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Application of stable isotope ratios of nitrogen and carbon as biomarkers to evaluate the assimilation of nutrients in juvenile sea cucumber Holothuria inornata (Semper, 1868) fed two species of macroalgae and biofloc

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Abstract

Studies aimed at exploring the digestive physiology of sea cucumbers are essential to expand the knowledge of alternative aquaculture species. In the present study, carbon and nitrogen stable isotope analysis, measured at natural abundance levels, were employed to determine the assimilation of nutrients supplied by two different macroalgae species, *Padina caulescens* and *Hypnea* sp. and pre-cultured bio-flocculated material (biofloc) in juvenile sea cucumbers *Holothuria inornata*. Feeding items were provided in four different regimes, namely, *Padina caulescens*, *Hypnea* sp, both combined macroalgae, and both macroalgae and biofloc. During the 70-day feeding trial, samples of the dietary sources and three sea cucumber tissues were collected for stable isotope analysis. At the end of the trial, there were no significant differences in the weight gain attained under the different feeding regimes. The isotopic data revealed that the metabolic turnover rates of carbon and nitrogen were the main driving force of isotopic change in sea cucumbers, followed by the characteristically slow growth rates. The assimilation of nutrients available in biofloc was lower than the established dietary proportions but still contributed structural carbon and nitrogen to sea cucumber growth. Residency half times in tissue were also estimated for carbon and nitrogen, the former being shorter than the latter due to differential allocation of nutrients. These findings provide new information on nutrient utilization by juvenile *H. inornata* and allow characterizing the potential use of macroalgae *Padina caulescens*, *Hypnea* sp. and biofloc as dietary resources for its culture.

1. Introduction

Holothuria inornata is an echinoderm belonging to the class Holothuroidea. It is commonly known as sand sea cucumber due to their habit of covering their bodies with sand, pebbles and mollusk seashells (Purcell et al. 2016). It is one of the most abundant sea cucumber species in the Mexican Pacific Ocean and its natural distribution ranges from the gulf of California to the north of Peru (Solís-Marín et al. 2009). Adults frequently reach up to 35 cm in length, and their characteristic cylindrical body presents ambulacral feet along the ventral surface (Pacheco-Vega et al. 2022; Zumaya-Higuera et al. 2025). Sea cucumbers are edible organisms having high market value and are exported to satisfy their demand in Europe, Japan, Malaysia and the United States (Sembiring et al. 2022). Over the last years, the high demand and value of sea cucumber have led to research efforts focusing on its controlled culture. *H. inornata* has potential to be farmed given their simple trophic habits once the juvenile stages have been reached. Sea cucumbers can utilize benthic organic matter as food and it has been demonstrated that these organisms have an important role in benthic bioremediation processes as they can use settled materials around aquaculture sites. The removal of this material leads to efficient nutrient recycling, favoring sustainable production and improving the sediment-water interface (Grosso et al. 2023; Cutajar et al. 2022; Contreras-Sillero et al. 2020). Unlike sea cucumber species as *Apostichopus japonicus* and *H. scabra*, the farming of *H. inornata* has not yet been upscaled. Sea cucumbers have been successfully incorporated into multi-trophic aquaculture systems, which highlights the potential these echinoderms have as part of sustainable and profitable systems (Huo et al. 2026; Senff et al. 2020; Hair et al. 2019). However, unlike *Apostichopus japonicus* and *Holothuria scabra* (Gorospe et al. 2026; Yuan et al. 2010),

the culture of *H. inornata* has not yet been developed on a comparable scale. Information on its nutritional requirements and optimal farming conditions also remains limited. Consequently, studies on nutrient utilization and the suitability of feeding regimes are needed to improve nutritional aspects related to this species. Different stages of the feeding process can be examined to determine the suitability of potential food sources. Food detection, ingestion, digestion, and nutrient assimilation can be assessed using different methodologies, each providing complementary information on nutrient utilization (Budaev et al. 2025). Stable isotope analysis (SIA) is an analytical alternative to evaluate the assimilation of dietary nutrients. Its application is based on the principle that animal consumers reflect the isotopic value(s) of their respective diet(s), although nutrient quality, assimilation, and metabolic turnover rates also play a role on the isotopic values of animal tissues (Boecklen et al. 2011; Post 2002). Stable isotope values can thus be used as nutritional biomarkers and have been widely applied to investigate feeding habits and trophic relationships in natural environments. Compared with complex natural ecosystems, aquaculture systems generally contain a fewer and better-defined number of potential food sources, and this facilitates the employment of SIA as a tool to estimate the relative assimilation of nutrients supplied by alternative ingredients or food sources (Gamboa-Delgado 2022). Carbon and nitrogen stable isotope ratios, expressed as $\delta^{13}C$ and $\delta^{15}N$, are particularly useful because dietary sources often have distinct isotopic values. These values can be compared with those measured in consumer tissues to trace the origin and fate of nutrients and to explore different aspects of the digestive physiology (García-Pérez et al., 2022; Barreto-Curiel et al. 2018). By measuring assimilation, it is possible to identify and quantify the dietary components that were selected, digested and incorporated into body tissues (or used for metabolic purposes), in this way complementing biometric and feed evaluation data in animal nutrition studies (Wirtz et al. 2022; Gamboa-Delgado et al. 2020). Isotopic data can be incorporated into mass-balance mixing models to estimate the relative contribution of two or more dietary sources to the growth of an organism (Phillips 2012). However, isotopic mixing models are applied under several assumptions that must be fulfilled to increase output resolution (Post 2002). The application of SIA in benthic organisms has revealed the source of nutrients derived from feeding items such as seagrass, macroalgae, phytoplankton and small animals (Skinner et al. 2022; Kang et al. 2003). Wen et al. (2016) employed isotopic values of carbon as trophic tracers to determine the ingestion of macroalgae in sea cucumber *Apostichopus japonicus*. Animals were reared for 70 days on diets consisting of dried powdered macroalgae (*Sargassum muticum, Gracilaria lemaneiformis* and *Ulva Lactuca*) and their mixtures. The isotopic values of *Apostichopus japonicus* were significantly affected by the isotopic profiles of macroalgae and it was possible to conclude that *U. lactuca* contributed higher amounts of dietary carbon, followed by *S. muticum*, while *G. lemaneiformis* was apparently not ingested. In this context, the aim of the present study was to evaluate the effect of four feeding regimes conformed by two species of macroalgae and biofloc on growth, survival, nutrient assimilation, and carbon and nitrogen metabolic turnover rates in juvenile *Holothuria inornata*.

