The effect of resource quality on the growth of *Holothuria scabra* during aquaculture waste bioremediation

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Abstract

Reducing dependency on environmentally unsustainable formulated feeds, most of which include limited reserves of fishmeal as a protein source, is a priority for the aquaculture industry, particularly for intensive culture systems. One approach is to increase nitrogen reuse within the system by feeding nitrogen-rich aquaculture effluent to deposit feeders, thereby closing the aquaculture nitrogen-loop. This study, for the first time and on a laboratory-scale, has reared juveniles of the sea cucumber *Holothuria scabra* at high densities solely on particulate organic waste from a commercial-scale land-based abalone recirculating aquaculture system. Furthermore, growth rates and biomass yields were increased significantly by adjusting the effluent C:N from 5:1 to 20:1 by adding exogenous organic carbon sources (glucose, starch and cellulose), so fuelling accelerated heterotrophic bacterial production within the redox-stratified tank sediment. Sea cucumber juveniles reared solely on effluent had a biomass density of 711 g m^{-2} after four months whereas animals reared on starch-amended effluent (the best performing treatment) had a final density of 1,011 g m^{-2}. Further optimisation of this approach could increase biomass yields and pave the way for the commercial cultivation of deposit feeding animals on waste streams, thus contributing to more environmentally sustainable aquaculture. Here, the nitrogen that originated from fishmeal is not lost through the discharge of aquaculture effluent; rather, it is immobilised into single cell biomass that is up-cycled into high-value secondary biomass. We demonstrate that sea cucumbers can be produced at high density through the manipulation of the C:N ratio of aquaculture effluent.

Keywords: C/N ratio; deposit feeder; stoichiometry; sustainable aquaculture; recirculating aquaculture system; sediment; sandfish

1. Introduction

Intensive aquaculture is generally characterised by the addition of nutritionally enriched diets that aim to satisfy the requirements of the culture species and completely replace natural food sources, a process not without its sustainability challenges (Naylor et al., 2009). Intensive aquaculture is associated with an enrichment of toxic wastes such as ammonia and other nitrogenous species, which are treated using biological filtration. High-protein feed inputs thus lead to an inefficient use of nitrogen, and the waste of the natural resources, mostly fishmeal, from which this nitrogen originated.

As aquaculture has intensified, there has been a shift towards recirculating aquaculture systems (RAS) (Badiola et al., 2012), from which the effluent streams are typically separated into high volume flows of dissolved inorganic effluents and low volume flows of suspended solids that accumulate as sludge. To advance the sustainability agenda and strengthen the economics of intensive aquaculture, there is a clear case for the industry developing culture strategies based on nitrogen reuse rather than removal.

Detritivores such as sea cucumbers and polychaete worms are ideal candidates for nitrogen reuse, yielding an additional commercial crop whilst bioremediating nitrogenous effluent (Cubillo et al., 2016; Zamora et al., 2016). Sea cucumbers are highly prized in Far Eastern markets and aquaculture is considered the only means of meeting demand, with production growing to ~130,000 tonnes per annum (Han et al., 2016). Culture technologies include sea ranching, sea pen farming, pond farming, production in co-culture and integrated multi-trophic aquaculture systems, and in intensive RAS (Purcell, 2010; Robinson, 2013).

Microorganisms play pivotal roles in aquaculture bioremediation technologies, which have evolved from exploiting autotrophic microbes (photoautotrophs and chemolithoautotrophs) to fully heterotrophic systems (Ebeling et al., 2006). This transition emphasises the re-use and recycling of feed residues within the culture system thereby

reducing feed, space, and energy requirements (Chávez-Crooker and Obreque-Contreras, 2010). Sediment microbial communities are primarily net heterotrophic systems that link energy transfer to higher trophic levels; therefore recycling nutrients *in situ* may provide a viable means to intensively culture deposit feeders with a higher overall efficiency (Schroeder, 1987). Furthermore, prior research (Robinson et al., 2015; Robinson et al., 2016) demonstrated that redox-stratified sediment supported faster growth rates and a higher biomass yield of *Holothuria scabra* relative to fully oxic sediment, indicating that heterotrophic systems are more favourable for deposit feeder growth.

Heterotrophs fundamentally differ from autotrophs due to their metabolic requirement for an organic source of carbon. Heterotrophic bacteria assimilate organic carbon and nitrogen in a stoichiometric balance based on the carbon to nitrogen ratio (C:N) of the bacterial cytoplasm (Goldman et al., 1987; Herbert, 1967). The C:N of organic substrates is an important parameter determining the degree of nitrogen regeneration as carbon and nitrogen are incorporated into bacteria at a fixed rate (Tezuka, 1990). For bacteria grown in an environment with a C:N of 5:1 and an average growth efficiency (the quantity of biomass produced per unit of assimilated organic carbon) of 50% under aerobic conditions, the threshold between net release and net immobilisation of nitrogen is 10:1 (Azim et al., 2008; Rittmann and McCarty, 2001). Increasing C:N beyond 10:1 provides sufficient carbon for heterotrophic bacteria to assimilate ammonium (NH_4^+) into biomass, thus mediating a shift from net NH_4^+ release (ammonification) to net immobilisation (assimilation) (Avnimelech, 1999; Avnimelech, 2014; Azim et al., 2008; Ebeling et al., 2006).

From a thermodynamic perspective, heterotrophic bacteria preferentially utilise reduced inorganic forms of nitrogen such as NH_4^+; however, NH_4^+ assimilation is dependent on the availability of carbon substrates (Church, 2008; Fenchel and Blackburn, 1979). Particulate organic wastes from aquaculture primarily comprise waste food and faeces, and are generally

deficient in organic carbon, with an average C:N of 7:1; thus, there is a net release of NH_4^+ during decomposition, often measured alongside ammonia as total ammonia nitrogen (TAN) (Avnimelech, 1999; Mirzoyan et al., 2012; Schneider et al., 2006). Particulate organic waste recovered from mechanical filtration has been used as substrate to produce heterotrophic bacteria and deposit feeding macrofauna, including polychaetes and sea cucumbers (Brown et al., 2011; MacDonald et al., 2013; Palmer, 2010; Schneider et al., 2007a; Schneider et al., 2007b; Schneider et al., 2006). Raising secondary livestock on aquaculture waste can provide a direct means of assimilating a proportion of the effluent nitrogen (Erler et al., 2004); however, detritivores are predicted to have poor nitrogen retention compared to other trophic groups (Schneider et al., 2005). Erler et al. (Erler et al., 2004) hypothesized that stimulated bacterial nitrogen processing during the production of secondary livestock on RAS effluents may be more important than direct assimilation. Schneider et al. (Schneider et al., 2007b) demonstrated that adding a source of labile organic carbon, e.g. molasses, could increase the conversion of inorganic nitrogenous wastes to heterotrophic bacterial biomass. Stimulating heterotrophic bacteria by manipulating the C:N may therefore offer an indirect means of increasing nitrogen retention in macrofauna reared on aquaculture effluents.

