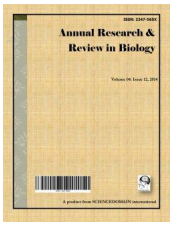

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Inferior Assimilation of Algae-based Diets by Sea Cucumber *Holothuria scabra* under Laboratory Condition Expressed by Stable Isotope Mixing Model

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Authors' contributions

This work was carried out in collaboration between both authors. Author KM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author AK managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To investigate the dietary preferences of sea cucumber *Holothuria scabra* fed with algal food sources like *Sargassum*, *Fucus*, *Spirulina*, *Ulva* in combination with marine pellet under laboratory condition. Carbon stable isotope technique ($\delta^{13}C$) was used to better understand the assimilation of different dietary food sources.
Study Design: A microcosm approach was undertaken where 24 experimental aquaria were set up, each having juvenile *H. scabra*. These aquaria were segregated into four different dietary treatments with six replicates per treatment. (SGP treatment= *Sargassum* + marine pellet, FCP treatment= *Fucus* + marine pellet, SPP treatment= *Spirulina* + marine pellet, ULP treatment= *Ulva* + marine pellet).

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Place and Duration of Study: Marine Experimental Ecology Unit (MAREE), Leibniz Centre for Tropical Marine Research, Bremen, Germany, between January and October 2017.
Methodology: We included 24 sea cucumber juveniles for conducting the feeding trial. CN contents, C/N ratio and carbon stable isotopes were measured in food sources as well as in *H. scabra* body wall. The growth of *H. scabra* was also monitored. Stable isotope mixing model was used to calculate the exact food preference under laboratory condition.
Results: The results of carbon stable isotopes ($\delta^{13}C$) of sea cucumber body wall exhibited depleted values that are significantly different (ANOVA, $P<0.05$) from all the dietary treatments (SGP, FCP, SPP, ULP) thereby suggesting inferior assimilation of food ingredients. The poor performance of selected food sources (algae and marine pellet) towards the growth of sea cucumber was clearly reflected in the data (ANOVA, $P>0.05$).
Conclusion: Therefore, it is assumed that some additional food source co-existed within the microcosm and could have contributed to their food uptake, probably sediment microbes that require further clarification.

Keywords: Algae; marine pellet; H. scabra; growth; carbon stable isotope.

1. INTRODUCTION

Sea cucumbers (Phylum: Echinodermata), close cousins to sea stars, sea urchins are exclusively marine and obtain food by ingesting marine sediment or by filtering seawater [1,2]. They usually feed on surface sediment containing microbes, meiofauna, decaying organic debris, inorganic components and thus play an important role in benthic nutrients recycling [3,4,5]. *Holothuria scabra* Jaeger 1833 commonly known as sandfish is one such commercially valuable tropical species of sea cucumber that fetches highest prices in international markets as bêche-de-mer [6] usually the dried and processed form. Proper scientific knowledge about *H. scabra* diet is important towards improving hatchery and their aquaculture. Under laboratory condition (microcosm) the selectivity and subsequent assimilation of food sources by *H. scabra* need further attention. Carbon stable isotope analysis (CSIA) is a useful technique to determine the exact food sources and feeding selectivity, trophic positions and movement patterns of both aquatic and terrestrial animals [7,8,9,10,11]. It implies diet information over a time-period depending on the tissue specific turnover rates [12,13,14]. The recent development of mixing isotope models further enables to investigate the relative contributions of each component towards the organism's food absorption [15,16]. A general principle applicable is that isotopic fractionation has similar values (0 - 1^{o}/oo for $\delta^{13}C$) regardless of food items or animal species, as evident from previous studies [7,17,18,19]. However certain studies showed that isotopic fractionation is species and tissue specific [20,21,22]. Isotopic studies conducted on various aquatic species, includes sea cucumbers [23,24,25,26,27,28,29, 30,31,32,33], shrimps [34,35], fish [36,37] and bivalves [10]. Therefore, the isotopic composition of sea cucumber body wall can reflect those of their dietary components because of growth and metabolic turnover [24,29]. Isotopic analysis related to acceptance or rejection of selected food sources by *H. scabra* under laboratory conditions has not been reported previously. In the present study, different food sources including *Sargassum*, *Fucus*, *Spirulina, Ulva* and marine pellet are offered as diet to feed juvenile *H. scabra* under laboratory conditions. The idea is to distinguish between the food qualities and to quantify the relative contributions of different ingredients to the food assimilation by *H. scabra*. The influence of diets on the growth of sea cucumber is also recorded to provide scientific evidence for optimizing the ingredients of artificial feed generally used in sea cucumber farming under captivity.

