of 28.2, 17.4, and 12.6 at the mouth, midstream and upstream region. Halo-tolerant retrievable count (HTRC) and limno-tolerant retrievable count (LTRC) of bacteria were in the order of $10^6\,L^{-1}$. Among the environmental parameters, a strong negative relationship between salinity and nitrate ($r = -0.806$; $p < 0.001$) suggested that 64% of the variation could be due to fresh water influence in the estuary. The limno-tolerant retrievable count (LTRC) brought about 23% variations in nitrate concentration. This influence was maximum during the monsoon ($r = 0.522$; $p < 0.05$) especially in the surface waters ($r = 0.624$; $p < 0.001$) suggesting nitrate reduction by LTRC. Measurements of nitrate reducing activity (NRA) in whole-water samples along the salinity gradient in the estuary also revealed higher reduction rates at lower salinity upstream. This was further confirmed by culture experiments where the limno-tolerant bacteria showed higher NRA than halo-tolerant forms. It is therefore suggested that LTRC is more actively involved in the variation of nitrate that enters the Mandovi estuary particularly during the monsoon.

1. Introduction

An estuary, as the meeting point of fresh and saline waters, is a dynamic environment which is subjected to a wide variation in physical and chemical parameters. In the estuary, the bacterial community may comprise physiologically versatile species belonging to the autochthonous estuarine bacterial population and/or the allochthonous fresh water or terrestrial populations. Although the fresh water bacteria are introduced with the nutrient rich waters, they could be adversely affected by an increase in salinity in the estuary (Hyun et al., 1999) compared to the autochthonous estuarine bacteria. Estuarine microorganisms thus require the capacity for adaptation to the continuous mixing of waters, the different residence times and transportation of materials from diverse sources (Igeno et al., 1995; Almeida et al., 2001). The adaptive response of the bacteria may be either by changing their abundance and/or their activity.

The Mandovi is a tide-dominated tropical estuary, located between 15°25′ to 15°31′N and 73°45′ to 73°59′E on the west coast of India and is well mixed throughout the year. Due to its location in the south-eastern Arabian Sea, it is subjected to wind-forcing resulting from the annual reversal of monsoon winds (Wiggert et al., 2005). The estuary receives an annual rainfall of the order 250–300 cm yr^{-1} during the southwest monsoon (June–September) and less than 10 cm yr^{-1} during the rest of the year (Shetye and Murthy, 1987). It shows a characteristic shift from an autotrophic phase during the non-monsoon period to heterotrophic mode during the monsoon (Ram et al., 2003).

The nitrate concentration is high (Sardessai and Sundar, 2007) in this estuary and the sources of nitrate are from terrestrial influx, the mangrove swamps (Qasim and Sengupta, 1981) and from the iron ore extraction-plant discharge (De Souza, 1983) located upstream. Do bacteria from these estuarine waters govern the level of nitrate? To address this question, we examined both autochthonous halo-tolerant and allochthonous limno-tolerant community along with nitrate concentrations in the Mandovi estuary. We hypothesize that the allochthonous limno-tolerant bacteria are responsible for governing the concentration of nitrate in the Mandovi estuary. Our hypothesis is further developed by laboratory experiments with whole-water sample and isolates of limno-tolerant and halo-tolerant forms on nitrate reducing activity at different salinities.

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2. Materials and methods

Surface and bottom waters were collected from the mouth (Lat 15°29′35.81″N; Long 73°48′35.33″E), midstream (Lat 15°30′13.19″N; Long 73°54′00.83″E) and upstream (Lat 15°32′35.21″N; Long 73°57′24.43″E) of the estuary at monthly intervals for a period of one year using Zobell water samplers (Fig. 1).

2.1. Physico-chemical parameters

Water temperature was measured immediately after collection of sample onboard using a field thermometer. All the other physico-chemical variables were estimated in triplicate in the laboratory after the transport of water samples in refrigerated conditions. pH was determined using pH meter (Elico LI 614 pH Analyser). Salinity, nitrate, and nitrite were estimated as described by Strickland and Parsons (1968). For oxygen estimation, the water samples that were fixed immediately on board and were then analyzed using Winkler's titrimetric method (Carpenter, 1965). Chlorophyll was estimated based on the fluorimetry method of Yentsch and Menzel (1963). Suspended load was determined gravimetrically on pre-weighed GF/F (Whatman) filters as described by Krey (1964).

2.2. Microbiological parameters

Total bacterial numbers were enumerated by the acridine orange direct count (AODC) method (Hobbie et al., 1977). Samples were fixed immediately with formaldehyde (2% final concentration). A fixed amount was filtered through 0.2 μm pore size black polycarbonate membrane filters (Nucleopore), stained with 0.01% acridine orange and enumerated using an epifluorescence microscope (*Olympus* BH). Total viable counts were estimated (Kogure et al., 1979) by incubating the sample with yeast extract for 6 h with subsequent filtration, staining, and enumeration as above. Abundance of limno-tolerant and halo-tolerant bacteria was estimated from the CFU (Colony Forming Units) retrieved on nutrient agar prepared in distilled water (salinity = 0) and aged estuarine water (salinity = 30) respectively (Krumbein, 1971). Enumeration was carried out after 48 h of incubation at 28 ± 2 °C and counts were expressed in numbers per liter. Interrelationships between bacterial and environmental parameters were statistically examined using Statistica and Analysis Tool Pack in Microsoft Excel. The microbial variables were log transformed before analyses.

2.3. Nitrate reduction along salinity gradients

Surface water samples were collected in sterile polypropylene bottles for the determination of nitrate reducing activity (NRA) along salinity gradients in the Mandovi estuary i.e. towards the upstream (salinity = 5), midstream (salinity = 17) and mouth (salinity = 34). Water samples were transferred to sterile conical flasks. Allylthiourea (ATU) at a final concentration of 86 μM was added to inhibit additional nitrate input through ammonium oxidation (Cinestet et al., 1998). Triplicates were maintained throughout the experiment and the flasks were incubated in the dark at room temperature in a static condition. The fall in nitrate content from each test flask was estimated at 0–1.5 and 3 h as described above. Cell counts were also done using a cell counting chamber under a bright field microscope (*Nikon* Eclipse 50i).

Nitrate reducing activity was estimated with representative halo-tolerant and limno-tolerant isolates. These isolates were grown on respective media. Pure cells harvested from culture plates were used. Cell suspension was vortexed and centrifuged at 8000 rpm for 10 min using a *REMI* R-24 centrifuge. The supernatant was discarded and washing procedure was repeated. The cell suspension with 0.1 OD_{600} was then added to 500 ml of test medium amended with ATU. Nitrate reducing ability in low (5) and high (34) saline conditions was determined as described above and are expressed as aM cell^{-1} h^{-1}. Data of only representative cultures are presented.

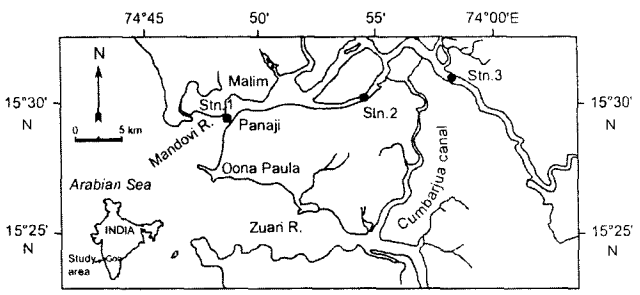

Fig. 1. Sampling locations along the Mandovi estuary (Stn 1: mouth; Stn 2: midstream; Stn 3: upstream).

3. Results

3.1. Variation in physico-chemical parameters

The temperature variation throughout the year was negligible with slight drop during cold seasons and rainy months. Annual average temperature of the estuary was 27.2 (±2.8) °C. The annual average salinity was 20.3 (±12.3) for the whole estuary. The average salinity varied from 12.6 (±11.2) in the upstream through 17.4 (±12) in the midstream to 28.2 (±9.25) in the mouth. During the monsoon period the salinity dropped to a low of 0.11, 0.11, and 12.7 at upstream, midstream and mouth respectively. The average concentration of nitrate during the monsoon was 6.2 (±3.1) μM NO_3–N whereas the value decreased drastically to 1.7 (±1.4) μM NO_3–N in the non-monsoon. Salinity and nitrate showed opposite trend (Fig. 2). In general, variation was more pronounced when data was analyzed seasonally (Table 1). In the surface waters salinity varied from 0.11 to 18.7 in the monsoon and from 0.9 to 34.7 in the non-monsoon. However, in the bottom waters the salinity ranged from 0.09 to 31.7 and from 13.6 to 34.9 during the monsoon

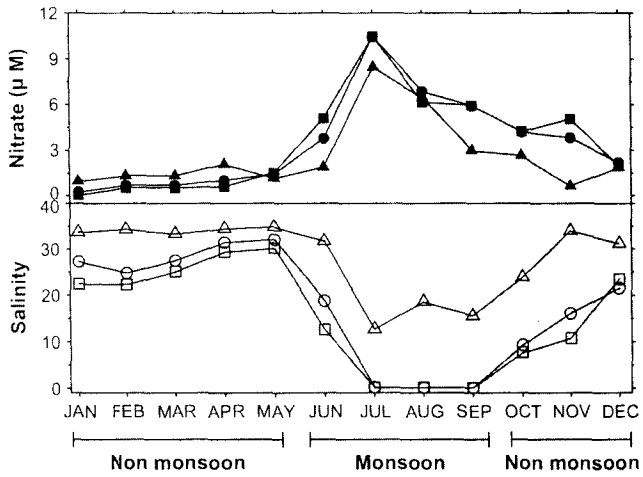

Fig. 2. Annual variation of salinity and nitrate in the estuary. Mouth (△), midstream (○) and upstream (□).

Table 1
Physico-chemical variables in surface and bottom waters during monsoon and non-monsoon periods. Values in parenthesis – mean ± SD.

Variables	Monsoon		Non-monsoon	
	Surface	Bottom	Surface	Bottom
Temperature (°C)	25–31[a] (28 ± 2.05)	24–30.5 (27 ± 2.1)	20.5–32.5 (26.5 ± 3)	19.5–32 (25 ± 3.5)
pH	6.8–7.8 (7 ± 0.31)	6.7–7.8 (7.3 ± 0.3)	7.2–8.2 (7.7 ± 0.26)	7.4–8.3 (7.87 ± 0.2)
Salinity	0.11–18.7 (9.4 ± 9.9)	0.09–31.7 (15 ± 12)	0.9–34.7 (17.8 ± 10.3)	13.6–34.9 (24 ± 6.2)
Dissolved oxygen saturation (%)	94–73 (84 ± 7.8)	104–53.7 (78.9 ± 15.4)	106–61 (83 ± 10)	111–62 (87 ± 13.3)
Nitrate (μM)	3.6–14.1 (8.8 ± 2.9)	1.17–13.3 (7.2 ± 3.4)	0.05–6.44 (3.2 ± 1.6)	0.09–3.6 (1.8 ± 1.1)
Nitrite (μM)	0.6–6.84 (3.7 ± 2.3)	0.72–8.9 (4.8 ± 2.8)	0.001–2.3 (1.1 ± 0.6)	0.001–2.2 (1.14 ± 0.6)
Suspended load ($g L^{-1}$)	0.01–0.1 (0.06 ± 0.04)	0.008–0.17 (0.09 ± 0.06)	0.008–0.29 (0.14 ± 0.08)	0.05–0.32 (0.18 ± 0.07)
Chlorophyll ($\mu g L^{-1}$)	2.6–0.04 (1.3 ± 0.86)	0.04–3.7 (1.8 ± 1.1)	0.04–10.59 (5.3 ± 2.8)	0.1–5.1 (2.6 ± 1.32)

[a] Range.

and non-monsoon respectively. The percentage of mean dissolved oxygen saturation during monsoon and non-monsoon were (84 ± 7.8) and (83 ± 10) respectively in the surface waters. At the surface, nitrate concentration ranged from 3.6 to 14.1 μM with an average value of 8.8 (±2.9) μM in the monsoon. The distribution in nitrite concentration followed similar trend. The suspended load was more during the monsoon compared to the non-monsoon period. Chlorophyll *a* concentration was higher during the monsoon period both at the surface and bottom.

3.2. Bacterial parameters

The variations in total count (TC), total viable count (TVC), limno-tolerant retrievable count (LTRC), and halo-tolerant retrievable count (HTRC) are shown in Table 2. The total count of bacteria ranged from 10^8 to 10^9 L^{-1}. The mean annual value of TC showed the maximum in the bottom waters during the non-monsoon season. The direct viable counts comprised 25% of the total bacterial abundance. These varied from the lowest 19.5% at the bottom in the non-monsoon season to the highest 51.7% at the surface in the monsoon season. The densities of LTRC and HTRC formed 0.008% and 0.014% respectively of the viable bacterial population in the estuary. On an average, the retrievability of halo-tolerant bacteria was double that of limno-tolerant bacteria at 10^6 CFU L^{-1} throughout the year (Fig. 3). The retrievability of LTRC during monsoon was of the order 10^6 CFU L^{-1}, while during the non-monsoon their abundance decreased by an order. The highest abundance of HTRC and LTRC was detected in the non-monsoon and monsoon season respectively. The LTRC/HTRC ratios were high in midstream. The highest value of 14.2 (±66.9) was recorded during the monsoon season (Table 3). The halo-tolerant forms did not show any significant relation to the variation in physico-chemical parameters. The variation in the limno-tolerant counts related significantly to the variation in nitrate by 23% ($p < 0.001$) on an annual basis. During monsoon, nitrate related negatively to salinity ($r = 0.536$; $p < 0.05$). Variation in the LTRC was found to be responsible for 27% ($p < 0.05$) of the variation in nitrate during this season particularly in the surface waters ($r = 0.624$; $p < 0.001$). During the non-monsoon, LTRC caused 27% variation in nitrite (Table 4).

