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Nitrogen-limited mangrove ecosystems conserve N through dissimilatory nitrate reduction to ammonium

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Earlier observations in mangrove sediments of Goa, India have shown denitrification to be a major pathway for N loss[1]. However, percentage of total nitrate transformed through complete denitrification accounted for <0–72% of the pore water nitrate reduced. Here, we show that up to 99% of nitrate removal in mangrove sediments is routed through dissimilatory nitrate reduction to ammonium (DNRA). The DNRA process was 2x higher at the relatively pristine site Tuvem compared to the anthropogenically-influenced Divar mangrove ecosystem. In systems receiving low extraneous nutrient inputs, this mechanism effectively conserves and re-circulates N minimizing nutrient loss that would otherwise occur through denitrification. In a global context, the occurrence of DNRA in mangroves has important implications for maintaining N levels and sustaining ecosystem productivity. For the first time, this study also highlights the significance of DNRA in buffering the climate by modulating the production of the greenhouse gas nitrous oxide.

M angroves play a major socio-economic role to human communities in developing countries. They not only provide protection from tidal erosion, storm surges and trap sediment for land accretion[2] but also play an important role in biogeochemical transformations in coastal ecosystems[3]. These transformations are mainly microbially-mediated which catalyze various steps of the oxidative and reductive phases of elemental cycles. Reducing conditions in mangrove sediments are known to favour alternate respiratory pathways like denitrification, sulfate reduction, etc. Recently, it has been shown that denitrification and anammox operate in tandem resulting in N loss in tropical mangrove sediments[1]. However, denitrification is a more important process which effectively reduces N load from the system. Though, denitrification is a major mechanism for NO_3^- removal in coastal sediments[4], it is also possible that its removal could proceed through other pathways. Nutrient regeneration could be important in N limited ecosystems like mangroves[5] wherein the microbial community could be competing with the vegetation for inorganic N requirements. Internal regeneration could act as an efficient mechanism to meet the N demand from both the microbial and plant communities. As a sequel to earlier findings[1], we examined the down-core variation (at every 2 cm interval within 0–10 cm depth range) in nitrate reducing activity (NRA), dissimilatory nitrate reduction to ammonium (DNRA) and net nitrous oxide (N_2O) production in two tropical mangrove systems of Goa, India. The Divar mangrove ecosystem which is influenced by NH_4NO_3 input from ferromanganese mines located upstream[6] was compared to Tuvem which is relatively pristine[7]. We hypothesize that mangroves are closed systems which efficiently conserve N through pathways like DNRA. Our observations reveal that ammonium is re-circulated within the mangrove systems through DNRA. This mechanism helps to overcome N limitation that could possibly arise due to demand from the biotic components.

Results

The Tuvem and Divar mangrove ecosystems are characterized by measurable pore water NO_3^- concentrations. Down-core variation in the concentraton of the nutrient showed a sub-surface maxima at Tuvem (36.62 ($\pm$2.91) μmol NO_3^--N L^{-1} at 2–4 cm) (Table 1). At Divar, the concentration of the nutrient was found to decrease with depth with a maximum of 19.90 ($\pm$1.66) μmol NO_3^--N L^{-1} at the surface. Examination of NRA at both the
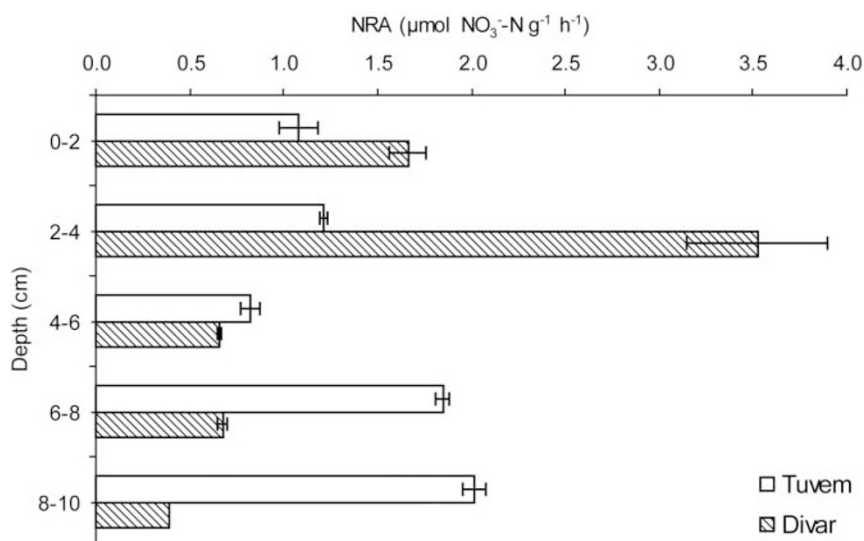
Figure 1 | Down-core variation in nitrate reducing activity (NRA) at the relatively pristine site Tuvem and anthropogenically-inflenced Divar. Error bars represent SDs.

