

Denitrification prevails over anammox in tropical mangrove sediments (Goa, India)

Sheryl Oliveira Fernandes^{a, b*}, Valérie D. Michotey^b, Sophie Guasco^b, Patricia C. Bonin^b, P. A. Loka Bharathi^{a, **}

^a Marine microbiology laboratory, National Institute of Oceanography, CSIR, Dona Paula, Goa, India.

^b Laboratoire de Microbiologie, de Géochimie et d'Ecologie Marines, CNRS-UMR 6117, Centre d'Océanologie de Marseille, Campus de Luminy, Case 901, 13288 Marseille Cedex 9, France.

* Present address

** Corresponding authors contact details:

Marine microbiology laboratory,
National Institute of Oceanography,
Dona Paula, Goa, India.
Tel.: +91-832-2450281
Fax: +91-832-2450606
E-mail address: loka@nio.org (P. A. Loka Bharathi)

ABSTRACT

Denitrification, anammox (Anx) and di-nitrogen fixation were examined in two mangrove ecosystems- the anthropogenically influenced Divar and the relatively pristine Tuvem. Stratified sampling at 2 cm increments from 0-10 cm depth revealed denitrification as the main process of N₂ production in mangrove sediments. At Divar, denitrification was ~3 times higher than at Tuvem with maximum activity of 224.51±6.63 nmol N₂ g⁻¹ h⁻¹ at 0-2 cm. Denitrifying genes (*nosZ*) numbered up to 2x10⁷ copies g⁻¹ sediment and belonged to uncultured microorganisms clustering within Proteobacteria. Anammox was more prominent at deeper depths (8-10 cm) mainly in Divar with highest activity of 101.15±87.73 nmol N₂ g⁻¹ h⁻¹ which was 5 times higher than at Tuvem. Di-nitrogen fixation was detected only at Tuvem with a maximum of 12.47±8.36 nmol N₂ g⁻¹ h⁻¹. Thus, in these estuarine habitats prone to high nutrient input, N₂-fixation is minimal and denitrification rather than Anx serves as an important mechanism for counteracting N loading.

Keywords: Anammox; bacteria; denitrification; denitrifiers; mangroves; nitrogen; sediments

1. Introduction

Mangroves constitute nearly 75% of tidal vegetation in tropical regions (Alongi et al., 1989). They protect the coast from tidal erosion, storm surges and trap sediment for land accretion (Pernetta, 1995). Their proximity to human inhabitation, aquaculture farms, waste discharge from industrial units, domestic sewage discharge-points, etc. makes them vulnerable to high nutrient inputs. Nitrogen is the critical limiting factor for primary production in some coastal systems (Howarth and Marino, 2006). The nitrogen cycle within mangrove forests is mediated pre-dominantly by microbial rather than chemical processes (Alongi et al., 1992). High litter fall, its degradation and re-mineralization is one of the factors contributing to high nitrogen concentrations (Ramos E Silva et al., 2007) in mangrove sediments. Benthic re-mineralisation is an important pathway in shallow ecosystems (Nixon, 1981; Zeitzschel, 1980) such as mangroves. The recycled N released from the sediment can substantially contribute to the nitrogen requirement of benthic and pelagic phytoplankton (Nixon, 1981) in mangroves and coastal waters. Thus, mangroves play an important role in the biogeochemical cycles of coastal ecosystems (Thorsten and José, 2001).

Microbial processes can transform N from these ecosystems by controlling its availability and fluxes. Di-nitrogen fixation and denitrification are two processes which are well-known to play a major role in the nitrogen balance in coastal marine sediments. Di-nitrogen fixation which is performed by some prokaryotes (diazotrophs) is a source of ammonium and may alleviate the N-limitation in some ecosystem (Vitousek and Howarth, 1991). 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 N_2O or N_2 (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 gamma Proteobacteria (Brettar et al., 2001). Culture-independent approaches have been employed to probe the diversity of denitrifying genes like *narG*, *nirK*, *nirS*, *norB*, *norC* and *nosZ* (Ward, 1995; Braker et al., 2000, 2001; Prieme et al., 2002; Liu et al., 2003; Jayakumar et al., 2004; Ward, 2005; Desnues et al., 2007). The *nosZ* gene encoding for nitrous oxide (N_2O) 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 (Horn et al., 2006). In the present study, the approach of enumerating the abundance and diversity of *nosZ* genes has been attempted with a view to understand their distribution and community composition in the mangrove sediments of Goa.

Recently, anaerobic ammonium oxidation (anammox) has gained importance in the marine nitrogen cycle (Devol, 2003; Dalsgaard et al., 2005; Nakajima et al., 2008). Anammox (Anx) is a process in which NH_4^+ is oxidized to N_2 at the expense of NO_2^- (Meyer et al., 2005). The NO_2^- could be produced by either heterotrophic NO_3^- reduction (Dalsgaard et al., 2003) or nitrification by aerobic ammonium oxidation (Francis et al., 2005). In mangrove systems, ~55% of the N loss (as NO, N_2O or N_2) is known to occur through denitrification (Chiu et al., 2004). However, Anx can account for up to 67% N removal (Thamdrup and Dalsgaard, 2002) in marine sediments by shunting nitrogen directly from NH_4^+ and NO_2^- under anaerobic conditions. In organically rich estuarine sediments, Anx has been shown to be significant under low concentrations of NO_2^- (Trimmer et al., 2003). Mangrove sediments could potentially present such characteristics since they are largely anaerobic and nitrate/nitrite availability is the factor controlling denitrification rates (Seitzinger, 1990). Besides, bioturbating fauna residing in the sediments can greatly influence the availability of inorganic N compounds in the benthic environment. Nitrate/nitrite can either be generated through intrinsic nitrification (Nielsen, 1992; Lohse et al., 1993), supplied extraneously through runoff from land (Naqvi et al., 2000) or through tidal pumping (Ji et al., 2008). Although denitrification could play a significant role in sediment ecology by mitigating excess nitrate from the system, we cannot exclude that the total N_2 production in mangrove ecosystems could be a result of the co-occurrence of Anx and denitrification.

This study is specifically aimed to (i) to assess the major pathway for N_2 production viz., denitrification and anammox in two mangrove ecosystems- the anthropogenically influenced Divar and the relatively pristine Tuvem (ii) to quantify the extent of N_2 -fixation activity in mangrove sediments (iii) to examine the down-core variation in denitrifier assemblage and their community composition at a molecular level in relation to denitrification rates and other environmental parameters.

2. Methods

2.1. Study area and sampling

Investigations were carried out at mangrove forests located along the Mandovi and Chapora rivers in Goa, west coast of India (Fernandes et al., 2010). The climate is tropical and 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 during peak monsoon.

