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Carbon supplementation promotes assimilation of aquaculture waste by the sea cucumber *Holothuria scabra*: evidence from stable isotope analysis

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Abstract

Cultivating high-value deposit feeders such as sea cucumbers on solid waste from intensive aquaculture is an innovative approach to turn potential nutrient pollution into a resource. Carbon supplementation to increase the carbon to nitrogen ratio (C:N) of the nitrogen-rich aquaculture waste has been demonstrated to support higher deposit feeder biomass, but commercially viable carbon sources remain to be tested. This

experiment investigated the performance of laboratory grade cellulose and bagasse, an agricultural waste product, to improve growth and nutrient utilization from milkfish aquaculture waste in juveniles of the sea cucumber *Holothuria scabra*. Supplementation with bagasse supported a significantly higher final biomass density (205.71 ± 11.08 g m^{-2}, mean ± SE) than waste without carbon supplementation ($p = 0.027$). For the first time, a species-specific trophic enrichment factor ($\Delta^{13}C_{org}$) was applied in stable isotope analysis of feed sources by *H. scabra* ($\Delta^{13}C_{org}$ of 2.56 ± 0.96 ‰ (mean ± SD) for the internal organs and 4.31 ± 0.96 ‰ for the body wall). Results indicated that sea cucumbers were able to assimilate carbon directly from the cellulose. Bagasse supported higher uptake of carbon from aquaculture waste, thus identifying it as a viable resource to improve bioremediation by deposit feeding sea cucumbers in integrated aquaculture. While illustrating the importance of nutrient balances for bioremediation, questions remain about the roles of direct feeding by deposit feeders vs. the decomposition by microbial communities.

Key words: bioremediation, integrated aquaculture, C/N ratio, sandfish, MixSIAR

1. Introduction

One of the biggest challenges of intensive aquaculture is the production of organic waste. When released to natural water bodies, it can negatively influence water and sediment quality and impact trophic balances (Holmer et al., 2007, 2003). Waste produced in closed systems needs to be filtered and disposed of, posing an economic challenge. Identification of sustainable ways to mitigate effluents from traditional aquaculture by integrating the co-culture of additional bioremediative species, which can also provide a second production output, is an important avenue of research. Widely studied extractive organisms include algae (Neori et al., 2004; Samocha et al., 2015) that take up dissolved inorganic nutrients and deposit feeding polychaetes, crustaceans and mollusks that feed on particulate matter (Bergström et al., 2017; Guo et al., 2017; MacDonald et al., 2011).

Sea cucumbers are a good candidate bioremediator, as they feed on organic matter in sediment. Culture of the sea cucumber *Holothuria scabra* on organic waste from aquaculture can provide additional benefits to the system they are grown in, as well as to the surrounding environment by balancing disrupted nutrient budgets through bioturbation and bioremediation (Lee et al., 2017). Experiments have shown that fish feces can be an effective source of feed in the culture of *Holothuria scabra* (Watanabe et al., 2012). However, the feces are likely not the main feed for *H. scabra* directly, but provide a substrate for the growth of microorganisms (Plotieau et al., 2014; Robinson et al., 2016). The key for efficient growth seems to be the supplementation with carbon to improve the utilization of nitrogen provided by the aquaculture waste (Robinson et al., 2019). The concept is similar to bioflocs, used in recirculating aquaculture, where

69 microbial growth in the water column of fish tanks or ponds is stimulated through the
70 supplementation with carbohydrates such as sugar or starch (Avnimelech, 2012). In
71 addition to carbon, bacteria require nitrogen to build proteins for growth, which they
72 take from the surrounding water, effectively transforming dissolved nitrogen species in
73 the water into bacterial biomass. Biofloc technology is commonly used in the
74 aquaculture of tilapia and shrimp (Avnimelech, 2007; Bauer et al., 2012; Kuhn et al.,
75 2009), but it requires knowledge and close monitoring of appropriate C:N and microbial
76 densities (Hende et al., 2014; Kuhn et al., 2010).
77
78 How bacteria use major nutrients (carbon, nitrogen and phosphorus) for growth is
79 dictated by their elemental composition and nitrogen use is thus stoichiometrically
80 dependent on the availability of carbon (Herbert, 1967). During mineralization of
81 organic matter, a net N uptake occurs at C:N of > 20:1 as inorganic nitrogen is taken
82 up to build bacterial biomass with a lower C:N (Blackburn and Blackburn, 1992;
83 Goldman et al., 1987). Increasing the C:N (from 5:1 to 20:1) of waste from abalone
84 aquaculture fed to *H. scabra* through the supplementation with starch significantly
85 improved sea cucumber growth, possibly by improving the nutritional value of the feed
86 mixture, while preventing build-up of organic carbon in the sediment (Robinson et al.,
87 2019). The authors also recommend using complex carbon sources with low
88 degradation rates, such as bagasse, available in agricultural waste streams. Bagasse is
89 high in cellulose and lignin and has been used as a substrate for bacterial growth to
90 control water quality in aquaculture ponds (Freeman et al., 1992; Gangadhar and
91 Keshavanath, 2012; Krishnani et al., 2006) and is readily available in many tropical
92 countries as a by-product of sugar production. Robinson et al. (2019) also suggest that
93 the C:N could be increased further to improve bacterial growth under reducing

conditions. Microbial activity in sediment or water is closely linked to redox potential as cell energetic pathways oxidize organic substrates. Aerobic bacteria reduce dioxygen to water during respiration, decreasing the redox potential of their environmental medium (Prévost and Brillet-Viel, 2014). Decomposition of organic matter and bacterial growth in marine sediments are generally considered to be more efficient under aerobic conditions (Andersen, 1996; Hedges et al., 1999).

Further research is necessary to optimize sea cucumber biomass production and bioremediation for practical integration into existing monoculture units and on sediments enriched by biodeposits from commercial marine fish farming (Chary et al., 2020). As limits to stocking densities are a challenge to their integration into intensive aquaculture operations, improving biomass density through carbon supplementations is an avenue to enable the successful integration of *H. scabra* as an extractive organism for tropical saline aquaculture (Chary et al., 2020; Robinson et al., 2019). The viability of economical and environment-friendly types of carbon for optimizing sea cucumber carrying capacity remains to be tested (Dumalan et al., 2019).

