Thank you for your revised manuscript. I would like to request further revision in order to avoid an emphasis on conclusions which do not seem fully supported.

Despite your response to reviewer comments, I am not comfortable with the emphasis of this study being on showing that C addition enhances bioremediation. I think that viewing the study through that lens is leading you to make statements that overstretch and are not fully supported by the results. The results presented are that C addition increases both N2 fixation and NH4 flux from the sediment, and it just seems rather contrived to say that these indicate enhanced bioremediation (even if the study were run for longer, and if holothurian biomass were measured, and increased – none of which were actually the case here). I take the argument that the concept of retaining N within the sediment is an alternative form of remediation, but surely NH4+ production does not achieve this (the ammonium would presumably be free to escape and cause eutrophication), and neither does adding N to the system through nitrogen fixation. I therefore think that the conclusion that carbon addition 'may result in greater retention of nitrogen within the system…' is a bit of a stretch, and masks the fact that amendment with C actually leads to N being added to the system.

Nonetheless, the introduction states that C addition is being practised in these new types of system, and that the resultant N cycling is not well understood. I think the manuscript would be much stronger if simply driven by an aim to understand the N cycling changes in response to the C addition practice. Then the full implications of the results can be properly acknowledged, and its impact on bioremediation can be discussed in a fully critical manner, i.e. there could be increased N retention, but there is probably also N addition, and also increased ammonium fluxes, and it is not clear that either of those things is entirely desirable.

Dear Dr Woulds,

Thank you for your insightful comments on our manuscript. We have sought to address these as best we could without compromising the overarching aims of our work. To present a more cautious interpretation of the data we have modified to abstract in two places: Line 43 now reads "These findings indicate that carbon addition may potentially result in greater retention of nitrogen within the system, however longer-term trials are necessary to determine whether this nitrogen retention is translated into improved sea cucumber biomass yields."

Further, we have added an additional two sentences to the end of the abstract that we hope reflect your concerns. "Whether this truly constitutes a remediation process is open for debate as there remains the risk that any increased nitrogen retention may be temporary, with

any subsequent release potentially raising the eutrophication risk. Longer and larger-scale trials are required before this approach may be validated with the complexities of the in-system nitrogen cycle being fully understood."

We hope that by expressing these points up front in the abstract that we will subtly shift the weight of interpretation of the paper than will allow the reader to focus more on the interesting nitrogen pathways that seem to be operating in the system. As you point out, the nitrogen cycle in these systems is not yet understood (hence the useful contribution that this study makes).

We have also modified our Conclusion text to provide a more cautious interpretation:

"The increase in NH_4^+ efflux combined with net uptake of N_2 into the sediment in response to carbon addition indicates that under nutrient loading rates consistent with hypereutrophic estuaries (400 mmol C m^{-2} day^{-1} and 240 N m^{-2} day^{-1}; Eyre and Ferguson, 2009), pathways that retained nitrogen could dominate over pathways of permanent removal."

Line 572: Please clarify the following statement 'For example, Castine et al. (2012) found that denitrification and anammox only removed 2.5 % of total nitrogen inputs to settlement ponds in intensive shrimp and barramundi farms'. How was the rest of the N removed, if not by a permanent removal mechanism? What 'counts' as 'removed' (stored in the sediment?).

We have clarified these points by making the following change to the text:

"For example, Castine et al. (2012) found that denitrification and anammox only removed 2.5 % of total nitrogen inputs (by N_2 production) to settlement ponds in intensive shrimp and barramundi farms. In this case denitrification was not carbon limited; rather the authors argue that inhibition of microbial metabolism by increased H_2S and NH^{4+} production limited the performance of the system."

I would be glad to receive another revised manuscript with these comments addressed.

Yours sincerely,

Clare Woulds

Carbon amendment stimulates benthic nitrogen cycling during the bioremediation of particulate aquaculture waste

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Abstract: The treatment of organic wastes remains one of the key sustainability challenges facing the growing global aquaculture industry. Bioremediation systems based on coupled bioturbation—microbial processing offer a promising route for waste management. We present, for the first time, a combined biogeochemical-molecular analysis of the short-term performance of one such system that is designed to receive nitrogen-rich particulate aquaculture wastes. Using sea cucumbers (*Holothuria scabra*) as a model bioturbator we provide evidence that adjusting the waste C:N from 5:1 to 20:1 promoted a shift in nitrogen cycling pathways towards the dissimilatory nitrate reduction to ammonium (DNRA), resulting in net NH_4^+ efflux from the sediment. The carbon amended treatment exhibited an overall net N_2 uptake whereas the control receiving only aquaculture waste exhibited net N_2 production, suggesting that carbon supplementation enhanced nitrogen fixation. The higher NH_4^+ efflux and N_2 uptake was further supported by metagenome predictions that indicate organic carbon addition stimulated DNRA over denitrification. These findings indicate that carbon addition may potentially result in greater retention of nitrogen within the system, however longer-term trials are necessary to determine whether this nitrogen retention is translated into improved sea cucumber biomass yields. Whether this truly constitutes a remediation process is open for debate as there remains the risk that any increased nitrogen retention may be temporary, with any subsequent release potentially raising the eutrophication risk. Longer and larger-scale trials are required before this approach may be validated with the complexities of the in-system nitrogen cycle being fully understood.

1. Introduction

Intensive land-based aquaculture produces nitrogen-rich effluent that may detrimentally impact water quality and other environmental parameters. In conventional recirculating aquaculture systems (RAS), biological filtration and water exchange are

commonly practiced for nitrogen removal; however, microbial nitrogen removal is limited by the supply of carbon as an electron donor (Castine, 2013). Carbon supplementation is employed in a number of treatment technologies to overcome this deficiency (Avnimelech, 1999; Hamlin et al., 2008; Schneider et al., 2006). The addition of exogenous carbon is a pre-requisite for the successful operation of denitrifying filters that permanently remove dissolved inorganic nitrogenous wastes by conversion to dinitrogen gas (Roy et al., 2010). Alternatively, in zero exchange biofloc systems, carbon to nitrogen ratios (C:N) are increased through the addition of labile carbon sources to promote ammonia assimilation from the water column by heterotrophic bacteria (Avnimelech, 1999; Crab et al., 2012). The fundamental difference between these approaches is the ultimate fate of nitrogen within the system i.e. removal versus retention. Technological advances are focused on the development of dissimilatory processes to permanently remove nitrogen from the system as N_2 gas, while ecological-based systems, such as biofloc, aim to re-cycle and re-use nitrogen within the culture system. This study aims to advance ecologically-based aquaculture bioremediation systems that may provide an alternative to closing the nitrogen cycle through the promotion of assimilatory processes (Robinson, *in review*).

The stoichiometric approach taken in C:N amendment in biofloc systems recognises that carbon and nitrogen cycles are coupled; therefore, the relative elemental abundances control the rate of nutrient cycling and energy flow within the treatment system (Dodds et al., 2004; Ebeling et al., 2006). The potential for C:N manipulation in sediment-based aquaculture effluent treatment systems containing deposit feeders (sea cucumbers) was previously demonstrated by Robinson et al., (*in review*), wherein the addition of soluble starch to aquaculture waste significantly improved sea cucumber growth rate and biomass density. Furthermore, redox-stratified sediments that harboured predominately heterotrophic microbial communities also supported higher sea cucumber yields, indicating that predominately reducing conditions are more favourable for deposit feeder growth (Robinson et al., 2015; Robinson et al., 2016). Since reducing conditions favour anaerobic respiratory and fermentative pathways, organic carbon supplementation may stimulate anaerobic bacterial metabolism by increasing the availability of electron donors and/or substrates for fermentation, in addition to increasing heterotrophic NH_4^+ assimilation (Fenchel et al., 2012; Oakes et al., 2011).

The C:N ratio affects the quantity of nitrogen released during mineralisation, with a net release of nitrogen occurring below a threshold of 20:1 (Cook et al., 2007; Blackburn, 1986). Robinson et al., (*in review*) hypothesised that C:N manipulation may alter the nitrogen

cycling pathways within the sediment microbial community by mediating a shift from ammonification (net release) to assimilation (net uptake) of NH_4^+ by heterotrophic bacteria; however, the effect of carbon supplementation on nitrogen cycling was not clearly elucidated. An improved understanding of how C:N manipulation influences benthic nitrogen cycling is necessary in order to improve nitrogen assimilation and incorporation into secondary biomass. In the current study, we applied a coupled biogeochemical-molecular approach to further investigate the effect of carbon supplementation on nitrogen cycling. Incubation experiments were conducted to quantify benthic fluxes, while sediment microbial communities were examined using 16S rRNA gene sequencing. The study aimed to test the hypothesis that increasing the C:N of particulate aquaculture waste from 5:1 to 20:1 would promote the assimilation of NH_4^+ by heterotrophic bacteria, drive shifts in microbial community composition and result in nitrogen retention in the culture system.

2. Materials and methods

2.1 Study site and experimental animals

The study was conducted in a purpose-built bio-secure heated conventional recirculating aquaculture system (RAS) described in Robinson et al. (2015). The experiment was conducted over a fifteen day period from January 30th (day -1) to February 14th (day 14) 2014 using juvenile sea cucumbers (*Holothuria scabra*) imported from a commercial hatchery (Research Institute for Aquaculture III, Vietnam) on September 5th 2013, that were quarantined and acclimated to the experimental system as described in Robinson et al. (*in review*).