The anthropogenically influenced site was located at Divar island (15°30'35"N and 73°52'28"E) which is separated from the mainland by the Mandovi estuary and is accessible by ferry. Mangroves that fringe the Divar Island consist mainly of species like *Rhizophora mucronata*, *Pongamia pinnata*, *Cyperus* spp., *Bruguiera gymnorrhiza*, *Avicennia officinalis*, *Caesalpinia* spp., *Sonneratia caseolaris* and *Acanthus illicifolius*. These mangroves support the livelihood of many islanders and are of immense ecological and economic value. The adjoining Mandovi estuary 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 Sousa, 1999). The relatively pristine site was located at Tuvem (15°39'09"N and 73°47'71"E) along the river Chapora. This ecosystem is set amidst verdant hills, banana and coconut plantations and is comparatively less influenced by anthropogenic activities (Krishnan et al., 2007). The site is fringed by lush mangroves mainly represented by *Acanthus illicifolius*, *Excoecaria agallocha*, *Caesalpinia* spp., *Avicennia officinalis* and *Clerodendrum inerme*.

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 length) and used for chemical and microbiological analysis. 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 as follows:

- (i) Three replicates (1 ml) stored at -20°C for molecular biology experiments
- (ii) Duplicates (10 ml) for immediate pore water analyses

(iii) Three replicates (1 ml) were dried at $60\pm 2^{\circ}\text{C}$, powdered and sieved through a 200 μm nylon mesh and stored in clean PVC vials until analysis

(iv) Duplicate laboratory replicates to determine an average value of microbial activities in each homogenized sample were maintained at every incubation interval (0, 2, 4, 6, 8, and 10 h; $n=12$). 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).

(v) Sediment sub-samples in duplicates for grain size analysis.

2.2. Grain size analysis

De-ionized water was used for desalination of 15 g 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 upon drying. 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 content.

2.3. Total organic carbon

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

2.4. Nutrient 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 pore water 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.

2.5. Denitrification and anammox rates

Experiments for Anx 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 ($^{15}\text{NO}_3^-$) and the production of single-labelled ($^{14}\text{N}^{15}\text{N}$) and double-labelled ($^{15}\text{N}^{15}\text{N}$) di-nitrogen which are detected using a mass spectrometer. Four ml of homogenised sediment from each section was transferred into 22 ml headspace vials. Four ml of filter sterilized seawater containing $10 \mu\text{mol NO}_3\text{-N l}^{-1}$ 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 $^{15}\text{NO}_3^-$ (97.4 atom%, Isotech Mathesson, USA) (Rich et al., 2008) to obtain final concentration of 50, 80 and $150 \mu\text{mol }^{15}\text{NO}_3^-$. The production of single-labelled ($^{14}\text{N}^{15}\text{N}$) and double-labelled ($^{15}\text{N}^{15}\text{N}$) 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 $^{29}\text{N}_2$ and $^{30}\text{N}_2$ produced in the incubation vials during the incubations was measured with a mass spectrometer (Quadrupole mass spectrometer *Anagaz 100*, MKS, England) (Minjeaud et al., 2008). Argon was used as an internal standard. The concentrations of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ 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. The values of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production were used in the equation described by Thamdrup and Dalsgaard (2002) with some modifications ($_{\text{mod-IPM}}$) as described by Minjeaud et al. (2008) to calculate Anx and total denitrification (D_{tot}) rates.

2.6. Di-nitrogen fixation rates

N_2 -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).

2.7. 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- μ m 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).

2.8. DNA extraction and PCR amplification for nosZ quantification and diversity

Genomic DNA was extracted in triplicates for each layer from 0.25 g (dry weight) sediment using a MOBIO PowerSoilTM DNA isolation kit (MoBio Laboratories, California) according to the manufacturer's protocol. 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 μ l) tube contained the following: Mg free buffer (2.5 μ l) (Promega, USA); dNTPs (2.5 μ l of 10mM Promega, USA); $MgCl_2$ (1.5 μ l of 25mM Promega, USA); *nosZ* Primer 0.5 μ l of each *nosZ* 1211F and *nosZ* 1897R (100 pmol. μ l⁻¹) (Rösch et al., 2002); Taq polymerase: 0.2 μ l (5 units/ μ l; 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).

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 120bp. Standards for *nosZ* quantification (6.8×10^{-4} to 6.8×10^{-2}) were prepared as a mixture of cloned *nosZ* fragments of *Pseudomonas*, *Marinobacter* and *Achromobacter* spp. Each replicate of DNA extract for a layer (n=3) were subject to PCR reactions in 25 μ l mixtures. The products were subjected to gel electrophoresis on a 1.5% agarose gel. The intensity of each band was measured using Quantity One software (BioRad).

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. PCR were run with each DNA extract from mangrove sediments having the same quantity of internal standard (3×10^3 *nosZ*) and quantification of *nosZ* per gram dry sediment was calculated from the standard curve.

Denaturing Gradient Gel electrophoresis (DGGE) was performed using a D-code Universal Mutation Detection System (Bio-Rad Laboratories Inc.). Samples containing approximately equal amounts (600 ng) of pooled PCR products of triplicate DNA extract were loaded onto 1 mm thick, 8% (wt/vol) polyacrylamide gel 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 buffer. Following electrophoresis, the gel was incubated for 30 minutes in 1X TAE buffer containing ethidium bromide (0.5 mg ml^{-1}) and then photographed on a UV transilluminator (GelDoc 2000, gel documentation system, Bio-Rad).

The DGGE bands comparison between samples and tree construction were realised with Gel Compar II software (Applied Maths) using the Jaccard method and the UPGMA algorithm. Representative bands from samples showing nearly identical alignments were used for phylogenetic analysis. Though nine bands were extracted from the polyacrylamide gel for DGGE analysis, only 4 were successfully cloned and sequenced. After allowing diffusion of the excised DNA in 50 μ L of sterile water overnight at 4°C, 5 μ l were used for re-amplification of *nosZ* by PCR and were cloned using the pGEM[®]-T Easy Vector (Promega Ltd., UK) as described by the manufacturer.

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). Bootstrapping (1000 replicates) was used to estimate the reproducibility of the trees. Sequences obtained in this study are available from the EMBL nucleotide sequence database under the accession numbers FN356193- FN356196.

2.9. Statistical analysis

The *nosZ* copy numbers were \log_{10} transformed before analyses. Pearson's correlation coefficients were used to assess inter-relationship between biotic and abiotic parameters. Only significant relationships have been discussed. Significant differences among chemical and bacteriological parameters were analysed using analysis of variance (ANOVA) in Analysis tool pack (*Microsoft Excel*).

Data for Principal component analysis (PCA) was normalized in Primer 6 software 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, France). The results of the ordination analyses are visualized as a bi-plot (Catell, 1966; Gabriel, 1971).