2. Materials and Methods

2.1. Experimental animals and facilities

The feeding experiment was carried out at the Live Feed Production Laboratory of the Escuela Nacional de Ingeniería Pesquera (Universidad Autónoma de Nayarit, Mexico). The facility is located on the eastern shore of Matanchén Bay, San Blas, Nayarit. The laboratory has been granted the status of Environmental Management Unit (UMA) under registry DGVS-UMA-IN-2025-NAY/22 issued by the Mexican Authorities in Environmental Affairs. The latter allows undertaking research activities employing the sea cucumber *H. inornata*, which is a species listed as protected under Mexican normativity (NOM-059-SEMARNAT-2010). Specimens of *H. inornata* were manually collected in rocky intertidal areas in the municipality of San Blas, Nayarit. Sea cucumbers ranging in size from 15 to 150 mm were selected and transported in portable coolers to the research facilities. Groups of 10 animals were placed in 30 l plastic containers holding treated seawater filtered through 1 µm-pore-size filters. Constant aeration was provided and partial water exchanges were applied every other day. Each container was fitted with sand substrates (approximately 2 kg), which were previously rinsed with distilled water and disinfected with sodium hypochlorite (6.0%) at 1 mL L^{-1} for 2 hours, to finally be neutralized using sodium thiosulfate ($Na_2S_2O_3$) at a ratio of 0.05 g per mL of added sodium hypochlorite. The previous treatment was as a sanitary measure and a method to eliminate any possible remaining organic feeding items before the start of the feeding experiment.

2.2. Experimental feeding regimes

Four feeding regimes were established and were randomly assigned to treatment triplicates within a completely randomized design. Two species of live marine macroalgae were employed and were offered either individually (Treatments 1 and 2) or as combined regimes (Treatments 3 and 4). The macroalgae *Hypnea* sp. (Rhodophyta, Cystocloniaceae) is a red macroalgae having global distribution. It is abundant in tropical and subtropical latitudes, inhabiting rocky substrates in the intertidal and sublittoral zones (Matsuda et al. 2026; Saber et al. 2024). *Padina caulescens* (Ochrophyta, Phaeophyceae) is a brown macroalgae dwelling in the same habitat as *Hypnea* sp. (Manh et al. 2024). Macroalgae were taxonomically identified following morphological characteristics to avoid interspecific bias. Treatment 3 consisted of a combination of both macroalgae supplied at a 50:50 proportion (on a wet weight basis). Treatment 4 supplied both macroalgae and biofloculated material (biofloc). The biofloccculated material was promoted using a lipid-rich microalgae (*Schizochytrium* sp.) and a probiotic, lactic acid bacteria following procedures by Pacheco-Vega et al. (2018). Macroalgae in feeding regimes were delivered as 10% of the observed sea cucumber standing weight in each replicate tank. Rations were adjusted every 4 days according to presence or absence of leftovers. In treatment 4, a suspension of 1 l of biofloc was daily supplied and provided 0.4 g of biofloccculated material, on a wet weight basis. Sea cucumbers were fed for 70 days on their respective regimes. Dissolved oxygen, temperature and pH were measured every third day at 09:00 and 15:00 h. Additionally, water alkalinity, total hardness, ionic concentration (Ca, Mg and K) and silicates were measured weekly using commercial kits (LYSA™).

2.3. Sampling procedure and sample pretreatment

A sampling schedule was designed to measure diet elicited isotopic changes in sea cucumber bodies after the experimental feeding regimes were provided. Such isotopic changes occur after organisms

undergo a dietary shift and tend to follow an exponential trend (Hesslein et al. 1993). Therefore, three sea cucumbers were randomly sampled from each treatment (one per replicate tank) on days 0, 7, 15, 30, 45, and 60, while six individuals were sampled on the final day (day 70). At the end of the feeding period, all remaining animals were individually measured (individual weight and total length), euthanized and eventually dissected to obtain conspicuous organs such as the respiratory tree, intestine and body wall. Euthanized individuals were accounted for to estimate survival in each treatment. Samples of live macroalgae and biofloc were also collected at different times to verify consistent elemental and isotopic values. Samples were dried in a convection oven (NOVATECH model HS35-AIA) at 60°C for 48 h and then ground using mortar and pestle. Ground samples were placed in labelled, 15 ml Falcon tube and stored in desiccators until stable isotope analysis.

2.4. Stable isotope analysis

Ground samples of macroalgal biomass, biofloc and sea cucumber tissues were weighted (1.00 ± 0.15 mg) and packed in 8 x 5 mm tin cups (Elemental Microanalysis Ltd., Okehampton, UK). The capsules were pressed and arranged in 96-well microplates for analysis. Elemental (carbon and nitrogen) and isotopic analysis at natural abundance levels, were conducted at the Stable Isotope Facility of the Department of Plant Sciences, University of California (Davis, USA) using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (IRMS, Sercon Ltd., Cheshire, UK). Triplicate samples were combusted at 1,000°C in a reactor packed with chromium oxide and silvered copper oxide. After combustion, oxides were removed in a reduction reactor containing reduced copper at 650°C. Helium carrier passed through a water trap containing magnesium perchlorate and phosphorus pentoxide. N_2 and CO_2 were separated on a Carbosieve GC column (65°C, 65 mL/min) before entering the IRMS. During isotopic analysis, experimental samples were interspersed with replicates of different reference materials (glutamic acid, caffeine and nylon). These reference materials were calibrated against international references, and the isotopic data were expressed in delta notation (δ), indicating *per mill* (‰) deviations from the isotopic values of the international standard reference materials: atmospheric nitrogen for $\delta^{15}N$ values, and Vienna Pee Dee belemnite (VPDB) for $\delta^{13}C$ values. Reported instrument precision (SD) was 0.09‰ for $\delta^{15}N$ values and 0.14‰ for $\delta^{13}C$ values.

