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Chemical fingerprint of plastic litter in sediments and holothurians from Croatia: Assessment & relation to different environmental factors

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A R T I C L E I N F O

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Sediment

A B S T R A C T

This paper increases knowledge on litter transfer from sediments towards the trophic web throughout sea cucumbers, key protected benthic species. In October, sediment and holothurian samples from seventeen sampling sites from Croatian Islands characterized by different levels of protection (Silba $n = 7$; Telašćica MPA $n = 10$) were collected. Collected particles ranged in sediments within 113.4–377.8 items/kg d.w., and in holothurians within 0.6–9.4 items/animal, showing sizes within 1.4–10,493 μm. In holothurians, cellulose and cellulose acetate (non-synthetic materials) mean percentages were within 5.0–12.7% of the total amount of particles. Nylon fibres ranged within 0–26.7%; while PP, PE, PA, and PS% were more abundant than in sediments. Among factors of variability tested, "island group" and "level of protection" resulted to affect plastic composition in sediments. Otherwise, other environmental factors (i.e. orientation, morphology of sampling site, *P. oceanica*) were significantly related to chemical composition of microplastic ingested by holothurians.

1. Introduction

Knowledge on dynamics affecting plastic transfer from aquatic environments towards trophic web represents a significant task to achieve by 2020 as reported in Marine Strategy Framework Directive principal purposes (2008/56/EC). Recent literature showed that plastic could penetrate the marine trophic web (Ivar Do Sul and Costa, 2014; Setälä et al., 2014). Large pelagic species such as cetaceans (Tonay et al., 2007), and fish (*Xiphias gladius*, *Thunnus* spp.) (Romeo et al., 2015a) ingest floating plastics. Small pelagic and planktivorous fish, such as sardines (Avio et al., 2015; Renzi et al., 2019a), and anchovies (Renzi et al., 2019a), show active predation of microplastics from the water column. Almost the 45% of biota from the Adriatic Sea is affected by the ingestion of MPs (Giani et al., 2019; Gomiero et al., 2019), while considering benthic species (i.e. flatfish *Solea solea*; Pellini et al., 2018) animals ingesting MPs increased up to 95% showing that benthic species are highly impacted by MPs stored in sediments. Literature shows as pelagic species ingested more particles than benthic ones, which, on the contrary, ingested more fibres (Neves et al., 2015). Nevertheless, recent researches on benthic sea cucumbers show that these animals tend to actively predate fragments rather than fibres in some marine ecosystems (Renzi et al., 2018a; Renzi et al., 2020) supporting results previously obtained by in vitro tests on holothurians (Graham and Thompson, 2009).

This research was developed on two different Croatian sites Silba and Telašćica subjected to different levels of protection and human pressures. Sediment samples and holothurians species were collected in October et al., 2017 from different sampling sites ($n = 17$) subjected to different levels of protection (MPA, designated MPA, and unprotected) and to different exposure to the Adriatic Sea inputs (completely exposed, lagoon, gulf).

The main aim of this study was to determine chemical fingerprint of plastic litter recorded in sediments and particles ingested by benthic species. Observed assessment was tested to evaluate significance of observed differences according to matrix, location, different levels of protection, and habitat types. Holothurians were selected as studied species because they are large-sized benthonic and deposit feeding protected species that selectively ingested microplastics.

2. Materials and methods

2.1. Principal features of study area

The study area is represented by two Croatian Silba and Telašćica Archipelagos settled in the Adriatic Sea (Geographical Sub-area GSA 17). Silba is an island, highly influenced by the Adriatic Sea and it is

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part of the Zara's Archipelago. Additionally, beside the Silba island there is also a small group of islands (ridges) called Grebena reefs. Grebena it is made up of 3 islands (ridges) which are called West (Južni greben), Medium (Srednji greben) and South Ridge (Sjeverni greben) which are designated for the institution of a new Marine Protected Area (MPA). Silba is a medium-large island (14.98 km^2 coastline length of 26.239 m) situated on an important maritime way that connect the Northern Adriatic Sea with the Southern (Gluščević, 2015). Including the Grebena reef (South Grebeni, North Grebeni, and Central Grebeni) the total amount of coastline is 31.850 m (Magaš and Brkić Vejmelka, 2013). The main island has only few inhabitants during the whole year (292 inhabitants). In the winter and in autumn the life on the island is peaceful and slow but in the summer the island become a popular touristic destination (Nikočević, 1983). On the other side Grebena are completely not inhabited. On Silba and, especially, on Grebena reefs the main activity is fishing and agriculture but maritime activities are, also, very important for the island (Tomljenović, 1983). The biodiversity of the island is very important, and a lot of studies have been made during the years, from history to cultural heritage, from flora to fauna (Durrigl et al., 2013). On the other side, the other study area is settled inside the MPA inside the Telaščica bay. Telaščica bay become first MPA in 1980 and after that a Nature Park in 1988 (total surface of 70.50 km^2) in winter it is very peaceful but in the summer it become a popular destination full of tutouristic and maritime activities.