3. Results

3.1. Physico-chemical characteristics of sediments

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 1) sand forms a major component ($\geq 70\%$) 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 32.45 (± 0.77) and 22.54 (± 0.17)% respectively.

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 ± 0.22 - 1.23 ± 0.10 %) for these layers. The TOC content at 6-10 cm was similar at both sites, i.e. a decrease with depth (Fig. 1).

Down-core nutrient profiles at Divar exhibited an overall decreasing trend with depth. Sediment extractable ammonium concentration at the surface was about $2.22 \pm 0.15 \text{ mmol l}^{-1}$ which steadily decreased to $1.21 \pm 0.08 \text{ mmol l}^{-1}$ at 8-10 cm depth (Fig. 2). Ammonium concentrations at Tuvem however showed an opposite trend, starting with $1.10 \pm 0.07 \text{ mmol l}^{-1}$ and increasing with depth. A maximum of $1.60 \pm 0.11 \text{ mmol l}^{-1}$ was observed at 8-10 cm.

Similarly at Divar, nitrite concentrations in the pore water decreased from $14.45 \pm 1.25 \text{ } \mu\text{mol l}^{-1}$ at the surface to $4.96 \pm 0.41 \text{ } \mu\text{mol l}^{-1}$ at the deeper layer while nitrate varied from $19.90 \pm 1.66 \text{ } \mu\text{mol l}^{-1}$ at 0-2 cm to $2.63 \pm 0.25 \text{ } \mu\text{mol l}^{-1}$ at 8-10 cm (Fig. 3). At Tuvem, nutrient concentrations were also found to decrease with depth. A maximum in the concentration of nitrite ($7.55 \pm 0.50 \text{ } \mu\text{mol l}^{-1}$) and nitrate ($36.62 \pm 2.91 \text{ } \mu\text{mol l}^{-1}$) was recorded at 2-4 cm.

3.2. Macrofaunal abundance

The mangrove sediments at Divar harbored a substantial macrofaunal population (Table 2) with a dominance of polychaetes (94%; 1376 nos. 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 nos. m^{-2}) at Tuvem. Crustaceans were absent while the minor fraction of the community were represented by oligochaetes and crustaceans.

3.3. Bacterial activities

Di-nitrogen production through Anx and denitrification was concomitantly determined (Fig. 4). Denitrification was observed to be the dominant process responsible for N-loss in mangrove sediments. At both locations, activity related negatively with depth ($n=10$; $r=-0.65$; $p<0.05$ at Divar and $r=-0.88$; $p<0.001$ at Tuvem) suggesting that the rate declined with depth. ANOVA showed no statistically significant difference in denitrification activity between treatments. The total N_2 production rates including denitrification and Anx 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 Divar, the observed rates in the upper layer was $224.51 \pm 6.63 \text{ nmol N g}^{-1} \text{ h}^{-1}$ which was three fold higher than that found for Tuvem for the same layer ($80.94 \pm 3.87 \text{ nmol N g}^{-1} \text{ h}^{-1}$). 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 Anx was calculated according to mod-IPM . Anammox activity was detected in mangrove sediments. Though the process was negligible in the upper layers (< 8 cm) at Divar, it was found to be the major source of N_2 in the deeper layer (8-10 cm) accounting for 67% of total N_2 production. Here, maximum Anx activity of $101.15 \pm 87.73 \text{ nmol N}_2 \text{ g}^{-1} \text{ h}^{-1}$ was recorded. At Tuvem, although the process was detected at all depths investigated, it was relatively weaker occurring at a rate of $1.48 \pm 1.16 - 18.31 \pm 5.79 \text{ nmol N}_2 \text{ g}^{-1} \text{ h}^{-1}$.

Di-nitrogen fixing activity (Fig. 5) was detected only at Tuvem with maximum activity of $12.47 \pm 8.36 \text{ nmol N}_2 \text{ g}^{-1} \text{ h}^{-1}$ occurring at 6-8 cm depth. At Divar, it was below detection limits at all depths. We calculated a net nitrogen balance by differences between N_2 production via denitrification plus Anx and consumption through N_2 -fixation. The loss of N_2 from the mangrove ecosystem far exceeded its fixation in these sediments (Fig. 6).

3.4. Quantification and phylogenetic analysis of *nosZ*

Abundance of *nosZ* genes ranged from $0.1-2 \times 10^7$ 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. 7).

The main component of the *nosZ* community was analysed by DGGE. Analysis showed distinct *nosZ* community at both the locations examined (Fig. 8). The band BD1 (a) was observed between 0-4 cm. The other two bands BD2 (b) and BD3 (c) were observed at all depths investigated. These bands were recorded at 8-10 and 6-8 cm at Tuvem respectively. At this site, the band BT1 (d) was prominent at all depths investigated and has not been observed in the Divar sediments. From comparison with *nosZ* of cultured strains, BD2 was found to cluster within the β -Proteobacterial *nosZ* and showed 62% similarity to *Ralstonia eutropha nosZ* (Fig. 9). The bands BD1 and BD3 were found to group within α Proteobacterial *nosZ* cluster and showed 74% and 77% similarity to *Azospirillum brasilense nosZ* and *Silicibacter pomeroyi nosZ* respectively. This band was also found to be in the Divar sediment mainly within 0-6 cm. The sequence of band BT1 fell within the γ Proteobacteria *nosZ* cluster and showed 96.5% similarity with *Shewanella loihica nosZ*.

3.5. Environmental controls on activity

The Principal Component Analysis (PCA) was applied to the matrix of correlation coefficients between biotic and abiotic parameters. The first three factors were taken into account which corresponded to 82.2% of total data variability (Table 3). The first component (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 second component (PC 2) explained 22.6% of data variability. The differences between sampling depths were clearly discriminated within the axis. 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 (Fig. 10). 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 Anx activity. The most significant variables in the third component (PC 3) were pore water NH_4^+ -N and abundance of *nosZ* genes which explained 21.5% of the variation.

4. Discussion

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 $224.51 \pm 6.63 \text{ nmol } N_2 \text{ g}^{-1} \text{ h}^{-1}$ ($\approx 2.08 \text{ mmol } N_2 \text{ m}^{-2} \text{ h}^{-1}$) have been measured in the Divar sediments. In sub-tropical mangrove sediments, denitrification has been reported to occur at a maximum rate of approximately $85 \text{ nmol } N \text{ cm}^{-3} \text{ h}^{-1}$ ($\approx 38 \text{ nmol } N \text{ g}^{-1} \text{ h}^{-1}$ or $240 \text{ } \mu\text{mol } N_2 \text{ m}^{-2} \text{ h}^{-1}$) (Meyer et al., 2005) which is about five times less than observed in our measurements. Further, benthic denitrification rates recorded in other mangrove areas (Table 4) are relatively less than those observed in the present study.