2.5. Nutritional contributions of macroalgae and biofloc to sea cucumber growth

A two-source, one isotope, isotope mixing model (Equations 1 and 2, IsoError, Phillips and Gregg 2003) was employed to estimate the proportional assimilation of dietary carbon and nitrogen supplied by macroalgae *Padina caulescens* and *Hypnea* sp. in the three types of sampled tissues. In the case of the feeding regime containing three dietary sources (*P. caulescens*, *Hypnea* sp. and biofloc), a three-source, two-isotope mass-balance mixing model was applied (Phillips and Koch 2002) to estimate the relative contribution of these sources to the sea cucumber body wall. Mixing models are applied under the assumption that (1) the isotopic values of the available feeding items are contrasting and (2) the mixture (animal consumer) is the weighted average of its components (dietary items). The two-source model

requires the $\delta^{13}C$ or $\delta^{15}N$ values, the number of samples analyzed and their respective standard errors. The output indicates the relative contributions of two dietary sources (f_1 and f_2) and their corresponding 95% confidence intervals. For the three-source model, $\delta^{13}C$ and $\delta^{15}N$ values of sea cucumbers and the three trophic items were simultaneously entered, hence, a third proportional contribution (f_3) is included to account for the additional dietary source.

$$f1 = \frac{(\delta^{13}C_{Sea\ cucumber} - \delta^{13}C_{source2})}{(\delta^{13}C_{Source1} - \delta^{13}C_{source2})} \text{ and f2 = 1 - f1 (1)}$$

$$f1 = \frac{(\delta^{15}N_{Sea\ cucumber} - \delta^{15}N_{source2})}{(\delta^{15}N_{Source1} - \delta^{15}N_{source2})} \text{ and f2 = 1 - f1 (2)}$$

Several assumptions required for the application of isotope mixing models to animal nutrition studies were considered, including the correction of differences in carbon and nitrogen content among dietary sources (Ballutaud et al. 2022). When organisms consume a constant diet, they tend to reach isotopic equilibrium with their respective diets; however, small isotopic differences are frequently observed between diet and consumers due to a preferential metabolic retention of the heavier isotopic forms (^{15}N and ^{13}C), while the lighter forms (^{14}N and ^{12}C) are excreted. These differences are referred as isotopic discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$). Sea cucumbers that were exclusively fed one type of macroalgae allowed estimating such isotopic differences, and these were in turn used as correction factors in the isotopic mixing models. As there was not a treatment having sea cucumbers fed only biofloc, for this feeding item, average isotopic discriminations factors reported in the literature were used ($\Delta^{15}N$ = 3.4‰ and $\Delta^{13}C$ = 1.0‰) (Stephens et al. 2022; Post 2002).

2.6. Elemental residency times in muscle tissue

In order to further explore the isotopic changes caused by the different feeding regimes, an exponential model (Eq. 3; Hesslein et al. 1993) was applied to assess the proportional roles of the somatic growth (*k*) and the metabolic turnover rates (*m*) in shifting the isotopic values in sea cucumbers.

$$C_{sample} = Cn + (C_o - C_n)\, e^{-(k+m)t}$$

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Where:

C_{sample} is the isotopic value determined in sea cucumbers at any given time *t*.

C_o is the isotope value caused by an initial diet (the natural sea cucumber diet in this study)

C_n represents the isotope value of sea cucumbers once isotopic equilibrium was reached or approached after receiving the experimental feeding regimes

The growth rate constant (*k*) was estimated for each treatment from sea cucumber weight data obtained over the experimental period according to $k = ln(Wf/Wi)/t$, where *Wf* and *Wi* represent final and initial body weights, respectively, and *t* is the duration of the feeding period in days. The metabolic turnover rate (*m*) was unknown and it was estimated by iterative nonlinear regression. The combined contribution of growth and metabolic turnover ($k + m$) was then used to estimate the time required for 50% of tissue carbon or nitrogen to be replaced in the sea cucumber body wall (t_{50}; Eq. 4), following MacAvoy et al. (2005).

$$t_{50} = In2 \,/\, m + \, k$$

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2.7. Statistical Analysis

Data were tested a priori for normality and homogeneity of variances by means of Shapiro-Wilk and Bartlett tests, respectively. Due to the lack of both assumptions, non-parametric, Kruskal-Wallis tests were applied to compare sea cucumber total length and final weight. The estimated proportions of assimilated carbon and nitrogen in sea cucumber tissues were compared with the expected dietary proportions established for the combined feeding regimes. Chi-square (χ^2) goodness-of-fit tests were used to compare the observed proportions in sea cucumber tissues with the expected proportions of carbon and nitrogen available from macroalgae and biofloc. Elemental residence half-times (t_{50}) in body wall tissue were compared among feeding regimes using Kruskal–Wallis tests. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc.), with statistical significance set at $P < 0.05$.

3. Results

3.1. Sea cucumber growth and survival

Animals readily accepted their respective feeding items and after 70 days of feeding, no significant differences in sea cucumber body size, mean wet weight or survival were detected among treatments ($p = 0.34$). Survival rates were high and ranged from 96.7 to 98.9% (Table 1). Sea cucumbers maintained under feeding regimes 1 and 3, receiving only *Hypnea* and combined *Hypnea* and *Padina caulescens*, showed slightly higher body length and wet weigh; however, the considerable variability determined in the variables weight and length prevented detection of significant differences. The latter was an effect of the heterogeneous initial sizes of the wild-caught sea cucumbers used in the feeding experiment.