In sediment-based sea cucumber culture, controlling inorganic nitrogen cycling by adding carbon may be particularly relevant due to the need to counteract additional sources of NH_4^+ within the system, including: i) net efflux from the sediment (Hargreaves, 1998); ii) excretion from the sea cucumbers (Uthicke and Klumpp, 1997); and, iii) decomposition of aquaculture waste and feeds (Avnimelech, 1999). Carbon supplementation may offer an indirect means to retain nitrogen safely within the system by immobilising NH_4^+ into microbial biomass that can be upcycled into high value secondary biomass. We therefore investigated whether, by carbon supplementation, the uptake of nitrogen from waste feed and faeces by sea cucumbers can be improved.

Applying C:N manipulation to sediment-based systems, where bacterial growth efficiencies are generally lower due to anoxia (Fenchel et al., 2012), is completely novel. As such, there is a need to test different carbon sources to determine their efficacy. This study compared a range of carbon sources of differing biochemical composition and degradation rates on the growth of *H. scabra* reared on particulate organic waste from an intensive abalone RAS.

2. Material and methods

2.1 Study site and experimental animals

The Ethics Panels of both Newcastle and Rhodes Universities approved the study, and no collections were made from wild populations to support it. The research was conducted in a purpose built, bio-secure, heated RAS between October 8th 2013 and January 28th 2014 at HIK Abalone Farm Pty (Ltd) in Hermanus, South Africa. The detailed system specifications are found in Robinson et al. (2015). Juvenile *Holothuria scabra* were imported from a commercial hatchery (Research Institute for Aquaculture III, Vietnam) on September 5th 2013, and quarantined in a bio-secure facility for six weeks in accordance with South African importation and scientific investigations licenses. Following the quarantine period and prior to experimentation, the sea cucumber juveniles were held in the RAS in tanks filled with 10 cm of calcium carbonate sand and were fed a 34% protein commercial abalone weaning diet (Abfeed®-S34, 1.0 mm sugar grain pellet; Marifeed Pty Ltd, South Africa). The proximate nutritional composition of the feed was; crude protein 33%, crude lipid 3.0%, vitamins (0.1%), gross energy 15.6 kJ g^{-1}.

2.2 Experimental design

The experiment was designed to test a range of carbon sources of differing biochemical composition on *H. scabra* growth in a sediment-based culture system. Two

soluble (D-glucose and starch) and one insoluble carbon sources (microcrystalline cellulose) representing a range of first-order decomposition rate constants and labilities were purchased from Merck Millipore, South Africa (Table 1). The carbon sources were tested in conjunction with aquaculture waste (comprising uneaten feed and faeces recovered by mechanical filtration from a land-based abalone RAS) at a C:N of 20:1; a fourth treatment receiving aquaculture waste only (C:N of 5:1) was included as a control (Table 1). The average C:N of the dried particulate aquaculture waste was 5.21 ± 0.55. The quantity of carbon necessary to increase the ratio to 20:1 was calculated from the proportion of carbon in the molecular composition of each compound. Carbon additions were standardised between treatments based on a daily addition of 200 mmol C m^{-2} d^{-1}, which is within the upper range tolerated by benthic animals under eutrophic conditions of 100-400 mmol C m^{-2} d^{-1} (Lehtoranta et al., 2009). At a C:N of 20:1, this equates to 2.4 g m^{-2} d^{-1} of carbon and 0.12 g m^{-2} d^{-1} of nitrogen, which is between a 'mid' and 'high' ration of 100 and 150 mg m^{-2} d^{-1} of nitrogen respectively for deposit feeders (Alongi and Hanson, 1985; Tenore and Chesney, 1985).

Abalone (*Haliotis midae*) waste comprising mainly uneaten feed and faeces was collected every morning at 09:00 from the backwash of a grow-out RAS sand filter over five days prior to the experiment. The total system volume was approximately 32,000 L, holding a maximum of 800 kg of adult abalone (Yearsley et al., 2009). The abalone were fed to satiation daily with a 34% protein commercial abalone weaning diet (S34 Abfeed®, 10 × 10 × 1.2 mm pellet; Marifeed Pty Ltd, South Africa). The water leaving the production tanks drained through a 500 L sand filter filled with BS8:16 silica filter media for particulate organic waste removal (1-2 mm) prior to foam fractionation and biofiltration. The sand filter was fitted with a Jetco 5-port valve to change the water flow direction and permit backwashing. A 50 mm flexihose pipe was connected from the sand filter outflow to a 100 L conical fibreglass tank to collect the waste during the first 30 seconds of back washing. The

166 waste was settled for one hour and the supernatant discarded. The particulate waste was then
167 concentrated by centrifuging in 50 mL at 10,000 *g* for 10 minutes (compact centrifuge Z 206
168 A, Hermle Labortechnik, Germany). The organic carbon and total nitrogen content of
169 triplicate pre-weighed and dried (105 °C, 24 h) waste samples were analysed on a LECO
170 TruSpec CHN elemental analyser.

171 The four experimental treatments were randomly allocated to one of sixteen tanks using
172 a randomised block design of four blocks with one replicate from each treatment represented
173 in each block. As such, there were four replicates per treatment and each replicate consisted of
174 one laboratory-scale tank with four sea cucumbers per tank (i.e. sixteen cucumbers per
175 treatment). These experimental tanks had an area of 0.125 m^{-2}, which resulted in a sea
176 cucumber stocking density equivalent to those used commercially. The tanks were filled with
177 calcium carbonate sand sourced from a commercial dune quarry (SSB Mining, Macassar, South
178 Africa) sieved to 125-250 μm. Tanks were supplied with heated (29.18 ± 0.26 °C) recirculating
179 seawater and aeration as described in Robinson et al. (2015).

