Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

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

Georgina Robinson[1,2*,#], Thomas MacTavish[3], Candida Savage[3,4], Gary S. Caldwell[1], Clifford L.W. Jones[2], Trevor Probyn[5], Bradley D. Eyre[6] and Selina M. Stead[1]

[1]School of Natural and Environmental Sciences, Newcastle University, Newcastle, NE1 7RU, UK.

[2]Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown 6140, South Africa.

[3]Department of Marine Science, University of Otago, Dunedin 9016, New Zealand.

[4]Department of Biological Sciences and Marine Research Institute, University of Cape Town, Rondebosch 7700, Cape Town, South Africa.

[5]Marine and Coastal Management, Private Bag X2, Rogge Bay 8012, Cape Town, South Africa

[6]Centre for Coastal Biogeochemistry, Southern Cross University, Lismore, NSW 2480, Australia

*Corresponding author. Tel +230 5982 4971; Email address Georgina.Robinson@sams.ac.uk (G. Robinson)

#Current address: The Scottish Association for Marine Science, Scottish Marine Institute, PA37 1QA, Oban, UK.

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 process 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 into the sediment and retention of nitrogen within the system. The carbon amended treatment exhibited an overall net N_2 uptake whereas the control receiving only aquaculture waste exhibited net N_2 production, indicating that carbon supplementation enhanced nitrogen fixation. The higher NH_4^+ efflux and N_2 uptake was further supported by metagenome predictions that indicate that organic carbon addition stimulated DNRA over denitrification. These findings indicate that carbon addition can provide a means to successfully bioremediate nitrogen-rich effluents. Longer-term trials are necessary to determine whether this nitrogen retention is translated into improved sea cucumber biomass yields.

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.

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.

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

2. Materials and methods

2.1 Study site and experimental animals

The study was conducted in a purpose-built bio-secure heated 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 (26.8 mg day^{-1} wet weight). The 'added carbon' treatment (+C) received aquaculture waste (26.8 mg day^{-1} wet weight) and carbon in the form of soluble starch (7.5 mg day^{-1} dry weight) to increase the C:N to 20:1 from day zero (Table 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.* in review 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).

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

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 and added daily to the incubation chambers at 16:00 from day zero to day 14 at a concentration of 400 mmol C m^{-2} d^{-1}.

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 ± 12.73 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

approximately two hours after sunrise (08:00 local time) and dark incubations were conducted approximately two hours after sunset (22:00 local time). When data were collected the flow from each chamber was interrupted, the stirrers were paused 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.

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

195 determination of nitrate and nitrite in seawater). All other nutrient samples were analysed
196 manually. Ammonium (0.01 µM) and dissolved inorganic phosphate (0.01 µM) were
197 determined using the methods of Grasshoff (1976) and Grasshoff et al. (1999) respectively,
198 and nitrite (NO_2-; 0.01 µM)) was determined according to Bendscheider and Robinson (1952).

199 ***2.9 Gas analyses***

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

206 Dinitrogen gas (N_2) was determined from N_2:Ar using membrane inlet mass
207 spectrometry (MIMS) with O_2 removal (± 0.01%). Measurement of direct N_2 fluxes using this
208 technique represents the net benthic flux of N_2 resulting from a combination of processes that
209 produce N_2, such as denitrification and anammox, and processes that consume N_2 such as
210 nitrogen fixation (Ferguson and Eyre, 2007;Eyre et al., 2013a).

211 Nutrient and gas fluxes across the sediment-water interface during light and dark
212 incubations were calculated using initial and final concentration data according to Equation 1.
213 Net flux rates, representing the net result of 13.57 h of dark fluxes and 10.43 h of light fluxes
214 were calculated according to Equation 2 (Veuger et al., 2007). Gross primary production was
215 calculated according to

216 Equation 3, where light O_2 fluxes represent net primary production and dark fluxes
217 represent respiration. Remineralisation ratios were calculated according to Equation 4 (Eyre et
218 al. (2013b).

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

220 where:

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

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

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

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

227

228 ***2.10 Sediment sectioning***

229 On days zero and 14, three sub-cores (internal diameter 30 mm) were extracted from
230 the In and experimental (-C and +C) chambers respectively. Each sub-core was sectioned into
231 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
232 analysis of sediment characteristics. One set of sub-cores was dried at 50 °C for 24 h for
233 analysis of total organic carbon and total nitrogen; the second set was frozen in sealed vials in
234 black bags for spectrophotometric analysis of total carbohydrates. Two sets of samples were
235 prepared from the third sub-core: sediment samples were frozen in 2 mL Eppendorf tubes for
236 subsequent deoxyribonucleic acid (DNA) extraction and sequencing. The remaining sediment
237 was added to 15 mL vials filled with 0.2 µm filtered, one percent buffered paraformaldeyde
238 and refrigerated for determination of bacterial abundance by flow cytometry.
239 The organic content measured as particulate organic carbon (OC) and total nitrogen
240 (TN) was determined on an elemental analyser after removal of carbonates by fuming
241 (Robinson et al., 2015). Total sediment carbohydrates were measured on defrosted samples
242 (Robinson et al., in review).

243 ***2.11 Flow cytometry***

244 Aliquots of preserved samples were prepared in duplicate by staining with
245 4′,6-diamidino-2-phenylindole (DAPI) for 15 minutes at 4 °C in darkness (Marie et al., 1999).
246 Bacterial abundance was analysed with a FACSCalibur flow cytometer (BD Biosciences,
247 Singapore), fitted with a 488 nm, 15 mW laser, using the FL1 detector (λ = 530 nm). TruCount
248 beads (BD Biosciences, Singapore) were used as an internal standard. All cytometric data were
249 logged and analysed using Cell Quest (Becton-Dickinson) using *Escherichia coli* cells as a
250 reference. Cell abundance was converted to cells g^{-1} of dry sediment.

251 ***2.12 Deoxyribonucleic acid extraction and importation***

252 Genomic DNA was extracted from approximately 250 mg of substrate samples using a
253 DNA isolation kit (ZR Soil Microbe DNA MiniPrep, Zymo Research, USA) yielding purified
254 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 1). 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 (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). 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 flux 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). Fluxes of oxygen and DIC clearly indicated 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 an influx of oxygen into the sediment 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 influx (-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 nitrogen removal pathways, such as denitrification or anaerobic ammonium oxidation (anammox), were slightly greater than nitrogen fixation.

There were no significant differences in the dark or net fluxes of any of the nutrients between treatments on day -1; however, the NH_4^+ fluxes during light incubations were significantly different (one-way ANOVA; $F_{(2, 9)}$ = 12.73, p = 0.002). The In chambers had a significantly higher NH_4^+ efflux of 115.32 ± 11.43 µmol m^{-2} h^{-1} compared with an influx 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.

