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Denitrification, anammox and nitrate reduction in sediments of the southern Great Barrier Reef lagoon

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ABSTRACT: We provide the first reported estimates of anammox activity in tropical continental shelf sediments (southern section of the Great Barrier Reef lagoon; GBRL). The measured contribution of anammox to total N_2 production was up to 70% but restricted to only 1 of the 4 (2 inshore and 2 offshore) sites assayed. Sediment characteristics (contents of total organic carbon [TOC] and manganese [Mn], C:N ratio) at this site appeared to favour anammox activity and the estimated maximum rate was 4.9 µmol m^{-2} h^{-1}. Anammox bacteria may be a significant contributor to N_2 production along the coastal zone of the GBRL. The availability of labile (low C:N) TOC seemed to drive denitrification to completion in the offshore sediments. However, rates of NO_3^- reduction to NH_4^+ at the offshore sites were comparable to or higher than denitrification rates. It was unclear whether dissimilatory or assimilatory processes were responsible for the observed reduction of NO_3^- to NH_4^+ at the offshore sites. At the 2 inshore sites, NO_3^- reduction to NH_4^+ was a larger sink for NO_3^- than denitrification. Anammox does exist in the tropical continental shelf sediments of the GBRL and should be studied further to determine its role in larger scale N cycling. The roles of assimilatory and dissimilatory nitrate reduction to ammonium also need to be assessed within the GBRL.

KEY WORDS: Great Barrier Reef · Anammox · Dissimilatory/assimilatory nitrate reduction to ammonia · Denitrification

INTRODUCTION

Continental shelf sediments are a conduit between terrestrially derived organic material and the open ocean. On shallow shelves, the water column is generally well mixed and there is much higher connectivity between benthic and pelagic nutrient cycling. As such, continental shelf sediments are major sinks for oceanic nitrogen (N) accounting for up to 67% of global denitrification (Codispoti et al. 2001). However, most continental shelf research has focussed on processes in fine grained sediments, largely ignoring N transformations in the 70% of continental shelf area covered by sand (Vance-Harris & Ingall 2005). Understanding N transformations in continental shelf sediments, in particular the magnitude of N sinks such as denitrification, is critical in constraining the discrepancies that currently exist in the global oceanic N budget (Brandes et al. 2007, Deutsch et al. 2007).

Research effort directed towards understanding N cycling in continental shelf sands has increased (Rao et al. 2007, Rao et al. 2008), but N transformations in tropical carbonate sediments remain relatively understudied. In the Great Barrier Reef lagoon (GBRL), terrestrially-derived matter is thought to be rapidly metabolised and recycled within sediments, preventing the accumulation of nutrients and the onset of widespread eutrophication (Alongi et al. 2007). Denitrification is one of the major processes responsible

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for maintaining the balance of nitrogen within the GBRL. Estimates of denitrification can account for all of the terrestrially-derived N entering the lagoon (Furnas et al. 2011). However, there are fundamental N cycling processes that remain unaccounted for in the current paradigm of nutrient cycling in the GBRL.

Anammox (anaerobic ammonium oxidation) is the chemolithotrophic production of N_2 from the oxidation of NH_4^+ with NO_2^-. It was first discovered in wastewater treatment systems (Mulder et al. 1995), but is now known to exist and often dominate in marine sediments worldwide (Thamdrup & Dalsgaard 2002, Risgaard-Petersen et al. 2004, Trimmer & Nicholls 2009). There is a comparative paucity of information regarding the anammox process in tropical shelf sediments. Two published studies (1 in tropical estuaries and 1 in GBR reef sediments) have measured for anammox in tropical sediments, but neither detected the process (Eyre et al. 2008, Dong et al. 2011). Anammox is favoured in sediments where NO_3^- and total organic C (TOC) are readily available (Trimmer et al. 2003, Engstrom et al. 2005). Under these conditions, there is a steady supply of NO_2^-, being reduced from NO_3^- by denitrifiers, and NH_4^+, from organic matter mineralisation (Engstrom et al. 2009). The oligotrophic waters of tropical oceans may preclude the development of anammox (Dong et al. 2011); however, as mentioned there have been very few studies that have attempted to measure anammox in tropical sediments. In tropical coastal estuaries, anammox is thought to be outcompeted by denitrification and dissimilatory nitrate reduction to ammonia (DNRA) for available NO_2^- (Dong et al. 2011).

The reduction of nitrate to ammonium (NRA) conserves N within sediments and should not be excluded in N budget calculations for the GBRL. Unlike anammox, DNRA (dissimilatory NRA) is known to be an important process in tropical and subtropical sediments (Gardner & McCarthy 2009, Dong et al. 2011). DNRA bacteria have a greater affinity for NO_3^- and the process is more energetically efficient at low NO_3^- concentrations (Dong et al. 2011). DNRA is prevalent in high sulphide environments (An & Gardner 2002) that are poisonous to anammox bacteria (Jensen et al. 2009), but DNRA does not necessarily outcompete denitrification. Assimilatory NRA can also shunt NO_3^- to NH_4^+ in sediments (Rysgaard et al. 1993, Cook et al. 2004). This is usually carried out by benthic autotrophs that assimilate NO_3^- and later release it as NH_4^+. Across the GBR shelf, there is a notable gradient in sediment geochemistry (Alongi et al. 2011), but it is unclear whether this is reflected in rates of NRA or denitrification.

The balance between the N_2 producing processes of denitrification and anammox and N conservation processes such as DNRA will have important implications for N budgets within the GBRL. The objectives of this study were to: (1) determine if anammox is present and, if so, investigate what conditions favour its development; and (2) establish the relative contributions of anammox, NRA and denitrification to N processing in the sediments of the GBRL. In order to address these objectives, a series of incubations were performed with sediment collected along a transect from inshore to the edge of the shelf in the southern section of the GBRL.

MATERIALS AND METHODS

Sampling sites and sediment collection

Sediment samples were collected from 3 to 14 April 2011 within the southern section of the GBRL (Fig. 1) during a cruise aboard the RV 'Cape Ferguson'. Sediments were collected from 4 sites, 2 within the Pompey reef complex (offshore sites: GR1 and PR1), and 2 within inshore locations (inshore sites: IS9 and WH1) (Fig. 1). The site names, except WH1, correspond to those described in Alongi et al. (2011). Site WH1 was located in the Whitsunday Islands (20° 18.9′ S, 148° 49.5′ E). The sediment grain size characteristics at each site are shown in Table 1. All experimental assays were performed in a temperature controlled lab (22°C). Sediments were collected using a Smith-MacIntyre grab and deposited into large plastic holding containers (25 l) immediately after being brought aboard. The grab sampler collects ~20 l of sediments (20 cm depth). At each site, a 10 l sample of bottom water was also collected using a Niskin bottle and kept in high-density polyethylene (HDPE) plastic drums until required. Sediments were deposited into the holding containers and were left to settle for at least 6 h under a constant flow of seawater (~5 l min^{-1}) to re-establish natural sediment gradients. After this initial settling period, the sediments were sub-sampled for a range of different assays. Three separate assays were performed. (1) A conventional sediment slurry type assay (at all sites) was used to detect the presence or absence of anammox and to calculate potential rates of denitrification. (2) We performed flow-through reactor type experiments with sediments from sites GR1 and PR1, where it was deemed that porewater advection may be important for N cycling. (3) We performed static core incubations at the inshore sites IS9 and WH1, where diffu-

