

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

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18 **Abstract**

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

39

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

## 42        **1. Introduction**

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

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

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

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

67 reducing feed, space, and energy requirements (Chávez-Crooker and Obreque-Contreras,  
68 2010). Sediment microbial communities are primarily net heterotrophic systems that link  
69 energy transfer to higher trophic levels; therefore recycling nutrients *in situ* may provide a  
70 viable means to intensively culture deposit feeders with a higher overall efficiency  
71 (Schroeder, 1987). Furthermore, prior research (Robinson et al., 2015; Robinson et al., 2016)  
72 demonstrated that redox-stratified sediment supported faster growth rates and a higher  
73 biomass yield of *Holothuria scabra* relative to fully oxic sediment, indicating that  
74 heterotrophic systems are more favourable for deposit feeder growth.

75 Heterotrophs fundamentally differ from autotrophs due to their metabolic requirement for  
76 an organic source of carbon. Heterotrophic bacteria assimilate organic carbon and nitrogen in  
77 a stoichiometric balance based on the carbon to nitrogen ratio (C:N) of the bacterial  
78 cytoplasm (Goldman et al., 1987; Herbert, 1967). The C:N of organic substrates is an  
79 important parameter determining the degree of nitrogen regeneration as carbon and nitrogen  
80 are incorporated into bacteria at a fixed rate (Tezuka, 1990). For bacteria grown in an  
81 environment with a C:N of 5:1 and an average growth efficiency (the quantity of biomass  
82 produced per unit of assimilated organic carbon) of 50% under aerobic conditions, the  
83 threshold between net release and net immobilisation of nitrogen is 10:1 (Azim et al., 2008;  
84 Rittmann and McCarty, 2001). Increasing C:N beyond 10:1 provides sufficient carbon for  
85 heterotrophic bacteria to assimilate ammonium ( $\text{NH}_4^+$ ) into biomass, thus mediating a shift  
86 from net  $\text{NH}_4^+$  release (ammonification) to net immobilisation (assimilation) (Avnimelech,  
87 1999; Avnimelech, 2014; Azim et al., 2008; Ebeling et al., 2006).

88 From a thermodynamic perspective, heterotrophic bacteria preferentially utilise reduced  
89 inorganic forms of nitrogen such as  $\text{NH}_4^+$ ; however,  $\text{NH}_4^+$  assimilation is dependent on the  
90 availability of carbon substrates (Church, 2008; Fenchel and Blackburn, 1979). Particulate  
91 organic wastes from aquaculture primarily comprise waste food and faeces, and are generally

92 deficient in organic carbon, with an average C:N of 7:1; thus, there is a net release of  $\text{NH}_4^+$   
93 during decomposition, often measured alongside ammonia as total ammonia nitrogen (TAN)  
94 (Avnimelech, 1999; Mirzoyan et al., 2012; Schneider et al., 2006). Particulate organic waste  
95 recovered from mechanical filtration has been used as substrate to produce heterotrophic  
96 bacteria and deposit feeding macrofauna, including polychaetes and sea cucumbers (Brown et  
97 al., 2011; MacDonald et al., 2013; Palmer, 2010; Schneider et al., 2007a; Schneider et al.,  
98 2007b; Schneider et al., 2006). Raising secondary livestock on aquaculture waste can provide  
99 a direct means of assimilating a proportion of the effluent nitrogen (Erler et al., 2004);  
100 however, detritivores are predicted to have poor nitrogen retention compared to other trophic  
101 groups (Schneider et al., 2005). Erler et al. (Erler et al., 2004) hypothesized that stimulated  
102 bacterial nitrogen processing during the production of secondary livestock on RAS effluents  
103 may be more important than direct assimilation. Schneider et al. (Schneider et al., 2007b)  
104 demonstrated that adding a source of labile organic carbon, e.g. molasses, could increase the  
105 conversion of inorganic nitrogenous wastes to heterotrophic bacterial biomass. Stimulating  
106 heterotrophic bacteria by manipulating the C:N may therefore offer an indirect means of  
107 increasing nitrogen retention in macrofauna reared on aquaculture effluents.