In this context, the present study investigated the growth and bioremediation performance of the sea cucumber *H. scabra* on sediment impacted by aquaculture waste. Specifically, it aimed to determine if the supplementation with carbon provided as either lab grade (microcrystalline) cellulose or agricultural waste (bagasse) improves sea cucumber biomass production, bioremediation and assimilation of carbon and nitrogen from aquaculture sludge. Further, it tested if the integration of *H. scabra* prevents the build-up of nitrogen in the sediment and the water column and allows for good sea cucumber growth.

2. Methods

2.1 Experimental design and setup

The experiment was conducted over 104 days (d) from 3rd December 2019 to 15th March 2020 at the Zanzibar Mariculture Project on Zanzibar, Tanzania (6°06'54"S 39°12'45"E). The tanks, 24 round plastic basins of 60 L volume, were filled with a 5 cm deep layer of sand with a final sand surface area of 0.44 m^2. The sand was sourced from the adjacent beach. Sediment particle size distribution was as follows: 0.7% > 2 mm, 4.2% >1 - 2 mm; 18.5% >500 µm – 1mm, 45.3% >250 – 500 µm, 30.6% >125 – 250 µm, 0.5% >63 – 125 µm. Before stocking, the sand was treated with 100 ppm chlorine solution to remove organic matter and sterilize the sediment. It was then rinsed with saline well water and the basins were filled with saline well water. Both sterilization of sand and use of saline well water are common practice at the hatchery facility as coastal waters can be polluted by sewage that is released from surrounding infrastructure. Continuous aeration was provided through tubes with attached airstones placed in the center of each tank. Partial water exchanges were done twice a week by adding more well water and letting the tanks overflow for 20 minutes.

Solid aquaculture waste, hereafter referred to as sludge, was sourced from the bottom of a 20 x 10 m concrete basin used to keep milkfish brood stock, fed daily to satiation with commercial feed. The sludge was pumped out, concentrated on 80 µm mesh gauze and stored frozen. Each tank received 7 g wet weight (1.3 g dry weight) of feces per

144 day. The “sludge” (S) treatment received aquaculture waste only, while for the “sludge
145 with cellulose” (SC) and “sludge with bagasse” (SB) treatments, the sludge was mixed
146 with microcrystalline cellulose or finely ground bagasse, respectively (Table 1). Before
147 each feeding, the frozen portions of sludge were thawed, mixed with the carbon source
148 into slurry and distributed throughout the tank. Aeration was disrupted during
149 administration of the organic matter and for 20 minutes after administration to allow it
150 to settle.
151
152 All experimental animals were hatchery-reared and no collections were made from wild
153 populations. Before stocking, 42 juvenile *Holothuria scabra* (16.8 ± 0.6 g mean ± SE
154 wet weight), sourced from the hatchery, were suspended in mesh bags for 24 hours to
155 allow the intestine to empty. Animals were photographed individually to allow for
156 identification based on natural marks. Their weight was recorded before distributing
157 three animals into each tank. The treatments containing sea cucumbers are indicated
158 with a plus sign (+). Tanks of the control treatments (“sludge control”, “bagasse
159 control” and “cellulose control”) that were not stocked with sea cucumbers are
160 indicated with a minus sign (-). Any animals that developed lesions or died within the
161 first month of the experiment were replaced with healthy individuals. Tanks in which
162 animals died after this point were excluded from analysis.
163
164 Table 1: Experimental treatments receiving waste from milkfish aquaculture with or without
165 the supplementation with different types of carbon sources and with or without the presence of
166 sea cucumber *Holothuria scabra*.

Treatment	Feed type	C:N	n	mmol C m^{-2} d^{-1}	mmol N m^{-2} d^{-1}	Number of sea cucumbers

Sludge (S +)	Sludge	8.5	4	43.6	4.4	3
Sludge with bagasse (SB +)	Sludge with bagasse	30	4	160.4	4.6	3
Sludge with cellulose (SC +)	Sludge with cellulose	30	4	153.4	4.4	3
Sludge control (S -)	Sludge	8.5	4	43.6	4.4	0
Sludge with bagasse control (SB -)	Sludge with bagasse	30	4	160.4	4.6	0
Sludge with cellulose control (SC -)	Sludge with cellulose	30	4	153.4	4.4	0

2.2 Sample collection and processing

2.2.1 Environmental samples

Water parameters (pH, dissolved oxygen, temperature, salinity) were measured weekly with a Multi 3430 probe (WTW). Every two weeks, water samples for the determination of dissolved inorganic nitrogen (DIN) species NH_4^+, NO_2^- and NO_3^- were taken through 0.45 µm filters and stored frozen in 20 ml polyethylene bottles until

177 analysis. Spectrophotometric analysis of dissolved inorganic nutrients was carried out
178 following the procedures of Strickland and Parsons (1972).
179
180 For elemental and stable isotope analysis, three sub-samples were taken of the sludge,
181 cellulose, bagasse and the prepared feed mixtures (sludge mixed with bagasse and
182 sludge mixed with cellulose), weighed, dried at 50 °C, weighed again and ground to a
183 fine powder with a mortar and pestle. The % moisture content of the sludge was
184 determined as the ((wet weight - dry weight) / wet weight) × 100. Five sediment
185 samples were taken and pooled per tank before stocking (initial sediment) and monthly
186 thereafter with a cut-off syringe corer of 5 mm diameter and dried at 50 °C for the
187 determination of nitrogen and organic carbon content as well as isotopic analysis.
188 Sediment samples were ground in a Pulverisette 7 ball mill (Fritsch). Samples of
189 sediment and sludge for the determination of the organic carbon content were pre-
190 treated with 150 µl 1M HCl and dried for 48 hours at 40 °C. Analysis to determine the
191 nitrogen and organic carbon (C_{org}) content was performed on a Euro EA3000 elemental
192 analyzer (Eurovector S.p.a.). Before stocking and on day 104, one sediment core of 5
193 mm diameter and 5 cm depth was taken with a cut-off syringe from each tank to record
194 the depth (mm) of the oxic-anoxic interface from the sediment surface as 100 % –
195 (depth of anoxic interface (cm) / (total depth of core (cm) * 100). A composite sample
196 was taken for the determination of sediment particle size distribution by pooling
197 approximately 100 g of sediment from each tank before stocking. The sample was
198 sieved for two minutes on a AS 200 sieve tower (63 – 2000 µm) (Retsch) at an
199 amplitude of 1.18.
200
201