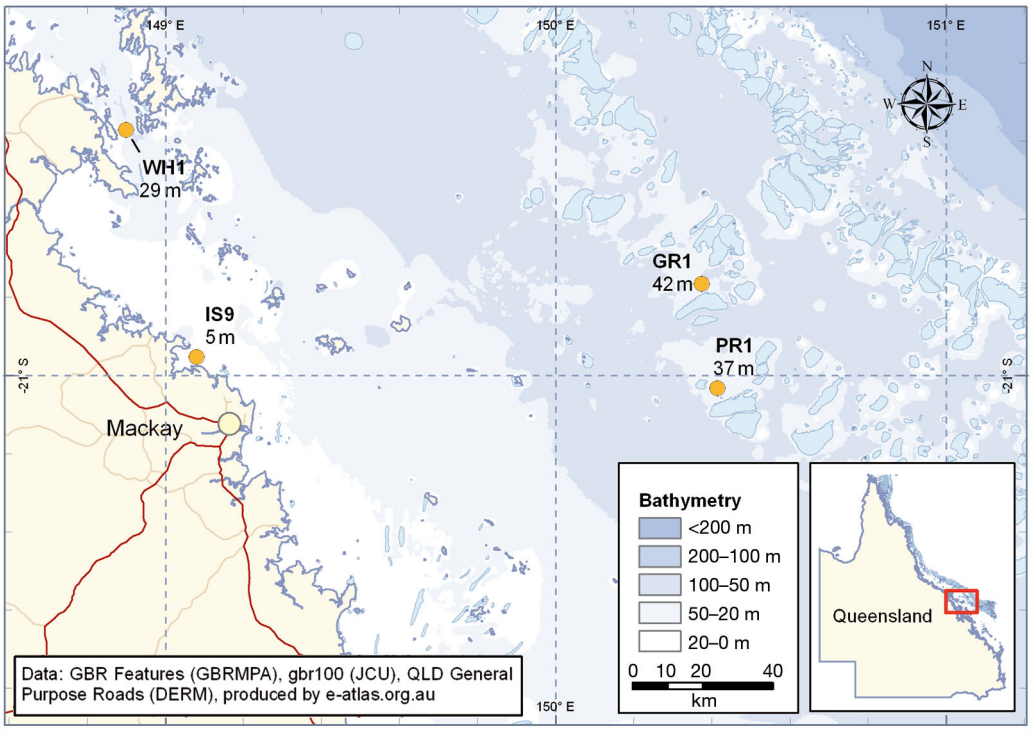

Fig. 1. Sampling sites (with depth) within the southern section of the Great Barrier Reef lagoon (GBRL)

Table 1. Location of sampling sites and their sediment characteristics (grain size as % weight in each fraction of samples collected in April 2011)

Site	Location		Grain size							
	S°	E°	(mm)				µm			
			>2	1–2	0.5–1	0.25–0.5	125–250	63–125	2–63	<2
GR1	20°45.9	150°22.4	2.3	8.9	14.8	16.0	18.5	24.2	10.1	5.3
PR1	20°45.9	150°22.4	10.2	18.9	22.9	28.4	12.2	5.0	0.8	1.7
WH1	20°45.9	150°22.4	32.3	18.9	23.8	17.1	4.4	2.1	0.7	0.8
IS9	20°45.9	150°22.4	3.0	3.5	9.3	19.8	47.1	12.8	1.7	2.8

sion was thought to be potentially more important than porewater advection. Three 10 cm long sediment cores were also collected from each plastic holding container for later analysis of C and N content. These samples were stored frozen.

Sediment slurry assay

A 5 cm diameter Perspex corer was used to collect 10 cm long sediment cores from the holding containers. Two cores were collected from each container and transferred to a graduated jug in a pure N_2 filled glove bag (except the top 2 cm of sediment from each core, which was discarded to try and remove microphytobenthos [MPB]). An equal volume of deoxygenated bottom water from the same site was added to the jug. The sediment slurry was deoxygenated for 6 h under a constant flow of pure N_2 gas (~60 ml min^{-1}) to remove traces of dissolved oxygen (DO) and also to facilitate the denitrification of any remnant NO_3^- within sediment porewater. A pipette was used to transfer ~2 ml of the slurry mixture into 60 glass headspace vials (13.5 ml) (Exetainer, Labco); the sediment slurry was continuously stirred during the transfer to keep the mixture uniform. The headspace vials were then filled with the deoxygenated water to within 2 ml of the surface. Each vial was then capped with a lid containing a rubber septum. The vials were separated into 4 groups of 12, and deoxygenated isotopic amendments were added through the septa of the vials using a syringe. The first group received 0.1 ml of 99.8% ^{15}N-NH_4Cl stock solution to give a final concentration of 50 µmol l^{-1}, the second group received 0.2 ml of 99.8% ^{15}N-NH_4Cl plus ^{14}N-KNO_3 stock solution to give a final concentration of 50 µmol l^{-1}, the third group received 0.2 ml of ^{15}N-KNO_3 stock solution to give a final concentration of 50 µmol l^{-1}

and the final group did not receive any amendment (controls). Three of the control vials contained an oxygen sensitive patch (Presense) that was used to monitor the DO concentration of the incubation water during the assay via a fibre optic cable and DO sensor (Presense). Once all amendments were added, the vials were shaken vigorously to mix the amendments into the slurry mixture. Three vials from each of the amendment groups, including the controls, were immediately preserved by adding 0.1 ml of saturated $HgCl_2$ through the septa. This was repeated for the remaining vials at roughly 6, 12 and 18 h. All vials were inverted, immersed in water and refrigerated for storage. The DO concentration in the control vials was checked repeatedly to ensure anoxic conditions remained during the entire incubation. At the completion of the assay, the height of the sediment and the water in each vial was measured to allow for the calculation of sediment and water volumes.

Flow-through reactor experiments

Perspex corers (5 cm diameter × 15 cm length) were used to collect 6 sediment samples from the holding containers. A cap containing a fluid injection port was attached to either end of the core and the sealed reactors were placed in an upright position. A media delivery tube (0.15 mm) was attached to the lower fluid injection port of each reactor, the tubes were connected via a peristaltic pump to two 6 l HDPE media drums (3 tubes into each drum) containing aerated bottom water collected from the same site. Another tube was connected to the upper port of each reactor, these outlet tubes led to a waste drain. Aerated bottom water was passed through the reactors at 0.5 ml min^{-1} for 6 h before the addition of an isotope tracer ($^{15}N\text{-}NO_3^-$) to one of the media drums (2 ml of concentrated 99.8% $^{15}N\text{-}KNO_3$ solution to reach a final concentration of 20 µmol l^{-1}). Water samples from both drums were collected for dissolved inorganic N (DIN) analysis before and after the isotope addition to determine the exact tracer concentration (see below for method). Both drums also received an amendment of the conservative tracer fluorescein (2.5 ml of a stock solution to give a final concentration of ~900 µg l^{-1}). Each reactor contained an oxygen sensitive patch on the reactor wall near the outlet that was used to monitor the DO concentration in the outgoing water. A fibre optic cable and DO detector (Presense) was used to measure DO near the reactor outlet every 2 h.

