



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

Georgina Robinson^{1,2,a}, Thomas MacTavish³, Candida Savage^{3,4}, Gary S. Caldwell¹, Clifford L. W. Jones², Trevor Probyn⁵, Bradley D. Eyre⁶, and Selina M. Stead¹

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

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

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

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

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

⁶Centre for Coastal Biogeochemistry, School of Environment, Science and Engineering, Southern Cross University, Lismore, NSW 2480, Australia

^acurrent address: The Scottish Association for Marine Science, Scottish Marine Institute, Oban, PA37 1QA, UK

Correspondence: Georgina Robinson (georgina.robinson@sams.ac.uk)

Received: 10 July 2017 – Discussion started: 14 August 2017

Revised: 28 December 2017 – Accepted: 11 January 2018 – Published: 29 March 2018

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 meta-genome predictions that indicate that 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 (RASs), 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 prerequisite 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 ecologically based systems, such as biofloc, aim to recycle 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, 2018).

The stoichiometric approach taken in C:N amendment in biofloc systems recognizes 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. (2018), 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, 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 mineralization, with a net release of nitrogen occurring below a threshold of 20:1 (Cook et al., 2007; Blackburn, 1986). Robinson et al. (2018) hypothesized 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 RAS described in Robinson et al. (2015). The experiment was conducted over a 15-day period from 30 January (day –1) to 14 February (day 14) 2014 using juvenile sea cucumbers (*Holothuria scabra*) imported from a commercial hatchery (Research Institute for Aquaculture III, Vietnam) on 5 September 2013 that were quarantined and acclimated to the experimental system as described in Robinson et al. (2018).

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 initial 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 ($215.06 \text{ mg day}^{-1}$ wet weight). The “added carbon” treatment (+C) received aquaculture waste ($215.06 \text{ mg day}^{-1}$ wet weight) and carbon in the form of soluble starch ($44.50 \text{ mg day}^{-1}$ dry weight) daily to increase the C:N to 20:1 (mass ratio) from day 0 (Table 1). The carbon addition treatments (+C) were standardized at a concentration of $400 \text{ mmol C m}^{-2} \text{ d}^{-1}$.

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 $16.34 \text{ exchanges h}^{-1}$. 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 preconditioned for 4 weeks in flow-through tanks prior to its transfer into the chambers. The sediment was allowed to condition and stabilize into redox-stratified layers for 14 days prior to the commencement of the experiment. No aeration was provided;

Table 1. Description of the experimental treatments. The presence (✓) or absence (×) from day 0 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	×	×	×	n/a
No added carbon	−C	5	✓	✓	×	5 : 1
Added carbon	+C	5	✓	✓	✓	20 : 1

n/a – not applicable

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 those causing 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., 2018) 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 0. 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 local time (UTC + 2) from day 0 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 0 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 (Robin-

son et al., 2015). Three juvenile *H. scabra* with a mean (\pm standard deviation) weight of 1.91 ± 0.36 g were added to each of 10 chambers (equivalent to a high stocking density of 1034.00 g m^{-2}) on day 0. 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 the 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 1 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 (~ 3 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 ($\pm 0.01 \text{ mg L}^{-1}$). The pH (± 0.01 pH units) was measured electrochemically (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 microfibre 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 1, 7 and 13) during dark incubations, as during daylight hours bubbles may form that interfere with the estimation of N_2 : Ar and thus lead to an over-estimation of N_2 production (Eyre et al., 2002). To minimize 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 recapped and the stirrers restarted. 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 min with an average duration of 104 min, 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 (NO_x ; $0.01 \mu\text{M}$) were determined colourimetrically by flow injection analysis (QuikChem[®] 8500 Automated Ion Analyzer, Hach Company, USA) 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 ($0.01 \mu\text{M}$) and dissolved inorganic phosphate ($0.01 \mu\text{M}$) were determined using the methods of Grasshoff (1976) and Grasshoff et al. (1999) respectively, and nitrite (NO_2^- ; $0.01 \mu\text{M}$) was determined according to Bendscheider and Robinson (1952).