108 In sediment-based sea cucumber culture, controlling inorganic nitrogen cycling by adding  
109 carbon may be particularly relevant due to the need to counteract additional sources of  $\text{NH}_4^+$   
110 within the system, including: i) net efflux from the sediment (Hargreaves, 1998); ii) excretion  
111 from the sea cucumbers (Uthicke and Klumpp, 1997); and, iii) decomposition of aquaculture  
112 waste and feeds (Avnimelech, 1999). Carbon supplementation may offer an indirect means to  
113 retain nitrogen safely within the system by immobilising  $\text{NH}_4^+$  into microbial biomass that  
114 can be upcycled into high value secondary biomass. We therefore investigated whether, by  
115 carbon supplementation, the uptake of nitrogen from waste feed and faeces by sea cucumbers  
116 can be improved.

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

122

## 123 **2. Material and methods**

### 124 ***2.1 Study site and experimental animals***

125 The Ethics Panels of both Newcastle and Rhodes Universities approved the study, and  
126 no collections were made from wild populations to support it. The research was conducted in  
127 a purpose built, bio-secure, heated RAS between October 8<sup>th</sup> 2013 and January 28<sup>th</sup> 2014 at  
128 HIK Abalone Farm Pty (Ltd) in Hermanus, South Africa. The detailed system specifications  
129 are found in Robinson et al. (2015). Juvenile *Holothuria scabra* were imported from a  
130 commercial hatchery (Research Institute for Aquaculture III, Vietnam) on September 5<sup>th</sup>  
131 2013, and quarantined in a bio-secure facility for six weeks in accordance with South African  
132 importation and scientific investigations licenses. Following the quarantine period and prior  
133 to experimentation, the sea cucumber juveniles were held in the RAS in tanks filled with  
134 10 cm of calcium carbonate sand and were fed a 34% protein commercial abalone weaning  
135 diet (Abfeed®-S34, 1.0 mm sugar grain pellet; Marifeed Pty Ltd, South Africa). The  
136 proximate nutritional composition of the feed was; crude protein 33%, crude lipid 3.0%,  
137 vitamins (0.1%), gross energy 15.6 kJ g<sup>-1</sup>.

### 138 ***2.2 Experimental design***

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

141 soluble (D-glucose and starch) and one insoluble carbon sources (microcrystalline cellulose)  
142 representing a range of first-order decomposition rate constants and labilities were purchased  
143 from Merck Millipore, South Africa (Table 1). The carbon sources were tested in conjunction  
144 with aquaculture waste (comprising uneaten feed and faeces recovered by mechanical  
145 filtration from a land-based abalone RAS) at a C:N of 20:1; a fourth treatment receiving  
146 aquaculture waste only (C:N of 5:1) was included as a control (Table 1). The average C:N of  
147 the dried particulate aquaculture waste was  $5.21 \pm 0.55$ . The quantity of carbon necessary to  
148 increase the ratio to 20:1 was calculated from the proportion of carbon in the molecular  
149 composition of each compound. Carbon additions were standardised between treatments  
150 based on a daily addition of  $200 \text{ mmol C m}^{-2} \text{ d}^{-1}$ , which is within the upper range tolerated by  
151 benthic animals under eutrophic conditions of  $100\text{-}400 \text{ mmol C m}^{-2} \text{ d}^{-1}$  (Lehtoranta et al.,  
152 2009). At a C:N of 20:1, this equates to  $2.4 \text{ g m}^{-2} \text{ d}^{-1}$  of carbon and  $0.12 \text{ g m}^{-2} \text{ d}^{-1}$  of nitrogen,  
153 which is between a ‘mid’ and ‘high’ ration of  $100$  and  $150 \text{ mg m}^{-2} \text{ d}^{-1}$  of nitrogen respectively  
154 for deposit feeders (Alongi and Hanson, 1985; Tenore and Chesney, 1985).