Table 1
Final mean body size and wet weight of sea cucumber *H. inornata* after receiving four feeding regimes containing two species of macroalgae and biofloc for 70 days. Values are presented as treatment mean values ± SD (n = 6 to 7 on final experimental day).

Feeding regime	Survival (%)	Size (mm)	Final Weight (g)	Weight gain (%)
Hypnea sp.	97.8 ± 3.8	86.5 ± 16.9	30.5 ± 16.9	31.3
Padina caulescens	96.7 ± 5.8	79.3 ± 19.0	25.9 ± 18.9	22.0
Hypnea sp. + *Padina caulescens*	98.9 ± 1.9	86.6 ± 24.2	32.0 ± 24.5	17.3
Hypnea sp. + *Padina caulescens* + Biofloc	96.7 ± 0.0	74.6 ± 16.3	23.1 ± 14.4	39.5
No significant differences were detected among treatments				

3.2. Effects of feeding regimes on tissue isotopic composition

The carbon and nitrogen isotopic composition of sea cucumber tissues reflected the isotopic signatures of the species of macroalgae and biofloc used as dietary sources (Table 2). Across feeding treatments, tissue $\delta^{13}C$ values indicated that isotopic equilibrium between sea cucumbers and their feeding items was reached approximately 30 days after the onset of the feeding trial. In turn, $\delta^{15}N$ values reached an equilibrium after approximately 60 days (Figs. 1a, b). Diet–tissue isotopic discrimination differed between elements, with smaller discrimination factors observed for carbon ($\Delta^{13}C$) than for nitrogen ($\Delta^{15}N$) (Table 3)

Table 2

Isotopic values measured at natural abundance levels (‰) in the supplied feeding items and different sea cucumber tissues. Animals were fed for 70 d with two types of macroalgae and biofloc. Mean values ± S.D. n = 6 samples.

Feeding item	**$\delta^{13}C$**		**$\delta^{15}N$**		**Protein content (%)[a]**			
Hypnea sp.	-18.42 ± 0.44		8.85 ± 0.38		15.0 ± 1.3			
Padina caulescens	-15.16 ± 0.74		11.36 ± 0.41		15.4 ± 2.1			
Biofloc	-13.81 ± 1.11		5.29 ± 0.94		31.2 ± 3.8			
Tissue type	*Hypnea* sp.		*Padina caulescens*		Hypnea sp. + *Padina caulescens*		*Hypnea* sp. + *Padina caulescens* + Biofloc	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Body wall	-17.44 ± 0.38	13.12 ± 0.32	-16.02 ± 0.31	15.46 ± 0.28	-16.97 ± 0.47	14.07 ± 0.51	-16.87 ± 0.51	13.86 ± 0.26
Intestine	-18.14 ± 0.54	15.26 ± 0.35	-17.81 ± 0.47	14.33 ± 0.24	-17.83 ± 0.52	15.22 ± 0.36	-16.88 ± 0.29	14.31 ± 0.32
Respiratory tree	-17.74 ± 0.67	15.62 ± 0.48	-17.87 ± 0.23	14.59 ± 0.38	-18.01 ± 0.30	15.99 ± 0.17	-18.49 ± 0.27	16.90 ± 0.40

[a] Estimated on a dry weight basis

Table 3

Isotopic discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$, ‰) determined in different sea cucumber tissues after feeding trial with the macroalgae *Hypnea* sp. and *Padina caulescens* supplied for 70 d. Values are expressed as mean values ± SD. n = 6 samples per dietary treatment.

Tissue type	***Hypnea* sp.**		***Padina caulescens***	
	$\Delta^{13}C$	**$\Delta^{15}N$**	**$\Delta^{13}C$**	**$\Delta^{15}N$**
Body wall	0.98 ± 0.06	4.27 ± 0.07	0.86 ± 0.08	4.10 ± 0.12
Intestine	0.28 ± 0.04	6.41 ± 0.11	2.65 ± 0.13	2.97 ± 0.09
Respiratory tree	0.68 ± 0.06	6.77 ± 0.09	2.71 ± 0.15	3.23 ± 0.10

3.3. Assimilated proportions of nutrients supplied by macroalgae and biofloc

Overall, the relative contributions of dietary carbon and nitrogen assimilated from macroalgae were higher than those derived from biofloc. When *Padina caulescens* and *Hypnea* ap. were provided together, chi-square goodness-of-fit tests showed no significant differences between the expected dietary

proportions of carbon and nitrogen and the estimated proportions assimilated into the body wall (p = 0.210). In contrast, significant differences between expected and assimilated proportions were detected for both carbon and nitrogen in the intestine and respiratory tree (Table 4). When biofloc was included as a dietary source (treatment 4), the proportion of assimilated nitrogen differed significantly from its expected dietary contribution (p = 0.012). No significant differences were detected for carbon (p = 0.147) or total dry matter (p = 0.089) between the expected dietary proportions and the estimated contributions from biofloc and macroalgae. *Hypnea* sp. contributed a greater proportion of assimilated nutrients than *Padina caulescens*, however a significant difference was detected only for nitrogen when the contributions of the two macroalgae species and biofloc were compared (Table 5).

Table 4
Relative assimilation of dietary nitrogen and carbon in different tissues of juvenile sea cucumber *Holothuria inornata* simultaneously fed with macroalgae *Hypnea* sp. and *Padina caulescens* Values are expressed as mean values ± confidence intervals (n = 6 samples per dietary treatment).

	Nutritional proportions			
	Expected	**Observed**		
	Nitrogen	Body wall min. mean. max.	Intestine min. mean. max.	Respiratory tree min. mean. max.
Hypnea sp.	50.8[a]	41.1 59.6[a] 78.0	57.7 69.3[b] 81.0	53.7 65.6[b] 77.4
Padina caulescens	49.2	22.0 40.4 58.9	19.0 30.7 42.3	22.6 34.4 46.3
	Carbon	Body wall min. mean. max.	Intestine min. mean. max.	Respiratory tree min. mean. max.
Hypnea sp.	50.7[a]	38.3 53.6[a] 68.8	66.2 81.4[b] 96.6	50.8 66.0[b] 81.0
Padina caulescens	49.3	31.2 46.4 61.7	3.4 18.6 33.8	19.0 34.0 49.2

Different letters indicate significant differences, row wise, between expected and observed relative contributions.