2.2.2. Sea cucumber samples

Sea cucumber wet weight was recorded during stocking (day 0) and subsequently on days 53, 78 and 104 of the experiment, each time after suspension for 24 h in mesh bags to evacuate their guts. The weight was determined to the nearest 0.01 g after leaving the animal out of the water for three minutes to expel water (Robinson et al., 2015).

For the determination of isotopic baseline values, three sea cucumbers from the same cohort and tank as the experimental animals were sampled at the start. All experimental animals were sampled at the termination of the experiment. The sea cucumbers were weighed and dissected to verify that the intestine was empty. Internal organs and the body wall were dried at 50 °C and subsequently processed separately. Sea cucumber internal organs were homogenized with a mortar and pestle, while the body walls were pulverized with a ZM 100 centrifugal mill (Retsch).

2.3. Stable isotope analysis

All samples for the determination of $\delta^{13}C_{org}$ were pre-treated with 150 µl 1M HCl and dried for 48 hours at 40 °C. Stable isotope analysis was performed with a Delta Plus mass spectrometer (Thermo Fisher Scientific) at an instrument precision (SD) of 0.04 ‰ for $\delta^{15}N$ and 0.07 ‰ for $\delta^{13}C_{org}$. Results are expressed in delta notation (δ) parts per thousand (‰), which is the deviation from the standard reference material (atmospheric nitrogen for $\delta^{15}N$ and Pee Dee belemnite for $\delta^{13}C$).

227 The two-source stable isotope mixing model IsoError was applied to verify the planned
228 proportional contribution of the two feed ingredients (sludge and bagasse or cellulose)
229 to the feed mixtures applied to the sea cucumber tanks as follows:
230
231 $(\delta^{13}C_X - \delta^{13}C_M)\, f_{X,B} + (\delta^{13}C_Y - \delta^{13}C_M)\, f_{Y,B} = 0$
232
233 $f_{X,B} + f_{Y,B} = 1$
234
235 where $f_{X,B}$ and $f_{Y,B}$ represent the fractions of assimilated biomass (B) of the feed
236 ingredients *X* and *Y*, respectively, in the feed mixture. *M* and *C* represent the C_{org}
237 isotopic signatures of the ingredients and mixture (Phillips, 2001).
238
239 The trophic enrichment factor (TEF, $\Delta^{13}C_{org}$) i.e. the change in $\delta^{13}C_{org}$ values from one
240 trophic level to the next, was calculated as the isotopic difference between the sea
241 cucumbers and their feed sources in treatment S. Despite cleaning the sand, a small
242 organic carbon content (0.04 ± 0.02 %, mean ± SD) remained in the substrate, but the
243 isotopic values of this organic carbon initially in the sediment (-18.21 ± 1.47 ‰) and
244 the aquaculture sludge (-19.57 ± 0.45 ‰) were very similar. A separate TEF was thus
245 calculated for sediment and sludge as the difference between each and the sea cucumber
246 tissue and averaged to obtain a mean TEF ± SD for internal organs and body wall. The
247 dietary contribution of C_{org} from the feed ingredients to sampled sea cucumber tissue
248 (internal organs or body wall) was determined via the Bayesian mixing model
249 MixSIAR (Stock and Semmens, 2016).
250
251

252 2.4. Statistical analysis

253

254 Data are reported as means ± standard error unless otherwise stated. Individual growth
255 rate was calculated for each animal and growth data and isotopic signatures of the three
256 sea cucumbers were averaged per tank. Water parameters and sediment nitrogen and
257 organic carbon content were averaged for each period of sea cucumber sampling,
258 referred to as days 53, 78 and 104. Data on water temperature, salinity, DIN, sea
259 cucumber growth rate and biomass density were tested for differences between
260 treatments with a linear mixed effects model (lme) allowing for the inclusion of a
261 random tank effect in the repeated measures design. Homogeneity of variance and
262 normal distribution of the model residuals were tested using Levene's and Shapiro
263 Wilk's tests, respectively and pairwise comparison was performed by estimated
264 marginal means. Data that did not meet the test assumptions, dissolved oxygen, pH and
265 sediment parameters, as well as sea cucumber stocking weight and final isotopic values
266 were analyzed for each sampling period individually. Data were tested by analysis of
267 variance (ANOVA) or Kruskal-Wallis ANOVA in case of non-parametric data,
268 followed by Tukey Honest Significant Difference or Dunn's Rank Sum Post Hoc tests,
269 respectively. Statistical analysis was done in *R* (R core team) and differences were
270 considered significant at alpha < 0.05.