3.3 Sediment characteristics and remineralisation ratios

The sediment organic carbon (OC) content decreased over the course of the experiment (Fig. 3a). The largest decrease was observed at the 1.0 – 2.0 cm and 2.0 – 4.0 cm depth intervals

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

3.3 Microbial community analysis and nitrogen metabolism functional gene prediction

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

Neither carbon addition, sediment depth nor the interaction between the factors (treatment × sediment depth) significantly affected the number of sequences, OTUs (observed species), community richness (Chao and ACE), or diversity measured as Simpson and Inverse Simpson indices (mixed model ANOVA; $p < 0.05$; Fig. 4). Sediment depth significantly influenced Shannon diversity, with the highest diversity of 2.85 recorded in the sediment

surface layer (0 - 0.5 cm) and the lowest (1.54) in the 4 - 6 cm layer (mixed model ANOVA; $F_{(4, 26)} = 3.14$, $p = 0.031$).

Flow cytometry data compared relatively well with the 16S rRNA amplicon sequencing data. Bacterial abundance (cells g^{-1}; Fig. 3e), the number of sequences and OTUs were higher in the In chambers than the experimental chambers sampled on day 14, presumably in response to grazing by the sea cucumbers. The number of OTUs decreased from 286.81 ± 128.13 in the In chambers to 176 ± 65.15 and 181.20 ± 45.90 in the +C and -C treatments respectively. Overall, the community diversity was low: Shannon diversity = 2.31 ± 0.13, Inverse Simpson = 5.79 ± 0.51. There was a marked increase in community richness at the 1 - 2 cm depth interval, coinciding with the oxic-anoxic interface. In the In chambers the number of OTUs was 778.00 ± 731.00, compared with 343.33 ± 199.25 and 322.67 ± 307.25 in the +C and -C treatments respectively. The Chao 1 richness indicator also followed this trend (Fig. 4).

The majority of sequences (99.8 %) were assigned to the Bacteria, with only 0.12 % assigned to Archaea. Taxa from four archaeal phyla were present, including Euryarchaeota, Thaumarchaeota and Woesearchaeota or were unassigned. *Natronorubrum* (Euryarchaeota), a halophilic aerobic chemoorganotroph, was the most abundant genus representing 14 of the 27 archaeal reads (Xu et al., 1999).

The bacterial community contained a total of 18 phyla, four candidate phyla and the candidate division WPS-2. Proteobacteria and Firmicutes were the two dominant phyla accounting for 47.64 and 34.71 % of the total sequences respectively, with Cyanobacteria accounting for 7.42 %. Planctomycetes (2.45 %), Actinobacteria (2.34 %), unclassified Bacteria (2.12 %) and Bacteroidetes (1.33 %) were minor components. The remainder of the phyla, candidate phyla and the candidate division WPS-2 each represented less than 1 % of the community. Candidate phyla included Hydrogenedentes (formerly NKB19), Latesbacteria (formerly WS3), Parcubacteria (formerly OD1) and Poribacteria.

Taxa within the Oxalobacteraceae and the genus *Herbaspirillum* were significantly more abundant in the -C treatment (Welch's two-sided t-test; $p < 0.05$; Fig. 5). In comparison, the genera *Blastopiellula* and *Litorilinea* were significantly enriched in the +C treatment. There were no significant differences in the mean proportion of taxa between experimental treatments at phylum, class or order levels, underscoring the high degree of similarity among the microbial communities between treatments (Fig. 6). Further, there was no correlation between the microbial community and environmental data (Mantel test; $r = 0.04$, $p = 0.27$). The first axis in the PCoA ordination explained 53.4 % of the variation and appeared to be associated with sediment depth, while the second axis (4.7 % of the variation) appeared to be associated with

experimental treatment. Treatment did not significantly influence microbial community structure (PERMANOVA; $p<0.05$; Table 2), which may be a function of the relatively short duration of the experiment. By contrast, there was a significant effect of sediment depth on the microbial community (PERMANOVA; $p=0.011$; Table 2).

There were no significant differences in the predicted relative abundance of genes involved in the six nitrogen transformation pathways (mixed model ANOVA; $p > 0.05$; Fig. 7). The relative abundance of predicted nitrification genes peaked at the 0.5 – 1.0 cm depth interval in the -C treatment, coinciding with the oxic zone. In the +C treatment, the relative abundance of predicted denitrification and DNRA genes were higher in the sediment layers sectioned at 1.0 – 2.0, 2.0 – 4.0 and 4.0 – 6.0 cm. Overall, DNRA was the dominant pathway (20.52 ± 0.01 %) predicted to occur in all treatments and sediment depths, with the exception of the surface layer (0.0 - 0.5 cm) in the +C treatment, where there was a higher predicted relative abundance of denitrification genes (Fig. 7). Denitrification was the second most abundant predicted pathway (18.02 ± 0.01 %), followed by complete nitrification (8.80 ± 0.43 %), indicating that the potential for coupled nitrification-denitrification was present in all treatments. Genes predicted to be involved in nitrogen fixation represented 2.85 ± 0.32 %.

4. Discussion

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

It was hypothesised that increasing the C:N would mediate a shift from ammonification (net release) to NH_4^+ assimilation (net uptake), leading to an overall decrease in NH_4^+ efflux, however, net NH_4^+ production was higher in +C treatments. NH_4^+ can originate from four nitrogen transformation pathways; ammonification (degradation of organic nitrogenous waste), nitrogen fixation, assimilatory reduction of nitrate to ammonia (ARNA), and dissimilatory nitrate reduction to ammonia (DNRA), in addition to sea cucumber excretion. ARNA and nitrogen fixation are both assimilatory pathways that occur within organisms, and therefore do

not contribute to an increase in NH_4^+ concentration at the sediment-water interface (Gardner et al., 2006). Ammonification and DNRA are therefore the only pathways with the potential to contribute to increased NH_4^+ production in the +C treatment, however the increased NH_4^+ concentration in the +C treatment is unlikely to have originated from ammonification since the waste was added on an isonitrogenous basis.

An increasing number of studies have demonstrated the importance, and indeed dominance of DNRA in nearshore shallow water coastal environments, particularly in tropical ecosystems (Decleyre et al., 2015;Fernandes et al., 2012;Gardner et al., 2006;Song et al., 2014;Erler et al., 2013). For example, Fernandes et al. (2012) showed that DNRA can account for 99 % of nitrate removal in nitrogen-limited mangrove ecosystems. In marine sediments, DNRA and denitrification compete for nitrate; however, denitrification results in the permanent removal of nitrogen from the system whereas DNRA retains bioavailable nitrogen in sediments by reducing nitrate to NH_4^+ (Gardner et al., 2006). Since these nitrogen transformation processes are reductive pathways, mediated by heterotrophic bacteria in the anaerobic zone of redox-stratified sediments, carbon addition can stimulate both denitrification and DNRA (Hardison et al., 2015). In some aquaculture systems the availability of organic carbon is known to limit N_2 production via denitrification (Castine et al., 2012); therefore, carbon supplementation is employed to successfully operate denitrifying filters (Castine, 2013;Roy et al., 2010). However, Castine (2013) found no significant differences in N_2 production when aquaculture slurries were amended with particulate organic matter or methanol as carbon sources. Other studies have found that high organic loading rates and/or the addition of exogenous carbon sources stimulated DNRA and concluded that high organic carbon loading is a pre-requisite for DNRA to be favoured over denitrification (Hardison et al., 2015;Capone, 2000). In the present study, the higher NH_4^+ efflux in the +C treatment, supported by the metagenome predictions and the influx of N_2 gas, would suggest that organic carbon addition stimulates DNRA over denitrification.