2.2. Target matrices

The principal target of this research was to determine the chemical fingerprint of microparticles recorded in sediments and in associated benthic species. Among benthic species, sea cucumbers were selected as target one in this study for a wide range of consolidated reasons: i) they are benthonic deposit-feeding or suspension-feeding animals and levels recorded in their stomach contents could be related to levels recorded in surrounding sediments; ii) they are larger animals among benthonic species. On the basis of our experience, their stomach content contains about 10–20 g of wet sediment per animals (unpublished data) and this allow us to record a relative solid number of items per animal to perform better chemical analyses on micro-particles; iii) they are actually protected species in some EU countries (i.e. Italy, Law n. 156 of 27/02/2018, Ministry of Agriculture and Forestry, actual deadline 31/12/2019) and reported data on these species could be of particular interest; iv) they are widely representative of marine benthic species and they are considered key benthic taxonomic group in marine ecosystems to preserve ecosystem integrity (Purcell et al., 2016); v) they represent a crucial chain in the food web through predation by star, crustaceans, gastropods, fishes (Francour, 1997; Dance et al., 2003); vi) they ingest microplastics both in vitro (Graham and Thompson, 2009) and in vivo (Renzi et al., 2018a). Holothurians collected in this study showed comparable body size with a mean value of 24.7 ± 5.0 cm of length (Silba Islands), and of 16.8 ± 2.6 cm of length (Telašćica).

2.3. Samplings

Samplings were performed in October, on seven sites from Silba Islands and ten sites from Telašćica (Fig. 1). Five sites (named Dražice bay, Južni Arat, Loišće bay, Smrdeča bay, Sotorišće) were located all around the Silba Island are considered unprotected; on the contrary, two sampling sites were located in Grebena reef's MPA (Južni Greben, Sjeverni Greben) and they are considered as designated MPA. Telaščica bay sampling sites are MPA since 1980; sampling stations inside the MPA were settled in Mala Proversa, Pod Stražica bay, Mir bay, Čuščica bay, Magrovica bay, Farfarikulac bay, Vela Proversa, Gmajnac, Loišče, and Lučice bay. Among them, Lučice and Loišče bay are situated on the external cliffs; all the others are settled inside the Telaščica bay.

Holothurians (*H. tubulosa*) were measured with an underwater meter and then collected from shallow water marine sediments (depth ranged within 5–18 m, Silba Islands; 4–10 m, Telašćica bay) by scientific scuba divers using wide mouth glass jars. In each sampling site, ten animals were collected and stored in pools of 5 animals in a glass jars submerged in ethanol (70%) to preserve animals until laboratory dissection and analyses (Renzi et al., 2018a). In parallel, three sediments replicates ($n = 3$) were collected to determine plastic levels in this matrix; samplings were performed using the same method performed by similar studies to reduce recorded variability (Blašković et al., 2017). During sampling environmental factors of specific interest (i.e. habitat morphology, bottom coverage by *P. oceanica* meadow, cardinal points) were collected to perform statistical correlations to chemical fingerprints of microplastic recorded in sediments and animals. Percentages of bottom coverage by *P. oceanica* meadow were determined in triplicates during samplings by scuba divers using a 50 × 50 cm square frame to obtain estimation on the mean coverage (%) of bottom per each sampling site.