Table 5. Assimilated proportions of dietary nitrogen, carbon, and total dry matter supplied by macroalgae *Hypnea* sp*., Padina caulescens* and biofloc simultaneously fed to juvenile sea cucumber *Holothuria inornata*. The mixing model integrates the elemental concentration and the isotopic values of the feeding items. Values are expressed as mean values ± truncated confidence intervals (n = 6).

	Expected contributions	Observed contributions to body wall		
		min.	mean.	max.
Nitrogen				
Hypnea sp.	42.9[a]	39.4	50.7[b]	62.0
Padina caulescens	41.5	36.6	45.9	55.2
Biofloc	15.6	0	3.4	12.4
Carbon				
Hypnea sp.	46.9[a]	38.9	49.6[a]	60.3
Padina caulescens	45.6	39.2	48.7	58.2
Biofloc	7.5	0	1.7	8.9
Total dry matter				
Hypnea sp.	46.3[a]	38.3	51.4[a]	64.5
Padina caulescens	45.0	35.9	46.8	57.7
Biofloc	8.7	0	1.8	8.2

Different letters indicate significant differences, row wise, between expected and observed relative contributions

3.4. Contribution of metabolic turnover rates to observed isotopic changes

Table 6 shows the growth rates (*k*), the metabolic turnover rates (*m*) measured at the end of the feeding period. Changes in the isotopic composition of the body wall were primarily driven by metabolic turnover, with m values ranging from 0.0250 to 0.1801 d^{-1}. In contrast, growth contributed comparatively less to isotopic change, as indicated by the lower k values, which ranged from 0.0022 to 0.0047 d^{-1}. Based on the estimated k and m values, the time required for 50% replacement of tissue carbon and nitrogen (t_{50}) was calculated. Model coefficients of determination (r^2) ranged from 0.58 to 0.95, indicating that the exponential models provided an adequate fit to the observed isotopic changes. Isotopic data and model estimates indicated that metabolic turnover accounted for 87–95% of the diet-induced change in nitrogen isotopic composition across the four feeding regimes. A similar pattern was observed for carbon, with metabolic turnover accounting for 92–98% of the isotopic change. The relative contribution of metabolic turnover to isotopic change was similar among feeding regimes, although higher nitrogen turnover rates were observed in sea cucumbers receiving the two combined diets. Estimated residence half-times (t_{50}) in the body wall were longer for nitrogen (9–24 d) than for carbon (4–18 d). In addition, t_{50} values were significantly shorter in sea cucumbers receiving the combined feeding regimes. Isotopic discrimination factors for carbon and nitrogen were estimated after tissue isotopic values approached equilibrium with the experimental diets. The $\Delta^{15}N$ values estimated for sea cucumbers fed *Padina caulescens* and *Hypnea* sp. were 0.6 and 1.2‰, respectively, whereas the corresponding $\Delta^{13}C$ values

were 0.3 and 0.6‰. These discrimination factors were subsequently used to correct the dietary source contributions estimated by the isotope mixing models.

Table 6
Growth rates (*k*), metabolic turnover rates (*m*) and elemental half times (t_{50}) in body wall of juvenile sea cucumbers *Holothuria inornata* fed four different dietary regimes consisting of macroalgae *Padina caulescens, Hypnea* sp. and biofloc. The estimated contributions of parameters *k* and *m* to the observed isotopic changes are indicated. n = 3 samples per dietary treatment, 6 on final day.

Treatment	***k*** **(d^{-1})**	***m*** **(d^{-1})**		***k*** **vs.*m*** **(%)**				t_{50} **(d)**	
		N	**C**	**Nitrogen**		**Carbon**		**N**	**C**
				k	***m***	***k***	***m***		
Padina caulescens	0.0028	0.026	0.035	10.8	89.2	7.4	92.6	24.0 ± 2.1[b]	18.3 ± 1.7[b]
Hypnea sp.	0.0038	0.025	0.104	13.2	86.8	3.5	96.5	23.9 ± 2.4[b]	6.4 ± 1.3[a]
Pad + Hyp	0.0022	0.078	0.180	4.9	95.1	1.2	98.8	8.6 ± 1.1[a]	3.8 ± 1.5[a]
Pad + Hyp+Biof	0.0047	0.082	0.061	7.2	92.8	7.7	92.3	10.5 ± 0.9[a]	7.9 ± 2.0[a]

Column wise superscripts indicate significant differences.

4. Discussion

4.1. Sea cucumber growth and survival

Sea cucumbers are ectothermic organisms with relatively slow growth rates, and their metabolic activity and energy allocation are strongly influenced by environmental conditions (Liu and Han 2025). In the present study, no significant differences were detected in terms of survival rates and mean final weight of sea cucumbers maintained under the different treatments. These results are different to observations reported by Wen et al. (2016) when supplying two types of macroalgae to sea cucumber *A. japonicus*. Authors reported higher growth rates in animals reared on macroalgae *Sargassum muticum* than in those fed *Gracilaria lemaneiformis.* In another *Holothuria* species, Su et al. (2026) also reported different growth performance after feeding *Holothuria leucospilota* with macroalgae *Ulva* sp., *Sargassum* sp., and *Laminaria japonica*, observing better growth and ingestion rate in sea cucumbers fed combined *Ulva* sp. and *Sargassum* sp. Differences in growth responses among studies may be associated with variable nutritional compositions and digestibility of the macroalgae. Species-specific physiological and metabolic characteristics of sea cucumbers may be also accountable for the different nutritional performance. In the present study, the similar final weights observed across the feeding treatments suggest that the dietary regimes promoted similar feed stimuli and nutrient uptake over the 70-day

experimental period. The comparable growth and survival responses indicate that the supplied dietary sources were nutritionally favorable under the experimental conditions.

