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Does denitrification occur within porous carbonate sand grains?

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Abstract. Carbonate sands form a major sediment type in coral reef environments and play a major role in organic matter recycling. It has previously been postulated that porosity of carbonate sand grains may lead to the formation of anoxic microniches that promote denitrification within these sediments. Under this conceptual model, we expect diffusion to exert an influence on process rates, which can be tested by determining their dependence to reactant concentrations. Here, we use two experiments in flow-through reactors to test this hypothesis in carbonate sediments collected from Heron Island, Australia. Denitrification was only observed to commence at substantial rates below 10 μM O_2, suggesting anoxic microniches do not exist. Furthermore, denitrification rates were constant above 18 μM nitrate, suggesting no diffusion limitation, as would be expected if significant rates of reaction were occurring within porous grains. Potential rates of denitrification rates relative to respiration were broadly consistent with those previously reported in silicate sands.

1. Introduction

Nitrogen is typically regarded as one of the key nutrients limiting production in the coastal environment (Howarth and Marino, 2006). Coral reefs are examples of highly oligotrophic environments that are coming under increasing threat from increased nutrient loads (De'ath et al., 2012). Denitrification is a key remedial process in the nitrogen cycle, leading to the conversion of bioavailable nitrate into relatively non bioavailable nitrogen gas. One of the dominant sediment types in reef environments are carbonate sands formed from the breakdown of carbonate produced by calcifying organisms (Eyre et al., 2014). Quantifying denitrification in sandy sediments is complicated by the fact that these sediments are permeable, allowing water movement through the sediment and affecting rates of denitrification (Cook et al., 2006). Reproducing these conditions whilst measuring denitrification is difficult and thus, models combined with a mechanistic understanding of the primary controls on denitrification offer a promising approach to quantifying denitrification in these environments (Kessler et al., 2012;Evrard et al., 2013).

For denitrification to take place anoxic conditions, a supply of nitrate and organic matter, are required. Advective flushing of permeable sediments leads to deeper oxygen penetration into these sediments, which will inhibit denitrification (Evrard et al., 2013). Nitrate may be supplied to the denitrification zone from either the water column or nitrification within the sediment. Recent work has shown that nitrification within the sediment may be a significant source of nitrate to fuel denitrificication (Marchant et al., 2016;Rao et al., 2007), although modeling studies suggest that even in the presence of nitrification, flow fields in permeable sediments may lead to little coupling between nitrification and denitrification (Kessler et al., 2013). In systems with high-bottom water nitrate concentrations, high rates of denitrification have been reported (Sokoll et al., 2016;Gao et al., 2012). Previous studies have also reported high rates of denitrification in carbonate sands (Eyre et al., 2008;Eyre et al., 2013b), which is surprising given the low nitrate concentrations in the overlying water and their highly oligotrophic nature. One possible explanation for high rates of denitrification is the coupling of nitrification and denitrification within microniches associated with sand grains (Rao et al., 2007;Santos et al., 2012;Jahnke, 1985). In this hypothesis, mineralization of organic matter within or on sand grains consumes oxygen at a rate greater than it can diffuse into the grain, causing anoxia within the grain. This allows the bulk sediment to be simultaneously oxic, which promotes nitrification, while anoxic sites within grains allow denitrification to take place closely coupled to nitrification. A recent study has shown that incorporating intra-granular porosity into model simulations of column experiments can lead to better agreement with observations (Kessler et al., 2014) in carbonate sediments, but direct experimental evidence for this phenomenon is lacking.

Direct measurement of oxygen concentrations and denitrification rates within sediment grains is not possible with current technologies. Nevertheless, there are means by which the hypothesis that denitrification takes place within sediment grains can be tested. Firstly, if denitrification is taking place within anoxic intra-granular niches, then it should be observed under low oxygen conditions within the pore channels (O_2 <50 uM but > 0). Second, if denitrification is taking place within the grain, then nitrate must diffuse into the grain, which means that diffusion may be a controlling factor on the rate observed. If diffusion is the controlling process, then the rates of transport of nitrate into the grain can be described by Ficks first law, which can be modified to describe the flux into the grain as follows

$$J = a \times D(C_{bulk}-C_{grain})/L \qquad (1)$$

Where J = flux, a = specific grain area, D = diffusion coefficient, C_{bulk} and C_{grain} = the concentration of nitrate in the bulk solution and within the grain, respectively, and L = length scale. Assuming the concentration in the grain is 0, the flux will depend linearly on the concentration of nitrate in the bulk solution.