2.4. Laboratory analyses

Sediment samples were analysed following the extraction and classification method reported by Fastelli et al. (2016) for the Aeolian Archipelago Islands to a better comparison of collected data with data reported previously by the literature. Extraction was adapted for better tissue dissolution because during the first extraction performed following literature (Renzi et al., 2018a) a percentage of undissolved tissue was observed in treated samples. For the first time, a basic solution was used for the extraction of microplastic from Holothurians. Animal extraction was performed in glass backer in two steps. First of all, 20 mL of 35% H_2O_2 per gram of tissue, backers were put in a Bain-marie at 50 °C till the complete tissue digestion (adapted Nuelle et al., 2014). After that, the supernatant containing dissolved tissue was divided from the undigested sediments that were contained in stomach contents. The liquid phase was added with 5 mL of 30% KOH/NaClO and the solution was mixed 2 h. Undigested sediments were extracted by adding 200 mL of a saturated NaCl solution (Fastelli et al., 2016). The basic solution was not added to the sediment phase because of its chemical reaction with the saturated NaCl solution inhibiting the extraction of microplastic. Extracts were filtered on paper fibres (0.45-μm filter disks). Glassware was accurately rinsed during the filtration of each sample to increase recovery efficiency of litter. Filters were stored in a glass Petri dish and dried in oven at 40 °C till constant weight. Collected litter were sorted by stereomicroscopy (Nikon SMZ-800 N) with micro tweezers and stored in Eppendorf tubes filled with distilled water for μFT-IR chemical analyses. Identified litter was divided by type (Macro, Meso, Microplastic), shape (filaments, fragments, pellet, film, foam), size, and colour according to the literature (Galgani et al., 2013; Alomar et al., 2016; Blašković et al., 2017; Fastelli et al., 2016). Maximum dimensions were determined measuring width for fragments and length for fibres. Chemical composition of microparticles recovered was performed by the microscopy associated to Fourier Transform Infrared Spectroscopy technique (Nicolet iN10 MX, μFT-IR, Thermo Fischer Scientific) equipped with liquid nitrogen cooled MCT-A operating within the spectral range 7800–650 cm^{-1} and with OMNIC Picta™ (Thermo Scientific) users' interface. Particles were analysed by the “tip and shoot” operating mode to improve focusing resolution by transmittance mode (spectral range 650–7800 cm^{-1}) if thinner than 35 μm. Thicker particles were identified by mid-infrared spectroscopy in ATR (Attenuated Total Reflection) using a germanium crystal (spectral range 1300–3000 cm^{-1}). Double peak system ranging between 2300 and 2350 cm^{-1} was corrected using the “atmospheric correction” mode available in the Omnic™ Picta™ software. Chemical identification was performed using a specific section of the Omnic™ Picta™ and Omnic™ Specta™ software which allow to identify collected spectrum by comparison with a specific reference library available in the Omnic™ Picta™ software. The threshold for IR spectra back-recognition was fixed over 65% of match among particles recovered from samples and libraries. A

Fig. 1. Sampling sites in Silba and Telaščica Islands are represented. Sampling sites are represented by geo-referenced sampling stations on pictures from Google Earth®; the figure is obtained by superimposing different images (Paint). Random sampling replicates within the same sampling station are not represented. Telašcica MPA and Silba Island are represented within the Northern Adriatic Sea. Sampling sites from Silba are reported in the white coloured frame while those from Telašcica are reported in the light blue coloured frame.
For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article. Silba sampling stations are: Dražice bay (DRA), Južni Arat (JAR), Loišće bay (LOI), Smrdeča bay (SMR), Sotorišće (SOI) and they were located all around the Silba Island (unprotected). Two sampling sites from Silba were located in the Grebena reef's; they are named Južni Greben (JGR), Sjeverni Greben (SGR) and they are considered as designated MPA. Sampling stations from Telaščica Island (MPA since 1980) are: Mala Proversa (MPR), Pod Stražica bay (STR), Mir bay (MIR), Čuščica bay (CUS), Magrovica bay (MAG), Farfarikulac bay (FAR), Vela Proversa (VPR), Gmajnac (GMA), Loišče (LOJ), and Lučice bay (LUC). LUC and LOJ are situated on the external cliffs; all the others are settled inside the Telaščica bay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relatively broader correspondence allows good recognition and takes into account the effect of aging on the spectrum of recovered microplastics. Limit of detection was 10 μm.

Data on sediments were normalized as items/kg d.w. while, data on holothurians, were expressed as items/individual. Chemical composition of collected particles was expressed as percentage of each chemical substance recorded compared to the total number of particles recovered in the tested sample.