2.2 Experimental design

Three experimental treatments were randomly allocated to 15 incubation chambers with five replicates per treatment. The 'initial' (In) treatment was included to ensure that there were no significant differences between treatments prior to the start of the experiment and as an intial reference point for evaluating the effect of the treatments. The 'no added carbon' treatment (-C) with a C:N of 5:1 received aquaculture waste only (215.06 mg day^{-1} wet weight). The 'added carbon' treatment (+C) received aquaculture waste (215.06 mg day^{-1} wet weight) and carbon in the form of soluble starch (44.50 mg day^{-1} dry weight) daily to increase the C:N to 20:1 (mass ratio) from day zero (Table 1). The carbon addition treatments (+C) were standardised at a concentration of 400 mmol C m^{-2} d^{-1}.

2.3 Experimental system and rearing conditions

Sediment incubation chambers were established by transferring unsieved $CaCO_3$ builder's sand sourced from a commercial dune quarry (SSB Mining, Macassar, South Africa) into Plexiglas® tubes (25 cm long, 8.4 cm internal diameter) sealed with a polyvinyl chloride (PVC) end cap to a depth of 7.5 cm. The incubation chambers were connected via 4.0 mm air tubing and 4.0 mm variflow valves to a manifold receiving seawater directly from a RAS biofilter (see Robinson *et al.,* 2015 for further details). The water flow rate was 50 mL min^{-1}, equivalent to 16.34 exchanges h^{-1}. The chamber outflows were routed into a main drainage channel and allowed to flow to waste to prevent soluble carbon sources from entering the RAS. Unsieved $CaCO_3$ was pre-conditioned for four weeks in flow-through tanks prior to its transfer into the chambers. The sediment was allowed to condition and stabilise into redox-stratified layers for 14 days prior to commencement of the experiment. No aeration was provided; however, water was continuously mixed at 60 rpm using a magnetic stirring rod positioned 15 cm above the sediment surface. Stirring rates were just below that which caused sediment re-suspension (Ferguson et al., 2004; Gongol and Savage, 2016).

The experimental area was fully shaded from direct sunlight. Light intensity was measured during daylight incubations using a light meter (LX-107, Lutron Electronic Enterprise Co. Ltd, Taipei, Taiwan) positioned 10 cm above each chamber. Additionally, a temperature/light logger (Hobo, UA-002-64, Onset, USA) was placed in an additional chamber positioned in the centre of the experimental treatments. The mean (hours) natural photoperiod was 13.34:10.26 (L:D).

2.4 Aquaculture waste and carbon additions

The aquaculture waste, used as feed for the sea cucumbers, comprised uneaten abalone (*Haliotis midae*) feed and faeces. It was collected daily from the backwash of a sand filter in a recirculating abalone grow-out system. Samples were sent for organic carbon and total nitrogen content analysis (Robinson et al., *in review*) and the mean C:N was 5.21:1. Soluble starch (Merck Millipore, Pretoria, South Africa) was used as an additional carbon source to increase the C:N to 20:1. Additions of waste with (+C) or without (-C) added carbon commenced on day zero. The aquaculture waste was mixed into a wet slurry while the starch was dissolved in seawater and added daily to the respective treatments at 16:00 from day zero to day 14.

2.5 Experimental timeline

Baseline data were collected at the start of the experiment (i.e. day -1), with fluxes measured in all 15 chambers under light and dark conditions. All replicates from the In treatment were sacrificed on day zero and sub-cored for analysis of sediment characteristics.

2.6 Sea cucumber growth

Animals (n = 30) previously acclimated in the RAS were suspended in mesh containers for 24 h to evacuate their guts prior to weighing and photo-identification (Robinson et al., 2015). Three juvenile *H. scabra* with a mean (± standard deviation) weight of 1.91 ± 0.36 g were added to each of 10 chambers (equivalent to a high stocking density of 1,034.00 gm^{-2}) on day zero. They were removed at the end of the experiment (day 14), gut-evacuated for 24 h and reweighed. Wet weight data were used to calculate growth rate ($g\ d^{-1}$; Robinson et al., 2015).

2.7 Benthic flux incubations

Benthic flux incubations were conducted on day -1 for all treatments (In, -C and +C) and on alternate days from day one to day 13 for the -C and +C treatments, after sacrifice of the In treatment. Light incubations were conducted during daylight hours, commencing after sunrise (08:00 local time) and dark incubations were conducted after sunset (22:00 local time). When data were collected the flow from each chamber was interrupted, the stirrers were paused (~ three min.) and the chambers were uncapped by removing the rubber bung. A portable optical meter (YSI ProODO, YSI Pty Ltd, USA) was inserted through the sampling port to measure temperature (± 0.01 °C) and dissolved oxygen (DO) concentrations (± 0.01 mg L^{-1}). The pH (± 0.01 pH units) was measured electro-chemically (Eutech Instruments pH 6+ portable meter, Singapore).

Water alkalinity and nutrient concentration (ammonia, nitrate/nitrite, nitrite and phosphate) were recorded at the start and end of each light/dark incubation period. To do this, samples were withdrawn using a 50 mL acid washed plastic syringe connected to the chamber outflow through 4.0 mm tubing and filtered (Whatman® glass microfiber filters grade GF/C, Sigma Aldrich, Johannesburg, South Africa) into 15 mL screw-capped polycarbonate vials. All nutrient samples were immediately frozen at –20 °C and alkalinity samples were kept cold at 4 °C. The N_2 samples were taken on three sampling occasions (days one, seven and 13) during dark incubations, as during daylight hours bubbles may form that interfere with the estimation of N_2:Ar and thus overestimate N_2 production (Eyre et al., 2002). To minimise bubble introduction, N_2 samples were collected by allowing the water to

flow by gravity from the chamber outflow directly into 7 mL gas-tight glass vials with glass stoppers filled to overflowing. The N_2 samples were poisoned with 20 µL of 5 % $HgCl_2$ and stored submerged at 20 °C. The N_2 samples were collected in duplicate or triplicate, thus the final values represent the mean value calculated for each replicate (Eyre and Ferguson, 2005).

After withdrawal of all water samples, replacement water was gravity fed into the chamber directly from the manifold and the chambers were re-capped and the stirrers re-started. All materials used for sample collection were acid washed, rinsed three times with distilled water and air dried prior to use. Total oxygen exchange was measured in three randomly selected chambers during incubations (one from each treatment) to ensure that the oxygen concentration did not decrease by more than 20 %. Incubation times were kept short, ranging from 68 to 146 minutes with an average duration of 104 minutes, to prevent oxygen depletion and ensure that flux rates were linear (Burford and Longmore, 2001; Glud, 2008).

2.8 Nutrient analyses

Dissolved nitrate and nitrite (NOx; 0.01 µM) were determined colourimetrically by flow injection analysis (QuikChem® 8500 Automated Ion Analyzer, Hach Company, U.S.A.) and a commercially available test kit (QuikChem® method 31-107-04-1-E for the determination of nitrate and nitrite in seawater). All other nutrient samples were analysed manually. Ammonium (0.01 µM) and dissolved inorganic phosphate (0.01 µM) were determined using the methods of Grasshoff (1976) and Grasshoff et al. (1999) respectively, and nitrite (NO_2-; 0.01 µM)) was determined according to Bendscheider and Robinson (1952).

2.9 Gas analyses

Alkalinity (0.01 mg L^{-1}) and total dissolved CO_2 (0.01 µM) concentrations were determined by potentiometric titration according to Edmond (1970) using an automated titrator system (876 Dosimat plus, Metrohm, USA). Total alkalinity was calculated according to the method of Snoeyink and Jenkins (1980). CO_2 concentrations were calculated from alkalinity and pH using the equations given in Almgren et al. (1983). Changes in pH and alkalinity were used to calculate dissolved inorganic carbon (DIC) fluxes.

Dinitrogen gas (N_2) was determined from N_2:Ar using membrane inlet mass spectrometry (MIMS) with O_2 removal (± 0.01%). Measurement of direct N_2 fluxes using this technique represents the net benthic flux of N_2 resulting from a combination of processes

that produce N_2, such as denitrification and anammox, and processes that consume N_2 such as nitrogen fixation (Ferguson and Eyre, 2007; Eyre et al., 2013a).

Nutrient and gas fluxes across the sediment-water interface during light and dark incubations were calculated using initial and final concentration data according to Equation 1. Net flux rates, representing the net result of 13.57 h of dark fluxes and 10.43 h of light fluxes were calculated according to Equation 2 (Veuger et al., 2007). Gross primary production was calculated according to Equation 3, where light O_2 fluxes represent net primary production and dark fluxes represent respiration. Remineralisation ratios were calculated according to Equation 4 (Eyre et al. 2013b).

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Equation 1 $$\text{Flux} = \frac{(C_n - C_0) \times V}{A \times t} \times 10{,}000$$

where:

Flux = flux (µmol m^{-2} h^{-1}), C_0 = concentration at time zero (µmol L^{-1}), C_n = concentration at time n (µmol L^{-1}), t = incubation time (h), A = area of sediment surface in chamber (cm^2), and V = volume of water in chamber (L).

Equation 2 $$\text{Net flux rates} = \frac{(\text{hourly dark rates} \times \text{hours of darkness}) + (\text{hourly light rates} \times \text{hours of daylight})}{24\text{h}}$$

Equation 3 $$\text{Gross primary production} = \text{light } O_2 \text{ flux (+ve)} - \text{dark } O_2 \text{ flux (-ve)}$$

Equation 4 $$\text{Remineralisation ratio} = \frac{\text{Dark } O_2 \text{ flux}}{N_2 + NH_4^+ + NO_x}$$

2.10 Sediment sectioning

On days zero and 14, three sub-cores (internal diameter 30 mm) were extracted from the In and experimental (-C and +C) chambers respectively. Each sub-core was sectioned into the following five depth intervals: 0.0 - 0.5, 0.5 - 1.0, 1.0 - 2.0, 2.0 - 4.0 and 4.0 - 6.0 cm for analysis of sediment characteristics. One set of sub-cores was dried at 50 °C for 24 h for analysis of total organic carbon and total nitrogen; the second set was frozen in sealed vials in black bags for spectrophotometric analysis of total carbohydrates. Two sets of samples were prepared from the third sub-core: sediment samples were frozen in 2 mL Eppendorf tubes for subsequent deoxyribonucleic acid (DNA) extraction and sequencing. The remaining sediment was added to 15 mL vials filled with 0.2 µm filtered, one percent buffered paraformaldeyde and refrigerated for determination of bacterial abundance by flow cytometry.