2.9 Gas analyses

Alkalinity (0.01 mg L^{-1}) and total dissolved CO_2 ($0.01 \mu\text{M}$) 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 ($\pm 0.01\%$). 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 Eq. (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 Eq. (3), where light O_2 fluxes represent net primary production and dark fluxes represent respiration. Remineralization ratios were calculated according to Eq. (4) (Eyre et al., 2013b).

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

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

$$\text{Net flux rates} = \quad (2)$$

$$\begin{aligned} & \left((\text{hourly dark rates} \times \text{hours of darkness}) \right. \\ & \left. + (\text{hourly light rates} \times \text{hours of daylight}) \right) / 24 \text{ h} \end{aligned}$$

$$\text{Gross primary production} = \quad (3)$$

$$\text{light O}_2 \text{ flux (+ve)} - \text{dark O}_2 \text{ flux (-ve)}$$

$$\text{Remineralization ratio} = \frac{\text{dark O}_2 \text{ flux}}{\text{N}_2 + \text{NH}_4^+ + \text{NO}_x} \quad (4)$$

2.10 Sediment sectioning

On days 0 and 14, three sub-cores (internal diameter 30 mm) were extracted from the In and experimental ($-C$ and $+C$) chambers. 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; this was done for the 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 $0.2 \mu\text{m}$ filtered, 1% buffered paraformaldehyde and refrigerated for the 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 the removal of carbonates by acid fumigation (Robinson et al., 2015). Total sediment carbohydrates were measured on defrosted samples using the phenol-sulfuric 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 min 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 ($\lambda = 530$ 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 per gram 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 microlitre of template DNA was arrayed into 96-well plate format with $17\ \mu\text{L}$ of Accuprime Pfx Supermix (ThermoFisher, UK), leaving two wells on each plate open for controls. Two microlitres of reconstituted indexed primers at $100\ \mu\text{M}$ 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 $1\ \mu\text{L}$ of PCR grade dH₂O and the positive control was $1\ \mu\text{L}$ 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 2 min followed by cycling parameters of 95°C for 20 s, 55°C for 15 s and 72°C for 5 min for 30 cycles. A final extension was done at 72°C for 10 min. The amplification of the PCR products was checked on a subset of 12 samples using gel electrophoresis on a 1 % 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 normalization using the Invitrogen SequalPrep Plate Normalization 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 homo-polymers 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 normalization. To standardize 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 meta-genome 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. 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 9 due to lowered O_2 concentrations in the chambers; therefore, light incubation data represent a mean of six values (days 1, 3, 5, 7, 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 remineralization ratios, and mean gross primary production measured during the experimental period (days 1–13) were analysed using a Student t test at al-

pha < 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 a Student *t* test at alpha < 0.05. Data are presented as mean \pm 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 visualized 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 visualized 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 the 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 meta-genomes based on pre-computed Kyoto Encyclopedia for Genes and Genomes (KEGG) ortholog reference profiles (Abhauer 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 characterized reactions listed under "nitrogen metabolism" (Table S2 in the Supplement). Anaerobic oxidation of ammonia (anammox) was not included, as although this process is recognized 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 9 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 0 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 to 1.62 ± 0.03 g; an overall mean growth rate of -0.02 ± 0.00 g day⁻¹). The biomass density decreased from $1,034.00 \pm 12.73$ to 874.97 ± 18.31 g m⁻², although the initial stocking density was comparable to the final densities (1011.46 ± 75.58 g m⁻²) achieved in previous carbon amended cultures standardized at 200 mmol C m⁻² day⁻¹ (Robinson et al., 2018).