2. METHODOLOGY

2.1 Experimental Diets

Four different algae *Sargassum*, *Fucus*, *Spirulina* and *Ulva* were selected in this experiment. Dried algae powder of *Sargassum* and *Spirulina* was obtained from Southeast Asian Fisheries Development Center (SEAFDEC), Iloilo, Philippines whereas fresh *Fucus* and *Ulva* were collected from Helgoland, Germany and then transferred to the laboratory where they were dried and processed into powder. Marine Pellet (First Bite Aqua Foods, UK) is also used in this experiment to meet the minimal nutritional requirements because a pure algae diet is not sufficient for sea cucumber. Four kinds of diets were prepared for *H. scabra* containing one

algae each mixed with marine pellet in the ratio of 7:3 i.e. SGP diet (*Sargassum* + marine pellet), SPP diet (*Spirulina* + marine pellet), FCP diet (*Fucus* + marine pellet) and ULP diet (*Ulva* + marine pellet) respectively. The prepared diets were stored for a few days only at 4°C until further feeding and analysis.

2.2 Sea Cucumber Feeding Trials

The experiment was conducted at Marine Experimental Ecology unit (MAREE) of Leibniz Centre for Tropical Marine Research, Bremen, Germany. *H. scabra* juveniles were imported from a commercial hatchery (Research Institute for Aquaculture III, Vietnam). After arrival, the juveniles were acclimatized under the rearing facility for four weeks prior to the start of feeding trial. Sea cucumbers with initial body weights of 4.38 ± 1.37 g were randomly segregated into 24 tanks (31×17×18 cm), in each of which 1 individual was kept and cultured. These tanks were divided into four diet groups (SGP, FCP, SPP, ULP, respectively) with six replicates for each group. Prior to the beginning of experiments, first sea cucumber samples were drawn and stored at -80°C for initial carbon stable isotope analysis. In a 90-days feeding trial, sea cucumbers were fed at 16:00 h with a ration of 1% of the body weight. The average water quality parameters were recorded during the experimental period, water temperature 25.7 ± 0.75°C, salinity 35.1 ± 1.0 ppt, dissolved oxygen 6.57 ± 0.26 mg/l and pH level 8.11 ± 0.31 respectively. 50% of the water volume in each tank was exchanged with filtered seawater every alternate day. Siphoning was carried out during water exchange usually to collect the left-over feed residues and faecal matter. At the end of the experiment, samples of sea cucumber were collected and stored at -80°C immediately for later analysis.

2.3 Sea Cucumber Growth

The following parameters were monitored:

Specific growth rate, SGR (% d^{-1}) = [(ln W_f - ln W_i)/ t] × 100

Where W_f and W_i are the average final and initial weight in time t.

Feed conversion ratio, FCR= Δf/Δb

Where Δf= total feed intake and Δb= total biomass gain

Ingestion rate, IR (gg^{-1} d^{-1}) = C/ [(W_2 +W_1)/2]

Where W_1 and W_2 are initial and final body weight of sea cucumbers in each tank and C is the dry weight of feed consumed.