locations revealed that the activity at Divar was comparatively higher within 0–4 cm occurring at a rate of 3.52($\pm$0.38) μmol g^{-1} h^{-1} ($\approx$1.07 μmol cm^{-3} h^{-1}) which is almost twice the rate recorded at Tuvem (Figure 1).

Labelling with ^{15}N to measure DNRA showed a steady increase in ^{15}NH$_4^+$ over time at all depths investigated at both the locations. Down-core observations showed NO$_3^-$ removal through DNRA occured $\sim$2x faster at the relatively pristine Tuvem than at the anthropogenically-influenced Divar ecosystem (Figure 2). Maximum NH$_4^+$-N retention in the Tuvem sediments was recorded at 2–4 cm occurring at a rate of 1.19 μmol g^{-1} h^{-1} i.e. $\sim$13.44 mmol m^{-2} h^{-1}. Co-occurring processes like anammox and denitrification were also measured[1] in conjunction with NRA and DNRA measurements. However, these results have been published separately (Table 1).

In the mangrove sediments of Goa, production of the greenhouse gas N$_2$O has been attributed to the denitrification pathway[8]. Apart from measuring N$_2$ production arising from anammox (Anx) and denitrification activity (DNT)[1], we measured net N$_2$O production to account for N loss in these systems. Nitrous oxide production at both

the locations was found to vary with depth. At Tuvem, maximum production of N$_2$O occurred between 2–6 cm (Figure 3). At Divar, a steady decrease in N$_2$O production with depth was observed. Here, N$_2$O production occurred at a rate of 2.71 nmol g^{-1} h^{-1} which was almost $\sim$2x lower than the rate recorded at Tuvem.

For a more holistic view of the N cycle processes at Tuvem and Divar, the range of activities measured have been illustrated in Figure 4. If the maximum rate of occurrence is considered, it can be observed that NH$_4^+$-N retention through DNRA is almost 15x higher than combined N$_2$O and N$_2$ loss through DNT in the Tuvem sediments (Figure 4). However, at Divar, NH$_4^+$-N retention through DNRA proceeds only 3x higher than denitrification.

To understand N retention versus loss at Tuvem and Divar, the percentage of NH$_4^+$-N retention through DNRA and loss as N$_2$O/N$_2$ through DNT and Anx was calculated based on the percentage of NO$_3^-$ reduced. Our observations reveal up to 99% N retention in the sub-surface layers at both the locations (Table 1). Of the total NO$_3^-$ reduced, the percentage of N loss was maximum at the deeper layer in Divar owing to elevated N$_2$ production through Anx.
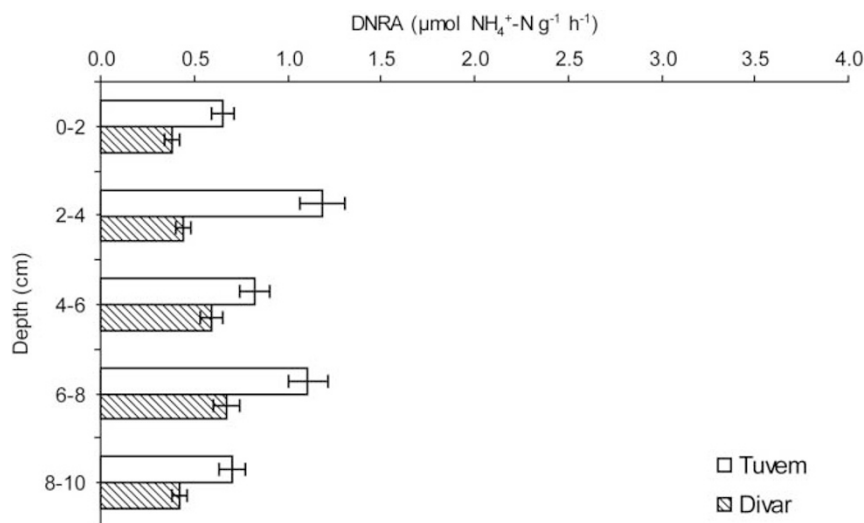