180 Feed additions (aquaculture waste with or without carbon sources) were made to all
181 tanks at 16:00 hours daily. All tanks received 2.41 g of concentrated aquaculture waste on a
182 wet weight basis per day. The experimental treatments received either 0.68 g of starch or
183 cellulose or 0.75 g of glucose per day on a dry weight basis respectively (Table 1). Prior to
184 feeding, the abalone waste was mixed with ambient seawater from the RAS into a wet slurry.
185 Similarly, the carbon sources were prepared by dissolving the soluble carbon sources in beakers
186 of ambient seawater while the insoluble cellulose was mixed in beakers to facilitate even
187 dispersal. Aeration was supplied continuously, except during feeding when the air and water
188 supplies were interrupted for 45 minutes. Polyvinylchloride end-caps (25 mm) were placed
189 over the tank standpipes and maintained in position for one hour to prevent the waste and
190 carbon sources from being washed out. The tank outflows were adapted to enable the tank

191 water to run to waste. This prevented the soluble carbon sources from entering the biological
192 filter.

193 Tanks were cleaned every two weeks to remove floating cyanobacteria colonies
194 (*Oscillatoria* sp.), epiphytic algae and biofilm. Tanks were subject to a natural photoperiod
195 which increased from 12.41: 11.19 L: D (06:10 hours to 18:51 hours, sunrise to sunset) to
196 13.53: 10.07 L: D (05:59 hours to 19:52 hours, sunrise to sunset) as day length increased during
197 the austral summer.

198 ***2.3 Water and sediment quality and environmental variables***

199 Light readings (aerial) were taken using a portable light meter (LX-107, Lutron
200 Electronic Enterprise Co. Ltd, Taipei, Taiwan) positioned 10 cm directly above the tank
201 outflow. Water quality parameters including salinity, temperature, pH, dissolved oxygen and
202 total ammonia nitrogen (NH_4-N; TAN) were recorded weekly as described in Robinson et al.
203 (2015). Nitrate concentration (± 0.01 mg L^{-1}) was measured weekly using a commercial test
204 kit (Merck Nitrate Test Kit, 109713, Merck, South Africa) and a spectrophotometer (Prim
205 Light, Secomam, 30319 Ales, France).

206 Sediment quality was monitored monthly to coincide with monthly sea cucumber
207 growth assessments. Sediment reduction-oxidation (redox) potential was measured with a
208 redox probe (Eutech Instruments pH 6+ portable meter) to the base of the sediment. Readings
209 were taken following stabilization (typically five minutes). Composite samples of the
210 sediment surface layers (upper 2-3 mm) were collected from all replicate tanks to determine
211 sediment characteristics. Chlorophyll *a* and phaeopigment concentrations were measured
212 using the spectrophotometric method described in Robinson et al. (2015). The organic
213 content measured as particulate organic carbon and total nitrogen was determined on an
214 elemental analyser after removal of carbonates by fuming with HCl. Total sediment
215 carbohydrates (µg g^{-1}) were measured using the phenol-sulphuric acid method (Underwood et

al., 1995). The absorbance of the supernatant was measured at 485 nm, quantified against a glucose standard and converted into the concentration of total carbohydrates using coefficients derived from the standard curves.

2.4 Sea cucumber growth

At the start of the experiment juvenile sea cucumbers (n = 64; 4.08 ± 0.58 g; mean ± standard deviation) were gut evacuated for 24 hours whilst suspended in mesh bags. They were drained on a damp cloth for one minute, weight and photographed, before being randomly allocated to tanks (four per tank), photo-identified and weighed. Each individual was re-weighed every four weeks (28 days) over the four-month experiment. Wet weight data were used to calculate biomass density (g m^{-2}) and growth rate (g d^{-1}; Robinson et al., 2015).

2.5 Statistical analyses

For all measured parameters, the data recorded per replicate tank were averaged and the mean value per tank was used. The light and water quality data were averaged to provide a mean value per month to give five time intervals for repeated measures analysis of variance (repeated measures ANOVA) and ensure consistency with the sediment quality and sea cucumber weight data. Units of pH were transformed prior to averaging using the antilog function in Microsoft Excel. Results are expressed as mean ± standard error unless otherwise stated.

Growth and environmental (light, water and sediment quality) data were tested for homogeneity of variance and for the normal distribution of the residuals using Levene's (Levene, 1960) and Shapiro Wilk's (Shapiro and Wilk, 1965) tests respectively. Initial weight data did not meet the assumptions of homogeneity of variance, therefore a non-parametric Kruskal-Wallis one-way ANOVA was used to test for significant differences between treatment medians. Data that met the test assumptions were analysed using repeated measures

ANOVA with treatment (carbon source) as the main factor and sampling time (month) as the repeated measure. Tukey's post-hoc honest significant difference (HSD) tests were used to compare differences among means. Multiple regression analysis was used to identify significant categorical predictors of sea cucumber biomass density. Differences were considered significant at alpha < 0.05. All statistical analyses were performed using Statistica version 13.

3. Results

3.1 Water and sediment quality and environmental variables

There were no significant differences in light, salinity, temperature, dissolved oxygen concentration, total ammonia or nitrate among treatments (repeated measures ANOVA: $p > 0.05$; Table 2 and Table S1). Salinity maintained a constant 35 g L^{-1} and dissolved oxygen concentration varied between 6.05 and 8.95 mg L^{-1} (7.26 ± 0.07 mg L^{-1}). The mean water temperature increased over the course of the experiment with the onset of the austral summer from a mean temperature of 25.49 ± 0.14 °C at the start, increasing to a mean of 32.30 ± 0.34 °C during the final month. pH differed significantly between treatments (repeated measures ANOVA; $F_{(12, 48)} = 2.79$, $p = 0.006$). The starch-amended treatment had the highest seawater pH between months one and three, peaking at 9.12 ± 0.05 in month three. In all treatments, pH tended to increase with time until the final month when values returned to those measured at the start of the experiment (Fig. 1A).