Body volume, V= 4/3π(L/2) $(B/2)^2$ assuming a spheroid body shape

Where, V= body volume, L is body length (cm), B is body breadth (cm)

2.4 C/N and Carbon Stable Isotope Measurements

The experimental samples were freeze dried at -65°C for 48 h to constant weight and processed into fine powder using a pulverizer. After pre-treatment, carbon isotope ratios of algae powder and sea cucumbers were determined using an elemental analyzer coupled with an isotope ratio mass spectrometer (Delta Plus flash EA 1112, ThermoFinnigan). The derived results of isotope ratios were expressed in standard δ-unit notation, which is defined as:

$$\delta^{13}C = [(R_{sample}/R_{standard})-1] \times 1000^{o}/oo$$

Where R is the $^{13}C/^{12}C$ ratio. The values were reported relative to the Vienna Pee Dee Belemnite (PDB) standard. A laboratory working standard (peptone) was run for every 7 samples. Analytical precision was ±0.1°/oo. The C/N contents of all experimental samples were also measured using Euro EA 3000 elemental analyzer (EuroVector). Food assimilation by *H. scabra* under laboratory condition was examined using carbon stable isotope in this experiment, however, nitrogen stable isotope was not used in the present experiment.

2.5 Stable Isotope Mixing Model

H. scabra diet was composed of two components i.e. algae + marine pellet, here a two-source mass balance isotope-mixing model was used to estimate the contribution of each component. It consists of the following equation: [38]

$$\delta^{13}C_{mix} = f_1\,\delta^{13}C_1 + f_2\,\delta^{13}C_2$$

$$f_1 + f_2 = 1$$

Where $\delta^{13}C_{mix}$, $\delta^{13}C_1$, $\delta^{13}C_2$ represents the mean isotopic signatures of the consumer (mixture) and sources 1 and 2 respectively. f_1 and f_2 represent the fractions of the assimilated

biomass of sources 1 and 2 respectively in the mixture. Isotopic signatures for the sources were corrected for trophic fractionation. Average fractionation effects of 1°/oo for carbon isotope was used to correct stable isotope shifts for each trophic level [7,24,39].

2.6 Statistical Analysis

The results from different diet groups were subjected to one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons at a significance level of 0.05 (P<0.05). The statistical analysis was done using SPSS software 16.0 for Windows.

3. RESULTS

3.1 C/N and Carbon Stable Isotope Ratios of Dietary Food Sources

C/N and carbon stable isotope ratios of the dietary food sources (*Sargassum*, *Fucus*, *Spirulina*, *Ulva* and marine pellet) are provided (Table 1). Significant differences in C/N and $\delta^{13}C$ ratios were observed (ANOVA, P<0.05). The $\delta^{13}C$ values exhibited the trend as *Fucus* (-16.64 °/oo) > *Ulva* (-17.30°/oo) > *Sargassum* (-19.37°/oo) > marine pellets (-21.83°/oo) > *Spirulina* (-33.32°/oo) whereas the C/N ratios were *Spirulina* (4.32) < marine pellets (4.95) < *Ulva* (10.64) < *Sargassum* (11.35) < *Fucus* (18.84) respectively.

3.2 Carbon Stable Isotopes of Sea Cucumber and Probable Food Contributions

The carbon stable isotope ratios ($\delta^{13}C$) and carbon contents of *H. scabra* fed with different diets are provided (Table 2). After 90 days of the feeding trial, $\delta^{13}C$ values of the consumer (*H. scabra*) from all the dietary treatments exhibited obvious ^{13}C depletion compared to the initial value (-10.92 °/oo). Normally such observation indicates assimilation of isotopically light fractions comprising the diet. It is also very interesting to note that none of the obtained isotope values of the sea cucumber tissue from different dietary treatments falls between those of the dietary food sources to be explained as a mixture of them. The values are found to be located well outside the mixing space in a linear interpolation of $\delta^{13}C$ of the consumer with that of