3.3. Nitrate reducing activity

The initial concentrations of nitrate were 4.31 (±0.2) μM NO_3–N at 5, 3.5 (±0.02) μM NO_3–N at 17, and 3.0 (±0.06) μM NO_3–N at 34 salinities (Fig. 4). Nitrification inhibited incubations with water collected along salinity gradients in the estuary revealed higher NRA in low saline waters upstream as compared to the higher salinity waters towards the mouth. Though increasing salinity generally inhibits the NRA activity, our observations show highest NRA of up to 8.72 (±0.1) aM $cell^{-1}$ h^{-1} in midstream (salinity = 17) where the ambient nitrate concentration was 3.5 (±0.02) μM. At salinity 5 specific NRA of limno-tolerant isolate was 55 aM $cell^{-1}$ h^{-1} whereas for halo-tolerant it was 9.87 aM NO_3–N $cell^{-1}$ h^{-1}. Specific NRA in the estuarine isolate showed 11–92% repression of nitrate reduction at higher salinity (Table 5).

4. Discussion

4.1. Physico-chemical parameters

Mandovi is a fresh water dominated estuary, considerably influenced by tides. Even during the pre-monsoon period there is enough fresh water influx to keep salinity close to zero upstream to about 40 km which prevents high salinity waters from intruding upstream (Shetye et al., 1995). In the present study, the average salinity of the estuary was 12.6 (±11.2) and 28.2 (±9.25) in the head and mouth respectively. The upstream and mid stations close to lower region of the estuary (Fig. 1) were selected to evaluate the effect of salinity on nitrate concentration and also to enumerate LTRC and HTRC.

The low salinity observed during the monsoon season in all the 3 stations was due to a considerable amount of run off from a larger tributary system and also because of its versatile topography (Qasim and Sengupta, 1981). During the season, the estuary is known to be stratified 2–3 m below surface depending on the depth. Two salt wedges are formed which extends 10 km from the mouth of the estuary (Qasim and Sengupta, 1981) though it remains well mixed during the non-monsoon (Varma et al., 1975). Therefore, the presence of lighter fresher water above and the denser saline water below leads to a lesser extent of mixing. The

Table 2
Total count (TC), total viable count (TVC), halo-tolerant retrievable count (HTRC) and limno-tolerant retrievable count (LTRC) of bacteria in surface and bottom waters during monsoon and non-monsoon periods. Values in parenthesis – mean ± SD.

Variables (L^{-1})	Monsoon		Non-monsoon	
	Surface	Bottom	Surface	Bottom
TC (10^9)	4.05–0.9[a] (2.4 ± 0.9)	12–0.6 (6.3 ± 3.3)	13.2–0.7 (7 ± 3)	41.4–0.74 (21 ± 13.7)
TVC (10^9)	1.8–0.2 (1 ± 0.5)	2.2–0.19 (1.2 ± 0.4)	2.8–0.3 (1.6 ± 0.6)	6.7–0.4 (3.6 ± 1.8)
HTRC (10^6)	9.2–0.3 (4.7 ± 2.5)	5.1–0.06 (2.6 ± 1.5)	13–0.06 (6.6 ± 3.2)	13.2–0.01 (6.6 ± 3.5)
LTRC (10^6)	3.3–0.1 (1.7 ± 1.05)	20–0.12 (10 ± 5.5)	1.5–0.06 (0.75 ± 0.35)	4.08–0.03 (2 ± 1.1)

[a] Range.

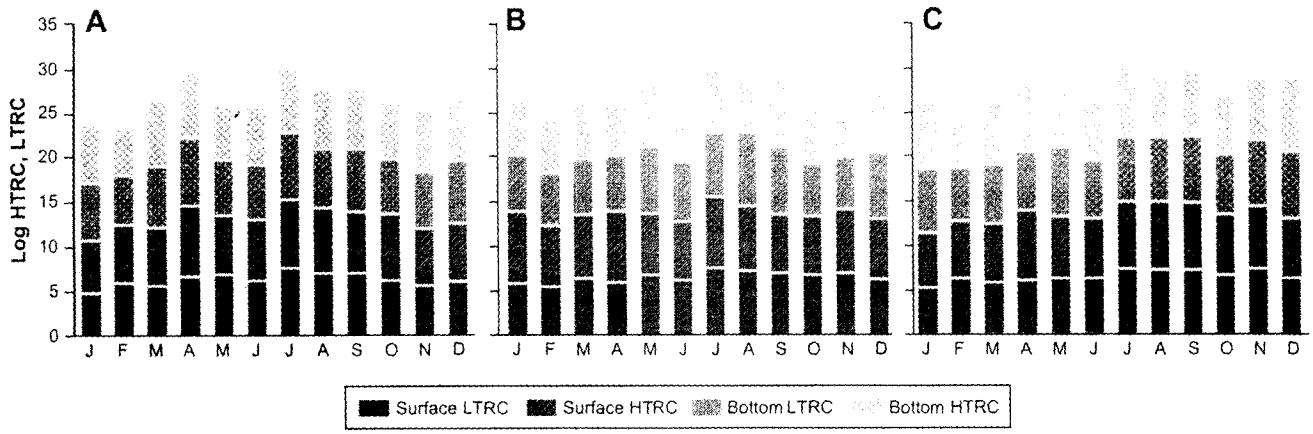

Fig. 3. Annual variation of halo-tolerant retrievable count (HTRC) and limno-tolerant retrievable count (LTRC) at the surface and bottom waters. A-mouth, B-midstream, C-upstream.

present study also indicates that nitrate concentrations decreased from the head towards the mouth of the estuary as has been reported elsewhere (Dong et al., 2000, 2002). Nitrate and nitrite concentrations in the estuary were higher during the monsoon compared to the non-monsoon period with surface water recording higher concentration than the bottom water. This is because the estuary is also a receptacle for continuous nutrient and terrigenous organic inputs from riverine run off and mixing of fresh water and seawater leading to chemical precipitation (Eckert and Sholkovitz, 1976; Karbassi et al., 2008) particularly during monsoons. The higher level of suspended load in the monsoon (0.14 gL^{-1}) compared to non-monsoon (0.068 gL^{-1}) would have contributed to the high level of nitrate. A similar observation was made in the Zuari estuary (De Souza, 1977) where nitrate levels were high in the surface waters and was correlated to the influence of fresh water influx following precipitation. The negative relationship between salinity and nitrate in the surface waters observed in the present study could be attributed to extraneous input of nitrate into the system by fresh water run off. The influx of fresh water in the head region throughout the year, especially during the monsoon would bring relatively high concentration of inorganic nitrogenous species into the estuary (Bhunia and Choudhary, 1982; Chandran and Ramamoorthy, 1984; Vijayakumar et al., 2000). The percent oxygen saturation in the estuary ranged from 53 to 111% annually with the least values during the monsoon which suggests a predominance of heterotrophic respiration. Increased allochthonous inputs have been shown to result in enhanced heterotrophic respiration and reduced primary production in the estuary (Ram et al., 2003). Intrinsic nitrification could also have a role in augmenting the levels of this parameter. In the non-monsoon season the variation in chlorophyll accounted for about 15% ($p < 0.001$) variation in nitrate concentration, while this relationship did not prevail during the monsoon. As there was no relationship between chlorophyll and nitrate it is suggested that excess of nitrate in the water did not limit the primary production during monsoon. Further, the significant relationship between chlorophyll and nitrate during the non-monsoon as observed in the present study suggests nitrate removal from the estuary could be largely due to phytoplankton uptake. Earlier studies by Dham et al. (2002) have also demonstrated that the nitrate uptake by phytoplankton was more during the non-monsoon than the monsoon. In addition, chlorophyll concentration was low during monsoon as compared to non-monsoon season suggesting reduced primary production due to increased turbidity during the monsoon as reported for Schelde estuary (Soetaert and Herman, 1995). However, the nitrate and nitrite concentrations in these waters are generally high due to enrichment caused by the discharge from extraneous sources like the iron ore screening plants throughout the year (De Souza, 1983).

4.2. Microbiological parameters

The bacterial abundance in this estuary is comparable to that of the earlier reports by De Souza et al. (2003) and Ram et al. (2003). About 25% of the total bacteria were viable in the whole of the estuary. This study also finds that the culturable forms of HTRC and LTRC are also high. The high amount of suspended load could provide the required microenvironments (Fletcher, 1991; Kirchman, 1993). Previous reports show that LTRC is known to be abundant when fresh water inflow is high (Nair and Bharathi, 1982). Interestingly, the present study reveals that the HTRC and LTRC were of similar magnitude. The culturability is different with the halo-tolerant forms being marginally higher than and limno-tolerant. This was in contrast to what has been reported in Gironde estuary where halo-tolerant far out-numbered limno-tolerant bacteria (Prieur et al., 1987). Thus, the limno-tolerant forms in the lower estuary seem to have adapted themselves to salinity fluctuations as the estuary maintained a salinity close 20 throughout the year except during monsoon thereby deriving the benefit of nutrients. Seasonal precipitation and salinity-induced stratification

Table 3
Spatial and seasonal variation in LTRC/HTRC ratio.

Location	Estuary	Season	
		Monsoon	Non-monsoon
Mouth	0.62 (±0.54)	0.8 (±0.4)	0.53 (±0.57)
Midstream	14.2 (±66.9)	41.8 (±115.8)	0.49 (±0.4)
Upstream	0.61 (±0.4)	0.6 (±0.31)	0.58 (±0.55)

Table 4
Pearson's correlation of variables with *r*-values in the estuary.

	Variables		Correlation –*r*
Estuary Whole	Nitrate	LTRC	0.479[b]
Estuary surface	Nitrate	Salinity	−0.806[b]
	Nitrate	LTRC	0.624[b]
Monsoon	Nitrate	Salinity	−0.536[a]
	Nitrate	LTRC	0.522[a]
Non-monsoon	Nitrate	Chlorophyll	0.383[b]
	Nitrite	LTRC	0.524[b]

[a] $p < 0.05$.
[b] $p < 0.001$.

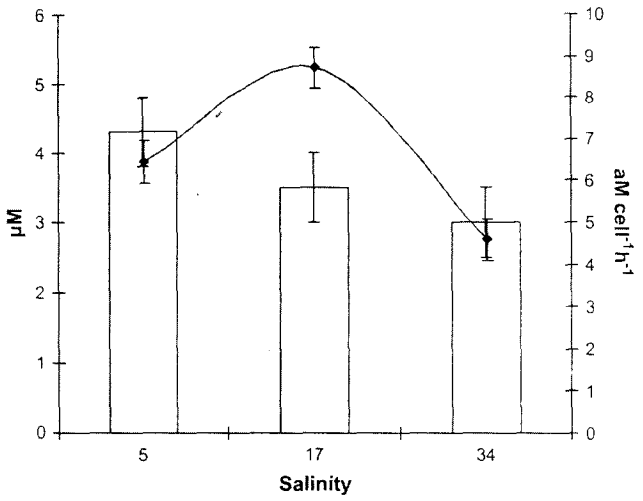

Fig. 4. Variation in nitrate concentration and specific nitrate reducing activity (NRA) along salinity gradients in the Mandovi estuary. Ambient nitrate: vertical bars; Specific nitrate reducing activity: smooth line.

apparently control the availability of the major nutrients in the water column of the estuary as elsewhere (Vareethiah et al., 1998).

Nitrate levels may be influenced by microbially driven processes (Smith et al., 2007) mediated by a diverse polyphyletic group of bacteria (Zumft, 1997). Nitrate in the Mandovi estuary could be removed through the reductive cycle by both halo-tolerant and limno-tolerant bacterial communities. LTRC was found to be responsible for the variation in nitrate during the monsoon season particularly in the surface waters. Even during the non-monsoon season, LTRC caused variation in nitrite. The significant relationship between LTRC and nitrite concentration observed in the present study emphasizes that the limno-tolerant bacteria could also be responsible for nitrite reduction in the estuary. Yoshie et al. (2004) have shown that salinity decreases the nitrite reductase gene diversity in denitrifiers. Quantification of nitrate/nitrite reducing phylotypes has shown that the gene copy numbers were detected the highest at the head of estuary where denitrification dominated (Smith et al., 2007). Consequently, this high nitrate input could trigger higher population of nitrate reducers and therefore nitrate reductase activity.