Ammonia concentrations increased in all treatments from 0.026 ± 0.001 mg L^{-1} at the start, peaking at 0.84 ± 0.15 mg L^{-1} in month two, and decreasing during the remaining two months to a mean of 0.19 ± 0.01 mg L^{-1} (Fig. 1B). Nitrate levels increased significantly over the experiment from 1.05 ± 0.08 mg L^{-1} at the start to 4.90 ± 0.23 mg L^{-1} in the final month

(repeated measures ANOVA; $F_{(4, 48)} = 84.45$, $p < 0.001$), although there were no significant differences between treatments (combined mean: 3.92 ± 0.20 mg L^{-1}; Table 2 and Table S1).

3.2 Sediment characteristics

The type of carbon source significantly affected sediment redox potential (repeated measures ANOVA, $F_{(12, 48)} = 4.76$, $p = <0.001$; Fig. 2A and Table S2). There were no significant differences between treatments at the start of the experiment with a positive reading of 173.06 ± 5.99 mV indicating fully oxic conditions. The redox potential decreased over time in all treatments; however, there was an apparent relatedness to the lability of the carbon source. The more refractory cellulose and starch had the greatest impact on redox potential, decreasing to -179.75 ± 18.93 mV and -109.50 ± 6.14 mV in the final month respectively. In the final month, the redox potential in tanks solely receiving aquaculture waste was marginally negative (-22.00 ± 12.19 mV) and not significantly different to glucose-amended tanks, the most labile carbon source (-45.75 ± 8.47 mV).

There were no significant differences in the levels of organic carbon, total nitrogen, C:N, total carbohydrate concentration, chlorophyll *a* or phaeopigment between treatments (repeated measures ANOVA: $p > 0.05$; Tables S2 and S3). Levels of organic carbon were initially low and increased in all treatments with time, with the highest levels of 0.20 ± 0.05% recorded in the cellulose-amended treatment (Fig. 2B and Table 2). Levels of total nitrogen were generally low (0.05 ± 0.00%) with minor fluctuations between treatments over time (Fig. 2C and Table 2). The surface sediment C:N of the starch-amended tanks was relatively constant over the experiment with a mean of 7.41 ± 0.23%. The C:N in the other three treatments fluctuated over time and between treatments with no clear trend, although there was a C:N increase in these treatments in the final month (Fig. 2D).

3.3 Growth and survival

There were no significant differences in mean wet weight or biomass density between treatments at the start of the experiment (130.59 ± 1.92 g m^{-2}; Kruskal-Wallis, $H_{(3, 16)} = 1.53$, $p = 0.68$ (Fig 3A and 3C). Survival was 100% in all treatments. Over the course of the growth trial, the mean growth rate (0.25 ± 0.02 g d^{-1}) and biomass density (1,011.46 ± 75.58 g m^{-2}) of *H. scabra* reared in the starch treatment was significantly higher than those reared on the waste alone (0.16 ± 0.01 g d^{-1} and 702.12 ± 35.93 g m^{-2}) repeated measures ANOVA, $F_{(12, 48)} = 2.49$, $p = 0.013$; Fig 3). Sea cucumbers in the starch treatment reached a final biomass density of 1,011.46 ± 75.58 g m^{-2} by month-4 compared to 702.12 ± 35.93 g m^{-2} in the control tanks. There were no significant differences in growth rate or biomass densities between the three carbon sources.

3.4 Multiple regression analysis

Sediment redox potential, light intensity and nitrate concentration were significant predictors of *H. scabra* biomass density (multiple regression, $F_{(13, 60)} = 36.26$, $r^2 = 0.89$; $p < 0.001$). Light and nitrate showed a positive relationship while sediment redox potential had a negative relationship with sea cucumber density.

4. Discussion

The current study indicated that increasing the C:N from 5:1 to 20:1 through carbon supplementation successfully increased the growth rate and biomass density of *H. scabra* juveniles. It is possible that increasing the C:N improved the nutritional value of the waste by increasing the quantity of organic carbon. It is commonly thought that deposit feeders gain energy for maintenance from carbon while nitrogen is predominantly used for growth (Lopez and Levinton, 1987). Deposit feeders are assumed to metabolise organic carbon and nitrogen in a 17:1 molar ratio (Russell-Hunter, 1970); therefore, carbon addition may have improved

growth rates by balancing the elemental stoichiometry of the food (Rice and Rhoads, 1989). An alternate explanation is that *H. scabra* was able to assimilate the carbon sources or their degradation products directly or indirectly through collaboration with microbial communities. Mechanisms for the uptake of organic molecules such as amino acids and carbohydrates are thought to exist in tissues such as the respiratory trees, body wall or tentacles, thereby enabling holothurians to assimilate dissolved organic matter directly from the water column (Fontaine and Chia, 1968; Jaeckle and Strathmann, 2013; Jangoux and Lawerence, 1982; Krishnan and Krishnaswamy, 1970; Lawerence, 1982).

Direct utilisation of starch for enhanced growth may not have been limited to the sea cucumbers. Although there was no significant difference in either chlorophyll *a* or phaeopigment concentrations, there were apparent differences (albeit qualitative) in the extent of phototroph biomass production between treatments. Carbohydrates such as glucose and polysaccharides can also be photo-oxidised with high quantum efficiencies in illuminated habitats leading to concomitant increases in microbial and algal productivity (Krishnan and Krishnaswamy, 1970; Stuart et al., 2016). The quality and quantity of organic matter supply to the sediment is modulated by sunlight, temperature and nutrient availability (Huettel et al., 2014). Light and nitrate were significant positive predictors of sea cucumber biomass density. Cyanobacteria may have supplied additional carbon to heterotrophic sediment bacteria and sea cucumbers as extracellular polymeric substances from photosynthesis (Stuart et al., 2016).