2.5. Statistical analyses

Statistics were performed to evaluate multivariate relationships among chemical compositions recorded in tested samples and environmental factors of specific interest (i.e. habitat morphology, bottom coverage by *P. oceanica* meadow, cardinal points). Univariate statistics (mean. 95% interval of confidence of mean, standard deviation, maximum, and minimum) on collected data are performed by GraphPad Prism v. 5.0 (GraphPad Software, San Diego, CA, www.graph pad.com). Multivariate statistics were performed using Primer-E package v6.0 (Plymouth Marine Laboratory, UK). Sediment and holothurians databases on chemical composition of microplastic recorded (% data) were analysed separately; in fact the ANOSIM test performed on the factor "matrix" showed significance. The Euclidean matrix of distance was calculated on normalized data of percentages (Clarke and Warwick, 2001). The ANOSIM test one-way was run to explore significance of observed segregation according to factor a priori selected (Clarke and Warwick, 2001; Anderson et al., 2008). Concerning possible factors able to affect measured variables distribution, were tested: Island group (two levels, factor fixed, Salina vs Telašćica); level of protection (three levels, factor fixed, anthropogenic stress or impact, designated MPA, MPA); morphology (three levels, factor fixed, open sea, lagoon, protected sea); percentage of bottom coverage by *P. oceanica* meadow (HP_BC; three levels, factor categorized, 0%, 1–50%, 51–100%), orientation with respect to the cardinal points (seven levels, factor fixed). Sampling replicates are considered as random factor of variability. Further details on factors (both fixed and random) considered in this study are reported in Table 1. Sediments represent a storage matrix for almost all macronutrients and chemical pollutants in marine environments (Hedges and Keil, 1995). Chemicals (i.e. TOC, TP, etc.) are usually positively correlated to the silty fraction of marine sediments (Magni et al., 2008; Lukawska-Matuszewska and Bolalek, 2008). Microplastics resulted not to correlate with grain-size of sediments by previous researches (Martins and Sobral, 2011; Romeo et al., 2015b; Fastelli et al., 2016; Blašković et al., 2017) and for this reason the factor of variability "grain-size of sediment" was not consider in this study. Principal Component Analyses (PCA) was performed on loadings values to evaluate similarities according chemical composition of microparticles, also, eigenvectors associated to PCA were reported.

Table 1
Considered factors of variability for statistical analyses. Notes: d_MPA = designated as future marine protected area (MPA), OAS = Open Adriatic Sea; IA = Inner sea; SEB = semi-enclosed bay/lagoon; HP_BC = percentage of bottom coverage by *P. oceanica* meadow.

	Code	Location	Level of Protection	Position	HP_BC (%)	Cardinal point
Sotorišće	sot	Silba	Touristic	OAS	0	NE
Dražice bay	dra	Silba	Touristic	OAS	50–89	E
Loišće bay	sloj	Silba	Touristic	OAS	50–89	S
Južni Arat	jar	Silba	Touristic	OAS	50–89	S
Smrdeča bay	smr	Silba	Touristic	OAS	< 50	NW
Južni Greben	jgr	Silba	d_MPA	OAS	90–100	S
Sjeverni Greben	sgr	Silba	d_MPA	OAS	90–100	S
Mala Proversa	mpr	Telaščica	MPA	IA	< 50	SE
Pod Stražica bay	str	Telaščica	MPA	SEB	0	N
Mir bay (Uvala)	mir	Telaščica	MPA	SEB	0	NW
Čuščica bay	cus	Telaščica	MPA	IA	≧50	SE
Magrovica bay	mag	Telaščica	MPA	SEB	0	SE
Farfarikulac bay	far	Telaščica	MPA	SEB	0	SE
Vela Proversa	vpr	Telaščica	MPA	IA	< 50	S
Gmajnac	gma	Telaščica	MPA	SEB	0	E
Loišče	loj	Telaščica	MPA	OAS	≧50	SW
Lučice bay	luc	Telaščica	MPA	OAS	< 50	SW

3. Results

3.1. Sediment features

The mean amount of sand (range: min-max, data expressed as % d.w.) is always over than 90% (93.9–98.9%) in almost all sediment samples with no differences related to the sampling area. Gravel represent a percentage of the total amount ranging within 0–3.6% while the silt fraction (mud + clay) resulted always lower than 4.0%.

