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Outcomes of feeding activity of the sea cucumber *Holothuria tubulosa* on quantity, biochemical composition, and nutritional quality of sedimentary organic matter

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Introduction: *Holothuria tubulosa* is one of the most common sea cucumbers in the Mediterranean Sea, generally associated with organically enriched coastal sediments and seagrass beds. As a deposit-feeder, it is responsible for strong bioturbation processes and plays a putative key role in sedimentary carbon cycling and benthic trophodynamics. With the aim of exploring the potential use of holothuroids as a tool for remediating eutrophicated sediments, we investigated the effects of *H. tubulosa* on sedimentary organic matter quantity, biochemical composition, and nutritional quality.

Methods: Holothuroids and associated samples of ambient sediments were collected in two sites located in the Central-Western Mediterranean Sea (Sardinia, Italy) and characterized by different trophic status backgrounds: the site of Oristano characterized by sandy-muddy sediments and the presence of mariculture plants (ranked as meso-eutrophic) and the site of Teulada characterized by sandy sediments and *Posidonia oceanica* meadows (ranked as oligo-mesotrophic). We compared the biochemical composition (proteins, carbohydrates, lipids) of ambient sediment *vs* sea cucumbers feces and the sedimentary protein content *vs* protein content in the sediments retrieved in different gut sections (esophagus, mid gut, end gut) of the holothuroid.

Results: Our results reveal that holothuroids feeding on meso-eutrophic sediments can increase protein (1.5 times) and lipid (1.3 times) content through their defecation, thus making these substrates a more labile food source for other benthic organisms. We report here that *H. tubulosa* feeding on meso-eutrophic sediment is most likely able to actively select particles rich in labile organic matter with buccal tentacles, as revealed by the protein content in the esophagus that is up to 2-folds higher than that in the source sediment. According to the inverse relationship between assimilation rates and availability of organic substrates and the optimal foraging theory, *H. tubulosa* feeding on oligo-mesotrophic sediments showed potential assimilation of proteins ca. 25% higher than that of specimens feeding on meso-eutrophic sediments.

Discussion: Our results reveal that *H. tubulosa* feeding on meso-eutrophic sediments can profoundly influence the benthic trophic status, specifically modifying the biochemical composition and nutritional quality of organic matter, thus paving the way to its possible use in bioremediation actions of eutrophicated sediments and in Integrated Multi-Trophic Aquaculture systems.

Introduction

Bioturbation mediated by benthic marine deposit-feeder plays a key role in carbon biogeochemical cycling from coastal to hadal depths (Uthicke and Karez, 1999; Roberts et al., 2000; Lohrer et al., 2004; Slater and Carton, 2009; Amaro et al., 2010; Slater et al., 2011a; Purcell et al., 2016). Bioturbation can indeed influence sediment permeability, chemical gradients in pore waters and sedimentary organic matter (OM) degradation rates (Reise, 2002; Lohrer et al., 2004; Solan et al., 2004; Meysman et al., 2006a; Meysman et al., 2006b; Schenone et al., 2019). Among the most effective bioturbators, sea cucumbers (Phylum Echinodermata), common members of marine benthic communities in world oceans at all depths, count more than 1,500 species (Horton et al., 2018). Deposit-feeding sea cucumbers, ingesting the whole sediment, and feeding on sedimentary OM, can rework large amounts of sediments (5.9 – 12.9 kg dw m^{-2} yr^{-1}) (Coulon and Jangoux, 1993; Uthicke and Karez, 1999; Mangion et al., 2004, Hartati et al., 2020), thus contributing to the redistribution and remineralization of sedimentary organic loads from hadal depth to coastal areas and coral reefs (Amaro et al., 2010; MacTavish et al., 2012; Purcell et al., 2016; Wolfe et al., 2017; Yamazaki et al., 2019).

Bioturbation caused by sea cucumbers extends from the upper layer of the seafloor to several centimeters' depth in the sediment, depending on the variable ability of the different species to dig into the sediment and their borrowing habits (Mercier et al., 1999; Roberts et al., 2000; Amaro et al., 2010; Ramón et al., 2019). During feeding, sediment particles are captured by the sea cucumber with the tentacles and released into the pharynx. Ingested particles are then mixed with the digestive enzymes and, once compressed into a plug that moves along the digestive system are released through the cloaca (Zamora and Jeffs, 2011). Thus, deposit-feeding sea cumbers, with their peculiar feeding activity, can reduce sedimentary OM loads (Slater and Carton, 2009; MacDonald et al., 2013; Neofitou et al., 2019). For this reason, sea cucumbers are considered one of the best candidates for Integrated Multi-Trophic Aquaculture (IMTA) practices (MacDonald et al., 2013; Purcell et al.,2014; Cubillo et al., 2016; Grosso et al., 2021). IMTA, in fact, can transform mariculture potential wastes (e.g., uneaten food and fish feces) into food sources for other reared species positioned at lower trophic levels (Zhou et al., 2006; Slater and Carton, 2007; Slater and Carton, 2009; Slater et al., 2009; Zamora and Jeffs, 2011; Zamora and Jeffs, 2012; Yuan et al., 2013; Lamprianidou et al., 2015; Shpigel et al., 2018). This sort of circular reuse of mariculture waste for producing extra commercial

biomass could help mitigate the well-documented impacts of marine aquaculture on the trophic status, biodiversity and functioning of coastal marine ecosystems (Kalantzi and Karakassis, 2006; Pusceddu et al., 2007; Holmer et al., 2008; Holmerm, 2010; Mirto et al., 2010).

So far, sea cucumbers used in IMTA and co-culture trials include high commercially valuable species such as *Apostichopus japonicus* Selenka, 1867 (Zhou et al., 2006; Yuan et al., 2013; Kim et al., 2015), *Australostichopus mollis* Hutton, 1872 (Slater and Carton, 2007; Slater and Carton, 2009; Slater et al., 2009; Zamora and Jeffs, 2011; Zamora and Jeffs, 2012), *Holothuria scabra* Jaeger, 1833 (Mathieu-Resuge et al., 2020), *Parastichopus californicus* Stimpson, 1857 (Paltzat et al., 2008) and *Holothuria tubulosa* Gmelin 1788 (Grosso et al., 2021). Recent experiments have shown that the Mediterranean Sea cucumber *Holothuria tubulosa* grows faster beneath finfish cages than in mariculture-free areas (Tolon et al., 2017). In another study, it was shown that the monthly reduction of organic carbon (OC) caused by *H. tubulosa* (ca. 10 ind m^{-2}) feeding in sediments beneath seabream and seabass cages has been estimated to account up to 62% (Neofitou et al., 2019).

Moreover, sea cucumbers have a high commercial value and are currently overexploited worldwide (Conand, 2006; Anderson et al., 2011a, b; Bordbar et al., 2011; Purcell et al., 2011; Conand et al., 2014; González-Wangüemert et al., 2018). Indeed, holothuroid fisheries have rapidly expanded in the last three decades, because of the increasing demand for food in international markets and biomedical research programs (Bordbar et al., 2011). Holothuroids, in fact, are a traditional food in China and other eastern regions, where they are considered gourmet and luxury seafood (Wen et al., 2010; Yang and Bai, 2015), and sold at prices as high as up to ca. 3000 US$ per dried kg (Purcell et al., 2012; Purcell et al., 2014). The large market demand for these animals has led to overfishing in worldwide oceans and seas, including the Mediterranean. Consequently, 16 species have been classified as threatened with extinction based on standard IUCN methodology (Conand et al., 2014) and 4 species are included in the Convention on International Trade in Endangered Species list (CITES, 2022). In this sense, restocking and stock enhancement practices of holothuroids, eventually also including hatchery production of juveniles could support their protection.