The fluorescein concentration in the outlet water from each reactor was measured at regular intervals using a handheld fluorometer (Turner Designs). Samples for concentration and isotope determination were collected at 12 and 24 h from the reactor outlets by inserting the outlet tubes into 40 ml glass vials, which were allowed to continuously overflow. Water was extracted from the vials with a syringe and 10 ml was filtered (0.45 µM - MiniSart, Sartorius) into 12 ml polypropylene vials for DIN analysis (in duplicate), a further 50 ml was filtered into 100 ml plastic HDPE bottles for $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ analysis. For $\delta^{15}N\text{-}N_2$ analysis the outlet tubes were placed into 13.5 ml headspace vials (Exetainers), which were allowed to overflow for at least 10 min. The vials were then capped without headspace and 0.1 ml of NaOH (2N) was added through the septa. Duplicate samples were collected from each reactor for $\delta^{15}N\text{-}N_2$ determination and stored submerged and refrigerated. At the completion of the incubation, all 6 reactors received aerated seawater for a further 1 h at an increased flow rate (5 ml min^{-1}) to remove any labelled porewater. The entire sediment column from each reactor was then collected in a plastic bag and frozen for later analysis of C and N content.

Static core incubations

At the 2 inshore sites, static core incubations were performed with the collected sediments. These incubations involved extracting sediment cores into 10 plastic corers (5 cm diameter × 30 cm length). A bottom cap was attached to each core and they were submerged in a bucket containing flow-through seawater (5 l min^{-1}). After 6 h, a sample of water from the bucket was collected and processed for DIN and $\delta^{15}N\text{-}NH_4^+$ and $\delta^{15}N\text{-}N_2$ analysis (see 'Flow-through reactor experiments' section above). A top cap, containing septa, was placed onto each of the cores. Increasing amounts of $^{15}N\text{-}NO_3^-$ (2.5, 5, 7.5, 10, 12.5 µmol) were injected, using a syringe through the septa, into duplicate cores to give final concentrations of ~20, 40, 60, 80 and 100 µmol l^{-1} (depending on the final volume of water in each core). The cores were incubated in flowing seawater in the dark. After 12 h, the top cap from each core was removed and a sample of the overlying water was collected with a 60 ml syringe and processed for DIN, $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}N_2$ analysis as described above. The top 5 cm from each sediment core was extruded into a plastic bag and frozen for sediment C and N analysis.

Sample analysis

DIN samples were analysed colourometrically on a Lachat Flow Injection Analyser (FIA) using standard protocols. The $\delta^{15}N\text{-}NH_4^+$ signature was determined following the method of Zhang et al. (2007). Briefly, NH_4^+ was converted to NO_2^- and then to N_2O following hypobromite and azide conversion, respectively. The N_2O was cryogenically trapped using liquid N_2 on a custom built purge and trap (PT) pre-concentrator. Our PT system was based on the design of McIlvin & Casciotti (2010), with the exclusion of the automated components. The $\delta^{15}N\text{-}N_2O$ signature of the trapped gas was determined on a gas chromatograph (Thermo Trace Ultra GC) interfaced to an isotope ratio mass spectrometer (IRMS, Thermo Delta V Plus IRMS). The IRMS was set to measure m/z 44, 45 and 46 and produced $\delta^{15}N$ values relative to a reference gas of pure N_2O (BOC gases). A set of NH_4^+ standards with known $\delta^{15}N$ (USGS 25 & 26) were analysed together with the samples and used to correct the $\delta^{15}N$ produced by the IRMS. Reported $\delta^{15}N$ values are relative to air and precision was >0.75 ‰. In samples where NO_2^- concentration was >0.1 µmol l^{-1}, we also measured the $\delta^{15}N\text{-}NO_2^-$ signature and corrected the $\delta^{15}N\text{-}NH_4^+$ signal for any contribution from $\delta^{15}N\text{-}NO_2^-$. The $\delta^{15}N\text{-}NO_2^-$ was determined using the method of McIlvin & Altabet (2005).

The $\delta^{15}N\text{-}N_2$ signature of N_2 in the headspace of the exetainer vials was determined via GC-IRMS with the instrument set to measure m/z 28, 29 and 30 (representing $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$, respectively). The mass of each isotopomer is given as a peak area. Pure analytical grade N_2 (BOC gases) was used as the reference gas and varying volumes (3–10 µl) of lab air were used to construct a standard curve and relate the peak areas to actual concentrations of N_2.

For sediment analysis, each collected sediment portion was thawed and mixed well. Weighed 10 g subsamples (n = 6) were added to 40 ml of 2 M KCl and shaken for 6 h (100 rpm) to extract loosely adsorbed DIN. After KCl extraction, the sediment subsamples were centrifuged and rinsed (distilled water) 3 times before being dried to constant weight. The difference in weight was used to calculate porosity. The volume of each 10 g of wet sediment was used to calculate the bulk density. Dried sediments were analysed for grain size distribution using the method of Lewis & McConchie (1994), sediment fractions <63 µm were measured using the hydrometer technique of Gee & Bauder (1986). Dried sediments were ground in a ball mill and weighed into tin capsules (100 mg) for total C and N via EA-IRMS. Samples were calibrated against IAEA-N-1 for N and NIST-141d for C. For TOC analysis, we followed the methods described by Ingalls et al. (2004). Briefly, samples of the dried sediments (100 mg) were placed into 20 ml vials, to which 2 ml of 6N HCl was added. After 12 h, the remaining solution was filtered (Whatman GF/F, nominal pore size 0.7 µm) to remove HCl insoluble particulate organic C, which was freeze dried and packaged for analysis via EA-IRMS. The filtrate was collected and the HCl soluble organic C concentration was measured on a Shimadzu DOC analyser. TOC content of the sediments was the sum of the HCl insoluble plus soluble fractions. Sediment carbonate content was assumed to be the difference between total C and TOC (Alongi et al. 2011). The total Mn, Fe and S content of dried ground sediments was determined by inductively coupled mass spectroscopy after acid digestion (Rayment & Higginson 1992).

Calculations

For the slurry assays, we first determined the concentrations of excess $^{29}N_2$ and $^{30}N_2$. These were calculated as the measured µmol of total N_2 in the exetainer multiplied by the ratio of peak area $^{29}N_2$, or $^{30}N_2$, to $^{28}N_2$ at each time step subtract the ratio of the peak area $^{29}N_2$, or $^{30}N_2$, to $^{28}N_2$ at t0. Excess concentration was normalized against the volume of the sediment slurry to give units of nmol cm^{-3}. The rate of production ($P^{29}N_2$ or $P^{30}N_2$) was the slope of the plot of excess $^{29}N_2$ or $^{30}N_2$ concentration vs. time (nmol cm^{-3} h^{-1}). All assays were first checked for oxygen contamination or the presence of anoxic nitrification, as indicated by a positive slope of $P^{29}N_2$ in the $^{15}NH_4^+$ treatment. Anammox activity in any particular sediment was identified as the production of $^{29}N_2$ in the treatment receiving both $^{15}N\text{-}NH_4^+$ and $^{14}N\text{-}NO_3^-$, relative to the treatment receiving $^{15}NH_4^+$ only. Where anammox was observed, we calculated anammox and denitrification rates using $P^{29}N_2$ and $P^{30}N_2$ in the $^{15}NO_3^-$ treatment following the procedure of Thamdrup & Dalsgaard (2002) using the variable F_N (i.e. $^{15}NO_3^-/^{14}NO_3^-$) of 0.95. Where anammox was absent, the potential rate of denitrification was simply the production rate of labeled $^{15}N\text{-}N_2$ (i.e. $P^{29}N_2 + 2 \times P^{30}N_2$) (Lansdown et al. 2012).