155         Abalone (*Haliotis midae*) waste comprising mainly uneaten feed and faeces was  
156 collected every morning at 09:00 from the backwash of a grow-out RAS sand filter over five  
157 days prior to the experiment. The total system volume was approximately 32,000 L, holding a  
158 maximum of 800 kg of adult abalone (Yearsley et al., 2009). The abalone were fed to  
159 satiation daily with a 34% protein commercial abalone weaning diet (S34 Abfeed®,  $10 \times 10$   
160  $\times 1.2$  mm pellet; Marifeed Pty Ltd, South Africa). The water leaving the production tanks  
161 drained through a 500 L sand filter filled with BS8:16 silica filter media for particulate  
162 organic waste removal (1-2 mm) prior to foam fractionation and biofiltration. The sand filter  
163 was fitted with a Jetco 5-port valve to change the water flow direction and permit  
164 backwashing. A 50 mm flexihose pipe was connected from the sand filter outflow to a 100 L  
165 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<sup>2</sup>, 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<sub>4</sub>-N; TAN) were recorded weekly as described in Robinson et al.  
203 (2015). Nitrate concentration ( $\pm 0.01$  mg L<sup>-1</sup>) 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 ( $\mu\text{g g}^{-1}$ ) were measured using the phenol-sulphuric acid method (Underwood et

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

#### 219 ***2.4 Sea cucumber growth***

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

#### 226 ***2.5 Statistical analyses***

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

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

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

### 246 **3. Results**

#### 247 ***3.1 Water and sediment quality and environmental variables***

248 There were no significant differences in light, salinity, temperature, dissolved oxygen  
249 concentration, total ammonia or nitrate among treatments (repeated measures ANOVA:  $p >$   
250  $0.05$ ; Table 2 and Table S1). Salinity maintained a constant  $35 \text{ g L}^{-1}$  and dissolved oxygen  
251 concentration varied between  $6.05$  and  $8.95 \text{ mg L}^{-1}$  ( $7.26 \pm 0.07 \text{ mg L}^{-1}$ ). The mean water  
252 temperature increased over the course of the experiment with the onset of the austral summer  
253 from a mean temperature of  $25.49 \pm 0.14 \text{ }^\circ\text{C}$  at the start, increasing to a mean of  $32.30 \pm 0.34$   
254  $^\circ\text{C}$  during the final month. pH differed significantly between treatments (repeated measures  
255 ANOVA;  $F_{(12, 48)} = 2.79$ ,  $p = 0.006$ ). The starch-amended treatment had the highest seawater  
256 pH between months one and three, peaking at  $9.12 \pm 0.05$  in month three. In all treatments,  
257 pH tended to increase with time until the final month when values returned to those measured  
258 at the start of the experiment (Fig. 1A).

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

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

### 265 **3.2 Sediment characteristics**

266 The type of carbon source significantly affected sediment redox potential (repeated measures  
267 ANOVA,  $F_{(12, 48)} = 4.76$ ,  $p = <0.001$ ; Fig. 2A and Table S2). There were no significant  
268 differences between treatments at the start of the experiment with a positive reading of  
269  $173.06 \pm 5.99 \text{ mV}$  indicating fully oxic conditions. The redox potential decreased over time  
270 in all treatments; however, there was an apparent relatedness to the lability of the carbon  
271 source. The more refractory cellulose and starch had the greatest impact on redox potential,  
272 decreasing to  $-179.75 \pm 18.93 \text{ mV}$  and  $-109.50 \pm 6.14 \text{ mV}$  in the final month respectively. In  
273 the final month, the redox potential in tanks solely receiving aquaculture waste was  
274 marginally negative ( $-22.00 \pm 12.19 \text{ mV}$ ) and not significantly different to glucose-amended  
275 tanks, the most labile carbon source ( $-45.75 \pm 8.47 \text{ mV}$ ).