Organic carbon in marine sediments is made of a variable combination of molecules that are differently prone to a rapid utilization by benthic detritivores (Pusceddu et al., 2009). The total organic C content of the sediments is, in this sense, a rather weak descriptor of the potential amount of food for the benthos. Since total

organic C content does not discriminate between refractory vs. labile fractions, its biopolymeric fraction (the sum of protein, carbohydrate and lipid C equivalents, *sensu* Fabiano et al., 1995) is currently used as a more reliable descriptor of the benthic trophic status, namely the availability of food available for the benthos in a variety of wetlands, coastal and even deep-sea sedimentary environments (Pusceddu et al., 2009; Pusceddu et al., 2010; Pusceddu et al., 2011; Dell'Anno et al., 2013). Nonetheless, studies dealing with the role of holothuroids on sedimentary organic matter dynamics have almost exclusively dealt with the mere measurement of total organic C only, with a very few exceptions (Amaro et al., 2010).

With an eye to the potential use of *H. tubulosa* for abating, also in an IMTA perspective, the consequences of benthic eutrophication, we investigated differences in quantity and biochemical composition of sedimentary OM and biochemical composition of *H. tubulosa* feces, along with changes in protein loads of ingested sediment in the different digestive tracts of *H. tubulosa*. More specifically, we posed the following questions: 1) does *H. tubulosa* influence sedimentary OM biochemical composition, and nutritional quality and, thus, the benthic trophic status? 2) does *H. tubulosa* has a specific influence on food availability of sedimentary proteins, supposed to represent the most labile fraction of OM? To answer these questions, we conducted experiments aimed at testing the following null hypotheses: 1) the biochemical composition of sediment OM hosting *H. tubulosa* is not different from that of sea cucumbers feces; 2) the protein loads of the sediment ingested by sea cucumbers do not vary among the digestive tracts and the feces.

Materials and methods

Model species, study sites and sampling strategy

Our study is focused on the sea cucumber *Holothuria tubulosa* Gmelin 1788, one of the most common and most exploited native species across the coastal Mediterranean Sea, where it can be pre-eminently found in organically enriched bottoms and seagrass beds (Tortonese, 1965; Bulteel et al., 1992; Koukouras et al., 2007; Costa et al., 2014; González-Wangüemert et al., 2014; González-Wangüemert et al., 2015; González-Wangüemert et al., 2018; Pasquini et al., 2021; Pasquini et al., 2022). Fishery of *H. tubulosa* is banned by the Italian Government that precautionarily prohibited its harvesting since 2018 (Ministerial decree 156/2018; Pasquini et al., 2021). In other areas of the Mediterranean, such as in Turkey, the fishery of *H. tubulosa* is governed since 2007 (González-Wangüemert et al., 2018; Dereli and Aydın, 2021).

H. tubulosa specimens and sediments were collected from two sites 80 nautical miles apart, in the Gulf of Teulada and the Gulf of Oristano, respectively (Sardinia, W Mediterranean Sea). Sediment grain size was determined by gravimetry after organic matter digestion with H_2O_2 and mesh sieving, using 63, 125, 250, 500, and 1000 µm meshes ICRAM, 2001). The sediments of the Oristano site (39°52'50"N; 8°28'54"E) were characterized by the dominance of sands (63-1000 µm; 75%), followed by mud (<63 µm; 14%) and gravel (>1000 µm; 11%) and by the presence of a nearby offshore mariculture fish farming plant for seabream and shi drum. The

Teulada site (38°55'46"N; 8°43'17"E) was characterized by the dominance of sandy sediments (74%), followed by gravel (24%), and mud (1%), and by the presence of wide *Posidonia oceanica* meadows interspersed with unvegetated sediments (Figure 1).

At both sites, holothuroid and sediment samples were collected by SCUBA divers at depths between 3 and 7 m. In order to include environmental conditions spanning from the worst conditions (in winter, January) to the most favorable ones (in spring and, to a lesser extent, in summer: May, June, July), data were collected in April, June, and August 2019 and January, May, and July 2020. At each site and on each sampling date, three metal frames 60x60 cm (sampling unit n=3) were placed on the sea floor having a single sea cucumber in the central sub-square (20x20 cm). From each sampling unit (60x60 frame), three sediment samples were obtained by scraping ca. 50 mL from the top 2 cm of the sea floor with a Falcon-type. One tube was collected from the central sub-square, in which the sea cucumber occurs, and two from other two random sub-squares (n=3 for each sampling unit), resulting in a total of 9 sediment samples per each site at each sampling date (Moccia et al., 2019) (Supplementary Figure 1). All sea cucumbers (one per each 60x60 cm squared frame, n=3) were placed separately into 3-L plastic bag filled with seawater collected *in situ* and maintained in a cooler box (at *in situ* temperature) during transportation to the laboratory (within 1.5 h from sampling). We notice here that the choice of sampling only one specimen per squared frame, despite the official authorization (see the acknowledgments for more details) was dictated by the need of limiting as much as possible the pressure on a locally protected species. Once in the laboratory, sea cucumbers were singularly placed in a tank filled with *in situ* seawater (15-L) kept at *in situ* temperature and salinity, deprived of sediment. The sea cucumbers feces were collected every 6-8 hours and until the complete evacuation of the digestive tract (usually within 3 days). Feces from each specimen were pooled together to ensure the storage of a sufficient amount of feces for the subsequent biochemical analyses. The sediments and the feces were stored at -20°C until the subsequent analyses.

Additional (2-3) sea cucumber specimens were collected at both sites in June 2019 and used for the analysis of the protein content of the sediment within the digestive tract. Sea cucumbers were weighed (± 0.1 g) before dissection and the sediments present in the intestine esophagus (ESO), mid-gut (MID), and end gut (END) were carefully collected to avoid contaminations from the digestive tissue (Amaro et al., 2010) and stored at -20°C until analysis. As for above, we sampled a low number of experimental individuals to limit as much as possible the pressure on a locally protected species.