The Divar mangrove ecosystem lies along the Mandovi estuary. This estuary receives about ~8-10 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 the important factors limiting denitrification in mangrove ecosystems. As reported in earlier studies by Fernandes and Loka Bharathi (2011), 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. Further, the PCA bi-plot also shows that ammonium, nitrite and nitrate clustered together suggesting a link between ammonium and nitrate and nitrite probably through nitrification. This finding is further strengthened by a strongly positive relationship observed between ammonium and nitrite concentration ($n=10$, $r=0.78$, $p<0.01$) especially at Divar.

Molecular investigations carried out in the present study reveal that the abundance of denitrifier genes in mangrove sediments numbered up to 2×10^7 target copies g^{-1} of dry sediment. 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 rates are 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.

Between the two sites investigated, the *nosZ* community was found to be different. The dominant denitrifiers at Tuvem 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. At Divar, the dominant Proteobacterial denitrifying communities were found from the surface till a depth of 10 cm indicating that the organisms were well dispersed. Grain size analysis has shown that the sediment at both the locations differed in composition. A high percentage of sand followed by silt and clay was observed at Divar. It is possible that sandy nature of the mangrove sediment and 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). Bioturbating 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 Penniford 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 Tuvem, Divar was dominated mainly by sand. The sandy nature of the sediments could be responsible for facilitating percolation of nutrients to deeper depths. In the Mandovi estuary, NH_4^+ is available in excess especially during the SW monsoon (Maya et al., 2011). In our 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. High denitrification rates supplied with NO_3 by a coupled nitrification process is known to be supported by O_2 presumably supplied by bioirrigation (Bissett et al., 2009). Nitrification/denitrification processes are generally known to be coupled either spatially or temporally. This coupling accounts for the homeostasis in an environment. Under eutrophicated conditions, the two processes tend to get de-coupled (Caffrey et al., 1993) with denitrification being more pronounced than nitrification. Our observations show that bioturbators (sediment destabilisers) like polychaetes comprise >95% of the macrofaunal population at Divar. These organisms may be playing a significant role in enhancing substrate availability for nitrification in these sediments consequently fuelling the denitrification process.

Anammox is known to co-occur along with denitrification in marine sediments (Rysgaard et al., 2004). In some marine environments, Anx is a major pathway for the removal of fixed inorganic nitrogen (Kuypers et al., 2003; Engstrom et al., 2005). The co-occurrence of Anx 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 r_a (percentage of Anx) values is thus a concern. Trimmer et al. (2006) compared rates of Anx and denitrification in slurries and intact cores. In sediments with low Anx activity ($r_a < 1\%$), slurries and intact core yielded similar results, but when Anx was more significant ($r_a > 5\%$), r_a was about 10-15% higher in intact core than in slurries (Trimmer et al., 2006). Consequently our slurry measurement probably accurately assessed the presence or absence of Anx activity, but the actual r_a in some layers with high activity may have been underestimated. Maximum Anx rates of up to $101.15 \pm 87.73 \text{ nmol } N_2 \text{ g}^{-1} \text{ h}^{-1}$ ($\approx 1.02 \text{ mmol } N_2 \text{ m}^{-2} \text{ h}^{-1}$) have been observed in the present study. In subtropical mangrove sediment, Meyer et al. (2005) have reported Anx rates varying between 0.5 to $8 \text{ nmol } N \text{ cm}^{-3} \text{ h}^{-1}$ (0.22 to $3.6 \text{ nmol } N \text{ g}^{-1} \text{ h}^{-1}$) which is approximately 25 times lower than those encountered in the present study. Reports by Rysgaard et al. (2004) in arctic marine sediments indicate Anx to occur at about 3 orders lower. In the north sea sediments, Anx rates were also relatively lower (Dalsgaard et al., 2005). Our observation that Anx could serve as an important mechanism for N_2 production was restricted to deeper depth of 8-10 cm where it accounted for 67-96% of the total N_2 production. High rates of Anx 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 Anx process. At pre-monsoon, nitrification occurs at a rate of up to $\sim 18 \text{ nmol } g^{-1} \text{ h}^{-1}$ at a depth of 8-10 cm at Divar (Krishnan and Loka Bharathi, 2009) which co-incidentally shows the highest Anx activity in our study. Anaerobic nitrification in metal-rich mangrove sediments (Krishnan et al., 2007) appears to be coupled to the reduction of Fe (Krishnan and Loka Bharathi, 2009). Thus, nitrite production through nitrification at deeper depths could fuel the Anx process. High NO_3^- concentrations (Rich et al., 2008) are also known to favour Anx rates. The mangrove sediments harbored fairly high concentrations of nitrate which could be favorable for anaerobic oxidation of ammonium to occur. At Tuvem, Anx was detected at all depths investigated in comparison to Divar where it was more restricted. It interesting to observe that Anx 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 Anx. 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, Anx 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 Anx bacteria in fluctuating environments. Reports from the Benguela upwelling system indicate that Anx bacteria are metabolically versatile and can function as nitrate reducers (Kartal et al., 2006). Thus, it is possible that these microbes could be mediating dissimilatory nitrate reduction to ammonium in mangrove ecosystems.

Mangroves ecosystems are generally known to be nitrate limited and the prevalence of diazotrophic bacteria in these systems (Zhang et al., 2008) helps counter this deficiency. The extent of N_2 -fixation is seen to be minimal especially in systems prone to anthropogenically-derived nutrient supply as in Divar. Unpublished data has shown C:N values of 14.6 ± 0.4 during the monsoon season at Divar indicating considerable terrestrial influence. As the aquatic system is being continuously replenished with nutrients, denitrification and anammox are more pronounced resulting in the transformation of nutrients consequently moderating the excess N input. However, results on net N_2 production (denitrification + Anx) in the present study showed N loss from the benthic environment which was not compensated by the process of N_2 -fixation.

5. Conclusion

In mangrove sediments, denitrification and Anx operate concurrently leading to N_2 production. However, the Anx 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 surficial layers and a coupling between nitrification and denitrification is suggested. Thus, in coastal ecosystems like mangroves which have low N_2 fixation but high inorganic N inputs through anthropogenic forcing, denitrification could be important. The process ameliorates the potential affects of extra N inputs and improves the "assimilative capacity" of these systems.

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Legend to tables and figures:

Table 1: Fractions of sand, silt and clay (\pm SD) at various depths in mangrove sediment

Table 2: Abundance of major macrofaunal groups in mangrove sediments

Table 3: Results of principal component analysis on the biogeochemical parameters and bacterial activities associated with the nitrogen cycle

Table 4: Denitrification rates in mangrove sediments

Fig. 1. Down-core total organic carbon profile (\pm se) at the anthropogenically-influenced site Divar and the relatively pristine Tuvem.

Fig. 2. Sediment extractable ammonium (\pm se) throughout the profile at Divar and Tuvem.