For the flow-through reactor experiments, the excess concentrations of $^{29}N_2$ and $^{30}N_2$ were calculated as the measured µmol of total N_2 in the reactor outlet water multiplied by the ratio of peak area $^{29}N_2$, or $^{30}N_2$, to $^{28}N_2$ subtract the ratio of the peak area $^{29}N_2$, or $^{30}N_2$, to $^{28}N_2$ in the inlet water. The excess

concentrations were multiplied by the flow rate through the reactors and divided by the sediment volume to give a ^{15}N-N_2 production rate in nmol cm^{-3} h^{-1}. The rate of NO_3^- reduction to NH_4^+ (NRA) in the flow through reactors was calculated as the sum of the excess $^{15}NH_4^+$ in the outflow water (i.e. excess $^{15}NH_4^+$ concentration in outflow water multiplied by flow rate and divided by sediment volume) plus the calculated amount of adsorbed ^{15}N in the sediment. Adsorbed ^{15}N in the sediment was calculated as the total ^{15}N content of the sediment (i.e bulk sediment ^{15}N) subtract the ^{15}N content of sediments that had been KCl extracted (i.e. the assimilated portion of sediment ^{15}N). The total amount of recovered ^{15}N-NH_4^+ is presented in nmol cm^{-3} h^{-1}. This rate underestimates the reduction of NO_3^- to NH_4^+ because it only includes reduction of NO_3^- present in the inlet water, not the reduction of any NO_3^- produced internally through nitrification. The inlet and outlet concentrations of ^{15}N-NO_3^- or ^{15}N-NH_4^+ were normalized to the sediment volume (i.e. concentration multiplied by flow rate divided by sediment volume) to allow a mass balance to be calculated.

Rates of N_2 production in the core assays were based on the measurement of excess concentrations of $^{29}N_2$ and $^{30}N_2$ in the overlying water (i.e. µmol of total N_2 in the overlying water multiplied by the ratio of peak area $^{29}N_2$, or $^{30}N_2$, to $^{28}N_2$ subtract the ratio of the peak area $^{29}N_2$, or $^{30}N_2$, to $^{28}N_2$ at the start of the incubation). Production rates were calculated by multiplying excess concentration by the core water volume and dividing by sediment surface area (µmol m^{-2} h^{-1}). When anammox was present, we used the amended IPT calculation of Risgaard-Petersen et al. (2003) to determine rates of anammox and denitrification. The parameter r_{14} was calculated using r_a (i.e. the contribution of anammox to total N_2 production) from the slurry assay at the same site. When anammox was absent, we calculated the rate of denitrification using the traditional IPT (Nielsen 1992). This calculation included the amount of coupled nitrification denitrification (CND) and the denitrification based on water column NO_3^- (*Dw*). The concentration of ^{15}N recovered in N_2 was calculated as D_{15} from Nielsen (1992) and was multiplied by the core water volume to give an amount of ^{15}N in N_2 (µmol). The amount (µmol) of ^{15}N-NO_3^- reduced to ^{15}N-NH_4^+ (NRA) in the core incubations was calculated as the excess concentration of ^{15}N-NH_4^+ multiplied by the volume of the cores. The total amount of ^{15}N adsorbed to sediment was also added to this value.

Any statistical comparison of parameters was made using analysis of variance (ANOVA) with post-hoc honest significant difference testing (SPSS). Any presented errors are standard deviations.

RESULTS

Sediment characteristics

Clear differences existed between the sediment geochemistry at the 4 sampling stations. Sediments from site IS9 had low carbonate content (30%), while the other inshore site, WH1, had a carbonate content of 50%. The 2 offshore sites had higher carbonate content (72%). The finest grain sediments were at site GR1, which had the highest proportion of sediment <125 µm. Sediment TOC content was highest ($p < 0.05$, ANOVA) at the inshore site IS9, followed by the WH1 site and then the 2 offshore sites GR1 and PR1. However, the HCl soluble fraction of TOC was higher at the offshore sites representing 22 and 34% of the sediment TOC at GR1 and PR1 respectively. At the 2 inshore sites, ~90% of TOC was HCl insoluble (Table 2). Total organic N content of the sediments increased from inshore to the offshore sites; consequently, the C:N ratio of sediments declined from inshore (~28) to offshore (~8). Total Mn and Fe content of the sediments was significantly

Table 2. Density (g wet sediment per cm^3), porosity, total organic C (TOC) content, HCl soluble and insoluble fractions of TOC (presented as a ratio of reactive soluble TOC (C_R) to total TOC and insoluble TOC (C_I) to TOC, respectively), total organic N (TON) content, C:N ratio and total Mn, Fe and S content of sediment at 4 sites across the southern Great Barrier Reef Lagune, super-scripts indicate significant differences between sites ($p < 0.05$)

	Density ($g\ cm^{-3}$)	Porosity (%)	TOC (%)	HCl digested		TON (%)	C:N	Mn (‰)	Fe (%)	S (‰)
				Sol (C_R:TOC)	Insol (C_I:TOC)					
GR1	$1.70^{a,b}$	0.68	0.54^{c}	0.22^{b}	0.78^{c}	0.057^{a}	9.5^{c}	0.02^{b}	0.09^{b}	4.06
PR1	1.65^{b}	0.74	0.46^{d}	0.34^{a}	0.66^{d}	0.057^{a}	8.0^{c}	0.01^{b}	0.04^{b}	4.16
WH1	1.90^{a}	0.69	0.62^{b}	0.11^{c}	0.89^{b}	0.038^{b}	16.5^{b}	0.46^{a}	1.77^{a}	3.85
IS9	$1.70^{a,b}$	0.71	1.04^{a}	0.06^{d}	0.94^{a}	0.037^{b}	27.9^{a}	0.35^{a}	1.80^{a}	3.26

higher inshore than offshore ($p < 0.05$, ANOVA; Table 2) whereas total S content was similar within all sediments.

Slurry assays

Anammox activity, which results in the production of $^{29}N_2$ in the treatment receiving both $^{15}N\text{-}NH_4^+$ and $^{14}N\text{-}NO_3^-$, was detected at the WH1 site only (Fig. 2C). The concentration of $^{30}N_2$ for the same treatment did not increase during the assay, which is a good indication that there was no O_2 contamination (O_2 would have led to nitrification of the added $^{15}N\text{-}NH_4^+$ and denitrification of the produced $^{15}N\text{-}NO_3^-$). There was also no labelled $^{15}N\text{-}N_2$ detected in this, or any of the $^{15}NH_4^+$ amendments (data not shown), a clear sign that anoxic nitrification, through for instance Mn(IV) reduction, did not occur in sediments from any site during the assay.

Rates of denitrification and anammox based on the anoxic slurry assay (Fig. 3) showed that the most active sediment in terms of total N_2 production was from site GR1 (1.43 ± 0.41 nmol cm^{-3} h^{-1}). Total N_2 production was significantly higher ($p < 0.05$) in the GR1 sediments relative to the other sites. Total rates of N_2 production were over 3 times higher in WH1 sediments relative to IS9 sediments. In the WH1 sediments, anammox accounted for 70% of N_2 production (0.55 ± 0.20 nmol cm^{-3} h^{-1}).