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

### 286 3.3 Growth and survival

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

### 297 3.4 Multiple regression analysis

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

## 302 4. Discussion

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

310 growth rates by balancing the elemental stoichiometry of the food (Rice and Rhoads, 1989).  
311 An alternate explanation is that *H. scabra* was able to assimilate the carbon sources or their  
312 degradation products directly or indirectly through collaboration with microbial communities.  
313 Mechanisms for the uptake of organic molecules such as amino acids and carbohydrates are  
314 thought to exist in tissues such as the respiratory trees, body wall or tentacles, thereby  
315 enabling holothurians to assimilate dissolved organic matter directly from the water column  
316 (Fontaine and Chia, 1968; Jaeckle and Strathmann, 2013; Jangoux and Lawrence, 1982;  
317 Krishnan and Krishnaswamy, 1970; Lawrence, 1982).  
318 Direct utilisation of starch for enhanced growth may not have been limited to the sea  
319 cucumbers. Although there was no significant difference in either chlorophyll *a* or  
320 phaeopigment concentrations, there were apparent differences (albeit qualitative) in the  
321 extent of phototroph biomass production between treatments. Carbohydrates such as glucose  
322 and polysaccharides can also be photo-oxidised with high quantum efficiencies in illuminated  
323 habitats leading to concomitant increases in microbial and algal productivity (Krishnan and  
324 Krishnaswamy, 1970; Stuart et al., 2016). The quality and quantity of organic matter supply  
325 to the sediment is modulated by sunlight, temperature and nutrient availability (Huettel et al.,  
326 2014). Light and nitrate were significant positive predictors of sea cucumber biomass density.  
327 Cyanobacteria may have supplied additional carbon to heterotrophic sediment bacteria and  
328 sea cucumbers as extracellular polymeric substances from photosynthesis (Stuart et al.,  
329 2016).  
330 Avnimelech (1999) hypothesised that manipulating C:N may change nitrogen cycling  
331 pathways in aquaculture systems by mediating a shift from ammonification (net release) to  
332 assimilation (net uptake) of  $\text{NH}_4^+$  by heterotrophic bacteria. For aerobic heterotrophic  
333 bacteria, the removal of  $\text{NH}_4^+$  by incorporation in cells is enhanced by carbohydrate addition  
334 (Ebeling et al., 2006). It was therefore expected in these experiments that carbon

335 supplementation would decrease total ammonia nitrogen (TAN) concentrations compared to  
336 the control. However, this was not the case, although levels were generally low throughout  
337 the study (averaging  $0.35 \pm 0.04 \text{ mg L}^{-1}$ ). This may have been due to assimilation by  
338 heterotrophic bacteria, which are responsible for a large fraction of  $\text{NH}_4^+$  assimilation in  
339 marine waters (Kirchman, 2012); however, the trends in the ammonia and nitrate data seem  
340 to indicate that nitrification rather than assimilation was the dominant  $\text{NH}_4^+$  conversion  
341 pathway (Wu et al., 2013). The increasing nitrate concentration from the start of the  
342 experiment to month two is consistent with an increasing nitrification capacity with the  
343 establishment of a community of nitrifying bacteria in the predominately oxic sediment  
344 layers (Wu et al., 2013). Autotrophic nitrifying bacteria use little energy for cell synthesis;  
345 consequently, nitrifiers are typically slow to establish due to slow growth rates compared to  
346 heterotrophic bacteria (Ebeling et al., 2006). The trends in the sediment redox data support  
347 this hypothesis, since the redox potential in the first two months of the experiment reflected  
348 conditions suitable for nitrification (Gerardi, 2002). The stabilization of nitrate concentrations  
349 between months two and three may indicate that the nitrification capacity reached steady  
350 state with the onset of coupled nitrification-denitrification as the redox potential indicated  
351 conditions suitable for denitrification (+50 mV to -50 mV) in month two (Gerardi, 2002). The  
352 quantity of organic carbon (i.e. carbon loading) is one of the main controls on the  
353 denitrification efficiencies of sediments since this is a heterotrophic pathway of anaerobic  
354 nitrate respiration (Blackburn and Blackburn, 1992; Joye and Anderson, 2008). The  
355 decreasing nitrate concentrations in the final month are consistent with the conversion to di-  
356 nitrogen gas by denitrifying bacteria under anoxic conditions indicated by the negative  
357 sediment oxidation potentials recorded at the end of the trial (Blackburn and Blackburn,  
358 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  $\text{NH}_4^+$  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  $\text{NH}_4^+$   
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  $1.15 \text{ d}^{-1}$ , to cellulose, a complex structural polysaccharide with a  
376 slow degradation rate of  $0.05 \text{ d}^{-1}$  (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,