Figure 2 | Down-core variation in rate of dissimilatory reduction of nitrate to ammonium (DNRA) at Tuvem and Divar. Error bars represent SDs.
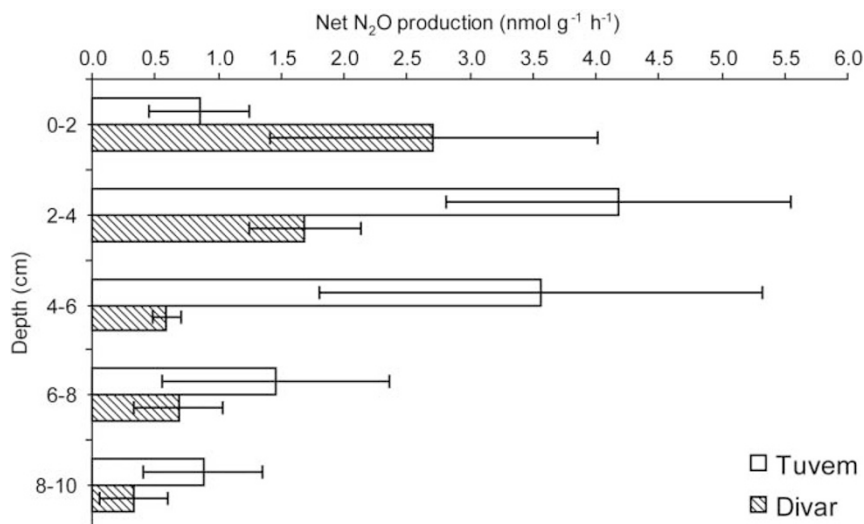
Figure 3 | Down-core profile of net nitrous oxide production at Tuvem and Divar. Error bars represent SDs.

Discussion

Sediment characterization carried out in present investigation revealed that though Tuvem is relatively free from extraneous nutrient input, it is characterized by NO_3^- accumulation at depth ≥ 2 cm which could be attributed to it's intrinsic production. As a result, elevated NRA was observed at ≥ 2 cm at this location. At Divar, high rates of NRA were recorded within the first 4 cm. The top layers of the sediment at Divar are constantly replenished with NO_3^- either originating from internal generation through nitrification or from external anthropogenic sources viz.; sewage outfall, mining rejects, land-runoff during monsoon, etc. Elevated NRA at ≤ 4 cm indicates efficient transformation of NO_3^- through the reductive phase of the N cycle which helps to maintain low levels of the nutrient in this system. Values recorded for NRA at Tuvem and Divar are also in close range to those reported in coastal sediments[9] where rates varying from 0.662–2.4 μmol cm^{-3} h^{-1} have been reported.

Dissimilatory nitrate reduction to ammonium is an important mechanism that supplies available N in the system[10]. Ammonium is known to be adsorbed easily onto clay particles[11]. In organically rich mangrove sediments[12], NH_4^+ released through degradation of organic compounds could easily get bound to clay particles making it unavailable for biological uptake. In some areas, DNRA can remove more NO_3^- than denitrification[13]. Our observations reveal that DNRA values recorded in the present investigation were 2–3 orders higher as compared to rates reported in other marine sediment[14]. In the mangroves ecosystems of Goa, the process accounts for up to 99% of the NO_3^- reduced. In marsh sediments, DNRA has been estimated to account for up to 23% of the NO_3^- reduced[15] which is far lower than observed in the current study. The DNRA process is probably responsible not only for NO_3^- removal, but also for a non-neglectable part of NH_4^+ production.