Experiments to determine nitrate reducing activity in ambient water samples have shown higher NRA to occur in low saline waters where nitrate concentrations were relatively high. Studies by Rysgaard et al. (1999) in estuarine sediments have shown that in situ nitrification and denitrification decreased with increasing salinities, with the most pronounced reduction of approximately 50% occurring when the salinity was raised from 0 to 10. Our study demonstrated that an increase in salinity up to 10 showed an increase of NRA by about 33% and this trend was observed up to 17. Nevertheless our experiments also indicated that when there was a 100% increase in salinity from 17 to 35 there was a decrease in NRA by 50%. Recently, Miranda et al. (2008) have also shown that nitrification rates are higher at intermediate salinities than in either fresh water or seawater. Moreover, the ratio of the distribution of LTRC/HTRC clearly shows that estuary is dominated by the limno-tolerant forms especially at the midstream and during the monsoon. Thus the higher NRA at the midstream is attributed to the higher ratio. Further, the tighter coupling of LTRC to nitrate concentration as revealed by lowered NRA in estuarine isolates under halo-tolerant conditions could be ascribed to repression of nitrate reductase at higher salinity. Igeno et al. (1995) have demonstrated that increase in salinity led to inhibition of nitrate uptake resulting in a drop in intracellular nitrate, thus repressing the nitrate reductase gene. Studies on the activity of nitrate reductase enzyme in a halo-tolerant cyanobacterium *Aphanothece halophytica* by Thaivanich and Incharoensakdi (2007), have also shown decline in NRA when sodium chloride concentrations were $\geq$300 mM. Though it is generally known that salinity can have a significant effect on inorganic nitrogen cycling in estuarine ecosystems, the differential inhibition in the reducing potential of estuarine bacteria observed in the present study is suggestive of optimal salinity favoring nitrate reduction. Although the nitrate reductase gene is known to be constitutive and widespread, the extent of influence of nitrate concentration on LTRC demonstrates that the environment could govern the degree of phenotypic expression in the LTRC community. Moreover, the nitrate reducing activity was found to be more widely expressed in LTRC especially during the monsoon during which the nitrate flux is pre-dominant. Thus, nitrogen cycling in estuarine waters could be maximal at optimal salinity levels. LTRC could therefore play a significant role in the variation of nitrate particularly in the surface waters where its concentration is greater.

Table 5
Specific nitrate reducing activity (NRA) of limno-tolerant and halo-tolerant bacteria at salinity 5 and 34.

Isolate	NRA (aM cell^{-1} h^{-1})		Percentage reduction in NRA
	Salinity		
	5	34	
Limno-tolerant	55.09 (±3.3)	4.40 (±3.12)	92.01
Halo-tolerant	9.87 (±0.08)	8.72 (±0.91)	11.65

5. Conclusion

Limno-tolerant bacteria, though less in abundance, are actively involved in the variation of nitrate that enters the Mandovi estuary particularly during the monsoon. Nitrate reduction in the estuary is enhanced at optimal salinity levels. Though the NRA activity is generally inhibited by increasing salinity, our observations show that different microbes exhibit different levels of inhibition varying between 11 and 95%. The Mandovi estuary could therefore act as a major sink of nitrogen throughout the year especially during the monsoon.

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Denitrification: An Important Pathway for Nitrous Oxide Production in Tropical Mangrove Sediments (Goa, India)

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Net nitrous oxide production and denitrification activity were measured in two mangrove ecosystems of Goa, India. The relatively pristine site Tuvem was compared to Divar, which is prone to high nutrient input. Stratified sampling at 2-cm intervals within the 0- to 10-cm depth range showed that N_2O production at both the locations decreased with depth. Elevated denitrification activity at Divar resulted in maximum production of up to 1.95 nmol N_2O-N g^{-1} h^{-1} at 2 to 4 cm, which was three times higher than at Tuvem. Detailed investigations to understand the major pathway contributing to N_2O production performed at Tuvem showed that incomplete denitrification was responsible for up to 43 to 93% of N_2O production. Nitrous oxide production rates closely correlated to nitrite concentration (n = 15; r = –0.47; p < 0.05) and denitrifier abundance (r = 0.55; p < 0.05), suggesting that nitrite utilization by microbial activity leads to N_2O production. Nitrous oxide production through nitrification was below detection, affirming that denitrification is the major pathway responsible for production of the greenhouse gas. Net N_2O production in these mangrove systems are comparatively higher than those reported from other natural estuarine sediments and therefore warrant mitigation measures.

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The marine environment is recognized as a net source of nitrous oxide (N_2O) to the atmosphere (Corredor et al., 1999). Estuaries and coastal regions account for approximately 60% of the total oceanic N_2O flux (Bange et al., 1996). The N_2O molecule is a precursor to compounds involved in the destruction of the stratospheric ozone layer (Yamagishi et al., 2007), which protects the Earth from harmful ultraviolet radiation. Since about 1750 onward, industrialization has increased the global atmospheric N_2O concentration from ~270 to 319 μL L^{-1} (IPCC, 2007). Although N_2O is responsible for 5 to 6% of the greenhouse effect (Houghton et al., 1996), its lifetime of about 114 yr makes the global warming potential of this biogenic gas 298 times greater than that of CO_2 over a 100-yr time frame (Forster et al., 2007). Nitrous oxide is produced as a by-product during several microbiological processes including nitrification, denitrification, and dissimilatory nitrate reduction to ammonium (De Wilde and De Bie, 2000). However, denitrification and chemolithotrophic nitrification appear to be the main biological sources of N_2O emission in natural systems (Bremner and Blackmer, 1978; Firestone and Davidson, 1989; Bonin et al., 2002).

In ecosystems with high inputs of nitrogen (N) such as estuaries, denitrification mediates reduction of N loadings and therefore contributes to control of eutrophication (Nogales et al., 2002). Mangroves ecosystems constitute nearly 75% of tidal vegetation in tropical regions (Alongi et al., 1989), and they play an important role in the biogeochemical cycles of coastal and marine ecosystems (Thorsten and José, 2001). The N cycle within mangrove forests is mediated predominantly by microbial rather than chemical processes (Alongi et al., 1992). Inorganic N and other parameters important for N turnover can fluctuate widely due to the position of mangroves in the intertidal zone (Meyer et al., 2008). Anthropogenic inputs such as effluents from sewage treatment plants (Corredor and Morell, 1994) increase the rate of N loading to mangroves (Muñoz-Hincapié et al., 2002). They function as efficient buffer zones mitigating large amounts of nutrients (Corredor and Morell, 1994) in the estuarine system and

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Abbreviations: DNT, denitrification activity; $^{D}N_2O$, incomplete denitrification; $^{D}N_2O$, percentage of N_2O through incomplete denitrification; MPN, most probable number; $^{N}N_2O$, nitrous oxide produced through nitrification; TOC, total organic carbon.

reduce water pollution. Chiu et al. (2004) stated that as much as 55% of the N loss in mangrove sediments occurs through the denitrification pathway. Earlier studies recorded a substantial benthic flux of N_2O in mangroves (Corredor et al., 1999; Muñoz-Hincapié et al., 2002; Kreuzwieser et al., 2003). Mangrove sediments are largely anaerobic and rich in organic matter, providing favorable conditions for denitrification. However, redox processes in the N cycle operate in tandem, stressing the importance of considering total N_2O production rates from nitrification and denitrification (Meyer et al., 2008). Further, N_2O production is dependent on a number of environmental factors, such as inorganic N concentrations (Dong et al., 2002), sediment redox potential (Van Cleemput and Samater, 1996), and organic carbon availability (Rosswall et al., 1989). Therefore, assessment of environmental parameters and their interrelationships with net N_2O production in mangrove sediments is crucial in determining the key parameters governing its formation.

In the Indian Ocean region, high N_2O emission has so far been reported within the oxygen minimum zones in the Arabian Sea (Naqvi et al., 2000; Bange et al., 2001). Recently, investigations by Krithika et al. (2008) in a South Indian mangrove system have shown that the benthic N_2O flux varies between 0.41 and 0.77 μmol m^{-2} h^{-1}, indicating that these wetlands are significant contributors of the radiative gas to the atmosphere. However, little is known about the net production or origin of N_2O in these environments. In the present study, we quantified down-core variation in denitrification activity and net N_2O production in two mangrove ecosystems of Goa, India—one relatively pristine and the other influenced by extraneous nutrient input. Detailed studies to assess the major pathway for N_2O production and environmental factors responsible for its generation have been restricted to the relatively undisturbed site. Because there is growing concern over the role of mangrove ecosystems with respect to increased N_2O fluxes to the atmosphere, the study will enhance our knowledge on the contribution of the N cycle processes in N_2O production. In addition, our study can help initiate the formulation of mitigatory measures to minimize N_2O production and its emission from estuarine zones of the Indian Ocean region.

Materials and Methods

Study Area and Sampling

Investigations were performed at mangrove forests located at Tuvem and Divar along the rivers Chapora and Mandovi, respectively, in Goa on the west coast of India (Fig. 1). The site at Tuvem (15°39′94″ N; 73°47′65″ E) is set amid coconut (*Cocos nucifera* L.), cashew (*Anacardium occidentale* L.), and banana (*Musa* L.) plantations and is comparatively less influenced by anthropogenic activities. The dominant species of mangroves found at Tuvem are mainly represented by *Acanthus ilicifolius*, *Excoecaria agallocha*, *Caesalpinia* spp., *Avicennia officinalis*, and *Clerodendrum inerme*. The Divar mangrove ecosystem (15°30′35″ N; 73°52′63″ E) is separated from the mainland by the river Mandovi. Here, the mangroves consist mainly of species like *Acanthus ilicifolius*, *Pongamia pinnata*, *Cyperus* spp., *Bruguiera gymnorrhiza*, *Avicennia officinalis*, *Caesalpinia* spp., *Sonneratia caseolaris*, and *Rhizophora mucronata*. The Mandovi

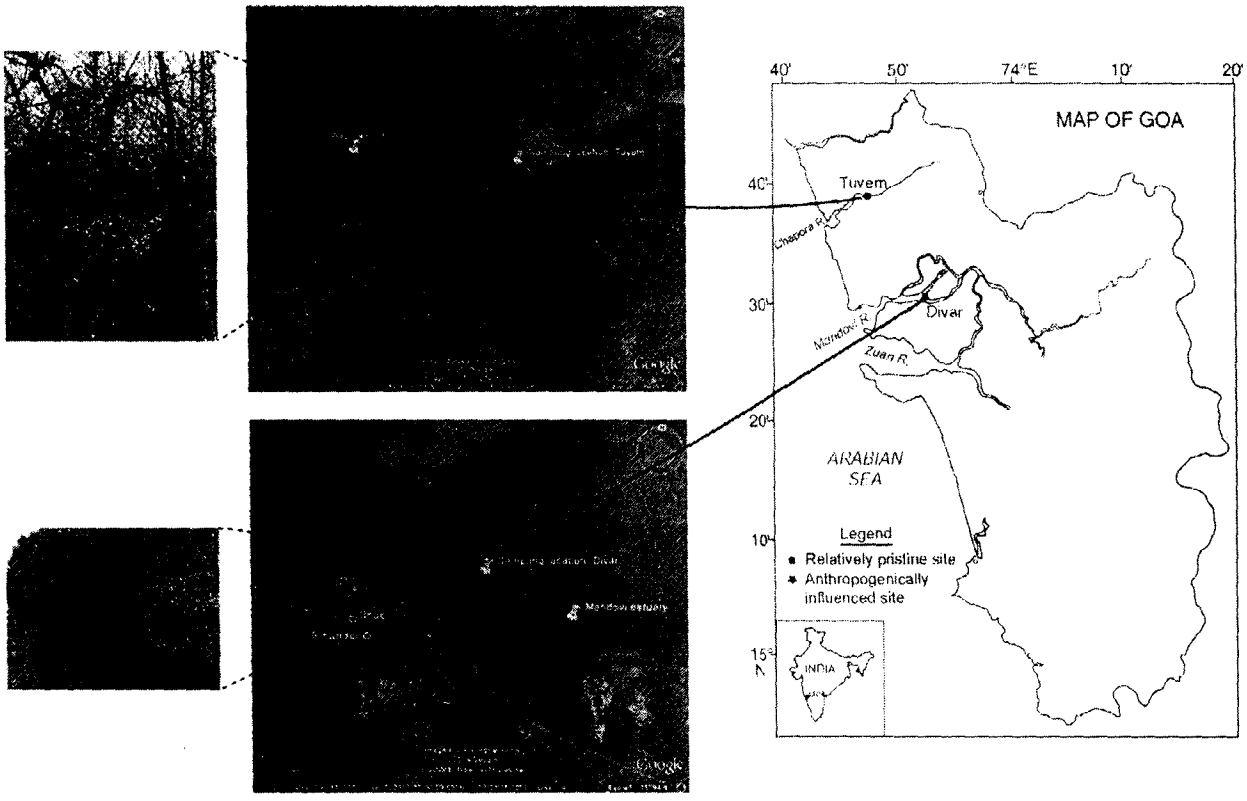

Fig. 1. Location of sampling sites along the Chapora and Mandovi estuary.

is prone to high nutrient concentrations, especially during the monsoon season, which has been attributed to riverine and land runoff (Divya et al., 2009) and anthropogenic sources (De Sousa, 1983). Approximately 10 Mg mo^{-1} of NH_4NO_3 is used as explosive in ferromanganese mining operations upstream of the Mandovi (De Sousa, 1999). Iron ore beneficiation plants situated on the riverbanks carry out treatment and upgradation of low grade ore fines. These plants use river water to wash the iron ore and discharge effluents directly into the aquatic system. This wastewater contains ~80 μmol NO_3–N L^{-1}, forming a major source of the nutrient to the estuary (De Sousa, 1999). Annual variation of NO_3^-–N concentrations along the Mandovi estuary has been reported by Divya et al. (2009). Further, Krishnan et al. (2007) showed that the Divar mangrove sediments are also enriched with metals (primarily Fe and Mn) as a result of ferromanganese mining upstream of the Mandovi. These metals, mainly Fe, have been shown to influence N transformations (Krishnan and Loka Bharathi, 2009).