384 carbon sources tend to be highly labile and soluble and include molasses, glucose, sucrose,  
385 glycerol and acetate (Avnimelech, 2014; Crab et al., 2010). In contrast, the biomass density  
386 curve of sea cucumber juveniles reared in tanks amended with cellulose did not show any  
387 signs of plateauing, indicating that the maximum system carrying capacity was not reached.  
388 Had the experiment continued for a further two months the cellulose treatment may have  
389 outperformed the starch treatment. In a redox-stratified sediment-based system where  
390 heterotrophic bacteria have lower growth efficiencies, more complex (i.e. refractory)  
391 polysaccharides may be a more suitable long-term substrate. Cellulose is more resistant to  
392 hydrolysis as its  $\beta$ -1,4 bonds form rigid, ribbon-like chains with crystalline structures  
393 (Fenchel et al., 2012). Since the slow hydrolysis of more stable polysaccharides such as  
394 cellulose, demands an incorporation of nitrogen and phosphorus into microbial cells  
395 (Schroeder, 1987), the use of a more complex compound may offer a more stable means of  
396 ensuring sufficient carbon availability to bacteria in the long-term. This is particularly  
397 relevant in microbial-deposit feeder aquaculture bioremediation systems (see (Robinson et  
398 al., 2018)), due to the need to balance the constant efflux of  $\text{NH}_4^+$  issuing from the redox-  
399 stratified sediment, sea cucumber excretion and ammonification following waste addition.  
400 The addition of cellulose would also approximate the natural habitat of *H. scabra* that  
401 comprises seagrass beds in areas with high terrigenous input (Hamel et al., 2001). This  
402 laboratory-scale observation highlights the potential to further increase the environmental and  
403 possibly the economic sustainability of integrated aquaculture production and bioremediation  
404 systems by utilising cheaper sources of complex carbon, including agricultural by-products  
405 such as bagasse or biochar (Srinivasva, 1987).

406

## 407 **5. Conclusions**

408 Carbon addition is utilised in other aquaculture treatment technologies including biofloc,  
409 denitrifying reactors and treatment of saline sludge by anaerobic digestion (Avnimelech,  
410 2014; Hamlin et al., 2008; Luo et al., 2015; van Rijn et al., 2006). While these technologies  
411 utilise low cost carbon sources, they focus on the permanent removal of nitrogen through  
412 denitrification, or generate additional solid wastes that require disposal. Carbon  
413 supplementation may offer a more sustainable alternative to retain nitrogen in the system by  
414 promoting the net immobilisation of  $\text{NH}_4^+$  into single cell biomass that can be up-cycled into  
415 high value secondary biomass. The potential for high-density culture is therefore an attractive  
416 approach to closing the nitrogen loop, especially if waste streams from aquaculture and  
417 agriculture are combined.