3.2 Gas and nutrient fluxes

Benthic fluxes of dissolved oxygen and 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 N₂ between treatments on day -1 (N₂ dark only; Fig. S1 in the Supplement). 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 -2905.84 ± 99.95 $\mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1}$ compared to -2511.31 ± 116.81 $\mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1}$ in the -C treatment (Fig. 1a). Oxygen and DIC fluxes clearly show that the sediment metabolism was net heterotrophic. During the day, DIC

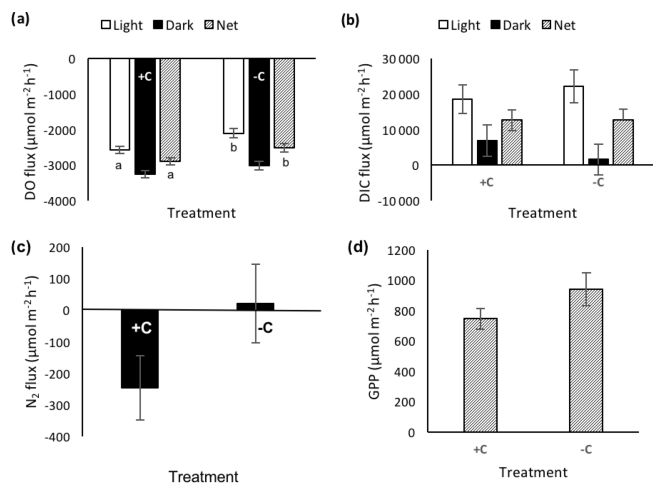


Figure 1. Mean (\pm standard error) net fluxes (in $\mu\text{mol m}^{-2} \text{h}^{-1}$; $n = 5$) of (a) dissolved oxygen (DO), (b) DIC, (c) dinitrogen gas (N_2) and (d) gross primary production (GPP) in incubation chambers containing sea cucumbers and aquaculture waste with (+C) or without (–C) carbon addition, incubated under light and dark conditions between day 1 and day 13.

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 $12\,732.34 \pm 2031.69 \mu\text{mol C m}^{-2} \text{h}^{-1}$ across the treatments (Fig. 1b). The assumed low rates of photosynthesis may have been due to shading and from turnover of the micro-phytobenthos standing stock due to grazing by sea cucumbers (Glud et al., 2008; MacTavish et al., 2012). In addition, DIC fluxes were 4-fold higher than oxygen fluxes, indicating that the majority of the organic carbon was oxidized by anaerobic pathways (Burford and Longmore, 2001; Eyre et al., 2011).

The mean dark N_2 flux on days 7 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 ($-142.96 \pm 107.90 \mu\text{mol m}^{-2} \text{h}^{-1}$), 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 ($17.33 \pm 36.20 \mu\text{mol m}^{-2} \text{h}^{-1}$), indicating that 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

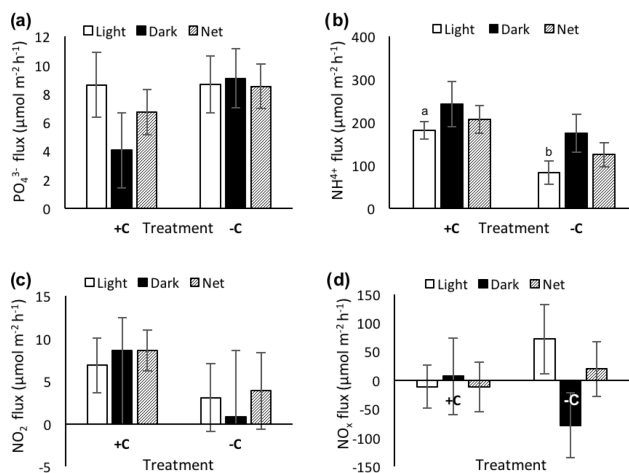


Figure 2. Mean (\pm standard error) benthic light, dark and net fluxes (in $\mu\text{mol m}^{-2} \text{h}^{-1}$; $n = 5$) of (a) phosphate (PO_4^{3-}), (b) ammonium (NH_4^+), (c) nitrite (NO_2^-), and (d) nitrate and nitrite (NO_x) in incubation chambers containing sea cucumbers and aquaculture waste with (+C) or without (–C) carbon addition, incubated under light and dark conditions between day 1 and day 13.