The working hypothesis of this study was that intra-granular porosity leads to the formation of anoxic micro-niches, that enhance denitrification rates in permeable carbonate sediments. This hypothesis was tested by applying sub-oxic and diffusion limitation tests to carbonate sediments of different grain size, recovered from Heron Island Australia, where intra-granular porosity has been suggested to enhance denitrification.

2. Methods

Sediments were collected from three sites at Heron Island, 23° 27'S, 151° 55'E in the Southern Great Barrier Reef, Australia. Site 1 is located adjacent to the Heron Island research station (23° 26' 37" S, 151° 54' 46" E, water depth 0.5 m at low tide), site 2 is located at Shark Bay (23° 26' 37" S, 151° 55' 09" E water depth 0.5 m low tide), and Site 3 is located in the lagoon approximately 4 km east of the island (23° 27' 04" S, 151° 57' 28" E, water depth 2 m at low tide). Site 3 was the most uniformly coarse and permeable site, and site 2 had the smallest median grain size and lowest permeability (Table 1). Sediments were packed into three replicate flow-through reactors (FTRs) for each experiment, as described by Evrard et al. (2013), within 2 hours of collection. Fresh unfiltered seawater was collected from in front of the research station and the columns were percolated at a flow rate of ~200 mL h^{-1}. The volume of the FTRs was 66 mL (~33 mL porosity corrected), giving a retention time of ~10 mins (corrected for sediment porosity). The flow velocity was 24 cm h^{-1} and was chosen as it was estimated to give a small but easily detectable change in $^{15}N-N_2$. FTRs for all experiments were collected with fresh unfiltered seawater collected from in front of the Heron Island Research Station. This is the upper end of those expected around ripples of 0.14-26 cm h^{-1} (Precht et al., 2004), and higher than those used by Santos et al. (2012). We deliberately did this to ensure any boundary layers at the grain surface were at a minimum and any effect observed here could be ascribed to intra-granular porosity. Diffusive chemical gradients were manipulated by changing oxygen and nitrate concentrations within the flow-through reactors.

Two experiments were undertaken as follows. First, the effect of NO_3^- concentration on denitrification was measured as described by Evrard et al. (2013). Repacked FTRs were percolated with anoxic seawater (30 mins purging with Ar), which was sequentially amended with 18, 37, 75, 150 and 300 μM $^{15}NO_3^-$. The oxygen concentration at the column inlets and outlets were monitored in real time using Firesting optical dissolved oxygen flow-through cells (Pyroscience) which had a detection limit of ~3 μM O_2 and a precision of ~1%. After ~3 retention times (~30 mins) at each nitrate concentration, a sample of the column effluent was collected directly into glass syringes, and transferred into an exetainer and preserved with 250 μL 50% w/v $ZnCl_2$. Second, the effect of oxygen on denitrification was measured as described by Evrard et al. (2013). Columns were percolated with unfiltered seawater amended with 150 μM $^{15}NO_3^-$ (99% Cambridge scientific), and the oxygen concentration was reduced incrementally in the reservoir by sporadic purging with Ar. Samples of column effluent were collected and preserved as described above.

Samples for $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ analysis had a 4 ml He headspace inserted into the exetainer, and were shaken for 5 minutes before the headspace gas was analysed on a Sercon 20-22 isotope ratio mass spectrometer, coupled to an autosampler and GC column to separate O_2 and N_2. Air was used as the calibration standard, and tests showed no false mass 30 signal compared to pure N_2 injections. The precision of the analysis of the ratios $^{29}N_2$ /$^{28}N_2$ and $^{30}N_2$ /$^{28}N_2$ was 0.2 and 5 %, respectively. For the analysis of 2 μmol N, this equates to an excess ^{15}N of 2.5×10^{-5} μmol for $^{29}N_2$ and 7.85×10^{-5} μmol for $^{30}N_2$. Assuming all of the N_2 production was in the form of $^{30}N_2$, this results in an equivalent detectable production rate of 0.014 nmol mL^{-1} h^{-1} in the column experiments. Sediment permeability was measured using the constant head method (Reynolds, 2008) and sediment porosity was measured by drying a known volume of sediment saturated with fresh water. Images of the grains were taken using a Motic dissecting microscope with a 5MP Moticam. The porosity of the sand grains was measured using mercury porosimetry at Particle & Surface Sciences Pty Ltd. Sediment grain size was measured using test sieves with mesh sizes of 2, 1.18, 0.5 and 0.125 mm.