Silba Islands show the absence of plastics > 2.5 cm while low levels of plastic within 0.5–2.5 cm were recorded (0.8–3.7 items/kg d.w.). Microparticles with maximum size lower than 0.5 cm (mean ± SD; range: min-max; data expressed as items/kg d.w.) are the most represented concerning the number of items recorded (86.0 ± 98.4: 113.4 smr–284.8 sloj). Among them cellulose and cellulose acetate (non-synthetic materials) represented a mean percentage of 13.3% (range 0–67.9%). Fibres represent the principal percentage of recorded microparticles in sediments ranging within 76.9% sloj–33.1% sot, followed by fragments and films that are equally represented respectively within 17.5% smr–55.6% jgr; and 0.2% sloj–29.7% sgr; granules were recorded occasionally.

Telašćica bay shows the absence of plastics > 2.5 cm while low levels of plastic within 0.5–2.5 cm (range: min–max) were recorded (0–27.9 items/kg d.w.). Microparticles lower than 0.5 cm (mean ± SD; range: min–max) are the most represented concerning number of items (177.6 ± 112.7 items/kg d.w.; 32.3 mag–377.8 mir items/kg d.w.). Among them cellulose and cellulose acetate (non-synthetic materials) represented a mean percentage of 21.0% (min–max range: 0.5–37.0%). Fibres represent the principal percentage of recorded microparticles in sediments ranging within 82.7% str–97.3% mpr, followed by films and fragments that are represented respectively within 1.4% mpr–33.5% gma and 0–7.4% str; granules were not recorded.

Chemical compositions of microparticles recorded in sediments are represented in Fig. 2; associated univariate statistics are reported in Table 2 showing mean, lower and upper 95% confidence intervals of mean, standard deviation, minimum and maximum values. Silba (Fig. 2a) shows high percentages of nylon (23.0%, range 0–60%) and PVC (26.9%, range 0–71.4%) in sediments associated to wide standard deviations. Telašćica chemical composition of recorded microplastics (Fig. 2b) shows a wide heterogeneity of plastic materials as PVC, PP, PE, PET, and nylon are recorded ranging within 12–18%. Comparing means, cellulose is larger recorded than in Silba sediments (mean 13.7% versus 9.7%).

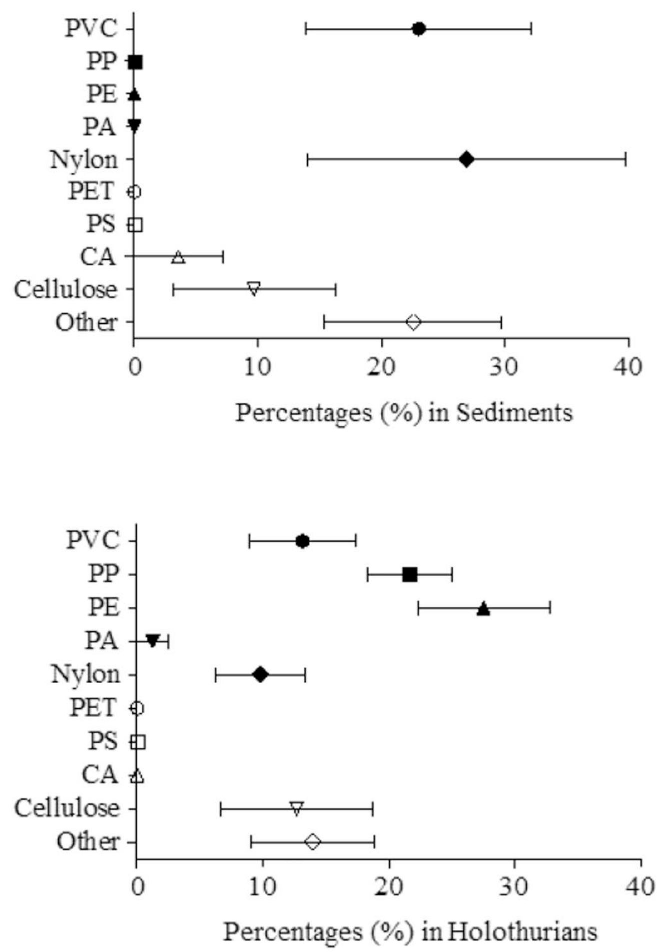

Fig. 2. Chemical composition of micro-particles collected in Silba samples.

