



The sea cucumber *Holothuria tubulosa* does not reduce the size of microplastics but enhances their resuspension in the water column

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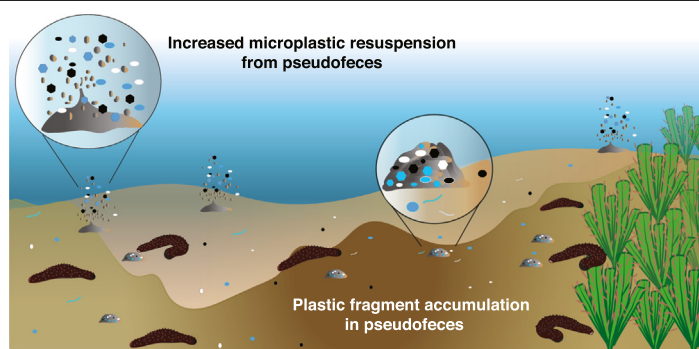
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HIGHLIGHTS

- Ingestion-egestion by *Holothuria tubulosa* does not alter microplastic size.
- *Holothuria tubulosa* pseudofeces are hotspots of microplastic concentration.
- Microplastic resuspension rates are higher from pseudofeces than sediments.
- *Holothuria tubulosa* sustains microplastic bioavailability.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic pollution is increasingly recognized as a prominent threat to marine life. Understanding the role of bioturbators is crucial to determine to what extent marine sediments can act as a microplastic sink. The presence of microplastics has been documented in holothurians, but no study has investigated how the ingestion-egestion process influences their bioavailability. Using the Mediterranean deposit-feeder, *Holothuria tubulosa*, as a model system, we assessed if, upon ingestion, plastic particles are accumulated in pseudofeces and if the passage through the digestive tract reduces their size. To this end, the number, shape and colour of plastic particles was compared between pseudofeces and surrounding surficial sediments collected along the edges of a seagrass meadow. Pseudofeces were enriched in plastic fragments with respect to surficial sediments, suggesting a selective ingestion of fragments over fibres. By contrast, there was no difference in the size or colour of plastic particles between pseudofeces and sediments. In addition, by means of a laboratory experiment, we evaluated how microplastic resuspension rates from pseudofeces compares with those from surficial sediments. Under standard water movement conditions, the resuspension of labelled microplastics from pseudofeces was much greater than that from sediments (i.e., about 92% and 26% at the end of the experimental trial). Greater relative abundance of fine material (i.e., pelite) in pseudofeces than sediments could explain their physical instability and, hence, their lower microplastic retention. Our results suggest that pseudofeces of *H. tubulosa* not only represent a hotspot for plastic fragment concentration, but, due to their surficial deposition and rapid dissolution, they could also promote their transfer to the water column. Ingestion and egestion of microplastics by this sea cucumber, although not altering their size, may thus enhance their bioavailability.

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1. Introduction

Plastic litter is ubiquitous across the global oceans (Thompson et al., 2004; Cole et al., 2011; Jambeck et al., 2015). Despite the broad recognition of plastic pollution as a plague of the Anthropocene (Galloway et al., 2017), inputs into the oceans were in the range of 4.8 to 12.7 million metric tonnes (Mt) year⁻¹ in 2010 (Jambeck et al., 2015) and set to rise as a consequence of the escalation in plastic production (Worm et al., 2017). Whether the effects of plastic litter on marine life have been investigated since the 1970s (Carpenter and Smith, 1972), microplastic pollution has emerged as a prominent environmental and human health concern in the last two decades (Thompson et al., 2004; Browne et al., 2007). Mesoplastic (5–200 mm) and microplastic (1 µm–5 mm) particles can be produced as such for manufacturing plastic goods or ingredients in cosmetic products (i.e., facial scrubs and toothpaste), but also originate from the biotic or physical breakdown of plastic litter and the release of fibres from clothes and textiles during washing (Browne et al., 2011; Worm et al., 2017).

A plethora of studies has shown that microplastics can be ingested by pelagic and benthic organisms, spanning a variety of feeding modes, i.e. planktivorous, detritivores, filter-, suspension- and deposit-feeders and taxonomic groups, such as mammals, fish, gastropods, echinoderms, porifera, crustaceans and polychaetes (Wright et al., 2013; Besseling et al., 2015; Setälä et al., 2016; Bour et al., 2018; Piarulli et al., 2020). Leaching of chemicals, release of contaminants adhered on their surfaces (POP) and physical impairment of digestion organs from ingested plastics can reduce the survival, growth and reproductive outcome of marine biota (Andrady, 2011; Besseling et al., 2013; Worm et al., 2017).

While high density plastic fragments tend to precipitate spontaneously, lower density particles can sink to the bottom through different pathways, including biofouling and marine snow (Wright et al., 2013). The presence of microplastics has been documented in sediments worldwide, with abundances increasing in densely populated areas (Thompson et al., 2004; Van Cauwenberghe et al., 2015; Alomar et al., 2016; Ling et al., 2017; Martin et al., 2017). Sediments may act as a long-term sink for microplastics (Cózar et al., 2014; Woodall et al., 2014; Näkki et al., 2019), although abiotic and biotic processes could regulate the redistribution of particles across sediment layers and their bioavailability. For instance, ingestion by benthic filter-feeders (e.g., bivalves and sea squirts) may act as collectors of microplastics from the water column, enhancing their availability to bioturbators (e.g. brittle stars) and deposit-feeders (e.g. polychaetes) that might facilitate their incorporation into sediments (Wright et al., 2013; Galloway et al., 2017).