3. Results

Sites 1 and 3 had the coarsest median grain size of 0.9 mm, whereas site 2 had a median grain size of 0.7 mm. All sites had 20-30 % sediment with grain sizes in the range of 1.18 – 3mm, the sediment permeability was also similar at all sites ranging from 24-30 × 10^{-12} m^2 and the bulk porosity was similar at all sites ranging from 0.48 – 0.56 at the 3 sites (Table 1). Images of the sand grains showed them to be porous (Figure 1), and this was confirmed by mercury porosimetry, which revealed the sand grains had a porosity of ~0.32 at sites 1 and 3, site 2 not measured (Table 1). Rates of denitrification were constant above NO_3^- concentrations of 18 μM at all three study sites, and were highest at site 3 which had the highest sediment oxygen consumption rates and lowest at site 2 which had the lowest (Figure 2). Plots of oxygen concentrations showed that concentrations of oxygen at the column inlets dropped in a stepwise manner when they were purged with Ar, and this that this was reflected at the column outlets with a delay of ~10 minutes consistent with the theoretical column retention time (Figure 3). Rates of denitrification were negligible at oxygen concentrations > 10 μM at the column outlets (Figure 4).

4. Discussion

4.1 Methodological considerations

Before discussing the results in detail, we briefly consider the methods used here and potential shortcomings. First, we only used one, relatively fast flow rate in these experiments. We chose this flow rate as we estimated this was the maximum flow rate we could use that would minimize boundary layers within the column, while giving detectable production of ^{15}N-N_2. These flow rates are in the upper range of those previously reported in sediments where porewater flow is driven by flow-topography interactions (Precht and Huettel, 2004), as we expected to be the case here. Second, we assumed that ^{15}N-N_2 production had reached a steady state after the manipulation of oxygen and nitrate concentrations within the column. Conceptually, nitrate from the bulk porewater will diffuse into the sediment grain, where denitrification will take place, and the produced ^{15}N-N_2 will diffuse out again before being washed out of the column. If we conservatively use a grain size of 2 mm (twice the median grain size), this means a maximum diffusion distance of ~1 mm to a putative denitrification zone within a sediment grain. The diffusion timescale for nitrate molecule can be calculated using equation 2

$$t = L^2/2D_s \quad (2)$$

where t= time, L= distances and D_s is the diffusion coefficient (Schulz and Zabel, 2005). For nitrate, with a diffusion coefficient of 1.7×10^{-5} cm^2 s^{-1} at 25°C and a salinity of 35 corrected for a grain porosity of 0.3 according to (Iversen and

Jørgensen, 1993) gives a timescale of ~10 mins. For nitrate to diffuse in and N_2 to diffuse out, we would therefore expect this to take a maximum of ~20 mins which is less than the time we waited before sampling. If there was a non steady-state artifact in our data, we would expect to see an upward drift in our denitrification rates as the concentrations increased (Figure 2) and a much less abrupt initiation of denitrification once anoxia commenced in the FTRs (Figure 3). We are therefore confident that a non-steady state artifact does not bias our results and conclusions.

Third, we used oxygen consumption as a proxy for respiration in these sediments. It has previously been shown that ~50% of oxygen consumption in sediments can be driven by the oxidation of reduced solutes (Cook et al., 2007). In this case, however, we believe that this was unlikely because, we waited >14 hours before oxygen consumption measurements commenced, after which we would expect all the reduced solutes to have either been washed out, or oxidized. We therefore believe that the vast majority of O_2 consumption was respiration as opposed to reduced solute oxidation. Finally, break-through curves are often used to quantify column retention time and dispersivity. In this instance, we did not undertake break-through curve measurements, as we have previously shown this column set up to give a very distinct plug flow (Evrard et al., 2013). The offset of 10 minutes between the purging of oxygen in the reservoir and the response at the column outlet qualitatively confirms that the theoretical retention time of the columns for these experiments.

4.2 No evidence for denitrification within carbonate sand grains

The results here do not support the hypothesis that intra-granular porosity leads to the formation of anoxic micro-niches, that enhance denitrification rates in permeable sediments. Firstly, our results clearly showed that denitrification rates were constant within experimental error at nitrate concentrations above 18 µM (Figure 2). Previous studies of enzyme kinetics in both cultures and column experiments have shown Km values for denitrification in the range of 2 – 18 µM nitrate (Murray et al., 1989;Evrard et al., 2013). We would therefore expect denitrification rates to remain constant above ~20-30 µM nitrate if enzyme kinetics were controlling denitrification, consistent with our observations. If diffusion was controlling denitrification, as is the case with the intra-granular porosity hypothesis, then we would expect to see some concentration dependence of the measured rates above ~20-30 µM nitrate, which we did not observe.