Biochemical composition of field sediment, holothuroids' feces, and sediment from the holothuroids' digestive tracts

Protein, carbohydrate, and lipid contents were determined spectrophotometrically (Danovaro, 2010). Proteins were determined according to Hartree (1972), as modified by Lowry et al. (1951) and Rice (1982) using the Folin-Ciocalteau reagent in a basic environment and expressed as bovine serum albumin equivalents. The procedure proposed by Gerchakov and Hatcher (1972), based on the phenol and concentrated sulfuric acid reaction with saccharides, was used to
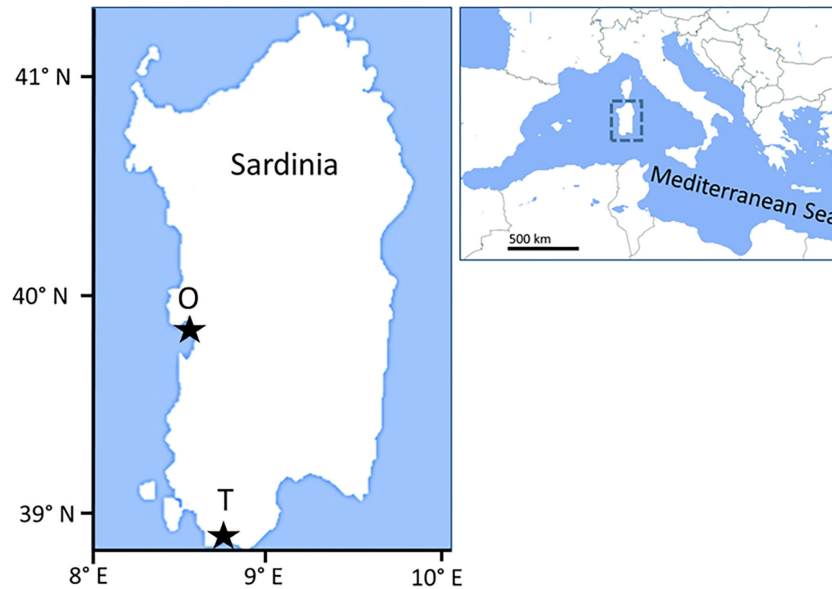
FIGURE 1
Location of the sampling sites in the Oristano (O) and Teulada (T) Gulfs.

determine carbohydrates, then expressed as D (+) Glucose equivalents. Lipids, after extraction in chloroform: methanol (1:1, vol:vol) (Bligh and Dyer, 1959), and evaporation in a dry hot bath at 80 to 100°C for 20 min, were determined after the sulfuric acid carbonization procedure (Marsh and Weinstein, 1966) and expressed as tripalmitin equivalents. For each biochemical assay, blanks were obtained using pre-calcinated sediments (450°C for 4 h). Protein, carbohydrate, and lipid concentrations were converted into C equivalents using the conversion factors 0.49, 0.40, and 0.75 mgC mg^{-1}, respectively, obtained from the C contents of the respective standard molecules (albumin, glucose and tripalmitin, respectively), and their sum was reported as the biopolymeric C (BPC) (Fabiano et al., 1995).

The percentage of proteins potential digestion was calculated as follows:

$$PD = 100 \ \times \ \frac{ESO\ PRT - END\ PRT}{ESO\ PRT}$$

where:

PD = potential proteins' digestion

ESO PRT= protein content (mg g^{-1}) in the sediment in the esophagus

END PRT= protein content (mg g^{-1}) in the sediment in the end gut

Chloroplastic pigments (chlorophyll-a and phaeopigments) in the ambient sediment were analysed fluorometrically according to Lorenzen and Jeffrey (1980). Pigments were extracted with 90% acetone (24 h in the dark at 4°C). After centrifugation (800 × g), the supernatant was used to determine the chlorophyll-a and, after acidification with 0.1 N HCl, to estimate the phaeopigments (Danovaro, 2010). The total algal C contribution (sum of chlorophyll-a and phaeopigments) to BPC was calculated as the percentage of phytopigment-to-BPC concentrations after converting

the total phytopigment concentrations into C equivalents using a mean value of 40 mgC mg^{-1} (Pusceddu et al., 2010). Although the C: Chla can vary from 10 to 100 (on average 35 for phytoplankton) (Cloern et al., 1995), we used the conversion factor proposed in Pusceddu et al. (2010) to allow comparability with other studies carried out in a variety of other shallow coastal aquatic environments (Pusceddu et al., 2009).

Statistical analyses

Differences in quantity and biochemical composition (protein, carbohydrates, lipids, chlorophyll-a, phaeopigments) of ambient sediments were investigated using Site (Si; 2 fixed levels: Oristano vs. Teulada) and Month (Mo; 6 fixed levels: April, June, August 2019, and January, May, July 2020) as orthogonal sources of variance, with n=9 for the combination of factors. Differences in OM quantity and biochemical composition in terms of protein, carbohydrates, lipids between sediment and holothuroid feces were investigated separately for the two sites (Oristano and Teulada) using Matrix (Ma; 2 fixed levels: sediment vs. feces) and Month (Mo; 6 fixed levels: April, June, August 2019, and January, May, July 2020) as orthogonal sources of variance. In both designs, as any sampled sediment portion around *H. tubulosa* specimens could have been visited by holothuroids before sampling, we chose to consider as independent replicates of ambient sediments either sediments without or with (in the center of the large frame) holothuroids. This choice generated an unbalanced design, which biases are, however, counterbalanced by the use of PERMANOVA, which is extended to accommodate, apart random effects, hierarchical models, mixed models, quantitative covariates, repeated measures, also unbalanced and/or asymmetrical designs (Anderson, 2017). Differences in protein content among sediment, sediment in the gut (three sectors: esophagus, mid gut, and end gut)

and feces were assessed by means of univariate PERMANOVA tests using the matrix (5 fixed levels: ambient sediment, esophagus, mid gut, end gut and feces) as the sole source of variation.

In all designs, differences were assessed with permutational analyses of variance (PERMANOVA), in both univariate and multivariate contexts. PERMANOVA is a semiparametric method described as a geometric partitioning of multivariate variation in the space of a chosen dissimilarity measure according to a given ANOVA design, with p-values obtained using appropriate distribution-free permutation techniques (Anderson, 2017). PERMANOVA on one response variable using Euclidean distance yields the classical univariate F statistic, so that it can also be used to do univariate ANOVA, but where p values are obtained by permutation (Anderson and Millar, 2004), thus avoiding the assumption of normality (Anderson, 2017). PERMANOVA tests were carried out on Euclidean distance-based resemblance matrixes of normalized data, using 999 random permutations of the appropriate units. When significant differences were observed, pairwise tests were also carried out to ascertain patterns of differences among treatments and/or sampling times.

Multivariate differences in sedimentary OM biochemical composition (in terms of protein, carbohydrate, lipid and phytopigment contents) between sites and months were visualized with a biplot after a canonical analysis of the principal coordinates (CAP) (Anderson and Willis, 2003). CAP allows identifying an axis through the multivariate cloud of points that is best at separating *a priori* groups. The motivation for the CAP routine arose as sometimes there are real differences among *a priori* groups in multivariate space that cannot be easily seen in an unconstrained ordination (e.g., PCA or MDS plots; Anderson and Gorley, 2008). All the statistical analyses were performed using the routines included in the PRIMER 7+ software (Anderson et al., 2008).

Results

Quantity and biochemical composition of organic matter in field sediments

Overall, contents of all organic compounds, but carbohydrates, differed significantly between sites (Supplementary Table 1), with values in the muddy organically enriched sediments at Oristano significantly higher (3-5 times) than those in the sandy poorer sediments at Teulada (Supplementary Figure 2). In both sites, significant temporal variations, though not consistent for the different classes of organic compounds, and significant effects of the Si×Mo interaction occurred only for protein, lipid, and chlorophyll-*a* contents. Results of the pairwise tests conducted to ascertain temporal differences are reported in Supplementary Table 2 and illustrated in Supplementary Figure 2.

In organically enriched sediments at Oristano, seasonal differences in the sedimentary protein contents varied between years: in 2019 values in spring (April) and early summer (June) were generally lower (1.5-2.9 times) than those in all other months, whereas in 2020 values in May were ca. 1.4 times higher than those in July. The lipid contents in this site, were significantly higher (3.5 – 5.4 times) in April 2019 than those in all other months, both in 2019 and

2020. The chlorophyll-*a* content of Oristano sediments in April was 1.7 times higher than those in January 2020 and 1.5 times lower than those in May 2020. Moreover, chlorophyll-*a* contents of Oristano sediments in June 2019 and January 2020 were significantly lower (1.5-3 times) than those in August 2019, May 2020, and July 2020 (Supplementary Figure 2).