Increasing the organic carbon availability can potentially stimulate all four nitrogen reduction pathways (supplementary Fig. 1). These pathways, with the exception of denitrification, result in ammonia production and therefore contribute to nitrogen retention within the system (Hardison et al., 2015). The factors regulating the balance between denitrification and nitrogen fixation are not well understood; however, the quality and quantity of organic carbon may influence the balance between these processes (Fulweiler et al., 2013). Historically, denitrification has been considered to be the main pathway of nitrogen loss, based on deficiencies in mass balance calculations (Seitzinger, 1988). However, in sediment-based

551 systems enriched with particulate organic waste (such as settlement ponds in aquaculture
552 systems) the processes of permanent nitrogen removal account for a very small fraction of the
553 total nitrogen that is permanently removed from the system. For example, Castine et al. (2012)
554 found that denitrification and anammox only removed 2.5 % of total nitrogen inputs to
555 settlement ponds in intensive shrimp and barramundi farms.

556 Sediment nitrogen fixation can equal or exceed N_2 loss in estuarine systems (Newell et
557 al., 2016a). The genetic potential for nitrogen fixation is widespread within the Bacteria and
558 Archaea (Newell et al., 2016b;Zehr and Paerl, 2008a). Heterotrophic nitrogen fixation has not
559 been widely demonstrated in sediments beyond the observation of N_2 uptake (Gardner et al.,
560 2006); however, recent studies provide direct evidence by measuring *in situ* N_2 production
561 combined with molecular and genomic tools to quantify the presence of the nitrogenase
562 reductase (*nifH*) gene (Newell et al., 2016b;Baker et al., 2015). Indirect evidence for nitrogen
563 fixation is provided in the present study by the presence of *nifH* (K02588) in all samples and
564 the taxonomic composition of the microbial communities.

565 Nitrogen fixation can be mediated by photoautotrophic and heterotrophic diazotrophs.
566 Heterotrophic diazotrophs, including Gammaproteobacteria and Group A cyanobacteria, are
567 the dominant nitrogen-fixing organisms in oceanic and estuarine systems (Halm et al.,
568 2012;Bentzon-Tilia et al., 2015). In this study, Cyanobacteria was the third most abundant
569 phylum. In the rhizosphere of seagrass beds most nitrogen fixation is mediated by sulphate-
570 reducing bacteria (Welsh et al., 1996). The Deltaproteobacteria, which contains most of the
571 sulphate-reducing bacteria, represented a very small proportion (<0.5 %) of the community;
572 however, Firmicutes were the second most abundant phylum, demonstrating that taxa capable
573 of nitrogen fixation were present (Zehr and Paerl, 2008b).

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

581 Benthic incubation chambers integrate the exchange of gases and nutrients across the
582 sediment-water interface; thus, while many reactions may be occurring within the sediments,
583 the net outcome of sediment reactions are translated into benthic fluxes. It was anticipated that
584 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 supplementation; however, no statistically significant differences in the microbial community or predicted nitrogen transformation pathways were observed. Robinson et al. (2016) showed that increasing the availability of rate-limiting electron acceptors (oxygen) had a marked effect on the sediment microbial taxonomic composition, structure, metabolic capacity and functional potential. In contrast, increasing the availability of potential electron donors through carbon supplementation did not significantly affect the microbial community structure. Significant variations at different sediment depths was likely due to the partitioning of processes within the oxic and anoxic layers. None of the environmental parameters, sediment characteristics, and gas or nutrient fluxes were significantly correlated with microbial community structure and no significant differences were observed in the relative abundance of predicted genes involved in the major nitrogen transformation pathways.

The benthic nitrogen cycle is one of the most complex biogeochemical cycles on earth, characterised by a diverse set of dissimilatory microbial processes (Thamdrup and Dalsgaard, 2008). The lack of significant changes in microbial community structure and functioning may indicate that processes that contribute NH_4^+ to the sediment were operating concurrently with transformations that removed NH_4^+ from the system, such as assimilation by heterotrophic bacteria, anammox and coupled nitrification-denitrification. Furthermore, organic carbon can fulfil many functions under reducing conditions: as an electron donor in redox reactions; a substrate for fermentation; or as an organic substrate assimilated by heterotrophic bacteria coupled with NH_4^+ uptake. The resulting effects may have been less discernible than originally predicted.

5. Conclusion

Pathways that support retention of nitrogen in sediments can dominate 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 influx of N_2 into the sediment in response to carbon addition indicates that even 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 dominated pathways of permanent removal, underscoring the immense capacity of sediments for assimilating nitrogen from land-based intensive aquaculture systems.

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 are recommended to fully elucidate the pathways of nitrogen cycling in response to C:N manipulation.

Acknowledgements: This research was funded by a Biotechnology and Biological Sciences Research Council (BBSRC) Industrial CASE Studentship to G.R. (Grant Code BB/J01141X/1) with HIK Abalone Farm Pty Ltd as the CASE partner, with additional contributions from the ARC Grant DP160100248. The work was conceptualised and funding 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.

References

Almgren, T., Dryssen, D., and Fonselius, S.: Determination of alkalinity and total carbonate, in: Methods of seawater analysis, 2nd ed., edited by: Grasshoff, K., Ehrhardt, M., and Kremling, K., Verlag Chemie, Weinheim, West Germany, 99-123, 1983.

Anderson, M. J.: A new method for non-parametric multivariate analysis of variance, Austral Ecology, 26, 32-46, 10.1111/j.1442-9993.2001.01070.pp.x, 2001.

Aßhauer, K. P., Wemheuer, B., Daniel, R., and Meinicke, P.: Tax4Fun: predicting functional profiles from metagenomic 16S rRNA data, Bioinformatics, 31, 2882-2884, 10.1093/bioinformatics/btv287, 2015.

Avnimelech, Y.: Carbon/nitrogen ratio as a control element in aquaculture systems, Aquaculture, 176, 227-235, 10.1016/s0044-8486(99)00085-x, 1999.

Baker, B. J., Lazar, C. S., Teske, A. P., and Dick, G. J.: Genomic resolution of linkages in carbon, nitrogen, and sulfur cycling among widespread estuary sediment bacteria, Microbiome, 3, 1-12, 10.1186/s40168-015-0077-6, 2015.

Bendscheider, K., and Robinson, R. J.: A new spectrophotometric method for the determination of nitrite in sea water, Journal of Marine Research, 11, 87-96, 1952.

Bentzon-Tilia, M., Traving, S. J., Mantikci, M., Knudsen-Leerbeck, H., Hansen, J. L. S., Markager, S., and Riemann, L.: Significant N_2 fixation by heterotrophs, photoheterotrophs and heterocystous cyanobacteria in two temperate estuaries, ISME J, 9, 273-285, 10.1038/ismej.2014.119, 2015.

Blackburn, T. H.: Nitrogen cycle in marine sediments, Ophelia, 26, 65-76, 10.1080/00785326.1986.10421979, 1986.