271

272

273 **3. Results**

274

275 3.1. Water parameters

276

277 Throughout the experiment, there was no significant difference between the treatments
278 in water temperature (28.10 ± 0.03 °C, mean ± SE) or salinity (29.20 ± 0.10 PSU). The
279 treatments did differ significantly in dissolved oxygen concentrations and pH (Table
280 S1, Fig. S1).
281
282 Ammonia concentrations peaked during the first sampling period at 0.18 ± 0.01 mg L^{-1}
283 and subsequently decreased without differences between treatments (lme; $\chi^2 = 3.84$,
284 df = 5; p = 0.573). Nitrite concentrations reached maximum concentrations of 0.10 ±
285 0.01 mg L^{-1} by day 53 and by day 78 remained significantly higher in the sludge
286 treatment compared to the sludge with bagasse (p = 0.015) and the sludge with cellulose
287 (p = 0.044) treatments. Nitrate concentrations between treatments differed significantly
288 between treatments throughout the experiment (lme; $\chi^2 = 132.93$, $p < 0.001$), with the
289 highest concentrations in the sludge with sea cucumbers and sludge without sea
290 cucumber treatments, reaching final values of 2.54 ± 0.34 and 2.73 ± 0.40 mg L^{-1}, while
291 all other treatments decreased to close to the detection limit (Figure 1).
292
293
294 3.2. Sediment parameters
295
296 At the start of the experiment, sediment nitrogen content in all tanks was below the
297 detection limit, then increased over the course of the experiment in all treatments. The
298 bagasse without sea cucumbers treatment had the highest increase in nitrogen content
299 with final values of 0.05 ± 0.00 % significantly higher than the sludge (0.02 ± 0.00 %,
300 p = 0.013) and sludge without sea cucumbers (0.03 ± 0.00 %, p = 0.040) treatments.
301 Content of organic carbon in the sediment also increased in all treatments and reached

significantly higher values in the two treatments receiving bagasse (SB+ = 0.76 ± 0.12 %, SB - = 0.85 ± 0.09 %) than in the treatments receiving sludge only (0.25 ± 0.02 %, all $p < 0.05$). Sediment nitrogen content was lower in the tanks with sea cucumbers than in their respective control treatments, but this difference was not statistically significant (Fig. 2).

Throughout the experiment, the bagasse treatment with sea cucumbers consistently showed the highest sediment C:N of 20.80 ± 0.87, while the bagasse control treatment without sea cucumbers had slightly lower values with 17.62 ± 0.91. The treatments receiving cellulose had overall lower values with 15.21 ± 1.96 in the tanks with sea cucumbers and 12.73 ± 1.20 in the control tanks. These tanks however showed an overall decrease from day 53 to day 104. The lowest values were in the treatments receiving sludge only with C:N of 10.00 ± 0.33 in the sea cucumber and 10.56 ± 0.61 in the sludge control tank. On days 53 and 78, both bagasse treatments had significantly higher C:N in the sediment than in the sludge treatment with sea cucumbers (all $p < 0.05$) (Fig. 2 iii).

Visual assessment of the depth of the oxic-anoxic interface revealed fully oxic sediments at the start of the experiment in all tanks. By the end of the experiment, the sludge with cellulose and sludge with cellulose control tanks had 45.00 ± 20.60 % and 55.00 ± 20.60 % anoxic sediment, respectively. The sludge control tank had 85.00 ± 15.00 % and the sludge with bagasse control tank had 32.50 ± 21.40 % anoxic sediment. Final values were significantly different between treatments (Kruskal-Wallis; $\chi^2=13.213$, df=5, p=0.021) with the sludge treatment having fully oxic sediment (0.00

± 0.00 %), while the sediment in the bagasse treatment was found to be 100.00 ± 0.00 % anoxic (Dunn Test, p= 0.036) (Fig. S2).

3.3. Sea cucumber survival and growth

Of the 36 sea cucumbers stocked at the start, 28 survived until completion of the experiment. Survival was 100.00 ± 0.00 % in the sludge treatment, 91.60 ± 8.38 % in the bagasse treatment and 75.00 ± 16.40 % in the cellulose treatment. All animals that died showed similar symptoms: reduced or no feeding activity/movement and development of white lesions. Within the first weeks of the experiment, all three sea cucumbers in a tank of the cellulose treatment died and were replaced with three healthy individuals from the same cohort on day 26. The animals then showed good health and feeding activity and the tank was included in subsequent analysis, adjusting the daily growth rate to account for the later starting date. Three sea cucumbers in another tank of the cellulose treatment and two in a tank of the bagasse treatment died as well. The replacement animals also showed low feeding activity and bad health. No irregularities in environmental factors could be determined and those replicate tanks were therefore excluded from all analyses, resulting in three replicates for the sludge with bagasse and Sludge with cellulose treatments with sea cucumbers

There were no significant differences between individual weight at the start of the experiment (ANOVA; $F_{(2,7)} = 2.049$, $p = 0.199$). The sea cucumbers grew in all treatments over the course of the experiment, but the growth rate in the sludge with cellulose treatment (0.30 ± 0.01 g d^{-1}) was significantly higher than that in the sludge

treatment (0.05 ± 0.01 g d^{-1}) by day 53 ($p = 0.006$). However, the sea cucumbers in the sludge with cellulose treatment subsequently showed negative growth rates for the remainder of the experiment, while growth in the sludge and sludge with bagasse treatments was relatively stable. The resulting biomass densities were significantly different between treatments (lme, $\chi^2=8.17$, $p=0.017$). By the end of the experiment, the sea cucumbers in the bagasse treatment had the highest final biomass density of 205.71 ± 11.08 g m^{-2} and values were significantly higher than for the sea cucumbers in the sludge treatment at 134.76 ± 14.09 g m^{-2} ($p = 0.027$) (Figure 3). There was no significant difference in the final nitrogen content of the sea cucumber body wall between treatments (ANOVA; $F_{(2,7)}=1.48$, $p=0.290$).

3.4. Stable isotopic values

3.4.1. Carbon isotopic values

The organic carbon isotopic signature of the feeds was significantly different between all treatments (Tukey HSD, $p < 0.05$): sludge was -19.36 ± 0.06 ‰, the mixture including bagasse was -14.86 ± 0.08 ‰ and the mixture with cellulose was -24.23 ± 0.02 ‰. Compared to the baseline samples, the sea cucumber organ values in the sludge with cellulose treatment had become significantly different by the end of the experiment (Dunn Test, $p = 0.039$), while the values of the body wall were significantly different both in the sludge and bagasse treatments (both Tukey HSD, $p = 0.001$).