Fig. 3. Nitrite and nitrate concentration (\pm se) profile for pore water at Divar (D) and Tuvem (T). The concentration of NO_2^- at Divar is represented by dark hatched bars while at Tuvem lighter hatched bars have been used. The concentration of NO_3^- at Divar is shown by darker dotted bars while those at Tuvem have been represented by lighter dotted bars.

Fig. 4. Di-nitrogen production profile from denitrification (hatched bars; \pm se) and anammox (dotted bars; \pm se) at Divar (D) and Tuvem (T). Denitrification at D is represented by darker hatched bars while at T, it has been shown using lighter shaded bars. Similarly, darker (at D) and lighter (at T) dotted bars have been used to distinguish anammox at the two sites.

Fig. 5. Di-nitrogen fixation profile (\pm se) at Tuvem. No activity was detectable at Divar.

Fig. 6. Down-core profile of di-nitrogen budget at Divar and Tuvem (\pm se) where consumption = N_2 fixation while N_2 production occurs through denitrification and anammox. N_2 fixation/consumption has not been observed to occur at Divar.

Fig. 7. Down-core variation in the abundance of *nosZ* genes (\pm se) at Divar and Tuvem.

Fig. 8. The *nosZ* DGGE profiles for mangrove sediments at Divar (D) and Tuvem (T); a= band BD1, b=BD2; c=BD3; d=BT1.

Fig. 9. 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. 10. 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), total denitrification (D_{tot}), anammox (Anx), N_2 production ($D_{\text{tot}}+\text{Anx}$)).

Table 1

Depth (cm)	Location: Divar			Location: Tuven		
	Sand (%)	Silt (%)	Clay (%)	Sand (%)	Silt (%)	Clay (%)
0-2	70.14 (±0.72)	16.69 (±0.89)	11.59 (±2.25)	48.42 (±1.46)	32.02 (±1.66)	16.31 (±1.40)
2-4	69.88 (±1.85)	14.32 (±0.77)	13.60 (±1.20)	44.56 (±0.70)	32.36 (±1.07)	20.05 (±0.96)
4-6	72.88 (±0.25)	12.37 (±0.70)	12.71 (±0.15)	43.07 (±0.44)	32.45 (±0.77)	20.10 (±1.51)
6-8	70.03 (±0.71)	13.16 (±0.40)	15.62 (±1.65)	47.93 (±1.49)	30.71 (±0.57)	19.62 (±0.72)
8-10	79.31 (±2.12)	8.68 (±0.71)	10.50 (±0.19)	44.16 (±0.74)	31.86 (±0.28)	22.54 (±0.17)
Texture	Sandy			Silty sand		

Table 2

Macrofaunal group	Density at Divar (nos. m ⁻²)	Density at Tuven (nos. m ⁻²)
Polychaeta	1376	224
Oligochaeta	32	16
Crustacea	32	16
Gastropoda	16	-

Table 3

Variable	Component 1 (PC1)	Component 2 (PC2)	Component 3 (PC3)
NH ₄ ⁺	0.634	0.291	0.643
NO ₂ ⁻	0.762	0.304	0.473
NO ₃ ⁻	0.101	0.692	-0.585
TOC	0.588	-0.616	0.387
D _{tot}	0.790	0.479	-0.281
Anx	0.179	-0.624	-0.512
Av. nosZ	-0.452	0.381	0.493
Nif	-0.789	0.450	0.046
N ₂ production	0.786	0.175	-0.464
Total variance (%)	38.2	22.6	21.5

*Correlation significant at 0.05 probability level; TOC= Total organic carbon, Nif= di-nitrogen fixation, D_{tot} = total denitrification, Anx= anammox, N₂ production= D_{tot}+Anx. The most significant variables in each component have been highlighted bold.

Table 4

<i>Sr. No.</i>	<i>Area</i>	<i>Denitrification rates</i>	<i>Method</i>	<i>Reference</i>
1	Terminos lagoon, Mexico	Fringe mangrove: 9.4 $\mu\text{mol m}^{-2} \text{h}^{-1}$	^{15}N isotope technique	Rivera-Monroy <i>et al.</i> , 1995
2	Mangrove forest, Phuket, Thailand	Basin mangrove: 1.9 to 4.5 $\mu\text{mol m}^{-2} \text{h}^{-1}$ Vegetated sediments: 1.91 $\mu\text{mol m}^{-2} \text{h}^{-1}$	^{15}N isotope technique	Kristensen <i>et al.</i> , 1998
3	Logan/Albert river, Queensland, Australia	Unvegetated sediments: 0.54 $\mu\text{mol m}^{-2} \text{h}^{-1}$ 85 $\text{nmol N cm}^{-3} \text{h}^{-1}$ *(38 $\text{nmol N g}^{-1} \text{h}^{-1}$ or 240 $\mu\text{mol N}_2 \text{m}^{-2} \text{h}^{-1}$)	^{15}N isotope technique	Meyer <i>et al.</i> , 2005
4	Matang mangrove forest reserve, Malaysia	16–458 $\mu\text{mol N}_2 \text{m}^{-2} \text{h}^{-1}$	N_2 -gas flux technique	Alongi <i>et al.</i> , 2004
5	Jiulongjiang estuary, China	46.08 to 157.5 $\mu\text{mol N}_2 \text{m}^{-2} \text{h}^{-1}$	N_2 -gas flux technique	Alongi <i>et al.</i> , 2005
6	Southwest coast of Puerto Rico	0.12 and 7.8 $\mu\text{mol N}_2\text{O m}^{-2} \text{h}^{-1}$	Gas chromatography	Corredor <i>et al.</i> , 1999
7	Goa, India	Divar: 1.58-224.51 $\text{nmol g}^{-1} \text{h}^{-1}$ *(10-2080 $\mu\text{mol N}_2 \text{m}^{-2} \text{h}^{-1}$) Tuvem: 0.05-77.13 $\text{nmol g}^{-1} \text{h}^{-1}$ *(4-660 $\mu\text{mol N}_2 \text{m}^{-2} \text{h}^{-1}$)	^{15}N isotope technique	Present study