Burford, M., A. , and Longmore, A., R.: High ammonium production from sediments in hypereutrophic shrimp ponds, Marine Ecology Progress Series, 224, 187-195, 2001.

Capone, D. G.: The marine nitrogen cycle, in: Microbial Ecology of the Oceans, 1st ed., edited by: Kirchman, D. L., Wiley-Liss, 455-493, 2000.

673 Castine, S. A., Erler, D. V., Trott, L. A., Paul, N. A., de Nys, R., and Eyre, B. D.: Denitrification
674 and anammox in tropical aquaculture settlement ponds: an isotope tracer approach for
675 evaluating N2 production, PLoS ONE, 7, 10.1371/journal.pone.0042810, 2012.

676 Castine, S. A.: Nitrogen removal and reuse in land-based intensive aquaculture, PhD, PhD
677 thesis. James Cook University. 173p, 173 pp., 2013.

678 Cook, P. L. M., Veuger, B., Boer, S., and Middelburg, J. J.: Effect of nutrient availability on
679 carbon and nitrogen incorporation and flows through benthic algae and bacteria in near-shore
680 sandy sediment, Aquatic Microbial Ecology, 49, 165-180, 10.3354/ame01142, 2007.

681 Crab, R., Defoirdt, T., Bossier, P., and Verstraete, W.: Biofloc technology in aquaculture:
682 beneficial effects and future challenges, Aquaculture, 356-357, 351-356,
683 10.1016/j.aquaculture.2012.04.046, 2012.

684 Decleyre, H., Heylen, K., Van Colen, C., and Willems, A.: Dissimilatory nitrogen reduction in
685 intertidal sediments of a temperate estuary: small scale heterogeneity and novel nitrate-to-
686 ammonium reducers, Frontiers in Microbiology, 6, 1124, 10.3389/fmicb.2015.01124, 2015.

687 Dodds, W. K., Marti, E., Tank, J. L., Pontius, J., Hamilton, S. K., Grimm, N. B., Bowden, W.
688 B., McDowell, W. H., Peterson, B. J., Valett, H. M., Webster, J. R., and Gregory, S.: Carbon
689 and nitrogen stoichiometry and nitrogen cycling rates in streams, Oecologia, 140, 458-467,
690 10.1007/s00442-004-1599-y, 2004.

691 Ebeling, J. M., Timmons, M. B., and Bisogni, J. J.: Engineering analysis of the stoichiometry
692 of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in
693 aquaculture systems, Aquaculture, 257, 346-358, 10.1016/j.aquaculture.2006.03.019, 2006.

694 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R.: UCHIME improves
695 sensitivity and speed of chimera detection, Bioinformatics, 27, 2194-2200,
696 10.1093/bioinformatics/btr381, 2011.

697 Edmond, J. M.: High precision determination of titration alkalinity and total carbon dioxide
698 content of sea water by potentiometric titration, Deep Sea Research and Oceanographic
699 Abstracts, 17, 737-750, 10.1016/0011-7471(70)90038-0, 1970.

Enrich-Prast, A., Figueiredo, V., De Esteves, F. A., and Nielsen, L. P.: Controls of sediment nitrogen dynamics in tropical coastal lagoons, PLoS ONE, 11, 10.1371/journal.pone.0155586, 2016.

Erler, D. V., Trott, L. A., Alongi, D. M., and Eyre, B. D.: Denitrification, anammox and nitrate reduction in sediments of the southern Great Barrier Reef lagoon, Marine Ecology Progress Series, 478, 57-70, 2013.

Eyre, B. D., Rysgaard, S., Dalsgaard, T., and Christensen, P. B.: Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification- assumption, modifications, and implications, Estuaries, 25, 1077-1087, 10.1007/BF02692205, 2002.

Eyre, B. D., and Ferguson, A. J. P.: Denitrification efficiency for defining critical loads of carbon in shallow coastal ecosystems, Hydrobiologia, 629, 137-146, 10.1007/s10750-009-9765-1, 2009.

Eyre, B. D., Ferguson, A. J., Webb, A., Maher, D., and Oakes, J. M.: Metabolism of different benthic habitats and their contribution to the carbon budget of a shallow oligotrophic sub-tropical coastal system (southern Moreton Bay, Australia), Biogeochemistry, 102, 87-110, 2011.

Eyre, B. D., Maher, D. T., and Squire, P.: Quantity and quality of organic matter (detritus) drives N2 effluxes (net denitrification) across seasons, benthic habitats, and estuaries, Global Biogeochemical Cycles, 27, 1083-1095, 10.1002/2013GB004631, 2013a.

Eyre, B. D., Santos, I. R., and Maher, D. T.: Seasonal, daily and diel N_2 effluxes in permeable carbonate sediments, Biogeosciences, 10, 2601-2615, 10.5194/bg-10-2601-2013, 2013b.

Fenchel, T., King, G. M., and Blackburn, T. H.: Bacterial Biogeochemistry. The Ecophysiology of Mineral Cycling, 3rd ed., Academic Press. 307 pp, San Diego, 2012.

Ferguson, A. J. P., Eyre, B. D., and Gay, M.: Benthic nutrient fluxes in euphotic sediments along shallow sub-tropical estuaries, northern New South Wales, Australia, Aquatic Microbial Ecology, 37, 219-235, 2004.

726 Ferguson, A. J. P., and Eyre, B. D.: Seasonal discrepancies in denitrification measured by
727 isotope pairing and N_2:Ar techniques, Marine Ecology Progress Series, 350, 19-27,
728 10.3354/meps07152, 2007.

729 Fernandes, S. O., Bonin, P. C., Michotey, V. D., Garcia, N., and LokaBharathi, P. A.: Nitrogen-
730 limited mangrove ecosystems conserve N through dissimilatory nitrate reduction to
731 ammonium, Scientific Reports, 2, 419, 10.1038/srep00419, 2012.

732 Fulweiler, R. W., Brown, S. M., Nixon, S. W., and Jenkins, B. D.: Evidence and a conceptual
733 model for the co‐occurrence of nitrogen fixation and denitrification in heterotrophic marine
734 sediments, Marine Ecology Progress Series, 482, 57-68, 2013.

735 Gardner, W. S., McCarthy, M. J., An, S., Sobolev, D., Sell, K. S., and Brock, D.: Nitrogen
736 fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics
737 in Texas estuaries, Limnology and Oceanography, 51, 558-568,
738 10.4319/lo.2006.51.1_part_2.0558, 2006.

739 Glud, R. N.: Oxygen dynamics of marine sediments, Marine Biology Research, 4, 243-289,
740 10.1080/17451000801888726, 2008.

741 Glud, R. N., Eyre, B. D., and Patten, N.: Biogeochemical responses to mass coral spawning at
742 the Great Barrier Reef: Effects on respiration and primary production, Limnology and
743 Oceanography, 53, 1014-1024, 2008.

744 Gongol, C., and Savage, C.: Spatial variation in rates of benthic denitrification and
745 environmental controls in four New Zealand estuaries, Marine Ecology Progress Series, 556,
746 59-77, 2016.