Sediment samples were collected at low tide during January 2008 (postmonsoon season) using hand-held polyvinyl chloride push cores (7.5 cm i.d., 15 cm length). The cores were immediately capped at both ends and transported to the laboratory in an ice box. Cores for pore water nutrient and denitrification activity measurements were maintained at 4°C until analysis. Water from the sampling sites was collected in carbuoys for media preparation.

Physicochemical Parameters

Hydrogen ion concentration (pH) was measured on sample arrival at the laboratory using an Orion 4-Star Plus benchtop pH/ISE meter (Thermo Fisher Scientific Inc., Waltham, MA). Sediment oxidation–reduction potential (Eh) was measured using an Orion platinum redox in combination with a Ag/$AgCl_2$ reference electrode (Thermo Fisher Scientific Inc.).

For nutrient analyses, triplicate subsamples from sediment cores were taken at 2 cm increments from 0- to 10-cm depth by careful sectioning of sediment. Each section (7.5 cm diam. and 2 cm thick) was transferred to 100 mL of sterile saline (8.5 g L^{-1} NaCl) and gently homogenized using a glass rod. The slurry was centrifuged at 4°C for 10 min at 5000 rpm (×1803 *g*) with a high speed cooling centrifuge (Model CPR-24; Remi Instrument Ltd., Mumbai, India). A low spin speed was maintained during centrifugation to ensure minimal change in nutrient concentrations due to lysis of benthic infauna. The supernatant was filtered through a 0.2-μm filter and stored at −20°C until analysis. Ammonium, NO_2^-–N and NO_3^-–N were measured colorimetrically (UV mini 1240 specrrophotometer; Shimadzu Corp., Tokyo, Japan) as described by Koroleff (1969), Bendschneider and Robinson (1952), and Wood et al. (1967). The weight of the sediment used in the extraction was estimated by drying the wet samples at 60°C for 48 h. Total organic carbon (TOC) was determined by wet oxidation method with a precision of 0.01% (El Wakeel and Riley, 1957).

Denitrification, Net Nitrous Oxide Production, and Its Origin

Sediment cores were demarcated into five sections (0–2, 2–4, 4–6, 6–8 and 8–10 cm). Then 1 mL of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20-mL headspace vials. Three milliliters of sterilized ambient sea water from the sampling site (containing 4.5 μmol NO_3 –N L^{-1}) was added. Sample preparations were also amended with chloramphenicol (1 g L^{-1}) to prevent de novo enzyme synthesis during the incubations (Bonin et al., 2002). No additional C or NO_3 –N was added as substrate. The vials were capped with butyl stoppers, sealed with Al crimps, and then briefly vortexed to form a slurry.

Denitrification activity (DNT) was measured by the acetylene inhibition technique based on the inhibition of the conversion of N_2O to N_2 (Sørensen, 1978). Over short incubation intervals, it is a cost-effective method for estimating denitrification, and rate estimates are comparable to those obtained by the membrane inlet mass spectrometry (Bernot et al., 2003). Thus, to measure DNT, some of the vials were made anaerobic by flushing with N_2 for 15 min. The headspace over these slurries was amended with acetylene at 20 kPa (Bonin et al., 2002) and the tubes were briefly vortexed.

To determine net N_2O production, aerobic conditions were maintained in the vials and no acetylene was added to the headspace. However, to measure N_2O produced by denitrifiers ($^{D}N_2O$), aerobic conditions were maintained and the headspace was adjusted to an acetylene concentration of 10 Pa to inhibit nitrification (Berg et al., 1982; Bonin et al., 2002). Triplicate measurements were performed at each depth, and the vials were incubated in the dark for 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h. Following incubation, each vial was treated with 0.1 mL of 1 M $HgCl_2$ solution and vigorously shaken for 2 min to stop the reaction.

Nitrous oxide in the headspace was analyzed using a gas chromatograph (Model 2010; Shimadzu Corp.) fitted with an electron capture detector and Poropak Q column (1/8" SS column, 3.05 m length, 80/100 mesh; Chromatopak Analytical Instrumentation, Mumbai, India). The oven and detector temperatures were 40 and 300°C, respectively. High purity nitrogen at a flow rate of 35 mL min^{-1} was used as a carrier gas. The gas chromatograph was calibrated using a secondary standard 44 ± 0.38 nmol N_2O in N_2 (National Physical Laboratory, New Delhi). The rate of N_2O production was determined based on its linear accumulation over time (Tiedje, 1982). The Bunsen solubility coefficient for the measured salinity and temperature in the microcosms was used to correct for dissolved N_2O (Weiss and Price, 1980). As the experiment was performed in microcosms, it was necessary to minimize error likely to be caused by variability during sediment transfer. Hence, sediment used in each microcosm was filtered through a laboratory grade filter paper and dried at 60°C for approximately 48 h. Average N_2O production and DNT were calculated as nmol N_2O-N g^{-1} h^{-1} of dry sediment.

Nitrous oxide produced through nitrification ($^{N}N_2O$) was calculated as

$$^{N}N_2O = \text{ner } N_2O \text{ production} - {}^{D}N_2O$$

where net N_2O production = N_2O production from denitrification plus nitrification (without nitrification inhibitor) and $^{D}N_2O$ = N_2O produced by denitrifiers only (nitrification inhibited in presence of 10 Pa C_2H_2).

Percentage of N_2O through incomplete denitrification ($^{I}N_2O$) was calculated as

$$\%\ ^{I}N_2O = {}^{D}N_2O/DNT \times 100/1$$

where DNT = denitrification activity (in the presence of 20 kPa C_2H_2).

Quantification of Denitrifiers

Denitrifiers were enumerated by the N_2O-most probable number (MPN) technique. The sediment core was thawed and sectioned at 2-cm intervals using a sterile core cutter to obtain representative samples at 0 to 2, 2 to 4, 4 to 6, 6 to 8 and 8 to 10 cm. Sediment from each depth was homogenized by mixing. Approximately 1 g of wet sediment was subsampled from each depth using sterile syringes. The subsamples were transferred to 9 mL of sterile culture medium to give a 10^{-1} dilution. The culture medium was prepared as described by Michotey et al. (2000). Tween 80 (20 µL) was added and the mixture was sonicated at 40 MHz for 15 s. Serial dilutions for each section of the core were prepared in triplicates. The vials were purged with high purity N_2 for 10 min to induce anaerobic conditions and supplemented with 20 kPa acetylene (Bonin et al., 1994). The vials were incubated at room temperature in the dark for 10 d, and the positive tubes were scored on the basis of the accumulation of N_2O. Subsequent quantification was made using standard McCready's table (Rodina, 1972). Denitrifier abundance has been expressed as MPN cells per gram of dry sediment.

Statistical Analyses

All analyses were performed using Statistica version 6 (StatSoft, Inc., Tulsa, OK). Bacterial numbers were $\log_{10}$ transformed before analysis. Nitrous oxide production rates were checked for normal distribution using the Kolmogorov–Smirnov test. As the data was normally distributed ($p > 0.2$), *t*-test was used to check for statistically significant differences in mean value of N_2O production between the two sites. Pearson's correlation coefficients were used to assess relationships between biotic and abiotic parameters. The correlation values were plotted using Cytoscape 2.6.3 software (http://www.cytoscape.org/), which enabled an open-source network visualization. Principal component analysis was used to examine the combined influence of environmental parameters on N_2O production.

Results

Physicochemical Characteristics

The mangrove sediments were acidic in nature with pH ranging from 5.8 to 6.1 (Table 1). Sediment redox potentials were consistently low, in the range of −27.8 (± 0.9) to −6 (± 16.45) mV at Tuvem, while the Divar sediments exhibited the lowest redox potential of −5.7 (± 25.10) at a depth of 8 to 10 cm.

Pore water nutrient content varied widely with depth with 15.1 (± 3.4) µmol NH_4^+–N L^{-1} recorded at 8- to 10-cm depth at Tuvem, while 31.34 (± 1.83) was recorded at Divar at a depth of 6 to 8 cm. Nitrite and nitrate concentration generally increased with depth with up to 11.7 (± 0.8) µmol NO_2^--N L^{-1} and 14.2 (± 0.4) µmol NO_3^-–N L^{-1}, respectively, at Tuvem, while at Divar the maximum levels were 14.2 (± 0.0) µmol NO_2^-–N L^{-1} 10.1 (± 0.2) µmol NO_3^--N L^{-1} at 6- to 8-cm depth, respectively. Total organic carbon at Tuvem varied from 2.1 to 4.5%, while at Divar it ranged between 2.5 and 4.0%.

Denitrification and Net Nitrous Oxide Production

The profile of DNT at Tuvem, showed a maxima at 2 to 4 cm (2.23 [± 0.34] nmol N_2O-N g^{-1} h^{-1}), which decreased with depth (Fig. 2). Similarly at Divar, the highest DNT activity was observed within 0 to 4 cm, which was ~5 times higher than at Tuvem.

Nitrous oxide production was significantly different (two-tailed *t* test, $P = 0.003$, $n = 15$) at both the locations and varied with depth. At Tuvem, a steady decrease in N_2O production with depth was observed. The highest production rate of 0.71 (± 0.11) nmol N_2O-N g^{-1} h^{-1} was recorded at 0 to 2 cm (Fig. 3). At the deepest layer investigated (8–10 cm), production of N_2O had decreased to a minimum of 0.12 (± 0.02) nmol N_2O-N g^{-1} h^{-1}. At Divar, the 2- to 4-cm layer had the maximum N_2O production rate of 1.95 (± 1.20) nmol N_2O-N g^{-1} h^{-1}. Depth integrated values for net N_2O production and denitrification activity at both the locations are shown in Table 2.

Table 1. Variation in the average values of physicochemical parameters (± SD; *n* = 15) in the Tuvem and Divar mangrove sediments.

Depth	pH	Eh	NH_4^+–N	NO_2^-–N	NO_3^-–N	TOC†
cm		mV	µmcl L^{-1}			%
			Location: Tuvem			
0–2	5.8 (± 0.2)	−27.8 (± 0.9)	14.7 (± 4.4)	4.5 (± 0.13)	10.4 (± 0.4)	2.1 (± 0.7)
2–4	5.9 (±0.2)	−23.2 (±7.39)	15.0 (±1.4)	8.6 (±0.33)	9.9 (±0.4)	2.9 (±1.4)
4–6	6.0 (± 0.6)	−9.5 (± 15.06)	7.8 (± 1.4)	8.1 (± 0.1)	10.9 (± 0.6)	4.5 (± 2.9)
6–8	6.0 (± 0.6)	−6 (± 16.45)	12.0 (± 0.8)	10.9 (± 0.6)	14.2 (± 0.4)	4.4 (± 2.8)
8–10	6.1 (± 0.5)	−6.3 (± 14.54)	15.1 (± 3.4)	11.7 (± 0.8)	11.4 (± 0.0)	4.0 (± 2.7)
			Location: Divar			
0–2	5.9 (± 0.3)	115 (± 64.7)	15.7 (± 3.4)	7.2 (± 0.0)	4.6 (± 0.7)	2.9 (± 1.6)
2–4	5.8 (± 0.1)	94.3 (± 62.6)	15.1 (± 2.0)	4.1 (± 0.2)	4.2 (± 0.2)	4.0 (± 2.1)
4–6	5.1 (± 0.1)	93.5 (± 45.18)	23.8 (± 2.3)	6.6 (± 0.6)	9.3 (± 0.2)	2.5 (± 1.0)
6–8	6.0 (± 0.1)	50.8 (± 61.38)	31.3 (± 1.8)	14.2 (± 0.1)	10.1 (± 0.2)	3.3 (± 1.7)
8–10	6.0 (± 0.0)	−5.7 (± 25.10)	22.1 (± 3.1)	8.1 (± 0.3)	10.0 (± 0.2)	3.1 (± 1.8)

† TOC, total organic carbon.

