Chapter 1

General Introduction
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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 NO₃⁻ 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.

$$\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_2\text{OH} \rightarrow \text{NH}_3 \rightarrow \text{R-NH}_2$$

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

$$\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$$

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: O₂ > NO₃⁻ > MnO₂ > FeO(OH) > SO₄²⁻ > CO₂ (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 (Drysdaile 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 (N₂O) instead of di-nitrogen (N₂) as the final product as they lack N₂O 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 O₂ or NO₃⁻ 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 NO₃⁻ and NO₂⁻.

Denitrifying microbial communities have been detected in marine and terrestrial habitats. In terrestrial ecosystems, denitrifier density ranges between 10⁵-6 cells g⁻¹ dry soil (Cheneby et al., 2000). In coastal sediments, culture based techniques have shown up to 10⁻⁴ cells/g of denitrifier abundance (Michotey et al., 2000; Nogales et al., 2002; Fan et al., 2006) whereas molecular techniques have detected up to 10⁶ 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-Fraterula, 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$_2$O 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 greenhouse gas. Though N₂O 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 CO₂ (Albritton et al., 1996). N₂O 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 N₂O flux (Bange et al., 1996). Nitric oxide (NO) and nitrous oxide (N₂O) 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 N₂O was reduced under anoxic conditions. The ability
denitrifiers to evolve N₂ as a denitrification product varies as many of the pre-
dominant isolates are not able to reduce N₂O (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, its 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, N2 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 N2O, 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