Holothurians ingest large amounts of sediments (9–82 kg ind⁻¹ y⁻¹) from which they extract organic matter, but also bacteria, cyanobacteria, diatoms, foraminifera and fungi, contributing to nutrient regeneration and mineralization of surface sediments (Costa et al., 2014; Purcell et al., 2016). Previous studies have found microplastics in the tissues of these animals, suggesting potential transfer to upper trophic levels (Graham and Thompson, 2009; Renzi et al., 2018; Mohsen et al., 2019). Indeed, some deposit-feeding holothurians displayed active selection of microplastics, since they ingested more than expected according to the plastic to sand ratio in sediments (Graham and Thompson, 2009). Nonetheless, no study has investigated how ingestion and egestion by holothurians may influence the size and vertical distribution of plastic fragments in sediments and their resuspension in the water column.

The transit through the digestive tract could allow the absorption of plastic leachates and adhered contaminants, but also reduce the size of plastic particles. For example, the transit through the digestive system of the Antarctic krill (*Euphausia superba*) transformed microplastics in nanoplastics (Dawson et al., 2018). In holothurians, plastic items ingested with sand grains could be grinded during their passage through the guts. In addition, as shown for the mussel *Mytilus*

galloprovincialis (Piarulli and Airoldi, 2020), due to selective pick up of plastics from sediments, holothurians may concentrate plastic particles in their pseudofeces that would function as hotspots of microplastic concentration. Microplastics and, in particular, microfibers, collected from the water via the respiratory tree, were found in the coelomic fluid of the holothurian *Apostichopus japonicus* (Mohsen et al., 2019). Nonetheless, the abundance of microplastics inside some holothurians (i.e., *A. japonicus* and *Holothuria tubulosa*) was generally lower than in sediments (Mohsen et al., 2019; Renzi et al., 2018; Renzi and Blaskovic, 2020), suggesting that most of the ingested items are incorporated into fecal pellets. Freshly egested feces of holothurians are generally richer in organic content than surrounding sediments (Amon and Herndl, 1991; Uthicke and Karez, 1999; Hudson et al., 2005; Costa et al., 2014) and might be preferentially used by either conspecifics or by other deposit-feeding species (Conde et al., 1991; Piarulli and Airoldi, 2020), potentially facilitating microplastic uptake. In addition, through the accumulation in pseudofeces, plastic particles might be transported and accumulated at the surface of sediments, increasing the rates of resuspension by waves and currents. This process might be facilitated by the fact that pseudofeces are often composed by finer sediment particles and organic matter (Mezali and Soualil, 2013; Costa et al., 2014; Ricart et al., 2015; Boncagni et al., 2019) and can, therefore, be rapidly disintegrated also by relatively weak hydrodynamic forces.

The aspirochirotid holothurian, *Holothuria tubulosa*, is a common and well-studied deposit feeder in Mediterranean soft-bottoms, feeding selectively on detritus from the seagrass *Posidonia oceanica* (Costa et al., 2014; Ricart et al., 2015). This species actively ingests plastic particles, including fragments, films, granules and fibres, mostly in the range 100–2000 µm, with a colour fingerprint reflecting that of sediments (Renzi et al., 2018). Thus, *H. tubulosa* represents an ideal species for investigating how the trophic activity of holothurians affects the fate of microplastics buried in sediments. Here, we compared the abundance, size and colour of plastic particles between *H. tubulosa* pseudofeces and surrounding surficial sediments to assess if the transit through their digestive tract reduces the size of plastic fragments and whether they are accumulated into fecal pellets egested at the surface of sediments. In addition, we experimentally tested the hypothesis that the rate of microplastic resuspension is greater when these are incorporated into pseudofeces than sediments.

2. Materials & methods

2.1. Study site

This study was carried out in the NW Mediterranean, about 10 km south of the town of Livorno (Antignano, 43° 29' N, 10° 19' E). At a depth of about 8 m, the southern margin of a dense *Posidonia oceanica* meadow (about 1 ha × 1.5 ha) is flanked by a sandy area extending for about 3.5 ha (Uyá et al., 2018). *Holothuria tubulosa* is relatively common at the interface between the seagrass meadow and the sandy bottom and, in particular, within patches of sediment-buried dead rhizomes of the seagrass (matte). Densities of *H. tubulosa*, quantified by means of six 10 × 2 m transects, about 20 m apart one from another, were largely variable and ranged between 0 and 14 (mean ± SE = 3.17 ± 2.19).

2.2. Comparison of plastic item abundance, size and colour between sediments and pseudofeces

In December 2018, surficial sediments and pseudofeces of *H. tubulosa* (hereafter also referred to as biodeposit sediments) were collected by hand while SCUBA diving. Freshly egested fecal pellets forming mounds at a distance shorter than 15 cm from the animal were gently scooped inside 50 ml Falcon tubes, using a steel-made spoon. Fecal mounds from 5 individuals (randomly chosen >5 m apart one from another) were necessary to fill in one 50 ml tube which was then treated as one sample. Surficial sediments were collected by gently

dragging the tube over the top 1-cm layer of sediments in areas without fecal mounds and macroscopic seagrass debris. For consistency with the pseudofeces sampling, one tube was filled by collecting sediments from five randomly chosen areas, at a minimum distance >5 m. A total of 20 and 12 samples (i.e. 60 fecal mounds) were collected for sediments and pseudofeces, respectively. One sediment sample was subsequently lost.