The organic content measured as particulate organic carbon (OC) and total nitrogen (TN) was determined on an elemental analyser after removal of carbonates by acid fumigation (Robinson et al., 2015). Total sediment carbohydrates were measured on defrosted samples using the phenol-sulphuric acid method (Underwood et al., 1995).

2.11 Flow cytometry

Aliquots of preserved samples were prepared in duplicate by staining with 4′,6-diamidino-2-phenylindole (DAPI) for 15 minutes at 4 °C in darkness (Marie et al., 1999). Bacterial abundance was analysed with a FACSCalibur flow cytometer (BD Biosciences, Singapore), fitted with a 488 nm, 15 mW laser, using the FL1 detector (λ = 530 nm). TruCount beads (BD Biosciences, Singapore) were used as an internal standard. All cytometric data were logged and analysed using Cell Quest (Becton-Dickinson) using *Escherichia coli* cells as a reference. Cell abundance was converted to cells g^{-1} of dry sediment.

2.12 Deoxyribonucleic acid extraction and importation

Genomic DNA was extracted from approximately 250 mg of substrate samples using a DNA isolation kit (ZR Soil Microbe DNA MiniPrep, Zymo Research, USA) yielding purified genomic DNA for use in polymerase chain reaction (PCR) amplification. Genomic DNA was stored in sealed, labelled Eppendorf tubes at -20 °C prior to being couriered from the Republic of South Africa to the United Kingdom. To comply with the Animal Health Act 1981, the samples were accompanied by a general import license (IMP/GEN/2008/03) for the importation of animal and poultry products, including DNA, from all non-EU countries.

2.13 Polymerase chain reaction and 16S rRNA sequencing

Library preparation was performed using a modified version of the MiSeq WetLab protocol (Kozich et al., 2013). One microliter of template DNA was arrayed into 96-well plate format with 17 µL of Accuprime Pfx Supermix (Thermofisher, UK), leaving two wells on each plate open for controls. Two microliters of reconstituted indexed primers at 100 µM were added to the samples to barcode them for identification. To identify any contaminating operational taxonomic units (OTUs), two control samples were included in the sequencing run. The negative control consisted of one microliter of PCR grade dH_2O and the positive control was one microliter of mock community (HM-278S, BEI Resources, Manassas, USA) at a 1:3 dilution. The primer pair 515F/806R was used to amplify the V4 region of the 16S rRNA gene. PCR was performed using the following conditions: initial enzyme activation

and DNA denaturation proceeded at 95 °C for two minutes followed by cycling parameters of 95 °C for 20 s, 55 °C for 15 s, 72 °C for five minutes for 30 cycles. A final extension was done at 72 °C for ten minutes. Amplification of the PCR products was checked on a subset of 12 samples using gel electrophoresis on a one percent agarose gel prior to library clean up. Samples from all plates were pooled and libraries were subjected to quality control including quantification using a KAPA Biosystems Q-PCR kit, obtaining a bioanalyser trace using the Agilent Technologies HS DNA kit and normalisation using the Invitrogen SequalPrep Plate Normalisation Kit (Thermofisher, UK). Amplicons were sequenced on an Illumina MiSeq platform by NU-OMICS (Northumbria University, UK).

2.14 Processing of raw sequence data

The raw fastq files were processed using Mothur (version 1.37.0) based on the Schloss MiSeq SOP with modifications. Raw forward and reverse sequence reads were merged to create contigs prior to quality filtering. The sequence reads were trimmed using a sliding window of five base pairs (bp) with an average window quality threshold (Q) of 22 or greater. Sequences containing an ambiguous (N) base, >8 homopolymers or that had a sequence length <275 bp were discarded. Quality-filtered sequences were aligned using a custom alignment created for the variable four (V4) region of the 16S rRNA gene using the Silva database (version 123; July 2015 release). The reads were screened to include only overlapping regions (based on alignment positions), pre-clustered (number of differences = 1) and checked for chimeras using the UCHIME algorithm (Edgar et al., 2011).

Taxons classified as 'Mitochondria', 'Eukaryota' or 'unknown' were specified during the remove.lineage command. The count.groups command was used to determine the minimum number of reads per sample for normalisation. To standardise sequencing effort, all samples were subsampled to 550 using the sub.sample command, to ensure that all replicate samples from the experimental treatments (+C and -C) were retained. The subsampled OTU table (shared file) and assigned consensus taxonomy (cons.taxonomy.file) were used in downstream analyses, including alpha and beta diversity, taxonomic composition and metagenome predictions of the microbial communities.

2.15 Statistical analyses and bioinformatics

Environmental (light, temperature, salinity) and flux rate data for nutrients (NH_4^+, NO_2^-, NO_x and PO_4^{3-}) and gases (DO, DIC and N_2 – night only) collected on day -1 during light and dark incubations were averaged to provide a mean value per replicate chamber for

each diurnal period respectively. The data were tested for homogeneity of variance and for the normal distribution of the residuals using Levene and Shapiro Wilk tests. One-way analysis of variance (ANOVA) tested for differences in the environmental, nutrient and gas flux data between the In, +C and -C treatments on day -1.

The light, water quality and flux rate data (days 1-13) for nutrients and gases were averaged to provide a mean value for each replicate incubation chamber. It was not possible to conduct daytime incubations on day nine due to lowered O_2 concentrations in the chambers, therefore light incubation data represents a mean of six values (days one, three, five, seven, 11 and 13), while the mean dark incubation data were calculated from the full set of seven incubations. The mean temperature, salinity and mean light, dark and net fluxes of nutrients and gas fluxes, mean remineralisation ratios and mean gross primary production measured during the experimental period (days 1-13) were analysed using a Student t-test at alpha <0.05. Sediment characteristics, including organic carbon, total nitrogen, C:N and bacterial cell abundance were compared using mixed-model ANOVA with treatment (+C and -C) and sediment depth as fixed factors. When a significant effect was observed, post hoc comparisons of means were conducted with a Tukey's honest significant difference test. Differences in *H. scabra* growth rate and biomass density were analysed by Student t-test at alpha <0.05. Data are presented as mean ± standard error unless otherwise stated. All statistical analyses were performed in Statistica v.13.

Alpha (within-sample) diversity metrics for the number of OTUs (observed), richness (Chao 1), abundance-coverage estimator (ACE) and diversity (Shannon, Simpson and Inverse Simpson) were calculated and visualised in the phyloseq package in R (McMurdie and Holmes, 2013). The diversity metrics were generated by the summary.single command by subsampling to the lowest number of reads per sample (n = 550) and compared across treatments and sediment depths using mixed model ANOVA.

Patterns in bacterial community structure between treatments and sediment depths were visualised using principal coordinates analysis (PCoA) based on a Bray–Curtis dissimilarity matrix calculated from the OTU table in R. In addition, a non-parametric multivariate analysis of variance (PERMANOVA) was performed on the community distance matrix based on Bray–Curtis dissimilarity index to test the null hypothesis that there was no difference in the structure of microbial communities between treatments (In vs. -C vs. +C) and sediment depth using the 'adonis' function of the vegan package in R (Oksanen et al., 2016).

Mantel correlation tests were performed on dissimilarity matrices of the community

and environmental data to provide an indication of how well microbial community data corresponded to the environmental data. The environmental distance matrix was calculated as Euclidean distances computed from a metadata table containing all of the data describing light, water quality, sediment characteristics and net flux rates for gases and nutrients. The significance of correlation coefficients was assessed using a permutation procedure. In addition, the correlation between environmental data and the sediment microbial communities was determined using the 'envfit' function of the 'vegan' package in R (Oksanen et al., 2016). Since none of the environmental characteristics were significantly correlated with the microbial community data, the environmental data were not plotted as vectors on the PCoA ordination.

The Tax4Fun package in R was used to predict the metabolic capacities of the microbial communities from the 16S rRNA sequences. The fctProfiling option was set to TRUE (default) to predict the metabolic capacities of the metagenomes based on pre-computed Kyoto Encyclopedia for Genes and Genomes (KEGG) Ortholog reference profiles (Aßhauer et al., 2015). Only KEGG Pathways within 'nitrogen metabolism' were retained for analysis. The KEGG pathway map 00910 for nitrogen metabolism and associated information was used to extract the KEGG ortholog reference numbers involved in the six fully characterised reactions listed under 'nitrogen metabolism' (supplementary Table 2). Anaerobic oxidation of ammonia (anammox) was not included, as although this process is recognised in the KEGG database it has yet to be assigned to a module or reference profile.

The relative abundance of functional genes predicted from the 16S rRNA sequences within each ortholog reference profile were summed to provide a mean value for each pathway module for each replicate sample from all sediment depths sampled in all treatments (n = 45). The relative abundance of functional genes in the In and experiment treatments was illustrated by graphically plotting vertical depth profiles and analysed statistically using a mixed-model ANOVA.