In the poorer Teulada sediments, the sedimentary protein contents varied significantly among sampling periods, with values in summer months (June, August) ca. 3-10 times higher than those in spring-winter ones (April, January), respectively of the study year. The lowest sedimentary lipid content was observed in spring 2019 (April), when values were 1.8 times lower than those in January 2020 and 6.5 times lower than those in June 2019. Lipid content in June 2019 was also significantly higher (ca. 3.3 times) than that in August 2019. Temporal variability of the chlorophyll-*a* contents in the poorer Teulada sediments was much less pronounced than in that in the organically enriched sediments in Oristano, with values in Summer 2019 (August) 2.5 times higher than those in Spring (April 2019) and Winter (January 2020). Phaeopigment contents differed significantly only between sites, with values in the richer Oristano sediments up to 7.7 times higher than those in the poorer Teulada ones (Supplementary Figure 2).

The biochemical composition of sediments differed spatially and temporally, as found in sedimentary OM levels (Supplementary Table 3). The pairwise tests revealed that significant differences occurred among sites in all the sampling months, but May and June (Supplementary Table 4A) and that in both sites the biochemical composition of sedimentary OM varied with time. More specifically, in the organically enriched sediments in Oristano proteins (46 – 52%) were the dominant class of organic compounds followed by carbohydrates (34 – 40%) and lipids (9 – 14%) in almost all samplings, with exceptions in April and June 2019, when carbohydrates were dominant class (54-56%) followed by lipids and proteins (April 2019, 32% and 11%, respectively; June 2019, 11% and 35%, respectively) (Supplementary Figure 3).

The biochemical composition of sediments from the poorer Teulada site was more variable (Figure 2) and significant differences among sampling occasions were observed between January 2020 *vs* April, June, and August 2019; and between April *vs* August 2019 (Supplementary Table 4B). Sediments collected in 2019 and in July 2020 were characterized by the dominance of carbohydrates (45 – 61%), followed by proteins (28 – 42%) and lipids (10 -17%); sediments collected in January (2020) showed a dominance of proteins (39%) and similar contributions of lipids and carbohydrates (32 and 29% respectively). The sediments collected in May 2020 were characterized by the dominance of proteins (46%), followed by carbohydrates (41%) and lipids (13%) (Supplementary Figure 3).

Effect of sea cucumbers on quantity and composition of the sedimentary organic matter

The effect of the sea cucumbers' feeding was considered separately for each site. In the organically enriched Oristano site (putatively impacted by mariculture effluents), protein contents were affected by a significant effect of the Mo×Ma interaction (Table 1A). More
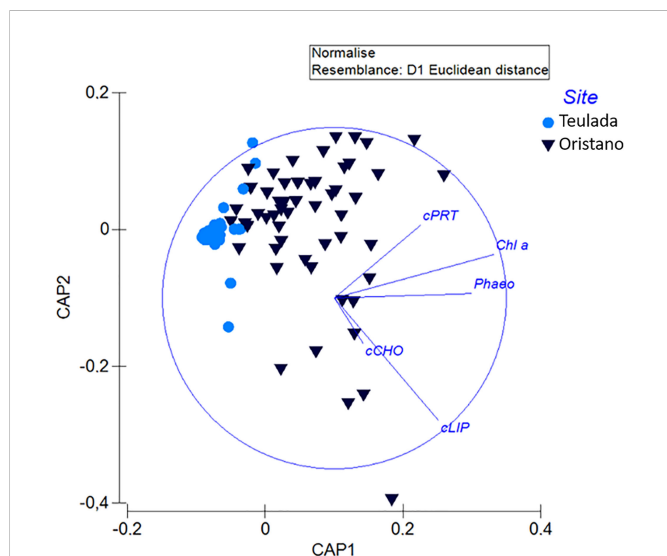
FIGURE 2
The biplot produced after canonical analysis of the principal coordinates (CAP) showing variations in the biochemical composition of sedimentary organic matter (chlorophyll: Chl a; phaeopigments: Phaeo; and proteins: cPRT; carbohydrates: cCHO; lipids: cLIP, converted in carbon equivalent) between the organically enriched site of Oristano and the poorer site of Teulada.

sediments) no significant differences between the sediment and the feces were observed (Table 1B; Figure 3B), but differences occurred only among months (Supplementary Table 5). Carbohydrate content of sediment and feces did not vary between the two matrixes in each of the sampling dates in both sites (Tables 1A, B and Figures 3C, D).

In the organically enriched Oristano site the lipid content was affected by a significant Mo×Ma interaction (Table 1A). In more details, the results of the *post-hoc* tests revealed that feces were strongly depleted (-83%) in lipids when compared with the surrounding sediment only in April 2019, whereas in all other sampling months, lipid contents in the feces were significantly higher (by 48 - 67%) than those in the surrounding sediment, with the exception for January 2020, when no significant differences were observed (Table 2 and Figure 3E). In the poorer Teulada site, the lipid content did not vary significantly between the matrices in each of the sampling dates (Table 1B and Figure 3F). The biopolymeric C contents were affected by a significant Mo×Ma interaction only in the organically enriched Oristano site (Table 1A), where the *post-hoc* test revealed that differences in biopolymeric contents between sediments and feces were significant only in summer 2020 (July), with values in feces 2 times higher than those in the sediment (Table 2 and Figure 3G). In the poorer Teulada site biopolymeric C contents did not vary among matrices in any of the sampling periods (Table 1B and Figure 3H).

The biochemical composition of OM was affected by a significant interaction of the factors Mo×Ma only in the organically enriched Oristano site (Table 3A). More in details, differences in OM composition between sediment and feces in Oristano occurred only in April 2019, when carbohydrate and protein contributions to

specifically, the results of the *post-hoc* tests revealed that the protein content of feces was significantly higher than that in the surrounding sediment in June 2019 (2.4 times) and in July 2020 (1.9 times) (Table 2; Figure 3A). In the poorer Teulada site (mariculture-free

TABLE 1 Results of univariate PERMANOVA testing for the effects of Months (Mo) and Matrices (Ma) and their interaction on protein, carbohydrate, lipid and BPC contents in sediments and feces from the two sites of Oristano (A) and Teulada (B).