4.2. Isotopic changes in different sea cucumber tissues

Sequential sampling of the body wall throughout the experiment, showed that $\delta^{13}C$ values changed faster than $\delta^{15}N$ values. Estimated *k* and *m* values indicated that the isotopic turnover was driven primarily by the metabolic replacement of tissular carbon and nitrogen, whereas growth played a lesser role in contributing to observed isotopic shifts. This pattern differs from commonly reported observations for fast-growing aquatic invertebrates, in which tissue accretion (and the associated isotopic dilution, *i.e.* the replacement of tissular molecules by the new dietary molecules) can account for a substantial proportion of isotopic change (Vander Zanden et al. 2015; Gamboa-Delgado et al. 2020). Previous studies on *Apostichopus japonicus* have also shown that their tissues reflect the isotopic composition of dietary macroalgae. For example, Wen et al. (2016) reported a relatively rapid equilibrium of $\delta^{13}C$ in sea cucumbers fed *Sargassum muticum* ($\delta^{13}C$ = − 15.49 ± 0.14‰), *Gracilaria lemaneiformis* ($\delta^{13}C$ = − 17.48 ± 0.11‰), and *Ulva lactuca* ($\delta^{13}C$ = − 14.42 ± 0.05‰). In contrast, Sun et al. (2012) indicated that in juvenile *A. japonicus* (5 to 36 g), the $\delta^{13}C$ values of body walls equilibrated after 56 to 84 d when fed powdered macroalgae *Laminaria* sp, *Gracilaria* sp. and sea mud. In the present study, $\delta^{13}C$ values measured in the body wall of *H. inornata* fed single macroalgae were similar to those reported for *A. japonicus* fed other macroalgae species: -$\delta^{13}C$= -17.44 ± 0.17‰ for *Hypnea* sp. and $\delta^{13}C$= -16.02 ± 0.31‰ for *Padina caulescens.* These values are within the range reported for *A. japonicus* fed other macroalgal species and indicate that the dietary carbon from both macroalgae was gradually incorporated into *H. inornata* tissues. However, in the present study, results indicate that the addition of biofloc did not significantly affect the $\delta^{13}C$ values of sea cucumbers, suggesting a limited contribution of dietary carbon from this source, which was later confirmed by the isotopic mixing model.

$\delta^{15}N$ values of trophic items can be used as reliable biomarkers to trace the available dietary protein in feeding items. $\delta^{15}N$ values of *Padina caulescens*, *Hypnea* sp. and biofloc were 11.36 ± 0.41, 8.85 ± 0.38 and 5.29 ± 0.94‰, respectively. $\delta^{15}N$ values in the three sampled tissues were higher than the $\delta^{15}N$ values of the supplied feeding sources. In treatment 4, the $\delta^{15}N$ value of biofloc had a small influence on the $\delta^{15}N$ values of sea cucumbers. The isotopic differences (for both elements, C and N) observed in sea cucumber body wall, respiratory tree and intestine can be attributed to the specific metabolic properties of each tissue type (Howland et al. 2003; Fogel and Torus 2003; Pinnegar and Polunin 1999). An isotopic equilibrium was reached after 30 d for $\delta^{13}C$ values and after 60 d for $\delta^{15}N$ values in all treatments and this is consistent with the higher metabolic turnover rates determined for carbon. The time required to reach isotopic equilibrium with a diet depends on animal size, environmental parameters and dietary characteristics (Zhou and Gu 2020; Mohan et al. 2016; Herzka 2005). Confirming isotopic equilibrium is important because allows estimating particular isotopic discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$) for each species and rearing conditions. In the present study, such values were small and positive for carbon, indicating that sea cucumbers and their tissues were slightly enriched in ^{13}C in comparison to their

respective feeding items. $\Delta^{15}N$ values were larger (2.97 to 6.77‰) than $\Delta^{13}C$ values and most values were above the published mean value $\Delta^{15}N$ = 3.4‰. Results reported for *Holothuria scabra* fed macroalgae, indicated $\Delta^{15}N$ values for body wall and intestine of 2.4 and 1.5‰, respectively (Watanabe et al. 2013). Interestingly, in nutritional studies, it has been discussed that the magnitude of $\Delta^{15}N$ and $\Delta^{13}C$ values seems to be associated to the quality and quantity of dietary protein animals consume (Le Vay and Gamboa-Delgado 2011; Martinez del Rio et al. 2009). Higher $\Delta^{15}N$ and $\Delta^{13}C$ values have been interpreted as indicators of tentative nutritional restrictions in some cases because the profiles and complementarity of essential amino acids affect the magnitude of the isotopic discrimination factors (Robbins et al. 2010). It has been argued that diets that consist of several protein sources do not necessarily cause isotopic discrimination factors equivalent to the mean value of the dietary items because the interaction between the feeding items can modify the availability and assimilation of nutrients (Caut et al. 2008; Phillips and Koch 2002).