Samples were dried at 40 °C for 36 h in a muffle oven and sieved through 1.4, 0.5 and 0.063 mm standard steel sieves. Activities were performed under a HEPA-II laminar flow hood and wearing a cotton laboratory coat and nitrile-coated gloves to avoid air-born contamination of samples. Sieves were thoroughly rinsed with ultrapure water to remove microplastic residuals between one sample and another. The material retained by 1.4-, and 0.5-mm sieves was examined under a stereomicroscopy and any item of unnatural appearance (due to colour, shape, dimensions) was collected and transferred into a petri dish which was immediately sealed. Confirmation of correct classification of plastic items was performed through chemical analysis using a microscopy associated with Fourier Transform Infrared Spectroscopy (μ FT-IR Nicolet iN 10 MX, ThermoFischer Scientific® Waltham, MA, USA Thermo Scientific), equipped with MCT-A detector, cooled with liquid nitrogen operating within the spectral range 7.800–650 cm^{-1} by both reflection and transmission detection techniques. Analyses were switched between the transmission or reflection modality according to the thickness of tested particles (limit of detection of 10 μm in size). Collected spectra of unknown particles that shall be identified were superimposed by determining the percentage of spectral match of targeted items with respect to referenced spectral libraries on microplastics (OMNIC™ Picta™ software libraries integrated with original laboratory spectral libraries collected on references materials). For each particle, spectra were collected at 10 different points to calculate the mean chemical spectrum associated. To increase spectral matches between libraries and unknown particles, a different support base for spectral acquisition operating in transmission mode (i.e., BaF_2) could be used. Particles <10 μm were not identified.

Plastic items within sediments retained by the 0.063 mm sieve were resuspended and extracted using a modified Munich Plastic Sediment Separator, following Coppock et al. (2017). The unit was constructed using two transparent PVC tubes (6 cm diameter x 15 cm high), connected by a PVC ball valve and fastened to a 8 x 8 cm PVC plate. Firstly, all unit components were rinsed with ultrapure water and the unit was filled with 700 ml of ZnCl_2 solution, at a density of 1.5 g cm^{-3} , to allow any externally-derived contaminant to float to the surface. After 5 min, the ZnCl_2 solution was filtered through a 0.45 μm paper fibre disks, by means of a syringe filter holder and a 60 ml syringe, into a clean flask to be reused, and this step was repeated prior to each extraction. To extract microplastic from the sedimentary matrices, each dry sample was added to the unit with the ball valve open, together with 700 ml of ZnCl_2 solution (1.5 g cm^{-3}) and a magnetic stirring bar. The top of the unit was closed with a clean aluminium foil, and the solution was stirred on a magnetic stirring plate for 5 min in order to allow the resuspension of plastic trapped into the sedimentary matrix. After mixing, the sediment was allowed to settle and the ball valve was closed. The supernatant, containing particles with a density less than 1.5 g cm^{-3} , was filtered through a 0.45 μm paper fibre disk, as described above. After each extraction, the unit and all the equipment were washed with ultra-pure water before processing the next sample. Procedural blanks were performed after the extraction of microplastics from each surficial sediment and pseudofeces sample, using only the ZnCl_2 extracting solution as a sample. Fibre filter disks from each sample and procedural blank were collected and stored in Petri dishes, sealed with Parafilm, to perform chemical analyses by μ FT-IR. The software Nikon ACT-1 was used for measuring the size of plastic items. In the case of fragments (i.e., irregular shape) the longest axis size was used as an estimate of size. All plastic items, retrieved in both sediments and pseudofeces could be classified as microplastics (63–5000 μm ; although one item

was 35.3 μm) (Galgani et al., 2013). Thus, plastic items were further classified according to their shape (fibres versus fragments, in this case) and colour, according to Galgani et al. (2013). In order to prevent contamination during microplastic extraction from the sedimentary matrices, all cleaned equipment (including unit, syringe filter holders, syringes, glassware) was placed inside a clean hood and covered with aluminium foil. All blanks resulted negative for the presence of microplastic.

The number of plastic items was analyzed using a Generalized Linear Mixed Model (GLMM), including Matrix (surficial sediment versus pseudofeces) and Shape (fibre versus fragment) as fixed factors, using the R package glmmTMB (Brooks et al., 2017). Since the same samples were used to quantify both types of plastic items, the sample was included as a random effect. In order to take into account variations in the amount of material collected in the field (surficial sediment or pseudofeces) among samples, the dry weight of each sample was used as the offset. Dealing with count data, the model was fitted using a Poisson distribution. The function *emmeans* in the R package *emmeans* (Lenth, 2020) was used for *post hoc* comparisons between treatment levels. Model assumptions were checked with QQ-plots and plots of standardized residuals versus the expected values (Fig. A1), using the R package DHARMa, which employs a simulation-based approach (Hartig, 2020). The same R package was used to run a Kolmorov-Smirnoff test to formally assess heteroscedasticity and goodness-of-fit tests on the simulated residuals to check for over-dispersion and outliers (Fig. A1).