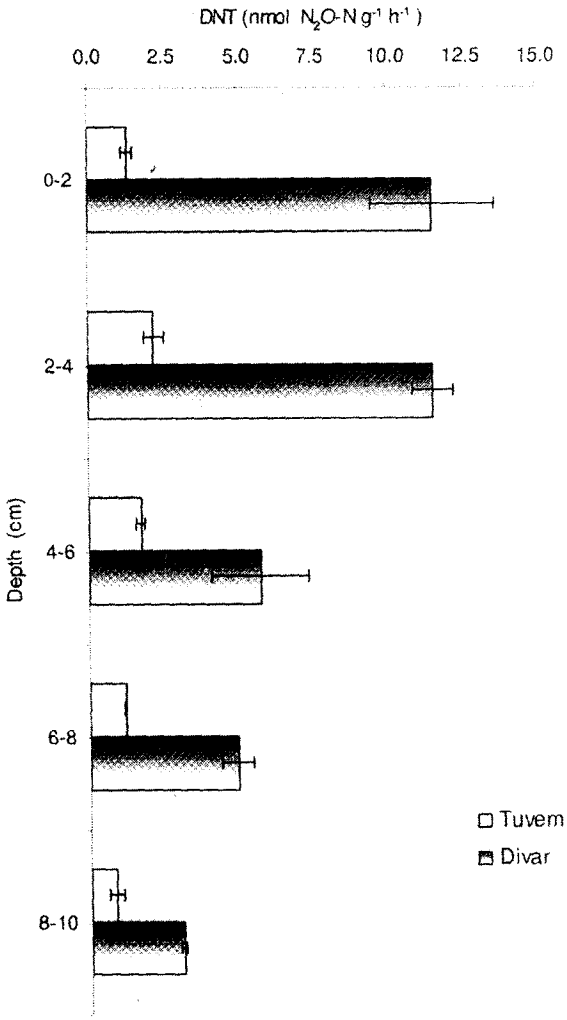

Fig. 2. Denitrification profile (± SD) over depth at Tuvem and Divar. DNT, denitrification activity.

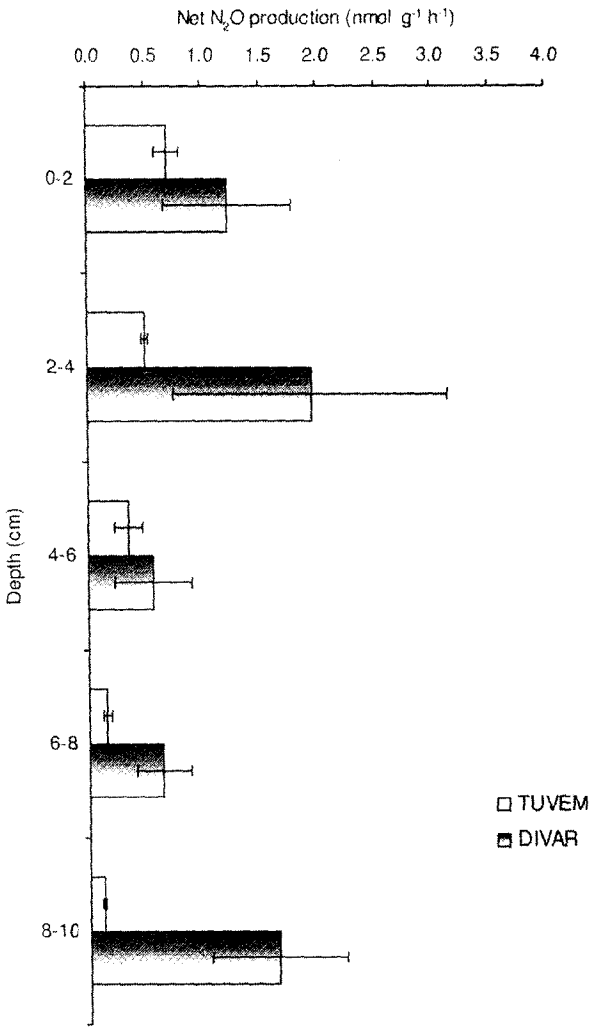

Fig. 3. Variation in net N_2O production (± SD) with depth from the Tuvem and Divar mangrove sediments.

Nitrous Oxide Production by Denitrifiers

Detailed investigations to elucidate the major pathway for N_2O production at the relatively pristine site Tuvem showed that these sediments harbored up to 10^7 denitrifiers g^{-1} sediment. Their abundance was maximum at 4 to 6 cm (7.14×10^7 cells g^{-1}) as compared to the other sections of the core where their number was relatively stable. In the Tuvem sediments, the denitrifiers significantly influenced N_2O production ($n = 15$; $r = 0.55$; $p < 0.05$) as illustrated in Fig. 4. They also showed an inverse relationship with pore water NH_4^+–N ($r = -0.57$; $p < 0.05$) and NO_2^-–N ($r = -0.55$; $p < 0.05$) concentrations.

In the presence of a nitrification inhibitor, denitrifiers produced a maximum of 1.26 (± 0.74) nmol N_2O-N g^{-1} h^{-1} at 0 to 2 cm (Fig. 5). Incomplete denitrification ($^I N_2O$) was responsible for 43 to 93% (Fig. 6) of the N_2O production, which accounts for about 13 to 52% (net N_2O/DNT × 100) of the N_2O produced through denitrification. Nitrous oxide production through nitrification ($^N N_2O$) was below detection.

Table 2. Depth integrated (0–10 cm) net N_2O production and denitrification activity in the Tuvem and Divar mangrove sediments.

Depth integrated activity	Tuvem	Divar
Denitrification (mmol N_2O-N m^{-2} h^{-1})	0.14	0.67
Net N_2O production (mmol N_2O-N m^{-2} h^{-1})	0.03	0.10

Environmental Factors Influencing Nitrous Oxide Production

The relationships of N_2O production rates with physicochemical parameters at Tuvem showed an inverse relationship between N_2O production and pore water NO_2^-–N concentration ($n = 15$; $r = -0.47$; $p < 0.05$). Up to 32% of the variation in N_2O production was negatively influenced by sediment pH ($r = -0.57$; $p < 0.05$). Even though the relationship between N_2O concentration and TOC was not statistically significant, a positive relationship existed between these parameters.

Principal component analysis on sediment variables resulted in four main components explaining nearly 80% of the total variance (Table 3) in N_2O production. The first component in the correlation plot explained 29% of the

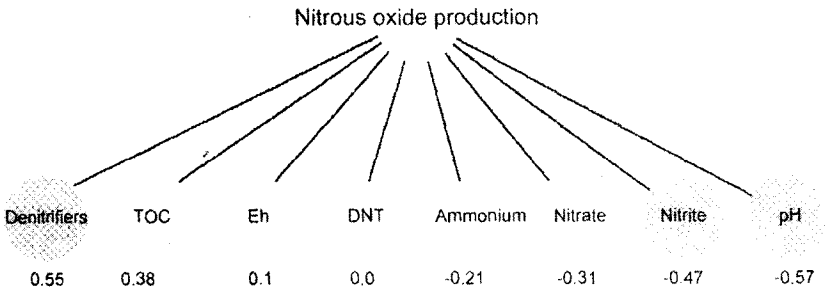

Fig. 4. A network visualization of factors influencing nitrous oxide production arranged based on the hierarchy of *r* values denoted at each node. Significant correlations at 0.05 probability level (n = 15) are highlighted in gray. DNT, denitrification activity; TOC, total organic carbon.

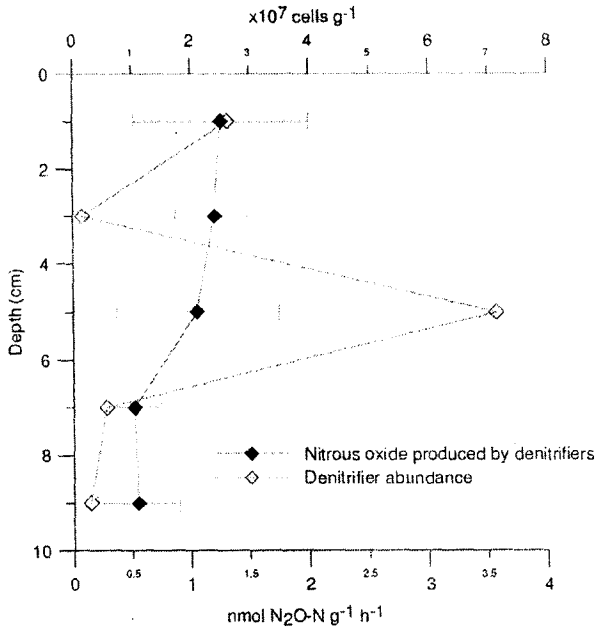

Fig. 5. Nitrous oxide production by denitrifiers under nitrification-inhibited conditions at Tuvem.

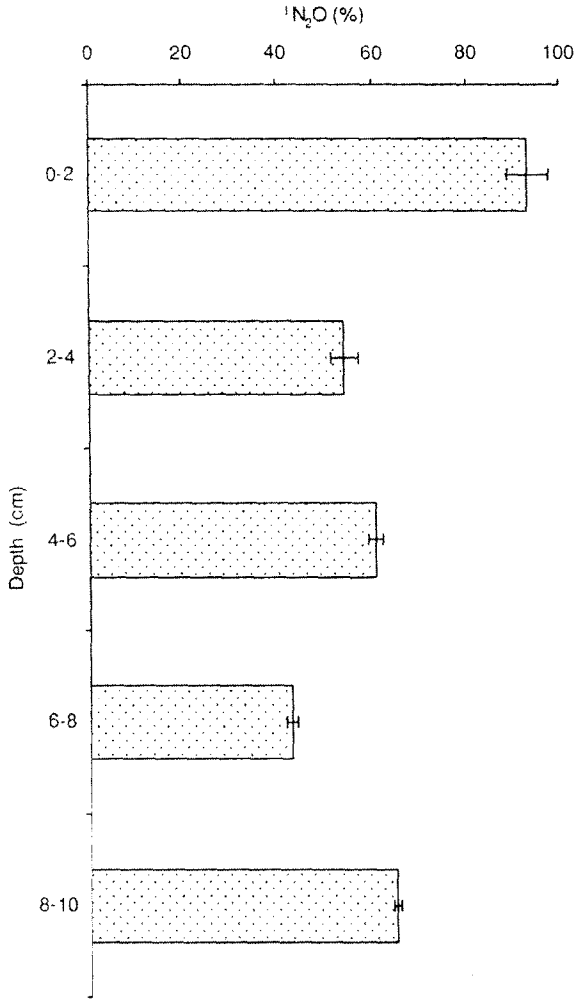

Fig. 6. Percentage of N_2O produced through incomplete denitrification (^1N_2O) at Tuvem.

observed variance. A forward regression (Fig. 7) of the reductive processes in the N cycle (DNT and N_2O production) was observed in the first component. Sediment pH, denitrifier abundance, and NO_2^-–N significantly influenced this component. The second component explained 22% of the variance with pore water NO_3^-–N and organic carbon concentrations correlating strongly with this component. The third component correlated with pore water NH_4^+–N and denitrification activity and explained 16% of the variation. The only and most significant variable in the fourth component was sediment redox potential, which explained 12% of the variation.

Discussion

The Mandovi estuary receives a considerable nutrient input from various sources including mining wastes (De Sousa, 1983, 1999), land runoff during the summer monsoon (Sardessai and Sundar, 2007; Divya et al., 2009), remineralization of organic matter (Pratihary et al., 2009), and sewage effluents (Ansari et al., 1986). Hence, we expected the adjoining Divar mangrove ecosystem fringing the estuary to also contain elevated inorganic N levels favoring denitrification and consequently, N_2O production. As hypothesized, down-core DNT at Divar was found to be comparatively higher than at the relatively pristine site Tuvem (Fig. 2). Low redox potentials (<115 mV) at the sampling sites (Table 1) are indicative of anaerobic conditions in the sediment, which are conducive to alternate respiratory pathways such as denitrification and sulfate reduction. Pore water profiles in the present study revealed that low NO_3^-–N concentrations within the 0- to 4-cm layer in the Divar sediments coincided with elevated DNT. Denitrification is dependent on NO_3^-–N supply either from nitrification (Klingensmith and Alexander, 1983) or availability of the nutrient from the ambient seawater. The surficial sediments, especially, are continuously replenished with nutrients from

Table 3. Results of principal component analysis of sediment variables and N_2O production.

Variable	Component 1	Component 2	Component 3	Component 4
N_2O production	0.856*	0.124	0.206	0.266
pH	−0.597*	−0.391	−0.541	0.134
Eh	−0.215	0.438	0.106	0.828*
Ammonium	−0.453	−0.420	0.640*	0.164
Nitrite	−0.687*	0.649	0.093	−0.004
Nitrate	−0.395	0.665*	−0.367	0.027
Denitrifiers	0.730*	−0.023	−0.496	0.326
TOC†	0.369	0.779*	0.286	−0.330
DNT†	0.021	0.133	−0.487*	−0.189
Total variance (%)	29.30	22.47	16.26	11.71

* Correlation significant at the 0.05 probability level.

† TOC, total organic carbon; DNT, denitrification activity.

the estuarine water. These sediments (≤4 cm) act as efficient traps to immobilize nutrients (Tam and Wong, 1993), which consequently enhances N metabolism within this depth range. Our observation is consistent with earlier studies by Jørgensen (1989), who showed that the denitrification capacity of estuarine sediments was always highest at the surface and declined with depth. To compare DNT measured using the acetylene block technique in the present study with other similar measurements in mangrove ecosystems, values were integrated to 10-cm depth. Denitrification occurred at a rate of up to 0.67 mmol N_2O-N m^{-2} h^{-1} in the sediments examined (Table 2). These values are similar in range to those reported from a mangrove system prone to secondary sewage effluents (Corredor and Morell, 1994) and in other estuaries (Barnes and Owens, 1998; Bernot et al., 2003). Although overenrichment of nutrients in coastal waters has ecological implications (Howarth et al., 2000), little is known about the fate of terrestrially derived nutrients in the Mandovi estuarine system. Pratihary et al. (2009) stated that benthic denitrification is responsible for 22% removal of riverine dissolved inorganic N in the Mandovi estuary. The high denitrifying capacity of the Divar sediments suggests that this ecosystem also acts as a buffer zone by reducing nutrient levels through the denitrification process and helps to maintain the water quality of the adjoining estuary.