Avnimelech (1999) hypothesised that manipulating C:N may change nitrogen cycling pathways in aquaculture systems by mediating a shift from ammonification (net release) to assimilation (net uptake) of NH_4^+ by heterotrophic bacteria. For aerobic heterotrophic bacteria, the removal of NH_4^+ by incorporation in cells is enhanced by carbohydrate addition (Ebeling et al., 2006). It was therefore expected in these experiments that carbon

supplementation would decrease total ammonia nitrogen (TAN) concentrations compared to the control. However, this was not the case, although levels were generally low throughout the study (averaging 0.35 ± 0.04 mg L^{-1}). This may have been due to assimilation by heterotrophic bacteria, which are responsible for a large fraction of NH_4^+ assimilation in marine waters (Kirchman, 2012); however, the trends in the ammonia and nitrate data seem to indicate that nitrification rather than assimilation was the dominant NH_4^+ conversion pathway (Wu et al., 2013). The increasing nitrate concentration from the start of the experiment to month two is consistent with an increasing nitrification capacity with the establishment of a community of nitrifying bacteria in the predominately oxic sediment layers (Wu et al., 2013). Autotrophic nitrifying bacteria use little energy for cell synthesis; consequently, nitrifiers are typically slow to establish due to slow growth rates compared to heterotrophic bacteria (Ebeling et al., 2006). The trends in the sediment redox data support this hypothesis, since the redox potential in the first two months of the experiment reflected conditions suitable for nitrification (Gerardi, 2002). The stabilization of nitrate concentrations between months two and three may indicate that the nitrification capacity reached steady state with the onset of coupled nitrification-denitrification as the redox potential indicated conditions suitable for denitrification (+50 mV to -50 mV) in month two (Gerardi, 2002). The quantity of organic carbon (i.e. carbon loading) is one of the main controls on the denitrification efficiencies of sediments since this is a heterotrophic pathway of anaerobic nitrate respiration (Blackburn and Blackburn, 1992; Joye and Anderson, 2008). The decreasing nitrate concentrations in the final month are consistent with the conversion to di-nitrogen gas by denitrifying bacteria under anoxic conditions indicated by the negative sediment oxidation potentials recorded at the end of the trial (Blackburn and Blackburn, 1992; Seitzinger, 1988).

359 The conditions under which the organic carbon source is degraded (oxic or anaerobic) will
360 also affect the decomposition rate since bacterial growth efficiencies are a direct function of
361 oxygen availability (Fenchel et al., 2012; Goldman et al., 1987; Tezuka, 1990). In the present
362 study, the C:N of the aquaculture waste was increased from 5:1 to 20:1. For heterotrophic
363 bacteria (average C:N of 5:1), a substrate C:N of 20:1 represents the threshold for net
364 removal or net regeneration of ammonium at a bacterial growth efficiency of 25%. Under
365 anaerobic conditions, such as redox-stratified sediments, bacterial growth efficiencies range
366 from 5–30% (Goldman et al., 1987). It is possible that the C:N of 20:1 was not sufficiently
367 high to mediate a shift in nitrogen cycling pathways to promote net NH_4^+ assimilation due to
368 lower bacterial growth efficiencies under reducing conditions. Knowledge of the sediment
369 redox potential and how it changes over time in response to waste and carbon addition is
370 therefore a pre-requisite for determining the optimal C:N to assure the assimilation of NH_4^+
371 into bacterial biomass.
372 The biochemical composition of substrate is an important factor in defining resource quality
373 since the degradability is linked to its structural complexity. The carbon sources tested in this
374 study differed in their biodegradability; from glucose, a labile carbon source with a first-order
375 degradation rate constant of 1.15 d^{-1}, to cellulose, a complex structural polysaccharide with a
376 slow degradation rate of 0.05 d^{-1} (Avnimelech et al., 1995; Reddy et al., 1986). Readily
377 biodegradable substrates such as glucose are effective in promoting heterotrophic bacterial
378 growth under aerated conditions in biofloc systems (Crab et al., 2010); however, in this study
379 glucose had little impact on sea cucumber growth. The fundamental concept of C:N control
380 underpins biofloc technology where carbon-rich substrates are added to aquaculture
381 production tanks to overcome carbon limitation in heterotrophic bacteria and control the
382 build-up of inorganic nitrogen in the water column (Avnimelech, 1999). Biofloc uses high
383 aeration rates to maintain dense flocs in suspension (Crab et al., 2012). For this reason,

carbon sources tend to be highly labile and soluble and include molasses, glucose, sucrose, glycerol and acetate (Avnimelech, 2014; Crab et al., 2010). In contrast, the biomass density curve of sea cucumber juveniles reared in tanks amended with cellulose did not show any signs of plateauing, indicating that the maximum system carrying capacity was not reached. Had the experiment continued for a further two months the cellulose treatment may have outperformed the starch treatment. In a redox-stratified sediment-based system where heterotrophic bacteria have lower growth efficiencies, more complex (i.e. refractory) polysaccharides may be a more suitable long-term substrate. Cellulose is more resistant to hydrolysis as its β-1,4 bonds form rigid, ribbon-like chains with crystalline structures (Fenchel et al., 2012). Since the slow hydrolysis of more stable polysaccharides such as cellulose, demands an incorporation of nitrogen and phosphorus into microbial cells (Schroeder, 1987), the use of a more complex compound may offer a more stable means of ensuring sufficient carbon availability to bacteria in the long-term. This is particularly relevant in microbial-deposit feeder aquaculture bioremediation systems (see (Robinson et al., 2018)), due to the need to balance the constant efflux of NH_4^+ issuing from the redox-stratified sediment, sea cucumber excretion and ammonification following waste addition. The addition of cellulose would also approximate the natural habitat of *H. scabra* that comprises seagrass beds in areas with high terrigenous input (Hamel et al., 2001). This laboratory-scale observation highlights the potential to further increase the environmental and possibly the economic sustainability of integrated aquaculture production and bioremediation systems by utilising cheaper sources of complex carbon, including agricultural by-products such as bagasse or biochar (Srinivasva, 1987).

5. Conclusions

Carbon addition is utilised in other aquaculture treatment technologies including biofloc, denitrifying reactors and treatment of saline sludge by anaerobic digestion (Avnimelech, 2014; Hamlin et al., 2008; Luo et al., 2015; van Rijn et al., 2006). While these technologies utilise low cost carbon sources, they focus on the permanent removal of nitrogen through denitrification, or generate additional solid wastes that require disposal. Carbon supplementation may offer a more sustainable alternative to retain nitrogen in the system by promoting the net immobilisation of NH_4^+ into single cell biomass that can be up-cycled into high value secondary biomass. The potential for high-density culture is therefore an attractive approach to closing the nitrogen loop, especially if waste streams from aquaculture and agriculture are combined.