Table 1. CN contents, C/N and $\delta^{13}C$ ratios of dietary food sources used for *H. scabra* feeding. Data are provided as mean ± S.D. (n=3). Different letters within the same column indicate significant differences (ANOVA, P<0.05)

Food Sources	%C	%N	C/N	$\delta^{13}C$ (°/oo)
Sargassum	25.63 ± 5.77^{c}	2.26 ± 0.58^{c}	11.35 ± 0.37^{b}	-19.37 ± 0.13^{c}
Fucus	33.44 ± 0.46^{bc}	1.77 ± 0.04^{c}	18.84 ± 0.22^{a}	-16.64 ± 0.31^{d}
Spirulina	44.83 ± 0.13^{a}	10.37 ± 0.04^{a}	4.32 ± 0.004^{c}	-33.32 ± 0.02^{a}
Ulva	26.80 ± 0.07^{c}	2.51 ± 0.02^{c}	10.64 ± 0.12^{b}	-17.30 ± 0.01^{d}
Marine pellet	33.98 ± 9.99^{b}	6.96 ± 2.55^{b}	4.96 ± 0.38^{c}	-21.83 ± 0.09^{b}

Table 2. Carbon stable isotope ratios ($\delta^{13}C$ °/oo) and carbon content (%) of *H. scabra* fed upon different diets. Data are provided as mean ± S.D. (n=6). Different letters within the same column indicate significant differences (ANOVA, P<0.05). For dietary treatments SGP, FCP, SPP and ULP, detailed description is provided in text

H. scabra fed diets	$\delta^{13}C$	%C
Sargassum	-19.37 ± 0.13^{c}	25.63 ± 5.77^{c}
Fucus	-16.64 ± 0.31^{d}	33.44 ± 0.46^{bc}
Spirulina	-33.32 ± 0.02^{a}	44.83 ± 0.13^{a}
Ulva	-17.30 ± 0.01^{d}	26.80 ± 0.07^{c}
Marine pellet	-21.83 ± 0.09^{b}	33.98 ± 9.99^{b}
Initial	-10.92 ± 0.22^{d}	17.61 ± 0.58^{a}
SGP treatment	-15.00 ± 0.00^{c}	12.10 ± 0.06^{c}
FCP treatment	-15.33 ± 0.57^{c}	12.06 ± 0.19^{c}
SPP treatment	-17.00 ± 0.00^{a}	13.74 ± 0.14^{b}
ULP treatment	-16.00 ± 0.00^{b}	13.60 ± 0.16^{b}

Table 3. Growth of *H. scabra* fed with different diets (mean ± S.D., n=6). No significant differences were observed between the diet groups (ANOVA, *P*>0.05). For diets SGP, FCP, SPP and ULP, detailed description is provided in text

Parameters	SGP diet	FCP diet	SPP diet	ULP diet
BW (g)	3.97 ± 0.83	3.84 ± 0.92	4.34 ± 1.72	3.41 ± 0.67
SGR (% day^{-1})	-0.79 ± 0.26	-0.42 ± 0.25	-0.54 ± 0.60	-0.07 ± 0.59
FCR	0.20	0.19	0.19	0.18
IR (g g^{-1} d^{-1})	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.00	0.05 ± 0.01
BV (cm^3)	3.09 ± 0.49	3.55 ± 0.43	3.59 ± 0.85	2.95 ± 0.31

the two source ingredients after correction of isotope fractionation of 1°/oo, thus resulting in a mathematical solution of diet fractions where one is negative and the other is >1, neither of which are biologically sensible.

3.3 Growth of Sea Cucumber

Performance of *H. scabra* fed with different diets is provided (Table 3). During the 90 days feeding trial, performance was monitored in terms of specific growth rate (SGR), feed conversion ratio (FCR), ingestion rate (IR), body volume (BV), body weight (BW). No significant differences were observed among the selected variables from the different diet groups (ANOVA, *P*>0.05).