3. Results

3.1 Sea cucumber growth and survival

Survival of sea cucumbers was 100 % in the +C treatment; however, one replicate chamber from the -C treatment was terminated on day nine following a period of water column hypoxia, caused by one animal preventing water exchange by blocking the outflow valve. This resulted in the mortality of all sea cucumbers in this chamber, reducing the

overall survival to 80 %. There was no significant difference between the mean sea cucumber wet weight on day zero or day 14 between treatments; however, despite the short duration of the experiment the sea cucumbers in both treatments lost mass (decreasing from 1.91 ± 0.02 g to 1.62 ± 0.03 g; an overall mean growth rate of -0.02 ± 0.00 g day^{-1}). The biomass density decreased from 1,034.00 ± 12.73 g m^{-2} to 874.97 ± 18.31 g m^{-2}, although the initial stocking density was comparable to the final densities (1,011.46 ± 75.58 g m^{-2}) achieved in previous carbon amended cultures standardised at 200 mmol C m^{-2} day^{-1} (Robinson et al., *in review*).

3.2 Gas and nutrient fluxes

Benthic fluxes of dissolved oxygen and dissolved inorganic carbon (DIC) can provide an indication of overall benthic metabolism in response to organic enrichment (Eyre et al., 2011). There were no significant differences in the light, dark or net fluxes of DO, DIC or N_2 between treatments on day -1 (N_2 dark only; Fig S1). Sediment oxygen consumption was significantly higher in the +C incubations throughout the experiment in both light and dark incubations (Student's t-test; t = -2.87, p = 0.006) resulting in a higher net consumption of -2,905.84 ± 99.95 µmol O_2 m^{-2} h^{-1} compared to -2,511.31 ± 116.81 µmol O_2 m^{-2} h^{-1} in the -C treatment (Fig. 1a). Oxygen and DIC fluxes clearly show that the sediment metabolism was net heterotrophic. During the day, DIC release from organic matter degradation exceeded DIC consumption from primary production (Fig. 1b). There was sediment oxygen consumption during light and dark incubations, indicating that respiration dominated over photosynthesis; supported by the lower gross primary production in the +C treatment (Fig. 1d). There were no significant differences in the light, dark or net fluxes of DIC with a mean net efflux of 12,732.34 ± 2,031.69 µmol C m^{-2} h^{-1} across the treatments (Fig. 1b). The assumed low rates of photosynthesis may have been due to shading and from turnover of the microphytobenthos standing stock due to grazing by sea cucumbers (Glud et al., 2008;Mactavish et al., 2012). In addition, DIC fluxes were four-fold higher than oxygen fluxes, indicating that the majority of the organic carbon was oxidised by anaerobic pathways (Burford and Longmore, 2001; Eyre et al., 2011).

The mean dark N_2 flux on days seven and 13 was not significantly different between treatments (Student's t-test; t = -1.29, p = 0.23; Fig. 1c). Carbon supplementation resulted in a net N_2 uptake (-142.96 ± 107.90 µmol m^{-2} h^{-1}), indicating that atmospheric nitrogen fixation dominated over denitrification and anammox during dark incubations. In contrast, the -C treatment had a small but positive net N_2 efflux (17.33 ± 36.20 µmol m^{-2} h^{-1}), indicating that

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nitrogen removal pathways, such as denitrification or anaerobic ammonium oxidation (anammox), were slightly greater than nitrogen fixation.

Ambient environmental conditions recorded in the incubation chambers at the start of the experiment on day -1, during light and dark periods, are presented in Table S1. There were no significant differences in the dark or net fluxes of any of the nutrients between treatments on day -1; except, the NH_4^+ fluxes during light incubations which were significantly different (one-way ANOVA; $F_{(2, 9)} = 12.73$, $p = 0.002$; Fig. S2). The In chambers had a significantly higher NH_4^+ efflux of 115.32 ± 11.43 µmol m^{-2} h^{-1} compared with an uptake of -9.77 ± 11.82 µmol m^{-2} h^{-1} in the -C treatment. The +C treatment had intermediary values with a mean NH_4^+ efflux of 56.03 ± 25.54 µmol m^{-2} h^{-1}. NH_4^+ had the highest flux rate throughout the experiment (Fig. 2b) with mean efflux significantly higher in the +C chambers during light incubations compared with the -C treatment (182.25 ± 120.77 vs. 83.90 ± 26.70 µmol m^{-2} h^{-1}, t-test; $t = 2.93$, $p = 0.005$; Fig. 2b). Sediment-water exchange of NO_2^-, NO_x and PO_4^{3-} were unaffected by carbon addition. Mean fluxes of NH_4^+, NO_2^- and PO_4^{3-} were positive irrespective of diel cycle, indicating net release from the sediment (Fig. 2a-c); however, NO_x fluxes were variable with opposing trends in light, dark and net fluxes between treatments (Fig. 2d). As both dissolved oxygen consumption and NH_4^+ production were higher in the +C chambers this indicates an overall increase in benthic metabolism during daylight.

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3.3 Sediment characteristics and remineralisation ratios

The sediment organic carbon (OC) content decreased in the experimental treatments after 14 days compared to the initial treatment (Fig. 3a). The largest decrease was observed at the 1.0 – 2.0 cm and 2.0 – 4.0 cm depth intervals spanning the approximate depth of the oxic-anoxic interface; one of the most active zones of organic matter mineralisation by heterotrophic microorganisms (Reimers et al., 2013). Vertical profiles of total nitrogen (TN) and the C:N on days zero and 14 followed a similar trend with the most marked changes occurring at the 1.0 – 2.0 cm and 2.0 – 4.0 cm depth intervals respectively. Carbon addition did not affect the OC or TN but sediment depth significantly influenced the OC (mixed model ANOVA, $F_{(4, 20)} = 3.54$, $p = 0.024$; Fig. 3a) and TN content (mixed model ANOVA, $F_{(4, 20)} = 3.37$, $p = 0.029$; Fig. 3b), being significantly lower at the 1.0 - 2.0 cm depth interval with mean values of 0.24 ± 0.02 % (OC) and 0.03 ± 0.00 % (TN) respectively. This confirms that the oxic-anoxic interface supported the highest rates of organic matter mineralisation. In

539 contrast, the deepest sectioned interval (4.0 – 6.0 cm) had significantly higher OC (0.51 ±
540 0.08 %) and TN content (0.07 ± 0.01 %) than the shallower intervals. Carbon addition did not
541 significantly increase the sediment C:N in the +C treatment (7.90 ± 0.27) compared to the -C
542 treatment (7.12 ± 0.24; mixed model ANOVA, $F_{(1, 20)} = 4.52$, $p = 0.054$; Fig. 3c). However,
543 carbon supplementation resulted in mean remineralisation ratios (after exclusion of outliers)
544 of 15.68 ± 7.43 that were approximately threefold higher than chambers receiving
545 aquaculture waste only (5.64 ± 4.50), although the difference was not significant (t-test; $t =$
546 1.08, $p = 0.32$). Remineralisation ratios were higher than the sediment C:N in the +C
547 treatment; a trend that is consistent with nitrogen assimilation by heterotrophic bacteria,
548 including nitrogen fixation (Eyre et al., 2013b). Conversely, in the -C treatment receiving raw
549 aquaculture waste at a C:N of 5:1, the remineralisation ratios were lower than the sediment
550 C:N, indicating net release of nitrogen.

551 ***3.4 Microbial community analysis and nitrogen metabolism functional gene prediction***

552 A total of 781,701 16S rRNA reads were generated. Four samples from one replicate
553 of the In treatment were removed during sub-sampling due to a low abundance of reads, and
554 therefore excluded from further analysis. A total of 780,612 sequences in the 41 samples
555 remained subsequent to quality control, primer trimming, size exclusion, and removal of
556 unassigned taxons, mitochondria and Eukaryota.

557 Neither carbon addition, sediment depth nor the interaction between the factors
558 (treatment × sediment depth) significantly affected the number of sequences, OTUs
559 (observed species), community richness (Chao and ACE), or diversity measured as Simpson
560 and Inverse Simpson indices (mixed model ANOVA; $p < 0.05$; Fig. 4). Sediment depth
561 significantly influenced Shannon diversity, with the highest diversity of 2.85 recorded in the
562 sediment surface layer (0 - 0.5 cm) and the lowest (1.54) in the 4 - 6 cm layer (mixed model
563 ANOVA; $F_{(4, 26)} = 3.14$, $p = 0.031$).

