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

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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 to 10 cm depth revealed denitrification as the main process of N_2 production in mangrove sediments. At Divar, denitrification was ~3 times higher than at Tuvem with maximum activity of 224.51 ± 6.63 nmol N_2 g^{-1} h^{-1} at 0–2 cm. Denitrifying genes (*nosZ*) numbered up to 2×10^7 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 N_2 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 N_2 g^{-1} h^{-1}. Thus, in these estuarine habitats prone to high nutrient input, N_2-fixation is minimal and denitrification rather than Anx serves as an important mechanism for counteracting N loading.

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, 1993). 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 predominantly 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 N-limitation in some ecosystems. 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;

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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 Anx 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 ferro-manganese mining operations (De Souza, 1999). The relatively pristine site was located at Tuvem (15°39′94″N and 73°47′65″E) along the river Chapora. This ecosystem is comparatively less influenced by anthropogenic activities (Krishnan et al., 2007). The site is fringed by lush mangroves mainly represented by *A. illicifolius*, *Excoecaria agallocha*, *Caesalpinia* spp., *A. 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 ± 2 °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 (63–2 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 2 M KCl were added to 1 ml sediment sub-samples 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 h with brief vortexing every 15 min. The samples were then centrifuged at 8000 rpm for 10 min using a Beckman GS-15R centrifuge. The