* Converted for comparison

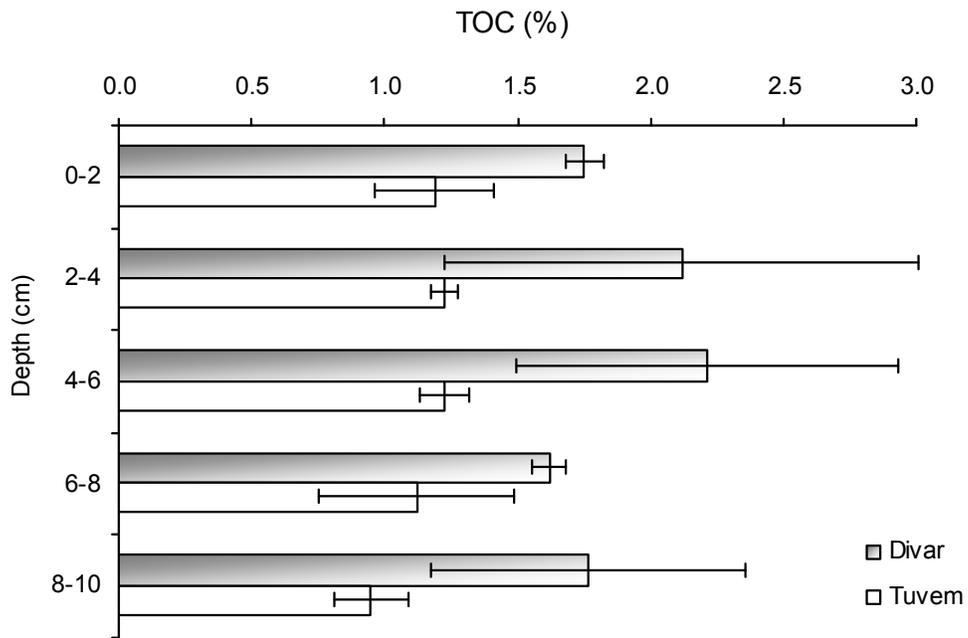


Fig. 1

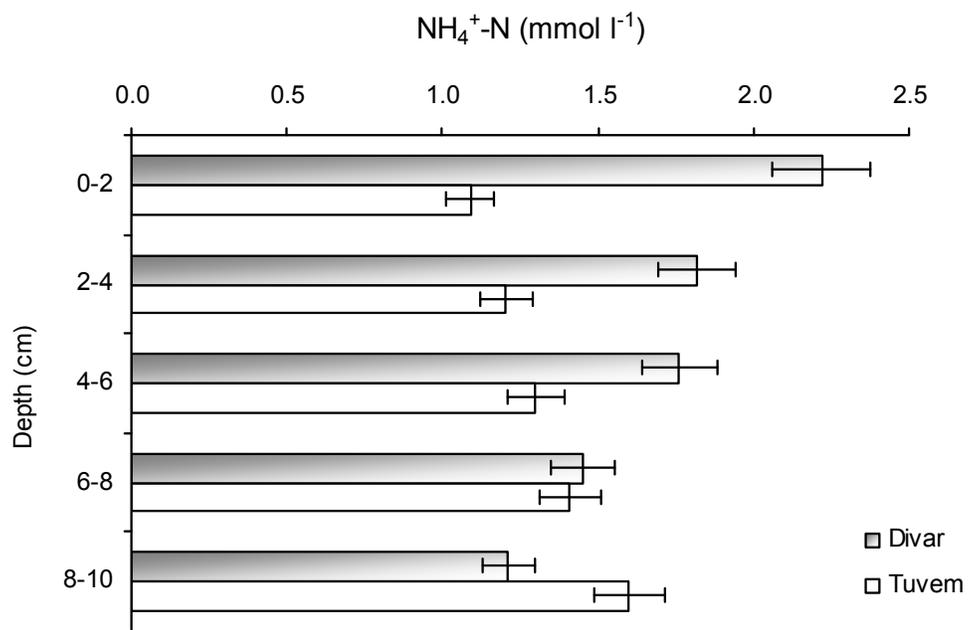


Fig. 2

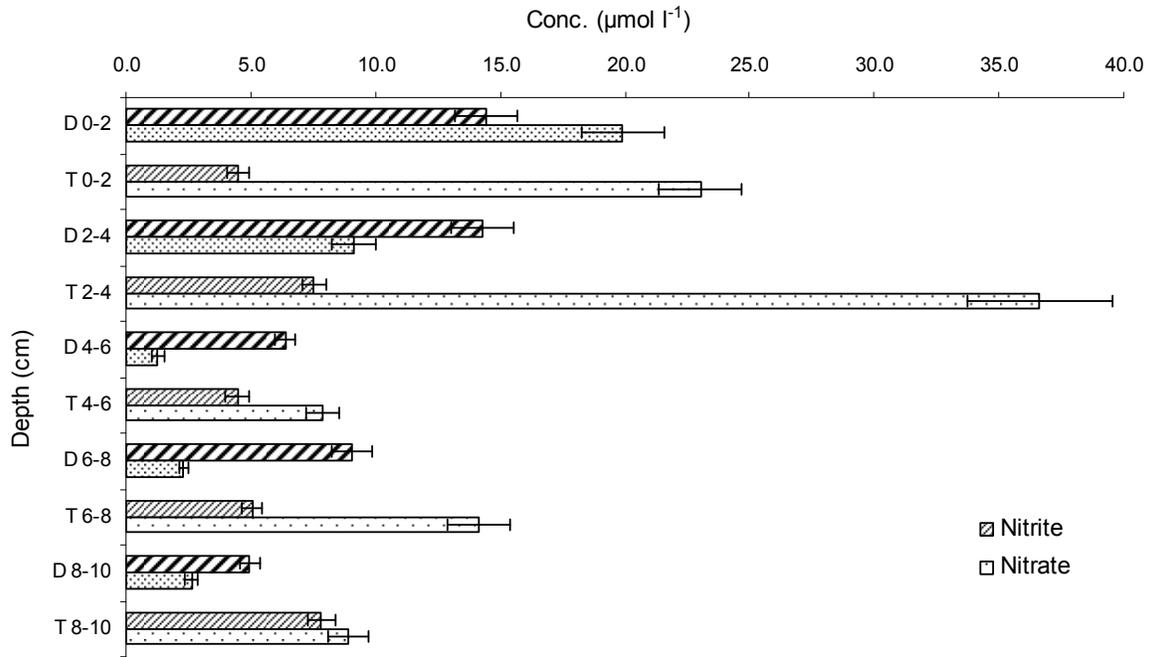


Fig. 3