4.3. Nutrient assimilation from macroalgae and biofloc

In their natural habitats, macroalgal species can exhibit distinct $\delta^{13}C$ and $\delta^{15}N$ signatures caused by differences in environmental conditions, available nutrients, and carbon and nitrogen acquisition pathways. The presence of isotopic differences in the trophic items offered to an animal consumer represents an advantage for the application of mixing models. When feeding items have similar isotopic values, the mixing models do not offer enough resolution to obtain feasible solutions on assimilation. In the present study, *Hypnea* sp., *Padina caulescens*, and biofloc had sufficiently distinct $\delta^{13}C$ and $\delta^{15}N$ values, which allowed estimating their relative nutritional contributions to *H. inornata*. Isotopic data from the dietary sources and sea cucumber tissues were incorporated into mass-balance mixing models to estimate the relative contributions of dietary carbon and nitrogen to the tissues Several assumptions associated with isotope mixing models were considered when estimating these contributions. Parameters such as diet–tissue discrimination factors are often difficult to determine under field conditions but can be estimated more directly in controlled feeding experiments. In the present study, discrimination factors obtained from sea cucumbers fed single-macroalga diets were used to correct the isotopic values of *Hypnea* sp. and *P. caulescens* before their inclusion in the mixing models. Because no treatment consisted exclusively of biofloc, the corresponding correction was based on discrimination factors reported in the literature, as described above. Accounting for diet–tissue discrimination is important because mixing models may under- or overestimate dietary contributions when $\Delta^{13}C$ and $\Delta^{15}N$ are not experimentally estimated (Gamboa-Delgado 2022; Caut et al. 2009). For this correction, the trophic sources incorporated into the model were not the isotopic dietary values of ingredients *per se*, instead, the isotopic values determined in sea cucumbers fed on macroalgae and biofloc were introduced as trophic sources, in this way correcting for their respective $\Delta^{15}N$ and $\Delta^{13}C$ values.

The macroalgae *Hypnea* sp. contributed higher proportions of dietary nitrogen and carbon to the growth of intestine and respiratory tree, but the nutritional contributions from *Hypnea* sp. and *Padina caulescens* to the body wall remained statistically similar in relation to the respective available nutrients. Observed differences in assimilated nutrients can be explained by the different metabolic properties of each tissue

type and by the nutritional profiles of both macroalgae. Although the protein content of both macroalgae was similar, other nutritional properties such as digestibility, ash and fiber content might have elicited a different assimilation of the dietary nitrogen and carbon in the sampled tissues. Previous studies have indicated that macroalgae belonging to the genus *Hypnea* sp. and *Padina caulescens* contain all the essential amino acids in their nutritional profiles. In addition to lysine, phenylalanine and aspartic acid, histidine occurs as a major constituent of *Padina* (Sethi 2012), while studies on species of *Hypnea* indicate that specimens from this genus contain all the essential amino acids, accounting for 52 to 54% of total amino acids (Siddique et al. 2013). In the present study, differences in these nutritional characteristics may partly explain the higher nutritional contributions derived from *Hypnea* sp., although direct measurements of digestibility and amino acid assimilation would be required to establish the mechanisms involved. Stable isotope analysis has previously demonstrated the importance of macroalgal-derived nutrients in the nutrition of sea cucumber. Sun et al. (2013) identified macroalgae as an important dietary source for cultured *Apostichopus japonicus*, together with small invertebrates, particulate organic matter, and benthic microalgae. Results derived from the present study indicate that biofloc also contributed structural carbon and nitrogen to the body wall of *H. inornata*, even though it was supplied at a lower dietary proportion than macroalgae. However, the estimated contribution of assimilated nutrients from biofloc was lower than the proportional dietary contribution. This result suggests that biofloc-derived nutrients were less effectively represented in the assimilation processes under the established experimental conditions. The relatively low contribution of biofloc may also have been caused by its availability in the rearing tanks. Rapid dispersion of the flocculated material after its addition to the tanks could have reduced the amount accessible to sea cucumbers, although this possibility was not directly evaluated in the present study. Biofloc represents a complex aggregate that may include bacteria, microalgae, protozoans, organic particles, and other microorganisms that can provide an additional nutritional resource in aquaculture systems (Khanjani et al. 2023). Its application in sea cucumber culture has also received increasing attention due to its potential contribution to nutrient recycling, water quality, growth, and health (Xiao et al. 2024). The present results indicate that *H. inornata* can assimilate carbon and nitrogen derived from biofloc, but under the conditions tested, its relative nutritional contribution was lower than the nutritional contributions from the macroalgal sources.

4.4. Elemental residency half times in sea cucumber

The isotopic changes observed during the feeding trial were fitted to the Hesslein model to estimate the metabolic turnover rate constant (*m*) for carbon and nitrogen. The relative contribution of *k* (growth) and *m* to the observed isotopic change in sea cucumbers was subsequently calculated. Results indicate that the metabolic turnover rate (*m*) of sea cucumbers was the main driving force of isotopic change for nitrogen in all treatments (87 to 95%), while growth (*k*) had a minor influence on such isotopic changes (5 to 13%). In the case of carbon, the contribution of turnover rate to the isotopic change was still higher (92 to 99%). Slow growing organisms change their somatic/tissue isotope profiles mainly through tissue reposition (Hesslein et al. 1993). In the present study, parameters *k* and *m* were utilized to estimate the residency half times (C and N) or t_{50} values in sea cucumber bodies. t_{50} values remained in a range of 8 to 24 d for nitrogen and from 4 to 18 d for carbon. For comparison, Sun et al. (2012) reported a carbon

t_{50} of approximately 21 days for juvenile *Apostichopus japonicus* with a mean body weight of 5 g. This value is slightly higher than the range estimated for *H. inornata* in the present study, suggesting a more rapid replacement of tissue carbon in *H. inornata* under the experimental conditions. However, direct comparisons between species should be interpreted cautiously because isotopic turnover can be influenced by diet composition, environmental conditions, body size, developmental stage, and species-specific physiology (Boecklen et al. 2011).