(A) Oristano		Proteins				Carbohydrates				Lipids				Biopolymeric C (BPC)			
Source	DF	MS	Pseudo-F	P (MC)	% EV	MS	Pseudo-F	P (MC)	% EV	MS	Pseudo-F	P (MC)	% EV	MS	Pseudo-F	P (MC)	% EV
Month (Mo)	5	4.813	9.761	***	32	1.047	1.141	ns	2	1.175	2.394	ns	5	0.338	0.396	ns	0
Matrix (Ma)	1	8.915	18.080	***	21	0.002	0.003	ns	0	1.561	3.180	ns	3	1.706	2.000	ns	3
Mo × Ma	5	1.382	2.802	*	13	0.688	0.750	ns	0	4.150	8.454	***	57	2.432	2.850	*	28
Residual	60	0.493			33	0.918			98	0.491			35	0.853			69
(B) Teulada		Proteins				Carbohydrates				Lipids				Biopolymeric C (BPC)			
Source	DF	MS	Pseudo-F	P (MC)	% EV	MS	Pseudo-F	P (MC)	% EV	MS	Pseudo-F	P (MC)	% EV	MS	Pseudo-F	P (MC)	% EV
Month (Mo)	5	2.105	2.509	*	13	1.477	1.484	ns	5	1.746	1.910	ns	9	1.661	1.749	ns	8
Matrix (Ma)	1	1.821	2.170	ns	3	0.913	0.918	ns	0	2.783	3.046	ns	7	1.308	1.377	ns	1
Mo × Ma	5	1.364	1.626	ns	11	0.115	0.116	ns	0	0.801	0.877	ns	0	0.297	0.313	ns	0
Residual	60	0.839			73	0.995			95	0.914			84	0.950			91

DF, degree of freedom; MS , mean squares; Pseudo-F, F; P(MC), probability level after Monte Carlo simulations. *P< 0.05; **P< 0.01; ***P< 0.001; ns, P>0.05; EV, % of explained variance.

TABLE 2 Results of the pairwise comparisons contrasting proteins and lipid contents between sediment and feces in the meso-eutrophic site (Oristano) in all sampling dates.

Site	Month	Proteins		Lipids		Biopolymeric C (BPC)	
		t	P(MC)	t	P(MC)	t	P(MC)
Oristano	Apr	1.003	ns	3.092	**	1.728	ns
	Jun	6.783	***	3.258	*	0.794	ns
	Aug	0.930	ns	3.282	**	1.962	ns
	Jan	2.150	ns	0.987	ns	1.503	ns
	May	0.178	ns	2.788	*	0.698	ns
	Jul	4.022	**	2.490	*	3.319	**

t, T value, P(MC), probability level after Monte Carlo simulations. *P< 0.05; **P< 0.01; ***P< 0.001; ns, not significant.

biopolymeric C were higher in feces than in the sediments, at the expense of lipids, and in Jul-Aug 2020 because of a higher lipid contribution in feces, at the expenses of proteins (Table 3B; Figure 4A). In the poorer Teulada, the biochemical composition did not vary between sediment and feces in all sampling periods (Table 3B; Figure 4B).

Proteins quantity in food sources, holothuroids' digestive tracts and feces

The results revealed that the protein content differed significantly among the different matrices (ambient sediment, digestive tracts, and feces) only in the organically enriched Oristano site (Table 4A), where

FIGURE 3
Protein (A, B), carbohydrate (C, D), lipid (E, F), and BPC (G, H) contents in sediment and feces from Oristano (organically enriched site) and Teulada (poorer site) during the study period. Error bars indicate standard error (sediments n=9; feces n=3).
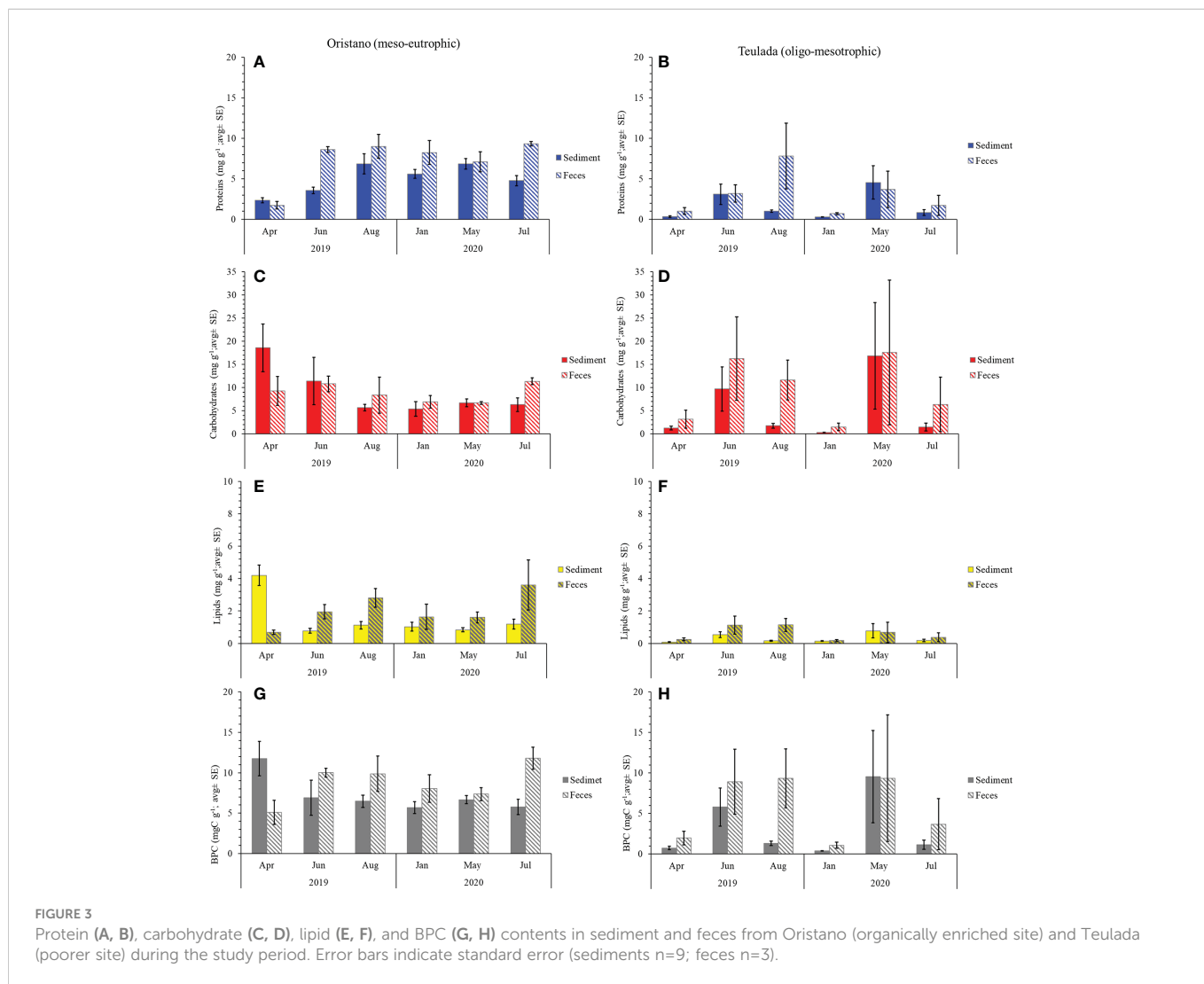
TABLE 3 Results of multivariate PERMANOVA testing for the effects of Month, Matrices and their interactions on the biochemical composition of OM in two study sites (A); and results of the pairwise comparisons testing for differences in the biochemical composition of OM between sediment and feces in the meso-eutrophic site (Oristano) during the study period (B).