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 $115.32 \pm 11.43 \mu\text{mol m}^{-2} \text{h}^{-1}$ compared with an uptake of $-9.77 \pm 11.82 \mu\text{mol m}^{-2} \text{h}^{-1}$ in the –C treatment. The +C treatment had intermediary values with a mean NH_4^+ efflux of $56.03 \pm 25.54 \mu\text{mol m}^{-2} \text{h}^{-1}$. 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. $83.90 \pm 26.70 \mu\text{mol m}^{-2} \text{h}^{-1}$, 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 remineralization ratios

The sediment 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 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 mineralization by heterotrophic mi-

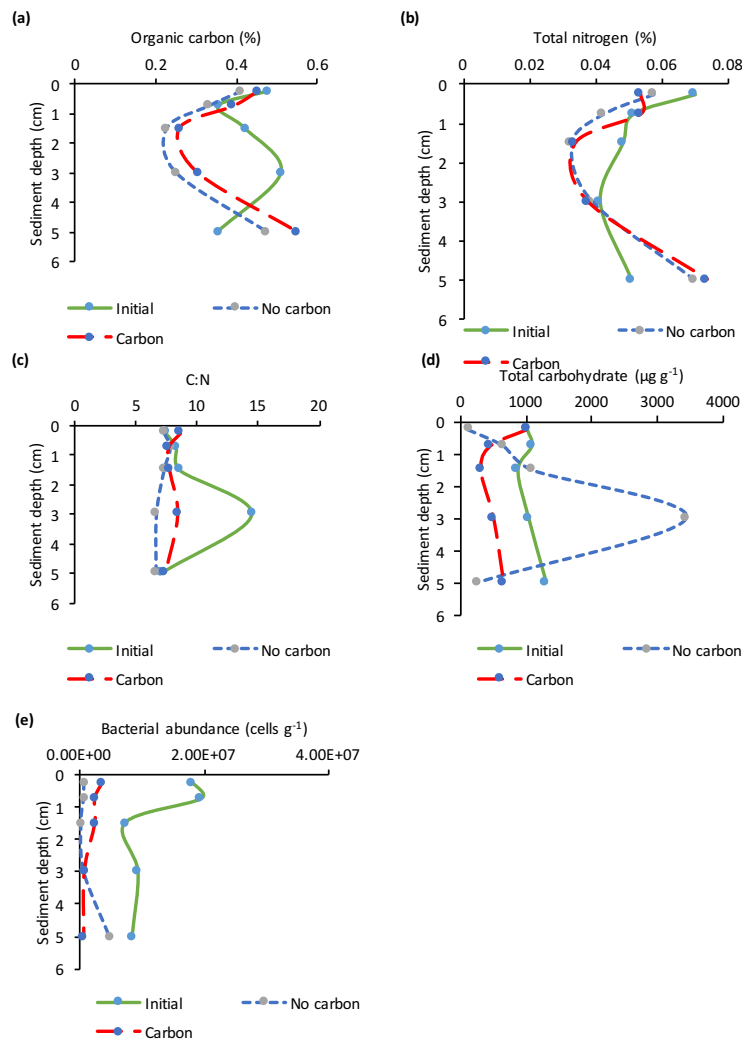


Figure 3. Vertical depth profiles of sediment characteristics: (a) organic carbon, (b) total nitrogen, (c) carbon-to-nitrogen ratio (C:N), (d) total carbohydrate and (e) bacterial abundance. Cores were sectioned on day 0 prior to the addition of aquaculture waste (initial; In) and after waste addition, both with and without carbon supplementation (carbon and no carbon respectively) on day 14.