418

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427

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429

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620 **Figure legends.**

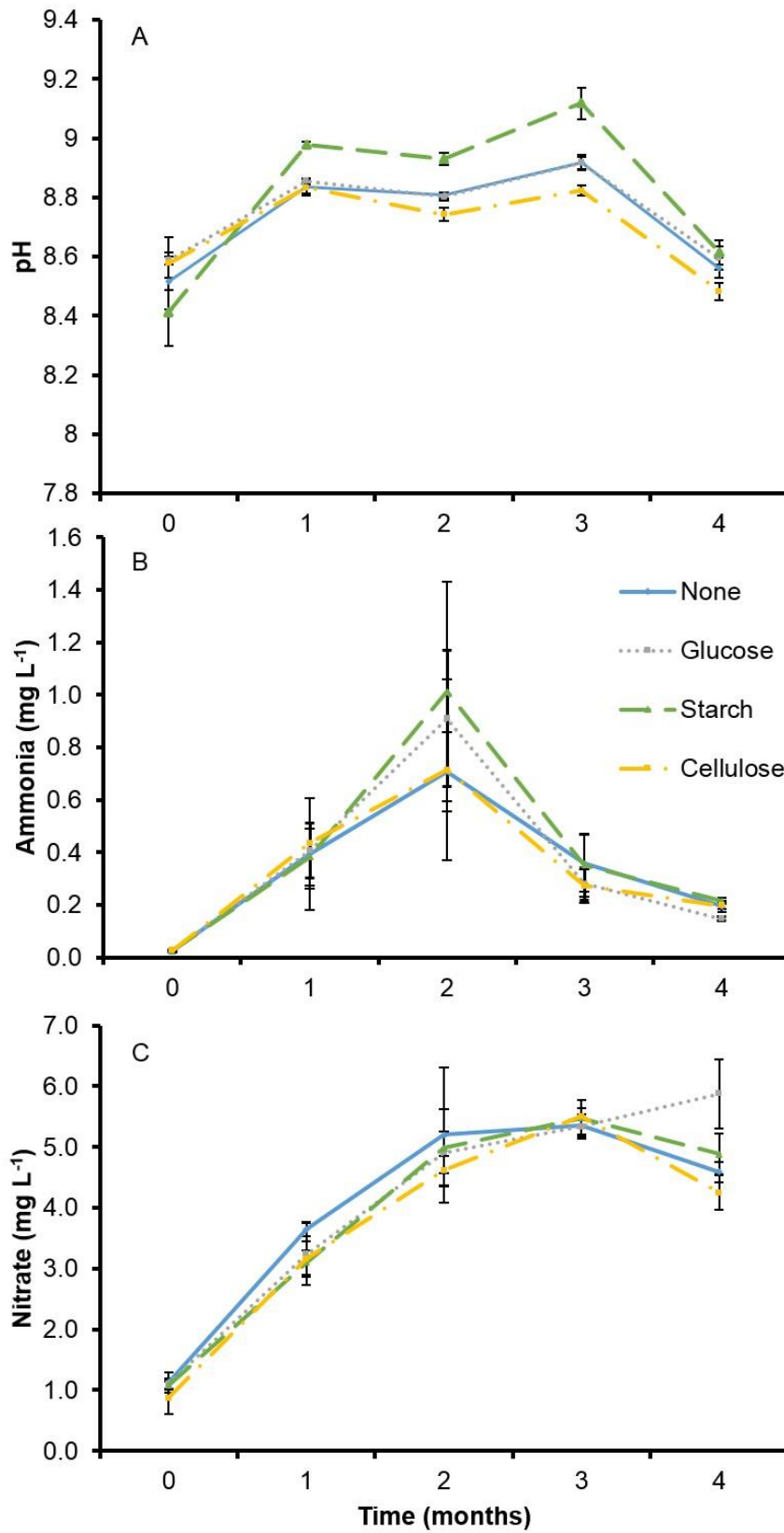
621 **Figure 1.** The mean ( $\pm$  standard error) (A) pH, (B) total ammonia-nitrogen (TAN) and (C)  
622 nitrate concentration of the water in sea cucumber tanks dosed with aquaculture waste only  
623 (none) or with aquaculture waste amended with various carbon sources (i.e. glucose, starch or  
624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