747 Grasshoff, K.: The automated determination of ammonia, in: Methods of Seawater Analysis,
748 edited by: Grasshoff, K., Verlag Chemie, Weinheim, 276–278, 1976.

749 Grasshoff, K., Ehrhardt, M., and Kremling, K.: Methods of seawater analysis, 3rd ed., Wiley-
750 VCH. 600 pp, Weinheim, 1999.

751 Halm, H., Lam, P., Ferdelman, T. G., Lavik, G., Dittmar, T., LaRoche, J., D'Hondt, S., and
752 Kuypers, M. M.: Heterotrophic organisms dominate nitrogen fixation in the South Pacific
753 Gyre, ISME J, 6, 1238-1249, 10.1038/ismej.2011.182, 2012.

754 Hamlin, H. J., Michaels, J. T., Beaulaton, C. M., Graham, W. F., Dutt, W., Steinbach, P.,
755 Losordo, T. M., Schrader, K. K., and Main, K. L.: Comparing denitrification rates and carbon
756 sources in commercial scale upflow denitrification biological filters in aquaculture,
757 Aquacultural Engineering, 38, 79-92, http://dx.doi.org/10.1016/j.aquaeng.2007.11.003, 2008.

758 Hardison, A. K., Algar, C. K., Giblin, A. E., and Rich, J. J.: Influence of organic carbon and
759 nitrate loading on partitioning between dissimilatory nitrate reduction to ammonium (DNRA)
760 and N_2 production, Geochimica et Cosmochimica Acta, 164, 146-160,
761 http://dx.doi.org/10.1016/j.gca.2015.04.049, 2015.

762 Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., and Schloss, P. D.: Development
763 of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence
764 data on the MiSeq Illumina sequencing platform, Applied and Environmental Microbiology,
765 10.1128/aem.01043-13, 2013.

766 Marie, D., Brussaard, C. P. D., Thyrhaug, R., Bratbak, G., and Vaulot, D.: Enumeration of
767 marine viruses in culture and natural samples by flow cytometry, Applied and Environmental
768 Microbiology, 65, 45-52, 1999.

769 McMurdie, P. J., and Holmes, S.: phyloseq: an R package for reproducible interactive analysis
770 and graphics of microbiome census data, PLoS One, 8, e61217,
771 10.1371/journal.pone.0061217, 2013.

772 Newell, S. E., McCarthy, M. J., Gardner, W. S., and Fulweiler, R. W.: Sediment nitrogen
773 fixation: a call for re-evaluating coastal N budgets, Estuaries and Coasts, 1-13,
774 10.1007/s12237-016-0116-y, 2016a.

775 Newell, S. E., Pritchard, K. R., Foster, S. Q., and Fulweiler, R. W.: Molecular evidence for
776 sediment nitrogen fixation in a temperate New England estuary, PeerJ, 4, e1615,
777 10.7717/peerj.1615, 2016b.

778 Oakes, J. M., Eyre, B. D., and Ross, D. J.: Short-Term Enhancement and Long-Term
779 Suppression of Denitrification in Estuarine Sediments Receiving Primary- and Secondary-
780 Treated Paper and Pulp Mill Discharge, Environmental Science & Technology, 45, 3400-3406,
781 10.1021/es103636d, 2011.

Oksanen, J., Guillaume Blanchet, F., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., and Wagner, H.: vegan: Community Ecology Package. R package version 2.3-4. http://cran.r-project.org/package=vegan, 2016.

Reimers, C. E., Alleau, Y., Bauer, J. E., Delaney, J., Girguis, P. R., Schrader, P. S., and Stecher, H. A.: Redox effects on the microbial degradation of refractory organic matter in marine sediments, Geochimica et Cosmochimica Acta, 121, 582-598, 2013.

Robinson, G., Caldwell, G. S., Jones, C. L. W., Slater, M. J., and Stead, S. M.: Redox stratification drives enhanced growth in a deposit-feeding invertebrate: implications for aquaculture bioremediation, Aquaculture Environment Interactions, 8, 1-13, 10.3354/aei00158, 2015.

Robinson, G., Caldwell, G. S., Wade, M. J., Free, A., Jones, C. L. W., and Stead, S. M.: Profiling bacterial communities associated with sediment-based aquaculture bioremediation systems under contrasting redox regimes, Scientific Reports, 6, 38850, 10.1038/srep38850, 2016.

Robinson, G., Caldwell, G. S., Jones, C. L. W., and Stead, S. M.: Manipulating the carbon to nitrogen ratio of aquaculture effluent is key to culturing sea cucumbers without formulated feeds, Aquaculture, in review.

Roy, D., Hassan, K., and Boopathy, R.: Effect of carbon to nitrogen (C:N) ratio on nitrogen removal from shrimp production waste water using sequencing batch reactor, Journal of industrial microbiology & biotechnology, 37, 1105-1110, 10.1007/s10295-010-0869-4, 2010.

Schneider, O., Sereti, V., Eding, E. H., and Verreth, J. A. J.: Molasses as C source for heterotrophic bacteria production on solid fish waste, Aquaculture, 261, 1239-1248, 10.1016/j.aquaculture.2006.08.053, 2006.

Seitzinger, S. P.: Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance, Limnology and Oceanography, 33, 702-724, 1988.

Snoeyink, V. L., and Jenkins, D.: Water Chemistry, John Wiley & Sons. 220 pp, New York, 1980.

Song, B., Lisa, J. A., and Tobias, C. R.: Linking DNRA community structure and activity in a shallow lagoonal estuarine system, Frontiers in Microbiology, 5, 460, 10.3389/fmicb.2014.00460, 2014.

Thamdrup, B., and Dalsgaard, T.: Nitrogen cycling in sediments, in: Microbial Ecology of the Oceans, John Wiley & Sons, Inc., 527-568, 2008.

Veuger, B., Eyre, B. D., Maher, D., and Middelburg, J. J.: Nitrogen incorporation and retention by bacteria, algae, and fauna in a subtropical intertidal sediment: an in situ ^{15}N-labeling study, Limnology and Oceanography, 52, 1930-1942, 2007.

Welsh, D. T., Bourgués, S., de Wit, R., and Herbert, R. A.: Seasonal variations in nitrogen-fixation (acetylene reduction) and sulphate-reduction rates in the rhizosphere of *Zostera noltii:* nitrogen fixation by sulphate-reducing bacteria, Marine Biology, 125, 619-628, 10.1007/bf00349243, 1996.

Welsh, D. T., Bourguès, S., De Wit, R., and Auby, I.: Effect of plant photosynthesis, carbon sources and ammonium availability on nitrogen fixation rates in the rhizosphere of *Zostera noltii*, Aquatic Microbial Ecology, 12, 285-290, 1997.

Xu, Y., Zhou, P., and Tian, X.: Characterization of two novel haloalkaliphilic archaea *Natronorubrum bangense* gen. nov., sp. nov. and *Natronorubrum tibetense* gen. nov., sp. nov, International journal of systematic bacteriology, 49 Pt 1, 261-266, 10.1099/00207713-49-1-261, 1999.

Zehr, J. P., and Paerl, H. W.: Biological nitrogen fixation in the marine environment, in: Microbial Ecology of the Oceans, 2nd ed., edited by: L., K. D., Wiley-Liss, Inc., 2008a.