3.2. Holothurians features

Silba Islands show the absence of plastics > 2.5 cm; while low levels of plastic within 0.5–2.5 cm were recorded (range min-max: 0.0–0.2 items/animal). Microparticles lower than 0.5 cm (mean ± SD; range: min–max) are the most represented concerning number of items (0.74 ± 3.7 items/animal; 0.9 smr–5.6 sloj items/animal). Among them cellulose and cellulose acetate (non-synthetic materials)

Table 2
Univariate statistics performed on recorded chemical composition of microparticles in sediment and holothurians. Data are reported as percentages (%).

			Plastic materials								Non-synthetic materials	
			PVC	PP	PE	PA	Nylon	PET	PS	Others	CA	Cellulose
Silba	Sediment	Mean	23.0	0.0	0.0	0.0	26.9	0.0	0.0	22.6	3.6	9.7
		Lower 95% CI mean	0.8	nc	nc	nc	0.0	nc	nc	4.9	0.0	0.0
		Upper 95% CI mean	45.2	nc	nc	nc	58.4	nc	nc	40.2	12.3	25.8
		Std. Deviation	24.0	0.0	0.0	0.0	34.1	0.0	0.0	19.1	9.4	17.4
		Minimum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Maximum	60.0	0.0	0.0	0.0	71.4	0.0	0.0	50.0	25.0	42.9
	Holothurians	Mean	13.2	21.6	27.5	1.2	9.8	0.0	0.0	13.9	0.0	12.7
		Lower 95% CI mean	2.7	13.4	14.8	0.0	1.1	0.0	0.0	2.1	0.0	0.0
		Upper 95% CI mean	23.6	29.9	40.3	4.3	18.6	0.0	0.0	25.8	0.0	27.5
		Std. Deviation	11.3	8.9	13.8	3.3	9.5	0.0	0.0	12.8	0.0	16.0
		Minimum	0.0	6.3	12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		Maximum	30.0	30.0	50.0	8.7	20.0	0.0	0.0	37.5	0.0	33.3
Telaščica	Sediment	Mean	18.0	14.0	12.2	0.0	17.2	16.2	1.2	6.8	0.8	13.7
		Lower 95% CI mean	10.3	9.2	7.1	0.0	11.3	9.4	0.4	3.0	0.0	5.8
		Upper 95% CI mean	25.7	18.9	17.2	0.0	23.1	22.9	2.0	10.6	1.8	21.5
		Std. Deviation	10.8	6.8	7.1	0.0	8.3	9.4	1.2	5.3	1.4	11.0
		Minimum	0.6	1.8	4.0	0.0	5.3	0.5	0.0	0.5	0.0	0.5
		Maximum	33.8	22.5	25.9	0.0	35.3	32.2	3.0	16.5	3.5	33.5
	Holothurians	Mean	17.6	12.8	12.7	0.0	11.6	12.9	5.3	25.8	0.0	1.4
		Lower 95% CI mean	13.6	8.7	7.1	0.0	6.1	5.6	0.1	18.1	0.0	0.0
		Upper 95% CI mean	21.5	17.0	18.3	0.0	17.1	20.2	10.5	33.5	0.0	4.7
		Std. Deviation	5.5	5.8	7.8	0.0	7.7	10.2	7.3	10.8	0.0	4.5
		Minimum	9.1	6.7	0.0	0.0	0.0	0.0	0.0	6.7	0.0	0.0
		Maximum	27.3	23.1	27.3	0.0	26.7	27.3	18.2	46.2	0.0	14.3

represented a mean percentage of about 12.7%. Fibres represent the principal percentage of recorded microparticles in holothurians ranging within 39.4% sloj–43.3% sot, followed by fragments and films that are represented respectively within 35.3%–40.2% jgr and 29.5–31.6% sot.

Telašćica bay shows the absence of plastics > 2.5 cm while low levels of plastic within 0.5–2.5 cm were recorded (range min–max: 0.0–0.4 items/animal; in far, vpr, mir). Microparticles lower than 0.5 cm are the most represented concerning number of items covering about 99% of the total. A mean (± SD; range: min-max) value of 4.5 ± 5.6 items/animal (range: 0.6 vpr–9.4 mir items/animal) was recorded. Among them cellulose and cellulose acetate (non-synthetic materials) represented a mean percentage < 5.0%. Microparticles size (μm) ranged within 1.4 mpr–10,493 vpr μm. Fibres represent the principal percentage of recorded microparticles in holothurians ranging within 35.7% mir–57.5% mag, followed by fragments and films that are represented respectively within 34.3% gma–53.9% loj; and 0.0 str–4.4% mpr; other shapes represented lower than 5% percentages.