375 The final sea cucumber organs differed significantly in their $\delta^{13}C_{org}$ values between all
376 treatments (ANOVA; $F_{(2,7)} = 28.71$, $p < 0.001$). The sea cucumbers in the bagasse
377 treatment showed the most enriched values (-14.61 ± 0.25 ‰), while the sludge
378 treatment had lower values (-16.33 ± 0.27 %) and the sludge with cellulose treatment
379 had the most depleted values (-20.59 ± 0.12 ‰). $\delta^{13}C_{org}$ values of the body wall showed
380 a similar trend and statistical differences (Kruskal-Wallis; $\chi^2 = 6.3$, df = 2, $p = 0.04$),
381 with the body wall of the sludge with cellulose treatment having lower values than the
382 bagasse treatment (Dunn Test, $p = 0.046$).
383
384 At the beginning of the experiment, the surface sediment had an average $\delta^{13}C_{org}$ of -
385 18.21 ± 1.47 ‰ and there were no significant differences between treatments (ANOVA;
386 $F_{(5,18)} = 0.733$, $p = 0.61$). Final $\delta^{13}C_{org}$ values differed significantly between treatments
387 (ANOVA; $F_{(5,16)} = 27.99$, $p < 0.001$; Fig. 6). Sediments in tanks with sea cucumbers
388 were not significantly different from their control tanks without sea cucumbers, while
389 sediment $\delta^{13}C_{org}$ values differed significantly between the cellulose, bagasse and sludge
390 treatments (Tukey HSD, all $p < 0.05$).
391
392
393 3.4.2. Nitrogen isotopic values
394
395 $\delta^{15}N$ values between the feeds differed significantly (Kruskal-Wallis; $\chi^2=6.4889$, df =
396 2, $p=0.039$). The bagasse mixture at 10.65 ± 0.27 ‰ was significantly different
397 compared to the cellulose mixture at 11.71 ± 0.10 ‰ ($p=0.004$), whilst the sludge alone
398 had a $\delta^{15}N$ value of 11.06 ± 0.06 ‰. Over the course of the experiment, $\delta^{15}N$ values of
399 the organs and body wall had become more enriched in all treatments. The isotopic

values of the organs of the sea cucumbers in the sludge treatment had become significantly different compared to the baseline values (Dunn Test, p = 0.011). Also, the final isotopic body wall values were significantly different in the sludge with cellulose treatment (Dunn Test, p=0.020). Comparison between the final $\delta^{15}N$ values of the organs of the sea cucumbers indicated significant differences between treatments (ANOVA; $F_{(2,7)}=6.26$, p=0.028) with the sludge treatment having higher values than the sludge with cellulose treatment (Tukey HSD, p=0.020). There was no significant difference between the $\delta^{15}N$ values of the body wall of the sea cucumbers at the end of the experiment (ANOVA; $F_{(2,7)}=4.63$, p=0.052).

At the start of the experiment, the sediment did not contain sufficient amounts of nitrogen to determine the $\delta^{15}N$ value, however at the end of the experiment, $\delta^{15}N$ values in the surface sediment differed significantly between treatments (Kruskal-Wallis; $\chi^2 = 15.189$, df = 5, p=0.010) with both cellulose treatments showing the most depleted and both bagasse treatments the most enriched $\delta^{15}N$ values (Table 2, Fig. 4).

Table 2: Final mean (±SE) stable isotope values of sediment, feeds and tissues of juvenile sea cucumber *Holothuria scabra* fed sludge from milkfish aquaculture (S+) or with sludge amended with bagasse (SB+) or microcrystalline cellulose (SC+) and their respective control tanks without sea cucumbers (S-, SB- and SC-).

Tissue/Sample	**Treatment**	**$\delta^{13}C_{org}$ (‰)**	**$\delta^{15}N$ (‰)**
Organs	Baseline	-14.12 ± 0.86	8.33 ± 1.05
	S+	-16.33 ± 0.16	15.16 ± 0.24
	SB+	-14.61 ± 0.16	14.46 ± 0.14
	SC+	-20.59 ± 0.13	13.61 ± 0.22

Body wall	Baseline	-18.29 ± 0.82	5.83 ± 0.67
	S+	-14.58 ± 0.26	10.47 ± 0.38
	SB+	-14.13 ± 0.21	11.34 ± 0.32
	SC+	-18.88 ± 0.31	11.71 ± 0.29
Sediment	S+	-21.51 ± 0.28	11.26 ± 0.16
	S-	-21.90 ± 0.35	11.21± 0.17
	SB+	-17.29 ± 0.22	10.56 ± 0.14
	SB-	-18.79 ± 0.39	10.81 ± 0.14
	SC+	-24.52 ± 0.84	9.94 ± 0.41
	SC-	-24.64 ± 0.84	11.29 ± 0.29

420

421

422

423 3.5. Trophic enrichment factor (TEF)

424

425 Calculations based on the $\delta^{13}C_{org}$ of sea cucumbers in the sludge treatment, the
426 isotopic values of the aquaculture waste and the organic carbon in the sediment at the
427 start of the experiment resulted in a TEF of 2.56 ± 0.96 ‰ (mean ± SD) for the
428 internal organs and 4.31 ± 0.96 ‰ for the body wall. The isotopic difference for
429 nitrogen ($\Delta^{15}N$) was 4.10 ‰ for organs and -0.59 ‰ for body wall in the sludge
430 treatment. In the bagasse treatment, the isotopic difference between the values of
431 organs and of the feed mixture was slightly lower, but higher for the body wall tissue.
432 The sludge with cellulose treatment had a much lower $\Delta^{15}N$ in the organ tissue, and
433 low but positive $\Delta^{15}N$ in the body wall (Table 3).

434

435 Table 3: Mean (± SE) difference in $\delta^{15}N$ between the feed mixtures and tissues of juvenile sea
436 cucumber *Holothuria scabra* fed sludge from milkfish aquaculture (S+) supplemented with
437 bagasse (SB+) or microcrystalline cellulose (SC+).