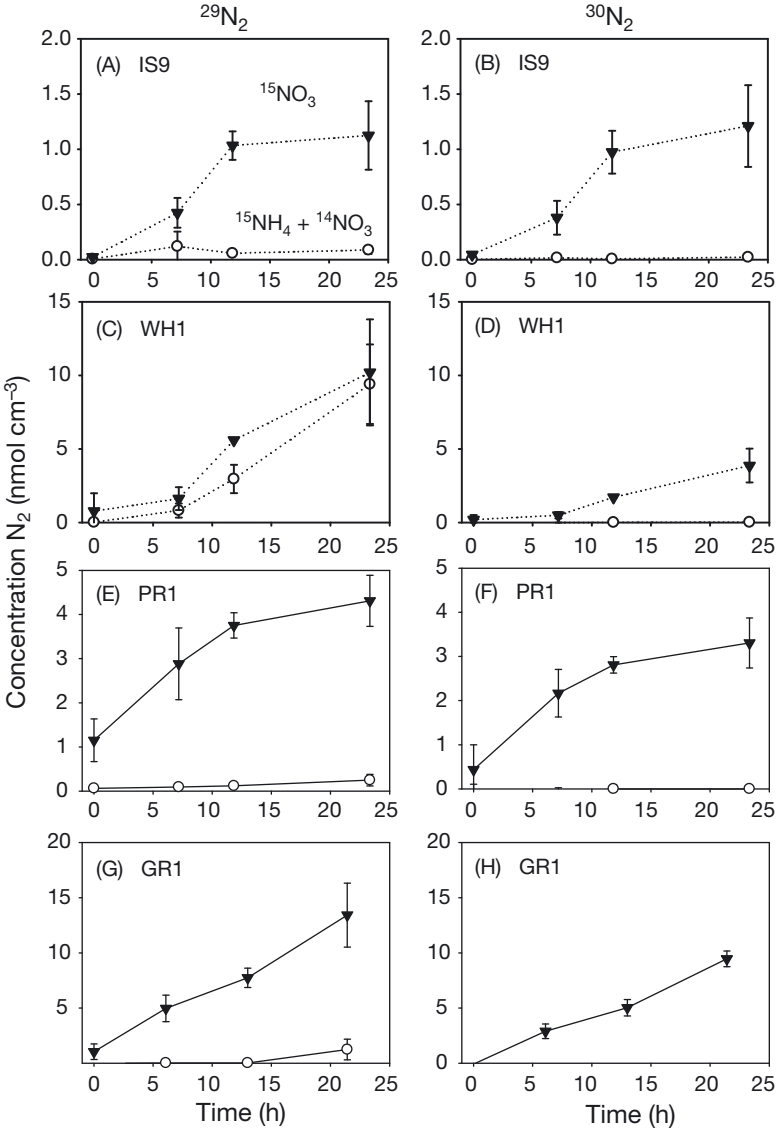

Fig. 2. Peak area ratios of $^{29}N_2$ to $^{28}N_2$ (A,C,E,G) and $^{30}N_2$ to $^{28}N_2$ (B,D,F,H) during anoxic slurry incubations of sediments from the 4 sampling sites: IS9 (A,B), WH1 (C,D), PR1 (E,F) and GR1 (G,H). Ratios from the amendments: ○ = $^{15}NH_4 + {}^{14}NO_3$; ▼ = $^{15}NO_3$

Flow-through reactors

In the flow-through reactor experiment, the rate of denitrification was similar between the 2 offshore sediments (Fig 4A). However, the rate of nitrate reduction to NH_4^+ was significantly ($p < 0.05$) higher for the PR1 sediments (0.91 ± 0.29 nmol cm^{-3} h^{-1}) relative to the GR1 sediments. The assimilation of added N into microbial biomass was also highest in the PR1 sediments and was the largest sink for added N. In the GR1 reactor, the bulk of the added N was not processed during advection (Fig. 4B). The flow of amendment through the reactors during the 2 experiments was not uniform (Fig. 5). In the GR1 reactors, there were clear signs of preferential flow with the concentration of fluorescein approaching that of the inlet media much quicker than in the PR1 reactors. There was complete consumption of available O_2 within the flow through reactors during the incubations.

Core experiment

For both the IS9 and WH1 sediments, the total retention of $^{15}N\text{-}NO_3^-$ (i.e. the sum of assimilation, N_2 production or

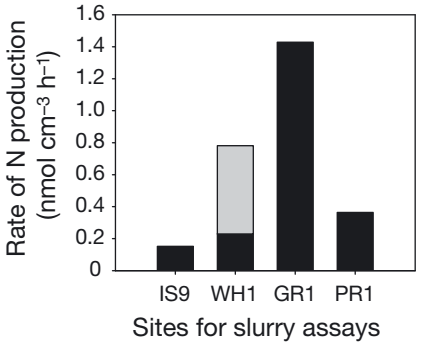

Fig. 3. Rates of denitrification (black) and anammox (grey) during the anoxic slurry assays with sediment from the 4 sites

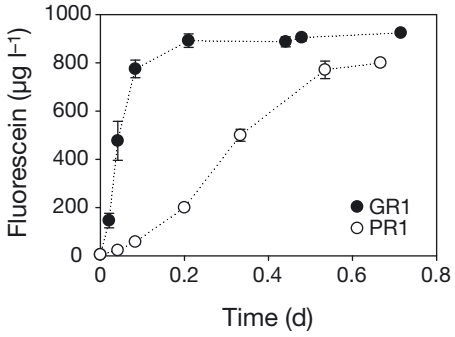

Fig. 5. Fluorescein concentration in the outlet water of the reactors from the GR1 and PR1 sites (means ± SD of the 6 reactors used at each site), inlet concentrations were 800 and 920 µg l^{-1} for PR1 and GR1 respectively

NRA) during the incubations was similar despite the increasing amendment concentration (Fig. 6). With the exception of the 2.5 µmol l^{-1} cores, most of the added ^{15}N-NO_3^- was not used and remained in the water column. Of the retained ^{15}N-NO_3^-, assimilation was the major uptake pathway. The movement of added ^{15}N-NO_3^- to ^{15}N-NH_4+ (i.e. NRA) was greater than the movement to ^{15}N-N_2 (i.e. via annamox and/or denitrification) in both inshore sediments. The largest recovery of ^{15}N-NH_4^+ was in the IS9 sediment cores receiving 12.5 µmol of ^{15}N-NO_3^- (Fig. 6). The production of ^{15}N-N_2 accounted for <1 % of the added ^{15}N-NO_3^-.

Despite the lack of significant production of ^{15}N-N_2, some trends were still observable between sites and amendment concentrations during the core incubations (Fig. 7). Namely, N_2 production, associated with anammox and denitrification of water column NO_3^-, only occurred in the WH1 sediments when the amendment was upwards of 7.5 µmol ^{15}N-NO_3^-. Also, there was an absence of nitrification coupled to denitrification in the WH1 sediments. For the IS9 sediments, the production of ^{15}N-N_2 did not vary greatly with changing amendment concentration (Fig. 7).