Zehr, J. P., and Paerl, H. W.: Molecular ecological aspects of nitrogen fixation in the marine environment, in: Microbial Ecology of the Oceans, 2nd ed., edited by: L., K. D., Wiley-Liss, Inc., 481-525, 2008b.

836 **Figure legends**

837 **Fig. 1.** Mean (± standard error) net fluxes (in µmol m^{-2} h^{-1}; n = 5) of: a) dissolved oxygen (DO);
838 b) dissolved inorganic carbon (DIC); c) dinitrogen gas (N_2); and, d) gross primary production
839 (GPP) in incubation chambers containing sea cucumbers and aquaculture waste with (+C) or
840 without (-C) carbon addition.

841 **Fig. 2.** Mean (± standard error) benthic light, dark and net fluxes (in µmol m^{-2} h^{-1}; n = 5) of: a)
842 phosphate (PO_4^{3-}); b) ammonium (NH_4^+); c) nitrite (NO_2^-); and d) nitrate and nitrite (NO_x) in
843 incubation chambers containing sea cucumbers and aquaculture waste with (+C) or without (-
844 C) carbon addition.

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

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

853 **Fig. 5.** The mean proportion (%) and the difference in the mean proportion of taxa at: a) family
854 and b) genus level between +C and -C treatments with 95 % confidence intervals. Significant
855 differences in mean proportions were determined using two-sided Welch's t-tests (alpha =
856 0.05).

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

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

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

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

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

869 **Table 1.** Description of the experimental treatments. The presence (✓) or absence (x) from day
870 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