564 Flow cytometry data compared relatively well with the 16S rRNA amplicon
565 sequencing data. Bacterial abundance (cells g^{-1}; Fig. 3e), the number of sequences and OTUs
566 were higher in the In chambers than the experimental chambers sampled on day 14,
567 presumably in response to grazing by the sea cucumbers. The number of OTUs decreased
568 from 286.81 ± 128.13 in the In chambers to 176 ± 65.15 and 181.20 ± 45.90 in the +C and -C
569 treatments respectively. Overall, the community diversity was low: Shannon diversity = 2.31
570 ± 0.13, Inverse Simpson = 5.79 ± 0.51. There was a marked increase in community richness
571 at the 1 - 2 cm depth interval, coinciding with the oxic-anoxic interface. In the In chambers

572 the number of OTUs was 778.00 ± 731.00, compared with 343.33 ± 199.25 and 322.67 ±
573 307.25 in the +C and -C treatments respectively. The Chao 1 richness indicator also followed
574 this trend (Fig. 4).
575 The majority of sequences (99.8 %) were assigned to the Bacteria, with only 0.12 %
576 assigned to Archaea. Taxa from three archaeal phyla were present, including Euryarchaeota,
577 Thaumarchaeota and Woesearchaeota. *Natronorubrum* (Euryarchaeota), a halophilic aerobic
578 chemoorganotroph (Xu et al., 1999), was the most abundant genus representing 14 of the 27
579 archaeal reads.
580 The bacterial community contained a total of 18 phyla, four candidate phyla and the
581 candidate division WPS-2. Proteobacteria and Firmicutes were the two dominant phyla
582 accounting for 47.64 and 34.71 % of the total sequences respectively, with Cyanobacteria
583 accounting for 7.42 %. Planctomycetes (2.45 %), Actinobacteria (2.34 %), unclassified
584 Bacteria (2.12 %) and Bacteroidetes (1.33 %) were minor components. The remainder of the
585 phyla, candidate phyla and the candidate division WPS-2 each represented less than 1 % of
586 the community. Candidate phyla included Hydrogenedentes (formerly NKB19),
587 Latesbacteria (formerly WS3), Parcubacteria (formerly OD1) and Poribacteria.
588 Taxa within the Oxalobacteraceae and the genus *Herbaspirillum* were significantly
589 more abundant in the -C treatment (Welch's two-sided t-test; $p < 0.05$; Fig. 5). In
590 comparison, the genera *Blastopiellula* and *Litorilinea* were significantly enriched in the +C
591 treatment. There were no significant differences in the mean proportion of taxa between
592 experimental treatments at phylum, class or order levels, underscoring the high degree of
593 similarity among the microbial communities between treatments (Fig. 6). Further, there was
594 no correlation between the microbial community and environmental data (Mantel test; $r =$
595 0.04, $p = 0.27$). The first axis in the PCoA ordination explained 53.4 % of the variation and
596 appeared to be associated with sediment depth, while the second axis (4.7 % of the variation)
597 appeared to be associated with experimental treatment. Treatment did not significantly
598 influence microbial community structure (PERMANOVA; $p<0.05$; Table 2), which may be a
599 function of the relatively short duration of the experiment. By contrast, there was a
600 significant effect of sediment depth on the microbial community (PERMANOVA; $p=0.011$;
601 Table 2).
602 There were no significant differences in the predicted relative abundance of genes
603 involved in the six nitrogen transformation pathways (mixed model ANOVA; $p > 0.05$; Fig.
604 7). The relative abundance of predicted nitrification genes peaked at the 0.5 – 1.0 cm depth
605 interval in the -C treatment, coinciding with the oxic zone. In the +C treatment, the relative

606 abundance of predicted denitrification and DNRA genes were higher in the sediment layers
607 sectioned at 1.0 – 2.0, 2.0 – 4.0 and 4.0 – 6.0 cm. Overall, DNRA was the dominant pathway
608 (20.52 ± 0.01 %) predicted to occur in all treatments and sediment depths, with the exception
609 of the surface layer (0.0 - 0.5 cm) in the +C treatment, where there was a higher predicted
610 relative abundance of denitrification genes (Fig. 7). Denitrification was the second most
611 abundant predicted pathway (18.02 ± 0.01 %), followed by complete nitrification (8.80 ±
612 0.43 %), indicating that the potential for coupled nitrification-denitrification was present in
613 all treatments. Genes predicted to be involved in nitrogen fixation represented 2.85 ± 0.32 %.

614 **4. Discussion**

615 Effluent (especially particulates) discharged from intensive land-based aquaculture
616 can impact the marine benthos through the organic enrichment of the underlying sediment. In
617 this study, the comparison of vertical sediment profiles before and after the experiment
618 indicated that the addition of particulate aquaculture waste to treatments with sea cucumbers
619 stocked at densities of >1 kg m^{-2} did not increase the organic carbon content, total nitrogen or
620 C:N. Overall, the values were generally lower after 14 days of daily waste addition than at
621 the start. This is consistent with previous studies that concluded that sea cucumbers are
622 efficient bioturbators that stimulate benthic microbial metabolism and organic matter
623 remineralisation and may partly ameliorate the effects of organic matter enrichment from
624 aquaculture effluent (MacTavish et al., 2012).

625 It was hypothesised that increasing the C:N would mediate a shift from
626 ammonification (net release) to NH_4^+ assimilation (net uptake), leading to an overall decrease
627 in NH_4^+ efflux, however, net NH_4^+ production was higher in +C treatment. In addition to sea
628 cucumber excretion, NH_4^+ can originate from four nitrogen transformation pathways;
629 ammonification (degradation of organic nitrogenous waste), nitrogen fixation, assimilatory
630 reduction of nitrate to ammonia (ARNA), and dissimilatory nitrate reduction to ammonia
631 (DNRA). ARNA and nitrogen fixation are both assimilatory pathways that occur within
632 organisms, and therefore do not contribute to an increase in NH_4^+ concentration at the
633 sediment-water interface (Gardner et al., 2006). Ammonification and DNRA are therefore the
634 only pathways with the potential to contribute to increased NH_4^+ production in the +C
635 treatment. The increased NH_4^+ concentration may have originated from an increase in
636 ammonification conisistent with the increase in metabolism observed in the +C treatment.

637 An increasing number of studies have demonstrated the importance, and indeed
638 dominance of DNRA in nearshore shallow water coastal environments, particularly in

639 tropical ecosystems (Decleyre et al., 2015; Fernandes et al., 2012; Gardner et al., 2006; Song
640 et al., 2014; Erler et al., 2013). For example, Fernandes et al. (2012) showed that DNRA can
641 account for 99 % of nitrate removal in nitrogen-limited mangrove ecosystems. In marine
642 sediments, DNRA and denitrification compete for nitrate; however, denitrification results in
643 the permanent removal of nitrogen from the system whereas DNRA retains bioavailable
644 nitrogen in sediments by reducing nitrate to NH_4^+ (Gardner et al., 2006). Since these nitrogen
645 transformation processes are reductive pathways, mediated by heterotrophic bacteria in the
646 anaerobic zone of redox-stratified sediments, carbon addition can stimulate both
647 denitrification and DNRA (Hardison et al., 2015). In some aquaculture systems the
648 availability of organic carbon is known to limit N_2 production via denitrification (Castine et
649 al., 2012); therefore, carbon supplementation is employed to successfully operate denitrifying
650 filters (Castine, 2013; Roy et al., 2010). However, Castine (2013) found no significant
651 differences in N_2 production when aquaculture slurries were amended with particulate
652 organic matter or methanol as carbon sources. Other studies have found that high organic
653 loading rates and/or the addition of exogenous carbon sources stimulated DNRA and
654 concluded that high organic carbon loading is a pre-requisite for DNRA to be favoured over
655 denitrification (Hardison et al., 2015; Capone, 2000). In the present study, the higher NH_4^+
656 efflux in the +C treatment, supported by the metagenome predictions and the uptake of N_2
657 gas, would suggest that organic carbon addition stimulates DNRA over denitrification.
658 Increasing the organic carbon availability can potentially stimulate all four nitrogen
659 reduction pathways (supplementary Fig. 2). These pathways, with the exception of
660 denitrification, result in ammonia production and therefore contribute to nitrogen retention
661 within the system (Hardison et al., 2015). The factors regulating the balance between the
662 different nitrogen processes are not well understood. For example, the quality and quantity of
663 organic carbon may influence the balance between denitrification and nitrogen fixation
664 (Fulweiler et al., 2013). Historically, denitrification has been considered to be the main
665 pathway of nitrogen loss, based on mass balance calculations (Seitzinger, 1988). However, in
666 sediment-based systems enriched with particulate organic waste (such as settlement ponds in
667 aquaculture systems), the processes of permanent nitrogen removal account for a very small
668 fraction of the total nitrogen that is permanently removed from the system. For example,
669 Castine et al. (2012) found that denitrification and anammox only removed 2.5 % of total
670 nitrogen inputs (by N_2 production) to settlement ponds in intensive shrimp and barramundi
671 farms. In this case denitrification was not carbon limited; rather the authors argue that

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inhibition of microbial metabolism by increased H_2S and NH_4^{4+} production limited the performance of the system.

Sediment nitrogen fixation can equal or exceed N_2 loss in estuarine systems (Newell et al., 2016a). The genetic potential for nitrogen fixation is widespread within the Bacteria and Archaea (Newell et al., 2016b; Zehr and Paerl, 2008a). Heterotrophic nitrogen fixation has not been widely demonstrated in sediments beyond the observation of N_2 uptake (Gardner et al., 2006); however, recent studies provide direct evidence by measuring *in situ* N_2 production combined with molecular and genomic tools to quantify the presence of the nitrogenase reductase (*nifH*) gene (Newell et al., 2016b; Baker et al., 2015). Indirect evidence for nitrogen fixation is provided in the present study by the presence of *nifH* (K02588) in all samples and the taxonomic composition of the microbial communities.

Nitrogen fixation can be mediated by photoautotrophic and heterotrophic diazotrophs. Heterotrophic diazotrophs, including Gammaproteobacteria and Group A cyanobacteria, are the dominant nitrogen-fixing organisms in oceanic and estuarine systems (Halm et al., 2012; Bentzon-Tilia et al., 2015). In this study, Cyanobacteria was the third most abundant phylum. In the rhizosphere of seagrass beds most nitrogen fixation is mediated by sulphate-reducing bacteria (Welsh et al., 1996). The Deltaproteobacteria, which contains most of the sulphate-reducing bacteria, represented a very small proportion (<0.5 %) of the community; however, Firmicutes were the second most abundant phylum, demonstrating that taxa capable of nitrogen fixation were present (Zehr and Paerl, 2008b).