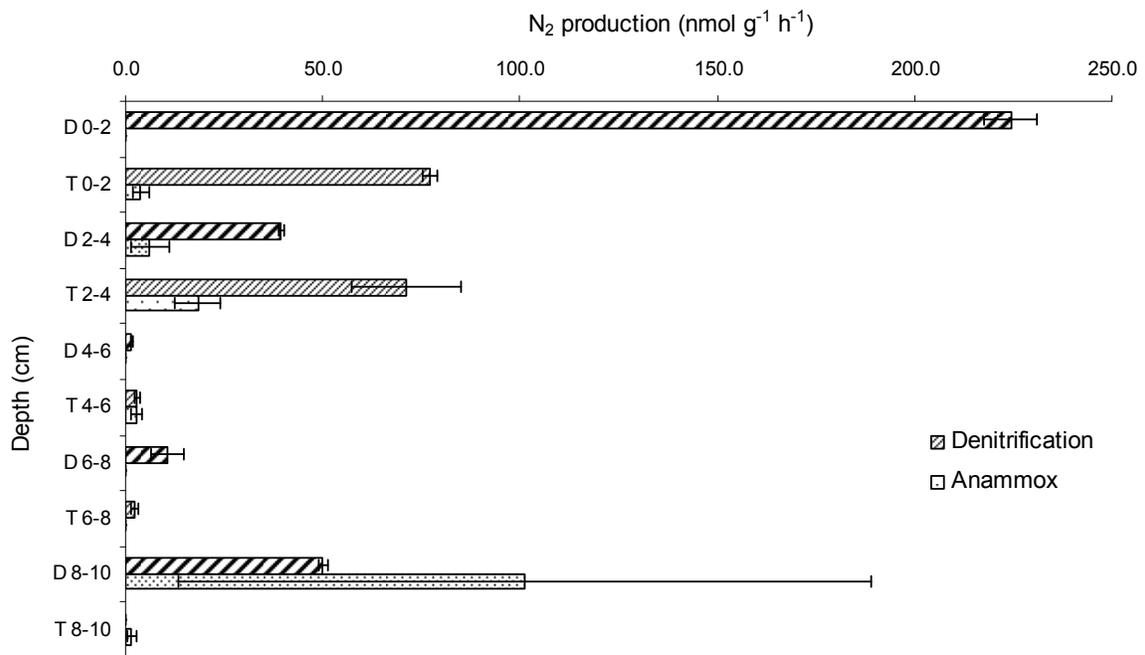


Fig. 4

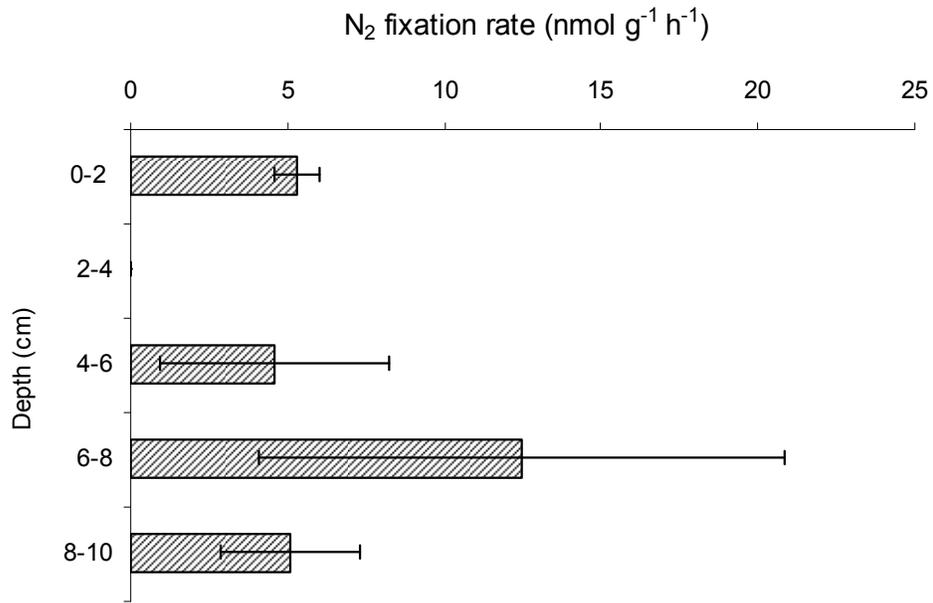


Fig. 5

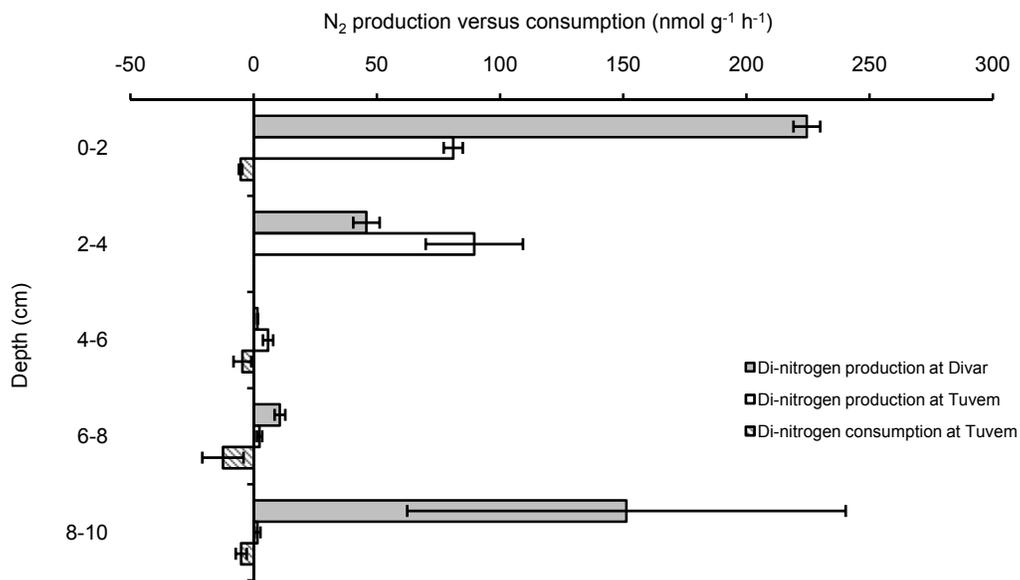


Fig. 6

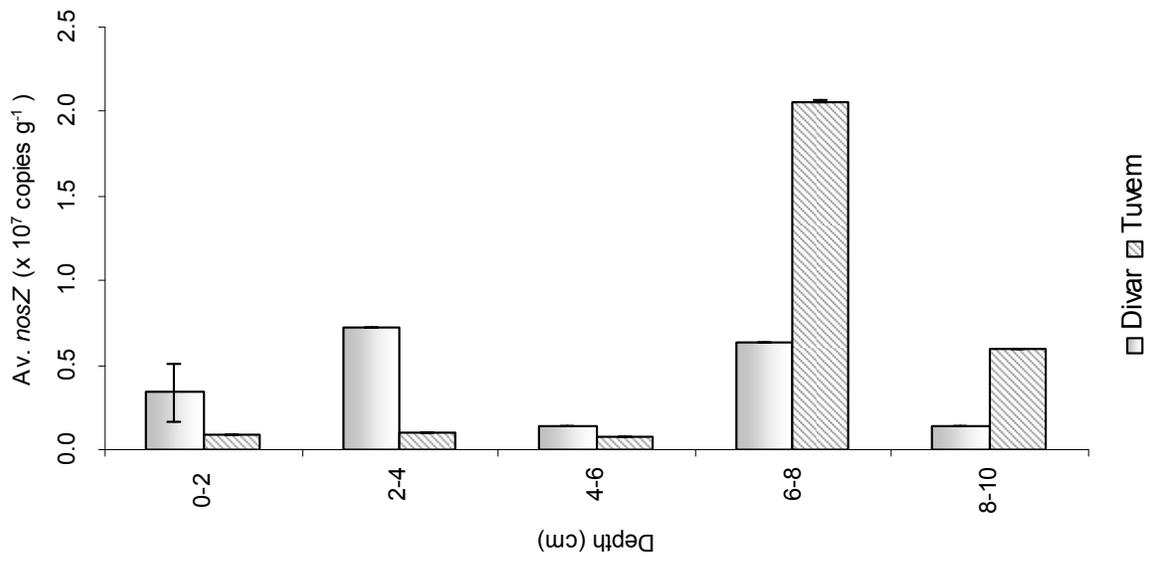