	Δ ^{15}N	
	Internal organs	**Body wall**
Sludge (S+)	4.10	-0.59
Sludge with bagasse (SB+)	3.81	0.68
Sludge with cellulose (SC+)	2.00	0.06

438

439

440 3.6. Assimilation of feed

441

442 The actual contributions of the two feed ingredients to carbon in the feed mixture of the
443 sludge with bagasse treatment were very close to the planned contributions. The feed
444 mixture in the sludge with cellulose treatment showed a slightly higher contribution of
445 cellulose (Table 4).

446

447 The mixing model for the body wall indicated that in the sludge treatment, the
448 aquaculture waste contributed the vast majority of carbon compared to the organic
449 carbon initially present in the sediment. The two sources were strongly negatively
450 correlated (Fig. S3). Samples of sea cucumbers in the sludge with bagasse treatment
451 indicated a larger contribution of organic carbon from sludge than from bagasse to sea
452 cucumber tissue, while in the sludge with cellulose treatment, cellulose contributed
453 more carbon than sludge (Fig. 5). As cellulose does not contain any nitrogen and it was
454 not possible to obtain $\delta^{15}N$ values for the bagasse, the contribution of nitrogen from
455 feed ingredients could not be determined by the isotopic mixing model using $\delta^{15}N$.

Table 4: Mean (± SE) proportional contribution of the feed ingredients to the organic carbon in feed mixtures containing sludge from milkfish aquaculture amended with bagasse (SB+) or microcrystalline cellulose (SC+) fed to juvenile sea cucumber *Holothuria scabra.* The contributions were derived by IsoError mixing model.

Treatment	**Ingredient**	**Planned contribution**	**Actual contribution ± SE**	**95% Confidence interval**
Sludge with bagasse (SB+)	Sludge	0.27	0.26 ± 0.02	0.21 – 0.31
	Bagasse	0.73	0.74 ± 0.02	0.69 – 0.79
Sludge with cellulose (SC+)	Sludge	0.28	0.19 ± 0.01	0.15 – 0.23
	Cellulose	0.72	0.81 ± 0.01	0.77 – 0.85

4. Discussion

4.1. Effect of carbon supplementation

The results of this study confirm that carbon supplementation to aquaculture sludge improves the growth of juvenile *Holothuria scabra*. Bagasse led to the highest biomass production over the course of the experiment. This supports the findings of similar experiments (Robinson et al., 2019, 2015) and indicates that carbon of low digestibility

sourced from agricultural waste streams is a suitable supplementation to nitrogenous waste.

Mixing-model results for the sludge and bagasse treatments showed a high assimilation of organic carbon from aquaculture sludge, identifying it as a good feed source for the sea cucumbers. Due to the strong negative correlation between the isotopic values of the aquaculture waste and the organic carbon in the initial sediment and the fact that source concentration was not taken into account by the mixing-model, assimilation of the sludge may even be underestimated as presumably much more of it was available to the sea cucumbers. The results further indicate that bagasse and cellulose functioned differently as feed supplementations. Organic carbon from cellulose was assimilated into sea cucumber tissue, while the bagasse contributed only to a small proportion of organic carbon in the sea cucumber tissue and most carbon from the sludge was assimilated. Combined with the significantly higher final sea cucumber biomass in the bagasse treatment compared to the sludge treatment, these results demonstrate that bagasse supplementation improved the assimilation of aquaculture waste. While cellulose supplementation also improved sea cucumber growth, it likely contributed directly as feed and did not improve the recovery of sludge-derived nutrients into secondary high-value biomass.

The different effects of the carbon supplementation could be due to their difference in digestibility. Feeds high in cellulose are not generally digestible for deposit feeding holothurians, as they lack cellulase, the enzyme to digest it directly, however the presence of cellobiase in their digestive tract suggests that they use the breakdown products of cellulose, made available to them by symbiotic gut bacteria (Roberts et al.,

2000; Yingst, 1976). Bagasse also contains high amounts of cellulose (42%), but is further composed of about 25 % lignin (Rocha et al., 2015, 2012; Triana et al., 1990). Lignin content is a limiting factor to microbial decomposition, especially under anaerobic conditions, slowing down decay (Benner et al., 1984; Harrison, 1989), which would delay its availability to sea cucumbers. Bagasse could play a similar role to seagrass detritus in the sea cucumbers' natural habitat, by providing a slowly digestible substrate for microbes, which combined with detritus, forms a food source for the detritivores and transports carbon and nitrogen into higher trophic levels (Harrison, 1989; Schneider et al., 2006; Siders et al., 2018).

The C:N of the milkfish sludge was 8.5:1, which is below the threshold of 10:1 to change from net nitrogen regeneration to assimilation (Goldman et al., 1987). Through supplementation with bagasse and cellulose, the C:N was increased considerably to 30:1. A functional shift in the bacterial community in aquaculture ponds has been found at a threshold of 10:1 in the water column and 12:1 in the sediment and heterotrophic pathways became superior when the ratio exceeded 15:1 (Zheng et al., 2018). In this experiment, only supplementation with bagasse was able to maintain C/N ratios above this threshold, increasing it to around 20:1. The supplementation with cellulose increased the C:N in the sediment during the first half of the experiment, but the tanks did not maintain this high value. During the same sampling period, high growth rates in the sludge with cellulose treatments were also seen which then decreased simultaneously with a decrease in sediment C:N values, providing further evidence that the growth of deposit feeding sea cucumbers is linked to the C:N of sedimentary organic matter. While the isotopic signatures and C:N values of the sediment samples could suggest that little cellulose actually reached the sediment, the sea cucumber tissue

samples indicate that the cellulose was available to the sea cucumber and incorporated from the feed and did not remain in the sediment. Bagasse would provide a favorable carbon supplementation compared to cellulose and it was confirmed that a C:N of 15 is sufficiently high to benefit nitrogen transformation activity (Lovett et al., 2004; Toi et al., 2013; Zhao et al., 2019).

4.2. *H. scabra* as an extractive species

Increases in sediment carbon and nitrogen content as well as changes in the $\delta^{13}C_{org}$ and $\delta^{15}N$ of the sediment towards the values of the feed indicate that the feed mixtures did accumulate in the tanks. The presence of sea cucumbers only led to a slight reduction in the accumulation of nitrogen and organic carbon in the sediment and the animals were not able to fully prevent the build-up of solid aquaculture waste. In experiments on sediment from Babylon snail culture, *H. scabra* juveniles were able to significantly reduce sediment total nitrogen, identifying them as a better bioremediator in the absence of a constant flux of particles from aquaculture (Dobson et al., 2020).