In order to test the hypothesis that the passage through the digestive tract of *H. tubulosa* reduces the size of plastic items, we compared their frequency distribution between surficial sediments and pseudofeces using a Kolmorov-Smirnoff test, using the R package dgof (Arnold and Emerson, 2011). In addition, the mean length of plastic items was compared using a GLMM, assuming a Gaussian distribution and including the Matrix (surficial sediment versus pseudofeces) and Shape (fibre versus fragment) as fixed effects, the sample as a random effect and the weight of samples as the offset. The same set of diagnostic tools described above were used (Fig. A2). All analyses were run in R version 4.0.2 (R Core Team, 2020).

Due to the relatively low numbers of fibres and fragments in pseudofeces and sediments, respectively, we did not attempt to test formally whether differences in particle colours between the two matrices varied according to their shape. Thus, variations in colour features of plastic items were compared between sediments and pseudofeces using a single-factor PERMANOVA on Bray-Curtis dissimilarities calculated on square root transformed data, including the factor Matrix (pseudofeces versus surficial sediments; fixed) and the weight of samples as a covariate. A Principal Coordinates Ordination (PCO) was used to visualize multivariate patterns, with overlaid vectors representing variables (i.e., colours) with a Spearman's correlation >0.3 with the PCO axes.

2.3. Analysis of granulometry

Using the same procedures described in the previous section, four samples of pseudofeces and four of surrounding surficial sediments were collected for assessing granulometry. Samples were dried in oven at 105 °C until no further weight loss occurred. Furthermore, about 20 g of wet sample was used to determine water content (%) of the two different environmental matrices according to the method reported in ASTM D2216–19. Grain-sized determination was performed using steel shives according to ICRAM 2001/3S. Particle sizes were determined using a stack of certified steel sieves ASTM shaken for 10 min by an automatic vibrating sieve to separate the percentages of material retained by $\frac{1}{2}$ phi progressive series within 0.063–2 mm dimensional range of meshes. The material was classified as pelite (< 0.063 mm), fine (0.063 < x < 1 mm) and coarse sand (1 mm < x < 2 mm). Sand grains larger than 2 mm were not found in the samples.

Granulometry was compared between sediments and pseudofeces by means of a PERMANOVA including the Matrix as a fixed factor.

2.4. Experimental evaluation of microplastic retention by pseudofeces and sediments

A specific experiment was set up to assess microplastic resuspension rates from surficial sediments and *H. tubulosa* pseudofeces. Five experimental replicates of both surficial and biodeposit sediments, collected using the same procedures described in the previous sections, were dried in an oven muffle at 105 °C until no further change in weight occurred. Each dried sample was poured into a separate cylindrical glass chamber (height = 150 cm; diameter = 4.8 cm) until a 0.5 cm thick layer on the bottom was obtained. For each sample, ten blue-labelled polypropylene microplastic fragments were then inserted within the sediment layer, using micro-tweezers. Particles were uniformly distributed across the sediment-covered bottom of chambers to avoid any potential interference among them. Before their introduction into chambers, each particle was photographed and their size recorded, using a Nikon stereomicroscope SMZ-800 N, 10–80× connected to a digital webcam DS fi3 managed by NIS-element D software (Nikon), in order to facilitate their tracking during the following stages of the resuspension experiment and, importantly, not to confound them with microplastics eventually present in the sediment matrices collected in the field. The average dimension of the added particles was within the size range detected in the analyzed samples. In particular, the values (mean ± SD) were 582.4 ± 887.5 µm for surficial sediments and 526.3 ± 757.9 µm for pseudofeces. After the addition of microplastics, each experimental chamber was filled drop-by-drop and slowly to a depth of 7 cm with 0.45 µm filtered seawater, taking care not to resuspend the microplastics during the filling phase. Chambers were then exposed to a series of four round-stirring cycles (at a constant speed of 5 RPM) of increasing duration (15, 25, 45, 60 s). There was a 1-min lag between successive cycles for data collection. The number of labelled microparticles floating on the water surface was recorded soon after filling in the container (hereafter referred to as 5 s) and at the end of each stirring interval. Detection of microplastics was performed at each time interval by inspection of the surface water layer and the glass walls of the test chamber by stereomicroscopy (1–80×).

Using the *coxph* function in the *survival* package (Therneau, 2020), a Cox proportional-hazards model (Cox, 1972) was fit to assess how the rate of resuspension of plastic particles from the sedimentary matrix into the water column (the hazard rate) happening at a particular point in time varied between surficial sediments and pseudofeces. In analogy with survival analysis, we assimilated the retention of a particle within the sedimentary matrix (either surficial sediments or pseudofeces) with survival and the resuspension of a particle into the water column with death. The resuspension into the water column was not observed for some plastic particles within the duration time of the experiment, generating censored observations at 60 s. The model can be written as: $h_x(t) = h(0) \exp(\beta x)$, where t represents the particle retention time, $h_x(t)$ the hazard function, $h(0)$ the baseline hazard, x the covariable (i.e., the type of sedimentary matrix) and the β value provides an estimate of the impact of the covariable on plastic particle retention. Since the covariable sedimentary matrix was encoded as a numerical vector in which surficial sediment = 1 and pseudofeces = 2, positive β values indicate decreased particle retention and hazard ratios $\exp(\beta)$ smaller than one would represent an increase in the probability of particle resuspension in pseudofeces with respect to surficial sediments. Retention curves were generated using the functions *survfit* and *ggsurvplot* in the packages *survival* (Therneau, 2020) and *survminer* (Kassambara et al., 2020), respectively. The Schoenfeld individual and global tests, performed using the function *cox.zph* in the package *survival*, indicated no violation ($p > 0.05$) of the proportional hazards assumption.