croorganisms (Reimers et al., 2013). Vertical profiles of TN and the C:N on days 0 and 14 followed a similar trend with the most marked changes occurring at the 1.0–2.0 and 2.0–4.0 cm depth intervals. 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 $0.24 \pm 0.02\%$ (OC) and $0.03 \pm 0.00\%$ (TN). This confirms that the oxic–anoxic interface supported the highest rates of organic-matter mineralization. In contrast, the deepest sectioned interval (4.0–6.0 cm) had significantly higher OC ($0.51 \pm 0.08\%$) and TN content ($0.07 \pm 0.01\%$) 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 remineralization ratios (after the exclusion of outliers) of 15.68 ± 7.43 that were approximately 3-fold higher than chambers receiving aquaculture waste only (5.64 ± 4.50), although the difference was not significant (t test; $t = 1.08$, $p = 0.32$). Remineralization ratios were higher than the sediment C:N in the +C treatment; 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 remineralization ratios were lower than the sediment C:N, indicating net release of nitrogen.

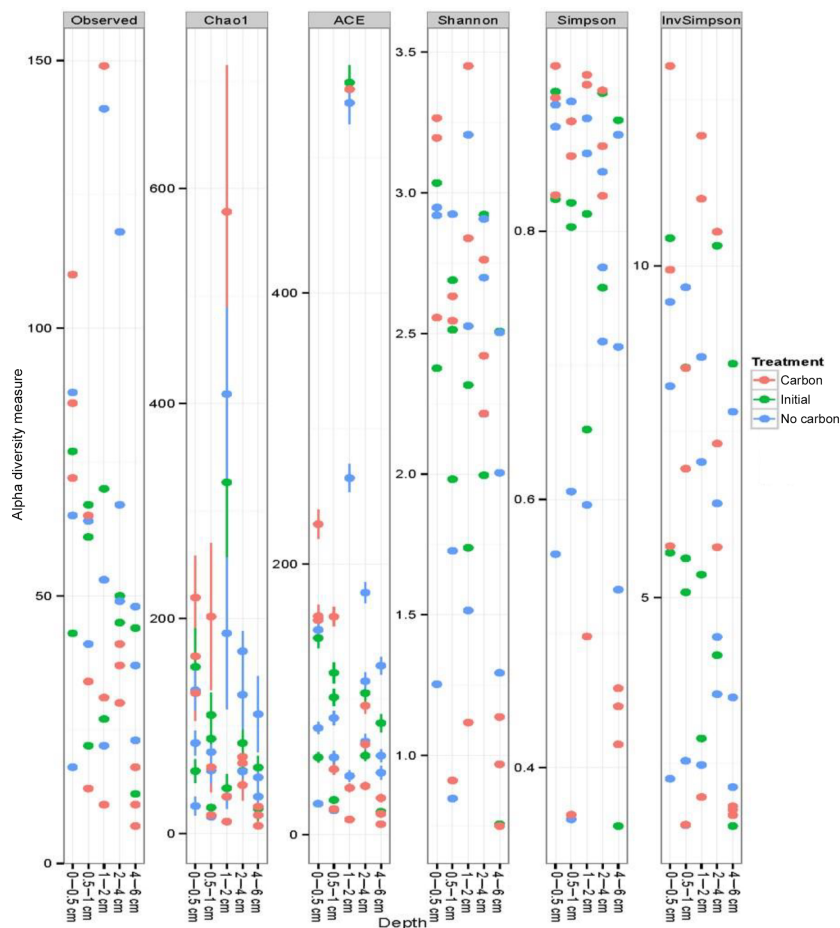


Figure 4. Alpha diversity metrics calculated on subsampled data. Observed: the number of operational taxonomic units (OTUs); ACE: abundance-coverage estimator; InvSimpson: Inverse Simpson diversity metric.

3.4 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 subsampling 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 the removal of unassigned taxa, mitochondria and Eukaryota.