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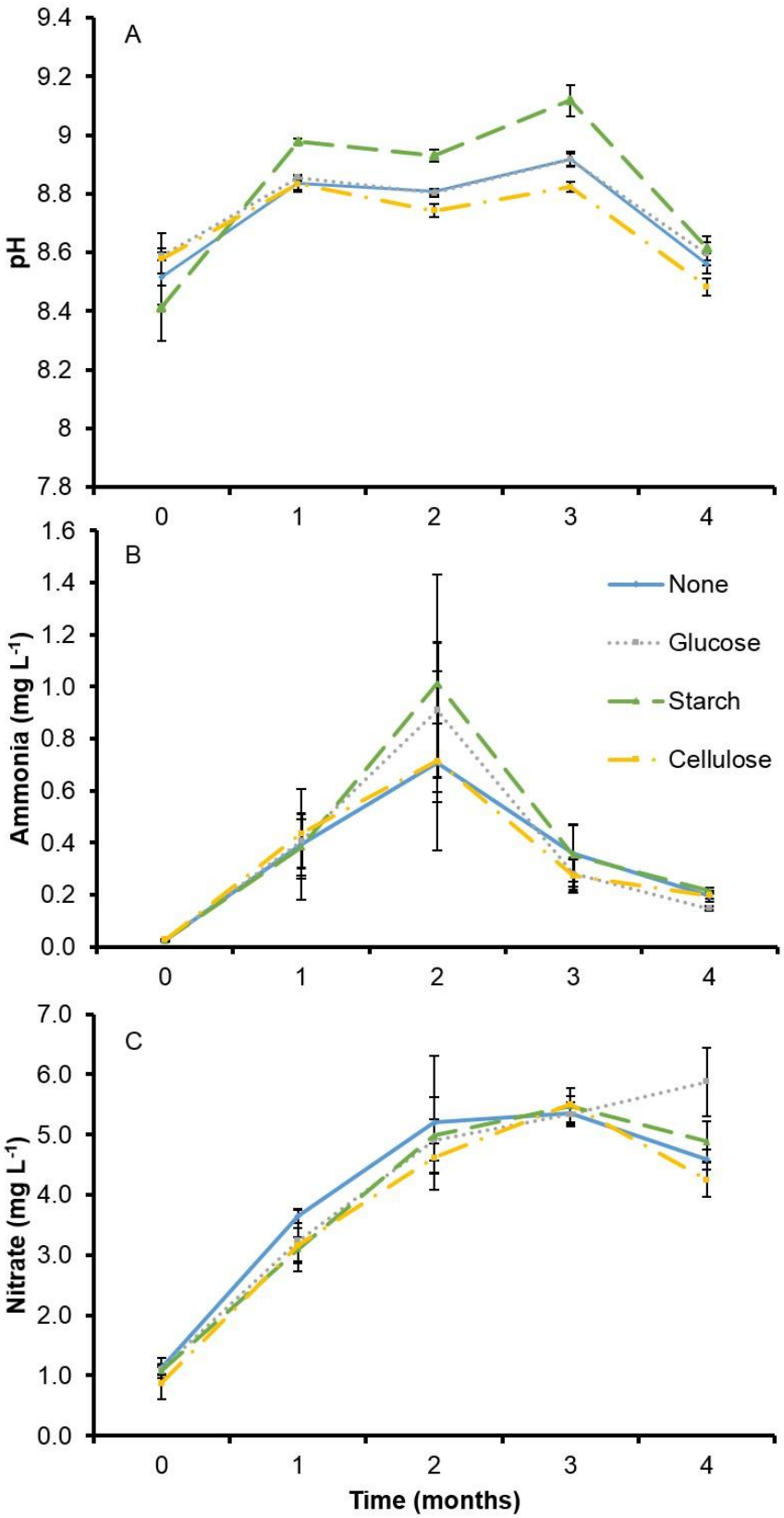
Figure legends.

Figure 1. The mean (± standard error) **(**A) pH, (B) total ammonia-nitrogen (TAN) and (C) nitrate concentration of the water in sea cucumber tanks dosed with aquaculture waste only (none) or with aquaculture waste amended with various carbon sources (i.e. glucose, starch or cellulose).

Figure 2. The mean (± standard error) (A) reduction-oxidation (redox) potential at the base of the sediment (4 cm deep), (B) organic carbon content (%) measured in the surface sediment, (C) total nitrogen content (%) measured in the surface sediment and (D) carbon to nitrogen ratios (C:N) measured in the surface sediment in sea cucumber tanks dosed with aquaculture waste only (none) or with aquaculture waste amended with various carbon sources (i.e. glucose, starch or cellulose).

Figure 3. The mean (± standard error) (A) growth rate, (B) cumulative biomass density and (C) wet weight of *Holothuria scabra* juveniles (n=4) reared on particulate aquaculture waste (none) or with aquaculture waste amended with various carbon sources (i.e. glucose, starch or cellulose) for four months.