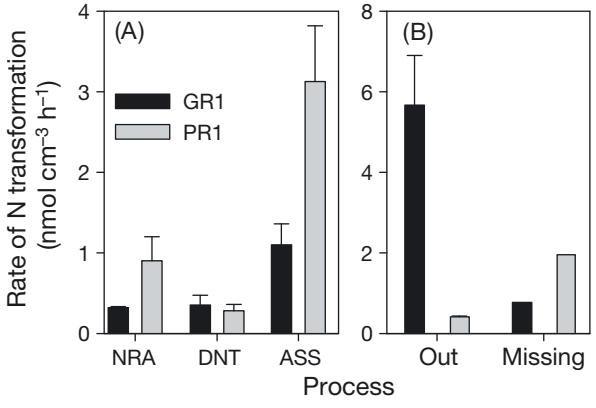

Fig. 4. (A) Rates of nitrate reduction to ammonium (NRA), denitrification (DNT) and assimilation (ASS) of added ^{15}N-NO_3^- in sediments from the PR1 and GR1 sites. (B) Amounts of added ^{15}N-NO_3^- that were recovered in the outlet water (Out) or unaccounted for (Missing) in the sediments from the PR1 and GR1 sites, amounts were normalised to nmol cm^{-3} h^{-1} to aid comparison. Means + SD, n = 3

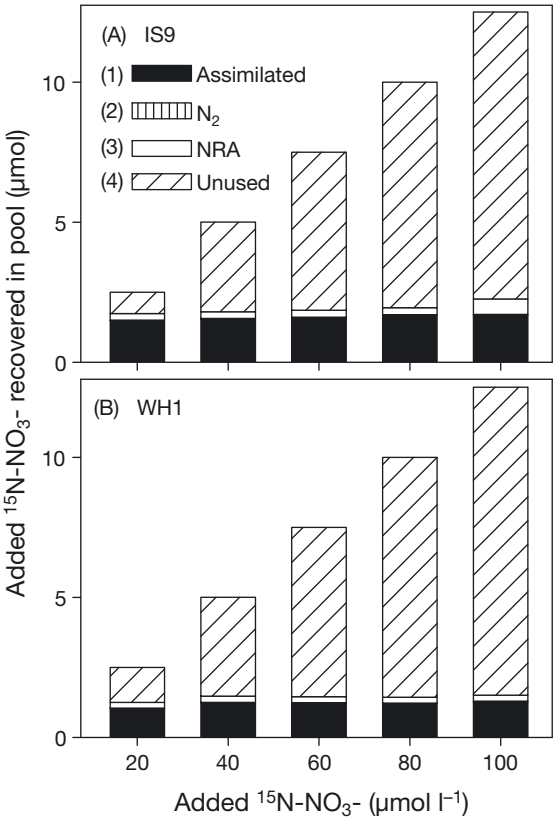

Fig. 6. Partitioning of added ^{15}N-NO_3^- into different pools during the core incubations for sites (A) IS9 and (B) WH1, pools include ^{15}N-NO_3^- that was (1) assimilated into microbial biomass, (2) recovered as ^{15}N-N_2, (3) recovered as ^{15}N-NH_4^+ (NRA), (4) or remained unused

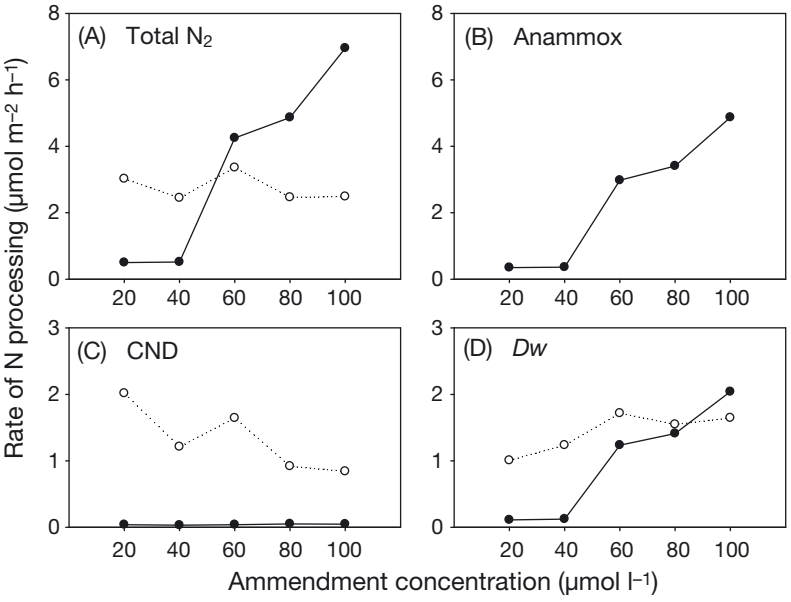

Fig. 7. Rates (µmol m^{-2} h^{-1}) of (A) *in situ* N_2 production, (B) anammox, (C) nitrification coupled to denitrification (CND), and (D) denitrification based on water column NO_3^- (*Dw*) at increasing amendment concentration during the core incubation of sediments from sites IS9 (○) and WH1 (●)

DISCUSSION

Anammox

This study provides the first evidence that anammox exists in tropical terrigenous and carbonate sand sediments. Anammox was detected at only 1 of the 4 sites, but at this site it was the dominant form of N_2 production (70% of total N_2). Our measured rates of anammox in the slurry incubation were similar to other reported literature values (Table 3) and also comparable to potential rates of denitrification at the offshore GBRL sites. In the core experiments, the highest calculated rate of anammox at the WH1 site was within the range reported for temperate sediments (Table 3). We suspect that anammox, where and when it occurs, is an important contributor to N_2 production within the GBRL.

Previous studies failed to detect anammox in tropical estuaries (Dong et al. 2011) or permeable carbonate reef sediments (Eyre et al. 2008), the explanation being that anammox bacteria in the estuaries were outcompeted for available NO_2^- in the low TOC sediments (Dong et al. 2011). The availability of NO_2^- has repeatedly been identified as the key determinant of anammox activity (Meyer et al. 2005, Trimmer et al. 2005). Anammox bacteria are not known to produce their own NO_2^- and are reliant on external sources, i.e. either NO_2^- diffusing from the overlying water, NO_3^- reduction or nitrification in the sediments (Meyer et al. 2005, Trimmer & Nicholls 2009). Of these, denitrification is thought to exert a crucial control on anammox by regulating the amount of NO_2^- available in sediment porewater (Thamdrup & Dalsgaard 2002). For NO_2^- to be available for anammox bacteria, it must first diffuse out from the cells of denitrifiers, a situation that can occur when labile (low C:N) TOC is limiting and denitrification does not proceed to completion (Trimmer & Nicholls 2009). When there is ample labile TOC available, denitrification of NO_3^- proceeds all the way to N_2 with less leakage of NO_2^- to the surrounding porewater. So, the reactivity of available TOC regulates the extent to which denitrification reaches completion. The C:N ratio often serves as a proxy for the reactivity of sediment organic material with low C:N material, generally representing fresh reactive organic matter (OM). This material favours denitrification over anammox, the latter tending to dominate under high C:N conditions (Trimmer & Nicholls 2009). Our data support this hypothesis, with anammox occurring within the sediments from site WH1, where the C:N ratio was >15. However, we did not detect anammox at the IS9 site despite the high C:N organic material found in the sediments. The answer may be related to the high S content of the IS9 sediments. Anammox is known to be inhibited by sulphide (Jensen et al. 2009) and at the inshore sites the sediment was distinctly black and sulphidic (pers. obs.) compared to the WH1 sediments.