Chemical composition of microparticles recorded in holothurians are represented in Fig. 3; associated univariate statistics are reported in Table 2 showing mean, lower and upper 95% confidence intervals of mean, standard deviation, minimum and maximum values. Holothurians in Silba (Fig. 3a) show lower percentages of nylon (9.8%, range: 0–20%) than in Telašćica (11.6%, range: 0–26.7%) (Fig. 3b). Holothurians shows a wide heterogeneity of plastic materials, in Telašćica PVC, PP, PE, PET, and nylon shows mean values ranging within 11.6–17.6%. Comparing means, cellulose is larger recorded in animals from Silba than those from Telašćica (12.7% versus 1.4% respectively). Animals from Silba show PP, PE, PA percentages higher than sediments from the same sites; on the contrary, animals from Telašćica show comparable chemical composition compared to sediments and only PS, and other particles are higher in animals than sediments.

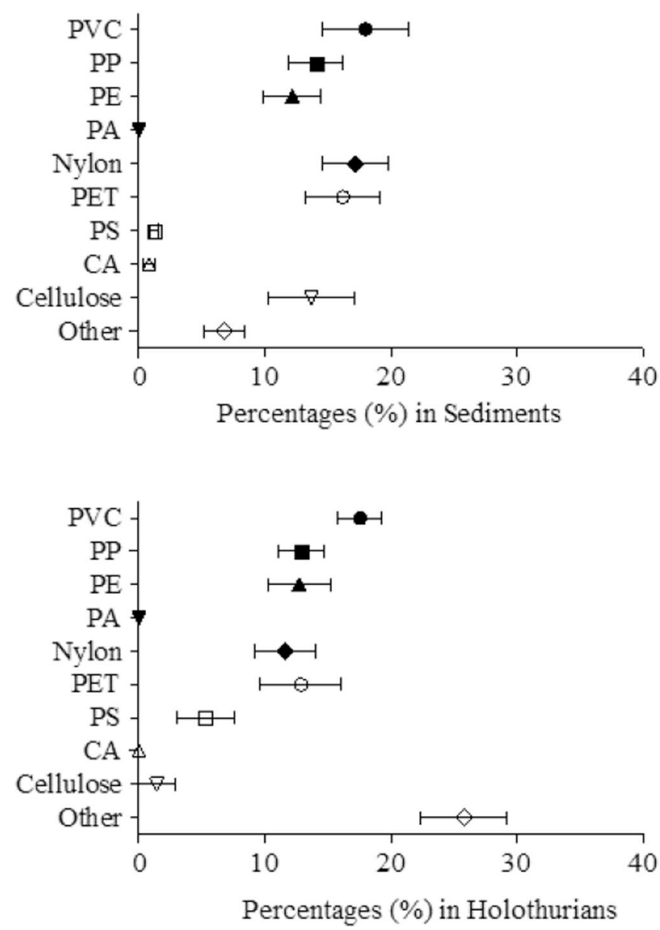

Fig. 3. Chemical composition of micro-particles collected in Telascica samples.

3.3. Statistical analyses

Principal component Analyses (PCA) performed on the whole dataset collected on chemical analyses performed on microparticles is reported in Fig. 4. The first three axes explained 51.8% (respectively 19.8%, 17.6%, and 14.5%) of the total variance. Eigenvectors, coefficients in the linear combinations of variables making up PC's, are reported in Table 3. The larger part of the variability related to the first axe is explained by a direct relation with cellulose acetate (0.359), and