The addition of exogenous carbon sources including glucose, sucrose and lactose, has been found to stimulate heterotrophic nitrogen fixation in cyanobacteria and sulphate reducing bacteria (Welsh et al., 1997; Newell et al., 2016a). The +C treatment exhibited an overall net N_2 uptake whereas the control receiving waste only exhibited net N_2 production, indicating that carbon supplementation enhanced nitrogen fixation. Similar to DNRA and denitrification, the rates of heterotrophic nitrogen fixation in coastal marine sediments are frequently limited by organic carbon availability (Welsh et al., 1997; Newell et al., 2016a).

Benthic incubation chambers integrate the exchange of gases and nutrients across the sediment-water interface; thus, while many reactions may be occurring within the sediments, the net outcome of sediment reactions are translated into benthic fluxes. It was anticipated that combining this traditional approach with next generation sequencing would elucidate the response of sediment microbial communities to carbon addition by highlighting shifts in taxonomy and functional potential. Benthic flux incubations detected a significant enhancement of NH_4^+ production during light incubations in response to carbon

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706 supplementation; however, no statistically significant differences in the microbial community
707 or predicted nitrogen transformation pathways were observed. Robinson et al. (2016) showed
708 that increasing the availability of rate-limiting electron acceptors (oxygen) had a marked
709 effect on the sediment microbial taxonomic composition, structure, metabolic capacity and
710 functional potential. In contrast, increasing the availability of potential electron donors
711 through carbon supplementation did not significantly affect the microbial community
712 structure. Significant variations at different sediment depths was likely due to the partitioning
713 of processes within the oxic and anoxic layers. None of the environmental parameters,
714 sediment characteristics, and gas or nutrient fluxes were significantly correlated with
715 microbial community structure and no significant differences were observed in the relative
716 abundance of predicted genes involved in the major nitrogen transformation pathways.
717 The benthic nitrogen cycle is one of the most complex biogeochemical cycles,
718 characterised by a diverse set of dissimilatory microbial processes (Thamdrup and Dalsgaard,
719 2008). The lack of significant changes in microbial community structure and functioning may
720 indicate that processes that contribute NH_4^+ to the sediment were operating concurrently with
721 transformations that removed NH_4^+ from the system, such as anammox and coupled
722 nitrification-denitrification. Furthermore, organic carbon can fulfil many functions under
723 reducing conditions: as an electron donor in redox reactions; a substrate for fermentation; or
724 as an organic substrate assimilated by heterotrophic bacteria coupled with NH_4^+ uptake. The
725 dual biogeochemical-molecular approach holds promise to further our understanding of
726 nitrogen cycling, the challenge remains to resolve net biogeochemical fluxes with molecular
727 tools that define microbial communities.
728 Our findings indicate that carbon addition may partly bioremediate nitrogen-rich
729 effluent by retaining nitrogen within the system, however longer-term trials are necessary to
730 determine whether this translates into improved sea cucumber biomass yields. In the current
731 study, the sea cucumbers decreased in mass with growth rates of 0.02 $g.day^{-1}$, however there
732 was no significant difference in mean wet weight of the sea cucumbers at the start or end of
733 the experiment. Two key factors are likely to have accounted for the differences in growth
734 performance of *Holothuria scabra* in the present study and the previous study of Robinson et
735 al. (*in review*). Firstly, chambers were shaded from direct sunlight in this experiment to
736 mitigate against water temperature spikes that would likely have caused hypoxia in the small
737 sealed chambers. However, because high light levels may be important for *Holothuria scabra*
738 growth (Battaglene et al. 1999), this may have resulted in the lower growth performance.
739 Secondly, the duration over which the sediment microbial community was allowed to

develop differed between the studies. In Robinson et al. (*in review*) the trials lasted 112 days compared with the current 28 day study (14 day preconditioning and 14 day experimental).

5. Conclusion

Pathways that support retention of nitrogen in sediments can dominate over pathways for permanent removal (Newell et al., 2016a), particularly in tropical ecosystems such as seagrass and mangrove systems (the natural habitat of *H. scabra*). This imbalance between denitrification and nitrogen fixation is partially responsible for nitrogen limitation in these systems (Fulweiler et al., 2013; Newell et al., 2016b). Thus, DNRA and heterotrophic nitrogen fixation are important processes for retaining nitrogen and sustaining ecosystem productivity (Fernandes et al., 2012; Enrich-Prast et al., 2016; Decleyre et al., 2015). In shallow euphotic sediments, these processes are likely important for overcoming nitrogen limitation and competition with benthic microalgae and cyanobacteria, by recycling and retaining NH_4^+ in the sediment. The increase in NH_4^+ efflux combined with net uptake of N_2 into the sediment in response to carbon addition indicates that under nutrient loading rates consistent with hypereutrophic estuaries (400 mmol C m^{-2} day^{-1} and 240 N m^{-2} day^{-1}; Eyre and Ferguson, 2009), pathways that retained nitrogen could dominate over pathways of permanent removal.

The coupled biogeochemical-molecular approach was useful in providing an overview of the functional potential for different nitrogen cycling pathways; however, given the complexity of nitrogen cycling in marine sediments, future studies should include more disparate C/N treatments of longer duration and measure all individual processes including denitrification, anammox, DNRA and nitrogen fixation. Furthermore, the use of more targeted molecular approaches, such as metagenomic shotgun sequencing or quantitative polymerase chain reaction (qPCR) in conjunction with stable isotope labelling studies (e.g. Eyre et al. 2016) are recommended to fully elucidate the pathways of nitrogen cycling in response to C:N manipulation.

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was secured by G.R., C.L.W.J., S.M.S, C.S., B. D. E. Experiments were performed by G.R and T.M with equipment provided by C.S., T.P and B.D.E and data analysed by G.R. The manuscript was written by G.R. and G.S.C. and edited by and B.D.E, C.L.W.J., C.S., T.M, T.P. and S.M.S. All authors have approved the final article.

The authors declare no competing financial interests.

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983

984 **Figure legends**

985 **Fig. 1.** Mean (± standard error) net fluxes (in μmol m^{-2} h^{-1}; n = 5) of: a) dissolved oxygen
986 (DO); b) dissolved inorganic carbon (DIC); c) dinitrogen gas (N_2); and, d) gross primary
987 production (GPP) in incubation chambers containing sea cucumbers and aquaculture waste
988 with (+C) or without (-C) carbon addition, incubated under light and dark conditions between
989 day 1 and day 13.

990 **Fig. 2.** Mean (± standard error) benthic light, dark and net fluxes (in μmol m^{-2} h^{-1}; n = 5) of:
991 a) phosphate (PO_4^{3-}); b) ammonium (NH_4^+); c) nitrite (NO_2^-); and d) nitrate and nitrite (NO_x)
992 in incubation chambers containing sea cucumbers and aquaculture waste with (+C) or
993 without (-C) carbon addition, incubated under light and dark conditions between day 1 and
994 day 13.

995 **Fig. 3.** Vertical depth profiles of sediment characteristics: a) organic carbon; b) total nitrogen;
996 c) carbon to nitrogen ratio (C:N); d) total carbohydrate; and, e) bacterial abundance. Cores
997 were sectioned on day zero prior to the addition of aquaculture waste (initial; In) and after
998 waste addition, both with and without carbon supplementation (carbon and no carbon
999 respectively) on day 14.

1000 **Fig. 4.** Alpha diversity metrics calculated on subsampled data. Observed = the number of
1001 operational taxonomic units (OTUs); ACE = abundance-coverage estimator; InvSimpson =
1002 Inverse Simpson diversity metric.

1003 **Fig. 5.** The mean proportion (%) and the difference in the mean proportion of taxa at: a)
1004 family and b) genus level between +C and -C treatments with 95 % confidence intervals.
1005 Significant differences in mean proportions were determined using two-sided Welch's t-tests
1006 (alpha = 0.05).

1007 **Fig. 6.** Principal Component Analysis ordination of the microbial community structure
1008 between the initial (In), +C and -C treatments at the five sediment depth intervals performed
1009 on a Bray-Curtis community dissimilarity matrix.

1010 **Fig. 7.** Vertical depth profiles of the predicted relative abundance of genes involved in the six
1011 nitrogen transformation pathways: a) nitrogen fixation; b) dissimilatory nitrate reduction to
1012 ammonium (DNRA); c) assimilatory nitrate reduction; d) denitrification; e) complete

nitrification; and, f) nitrification, under the pathway module of nitrogen metabolism in the Kyoto Encyclopaedia for Genes and Genomes (KEGG) database.

1019 **Table 1.** Description of the experimental treatments. The presence (✓) or absence (x) from
1020 day zero of aquaculture waste, added carbon source or sea cucumbers is indicated.