3. Results

3.1. Comparison of plastic item abundance, size and colour between sediments and pseudofeces

A total of 59 plastic items were retrieved from surficial sediment and pseudofeces samples. No microsphere was detected, but only fibres and fragments. Microfibres were mostly made of PET and nylon, while most of the fragments by PET, PE and PVC (Table 1). The GLMM model showed a significant effects of the interaction between the shape and the sedimentary matrix on the abundance of microplastics (Table A1). Post-hoc comparisons indicated a greater abundance of fragments in pseudofeces than surficial sediments ($p < 0.001$), but no difference in that of fibres (Fig. 1).

The density distribution of plastic particle size in pseudofeces had a greater dispersion than that in sediments. A longer right tail indicates that pseudofeces contained a few larger particles, which size was not represented in sediments. Nonetheless, differences in the frequency distribution of plastic item size between sediments and pseudofeces were not significant (K-S test: $D = 0.337$; $p > 0.05$, Fig. 2). Likewise, the GLMM showed no significant differences between surficial sediments and pseudofeces (Table A2) in the mean size of neither fibres (mean length ± SE: sediments = 806.93 ± 80.81 µm; pseudofeces = 775.08 ± 376.58 µm) nor fragments (mean length ± SE: sediments = 850.34 ± 191.84 µm; pseudofeces = 1295.17 ± 297.99 µm).

Patterns in colour of fibres were slightly different between surficial sediments and pseudofeces (Fig. 3). Both matrices had a large proportion of blue fibres, but sediments were also characterized by the dominance of light blue fibres, while pseudofeces by that of white ones. Such mismatch might be due to the fact that only few fibres were retrieved in pseudofeces (i.e., six). By contrast, white, blue and black plastic fragments were predominant in both matrices (Fig. 3).

The PERMANOVA showed that the composition in colours of the pool of plastic items found in pseudofeces did not differ from that in surficial sediments ($MS_{\text{Matrix}} = 5593.1$; $MS_{\text{Residual}} = 2723.9$, Pseudo- $F_{1,25} = 2.053$, $p = 0.122$). Only 3 colours, namely the blue, light blue and white, had a Pearson correlation with PCO axes >0.3 (Fig. 4). The first PCO axis explained about 48% of the total variation and differentiated samples in which plastic items were predominantly white or light blue from those in which the blue was the most common colour. The second PCO axis explained accounted for about 35% of the total variation and differentiated samples dominated by each of these three colours.

3.2. Analysis of granulometry

The PERMANOVA showed significant differences in granulometry between pseudofeces and surficial sediments ($MS_{\text{Matrix}} = 1082.7$; $MS_{\text{Residual}} = 8.865$, Pseudo- $F_{1,6} = 122.13$, $p = 0.04$). Sediments were mostly composed by fine sand (92.22%) and had a small proportion of both pelite (4.92%) and coarse sand (2.86). By contrast, pseudofeces had a smaller amount of fine sand (68.85%) and higher contents of pelite

Table 1

Number and chemical composition of fibres and fragments retrieved in sediments and pseudofeces. The mean weight across samples is calculated with $n = 19$ for sediments and $n = 12$ for pseudofeces. PET = Polyethylene terephthalate, PE = Polyethylene, PP = Polypropylene, PVC = Polyvinyl chloride, PA = Polyamide.

Matrix	Shape	Chemical composition						Mean sample weight gr. ± SE
		Nylon	PET	PE	PP	PVC	PA	
Sediment	Fibres	7	22	0	0	0	0	79.61 ± 1.99
	Fragments	0	3	2	1	2	0	
Pseudofeces	Fibres	2	1	1	1	1	0	52.59 ± 1.22
	Fragments	0	6	4	0	4	2	

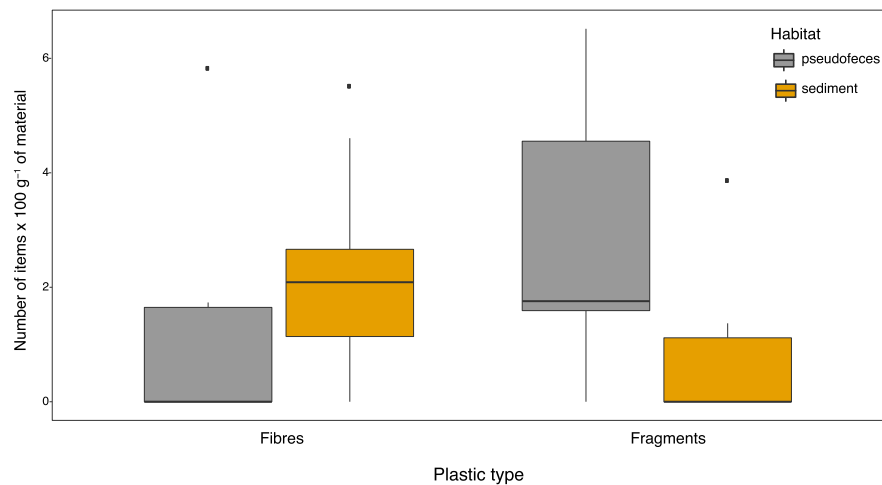


Fig. 1. Comparison of the abundance of microplastics between sedimentary matrices. Boxplots of the number of microplastic items $\times 100 \text{ g}^{-1}$ of either surficial sediments or pseudofeces, by plastic type (fibres and fragments).

