Chapter 5
Discussion
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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 N₂O or N₂ 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 N₂O-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 N₂O-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 NH₄⁺. 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, NO₃⁻, 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 $3.64 \times 10^4$ 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 McMahon 1997; McCarty 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):

$$5\text{Mn}^{2+} + 2\text{NO}_3^- + 8\text{OH}^- \rightarrow 5\text{MnO}_2 + \text{N}_2 + 4\text{H}_2\text{O}$$

Experiments using denitifying 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 an 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 $\leq 6$ 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 N₂O 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 N₂O 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 N₂O 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). N₂O 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 N₂O 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), N₂O 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 NO₃⁻ concentration. Intertidal rocky biofilms have also shown N₂O 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 N₂O production and its consequent flux to the atmosphere.

Experiments to examine the major pathway for N₂O 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 N₂O. Studies by Robinson et al., (1998) in hypernutrified estuarine sediments have also shown higher N₂O concentrations in the surface layer (<2 cm) attributing it to denitrification fuelled by NO₃⁻ 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 N₂O 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 N₂O consumption rates in the sediment layers it passes. A microsensor approach by Meyer et al., (2008) has shown that in sub-tropical mangrove sediments, N₂O 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 N₂O 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 N₂O 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 Tuven 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. N₂O 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 N₂O production. Both denitrification rate and N₂O production followed a decreasing trend with depth, however no significant relationship was observed between the two parameters. The production of N₂O could be thus be governed by the availability of electron donors and acceptors like NH₄⁺, NO₃⁻, 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 10⁷ 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 N₂ to partially reduced ones such as nitrite and N₂O (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

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Area</th>
<th>Denitrification rates</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Terminos Lagoon, Mexico</td>
<td>Fringe mangrove: 9.4 μmol m⁻² h⁻¹</td>
<td>¹⁵N isotope technique</td>
<td>Rivera-Monroy et al., 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basin mangrove: 1.9 to 4.5 μmol m⁻² h⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vegetated sediments: 1.91 μmol m⁻² h⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mangrove forest, Phuket, Thailand</td>
<td>Unvegetated sediments: 0.54 μmol m⁻² h⁻¹</td>
<td>¹⁵N isotope technique</td>
<td>Kristensen et al., 1998</td>
</tr>
<tr>
<td>3</td>
<td>Logan/Albert River, Queensland, Australia</td>
<td>85 nmol N cm⁻³ h⁻¹ or 38 nmol N g⁻¹ h⁻¹</td>
<td>¹⁵N isotope technique</td>
<td>Meyer et al., 2005</td>
</tr>
<tr>
<td>4</td>
<td>Matang Mangrove Forest Reserve, Malaysia</td>
<td>16-458 μmol N₂ m⁻² h⁻¹</td>
<td>N₂-gas flux technique</td>
<td>Alongi et al., 2004</td>
</tr>
<tr>
<td>5</td>
<td>Jiulongjiang Estuary, China</td>
<td>46.08 to 157.5 μmol N₂ m⁻² h⁻¹</td>
<td>N₂-gas flux technique</td>
<td>Alongi et al., 2005</td>
</tr>
<tr>
<td>6</td>
<td>Southwest coast of Puerto Rico</td>
<td>0.12 and 7.8 μmol N₂O m⁻² h⁻¹</td>
<td>Gas chromatography</td>
<td>Corredor et al., 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tuve: 0.03-74.88 nmol g⁻¹ h⁻¹ or 0.339-846.14 μmol N₂ m⁻² h⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Goa, India</td>
<td>Divar: 1.47-222.59 nmol g⁻¹ h⁻¹ or 16.61-2515.26 μmol N₂ m⁻² h⁻¹</td>
<td>¹⁵N isotope technique</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Note: *For conversion of DNT activity in nmol N₂ g⁻¹ h⁻¹ to μmol N₂ m⁻² h⁻¹:

\[(\text{DNT activity in nmol N}_2 \text{ g}^{-1} \text{ h}^{-1} \times 1.13 \times 10^4) / 10^3\]
initiated to minimize N loading in adjoining estuarine systems in order to lower environmental pollution but also simultaneously result in decreased N$_2$O 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 Tu vem. 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 (\(D_{\text{no}}\)) 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 \(\text{nosZ}\) 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 \(\text{nosZ}\) were most abundant at 6-8 cm. Experiments using the bacterial strain \(Pseudomonas mandelii\) has shown that \(\text{nosZ}\) gene expression did not respond to increasing \(\text{NO}_3^-\) 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 \(\text{nosZ}\) 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 N₂ 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 N₂ 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 N₂ production from denitrification and anammox together.

Though denitrification is a major mechanism for NO₃⁻ removal in coastal sediments (Tuerk and Aelion, 2005), N₂ produced through complete denitrification of nitrate accounted for ≤1% of the total pore water nitrate reduced. The overall low contribution of denitrification in NO₃⁻ 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 N₂ 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 NH₄NO₃ 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\textsuperscript{-3} h\textsuperscript{-1} (Lavernman et al., 2006).

DNRA showed a steady increase in \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} 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\textsubscript{3}\textsuperscript{-} to H\textsubscript{2}S/S\textsubscript{2}\textsuperscript{−} 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\textsubscript{2}O and N\textsubscript{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\textsubscript{2}O flux (Bange et al., 1996). The present observations reveal that N\textsubscript{2}O production in mangrove sediments is relatively small highlighting the capacity of mangroves to buffer the climate against the greenhouse 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\textsubscript{3}\textsuperscript{-} 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=Tuven; 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 greenhouse gas N2O 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 N2O 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 N2O-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$_2$O or N$_2$ whereas organic C gets oxidized to CO$_2$ and H$_2$O. 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$_2$O-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$_2$O 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,
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.