4. DISCUSSION

Stable isotope analysis based on the predictable trophic enrichment between the isotopic signatures of organisms and their food source is a more advanced way to characterize the feeding patterns and trophic positions of animals in food webs [11,40]. The carbon isotope signature ($\delta^{13}C$) generally exhibits a proximity between consumers and their food source with 1°/oo of fractionation effects [41,42]. Numerous scientific data reveal the use of stable isotopes in studying the food source and their uptake by aquatic invertebrates like bivalve molluscs [10,43], shrimps [35,44] and sea cucumbers [23,26,28, 29,30,45]. Therefore, an attempt was made to examine the assimilation of different food sources like *Sargassum*, *Fucus*, *Spirulina*, *Ulva* and marine pellet by *H. scabra* under laboratory condition (in microcosm) and the subsequent growth responses with an idea to further optimize the ingredients of artificial diets. There were significant differences in $\delta^{13}C$ values observed between the food sources (mainly brown and green algae). This variation could be attributed to taxonomic differences, probably due to different mechanisms adopted by the primary producers in absorbing CO_2 and HCO_3^- for photosynthesis [46,47,48], also the physico-chemical and biogeochemical properties of the geographical location particularly with respect to concentrations of dissolved inorganic carbon and flow rates [49,50].

After 90 days of the feeding trial, *H. scabra* showed reduced growth probably due to poor isotopic turnover of existing tissues. In simple terms, the isotopic composition of the consumer *H. scabra* could be best explained as a mixture of the isotopic composition of its assimilated diet based on isotopic mass balance and that the isotope value should lie between those of dietary components [38]. The present study provided no such evidence, the $\delta^{13}C$ values obtained from *H. scabra* body wall post-experiment in each dietary treatment were significant (*P*<0.05) but could not be expressed in terms of percentage contribution of assimilated ingredients. Hence it is assumed that an additional food source co-existed which was not considered or could be the use of inappropriate diet-tissue discrimination corrections. Another probable reason could be non-supplementation of sea mud to the diet of sea cucumber that could have contributed towards growth, digestion and tissue isotopic turnover rate compared to pure algae powder [51]. Studies indicate that isotopic fractionation is species and tissue specific [21,22]. Diet-tissue isotopic fractionation studies are still in its nascent stage with reference to *H. scabra*. The results obtained are not consistent with published scientific data from other sea cucumber species. The algal food sources *Sargassum*, *Fucus*, *Spirulina*, *Ulva* and marine pellet tend to be rejected by *H. scabra*, which is surprising when compared to *Apostichopus japonicus* that shows more affinity towards brown and green algae [23,28,30,31]. It is assumed that *H. scabra* is more selective in assimilating some other carbon sources from the experimental system, possibly bacterial carbon. Plotieau et al. [52] demonstrated that the elements assimilated into the tissues of *H. scabra* are largely derived from a mixture of dissolved nutrients, heterotrophic

bacteria and autotrophic microorganisms. In this context determining an exact diet for *H. scabra* is more complex than our general assumption. Adsorption of fungi and bacteria attached to the sand particles and pellets may also provide some useful information.

5. CONCLUSION

The diets provided to *H. scabra* could not promote growth under laboratory condition because they were poorly assimilated and tend to be rejected, as expressed through the stable isotope-mixing model. It is assumed that the isotopic turnover of tissues is relatively poor that is not clearly understood. Further input is needed to investigate the interactions between sediment microbiota and the consumer organism in the culture system. This may provide a clue to their selective source of nutrition and in optimizing better farming techniques. Thus, $\delta^{13}C$ analysis is an important criterion in determining how the energy is transferred in a culture system in context to the present work and would provide insights to the isotopic relation among various tissues, specific tissue algorithm in future.

ETHICAL APPROVAL

Written ethical approval has been collected and preserved by the authors as an institutional standard.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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