636

637

638 Fig 1.

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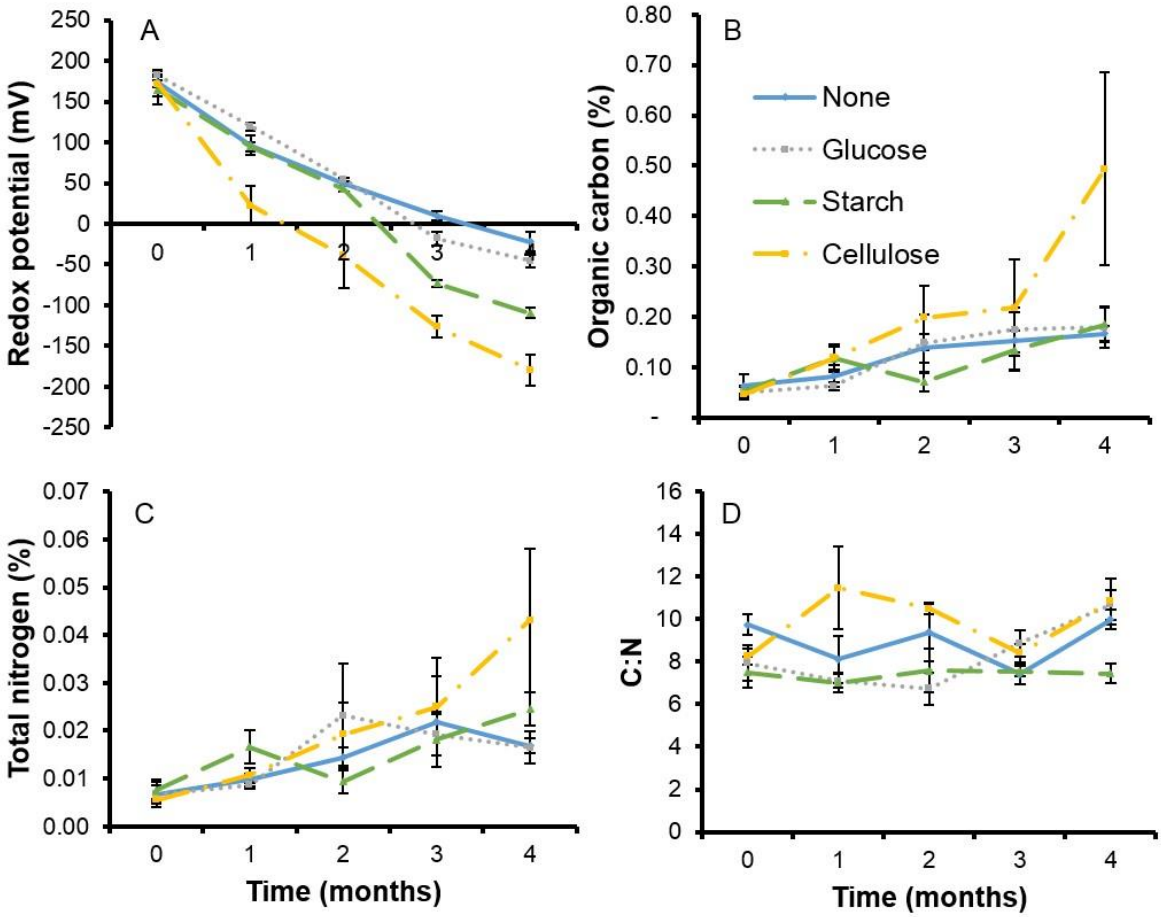
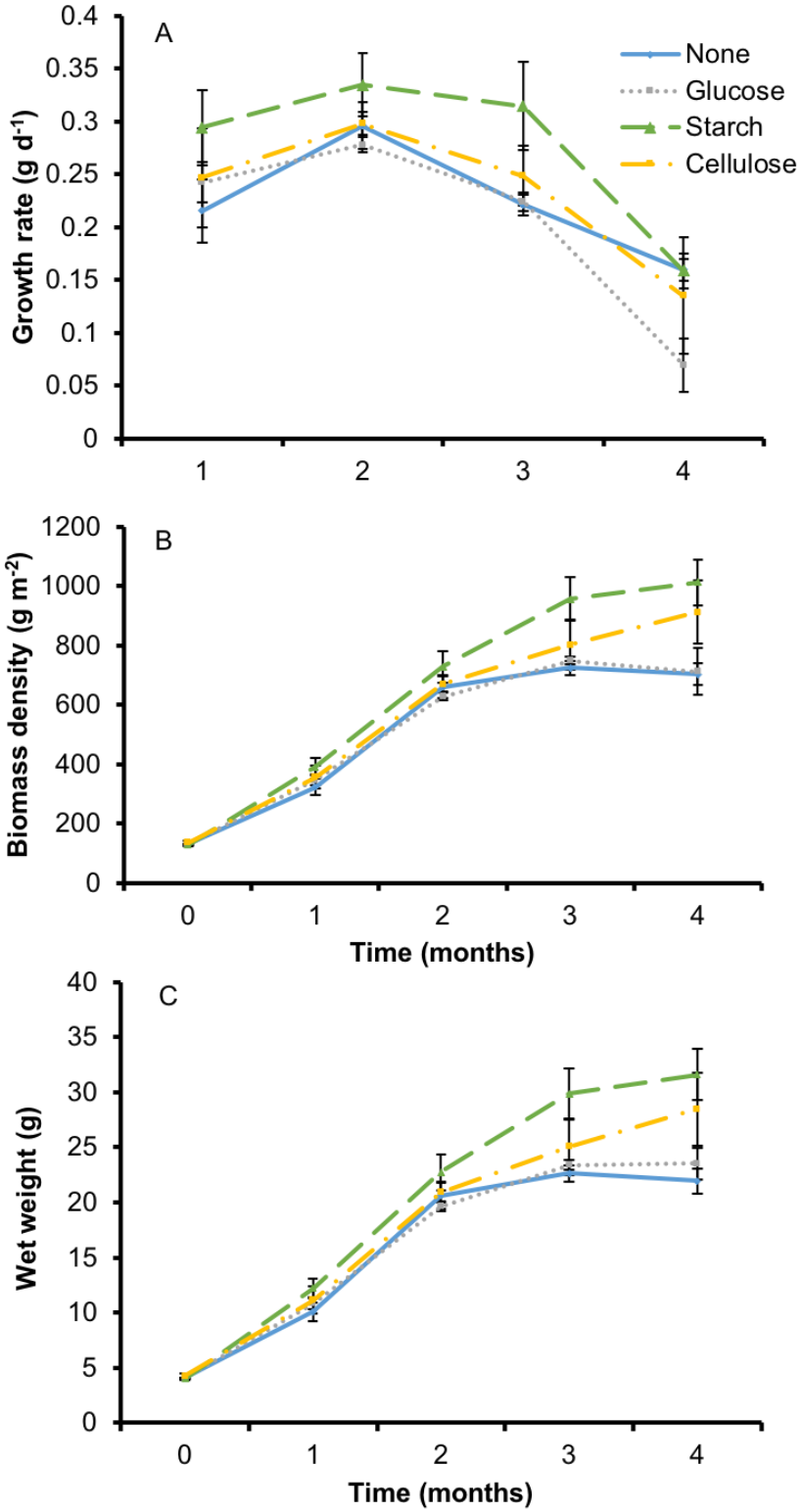


640
641 Fig 2.

642


A
None
Glucose
Starch
Cellulose
Growth rate (g d-1)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
B
Biomass density (g m-2)
1200
1000
800
600
400
200
0
0
1
2
3
4
Time (months)
C
Wet weight (g)
40
35
30
25
20
15
10
5
0
0
1
2
3
4
Time (months)

643

644 Fig 3.

Table 1. Description of experimental treatments including daily additions of particulate aquaculture waste (2.41 g wet weight) and the different carbon sources standardised to 200 mmol C m^{-2} d^{-1}. *First-order decomposition rate constant is the percentage of a given compound that degrades each day; **Half-life is the time it takes to reach 50% of the complete degradation of a given substrate concentration.