871

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

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	

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

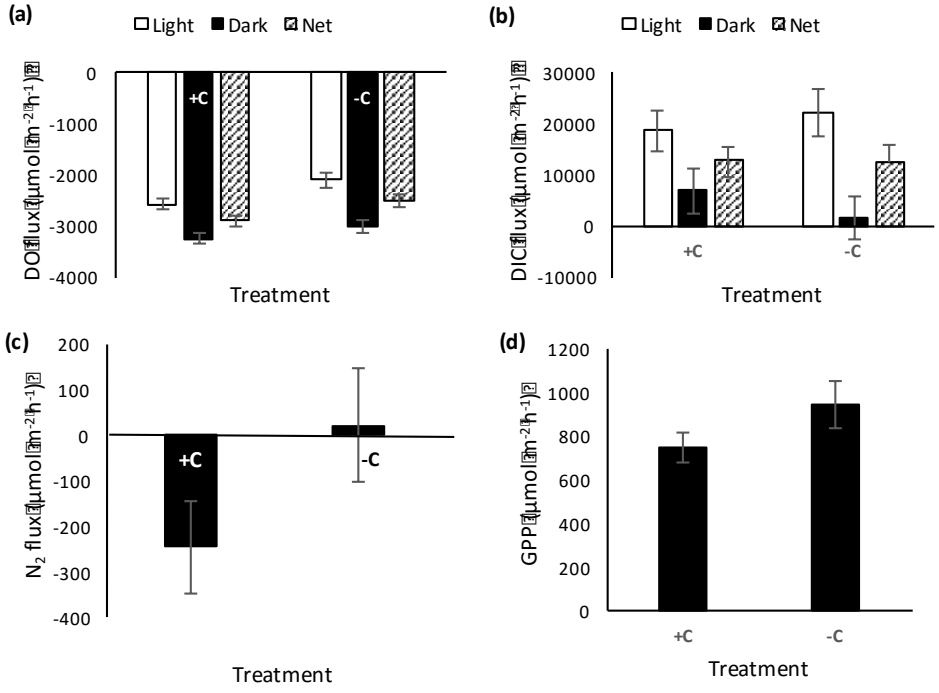

Fig. 1

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

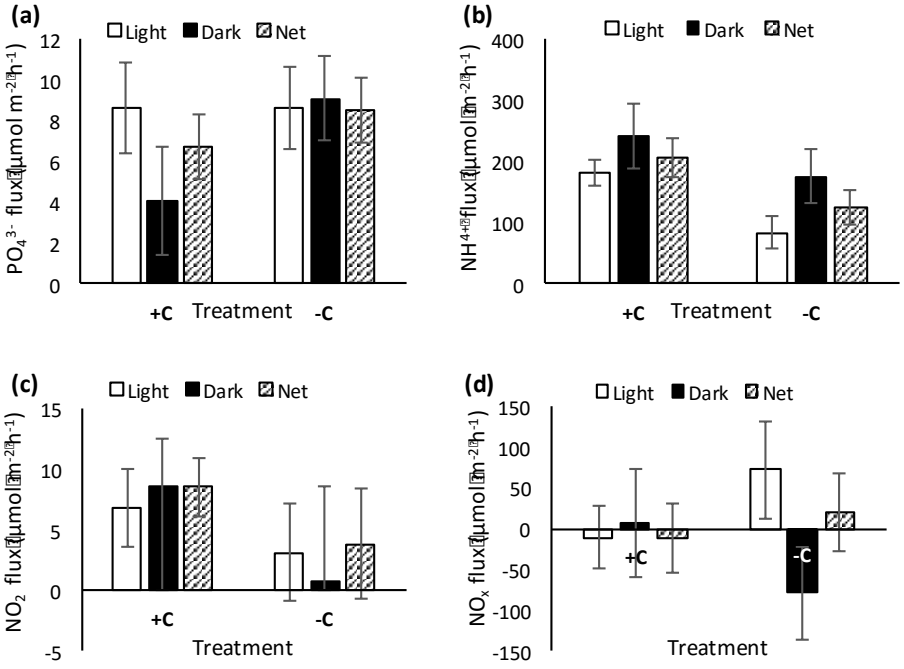

Fig. 2

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

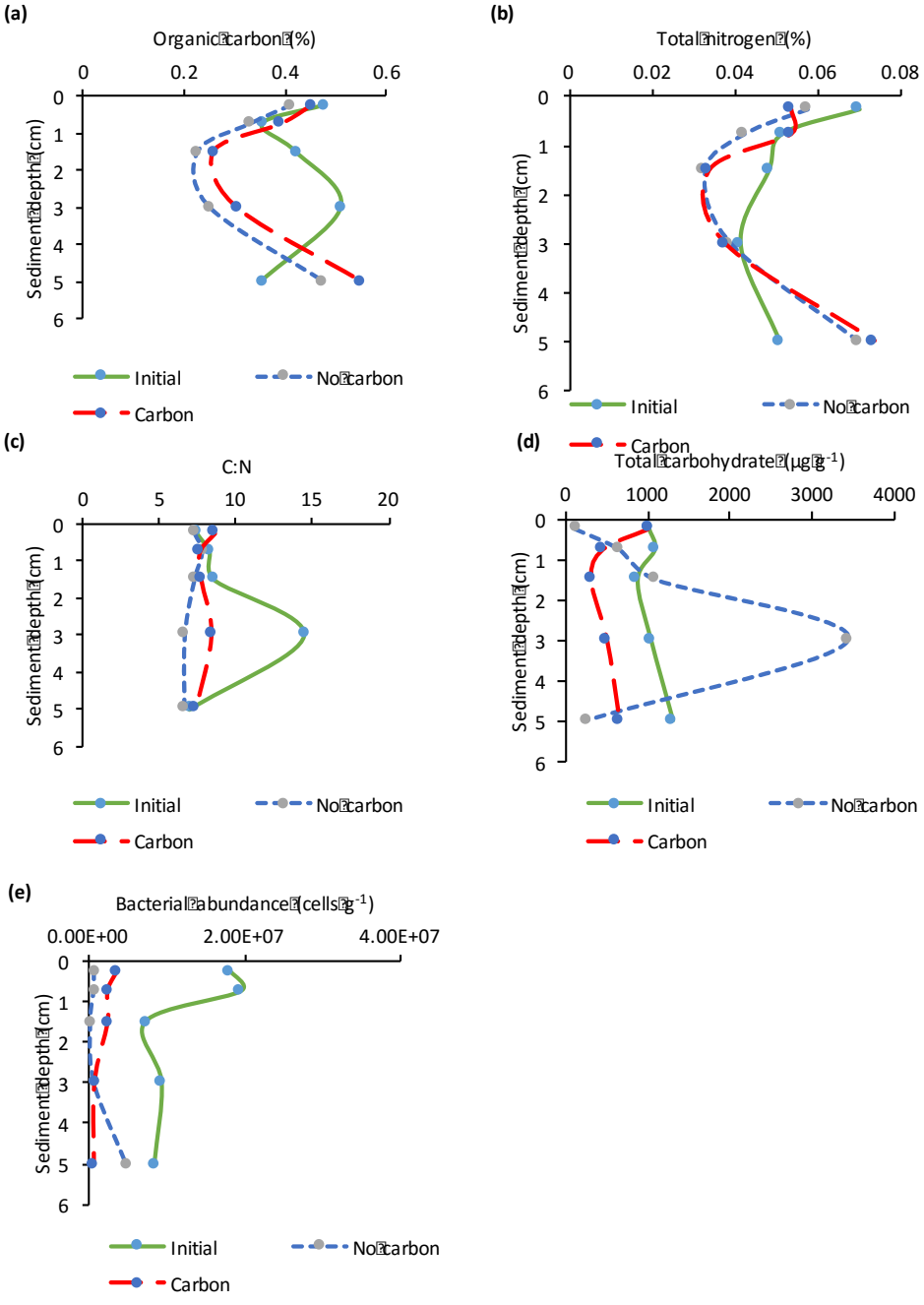

Fig. 3

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