Neither carbon addition, sediment depth nor the interaction between the factors (treatment \times 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 per gram; 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 three archaeal phyla were present, including Euryarchaeota, Thaumarchaeota and Woesearchaeota. *Natronorubrum* (Euryarchaeota), a halophilic aerobic chemoorganotroph (Xu et

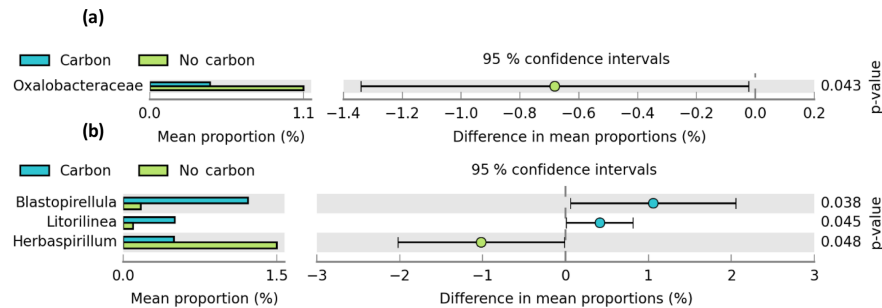


Figure 5. The mean proportion (%) and the difference in the mean proportion of taxa at (a) family and (b) genus level between +C and –C treatments with 95 % confidence intervals. Significant differences in mean proportions were determined using two-sided Welch’s *t* tests ($\alpha = 0.05$).

Table 2. Results of a 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	Sum of squares	Mean squares	<i>F</i> model	<i>R</i> ²	<i>p</i>
Treatment (<i>T</i>)	2	0.797	0.399	1.195	0.058	0.115
Sediment depth (<i>D</i>)	4	1.705	0.426	1.278	0.123	0.011
<i>T</i> × <i>D</i>	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	

al., 1999), was the most abundant genus, representing 14 of the 27 archaeal reads.

The bacterial community contained a total of 18 phyla, 4 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 *Blastopirellula* 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 %.