5. Conclusions

The present study explored the nutritional role of macroalgae *Hypnea* sp. and *Padina caulescens*, and bioflocculated material as feeding items for the sea cucumber *Holothuria inornata*. Final body weight and survival did not differ significantly among feeding treatments, and the high individual variability associated to the wild-caught juveniles should be considered when interpreting growth responses. When *Hypnea* sp. was supplied as sole feeding item, and in conjunction with *Padina caulescens*, higher weight gain was promoted in sea cucumbers, although differences were not statistically significant. Stable isotope analysis provided evidence of differences in the assimilation of nutrients supplied by the three dietary sources. *Hypnea* sp. generally contributed greater proportions of assimilated carbon and nitrogen to the body wall, respiratory tree, and intestine than *P. caulescens* and biofloc. Both macroalgae contributed nutrients to *H. inornata* tissues, supporting their potential as dietary components for the culture of this species. Biofloc also contributed carbon and nitrogen to sea cucumber tissues, although the estimated contributions were lower than those derived from both macroalgae. Overall, the results demonstrate the usefulness of stable isotope analysis for identifying dietary sources contributing carbon and nitrogen to the biosynthesis of *H. inornata* tissues, even when differences in growth were not statistically detectable. Further studies should focus on evaluating different proportions and delivery methods of macroalgae and biofloc. Longer feeding periods and more homogeneous experimental animals will assist in defining enhanced feeding strategies for the future aquaculture management of the sea cucumber *H. inornata*.

Declarations

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Competing Interests

The authors declare no conflict of interest.

Author Contribution

All authors contributed to the study conception and design. Material preparation and data collection were performed by Miriam Guadalupe Zumaya-Higuera, José Trinidad Nieto-Navarro and Erika Torres-Ochoa. Biometric data analysis, and interpretations of isotopic data were conducted by Miriam Guadalupe Zumaya-Higuera and Julián Gamboa-Delgado. The first draft of the manuscript was written by Julián Gamboa-Delgado. Writing-review and editing of the manuscript was done by Juan Manuel Pacheco-Vega. All authors read and approved the final manuscript

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Data Availability

Isotopic values and elemental content of samples will be made available (after administrator approval) on the Universidad Autónoma de Nuevo León´s institutional repositoryhttp://eprints.uanl.mx/id/eprint/31831

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Figures

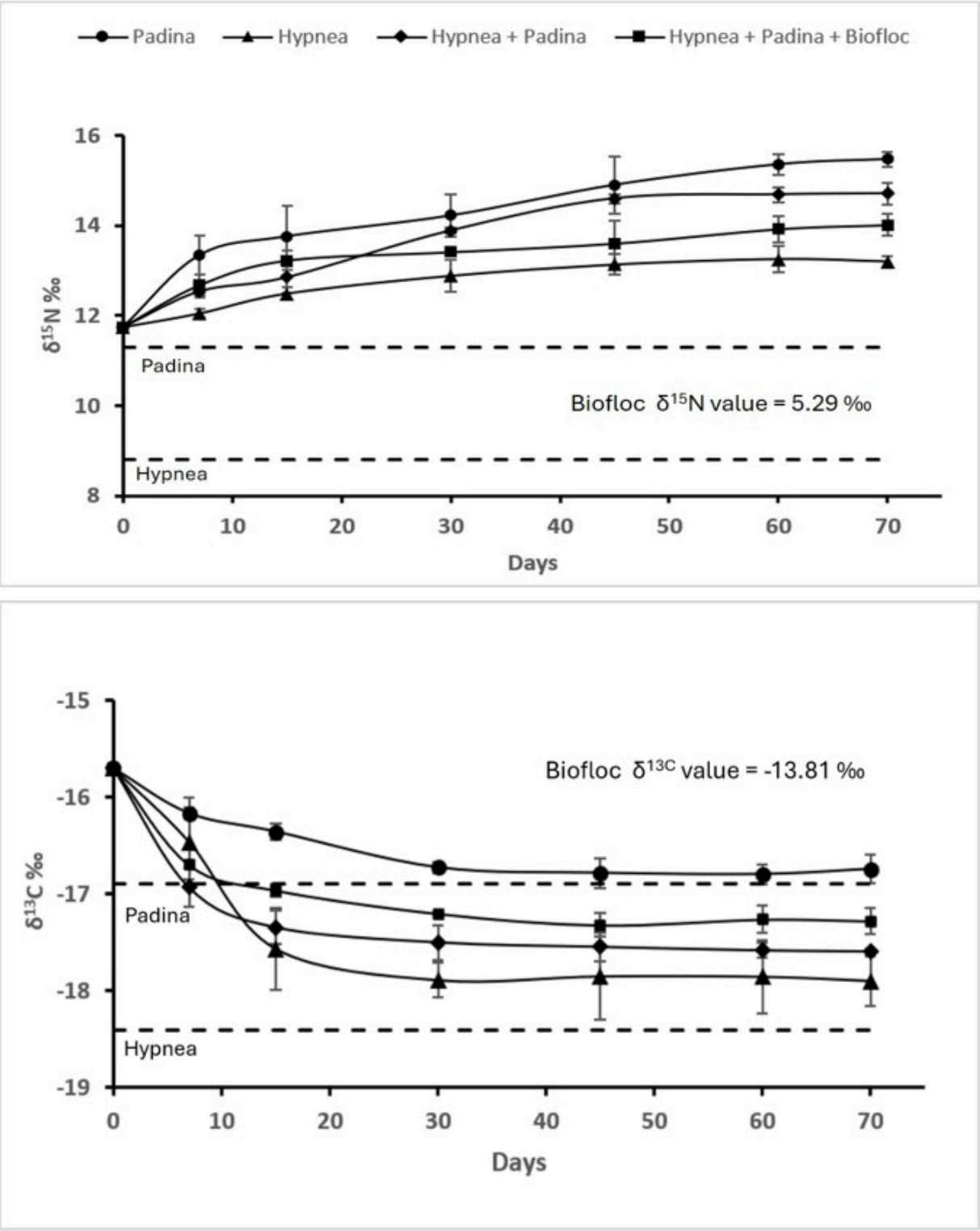

Figure 1

Changes in (a) nitrogen ($\delta^{15}N$) and (b) carbon ($\delta^{13}C$) stable isotope values in the body wall of wild-caught juvenile *Holothuria inornata* during a 70-day feeding trial using macroalgae *Hypnea* sp., *Padina caulescens* and biofloc as feeding items. Dotted lines indicate the isotopic values of *Hypnea* sp. and *P. caulescens*. Values are expressed as mean ± SD (n = 3 per sampling day; n = 6 on day 70).