High DNT at Divar was accompanied by elevated N_2O production (Fig. 3) compared with Tuvem. Net N_2O production at Divar occurred at a rate of up to 1.95 nmol g^{-1} h^{-1}, which is almost three times higher than the relatively pristine site Tuvem. Natural N_2O production rates in estuarine sediments range from 0.1 to 8.5 μmol m^{-2} h^{-1} (Wang et al., 2007). At NO_3^-–N concentrations of ~10–15 μmol (in situ + ambient seawater used in medium), depth integrated N_2O production values of up to 0.1 mmol N_2O-N m^{-2} h^{-1} (Table 2) recorded in our study are far greater than those reported by Dong et al. (2002) from the anthropogenically influenced Colne estuary at similar NO_3^- concentration. A microsensor approach by Meyer et al. (2008) has shown that in subtropical mangrove sediments, anaerobic N_2O production (through denitrification) under eutrophicated conditions occurs at a rate of 0.1 mmol m^{-2} h^{-1}. These values are comparable to those recorded in the current study. Our findings emphasize that ecosystems prone to higher N loading can have a detrimental effect on the environment through increased N_2O production. Elevated levels

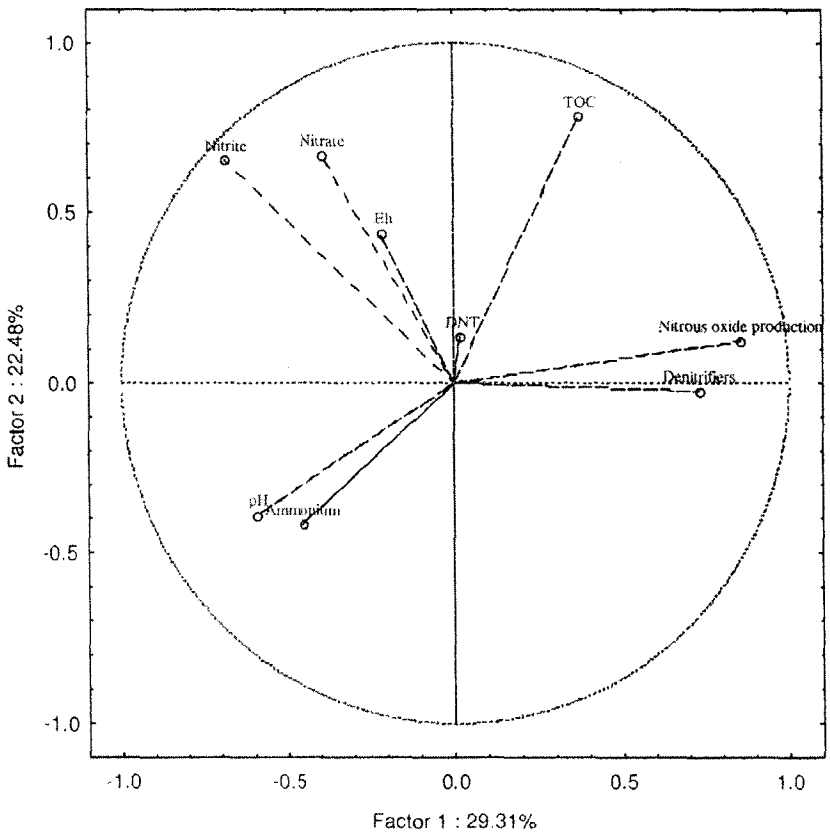

Fig. 7. Correlation plot from standardized principal component analysis (PCA) on sediment variables. Variables DNT, denitrification activity; TOC, total organic carbon.

of greenhouse gas emissions from these sediments would thus pose a major environmental issue.

Experiments to examine the major pathway for N_2O production in the present study indicate that up to 93% incomplete denitrification at the surface of mangrove sediments (Fig. 6) could contribute substantially to an increase in atmospheric N_2O. Studies by Robinson et al. (1998) in hypernutrified estuarine sediments have also shown higher N_2O concentrations in the surface layer (≤2 cm) attributing it to denitrification fuelled by NO_3^-–N availability. Similarly, Koike and Terauchi (1996) reported their highest concentration of N_2O in the top 1 cm of the marine sediments studied. Stratified sampling in our study showed that benthic N_2O production was found to generally decrease with depth. The flux of the radiative gas to the atmosphere would however be dependent on the diffusion coefficient and N_2O consumption rates in the sediment layers it passes through.

A number of factors are known to influence the production of N_2O in marine sediments. Physical, chemical, biological, and environmental factors like temperature, pH, sediment redox potential (Van Cleemput and Samater, 1996), organic C availability (Rosswall et al., 1989), NO_2^-–N concentration (Dong et al., 2002), and denitrifying communities play an important role in N_2O production. Statistical analysis showed that pore water nutrient concentrations, organic carbon availability, and denitrifiers were some of the important factors influencing the production of N_2O in the current study. Degradation of sediment organic matter results in acidic conditions. Although the relationship between N_2O production and TOC content in these sediments was not significant, a positive relationship existed and is thus indicative of its influence on the gas. Although the water soluble fraction of organic C was not estimated during the study, it is possible that it stimulated denitrifier activity and consequently, N_2O production. Both DNT and N_2O production followed a decreasing trend with depth; however, no significant relationship was observed between the two parameters. The production of N_2O could be thus be governed by the availability of electron donors and acceptors like NH_4^+, NO_3^-, and organic carbon rather than the rate of denitrification (Usui et al., 2001; Mathieu et al., 2006).

The denitrifier community was also found to influence N_2O production in mangrove sediments (Fig. 4, 5). They numbered up to 10^7 cells g^{-1}, and their abundance could be regulated by the amount of organic matter available for their growth (Fig. 7). This can be easily explained by the fact that the denitrifiers are facultative aerobic microorganisms and their activity is limited by the amount of NO_3^-–N available. Estuaries are generally heterotrophic systems, with bacterial respiration exceeding primary production (Heip et al., 1995, Gattuso et al., 1998). Consequently, removal of dissolved inorganic N from estuaries occurs through sedimentary denitrification and/or burial in the sediment (Middelburg and Nieuwenhuize, 2000). An inverse relationship observed between denitrifiers and pore water nutrient concentrations in the present study suggests that denitrification could play an important role in mitigating excess nutrients within the aquatic system, preventing eutrophication.

In sulfidic sediments, the denitrification end-product is known to shift from N_2 to partially reduced inorganic N forms such as NO_2^- and N_2O (Ebrahimipour et al., 2000). This could explain the high NO_2^-–N pool in the largely anaerobic mangrove sediments studied. About 22% variation in N_2O production was caused by the variation in NO_2^-–N concentrations, suggesting that it was one of the important and statistically significant parameters regulating the production of N_2O in mangrove sediments. Many other studies have shown a correlation between N_2O production and NO_2^-–N concentration (He et al., 2001; Dong et al., 2004; Alinsafi et al., 2008). Denitrification activity in estuarine sediments is dependent on NO_3^-–N availability (Kana et al., 1998), consequently leading to NO_2^- and N_2O production. Dong et al. (2002) stated that formation of N_2O from NO_2^-–N is thermodynamically favorable compared to nitrate, suggesting that it may be a critical factor regulating N_2O formation. Bauza et al. (2002) reported N_2O production mainly through nitrification in red mangrove forests, which are characterized by oxic conditions (redox potentials: 159–377 mV) and NH_4^+–N concentrations varying from 0.188 to 0.273 mmol L^{-1}. On the contrary, Meyer et al. (2008) showed that nitrification and denitrification contributed almost equally to N_2O production under NH_4^+-amended conditions. In the present study, although ammonium concentrations at Tuvem were <15 μmol L^{-1}, it did not appear to assert a strong influence on N_2O production. Nitrification could be a significant source of N_2O. In organically rich mangrove sediments, NH_4^+–N released through remineralization of organic matter could easily get bound to clay particles, making it unavailable for biological uptake. Thus, when NO_3^-–N and NO_2^-–N predominate, N_2O arises from microbial denitrification (Corredor et al., 1999). Nitrate respiration is kinetically and thermodynamically favorable (Aivasidis et al., 2005) in oxygen-depleted environments and is preferred over other electron acceptors (Canfield et al., 2005). Denitrification activity also enhances NO_2^- uptake, preventing its accumulation to toxic levels. Experimental results reveal that denitrification was the major pathway for N_2O production in the mangrove ecosystems of Goa, India. Close grid measurements at submillimeter intervals by Meyer et al. (2008) have shown that in subtropical mangrove sediments, N_2O production through nitrification occurs very close to the surface, while denitrification is responsible for its production in the deeper anaerobic layers. The production of N_2O through nitrification could be more prominent when the oxidative process is more pronounced. However, N_2O production through nitrification was not detected, indicating that the reductive phase of the N cycle was predominant at the time of sampling (postmonsoon). Despite denitrification showing highest activity during the premonsoon season (data not shown), measurements have shown that the mechanism of N_2O production in mangrove sediments of Goa during all the three seasons is essentially the same.

Conclusions

Denitrification and N_2O producing capacity of anthropogenically influenced mangrove ecosystems was clearly higher when compared with relatively pristine locations. Nitrous oxide production in the mangrove sediments of Goa was associated mainly with denitrification, whereas its production through nitrification was nondetectable. The highest percentage of N_2O

production through incomplete denitrification occurred within the first 2 cm of the sediment, a fraction of which could be lost to the atmosphere. Nitrite concentration and denitrifier abundance were the two most important environmental parameters governing the production of N_2O in these sediments, indicative of active nutrient uptake by the autochthonous denitrifier community. Although mangroves have the ability to efficiently moderate elevated nutrient concentrations in the estuarine system through the denitrification pathway, they also pose a threat by increasing greenhouse gas production. Our study shows that in mangroves prone to elevated nutrient levels, benthic N_2O production was three orders higher than natural production rates in estuarine sediments elsewhere. Thus, adequate measures such as lowering use of NH_4NO_3 in mining activities and building predictive models (Valiela et al., 2000, 2004; Bowen et al., 2007) for tracking the fate of N inputs could be initiated to minimize N loading in adjoining estuarine systems. These strategies would not only help to lower N pollution but also simultaneously result in decreased N_2O emission to the atmosphere.

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Nitrate levels modulate denitrification activity in tropical mangrove sediments (Goa, India)

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Abstract A study to examine the short-term effect of nitrate and organic carbon addition on denitrification activity was carried out on sediments from a mangrove ecosystem prone to anthropogenic activities (Divar, Goa, India). Laboratory microcosms were prepared using sediment sectioned at every 2-cm-depth interval from the surface to 10 cm. The incubations were subjected to varying nitrate amendments at concentrations ranging from 0, 5, 10, 20, 40 to 60 μmol l^{-1} (up to three times more than measured in field). Nitrous oxide production rates increased significantly ($n = 15$; $p < 0.001$) on addition of the nutrient at all depths investigated indicating that denitrification in mangrove sediments was NO_3^- limited. Incubations amended with organic carbon were prepared using glucose as a substrate with concentrations ranging from 0%, 0.1%, 0.3%, 0.5%, 0.75% to 1%. No significant increase in N_2O production was observed on organic C addition. When both the substrates were in excess (1 mmol KNO_3 + 1 mmol glucose), potential denitrification rates decreased with depth and were up to 38 times higher than the in situ denitrification activity varying from 81.26 to 304.09 μmol N_2O-N m^{-2} h^{-1}. These results reveal that mangrove sediments could act as a sink for nitrate and microbially mediated denitrification could effectively reduce N load controlling any adverse environmental impact in the adjoining estuarine system.

Keywords Denitrification · Organic carbon · Nitrate · Mangrove · Sediment

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Introduction

In ecosystems with high inputs of nitrogen (N) such as estuaries, denitrification mediates reduction of nitrogen load and therefore contributes to eutrophication control (Nogales et al. 2002). One such ecosystem is the mangroves which constitute nearly 75% of tidal vegetation in tropical regions (Alongi et al. 1989). These habitats lie in close proximity to areas prone to anthropogenic activity (Muñoz-Hincapié et al. 2002). Mangroves play an important role in the biogeochemical cycles of coastal and marine ecosystems (Thorsten and José 2001). They function as efficient buffer zones mitigating large amounts of excess nutrients in the estuarine system reducing water pollution.