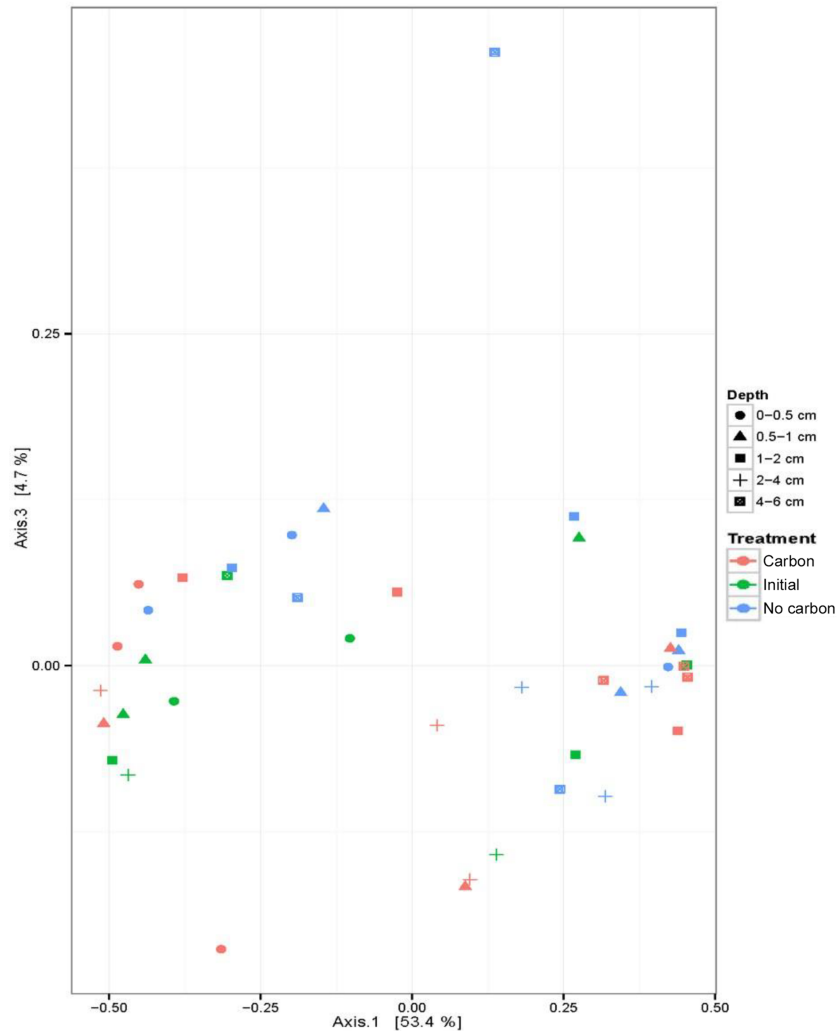


Figure 6. Principal component analysis ordination of the microbial community structure between the initial (In), +C and –C treatments at the five sediment depth intervals performed on a Bray–Curtis community dissimilarity matrix.

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 $> 1 \text{ kg m}^{-2}$ 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 remineralization and may partly ameliorate the effects of organic-matter enrichment from aquaculture effluent (MacTavish et al., 2012).

It was hypothesized 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 treatment. In addition to sea cucumber excretion, 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). 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. The increased NH_4^+ concentration may have originated from an increase in ammonification consis-

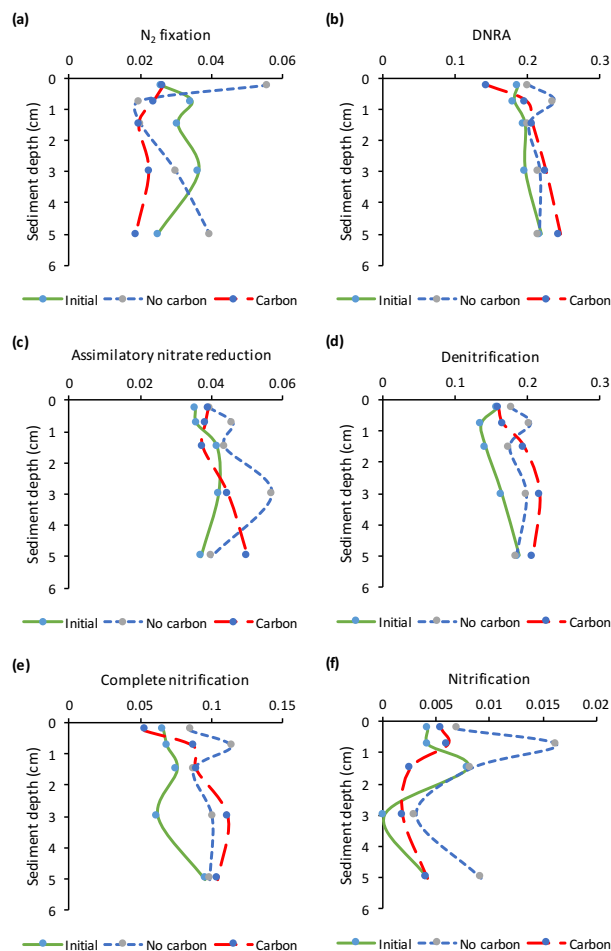


Figure 7. Vertical depth profiles of the predicted relative abundance of genes involved in the six nitrogen transformation pathways: (a) nitrogen fixation, (b) dissimilatory nitrate reduction to ammonium (DNRA), (c) assimilatory nitrate reduction, (d) denitrification, (e) complete nitrification and (f) nitrification, under the pathway module of nitrogen metabolism in the KEGG database.

tent with the increase in metabolism observed in the +C treatment.

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 anaero-

bic 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 prerequisite 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 meta-genome predictions and the uptake 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 (Fig. S2). 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 the different nitrogen processes are not well understood. For example, the quality and quantity of organic carbon may influence the balance between denitrification and nitrogen fixation (Fulweiler et al., 2013). Historically, denitrification has been considered to be the main pathway of nitrogen loss, based on mass balance calculations (Seitzinger, 1988). However, in sediment-based systems enriched with particulate organic waste (such as settlement ponds in aquaculture systems), the processes of permanent nitrogen removal account for a very small fraction of the total nitrogen that is permanently removed from the system. 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.