(A)	Site	Source	DF	MS	Pseudo-F	P(MC)	%EV
	Oristano	Month (Mo)	5	7.035	3.699	**	15
		Matrix (Ma)	1	10.478	5.510	**	8
		Mo × Ma	5	6.220	3.271	***	26
		Residual	60	1.902			51
	Teulada	Month (Mo)	5	5.327	1.939	ns	9
		Matrix (Ma)	1	5.517	2.008	ns	3
		Mo × Ma	5	2.280	0.830	ns	2
		Residual	60	2.748			87
(B)	Site		Month	t	P(MC)		
	Oristano		Apr	1.943	*		
			Jun	1.768	ns		
			Aug	1.492	ns		
			Jan	1.512	ns		
			May	0.959	ns		
			Jul	2.869	**		

DF, degree of freedom; MS, mean squares; Pseudo-F, F; P(MC), probability level after Monte Carlo simulations. *P< 0.05; **P< 0.01; ***P<0.001; ns, P>0.05; EV, % of explained variance, t, T value.

proteins' content of sediment in the esophagus was ca. 2 times higher than that in the ambient sediment, and slightly decreased along the other digestive tracts (Table 4B; Figure 5). The estimated digestion rate of proteins along the digestive tracts of *H. tubulosa* (Figure 5A) was slightly higher (40%) in specimens from the poorer Teulada site than that in specimens from the organically enriched Oristano site (30%). As a result, the amounts of proteins accumulated in the esophagus in specimens from Oristano are so high that feces are enriched in proteins when compared to the ambient sediment, whereas in the poorer Teulada site feces have protein contents similar to those measured in the ambient sediment (Figures 5B, C).

Discussion

Trophic status of ambient sediments

We report here that, as expected, the two investigated sites were characterized by well-delineated differences in benthic trophic status, as assessed in terms of quantity and composition of sedimentary OM. The sandy-mud sediments of the Oristano site, influenced by putative organic inputs released by a close mariculture plant, were in fact characterized by biopolymeric C contents (dominated by proteins) generally > 3 mgC g^{-1} and by a limited temporal variability of either quantity or composition of sedimentary OM. The sandy sediments of the Teulada site were characterized by biopolymeric C contents varying from<1 mgC g^{-1} in Winter and early Spring to 1-3 (or higher) mgCg^{-1} in Summer, and a much wider temporal variability

of OM biochemical composition (though generally characterized by the dominance of carbohydrates). Overall, chlorophyll-*a* contents and the algal contribution to biopolymeric C (estimated as the percentage fraction of total phytopigments transformed in C equivalents over biopolymeric C contents) were higher in the Oristano than in the Teulada sediments. Meso-eutrophic sediments are typically characterized by biopolymeric C contents > 3 mgC g^{-1} (Pusceddu et al., 2011) and a general dominance of proteins over carbohydrates (Pusceddu et al., 2009; Pusceddu et al., 2011), which makes them a more labile source of food for benthic (especially deposit-feeders) consumers (Pusceddu et al., 2009). According to this, we can rank the sediments from Oristano as meso-eutrophic, and those from Teulada as oligo-mesotrophic. Moreover, the contribution of total phytopigments (as a proxy of OM of primary origin) to biopolymeric C was slightly higher in Oristano (ca. 14 ± 4%) than in Teulada (ca. 10 ± 4%). Since the higher the algal fraction of biopolymeric C, the higher the lability of sediment OM for benthic consumers (Pusceddu et al., 2003), altogether our results indicate that the two sites offered totally different trophic conditions (i.e., food availability levels) to the holothuroids.

Effects of *H. tubulosa* on the benthic trophic status

Deposit-feeders sea cucumbers are generally associated with soft organic-rich bottoms, where they rework the upper sediment layers feeding on the most readily utilizable OM (Yingst, 1982; Roberts et al.,
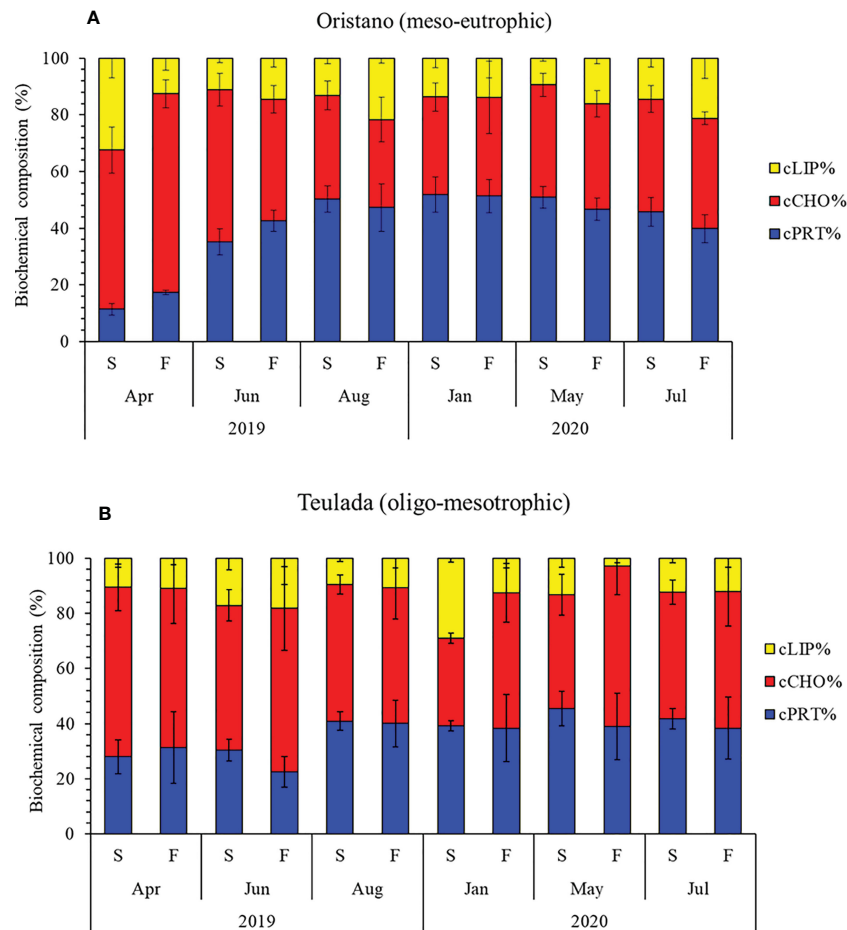
FIGURE 4
Variations in the percentage contribution of proteins, carbohydrates, and lipids to the BPC in sediments (S) and feces (F) in the organically enriched site of Oristano **(A)** and in the poorer site of Teulada **(B)**. Error bars indicate the standard error (sediments n=9; feces n=3). cLIP, lipid; cCHO, carbohydrate; cPRT, protein.

2000; Purcell et al., 2016). The studies conducted so far on the feeding behavior of coastal sea cucumbers investigated the fate of total organic matter (TOM, as obtained by the loss on ignition technique) and/or total organic carbon (TOC), without considering the biochemical composition and the biopolymeric C content of the sediments. Both TOM and TOC include any organic molecule and do not allow a distinction between labile (i.e., nutritionally available) vs. refractory fractions of OM (Pusceddu et al., 2009). In this regard, most recently the biochemical gross composition of sedimentary OM (in terms of protein, carbohydrate, and lipid contents) has been used as a proxy of OM availability for benthic consumers (Danovaro et al., 2001; Gambi et al., 2014; Gambi et al., 2017).