More descriptive of the TOC reactivity is perhaps the ratio of HCl soluble TOC to total TOC (C_R:C). Strictly speaking, this represents chemical reactivity, although it may still serve as a useful proxy of the biological reactivity of sediment TOC. In the sediments from the WH1 site, the C_R:C was 0.11 relative to 0.22 in the shelf sediments. When C_R:C is high, denitrification is complete and less NO_2^- is released to porewaters for anammox. In the core experiment data for the WH1 sediments, we observed coupling between denitrification and anammox. Here, increasing amendment concentrations increased denitrification but also the rate of anammox. The variable quality of TOC in these sediments must have limited the extent to which denitrification was completed

Table 3. Rates of anammox (ANX), denitrification (DNT) and nitrate reduction to ammonium (NRA) (either DNRA or ANRA) in sediments from different regions and habitats. Rates are in µmol m^{-2} h^{-1}, except for *: nmol cm^{-3} h^{-1}. Method = procedure of measurement. Isotope = core or reactor incubations where ^{15}N tracer is used. Slurry = anoxic slurry incubations. N_2 flux and N_2:Ar = measurement of total N_2 production (i.e. includes both anammox and denitrification)

Region	Habitat	ANX	DNT	NRA	Method	Source
Tropical*	GBRL		0.35	0.90	Isotope	Present study
Tropical*	GBRL	0.55	1.43		Slurry	Present study
Tropical	GBRL	4.86	2.08		Isotope	Present study
Tropical	GBRL		4.1–154		N_2 flux	Alongi et al. (2007)
Tropical	GBRL		20–105		N_2 flux	Alongi et al. (2011)
Tropical	GBRL		18–240		N_2:Ar	Eyre et al. (2008)
Tropical	Estuary		2.6–103	1137	Isotope	Dong et al. (2011)
Subtropical*	Mangrove	0.5–8	90		Slurry	Meyer et al. (2005)
Temperate	Shelf seds	0.1–2.5	0.2–5.8		Isotope	Trimmer & Nicholls (2009)
Temperate	Estuary/fjord	6–49	6 –192		Isotope	Trimmer et al. (2006)
Temperate	Shelf	32–82	50–110		Isotope	Engstrom et al. (2009)
Temperate	Estuary		3–394	4–319	Isotope	Dong et al. (2009)
Temperate	Estuary		2.8–294	3.9–307	Isotope	Koop-Jakobsen & Giblin (2010)
Temperate	Estuary		0–90	0–80	Isotope	Gardner et al. (2006)
Temperate	Polluted fjord		166	291	Isotope	Christensen et al. (2000)
Arctic	Coastal	0.05–3.8	1.4–11		Isotope	Rysgaard et al. (2004)

allowing NO_2^- to leach to the sediments, where it could fuel anammox. Interestingly, there was no observable coupled nitrification-denitrification within the WH1 sediments. We suspect that partial nitrification in the surface sediments may have produced NO_2^- that diffused down and was used by anammox bacteria. The supply of NO_2^- to anammox bacteria from nitrifiers is uncommon because most aerobically produced NO_2^- supposedly diffuses to the overlying water or is oxidised to NO_3^- as it diffuses deeper into the sediments (Meyer et al. 2005). However, Trimmer & Nicholls (2009) found in Irish sea sediments that nitrification could, in fact, fuel high rates of anammox.

The use of chemically (HCl) soluble TOC as a measure of biological availability is not ideal and more common is the use of DIC flux. We did not measure DIC flux during this study, but our concept of increased TOC reactivity are well supported by DIC flux calculations made by Alongi et al. (2011) at the same sampling sites. In their study, DIC and NH_4^+ fluxes were greater in sediments from mid-shelf sites relative to the inshore sediments, indicating the presence of readily degradable organic material probably from marine algae. Our study provides a tropical carbonate sediment example of the relationship between anammox and a low sediment reactivity that has been well established for temperate sediments (Engstrom et al. 2005, Nicholls & Trimmer 2009).

Sediment reactivity is not, however, the only regulator of anammox activity. In temperate sediments, a clear positive relationship was found between the presence of Mn-oxide and anammox activity (Engstrom et al. 2005). The presence of Mn(IV) provides a potential oxidant for TOC, reducing the availability of electron donors for denitrification and subsequently favouring anammox activity. The inshore sediments from WH1 had the highest concentration of total Mn among all sediments. Alongi et al. (2011) showed that Mn was mostly present as Mn(IV) across the shelf; therefore, the high total Mn concentrations in the WH1 sediments likely reflect a high Mn-oxide content. Again, this raises the question of why anammox was not detected in the IS9 sediments where Mn content was also high and reactive TOC content low. At this stage, we suggest that sulphide may play a role in limiting the extent to which anammox occurs. The difference between the sulphide content at the 2 inshore sediments is most likely related to the hydrodynamics at each site. The WH1 site is subject to strong currents that continuously scour the sediment, preventing buildup of TOC and increasing oxygen penetration to the sediments. The IS9 site is located within a relatively protected bay where sediments can accumulate and become anoxic just below the sediment surface. The sediment at the IS9 site did have the highest TOC content of all the studied sediment, suggesting that there were more favourable conditions for sediment accumulation and potentially SO_4^{-2} reduction. Sulphate SO_4^{2-} reduction is relatively high at site IS9 (Alongi et al. 2011), suggesting that sulphide presence may be a limiting factor for anammox there.

Denitrification

While anammox rates were highest in the inshore WH1 sediments, the slurry assays showed that the highest rates of N_2 production were found in offshore sediments. This can be explained by the high amount of reactive TOC (higher C_L:C) in the offshore sediments relative to the inshore sediments. The offshore sediments may have contained more reactive TOC because of the rapid turnover of fresh water-column OM with the sediment, driven in part by high advective flow associated with strong currents. The isotope data of Alongi et al. (2011) show a tendency for the inshore sediment to contain more terrigenous material (lower $\delta^{13}C$) with higher C:N than the shelf sediments. Greater amounts of reactive TOC appear to have driven denitrification to completion in the offshore sediments.

The production of N_2 was particularly high in the GR1 sediments during the slurry assay. These sediments are located in a relatively sheltered region enhancing the settlement of material to the sediment surface; hence, the sediments at the GR1 site had a higher TOC content than the more exposed sediments from the PR1 site. The GR1 sediments were also finer than the PR1 sediments and could be classified as carbonate muds. These fine sediments may provide a high surface area for attached microbial growth. Hence, denitrification may have been higher in these sediments simply because more bacteria are present. Another possibility is that the sediments from the PR1 site may have contained an active benthic microalgal community that could have rapidly assimilated the added $^{15}N\text{-}NO_3^-$, making it less available for denitrification. We found that in the flow-through reactor experiments, the sediments from PR1 assimilated most of the added $^{15}N\text{-}NO_3^-$. Assuming this also occurred in the slurry assay, it would explain why the denitrification rate was less in the PR1 sediments relative to the GR1 sediments. The question arises as to why the rate of denitrification in the GR1 sediments during the reactor experiment was less than in the PR1 sediments. The answer is probably related to the short-circuiting of water flow in the GR1 sediments. This is seen in Fig. 5 and probably resulted in the reduced delivery of the added $^{15}N\text{-}NO_3^-$ to all parts of the sediment cores. Hence, less denitrification was observed in the GR1 sediments.

Based on the slurry assays the inshore sediments appeared relatively inactive with regard to denitrification, particularly the IS9 sediments. This appears to be linked to the lack of labile C in the IS9 sediments. This sediment had the highest amount of TOC of all the sites, but the organic material associated with this C had the highest C:N ratio and, therefore, can be considered as the least labile.

The core experiments yielded denitrification rates that were at the lower end of other measurements found in the literature, including estimates made for GBRL sediments (Table 3). For the IS9 core experiment, the increased addition of $^{15}N\text{-}NO_3^-$ did not result in increased denitrification rates, suggesting again that labile C was limiting the process. Assimilation was the main contributor to $^{15}N\text{-}NO_3^-$ uptake in both inshore sediments, but did not show a significant increase with increasing amendment addition. This suggests that the uptake of the added $^{15}N\text{-}NO_3^-$ by the surface microbial community had reached a maximum for the given incubation time. A longer incubation may have increased diffusion and, therefore, assimilation of added $^{15}N\text{-}NO_3^-$ in these sediments. A longer incubation period then would have increased the processing of the assimilated $^{15}N\text{-}NO_3^-$, perhaps resulting in greater release of $^{15}N\text{-}N_2$ to the porewater. However, the rate of denitrification would probably not have increased markedly given the C limitation previously discussed. In the WH1 sediments, there was an increase in the rate of denitrification with increasing amendment concentration, suggesting that there was enough TOC to fuel the process. As discussed earlier, anammox rates also increased most likely as a result of TOC limitation on complete denitrification.