Second, denitrification rates were negligible above oxygen concentrations of ~10 µM in the column effluent (Figure 4). If appreciable anoxic microniches were forming within the grains, we would expect to see denitrification occurring under bulk oxic conditions. Theoretically, the critical radius (r) of a particle at which anoxia will occur in the centre can be calculated from equation 3 (Jørgensen, 1977):

$$r = (6D_sC/J)^{1/2} \qquad (3)$$

where D_s is the diffusion coefficient (corrected for tortuosity), C is the oxygen concentration at the particle surface and J is the volumetric oxygen consumption rate. At sediment respiration rates of 270 – 460 µmol L^{-1} h^{-1} observed in this study, we would expect to see the centre of particles >1 mm in diameter become anoxic only at oxygen concentrations <5 µM and particles at 3 mm diameter become anoxic at oxygen concentrations of 30 – 50 µM. Given that ~20 - 30% of the particles fell in this size range, we would expect denitrification to have commenced at O_2 concentrations < 50 µM at site 3 and < 30 µM at site 2 if significant rates of denitrification were taking place within the particles. This finding is in agreement with a previous study in silicate sediments that showed a very similar relationship between oxygen concentration and denitrification (Evrard et al., 2013). It has previously been hypothesised that intra-granular porosity plays a role in nitrogen cycling, which arose from observations that denitrification rates peaked at intermediate flow rates in column experiments (Santos et al., 2012). At low flow rates, oxygen is rapidly consumed within the FTRs, leading to limited coupled nitrification-denitrification. At intermediate flow rates, anoxic environments develop within the grains, thus allowing denitrification to take place. At high flow rates, oxygen concentrations are so high throughout the FTR that anoxic zones no longer exist within the grains, thus limiting denitrification. A previous study has, however, also observed this same behavior in silicate sands (Bourke et al., 2014) which do not have intra-granular porosity and where simultaneous planar optode experiments showed no anoxic microsites, suggesting another mechanism which we explain as follows. At low flow rates, denitrification is limited by the rate of nitrate transport into the anoxic zone of the column. As the flow rate increases, so does denitrification, but at the same time the increased flow rate reduces the volume of anoxic sediment, which eventually results in denitrification being reduced. This broad pattern was also reproduced in model simulations of columns containing non-porous sand grains. Including intra-granular porosity, however, led to better agreement between the measured and model runs, leading Kessler et al. (2014) to conclude that intra-granular porosity was a plausible hypothesis. We note, however, that the study was able to produce a similar peak in denitrification at intermediate flow rates in a simulation with no intra-granular porosity, albeit with a smaller magnitude (factor of ~3 lower than observed in the experiments).

4.3 Comparison to denitrification rates with previous studies

The denitrification rates measured in the present study spanned the range of flow through reactor rates rates (~<1 – 32 µmol L^{-1} h^{-1}) previously observed in silicate sands (Rao et al., 2007;Evrard et al., 2013;Marchant et al., 2014;Kessler et al., 2012). The availability, and composition, of organic matter is expected to be a key factor controlling potential denitrification rates (Seitzinger, 1988;Eyre et al., 2013a), and the importance of this in permeable sediments has also been recently underscored by Marchant et al. (2016) who observed a strong relationship between potential denitrification rate and sediment oxygen consumption. If the results of the previous studies are plotted versus sediment oxygen consumption rate, a significant relationship is observed with an r^2 of 0.92 (Figure 5). Sites 2 and 3 in the present study seemed to deviate significantly from this relationship, as they were the only data points to lie outside the 99% prediction interval, while site 1 sat close to the line of best fit. Omitting the study of Kessler et al. (2012), still led to sites 2 and 3 being outside the prediction intervals with site 2 being below, and site 3 above the relationship observed for silicate sands. This suggests that the slope of the relationship between denitrification and oxygen consumption rate in this study differs from previous studies. It has recently been shown that much of the metabolism in permeable sediments is dominated by algae, rather than bacteria (Bourke et al., 2016). Given that bacteria undertake denitrification it is likely that there was relatively more algal respiration occurring at site 2. We speculate that site 2 which was the most sheltered and had the finest sediment was dominated by microphytobenthos, while site 3, which had coarser sediment and higher turbulence in the outer lagoon had a larger advective supply of phyto-detritus from the water column owing to higher flushing rates (Huettel and Rusch, 2000).