The presence of the sea cucumbers also had no effect on concentrations of dissolved nitrogen. Nitrate concentrations were however lower in the systems receiving carbon supplementation and a nitrite peak occurred earlier, as would be expected if environmental conditions favored immobilization or denitrification by microbes (Zheng et al., 2018). Dissolved nitrogen accumulated in the sludge treatments and led to significantly higher nitrate concentrations. Significantly higher sedimentary nitrogen concentrations compared to the sludge treatment could be caused by dissolved nitrogen assimilated into microbial biomass. This resource would then provide an improved feed

source to the grazing sea cucumbers. Further experiments applying pulse chase with dissolved nitrogen labeling with high ^{15}N could shed further light onto these pathways.

4.3. Sea cucumber biomass production

Small weight gain was measured in the animals fed sludge alone. One reason could be that milkfish waste did not provide enough nutrients. Sea cucumbers require a certain level of protein in their diet (>15%) (Zacarias-Soto and Olvera-Novoa, 2015). While the 43.6 mmol C m^{-2} d^{-1} that were added through sludge could be considered high under natural conditions, the 61 mg N m^{-2} d^{-1} that it supplied may have been too low for good deposit feeder growth (Alongi and Hanson, 1985; Lehtoranta et al., 2009; Robinson et al., 2019; Tenore and Chesney, 1985). The average 156.9 mmol C m^{-2} d^{-1} supplied through carbon supplementation improved sea cucumber biomass production. Not only higher C:N, but also increased supplies of total organic carbon lead to higher abundance of deposit feeders along with meio- and macrofauna (Campanyà-Llovet et al., 2017).

$\delta^{15}N$ isotopic values of sea cucumber tissues changed compared to the signatures of the animals sampled for the determination of baseline values, showing assimilation of nitrogen from the aquaculture sludge. In the sea cucumber organs, a higher isotopic fractionation in the sludge with bagasse treatment compared to the sludge with cellulose treatment would further support that sludge derived nitrogen was assimilated better if mixed with bagasse than with cellulose. Final $\delta^{15}N$ values of the body wall in the Sludge treatment were below the value of the feed. Previous findings show that internal organs have faster turnover rates than the body wall, but the length of the experiment provided

570 ample time for full tissue turnover in sea cucumbers of this size (Sun et al., 2012; Xia
571 et al., 2015). Presumably, not enough nitrogen was available to reach isotopic
572 equilibrium as maintenance rather than growth metabolism is the main use of energy in
573 sea cucumbers (Sun et al., 2012). $\delta^{15}N$ in the treatments with added carbon was higher,
574 with the bagasse treatment supporting the highest assimilation. The overall greater
575 availability of nutrients may have supported increased transport of nitrogen into the
576 body wall - a site of nutrient storage.
577
578 Observed growth rates generally decrease over time, as animals' size and biomass
579 density increase. Density limitations to growth around 220 g m^{-2} are common
580 (Battaglene et al., 1999; Mathieu-Resuge et al., 2020). Some experiments have also
581 been able to achieve much higher biomass densities in the range of 600 to 900 g m^{-2}
582 (Robinson et al., 2019, 2015; Watanabe et al., 2014) and Mathieu-Resuge and
583 colleagues (2020) hypothesize that reasons for these different outcomes are initial
584 conditions, controlled water and sediment inputs and manipulated C/N ratios. Robinson
585 et al. (2015) found high growth rates of sea cucumbers reared in oxic-anoxic treatments,
586 while we only observed fully anoxic sediments in the tanks receiving bagasse. The use
587 of unfiltered saline well water may have caused sufficiently different environmental
588 conditions for the observed outcome. Yang et al. (2019) recommended the use of
589 underground seawater instead of coastal water due to lower dissolved nutrient
590 concentrations, fewer microorganisms and pathogens as well as more denitrifying
591 bacteria. High nitrate concentrations in the well water used in this experiment suggests
592 that this water source was not pristine. It is possible that using unfiltered well water as
593 opposed to coastal seawater introduced different microbes than usually found in *H.*

scabra habitats that may not readily break down organic material, provide a food source and leave the systems unable to cope with the influx of high amounts of organic matter.

Besides influencing the microbial community in the water, the source water can also determine the gut microbes of cultured animals (Moss et al., 2000). The role of the sediment microbial community is still an important research subject in sea cucumber aquaculture (Gao et al., 2014; Yamazaki et al., 2020). Administering probiotics with aquaculture feeds is considered as an approach to support sea cucumber growth and immune response (Ma et al., 2019; Wang et al., 2020, 2015), but it is questionable if treating the entire culture system can prevent the establishment of pathogenic communities (Zheng et al., 2019). Given the environmental circumstances (under "realistic" non-sterile conditions), desirable microbial bioremediators can be easily outcompeted by undesired microorganisms (Tal et al., 2001). However, increasing C:N could benefit both potential probiotic bacteria and opportunistic pathogens at the same time (Zheng et al., 2018).

4.4. Species-specific trophic enrichment factor

This is the first study that uses a species-specific TEF in the stable isotope analysis of feed use in *H. scabra*. Several feeding experiments with sea cucumbers have adjusted their values by 1 ‰ for use in different mixing models, in reference to literature unrelated to holothurians (Gao et al., 2011; Hou et al., 2017; Mondal and Kunzmann, 2018; Song et al., 2018; Sun et al., 2013). Trophic enrichment can however vary greatly depending on species, feed, tissue and sample treatment (Bodin et al., 2007; Caut et al.,

2009; Watanabe et al., 2013). Higher $\Delta^{13}C$ close to the values found in this study have been experimentally determined for sea cucumbers (Sun et al., 2020, 2012; Watanabe et al., 2013), calling into question the appropriateness of using a TEF of 1 ‰ to adjust isotopic values of holothurians. This also points to the microbial degradation of organic matter before consumption by sea cucumbers, which would add additional trophic steps to the processes of carbon assimilation. As the assumptions on trophic enrichment greatly influence the outcome of experiments and conclusions based on isotopic mixing-models, this study further underlines the need to determine and apply appropriate TEF for stable isotope studies of sea cucumbers (Phillips et al., 2014).