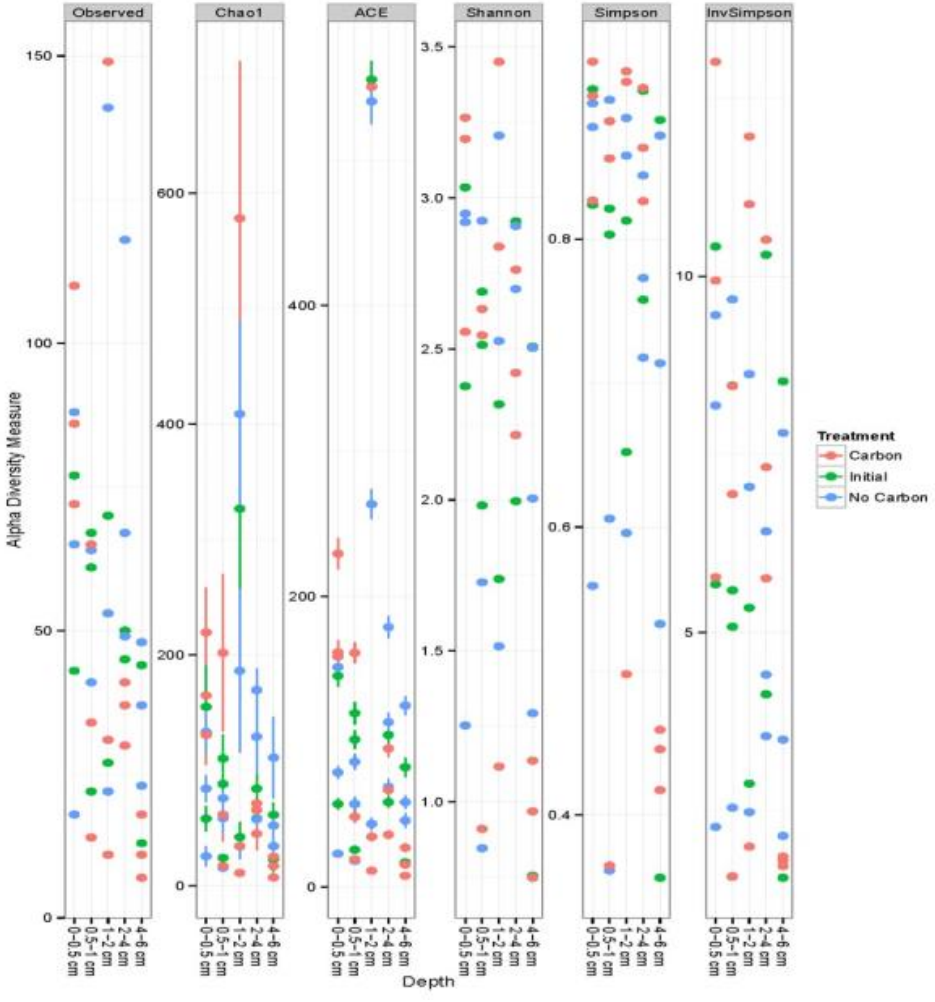

Fig. 4

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

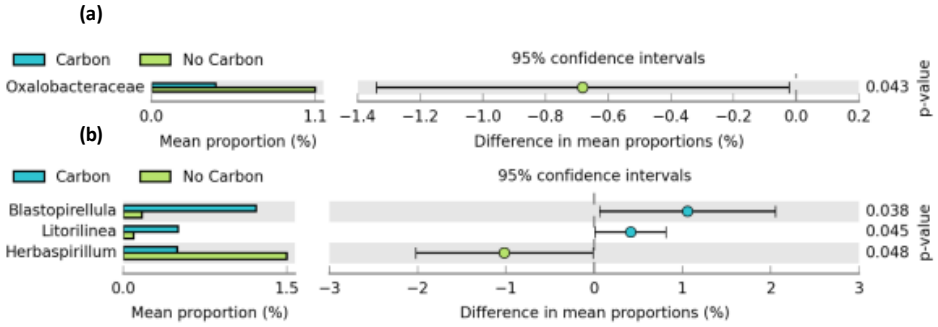

Fig. 5

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

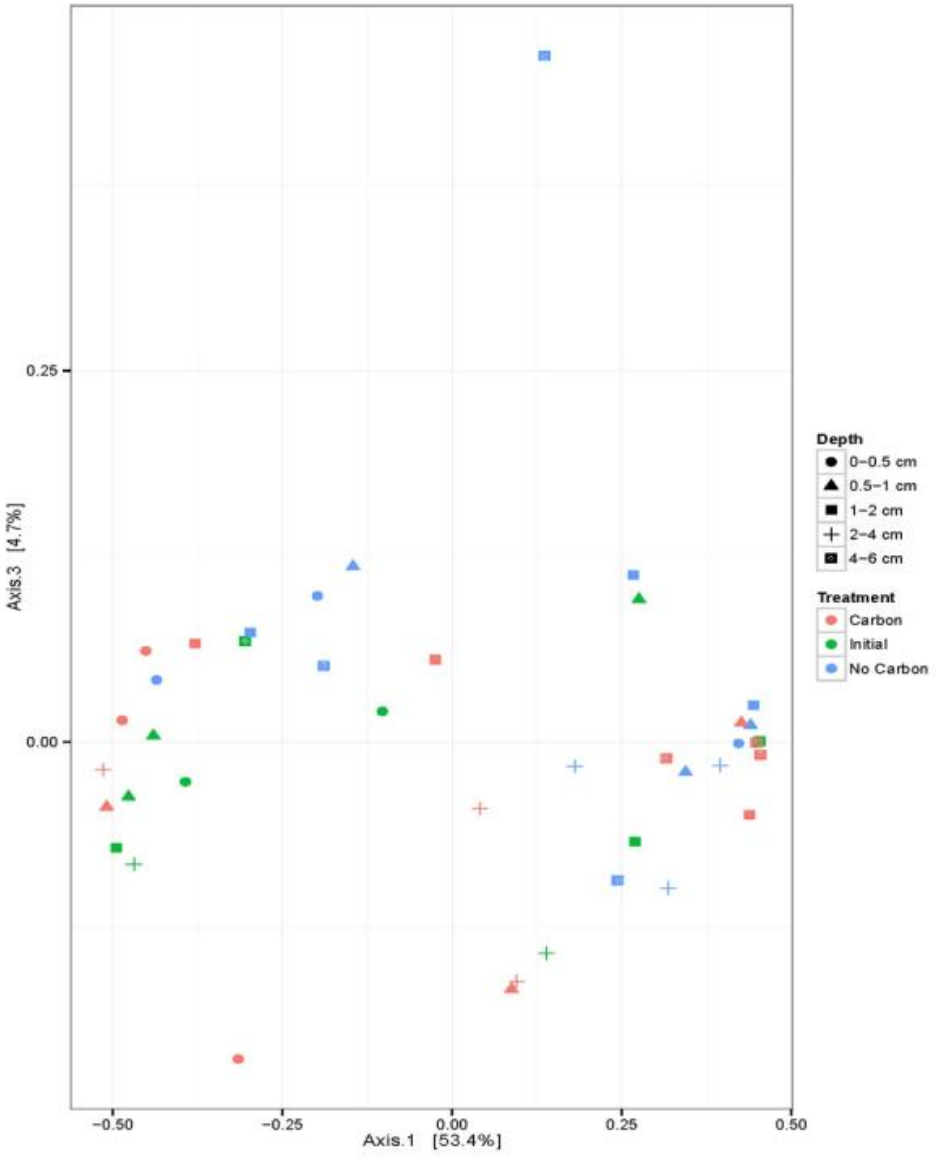

Fig. 6

Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-293
Manuscript under review for journal Biogeosciences
Discussion started: 14 August 2017

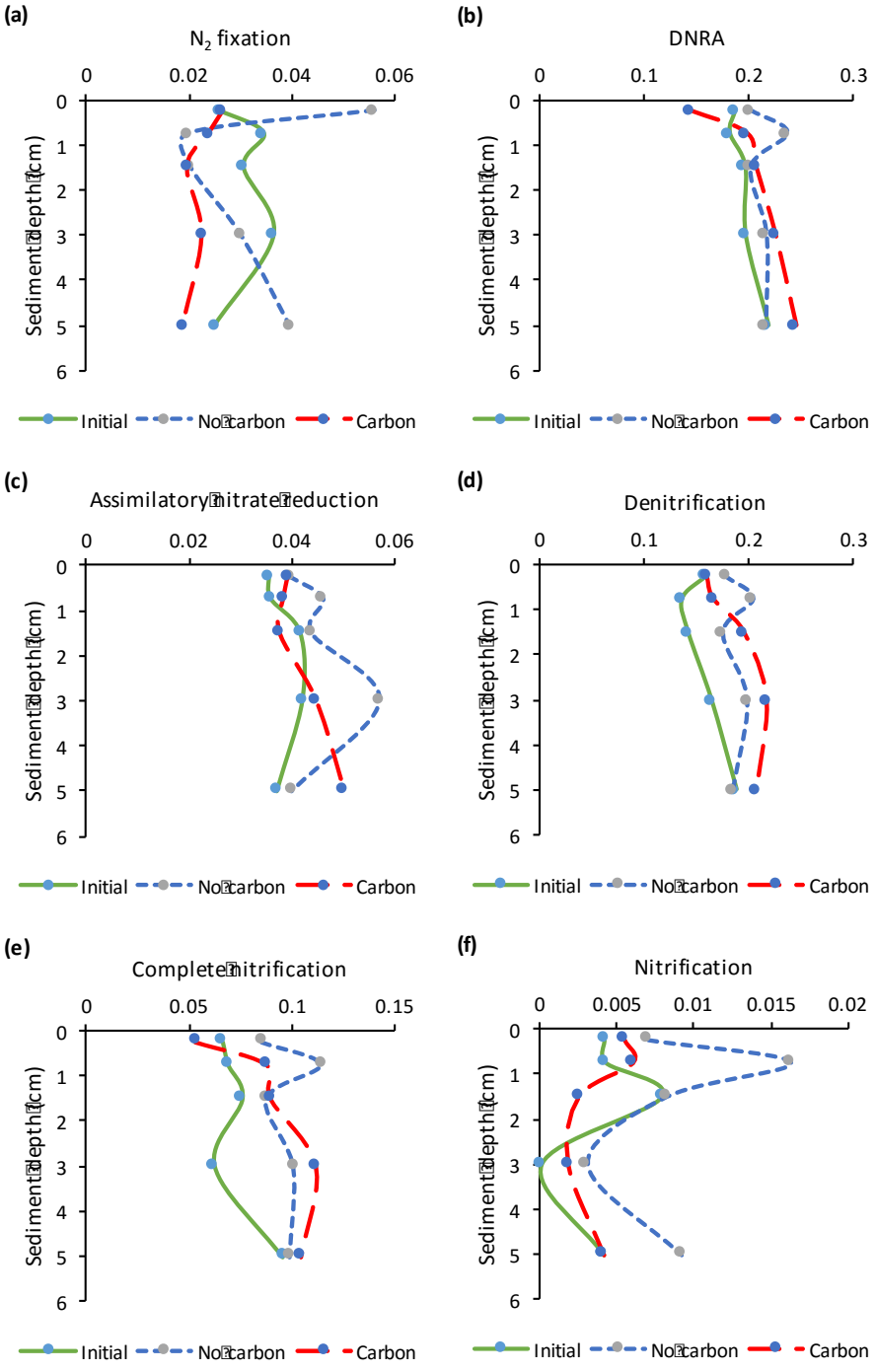

Fig. 7