Treatment	Treatment code	No of replicates	Aquaculture waste	Sea cucumber	Carbon source	C:N
Initial	In	5	x	x	x	n/a
No added carbon	-C	5	✓	✓	x	5:1
Added carbon	+C	5	✓	✓	✓	20:1

1021

Table 2. Results of a non-parametric multivariate analysis of variance (PERMANOVA) testing the differences in microbial community structure at the five sediment depths prior to the addition of aquaculture waste (In) and after waste addition, both with and without carbon supplementation.

	df	SS	Mean squares	F model	R^2	p
Treatment (T)	2	0.797	0.399	1.195	0.058	0.115
Sediment depth (D)	4	1.705	0.426	1.278	0.123	0.011
T × D	8	2.656	0.332	0.996	0.192	0.494
Residuals	26	8.672	0.334		0.627	
Total	40	13.830			1.000	

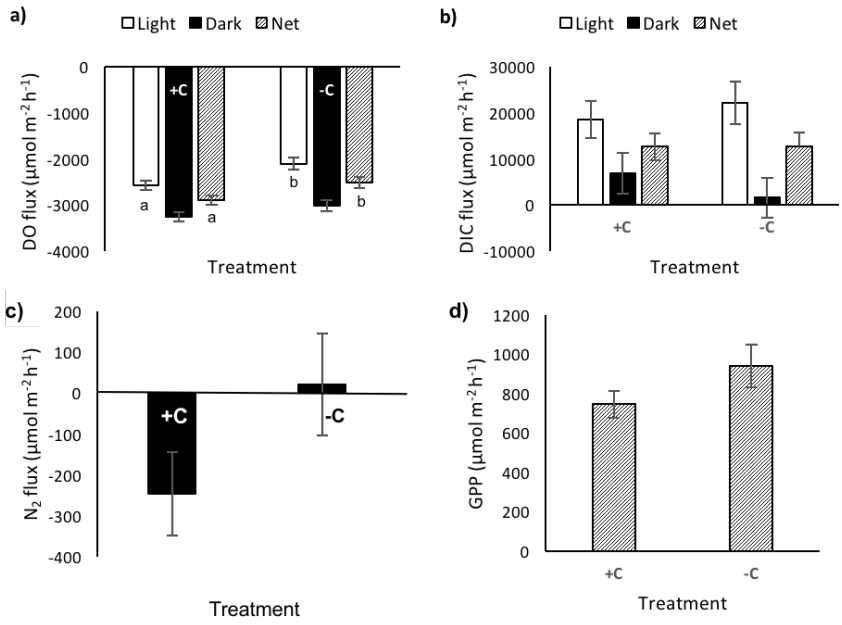
a)
Light
Dark
Net
DO flux (µmol m-2 h-1)
0
-1000
-2000
-3000
-4000
+C
-C
a
a
b
b
Treatment
b)
Light
Dark
Net
DIC flux (µmol m-2 h-1)
30000
20000
10000
0
-10000
+C
-C
Treatment
c)
N2 flux (µmol m-2 h-1)
200
100
0
-100
-200
-300
-400
+C
-C
Treatment
d)
GPP (µmol m-2 h-1)
1200
1000
800
600
400
200
0
+C
-C
Treatment

Fig. 1

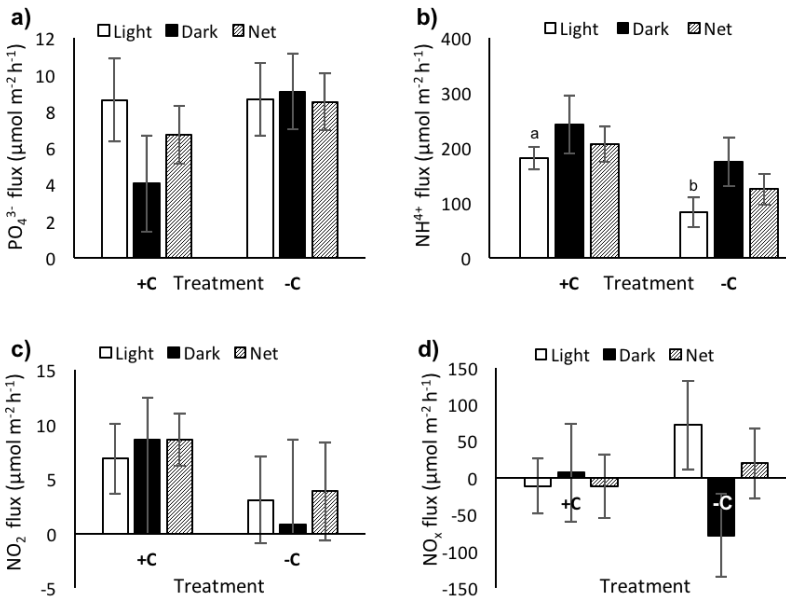
a)
Light
Dark
Net
PO_4^{3-} flux (µmol m^{-2} h^{-1})
12
10
8
6
4
2
0
+C
Treatment
-C
b)
Light
Dark
Net
NH^{4+} flux (µmol m^{-2} h^{-1})
400
300
200
100
0
a
b
+C
Treatment
-C
c)
Light
Dark
Net
NO_2 flux (µmol m^{-2} h^{-1})
15
10
5
0
-5
+C
-C
Treatment
d)
Light
Dark
Net
NO_x flux (µmol m^{-2} h^{-1})
150
100
50
0
-50
-100
-150
+C
-C
Treatment

Fig. 2

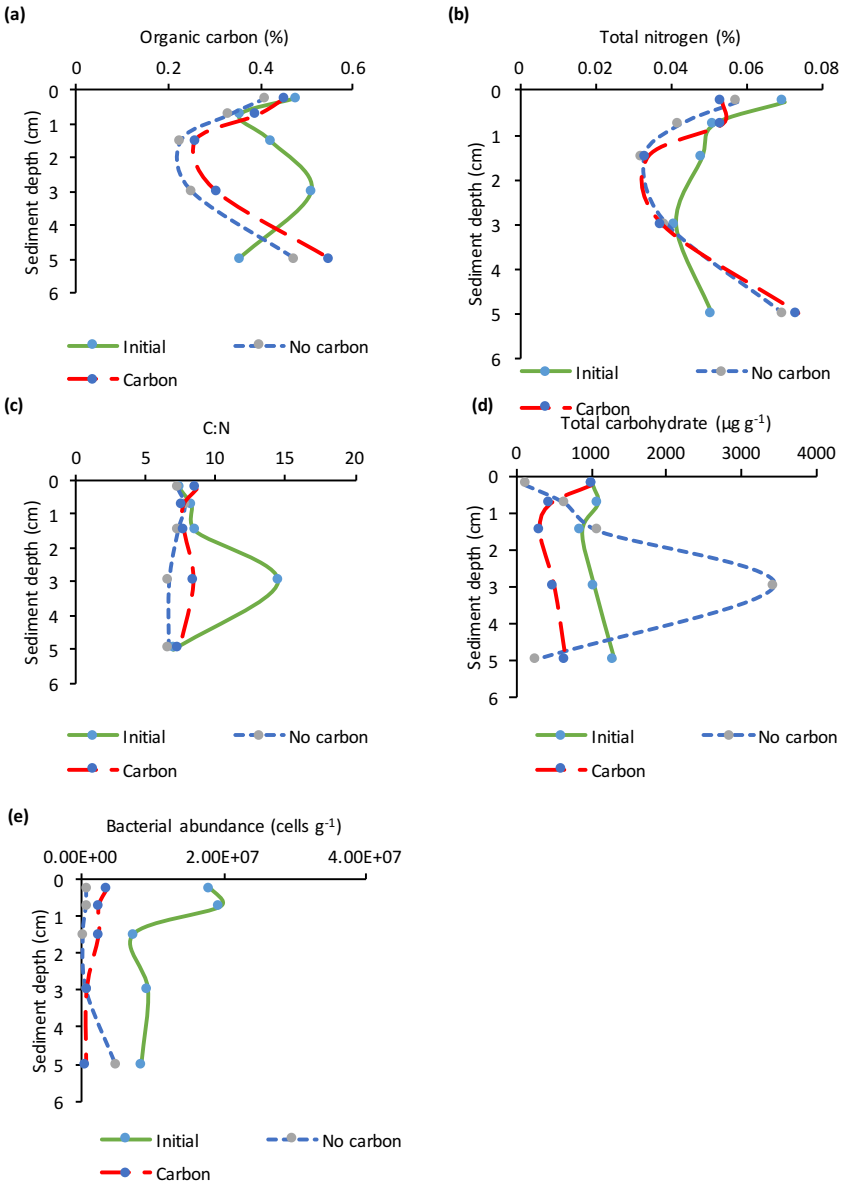
(a)
Organic carbon (%)
0
0.2
0.4
0.6
Sediment depth (cm)
0
1
2
3
4
5
6
Initial
No carbon
Carbon
(b)
Total nitrogen (%)
0
0.02
0.04
0.06
0.08
Sediment depth (cm)
Initial
No carbon
Carbon
(c)
C:N
0
5
10
15
20
Sediment depth (cm)
Initial
No carbon
Carbon
(d)
Total carbohydrate (μg g-1)
0
1000
2000
3000
4000
Sediment depth (cm)
Initial
No carbon
Carbon
(e)
Bacterial abundance (cells g-1)
0.00E+00
2.00E+07
4.00E+07
Sediment depth (cm)
Initial
No carbon
Carbon

Fig. 3

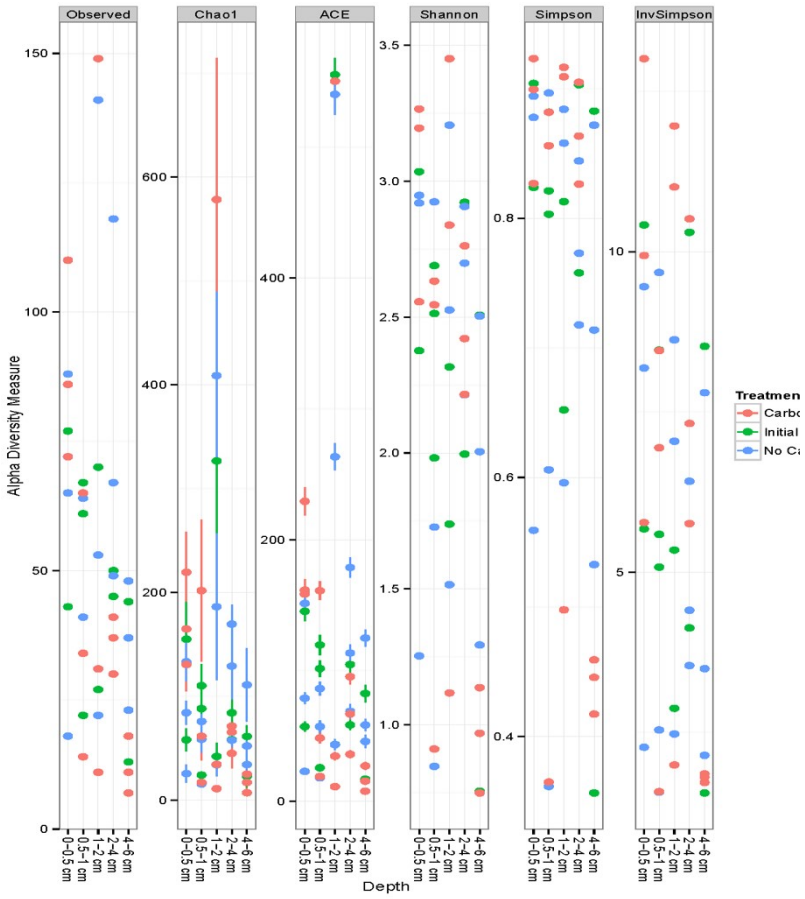
Observed
Chao1
ACE
Shannon
Simpson
InvSimpson
Alpha Diversity Measure
Depth
Treatmen
Carbo
Initial
No Ca
0–0.5 cm
0.5–1 cm
1–2 cm
2–4 cm
4–6 cm