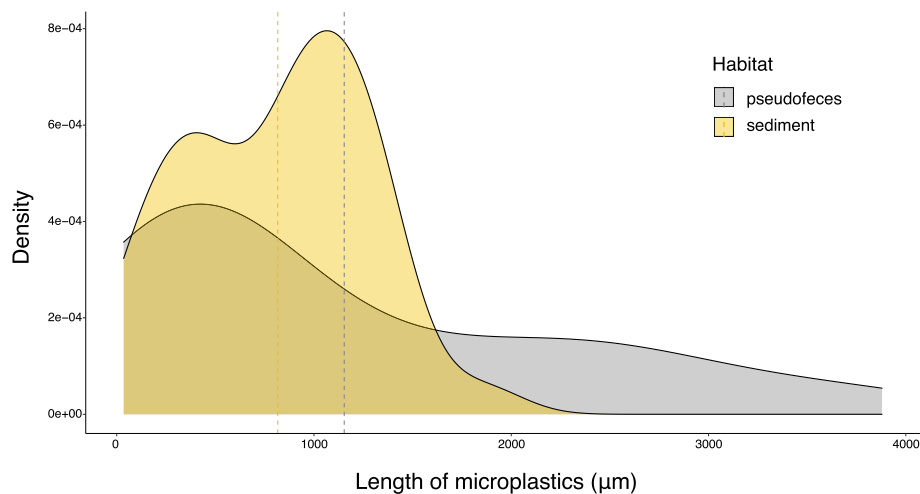


Fig. 2. Size of microplastics in sedimentary matrices. Smoothed density curves (via Kernel density estimation) of the distribution of plastic particle size in surficial sediments and pseudofeces. Vertical dashed lines represent the mean size of particles in each of the two matrices.

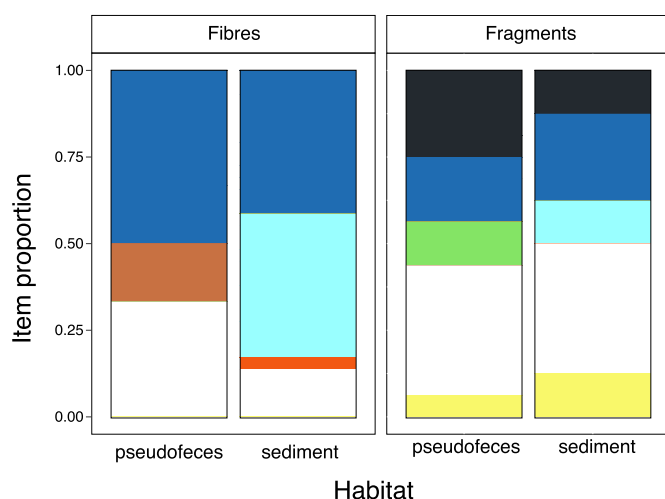


Fig. 3. Relative abundance of microplastics of different colours in surficial sediments and pseudofeces, separately for fibres and fragments. The colours reported in bars correspond to the real colours of microplastics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(22.47%) and coarse sand (8.69%). Water content was 58.01% in pseudofeces and 28.06% in sediments.

3.3. Experimental evaluation of microplastic retention by pseudofeces and sediments

The Cox regression indicated that plastic particle retention was significantly different between surficial sediments and pseudofeces ($p < 0.001$). A concordance of 0.732 suggests a good predictive ability of the model. A coefficient $\beta > 1$ (1.938) indicates that particle resuspension increased from surficial sediments to pseudofeces and, accordingly, a hazard ratio smaller than one (0.144) shows an increased probability of particle resuspension in pseudofeces (Fig. 5). Indeed, at the end of the experiment (i.e., after a 60 s stirring), 92% of the microplastics in pseudofeces were resuspended, while only 26% of added microplastics were released from surficial sediments.

4. Discussion

Pseudofeces of *H. tubulosa* were enriched in microplastic fragments with respect of the surrounding sediments deposited over dead *P. oceanica* rhizomes. By contrast, although not significantly different,

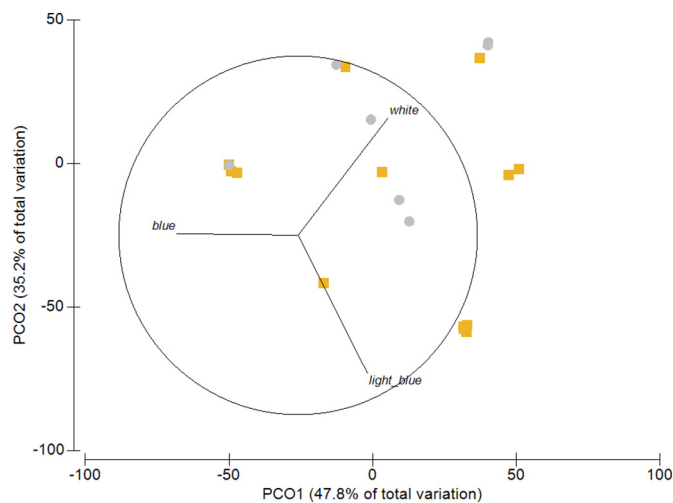


Fig. 4. Multivariate patterns of microplastic colours. Principal coordinate ordination (PCO) of the colour composition of the microplastics found in surficial sediments and pseudofeces. Vector overlay, based on the Pearson correlation ($r > 0.3$), shows the main colours contributing to the multivariate pattern. Orange squares = surficial sediments; grey circles = pseudofeces. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the abundance of microfibers in pseudofeces tended to be smaller than that in surficial sediments. Our results support the findings of previous studies that documented the presence of plastic litter in the tissues of holothurians and a selective intake of plastic particles during their feeding activity (Graham and Thompson, 2009; Renzi et al., 2018; Mohsen et al., 2019).