The nitrogen cycle within mangrove forests is mediated predominantly by microbial rather than chemical processes (Alongi et al. 1992). Inorganic N and other parameters important for its turnover

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can fluctuate widely due to the position of mangroves in the intertidal zone (Meyer et al. 2008). Chiu et al. (2004) state that as much as 55% of the N loss in mangrove sediments occurs through denitrification. Mangrove sediments are largely anaerobic and rich in organic matter providing favorable conditions for denitrification. The process is mediated mainly by facultatively anaerobic heterotrophic bacteria which utilize oxidized N compounds for respiration under oxygen deficient conditions (Zumft 1997). Earlier studies have highlighted nitrate (Morell and Corredor 1993; Corredor and Morell 1994; Rivera-Monroy and Twilley 1996; Kana et al. 1998) and organic carbon (McCarty and Bremner 1993; Pfenning and McMahon 1997; Hill and Cardaci 2004) as important factors affecting denitrification. In sediment, denitrifying activity is highly correlated with water-extractable organic carbon and is frequently stimulated by the addition of exogenous carbon (Knowles 1982; Hahndel and Isermann 1992). Sufficient organic carbon is required for denitrification to occur as it is a source of energy for the conversion of nitrate to nitrogen gas. In the marine environment, high denitrifying activity has been reported to occur within the oxygen minimum zones (OMZs) contributing to 30–50% of the total nitrogen loss and has been commonly attributed to heterotrophic denitrification (Gruber and Sarmiento 1997; Codispoti et al. 2001). In the OMZs of the Eastern Tropical North and South Pacific, denitrification appeared to be limited by organic carbon (Ward et al. 2008). In the Indian Ocean region, denitrification in the Arabian Sea has also been shown to be dependent on the organic carbon regime (Anderson et al. 2007). So far, there have been no reports on the factors limiting denitrification from nearby estuarine zones.

In the present study, we examined the short-term effect of nitrate and organic carbon addition on denitrification activity in mangrove sediments prone to nutrient input from anthropogenic activities. We hypothesize that in organically rich mangrove sediments, denitrification could be more dependent on nitrate availability. The significant increase in denitrification activity on nitrate amendments observed in the present study further corroborates our hypothesis suggesting that nitrate is the main limiting factor for denitrification in mangrove sediments.

Materials and methods

Study area and sampling

Investigations were carried out at fringing mangrove forest located at Divar in Goa, west coast of India (Fig. 1) during January 2008 (post-monsoon

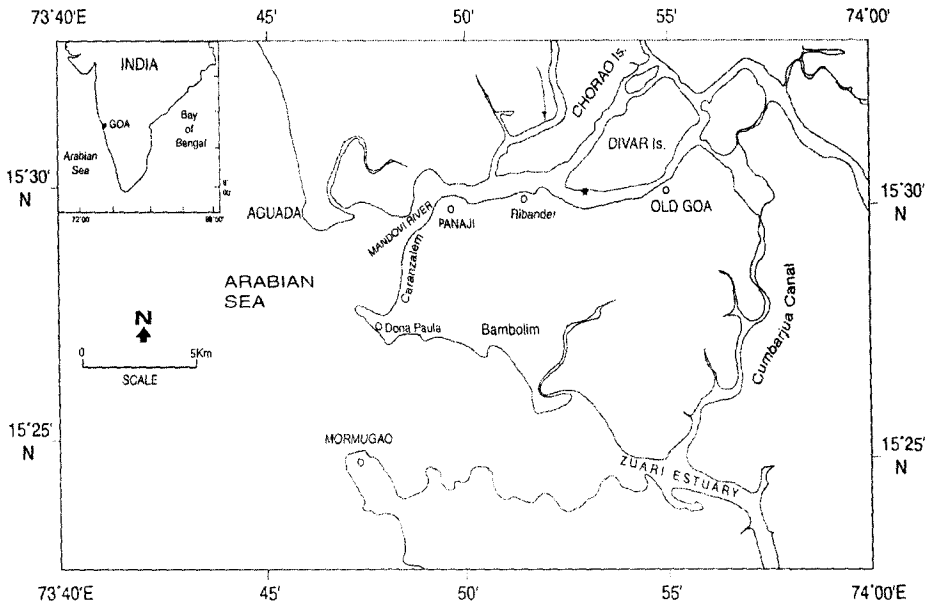

Fig. 1 Sampling location at Divar Island, Goa

season). The Divar mangrove ecosystem (15°30′35″ N and 73°52′63″ E) is separated from the mainland by the river Mandovi and is accessible by ferry. A significant number of inhabitants colonize along the banks. The Mandovi is important for the economy of the territory as it is heavily used for transportation of iron ore from mines located upstream. These iron ore beneficiation plants situated on the riverbank discharge effluents directly into the estuary. This discharge contains high quantities of NH_4NO_3 used as explosive in ferromanganese mining operations (De Souza 1999).

Sediment cores were collected at low tide and transported in an ice box. Cores for activity measurements were maintained at 4°C. For measurement of labile organic matter (LOM), cores were immediately sectioned at 0–2-, 2–4-, 4–6-, 6–8-, and 8–10-cm interval. The sections were dried at 60°C ±2, powdered and sieved through a 200-μm sieve and stored in clean polyvinyl chloride vials until analysis.

Pore water nitrate

For the estimation of pore water nitrate concentration, subsamples were taken at every 2 cm intervals from surface to 10 cm by carefully sectioning the core. Each section (7.5 cm diameter and 2 cm thick) was transferred to 100 ml of sterile saline and homogenized using a glass rod. The slurry was centrifuged at 5,000 rpm for 10 min and 4°C. Low spin was maintained during centrifugation to ensure minimal change in nutrient concentration due to lysis of benthic infauna. The supernatant was filtered through a 0.2-μm filter and stored at −20°C until analysis. Nitrate was measured colorimetrically (*Shimadzu* UV/VIS spectrophotometer; precision ± 0.01 μmol l^{-1}) as described by Wood et al. (1967). Weight of the sediment used in the extraction was estimated by drying in a hot air oven at 60°C for 48 h.

Total organic carbon

Total organic carbon (TOC) was determined by wet oxidation method with a precision of 0.01% (El Wakeel and Riley 1957).

Labile organic matter

The LOM was measured as a sum of proteins, carbohydrate and lipid content in the sediments. Proteins were estimated as described by Lowry et al. (1951). Carbohydrate was estimated by phenol–sulfuric acid method (Dubois et al. 1956) using glucose as standard. Lipid content in the sediment was estimated by using the acid dichromate method outlined by Parsons et al. (1984).

Effect of NO_3^- addition on denitrification

Sediment cores were demarcated into five sections (0–2, 2–4, 4–6, 6–8, and 8–10 cm). About 1 cm^3 of sediment was extruded from each section using a syringe core and transferred aseptically to sterile 20-ml headspace vials. Three milliliters of sterilized ambient seawater from the sampling site was added to the sediment. This seawater used for slurry preparation was spiked with a KNO_3 solution to give final concentrations of 0 (unamended to reflect in situ denitrification activity), 5, 10, 20, 40, and 60 μmol NO_3-N l^{-1}. The seawater was also amended with chloramphenicol at a final concentration of 1 g l^{-1} (Bonin et al. 2002) to inhibit de novo synthesis of denitrifying enzymes thus reflecting in situ activity at the time of sampling (Brooks et al. 1992). No additional carbon substrates were added. The vials were capped with butyl stoppers and were briefly vortexed to form slurry. The vials were purged with high purity N_2 for 10 min to induce anaerobic conditions. Acetylene gas at 20 kPa (Bonin et al. 2002) was injected into the headspace to inhibit N_2O production by nitrification and its reduction by denitrification (Castro-González and Farías 2004). Triplicates were maintained at each depth and the vials were incubated in the dark for 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h. At the end of the incubation period, bacterial activity was terminated using 0.1 ml of 1 M $HgCl_2$.

Nitrous oxide in the headspace was analyzed using a *Shimadzu* 2010 gas chromatograph fitted with a electron capture detector and Porapak Q column (1/8″ SS column, 3.05 m length, 80/100 mesh). The oven and detector temperatures were

40°C and 300°C, respectively. High-purity nitrogen at a flow rate of 35 ml min^{-1} was used as a carrier gas. The gas chromatograph was calibrated using a secondary standard 44 ± 0.38 nmol N_2O in nitrogen (National Physical Laboratory, New Delhi, India). Denitrification activity was calculated based on the linear accumulation of N_2O over time. The solubility coefficients of N_2O were used to correct for dissolved N_2O in the microcosms (Weiss and Price 1980). Sediment used in each microcosm was filtered through a laboratory grade filter paper, dried at 60°C for approximately 48 h, and weighed. Denitrification activity was calculated as nanomoles of N_2O-N per gram per hour of dry sediment, extrapolated to a unit area basis (per square meter) and expressed as micromoles of N_2O-N per square meter per hour.

Effect of organic C addition

Sterilized seawater used for slurry preparation was amended with 1 g l^{-1} chloramphenicol and glucose solution at a final concentration of 0%, 0.1%, 0.3%, 0.5%, 0.75%, and 1%. Samples were prepared and analyzed as described above. No additional nitrate was added.

Potential denitrification rates

Samples were prepared and analyzed as described above except that the seawater used for slurry preparation was amended with KNO_3 and glucose at a final concentration of 1 mmol l^{-1} in addition to chloramphenicol.

Statistical analyses

Statistical analyses have been carried out using analysis tool pack in *Microsoft Excel*. Significant differences in denitrification rates have been determined using analysis of variance (ANOVA).

Results and discussion

Estuarine sediments are known to have considerable nutrient loading mostly derived from extraneous inputs like sewage outfall (King and Nedwell 1987). The present study showed that the Divar sediments harbored measurable pore water nitrate content which increased with depth (Fig. 2) ranging from 4.15 (±0.21) to 18.71 (±0.28) μmol l^{-1}. Down-core profiling of denitrification showed a subsurface maxima at 2–4 cm with a rate of 20.08 (±4.37) μmol N_2O-N m^{-2} h^{-1} (1.43 ± 0.66 nmol g^{-1} h^{-1}). Isotopic measurements by Chiu et al. (2004) have shown much higher denitrification activity in surficial mangrove sediments as compared to the deeper nonrhizosphere soil with rates of up to 120 nmol g^{-1} h^{-1}. Thomas and Lloyd (1994) have also reported maximum denitrification to occur at the surface in estuarine sediment. The process is known to be dependent on nitrate availability (Seitzinger 1990). Low nitrate values encountered

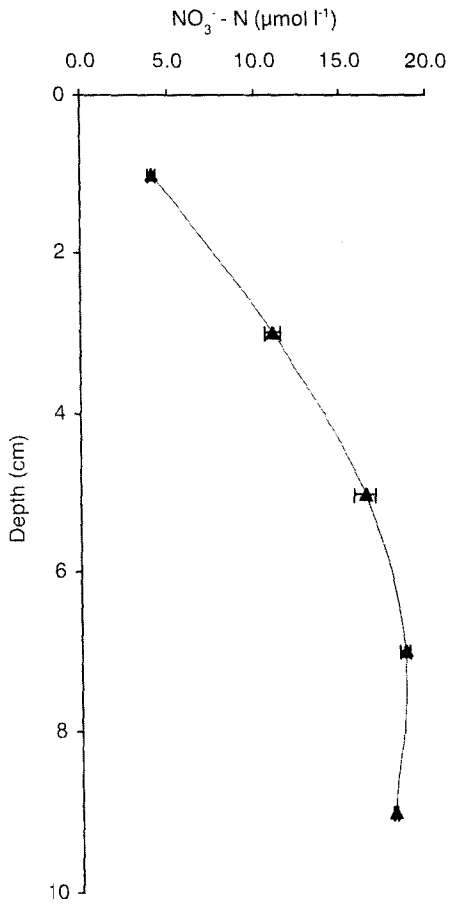

Fig. 2 Down-core variation in pore water nitrate (±SD) at Divar

at the surface could thus be attributed to higher nitrate removal in the upper few centimeters of the sediment.

High denitrification activity at 2–4 cm coincided with increased organic carbon availability at this depth which varied from 2.88% at 4–6 cm to 4.95% at 2–4 cm (Fig. 3). Naturally occurring organic carbon in sediments is critical because it can influence nutrient availability (Moore 1989), enhance biological activity, and can increase acidity of ambient waters through organic acids (Eshleman and Hemond 1985; Kerekes et al. 1986). The primary source of TOC in mangrove swamps is plant material which is decomposed by sediment organisms and converted to organic compounds viz., carbohydrates, proteins and lipids. A sizeable fraction of organic matter could be transported to deeper depths by bioturbating infauna and retained within the system. Natural processes and human activities can also result in elevated content of TOC in the area. Sardessai (1993) have shown that decomposition of mangrove litter and influx of freshwater during monsoons contributes to organic matter derived from humic acids. In the present investigation, labile organic matter showed a similar depth-wise distribution like TOC with a maximum concentration of 0.68% LOM at 2–4 cm (Fig. 4). Labile organic matter is known to limit denitrification (McCutchan and Lewis 2008). In the reductive phase of the N cycle, NO_3^- ions are reduced to N_2O or N_2 whereas organic C gets oxidized to CO_2 and H_2O. Our findings suggest that about 11–19% of the TOC is present in readily utilizable form (LOM) in mangrove sediments and could be important for heterotrophic metabolism.