The partitioning of NO_3^- between denitrification and DNRA is affected by it's concentration and the quantity of carbon[16]. In carbon rich and N limited systems like Tuvem, the larger contribution of DNRA indicates that this ecosystem efficiently re-circulates available N and conserves it to overcome limitation. A similar scenario could
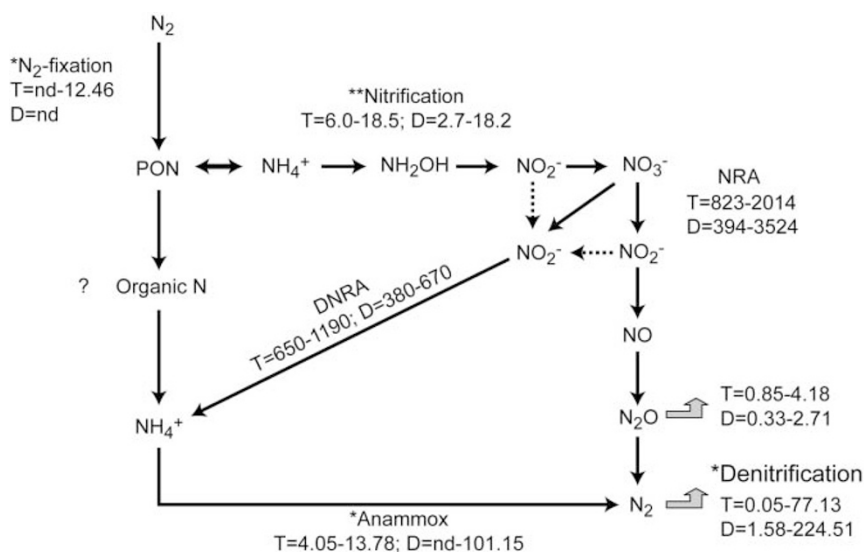
Figure 4 | Nitrogen cycling in mangrove sediments where microbially-mediated activity has been expressed as nmol g^{-1} h^{-1}. Tuvem= (T); Divar= (D); nd= not detected; *Fernandes et al.[1] **Krishnan & Loka Bharathi[12]. At Tuvem, rates of N retention through DNRA are 15x higher than N loss through denitrification while at Divar they were only 3x higher. This observation indicates that NO_3^- reduction to NH_4^+ results in N conservation especially in mangrove ecosystems that are not prone to anthropogenically derived N inputs.

Table	Percentage of ammonium retention and N loss in mangrove sediments							
Depth (cm)	NO_3^--N in pore water (µmol L^{-1})	NRA (µmol NO_3^--N g^{-1} h^{-1})	Anx (µmol N$_2$ g^{-1} h^{-1})	DNT (µmol N$_2$ g^{-1} h^{-1})	DNRA (µmol NH_4^+-N g^{-1} h^{-1})	♦N loss (µmol N$_2$-N g^{-1} h^{-1})	♦NH_4^+-N (% NRA)	♠N loss (% NRA)
Location: Tuvem								
0–2	23.02 (±1.66)	1.08 (±0.10)	0.00 (±0.00)	0.08 (±0.00)	0.65 (±0.06)	0.16 (±0.01)	60	15
2–4	36.62 (±2.91)	1.21 (±0.02)	0.02 (±0.01)	0.07 (±0.01)	1.19 (±0.12)	0.19 (±0.04)	98	15
4–6	7.89 (±0.65)	0.82 (±0.05)	0.00 (±0.00)	0.00 (±0.00)	0.82 (±0.08)	0.02 (±0.01)	99	2
6–8	14.15 (±1.25)	1.85 (±0.03)	0.00 (±0.00)	0.00 (±0.00)	1.11 (±0.11)	0.00 (±0.00)	60	0
8–10	8.92 (±0.83)	2.01 (±0.06)	0.00 (±0.00)	0.00 (±0.00)	0.70 (±0.07)	0.00 (±0.00)	35	0
Location: Divar								
0–2	19.90 (±1.66)	1.66 (±0.10)	0.00 (±0.00)	0.22 (±0.01)	0.38 (±0.04)	0.45 (±0.02)	23	27
2–4	9.14 (±0.91)	3.52 (±0.38)	0.01 (±0.00)	0.04 (±0.00)	0.44 (±0.04)	0.09 (±0.01)	13	3
4–6	1.28 (±0.25)	0.66 (±0.01)	0.00 (±0.00)	0.00 (±0.00)	0.59 (±0.06)	0.00 (±0.00)	90	1
6–8	2.31 (±0.20)	0.68 (±0.03)	0.00 (±0.00)	0.01 (±0.00)	0.67 (±0.07)	0.02 (±0.01)	99	3
8–10	2.63 (±0.25)	0.39 (±0.00)	0.01 (±0.09)	0.05 (±0.00)	0.42 (±0.04)	0.30 (±0.18)	99	72