Microfibers were the most common type of microplastics in surficial sediments (about 78% of the total), a pattern reported in the Mediterranean and elsewhere (Browne et al., 2011; do Sul and Costa, 2014; Frias et al., 2016; Taylor et al., 2016; Renzi et al., 2018; Mohsen et al., 2019). The study area is at the southern periphery of the town of Livorno and is, thus, exposed to inputs of wastewater from domestic washing machines, a primary source of microfibers (Browne et al., 2011). Nonetheless, fibres were poorly represented in pseudofeces (about 27% of total microplastics). Plastic particles isolated in the sediment were always larger than 20 μm and, hence, not susceptible to translocation from the intestine to the coelomic fluid (Mohsen et al., 2019). Thus, the low content of fibres in pseudofeces was unlikely the result of their retention by animals, but rather an active avoidance of these items. By

contrast, the abundance of fragments in pseudofeces was higher than that at which they are found in sediments. Our analysis of granulometry clearly indicates the ability of *H. tubulosa* to select sand and food particles according to their size. Selectivity in *H. tubulosa* might be not limited to the size of particles, but also extend to their shape. Differently, microplastic fragments made of PET, PE, PP, PVC and PA were found in both sediments and pseudofeces, suggesting little selectivity with respect to the chemical composition.

Patterns of microplastic colour did not differ between surficial sediments and *H. tubulosa* pseudofeces. The similarity in colour between sedimentary matrices was greater in the case of fragments, as patterns in biodeposits closely mirrored those in surficial sediments. Selective ingestion of microplastic of certain colours has been documented in other species of pelagic fish and invertebrates (Wright et al., 2013; de Sa et al., 2015). Other studies have shown a strong correlation of microplastic colour between sediments or water and the guts of pelagic and demersal fish. Our results are in line with those of Renzi et al. (2018), who found that the proportion of plastic particles of different colours in *H. tubulosa* reflected patterns in sediments collected from different habitats, namely rocky slides, cliffs and banks. Thus, this holothurian appears to express no selectivity for the colour of plastic particles.

Contrary to our predictions, the frequency distribution and mean size of plastic particles in *H. tubulosa* pseudofeces was not different from that in sediments. Previous studies have shown that the transit through the digestive tracts of some crustaceans can reduce the size of plastic particles (Dawson et al., 2018; Cau et al., 2020). The anatomy of species' buccal and digestive tracts is likely to determine the extent of fragmentation of ingested particles. For instance, food items ingested by the Antarctic krill, *Euphausia superba*, studied by Dawson et al. (2018), pass through mandibles equipped with a cutting and grinding surface and are then moved for further mastication and exposure to digestive enzymes in the gastric mill and stomach. Likewise, the gastric mill of the Norwegian langoustine, *Nephrops norvegicus*, investigated by Cau et al. (2020) has a complex of calcified plates for food trituration. Thus, both species have anatomic structures along their feeding and digestive apparatus that could efficiently shred plastic particles. By contrast, *H. tubulosa* uses flattened oral tentacles to shovel and push sediments into their mouth food particles and does not have structures for mechanical trituration of food particles. Our study indicates that the transit through the *H. tubulosa* guts, amidst sand grains, does not cause a reduction of plastic particles.

We would, however, caution to not extend our results to microplastic items with a different shape or chemical composition. These features of plastic particles might influence their susceptibility

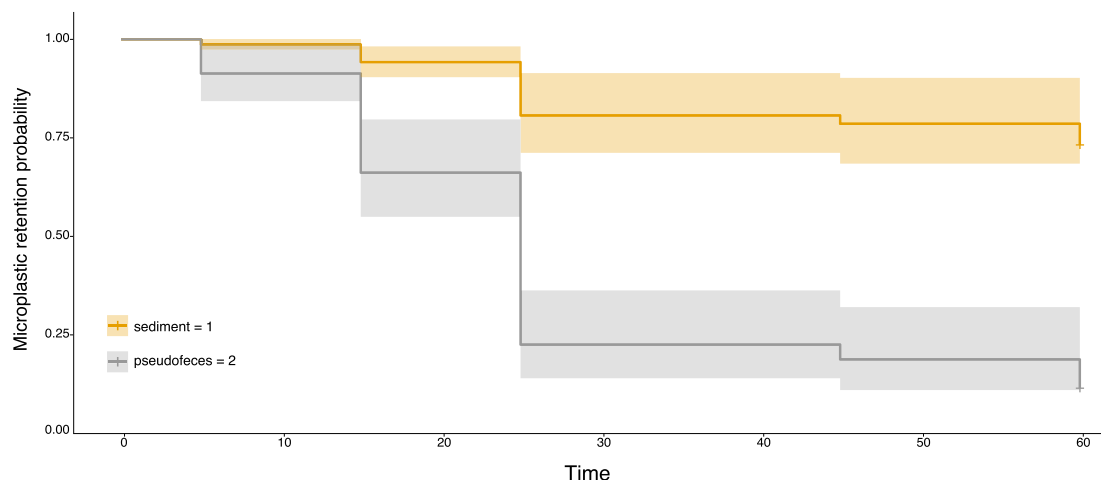


Fig. 5. Comparison of labelled microplastic retention between surficial sediments and pseudofeces. Curves showing the estimated probability of microplastic retention by surficial sediments and pseudofeces over time (seconds).