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

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

635



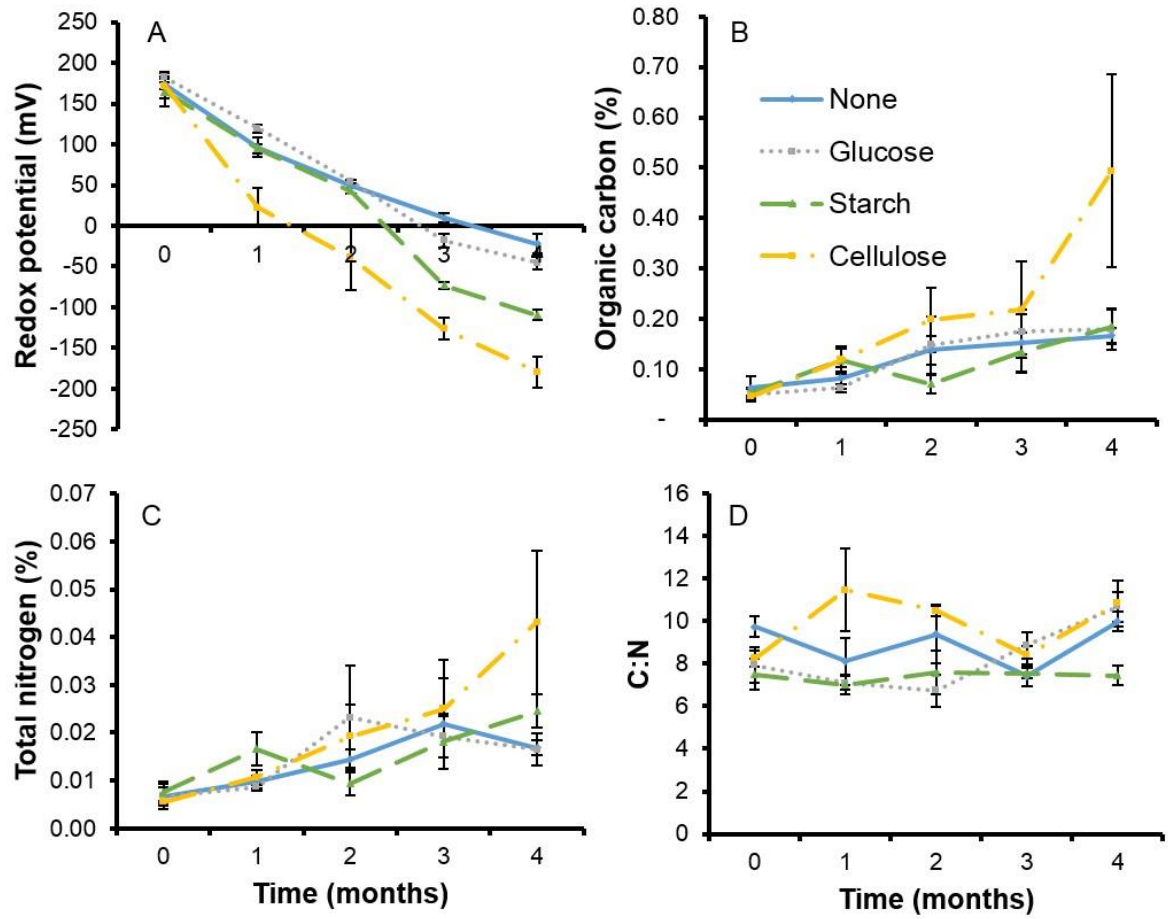


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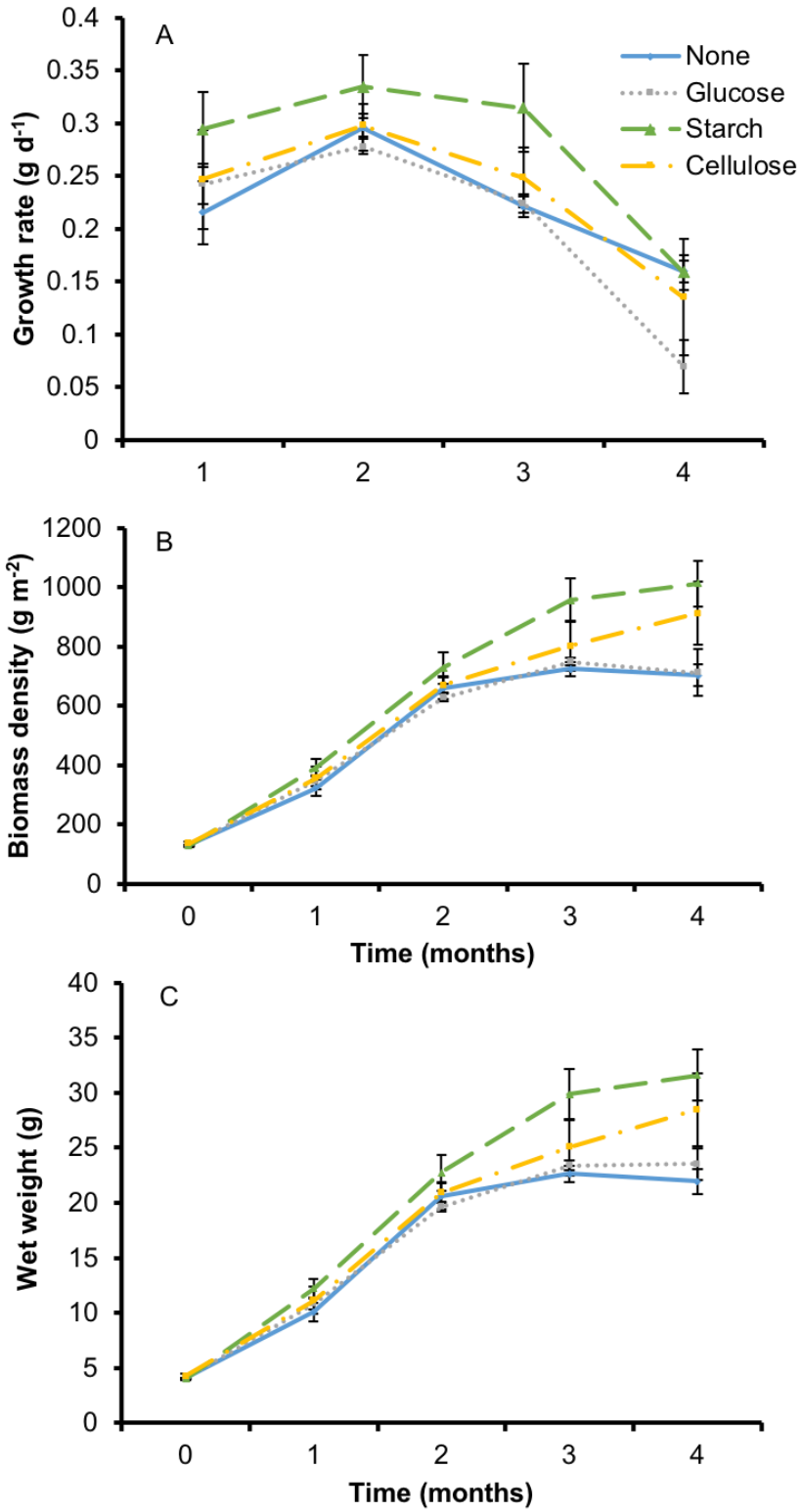
638 Fig 1.