4.4 implications for nitrogen cycling

In the absence of any intra-granular porosity effect denitrification rates of only ~5 µmol m^{-2} h^{-1} were modeled (Kessler et al., 2014), which is 5-10 times lower than denitrification rates previously measured in coral reef sands in static chambers using the direct N_2 flux method (Alongi et al., 2011;Alongi et al., 2008) and advective chambers using the N_2/Ar method (Eyre et al., 2013b). An uncertainty in the advective chamber approach is inducing a realistic advection rate in chambers, and also 'wash out' of nitrogen accumulated within porewaters which can enhance measured nitrogen fluxes (Cook et al., 2006) possibly explaining the higher chamber rates. However, this doesn't explain the high rates measured in the non-advective chambers. In addition, the high in situ measured denitrification rates across all carbonate sand studies are highly correlated with sediment oxygen consumption, which can easily support the high denitrification rates in terms of a supply of carbon and nitrogen from the decomposition of organic matter (Eyre et al., 2013b). Alternatively, it is also possible that factors not included in the model such as bio-irrigation could lead the model to under-estimate denitrification rates, however, these factors are unlikely to explain such a large discrepancy. These results suggest we have an incomplete conceptual understanding of denitrification in permeable carbonate sediments and that further work, such as with flumes and isotope tracers are required to provide mechanistic insight into the rates and controls of denitrification.

5. Author contributions

All authors contributed to the design, undertaking the experiments, data interpretation and manuscript preparation.

6. Acknowledgements

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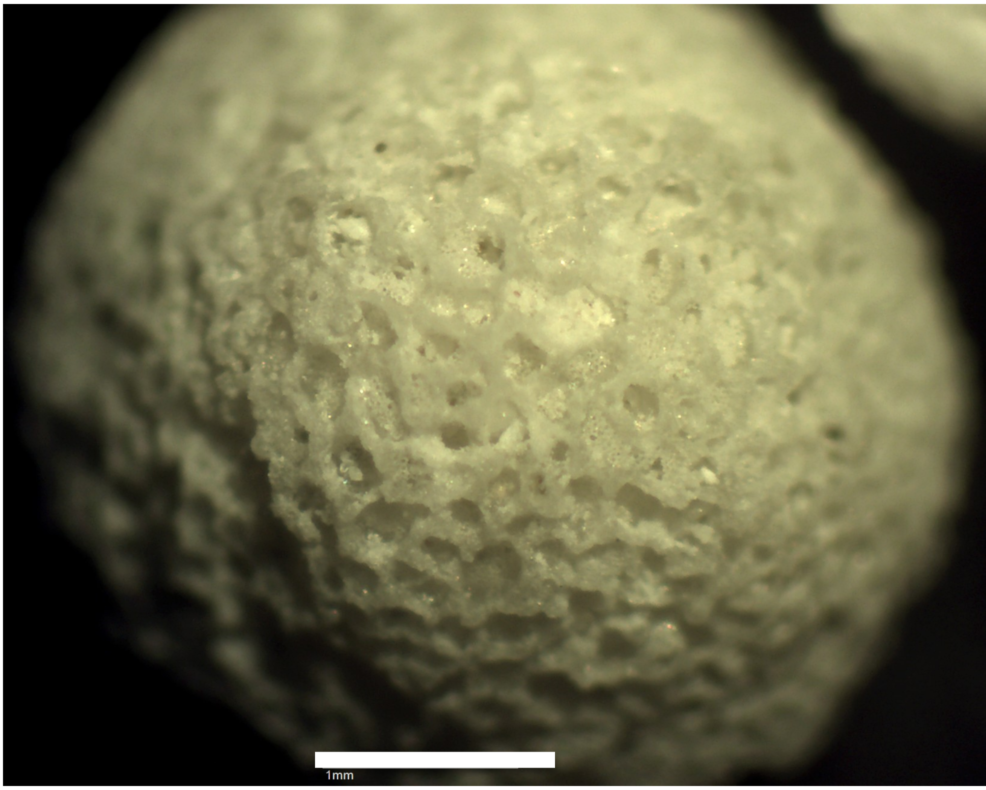
Table 1 shows sediment grain size, permeability, grain porosity and sediment oxygen consumption rate (with S.D.) at the 3 study sites.