To shed light on the potential role of *H. tubulosa* on the benthic trophic status, we investigated differences in quantity and biochemical composition of ambient sediments and sea cucumbers' feces in two distinct sites characterized by the above-described different background trophic status. Although the analysis refers to a relatively limited number of individuals in each sampling unit, our data suggest that the feces produced by *H. tubulosa* feeding on sediment from the less productive "oligo-mesotrophic" site showed

the same OM contents as the ambient sediment. Instead, sea cucumbers feeding on richer (meso-eutrophic) sediments (Oristano) produced, especially in Summer, feces with OM contents significantly higher than those of the surrounding sediment. These results are consistent with previous studies conducted by comparing when OM contents in sediment and feces of other holothuroids (Mercier et al., 1999). These results are apparently in contrast with those from previous studies showing that holothuroids feeding can abate OM sedimentary contents on long time scales (Paltzat et al., 2008; Slater and Carton, 2009; MacDonald et al., 2013; Neofitou et al., 2019). At a first glance, such a discrepancy could be related to the different proxy of OM quantity used in this study (biopolymeric C) and that (TOM) used in previous studies. Although we did not estimate TOM contents in the sediment and the feces, such discrepancy suggests that *H. tubulosa* consumes TOM, but releases feces enriched in biopolymeric compounds. Since biopolymeric C is assumed to represent the most labile fraction of OM, our results suggest that other mechanisms, besides nutrition and assimilation, could be responsible for increasing BPC contents in feces. For instance, based on the results of previous studies which identified
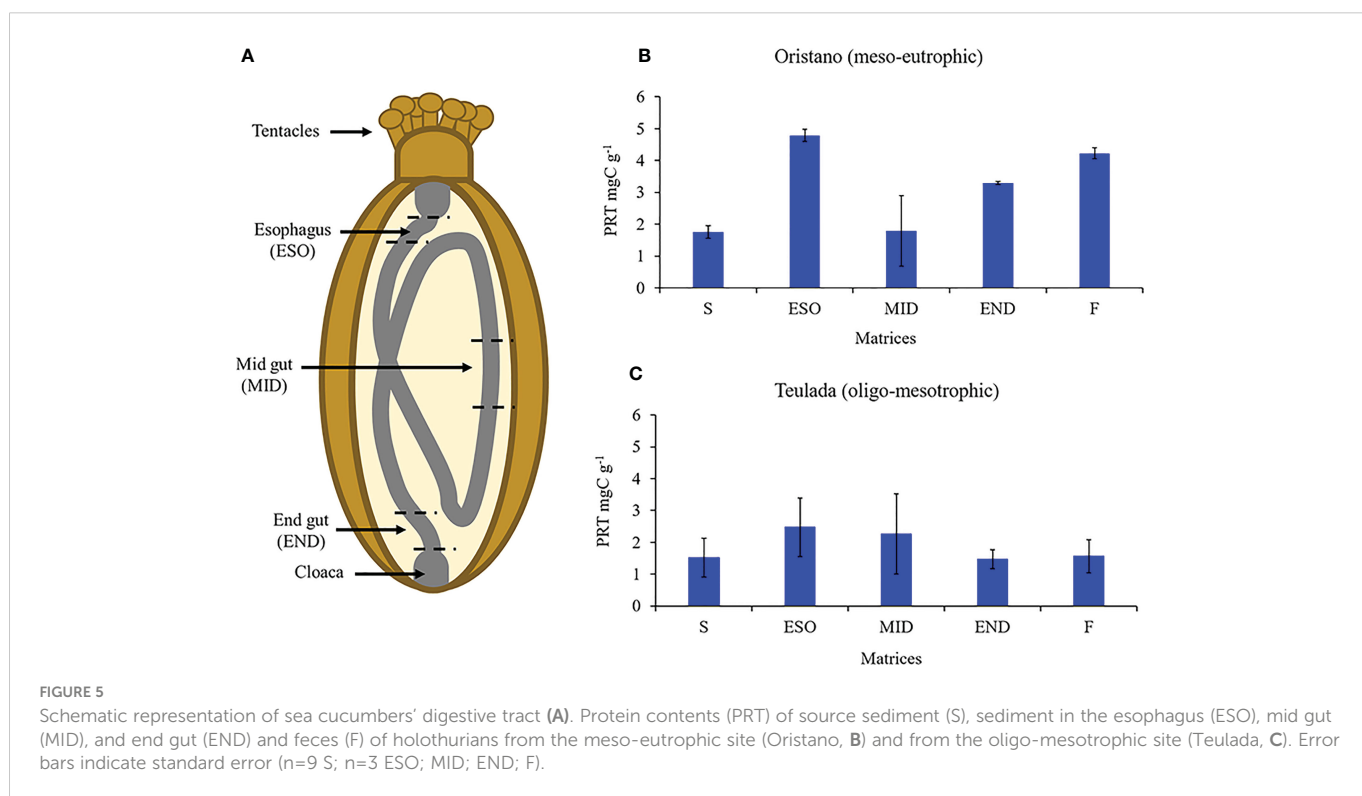
TABLE 4 Results of the univariate PERMANOVA testing differences in the protein content of the source sediment, digestive tract and feces of *H. tubulosa* in the two study sites (A); and results of the pairwise comparison testing for difference in protein contents in the source sediment (S), digestive tracts (esophagus = ESO; mid gut = MID; end gut = END) and feces (F) in the meso-eutrophic site (Oristano) (B).

(A)	Site	Source	DF	MS	Pseudo-F	P(MC)	%EV
	Oristano	Matrix (Ma)	4	3.512	15.458	***	82
		Residual	13	0.227			16
	Teulada	Matrix (Ma)	4	1.077	0.269	ns	0
		Residual	13	15.923			100

(B)	Site	Contrast	t	P(MC)		
	Oristano	S, F	6.783	***		
		S, ESO	6.860	***		
		S, MID	0.061	ns		
		S, END	3.537	**		
		F, ESO	2.100	ns		
		F, MID	2.862	ns		
		F, END	4.063	*		
		ESO, MID	2.682	ns		
		ESO, END	7.475	*		
		MID, END	1.369	ns		

DF, degrees of freedom; MS, mean square; Pseudo-F, F statistic; P(MC), probability level after Monte Carlo simulations; %EV, percentage of explained variation; ***P< 0.001; ns, not significant; t , t value. *P< 0.05; **P< 0.01.

meiofauna as a food source for holothuroids (Wolfe and Byrne, 2017; Wolfe et al., 2018; Wolfe et al., 2021), we cannot exclude that some of the observed patterns could be also due to the different intake of meiofauna in the two different environmental assets. Moreover, we have to consider that seasonal variations in the intake of organic C by holothuroids, caused, for instance, by aestivation in Summer and hibernation in Winter as observed in other temperate species (e.g., *A. japonicus*; Ji et al., 2008; Yu et al., 2014) could cause variations in their

FIGURE 5
Schematic representation of sea cucumbers' digestive tract **(A)**. Protein contents (PRT) of source sediment (S), sediment in the esophagus (ESO), mid gut (MID), and end gut (END) and feces (F) of holothurians from the meso-eutrophic site (Oristano, **B**) and from the oligo-mesotrophic site (Teulada, **C**). Error bars indicate standard error (n=9 S; n=3 ESO; MID; END; F).

behavior and effects on sedimentary OM. Nevertheless, nor previous studies (Günay et al., 2019) nor our study have observed this in *H. tubulosa*.

We notice also that the overall increase in BPC contents of feces produced by holothuroids feeding on OM-rich sediments (Oristano), was accompanied by an increase of the most labile components of OM (i.e., proteins and lipids). Since both proteins and lipids are high-energy compounds putatively used by holothuroids for their somatic growth and gonad development, respectively (Yang et al., 2006; Slater et al., 2011b), their enrichment in the feces appears counterintuitive. Whatever the potential explanations (see below), this result first indicates that feces released by holothuroids during their feeding can enhance locally and temporarily the nutritional quality of the ambient sediment. Since this enrichment process is not detected in the more oligotrophic site, we contend that the efficacy of sea cucumbers as bioreactors (whatever the effect on the sediment trophic status) is most likely modulated by the actual quantity and nutritional quality of the sedimentary OM.