Nitrate reduction to ammonium (NRA)

The addition of $^{15}N\text{-}NO_3^-$ resulted in the production of $^{15}N\text{-}NH_4^+$ in all studied sediments during the flow-through reactor (FTR) and core experiments. For the offshore sites, the PR1 sediments had a higher rate of $^{15}N\text{-}NH_4^+$ production than the GR1 sediments. The PR1 site was characterised by a low percentage of TOC, but a relatively high ratio of HCl digestible C to TOC. The C:N ratio of the sediment organic material was also lowest of all the sites and the total amount of S was also highest. Other studies have shown there to be high fluxes of DIC and NH_4^+ from PR1 sediments suggesting that organic matter is both present and available to the microbial community (Alongi et al. 2011). Production of labelled $^{15}N\text{-}NH_4^+$ following a $^{15}N\text{-}NO_3^-$ amendment usually suggests the occurrence of DNRA. However, we have been very careful to call this nitrate reduction to ammo-

nium, NRA, to ensure we do not ignore assimilatory nitrate reduction to ammonia. The current paradigm suggests that DNRA is favoured over denitrification under low NO_3^- conditions. However, in the PR1 sediments there was ample NO_3^- available to the microbial community and also the presence of readily degradable organic material. This raises the possibility that assimilation of $^{15}N\text{-}NO_3^-$ and subsequent release as $^{15}N\text{-}NH_4^+$ by MPB, i.e. assimilatory nitrate reduction to ammonia (ANRA). MPB can continue to assimilate NO_3^- in the dark, potentially releasing it to the porewater as NH_4^+ (Rysgaard et al. 1993, Cook et al. 2004). In the PR1 sediments, the assimilation of added $^{15}N\text{-}NO_3^-$ into microbial biomass, as well as the adsorption of $^{15}N\text{-}NH_4^+$ onto sediment particles, were higher than in the GR1 sediments, which is consistent with ANRA. However, ANRA is assimilatory; hence, there would have to be some turnover of MPB in order for ^{15}N locked up in proteins to be released as $^{15}N\text{-}NH_4^+$. It is unclear whether there was enough time for this to occur during the FTR experiments.

An alternative explanation for the occurrence of NRA under high NO_3^- conditions in the PR1 sediments may be DNRA in the presence of free sulphide. In the NO_3^- limited offshore waters, SO_4^{2-} reduction can be an important pathway for reactive TOC oxidation and Alongi et al. (2011) found that sediments from PR1 did indeed have high rates of SO_4^{2-} reduction as a result of the presence of readily degradable organic C. Sulphate reduction produces sulphide, which is a requirement for chemolithotrophic DNRA (Burgin & Hamilton 2007). Hence, in the PR1 sediments DNRA may have been largely chemolithotrophic rather than fermentative. While it is unclear whether the production of $^{15}N\text{-}NH_4^+$ in the PR1 sediments was a result of ANRA or DNRA, the end result was retention of N within the system rather than a loss via denitrification. This is particularly true for the PR1 sediments, where the rate of nitrate reduction to ammonium was over twice the rate of denitrification. The PR1 sediments were more porous than the GR1 sediments and resulted in greater mixing of amendment with the porewater during the flow through reactor experiments. Hence, the overall processing of inlet $^{15}N\text{-}NO_3^-$ was markedly less in the GR1 relative to PR1 sediments. Without molecular techniques, it is difficult to distinguish between DNRA and ANRA, particularly in euphotic systems like the GBR shelf. Hence, we prefer to call the production of $^{15}N\text{-}NH_4^+$ following $^{15}N\text{-}NO_3^-$ addition NRA.

The inshore sediments also showed the production of $^{15}N\text{-}NH_4^+$ following the addition of $^{15}N\text{-}NO_3^-$. Consistently high turbidity at the IS9 site limits MPB activity relative to the offshore sites and, therefore, most of the $^{15}N\text{-}NH_4^+$ production here can probably be attributed to DNRA. With this in mind, DNRA activity was higher in the IS9 sediments than the WH1 sediments, it is likely that DNRA activity within the IS9 sediments was largely chemoautotrophic, rather than fermentative, given the sulphidic nature of the inshore sediments (Alongi et al. 2011) and lack of labile C. Importantly, DNRA activity was much more significant than total N_2 production in the IS9 sediments, as can be seen in the core experiments. DNRA can outcompete denitrification at low NO_3^- concentrations because DNRA bacteria generally have a greater affinity (lower half saturation constant) for NO_3^- and derive more energy from NO_3^- reduction relative to denitrifiers (Dong et al. 2011). However, in the core experiments NO_3^- was not limiting. Consequently, increasing concentrations of NO_3^- should have resulted in increasing rates of denitrification relative to DNRA. We suspect that the presence of free sulphide favoured DNRA over denitrification. It should be noted that denitrification can also occur with sulphide as the electron donor; however, if it was occurring it did not do so at a rate comparable to the rate of DNRA.

Within the WH1 sediments, there was also clear reduction of production of $^{15}N\text{-}NH_4^+$ following the addition of $^{15}N\text{-}NO_3^-$. Again, this could be attributed to DNRA; however, it is also possible that ANRA was also occurring. The waters of the Whitsunday Islands are generally clearer than the turbid inshore waters at the IS9 site, due largely to strong tidal currents that flush clear oceanic water through the island passages. While the presence of ANRA is speculative, it would make sense in light of the presence of anammox in the WH1 sediments. Anammox is inhibited by sulphide and we argue that the presence of sulphide in the IS9 sediments promoted DNRA. In the WH1 sediments, denitrification was prevented from proceeding to completion because of a lack of labile C; hence, if DNRA was occurring it would have been through sulphide oxidation, not C fermentation because fermentative DNRA requires a labile C source (Kelso et al. 1997). However, if sulphide driven DNRA was occurring then anammox should be absent. Hence, we suspect that ANRA was possibly responsible for the production of $^{15}N\text{-}NH_4^+$ following the addition of $^{15}N\text{-}NO_3^-$. It should be noted that our separation of DNRA from ANRA is largely speculative; hence, we prefer to use the term nitrate assimilation to ammonium (NRA).

Summary

The complex N processing that occurs within the GBRL sediments can be summarised as follows. Inshore sediments receive terrigenous material, which can build up under quiescent conditions, reducing oxygen penetration, enhancing sulphide production and promoting the growth of chemolithotrophic DNRA (assuming NO_3^- is available and labile TOC is limiting). As sediment disturbance increases, the amount of TOC accumulation declines and sulphide production diminishes. The amount of labile TOC increases but is still relatively low, preventing denitrification from reaching completion. This results in the release of NO_2^- and the occurrence of anammox. The presence of Mn-oxides may also help to reduce the reactive TOC content of the sediments, further promoting anammox over denitrification. Nitrate reduction to ammonium occurs through NRA (i.e. DNRA and/or ANRA) and nitrification is linked to the supply of NO_2^- for anammox. The offshore sites are characterised by high proportions of reactive TOC of marine origin, this fuels denitrification at the expense of anammox. In the offshore sediments, there is appreciable NRA occurring either through assimilatory or dissimilatory pathways. This retains N within the sediments.

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