Site	Coordinates	Median grain size mm	% 1.18 - 3 mm	% 0.5 – 1.18 mm	% 0.125 – 0.5 mm	% <0.124 mm	Permeability × 10^{-12} m^2	Bulk Sediment porosity	Grain Porosity Vol/vol	O_2 Consumption µmol L^{-1} h^{-1}	Vmax denitrification rate µmol L^{-1} h^{-1}
1	23° 26’ 37”S 151° 54’ 46” E	0.9	32	54	13.4	0.6	27	0.55	0.31	350 (50)	11.2 (0.3)
2	23° 26’ 37” S 151° 55’ 09”E	0.7	20	44	29	8	24	0.48	N/A	270 (80)*	2 (0.5)
3	23° 27’ 04” S, 151° 57’ 28” E	0.9	32	52	16	1	30	0.56	0.32	466 (9)	28 (2)

N/A = not analysed, *n= 2, range shown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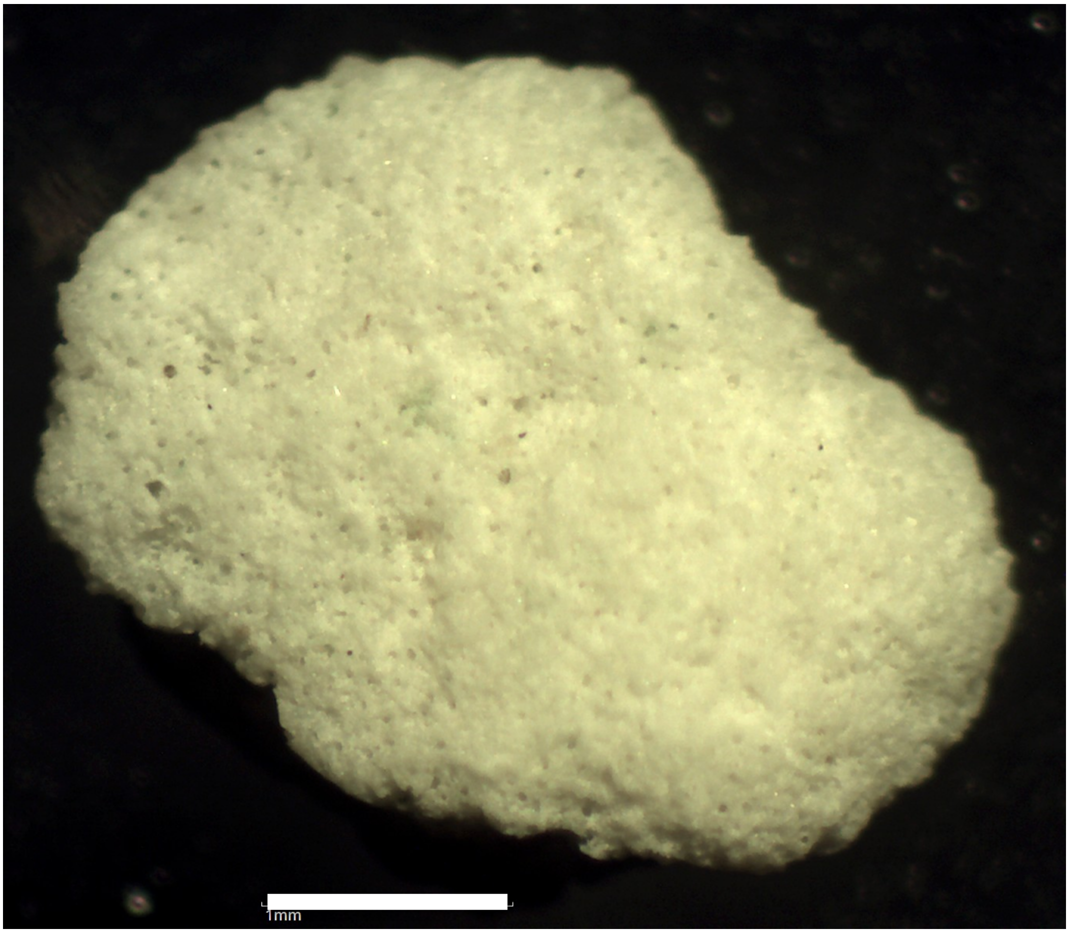

Figure 1. Images of carbonate grains from Site 3 at Heron Island. Scale bar is 1mm.