Fig. 7

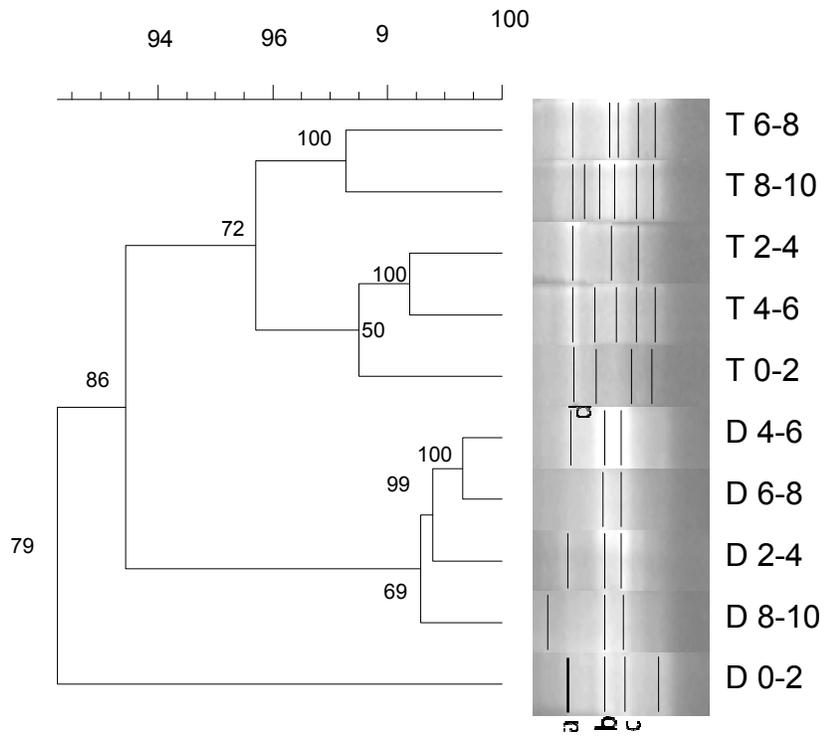


Fig. 8

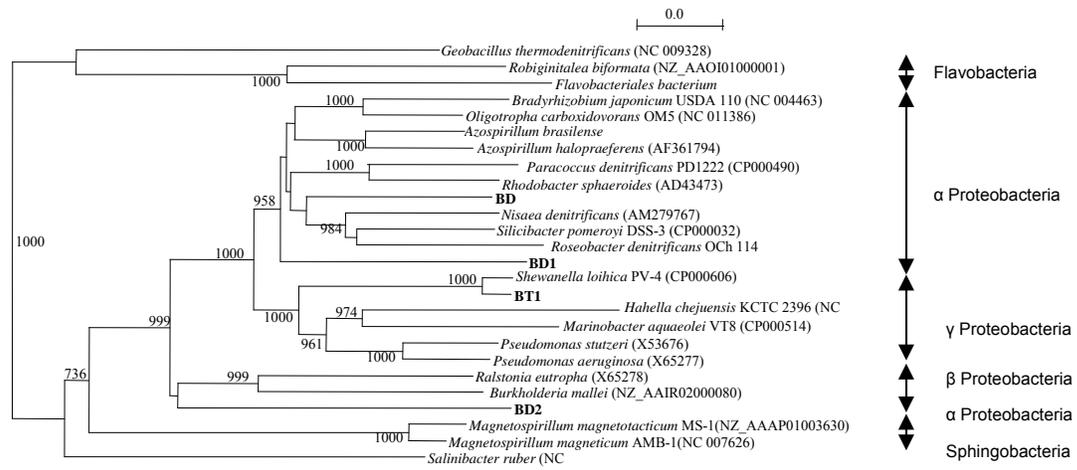


Fig. 9

Biplot (axes F1 and F2: 60.78 %)

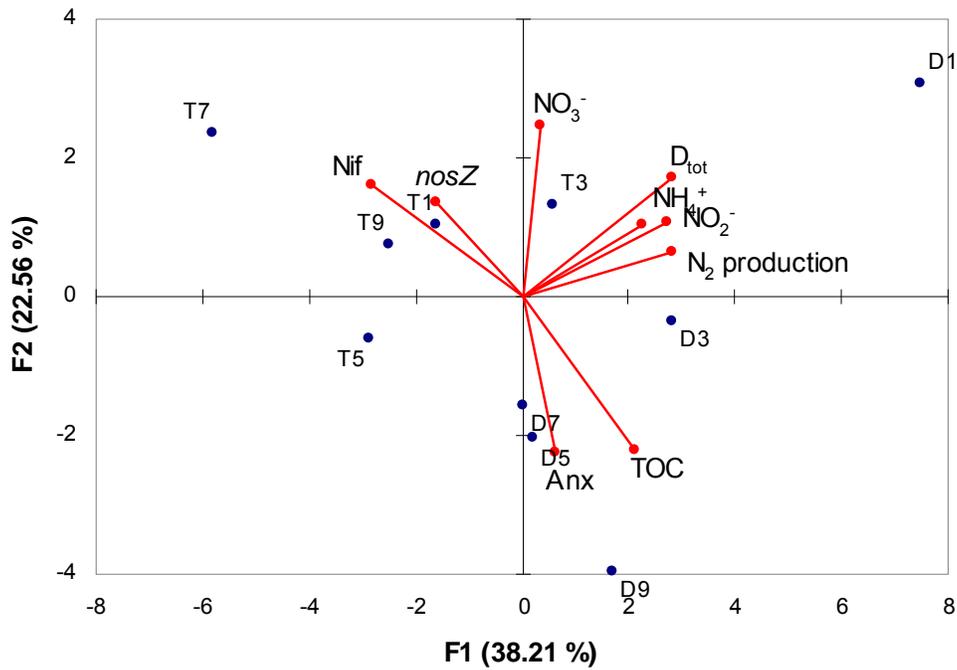


Fig. 10