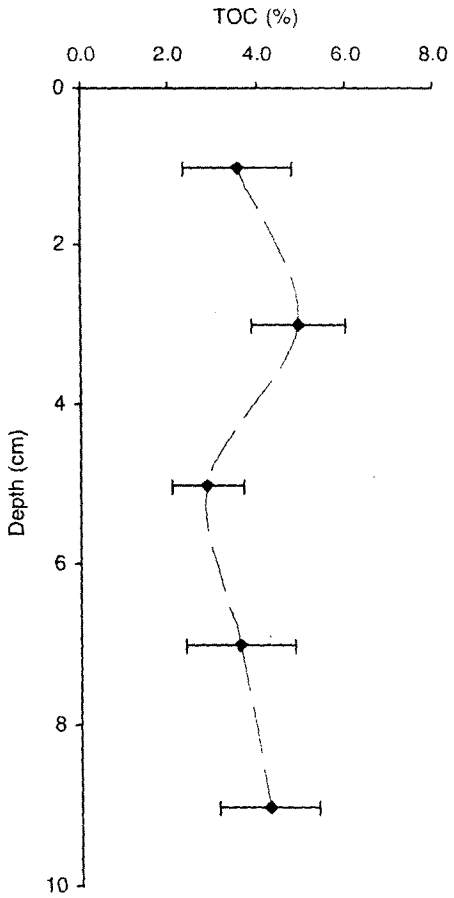

Fig. 3 Down-core variation in total organic carbon (*TOC*; ±SD) at Divar

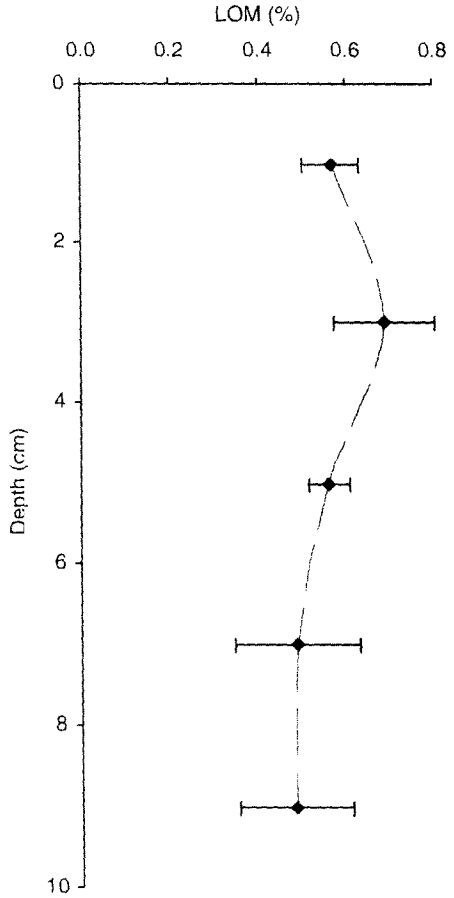

Fig. 4 Down-core variation in labile organic matter (*LOM*; ±SD) at Divar

Denitrification activity in mangrove sediments was monitored when subjected to a wide range of nitrate amendments (three times higher than the ambient concentration). Denitrification activity was stimulated in microcosms containing seawater with all combinations of nitrate amendments, i.e., 5, 10, 20, 40, and 60 μmol NO_3-N l^{-1}. Higher rates were observed especially at depth ≤4 cm. Highest activity of 129.22 (±31.94) μmol N_2O-N m^{-2} h^{-1} at 0–2 cm was observed at nitrate amendment of 40 μmol NO_3-N l^{-1} (Fig. 5). At depths >4 cm, the activity was less pronounced. In estuarine sediments prone to high nitrate inputs, the bacterial communities adapt to changes in the concentration exhibiting higher rates of nitrate reduction and also increasing the proportion of nitrate reduced to gaseous products (King and Nedwell 1987). Laverman et al. (2007) have shown that denitrification in estuarine sediments is nitrate-limited and the resident denitrifying community rapidly adjusts its level of activity to increased nitrate availability. Denitrification accounts for 27% to 57% of the nitrate consumption in estuarine and coastal sediments (Nishio et al. 1982). The Divar mangrove ecosystem is prone to high nutrient input from mining rejects, land runoff and domestic sewage discharge. The increase in denitrification activity especially in the first few centimeters is indicative of the high nitrate removal capacity of these sediments. Corredor and Morell (1994) have confirmed that mangrove sediment–microbial communities are capable of depurating up to 10–15 times the nitrate added. Recently, Krishnan and Loka Bharathi (2009) have shown that nitrification rates in the Divar sediments vary between 2.7 to 18.2 nmol g^{-1} h^{-1}. A strong coupling between redox processes of the N cycle could exist in these sediments wherein nitrate supplied continuously through the nitrification process could be fueling denitrification especially in the upper few centimeters.

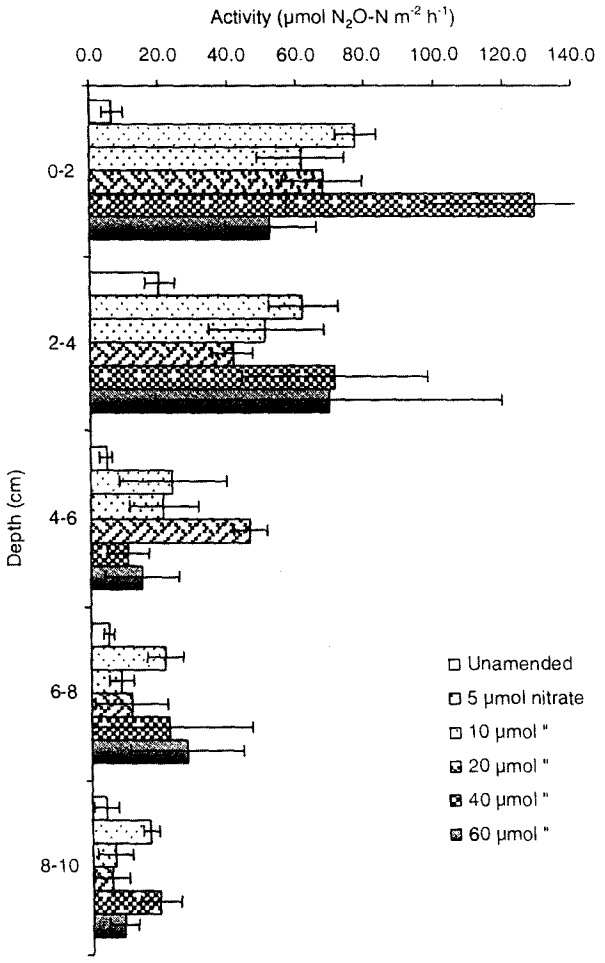

Fig. 5 Effect of nitrate addition on denitrification activity (±SD)

Organic carbon addition stimulated denitrification activity mostly at depths ≥4 cm (Fig. 6). However, maximum activity of only 35.24 (±9.93) μmol N_2O-N m^{-2} h^{-1} was recorded at 4–6 cm depth. A 0.5% amendment of labile organic carbon (glucose) was found to effectively stimulate denitrification activity at all depths suggesting that the process is optimal at this concentration. Statistical analyses did not show significant increase in denitrification activity on organic carbon addition as compared to amendments with nitrate (one-way ANOVA; $n = 15$; $p < 0.001$) at all depths indicating that organic carbon was not a limiting factor for denitrification in mangrove sediments. Denitrification in oxygen minimum zones is known to be fueled almost entirely by organic matter supplied by particles sinking vertically from the euphotic zone (Anderson et al. 2007; Ward et al. 2008). In these environs, organic carbon is the main limiting factor controlling denitrification. In contrast, estuarine systems have considerable organic C loading and labile organic matter is readily available for metabolic activity. In such circumstances, denitrification is

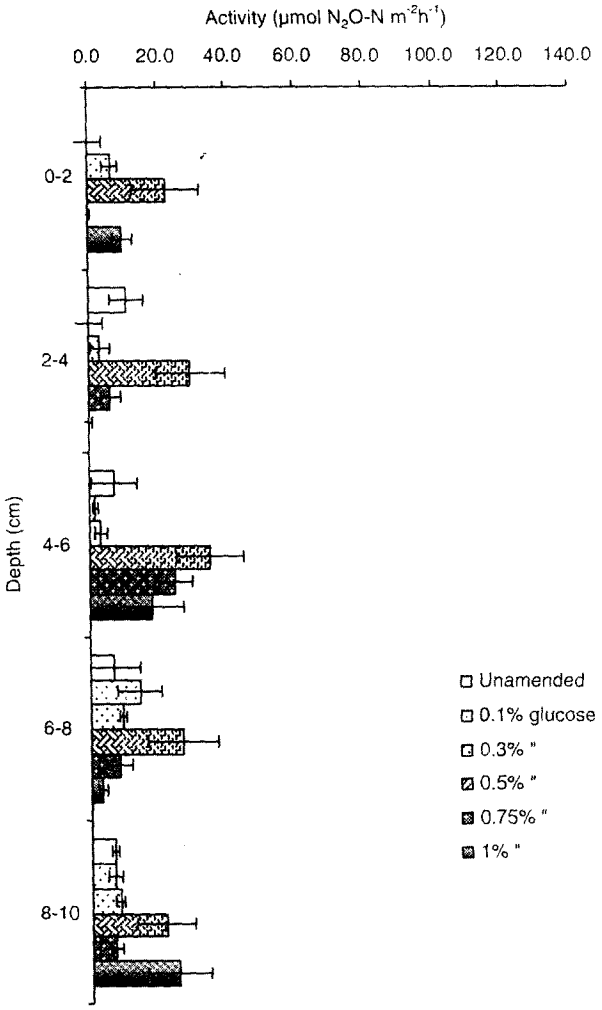

Fig. 6 Effect of varying organic carbon amendments on denitrification (±SD)

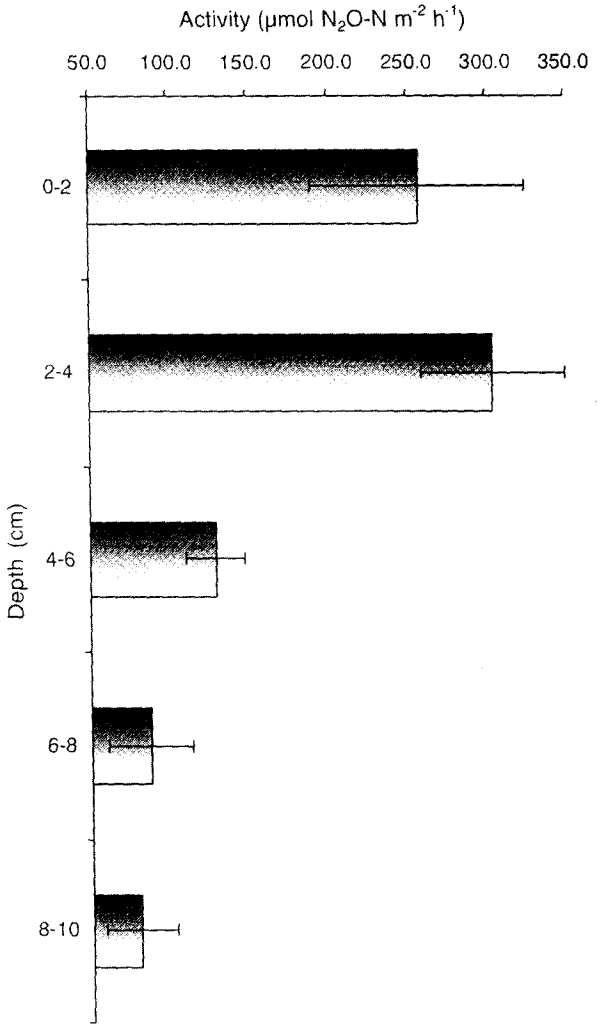

Fig. 7 Down-core variation in potential denitrification activity (±SD)

more dependent on nitrate availability. Similar observations have been made by Davidsson and Leonardson (1996) using peaty and sandy soil in which NO_3^- has been shown to be a stronger regulator of denitrification than organic carbon.

Potential denitrification rates decreased with depth and were 15–38 times higher (within 0–10 cm core) than in situ denitrification activity when both nitrate and organic carbon were in excess. Highest activity of up to 304.09 (±47.6) µmol N_2O-N m^{-2} h^{-1} (Fig. 7) was recorded at 2–4 cm. At 8–10 cm, the activity decreased to 81.25 (±22.58) µmol N_2O-N m^{-2} h^{-1}. Flemer et al. (1998) have reported relatively high potential denitrification rates in estuarine sediments ranging between 500 to 1,000 µmol N m^{-2} h^{-1} which were also limited by nitrate availability. Laverman et al. (2007) have encountered maximum in situ rates two-fold lower than the maximum potential rate in surficial sediment indicating that in situ denitrification was nitrate limited. Our observations indicate that the surficial sediments have a comparatively higher denitrifying potential. This could be possible since the denitrifying communities at depths <4 cm get a continuous availability of electron acceptors through lateral supply from the ambient seawater in addition to relatively intense nitrification in the surficial sediments. Thus, these microbes are

able to eliminate elevated nitrate concentration through the denitrification pathway.

Conclusion

The Divar mangrove sediments harbored sufficient amount of labile organic matter to support denitrification activity. Microcosm experiments have indicated that nitrate addition enhanced denitrification at in situ organic carbon concentration. The benthic denitrifying community in these habitats respond rapidly to episodic events of elevated nutrient supply by increasing the rate of nitrate removal through the denitrification pathway. Thus, these sediments could act as a sink for nitrate and this nutrient is more crucial in controlling denitrification activity. Potential denitrification rates were up to 38 times higher than the in situ denitrification activity indicating that the process was important in maintaining low concentration of nitrate and helps to maintain the water quality of the adjoining aquatic system.

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