Note: NRA= nitrate reducing activity measured in nitrification blocked experiments; DNRA= Dissimilatory nitrate reduction to ammonium; Anx= Anammox; DNT= Denitrification activity. Though data for net N$_2$O production has been used in calculating N loss, it has not been included in the above table as it is 2–3 orders lower in magnitude. A figure for the down-core variation in net N$_2$O production has been provided. Measurements for Anx and DNT were carried out during the same sampling time but have been published in Fernandes et al.[1]. The higher percentage of N loss observed at 8–10 cm in Divar is due to elevated N$_2$ production through Anx at this depth.
♦N loss = (Rate of N$_2$O production + DNT + Anx) x 2.
♦NH_4^+-N retention (% NRA) = (NH_4^+-N produced through DNRA / NRA) x 100.
♠N loss (% NRA) = (N$_2$ production through DNT + Anx / NRA) x 100.

be expected at Divar. However, this ecosystem receives extraneously-derived nutrients. As a result, the contribution of DNRA is relatively less as compared to ecosystems that need to conserve N. In anoxic estuarine sediments, degradation of organic matter results in sulfide enrichment[17,18]. Chemolithoautotrophic DNRA is also known to couple the reduction of NO_3^- to the oxidation of H_2S/S^{2-} for the generation of NH_4^+ which is a more readily utilisable form than NO_3^- and is less toxic than H_2S. As DNRA provides an electron donor[19], the process could be linked to lowering levels of reduced sulfur forms in the aquatic system.

Nitrous oxide production was also seen to occur in the Tuvem and Divar sediments. As NO_3^- removal is mainly routed through the DNRA pathway, it highlights the capacity of mangroves to buffer the climate against the production of N$_2$O through incomplete denitrification and it's consequent flux to the atmosphere.

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[20] through denitrification. Our study shows that mangroves have the potential to buffer the climate by modulating the production of N$_2$O resulting from incomplete denitrification. This is achieved in exchange for NH_4^+ that gets retained in the system perhaps within biologically acceptable limits through the DNRA pathway. The DNRA process is a major mechanism for NO_3^- removal rather than denitrification especially in N limited mangrove systems than those receiving nutrients through extraneous sources. Thus, these mangrove ecosystems have the potential to make a significant contribution to the N pool in coastal waters by accumulating and exporting inorganic N.

In many ecosystems, a possibility of N limitation has been suggested to occur in the near future[21]. This is mainly attributed to elevated atmospheric CO$_2$ concentrations which can reduce N mineralization[22], consequently limiting the nutrient supply to plants. Besides, mangrove ecosystems are generally known to be rich in carbon but limited in N. Hence, N retention through DNRA could be an important strategy to overcome this constraint for ecosystem productivity. Most recent estimates using Global Land Survey (GLS) data and Landsat imagery have shown that the worldwide distribution of mangrove forests amounts to approximately 15x10^6 ha[23]. Thus, on a global scale, the prevalence of DNRA in mangrove and other carbon rich systems has critical implications for sustaining ecosystem productivity. We strongly recommend considering DNRA as a relevant process in future N cycling studies in mangrove ecosystems.