Fig. 4

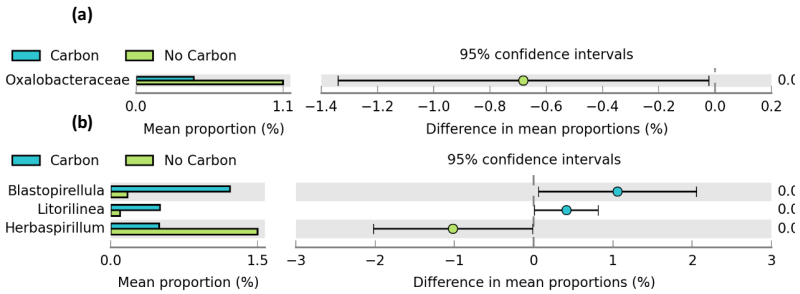
(a)
Carbon
No Carbon
Oxalobacteraceae
0.0
1.1
Mean proportion (%)
95% confidence intervals
−1.4
−1.2
−1.0
−0.8
−0.6
−0.4
−0.2
0.0
0.2
Difference in mean proportions (%)
(b)
Carbon
No Carbon
Blastopirellula
Litorilinea
Herbaspirillum
0.0
1.5
Mean proportion (%)
95% confidence intervals
−3
−2
−1
0
1
2
3
Difference in mean proportions (%)

Fig. 5

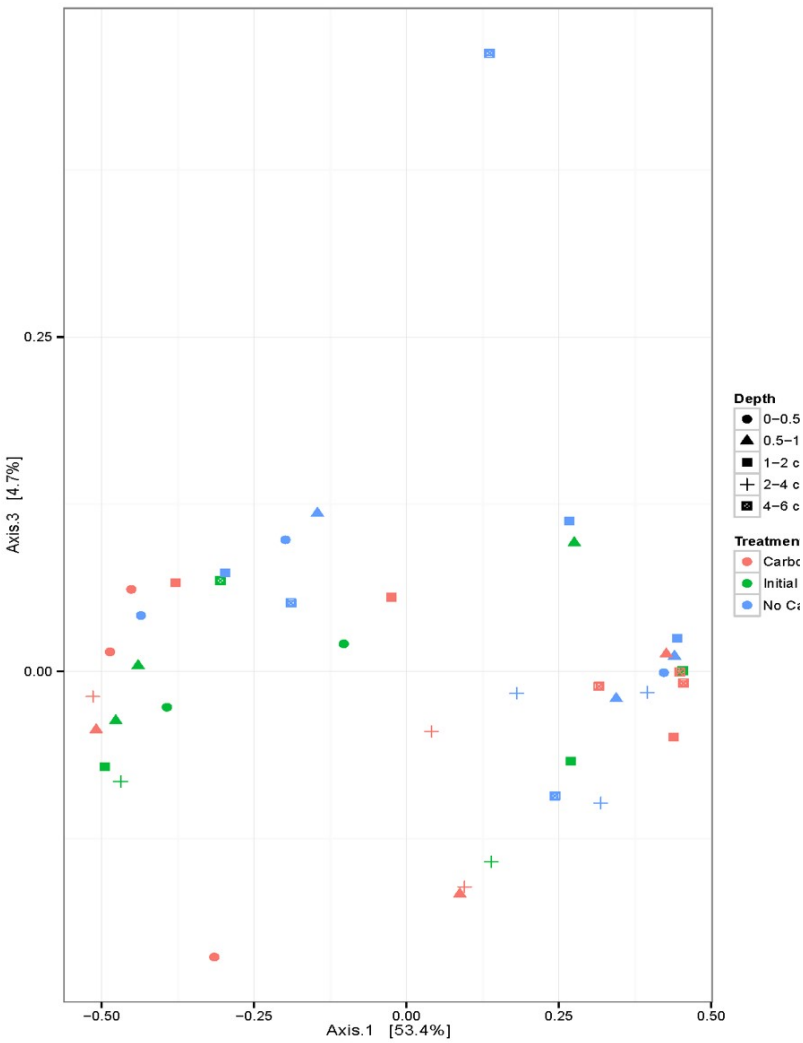
0.25
0.00
Axis.3 [4.7%]
−0.50
−0.25
0.00
0.25
0.50
Axis.1 [53.4%]
Depth
0–0.5
0.5–1
1–2 c
2–4 c
4–6 c
Treatmen
Carbo
Initial
No Ca

Fig. 6

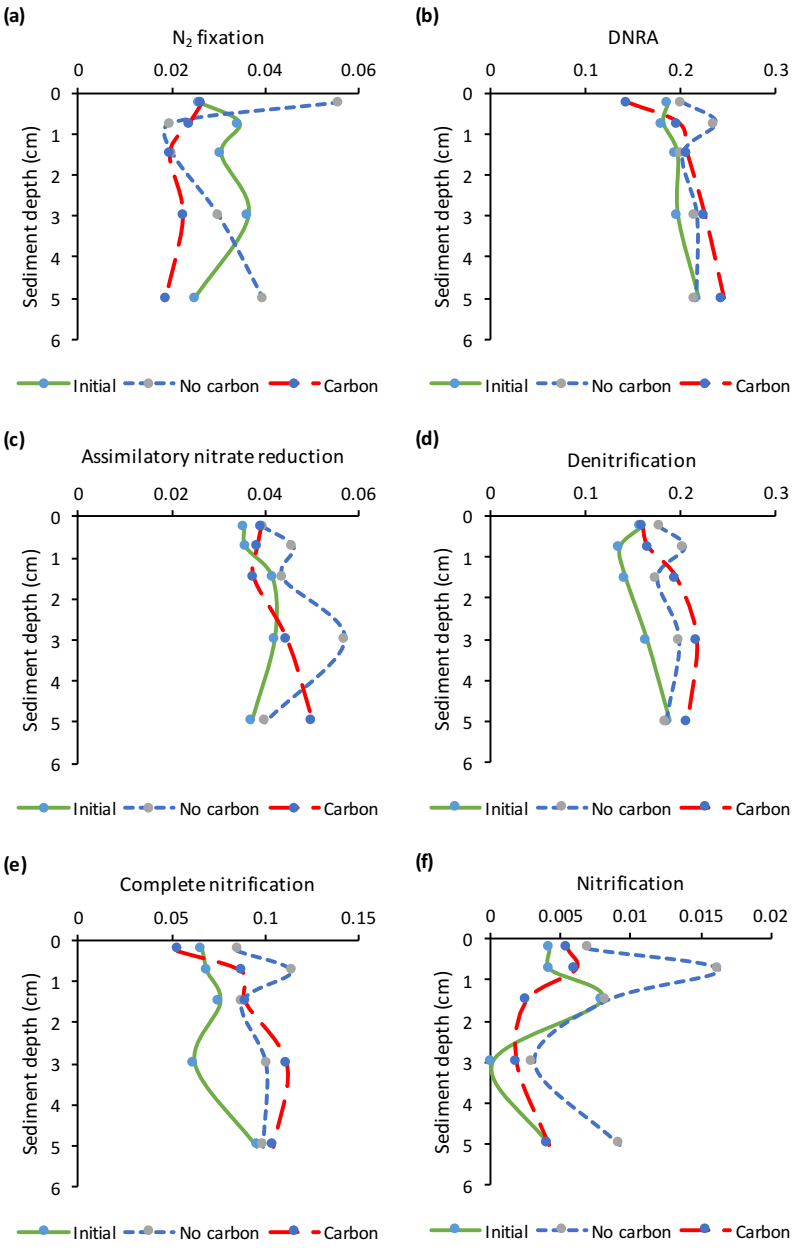
(a)
N2 fixation
0
0.02
0.04
0.06
Sediment depth (cm)
0
1
2
3
4
5
6
Initial
No carbon
Carbon
(b)
DNRA
0
0.1
0.2
0.3
Sediment depth (cm)
Initial
No carbon
Carbon
(c)
Assimilatory nitrate reduction
0
0.02
0.04
0.06
Sediment depth (cm)
Initial
No carbon
Carbon
(d)
Denitrification
0
0.1
0.2
0.3
Sediment depth (cm)
Initial
No carbon
Carbon
(e)
Complete nitrification
0
0.05
0.1
0.15
Sediment depth (cm)
Initial
No carbon
Carbon
(f)
Nitrification
0
0.005
0.01
0.015
0.02
Sediment depth (cm)
Initial
No carbon
Carbon

Fig. 7