5. Conclusions

This study verified the supplementation with bagasse as a suitable carbon source to improve the cultivation of juvenile sea cucumber *Holothuria scabra* on sediment impacted by milkfish aquaculture. Carbon supplementation also prevented the accumulation of dissolved inorganic nitrogen and the sea cucumbers slightly reduced the build-up of organic matter from aquaculture waste in the sediment. Our findings support the benefit of slowly degrading carbon sources to sea cucumbers cultivated in detritivore-bioremediation systems, as stable isotope analysis indicated improved nutrient assimilation from aquaculture waste. The results however also hint at the complexity of the interplay between microbes and detritivores. A well-established bacterial community in the sediment seems to be a key factor for successful bioremediation, and the respective role that these components play still remains to be investigated further. Stable isotope analysis is a valuable tool to investigate nutrient

assimilation by deposit feeding holothurians, but species- or context-specific trophic enrichment factors, as presented in this study for two different tissues of *H. scabra*, are needed to produce reliable results.

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938 **Figure captions**

939

940 Fig.1. Concentrations (mean ± SE mg L^{-1}) of i) ammonia, ii) nitrite and iii) nitrate in the water
941 of tanks with juvenile *Holothuria scabra* dosed with sludge from milkfish aquaculture only
942 (S+) or with sludge supplemented with bagasse (SB+) or microcrystalline cellulose (SC+) and
943 their respective control tanks without sea cucumbers (S-, SB- and SC-). Different lowercase
944 letters indicate significant differences; see text for detail on the treatments.

945

946 Fig. 2. The mean (± standard error) i) nitrogen content, ii) organic carbon content and iii) carbon
947 to nitrogen ratio of sediment in tanks with juvenile *Holothuria scabra* dosed with sludge from
948 milkfish aquaculture only (S+) or with sludge supplemented with bagasse (SB+) or
949 microcrystalline cellulose (SC+) and their respective control tanks without sea cucumbers (S-,
950 SB- and SC-). Different lowercase letters indicate significant differences between treatments
951 based on Kruskal-Wallis and Dunn Post Hoc Tests ($p < 0.05$).

952

953 Fig. 3. The mean (± SE) i) growth rate, ii) biomass density and wet weight of juvenile
954 *Holothuria scabra* in tanks dosed with sludge from milkfish aquaculture only (S+) or with
955 sludge supplemented with bagasse (SB+) or microcrystalline cellulose (SC+). Different
956 lowercase letters indicate significant differences between treatments.

957

958 Fig. 4: Mean (± SD) stable isotope values of sediment, and tissues of juvenile sea cucumber
959 *Holothuria scabra* fed sludge from milkfish aquaculture (S+) or with sludge supplemented with
960 bagasse (SB+) or microcrystalline cellulose (SC+). Dashed lines indicate pure endmembers of
961 sources: sludge (red) and bagasse (green), cellulose (blue) and initial sediment (grey) for which
962 no $\delta^{15}N$ values are available.

963

Fig. 5: Proportional contribution of the feed ingredients to the tissues (internal organs or body wall) of juvenile sea cucumber *Holothuria scabra* fed sludge from milkfish aquaculture (S+) amended with bagasse (SB+) or microcrystalline cellulose (SC+). Contributions are derived by Bayesian mixing model MixSIAR.

969 **Supplementary material**

970

971 Table S1: Results of statistical analysis of water parameters between treatments.

Parameter	**Test statistics**
Temperature	(lme; χ^2=3.12, df=5, p=0.68)
Salinity	(lme; χ^2=1.72, df=5, p=0.89)
DO	Day 53 (ANOVA; $F_{(5,16)}$=5.52, p=0.004) Day 78 (Kruskal-Wallis; χ^2=14.21, df=5, p=0.014)
pH	Day 53 (ANOVA; $F_{(5, 16)}$=5.63, p=0.004) Day 78 (ANOVA; $F_{(5, 16)}$=4.16, p= 0.013) Day 104 (Kruskal-Wallis, χ^2=11.43, p=0.043)

972

973 Fig. S1: The mean (± standard error) i) water temperature, ii) salinity, iii) dissolved oxygen
974 and iv) pH in the water column of tanks containing sea cucumbers or control treatments
975 without sea cucumbers dosed with sludge from milkfish aquaculture only (S+) or with sludge
976 supplemented with bagasse (SB+) or microcrystalline cellulose (SC+) and their respective
977 control tanks without sea cucumbers (S-, SB- and SC-). Different lowercase letters indicate
978 significant differences between treatments.

979

980 Fig. S2: Pictures of sediment cores for the visual assessment of the oxic-anoxic interface
981 taken from tanks with juvenile sea cucumbers *Holothuria scabra* fed sludge from milkfish
982 aquaculture (S+) or sludge amended with bagasse (SB+) or microcrystalline cellulose (SC+)
983 and their respective control tanks without sea cucumbers (S-, SB- and SC-). The fourth
984 replicate of treatments SB+ and SC+ were excluded from analysis due to high rates of sea
985 cucumber disease and mortality, see Results section for details.

986

Fig. S3: Matrix plot of food sources for juvenile sea cucumbers *Holothuria scabra* fed sludge from milkfish aquaculture (S+) or sludge amended with bagasse (SB+) or microcrystalline cellulose (SC+) based on samples of internal organs or body wall. The diagonal cells show graphs of the posterior probability distribution for each food source. The cells below the diagonal show the correlations between contributions for pairs of food sources. More negative values in larger print indicate a strong negative correlation. The cells above the diagonal show contours of the joint posterior probability distribution for contributions for pairs of food sources.