to fragmentation throughout the ingestion-egestion processes. Indeed, Antarctic krill were exposed to polyethylene beads (Dawson et al. (2018)) and fragments and films were the most common microplastics found in *N. norvegicus* by Cau et al. (2020). Further experimental studies appear warranted to determine variation in the susceptibility of plastic particles differing in composition and shape to mechanical fragmentation due to the passage through the digestive tract of holothurians. Likewise, the role of sediment characteristics (e.g. granulometry and composition) in determining the shredding of ingested microplastics is yet to be explored.

Our results suggest that the ingestion by *H. tubulosa* does not reduce the size of plastic particles, but alters their horizontal distribution through the accumulation of fragments in fecal pellets. The transfer of microplastics from sediments surrounding dead seagrass rhizomes to fecal mounds can have important implications for their resuspension and, hence, bioavailability. Bioturbation by some species, such as the polychaetae, *Arenicola marina*, and brittle stars has been hypothesized to promote long-term burial of microplastics into deeper sediment layers (Galloway et al., 2017; Gebhardt and Forster, 2018). Another study found weak effects of bioturbators, such as clams, gammarids and polychaetes, in the upward transport of microplastic buried in sediments (Näkki et al., 2019). In contrast, in the case of *H. tubulosa*, ingested plastic particles are incorporated into mounds of fecal pellets deposited above the surface of sediments or dead seagrass rhizomes. Contrasting findings among studies focusing on different taxa would suggest that life-traits (e.g., feeding habit, buccal anatomy, burrowing behaviour) of bioturbators may play an important role in determining their influence on the fate of microplastics and, ultimately, the effectiveness of sedimentary environments to act as a microplastic sink.

Fecal mounds of holothurians promote organic resuspension in the water column since they are physically unstable and they can be rapidly eroded also by relatively weak water currents (Rhoads and Young, 1970; Conde et al., 1991). For example, fecal pellets of *Isostichopus badiototus* dissolved completely in about 6 h (Conde et al., 1991). High organic content, accelerating the breakdown of the external mucous membrane by bacteria and fungi, favors the rapid wearing of fecal pellets (Honjo and Roman, 1978). Our experiment shows that physical instability of fecal pellets, likely due to the high content of very fine particles (i.e., pelite), enhances the resuspension of the microplastics they contain. Indeed, labelled microplastic added to pseudofeces had a resuspension likelihood that was about three times that of microplastics added to sediments, under a relatively short period of exposure to a moderate water movement. It is important to note that, due to technical constraints, we could not add labelled microplastics to fecal pellets without altering their integrity and arrangement in mounds. Thus, our experimental results are not indicative of microplastic resuspension from freshly egested fecal mounds, but rather during the period of time following their disintegration (i.e., after a few hours since deposition). Thus, further experimentation encompassing the different phases of fecal pellet wearing, multiple levels of water movement and plastic particle features (i.e., size, shape, floatability) is necessary to assess variations in microplastic resuspension between pseudofeces and surrounding sediments.

Nonetheless, our study brings some evidence that pseudofeces of *H. tubulosa* could not only represent hotspots of microplastic concentration but also accelerate their transfer to the water column. Similarly, previous studies have demonstrated that filter-feeders (e.g., mussels) or pelagic biota (e.g., fish, salps) can facilitate the sinking of microplastics to the bottom through their incorporation into fecal matter, enhancing their availability to benthic invertebrates (Clark et al., 2016; Piarulli and Airolidi, 2020). Our results show that invertebrates can also operate the opposite process and suggests that microplastic transfer from the benthic to the pelagic compartment is not necessarily driven by trophic interactions.

Holothurian's pseudofeces, including those of *H. tubulosa* (Costa et al., 2014), are generally enriched in organic matter and represent

elective feeding substrata for coprophagous species (Sloan and Vonbodungen, 1980). Coprophagy has been shown to facilitate vertical movements of microplastics in the water column and, thereby, their transfer between copepod species (Cole et al., 2016). Although yet to be explored, fecal pellets of *H. tubulosa* could be a vector for the transfer of microplastics to coprophagous benthos. Thus, coprophagy would reduce microplastic resuspension and availability for the pelagic biota.

5. Conclusions

Our study suggests that *H. tubulosa*, although not altering the size of microplastics, concentrates them into fecal pellets composed by fine material and deposited in mounds upon the surface of sediments. Physical instability of such fecal mounds would facilitate microplastic resuspension into the water column even under relatively weak hydrodynamic forces. In this case, due to the large per-capita volume of sediments egested (up to 17.5 kg dw sediment per year; Coulon and Jangoux, 1993), *H. tubulosa* may reduce the effectiveness of sedimentary bottoms as microplastic sinks.

CRediT authorship contribution statement

Fabio Bulleri: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Chiara Ravaglioli:** Conceptualization, Investigation, Writing – review & editing. **Serena Anselmi:** Investigation, Writing – review & editing. **Monia Renzi:** Conceptualization, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2021.146650>.

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