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640  
641 Fig 2.

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644 Fig 3.

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

Carbon source	Solubility	*First-order decomposition rate (d <sup>-1</sup> )	**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 <sup>-1</sup> 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

650  
651

**Table 2.** Mean ( $\pm$  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
<i>Water quality</i>					
Light (lux)	139.25 $\pm$ 5.22	141.25 $\pm$ 5.22	141.25 $\pm$ 5.22	136.35 $\pm$ 5.41	139.53 $\pm$ 2.59
Temperature (°C)	29.07 $\pm$ 0.50	29.24 $\pm$ 0.53	29.21 $\pm$ 0.55	29.19 $\pm$ 0.51	29.18 $\pm$ 0.26
Dissolved oxygen (mg L <sup>-1</sup> )	7.28 $\pm$ 0.17	7.19 $\pm$ 0.12	7.47 $\pm$ 0.14	7.11 $\pm$ 0.14	7.26 $\pm$ 0.07
Ammonia (mg L <sup>-1</sup> )	0.34 $\pm$ 0.07	0.35 $\pm$ 0.09	0.40 $\pm$ 0.11	0.33 $\pm$ 0.09	0.35 $\pm$ 0.04
Nitrate (mg L <sup>-1</sup> )	3.98 $\pm$ 0.41	4.09 $\pm$ 0.43	3.91 $\pm$ 0.40	3.68 $\pm$ 0.38	3.92 $\pm$ 0.20
<i>Sediment quality</i>					
Total carbohydrate ( $\mu\text{g g}^{-1}$ )	64.27 $\pm$ 28.08	49.90 $\pm$ 19.37	41.11 $\pm$ 21.96	96.08 $\pm$ 40.80	62.84 $\pm$ 14.30
Chlorophyll <i>a</i> ( $\mu\text{g g}^{-1}$ )	0.86 $\pm$ 0.56	0.85 $\pm$ 0.37	1.29 $\pm$ 0.54	2.67 $\pm$ 1.19	1.42 $\pm$ 0.37
Phaeopigment ( $\mu\text{g g}^{-1}$ )	1.08 $\pm$ 0.77	0.97 $\pm$ 0.49	3.04 $\pm$ 1.10	4.76 $\pm$ 2.15	2.46 $\pm$ 0.66
Organic carbon (%)	0.12 $\pm$ 0.02	0.12 $\pm$ 0.02	0.11 $\pm$ 0.02	0.20 $\pm$ 0.05	0.14 $\pm$ 0.01
Nitrogen (%)	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00
C:N	8.97 $\pm$ 0.44	8.43 $\pm$ 0.43	7.41 $\pm$ 0.23	9.84 $\pm$ 0.51	8.66 $\pm$ 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 <sup>-1</sup> )			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 <sup>-1</sup> )			Chlorophyll <i>a</i> (µg g <sup>-1</sup> )			Phaeopigment (µg g <sup>-1</sup> )	
		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 <sup>-2</sup> )			Growth rate (g d <sup>-1</sup> )					
	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