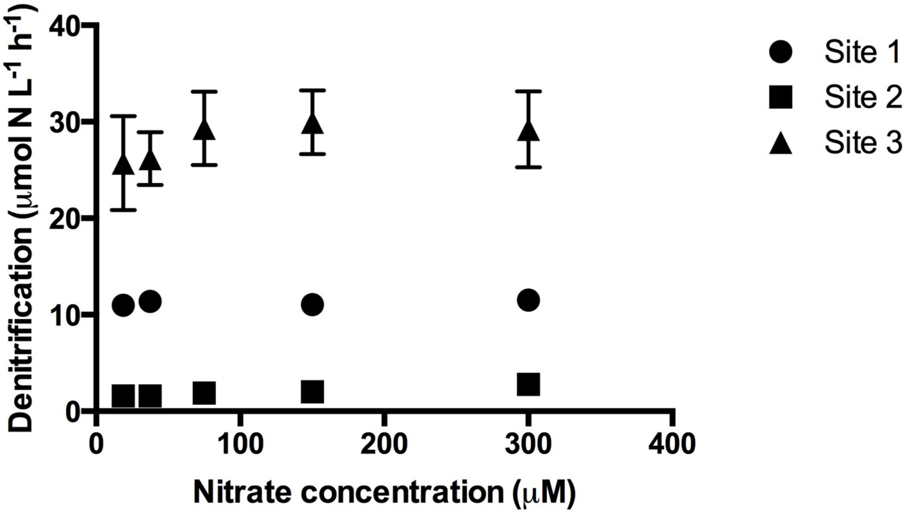

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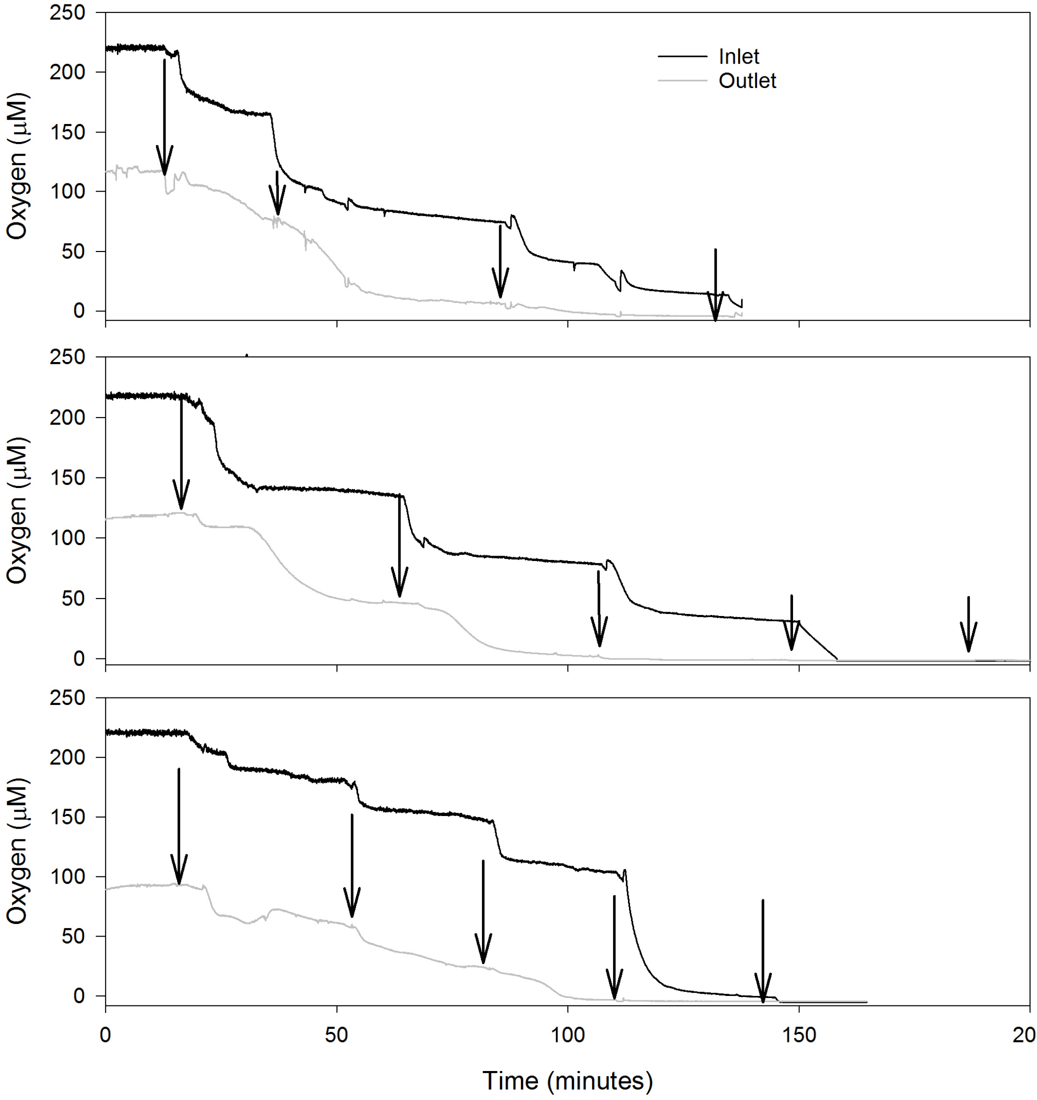
Figure 2. Denitrification rates as a function of nitrate concentration at the inlet of the columns at the 3 sites studied.