Fate of proteins ingested by *H. tubulosa*: Role of food particle selection and estimate of potential assimilation of proteins

As delineated above, the protein content in *H. tubulosa* feces collected from the meso-eutrophic site (Oristano) is higher than that in the ambient sediment. This apparently counterintuitive result could be caused by the peculiar feeding behavior of the sea cucumbers and, to shed light on the mechanism behind such a phenomenon, we compared protein contents in ambient sediment with those in sediment remains within the holothuroids' gut and feces from the two sites. The protein content in the esophagus of *H. tubulosa* is significantly higher than that in the source sediment only in specimens collected from the meso-eutrophic site (Oristano), suggesting, in this trophic asset, the presence of a possible accumulation or a concentration mechanism of proteins in the esophagus. This result is consistent with the results reported on the protein contents of the deep-sea holothuroid *Molpadia musculus* gut, in which the concentration factor was estimated to be 3.1 (Amaro et al., 2010). Other studies, based on total N or total OM contents reported a similar concentration mechanism in *Australostichopus mollis* (Slater et al., 2011a, b; Zamora and Jeffs, 2011) and *Parastichopus californicus* (Paltzat et al., 2008). The increase in proteins (or total OM) contents in sediment remains retrieved from the esophagus can be explained by the presence of a selection mechanism of protein-rich particles ingested by holothuroids. Several studies have indeed proven that holothuroids, rather than simply packing up sediment particles with mucus and digestive fluids, can select, possibly by chemo-selection mechanisms, food particles with their tentacles (Uthicke and Karez, 1999; Roberts et al., 2000; Dar and Ahmad, 2006; Paltzat et al., 2008; Slater et al., 2011a; Navarro et al., 2013; Ramón et al., 2019; Hartati et al., 2020). However, this hypothesis is not supported everywhere as, in our study, the concentration mechanism was reported only for holothuroids from the OM-enriched sediments (Oristano). Nevertheless, we must notice that the protein and lipid enrichment of gut sediments and feces of holothuroids, when compared to ambient sediments, could be also the

result of the presence of mucus coating the feces before their release (Mercier et al., 1999; Slater et al., 2011b). Based on our sampling approach, we cannot exclude this, but we notice here that such enrichment is documented only from OM-enriched sediments, giving room to consider this process the result of mixed effects associated with either the physiology of feces production or with the process of food particles selection.

The apparently different behavior of holothuroids feeding on sediments with different OM contents could be related to the different sediment grain size and organic nutritional quality observed in the two sites. Sediments from the meso-eutrophic site (Oristano) show a fraction of mud and fine sand, including the particles typically selected with buccal tentacles (Mezali and Soualili, 2013), higher than that in the oligo-mesotrophic site (Teulada). Moreover, we report here also that the estimated protein assimilation rate of holothuroids from the oligo-mesotrophic site (ca. 40%) is higher than that (ca. 30%) of holothuroids from the meso-eutrophic one. This result fits the known inverse relationship between assimilation rates and availability of organic substrates (Roberts et al., 2000; Zamora and Jeffs, 2011) and the optimal foraging theory, which foresees that consumers tend to optimize energy intake by means of a trade-off between the quantity of energy necessary for survival and that spent to acquire it (Stephens and Krebs, 1986). Our contention, indeed, corroborated by the finding of higher defecation rates of the holothuroid *Thelenota anax* in sediments with lower organic matter levels (Hammond et al., 2020), suggests that feces produced by holothuroids in oligo-mesotrophic bottoms are poorer in protein contents than those from meso-eutrophic sediments because of their larger needs of accumulating energy from a poorer food source. Although on a merely speculative basis, due to the restricted number of specimens considered here, our results support the hypothesis that the sea cucumber *H. tubulosa* can select food particles from the surrounding sediment and concentrate them in the esophagus, but also that the potential assimilation can be strongly dependent upon the relative amount of available food and its nutritional quality.

Protecting and harnessing sea cucumbers' environmental services

With an eye to the possible use of sea cucumbers as a bioremediation tool of eutrophicated sediments, since proteins are N-rich compounds, and N is the most limiting factor for heterotrophic nutrition (Dell'Anno et al., 2002; Pusceddu et al., 2009), we conclude that the availability of protein-enriched feces could foster and facilitate the transfer of energy towards higher trophic levels, thus limiting benthic eutrophication (i.e., the accumulation of huge amounts of labile organic C in sediments below fish cages; Pusceddu et al., 2007). In this sense, *H. tubulosa*, by accelerating the detritus cycling, especially in OM-enriched sea floors, could be also potentially used to condition the benthic trophic status for additional components (including reared species, such as small scavenging crustaceans) of the 'manipulated' food webs in Integrated Multi-Trophic Aquaculture (IMTA) (Neofitou et al., 2019). Encouraging results from recent studies on breeding Mediterranean holothuroids, including *H. tubulosa*, pave the way for the possibility of using captive-bred specimens as tools for

bioremediation and IMTA purposes, at the same time limiting the exploitation of this protected species (Rakaj et al., 2018; Pasquini, 2022).

Conclusions

H. tubulosa is a "continuous" feeder that can ingest and rework large amounts of sediment and can have a role on the recycling of seagrass detritus in *Posidonia oceanica* meadows (Costa et al., 2014; Boncagni et al., 2019). Here we showed that the sea cucumber *H. tubulosa* can influence the benthic trophic status of marine sediments, specifically by modifying the quantity, biochemical composition, and nutritional quality of sedimentary OM. Our results proved also that *H. tubulosa* influences particularly the sedimentary contents of the most labile molecules (i.e., proteins and lipids) and that, by producing protein- and lipid-enriched feces (especially in meso-eutrophic assets), can contribute making labile substrates more available for higher trophic levels.

Holothuroid fisheries had rapidly grown and expanded in the last three decades and large market demand for these animals has led to overfishing in worldwide oceans and seas, including the Mediterranean. Since our results have shown that holothuroids play an important role in conditioning sediment OM content, possibly interacting with species from different trophic levels, we anticipate that their overfishing, besides posing their populations at risk, might have severe consequences on the structure of benthic communities and ecosystem functioning.

Based on the changes in protein concentration of sediment within the digestive tract we also provided elements in support of the hypothesis of an active selection of labile food particles by holothuroids and that this mechanism could be dependent upon sediment grain size and the actual bioavailability of organic substrates. Finally, based on our results, we conclude that, since the 'bioreactor' efficacy of *H. tubulosa* can vary according to the OM quantity biochemical composition and nutritional quality of the ambient sediment, their use in Integrated Multi-trophic Aquaculture and bioremediation actions should be modulated accordingly.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

VP, AP, and PA conceived and planned the experiments. VP, AG and DM carried out the sampling and laboratory analyses, VP and AP carried out the statistical analyses. All authors contributed to the interpretation of the results. VP and PA took the lead in writing the manuscript followed by extensive revision by AP to achieve the final version. All authors provided critical feedback and helped shape the research, analyses, and manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1010014/full#supplementary-material

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