Methods

Sampling was carried out at two mangrove forests located along the Mandovi and Chapora rivers in Goa, west coast of India[8]. Sediment cores (inner diameter 7.5 cm, 20 cm length) for activity measurements were collected at low tide during May, 2008 from anthropogenically-inflenced site Divar (15°30′35′′ N and 73°52′63′′ E) which lies along the river Mandovi and the relatively pristine Tuvem (15°39′94′′ N and 73°47′65′′ E) along the river Chapora. The cores were maintained at 4°C until analyses. The cores were sectioned aseptically at 2 cm intervals to obtain representative samples at 0–2, 2–4, 4–6, 6–8 and 8–10 cm. For each sampling site, sediment corresponding to the same depth were pooled and homogenized. Each homogenized sample was further sub-divided as follows:

(i) Duplicates (10 mL) for immediate analysis of NO_3^--N in pore water.

(ii) For NRA and net N$_2$O production, triplicate measurements were done at every time interval (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h; n=21 for each section of the core).

(iii) For DNRA measurement, duplicate samples were maintained at every time interval (0, 2, 4, 6, 8, and 10 h; n= 12 for each section of the core).

To measure nitrate reducing activity (NRA), approximately 1 g wet weight sediment obtained from each representative section was transferred to 60 mL serum bottles. Ambient seawater was collected from site for media preparation. This seawater contained approximately 4.5 µmol NO_3^--N L^{-1}. The seawater was amended with allythiourea (ATU) at a pre-standardized concentration of 125 µmol L^{-1} to inhibit nitrification[24]. The sediment slurry was briefly vortexed and the bottles were then filled with filter sterilized seawater up to the brim to create micro-aerophilic conditions. The bottles were capped with butyl stoppers and the slurry was gently mixed and incubated in triplicates under static conditions for up to 3 h as the nitrification inhibitor used became ineffective beyond this period. At the end of the sampling period, the bottles were gently swirled. The contents were transferred to 50 mL centrifuge tubes and centrifuged (*REMI Compufuge* CPR-24) at 5000 rpm and 4°C for 10 min. Nitrate in the supernatant was measured spectrophotometrically[25]. The NRA was determined from the fall in NO_3^- level over time and has been expressed on a dry weight basis as µmol NO_3^--N g^{-1} h^{-1}. Net N$_2$O production was measured as described elsewhere[8].

DNRA measurements were carried out in conjunction with pore water nutrient analysis and measurement of N$_2$-fixation, Anx and DNT by mass spectrometry[1]. Four mL of homogenised sediment from each section was transferred into 22 mL headspace vials. Four mL of filter sterilized seawater containing NO_3^--N at a final concentration of 10 µmol L^{-1} was added. The vials were sealed with butyl stoppers, purged with He and pre-incubated for about an hour before addition of stock solution of $^{15}NO_3^-$ (97.4 atom%, Isotech Mathesson, USA)[26] to obtain a final concentration of 80 µmol L^{-1}. DNRA was measured by monitoring the progressive isotopic enrichment of $^{15}NH_4^+$ for up to 10 h in the dark. Two or three vials were sacrificed by adding HgCl$_2$ (final concentration of 10 mmol L^{-1}) at each point of the time series (0, 2, 4, 6,

8 and 10 h). NH_4^+-N in pore water and sediment was extracted by microdiffusion[27] and the N was analyzed by mass spectrometry. Unlabelled ammonium (1 μmol L^{-1}) was added to the filters after microdiffusion and this quantity was taken into account when calculating the DNRA activity. The samples were treated with a mild alkali (MgO) to convert NH_4^+ to NH_3, which was trapped on acidified (50 μL, 0.5 N H_2SO_4) pre-combusted Whatman GF/C filters. To calculate the rate of flux from dissolved NO_3^- to dissolved NH_4^+, equations derived by analogy with that of Dugdale & Goering[28] were used. DNRA was calculated using previously described equations[27] and the rate has been expressed as μmol NH_4^+-N g^{-1} h^{-1}.

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Author contributions

SOF, PCB & PAL conceived the study. SOF, PCB & VDM prepared the samples. NG assisted in analysis of nutrients. SOF carried out the experiments, data analyses and wrote the manuscript. PAL, PCB and VAL reviewed the manuscript.

Additional information