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, 2008). 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 of 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 sulfate-reducing bacteria (Welsh et al., 1996). The Deltaproteobacteria, which contain most of the sulfate-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, 2008).

The addition of exogenous carbon sources, including glucose, sucrose and lactose, has been found to stimulate heterotrophic nitrogen fixation in cyanobacteria and sulfate-reducing bacteria (Welsh et al., 1997; Newell et al., 2016a). The +C treatment exhibited an overall net N₂ uptake, whereas the control receiving waste only exhibited net N₂ 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 NH₄⁺ 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 were 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, characterized by a diverse set of dissimi-

latory microbial processes (Thamdrup and Dalsgaard, 2008). The lack of significant changes in microbial community structure and functioning may indicate that processes that contribute NH₄⁺ to the sediment were operating concurrently with transformations that removed NH₄⁺ from the system, such as anammox and coupled nitrification–denitrification. Furthermore, organic carbon can fulfil many functions under reducing conditions: it can be an electron donor in redox reactions, a substrate for fermentation or as organic substrate assimilated by heterotrophic bacteria coupled with NH₄⁺ uptake. The dual biogeochemical–molecular approach holds promise to further our understanding of nitrogen cycling; the challenge remains to resolve net biogeochemical fluxes with molecular tools that define microbial communities.

Our findings indicate that carbon addition may partly bioremediate nitrogen-rich effluent by retaining nitrogen within the system; however, longer-term trials are necessary to determine whether this translates into improved sea cucumber biomass yields. In the current study, the sea cucumbers decreased in mass with growth rates of 0.02 g day⁻¹; however, there was no significant difference in mean wet weight of the sea cucumbers at the start or end of the experiment. Two key factors are likely to have accounted for the differences in growth performance of *Holothuria scabra* in the present study and the previous study of Robinson et al. (2018). Firstly, chambers were shaded from direct sunlight in this experiment to mitigate against water temperature spikes that would likely have caused hypoxia in the small sealed chambers. However, because high light levels may be important for *Holothuria scabra* growth (Battaglione et al., 1999), this may have resulted in the lower growth performance. Secondly, the duration over which the sediment microbial community was allowed to develop differed between the studies. In Robinson et al. (2018) the trials lasted 112 days compared with the current 28-day study (14-day preconditioning and 14-day experimental).

5 Conclusion

Pathways that support the 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 NH₄⁺ in the sediment. The increase in NH₄⁺ efflux combined with net up-

take of N_2 into the sediment in response to carbon addition indicates that under nutrient loading rates consistent with hypereutrophic estuaries ($400 \text{ mmol C m}^{-2} \text{ day}^{-1}$ and $240 \text{ N m}^{-2} \text{ day}^{-1}$; 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 meta-genomic 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.

Data availability. Access to the original data can be requested from the corresponding author (Georgina Robinson).

The Supplement related to this article is available online at <https://doi.org/10.5194/bg-15-1863-2018-supplement>.

Author contributions. The work was conceptualized and funding was secured by GR, CLWJ, SMS, CS and BDE. Experiments were performed by GR and TM with equipment provided by CS, TP and BDE, and data were analysed by GR. The manuscript was written by GR and GSC and edited by and BDE, CLWJ, CS, TM, TP and SMS. All authors have approved the final article.

Competing interests. The authors declare that they have no conflict of interest.

Acknowledgements. This research was funded by a Biotechnology and Biological Sciences Research Council (BBSRC) Industrial CASE Studentship to Georgina Robinson (grant code BB/J01141X/1) with HIK Abalone Farm Pty Ltd as the CASE partner, with additional contributions from the ARC Grant DP160100248.

Edited by: Clare Woulds

Reviewed by: two anonymous referees

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