Carbon source	Solubility	*First-order decomposition rate (d^{-1})	**Half-life (d)	Carbon content (%)	Dry weight (g)	C:N
None	N/A	N/A	N/A	N/A	N/A	5:1
D-glucose	Soluble	1.1500	0.6	39.99	0.75	20:1
Starch	50 g L^{-1} at 90 °C	0.8000	5.0	44.44	0.68	20:1
Cellulose, microcrystalline	Insoluble (20 °C)	0.0495	14.0	44.44	0.68	20:1

Table 2. Mean (± standard error) of water and sediment quality parameters recorded during the four-month experiment. C:N = carbon to nitrogen ratio.

	None	Glucose	Starch	Cellulose	Overall
Water quality					
Light (lux)	139.25 ± 5.22	141.25 ± 5.22	141.25 ± 5.22	136.35 ± 5.41	139.53 ± 2.59
Temperature (℃)	29.07 ± 0.50	29.24 ± 0.53	29.21 ± 0.55	29.19 ± 0.51	29.18 ± 0.26
Dissolved oxygen (mg L^{-1})	7.28 ± 0.17	7.19 ± 0.12	7.47 ± 0.14	7.11 ± 0.14	7.26 ± 0.07
Ammonia (mg L^{-1})	0.34 ± 0.07	0.35 ± 0.09	0.40 ± 0.11	0.33 ± 0.09	0.35 ± 0.04
Nitrate (mg L^{-1})	3.98 ± 0.41	4.09 ± 0.43	3.91 ± 0.40	3.68 ± 0.38	3.92 ± 0.20
Sediment quality					
Total carbohydrate (μg g^{-1})	64.27 ± 28.08	49.90 ± 19.37	41.11 ± 21.96	96.08 ± 40.80	62.84 ± 14.30
Chlorophyll *a* (μg g^{-1})	0.86 ± 0.56	0.85 ± 0.37	1.29 ± 0.54	2.67 ± 1.19	1.42 ± 0.37
Phaeopigment (μg g^{-1})	1.08 ± 0.77	0.97 ± 0.49	3.04 ± 1.10	4.76 ± 2.15	2.46 ± 0.66
Organic carbon (%)	0.12 ± 0.02	0.12 ± 0.02	0.11 ± 0.02	0.20 ± 0.05	0.14 ± 0.01
Nitrogen (%)	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C:N	8.97 ± 0.44	8.43 ± 0.43	7.41 ± 0.23	9.84 ± 0.51	8.66 ± 0.23

Captions for supplementary tables:

Table S1: Supporting statistics for environmental and water quality parameter data analysed by repeated measures ANOVA, $p<0.05$ (* indicates significant differences).

Table S2: Supporting statistics for sediment reduction-oxidation potential, total carbohydrate, chlorophyll *a* and phaeopigment data analysed by repeated measures ANOVA, $p<0.05$ (* indicates significant differences).

Table S3: Supporting statistics for sediment organic carbon, nitrogen and carbon to nitrogen ratio data analysed by repeated measures ANOVA, $p<0.05$ (* indicates significant differences).

Table S4: Supporting statistics for sea cucumber juvenile growth data analysed by repeated measures ANOVA, $p<0.05$ (* indicates significant differences).

Supplementary:

The effect of resource quality on the growth of *Holothuria scabra* during aquaculture waste bioremediation

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Supplementary information

Table S1: Supporting statistics for environmental and water quality parameter data analysed by repeated measures ANOVA, p<0.05 (* indicates significant differences).

Source of variation	*df*	Light (lux)			Temperature (˚C)			Dissolved oxygen (mg L^{-1})			pH		
		MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*P*	MS	*F*	*p*
Carbon source (C)	3	8.75E+07	0.226	0.877	0.11	0.1	0.972	0.485	1.61	0.240	0.05	5.62	0.012*
Time (T)	4	4.02E+08	3.038	0.026*	93.44	190.7	0.000*	5.444	52.52	0.000*	0.575	67.4	0.000*
C x T	12	3.52E+07	0.265	0.992	0.06	0.1	1.000	0.081	0.78	0.663	0.024	2.79	0.006*

Table S2: Supporting statistics for sediment reduction-oxidation potential, total carbohydrate, chlorophyll *a* and phaeopigment data analysed by repeated measures ANOVA, p<0.05 (* indicates significant differences).

Source of variation	*df*	Reduction-oxidation potential (mV)			Total carbohydrate (µg g^{-1})			Chlorophyll *a* (µg g^{-1})			Phaeopigme (µg g^{-1})		
		MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*	MS	*F*	
Carbon source (C)	3	3.62E+04	30.1	0.000*	1.16E+04	0.985	0.432	14.81	1.530	0.257	64.9	1.636	
Time (T)	4	1.77E+05	251.9	0.000*	8.32E+04	5.373	0.001*	48.62	5.593	0.001*	136.3	5.244	
C x T	12	3347	4.8	0.000*	3,323.00	0.214	0.997	7.51	0.864	0.587	21.8	0.840	

Table S3: Supporting statistics for sediment organic carbon, nitrogen and carbon to nitrogen ratio data analysed by repeated measures ANOVA, p<0.05 (* indicates significant differences).

Source of variation	*df*	Organic carbon (%)			Nitrogen (%)			C/N ratio		
		MS	*F*	*p*	MS	*F*	*p*	MS	*F*	*p*
Carbon source (C)	3	0.048	1.852	0.238	0.000	1.237	0.376	16.45	4.211	0.064
Time (T)	4	0.060	6.061	0.002*	0.001	5.877	0.002*	5.43	2.695	0.055
C x T	12	0.015	1.548	0.175	0.000	1.422	0.223	4.38	2.174	0.051

Table S4: Supporting statistics for sea cucumber growth data analysed by repeated measures ANOVA, p<0.05 (* indicates significant differences).

Source of variation	Wet weight (g)				Biomass density (g m^{-2})				Growth rate (g d^{-1})			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*	*df*	MS	*F*	*P*
Carbon source (C)	3	1.16E+04	0.985	0.432	3	7.98E+04	4.1	0.031*	3	0.023	4.22	0.030*
Time (T)	4	8.32E+04	5.373	0.001*	4	1.50E+06	224.2	0.000*	3	0.126	44.53	0.000*
C x T	12	3,323.00	0.214	0.997	12	1.67E+04	2.5	0.013*	9	0.002	0.72	0.690