265 **Figure 3. Examples time series of oxygen concentrations at the FTR inlet and outlet during experiments 1 (top panel) 2 (middle panel) and 3 (bottom panel). Arrows mark sampling points for ^{15}N-N_2, note $^{15}NO_3^-$ tracer was added > 30minutes before the first sampling point (ie before the O_2 trace commences).**

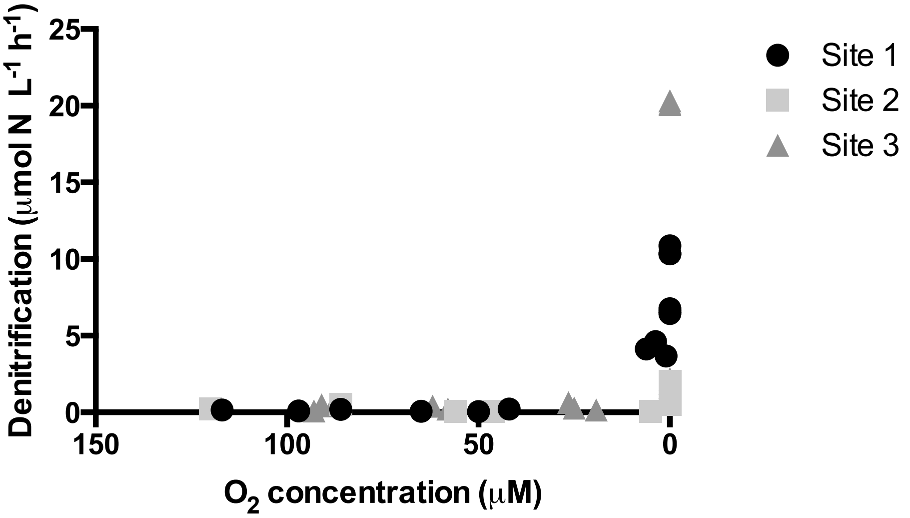


Figure 4. Denitrification rate as a function of oxygen concentration at the outlet of the columns at the 3 study sites.

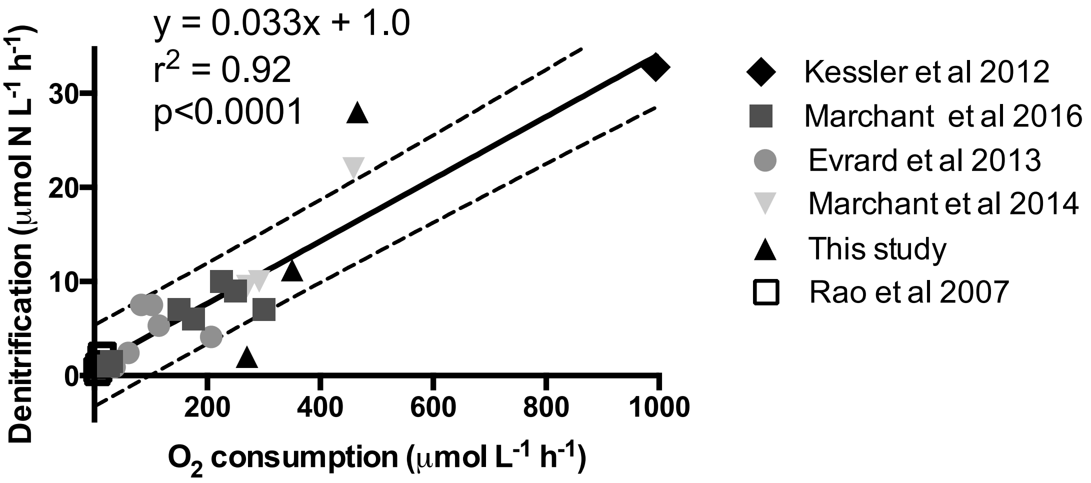


Figure 5. Denitrification rate as a function of oxygen consumption in this, and previously published studies. The solid line is the line of best fit for previous studies in silicate sands, the dashed lined are the 99% prediction interval.

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