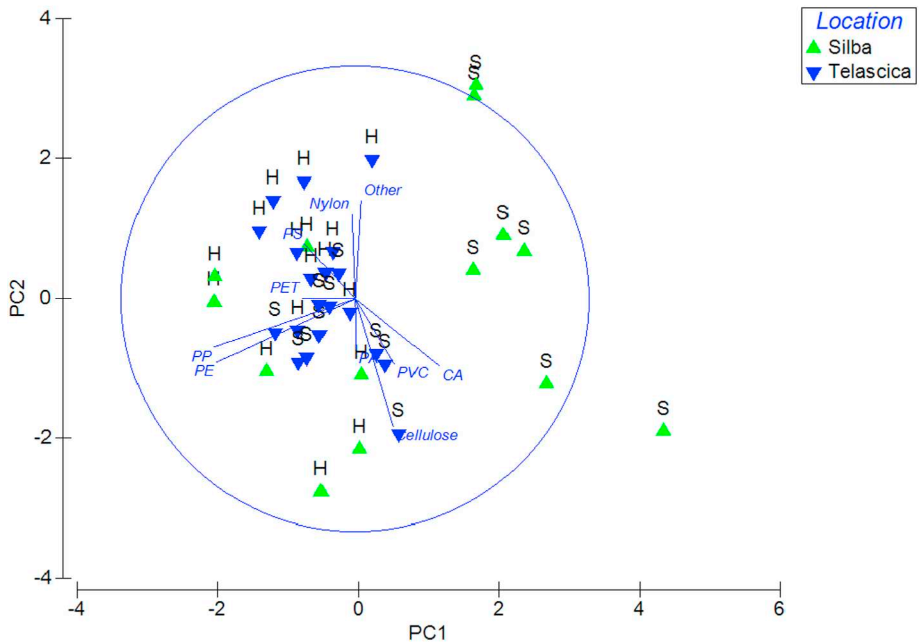

Fig. 4. Principal Component Analysis performed to evaluate similarities according chemical composition of detected particles. Data used to plot PCA are related to loadings (percentage).

Table 3
Eigenvectors (Coefficients in the linear combinations of variables making up PC's for the first three axes) describing the fraction of total variance explained by each PCs associated to the PCA reported in Fig. 3.

	PC1	PC2	PC3
PVC	0.167	−0.280	0.464
PP	−0.606	−0.208	0.067
PE	−0.591	−0.272	−0.133
PA	0.002	−0.217	−0.472
Nylon	−0.014	0.367	−0.183
PET	−0.228	0.001	0.512
PS	−0.217	0.237	0.394
CA	0.359	−0.289	0.282
Cellulose	0.163	−0.550	−0.099
Other	0.024	0.425	0.012

by indirect relation with PP (−0.606), and PE (−0.591) levels. On the contrary, concerning the second axe, the larger part of the recorded variability is directly related to nylon (0.367), PS (0.237), and others (i.e. IBVE, etc., 0.425) and indirectly related to cellulose (−0.550), and PVC (−0.280). Results obtained by the ANOSIM test one-way performed on the whole database and separately on sediments and holothurians are reported in Table 4. Tested matrices show a clear significance different chemical fingerprint. Both sediments and holothurians showed significance differences concerning tested factors Island and level of protection. On the contrary the factor "morphology" do not significantly affected the chemical composition of microparticles recorded. On the contrary, the "geographic orientation" of sampling site resulted to slightly affect chemical composition in holothurians. The percentage of bottom coverage by *P. oceanica* resulted not significance even if a level of 6.3% is recorded.

The pairwise tests showed a high significance differences between the couple of factor Touristic – MPA; slight significance differences are observed between the couple designated-MPA - MPA in both of the considered matrices sediments and holothurians. The couple semi enclosed basins – Open Adriatic Sea (for the factor "morphology") and the couple 0– > 50% (for the factor "coverage of bottom by *P. oceanica*") significantly affect chemical fingerprints in holothurians.

4. Discussion

4.1. General features

The largest part of particles recorded in both sediments and holothurians were microparticles. Levels recorded in this study are similar to those reported by the same study area (Blašković et al., 2017). Literature reported macroplastics in sediments from high-polluted harbour sites (i.e. Reddy et al., 2006; Claessens et al., 2011; Romeo et al., 2015a) but, also, from designated-AMP (Salina Island, south Tyrrhenian Sea; Fastelli et al., 2016). Any macroplastic was recorded in this study, but low levels of mesoplastics (size within 0.5–2.5 cm) resulted from both Silba and Telašćica Island groups. Confirmed microplastic recorded in this study are similar but lower compared to the literature for the same study area (Blašković et al., 2017), and in designated-MPA (Fastelli et al., 2016; Renzi et al., 2018a; Renzi et al., 2020); this is probably due to the different method of MPs identification of this study that reduces overestimations. This is supported by the large percentages of cellulose and cellulose acetate (non-synthetic materials) recorded. In particular, fibres represented the larger amount of recorded microplastics in sediments from both Silba and Telašćica. In this study, MPs recorded in sediments (mean 86.0 ± 98.4; min–max: 113.4–284.8 items/kg d.w. in Silba; mean 177.6 ± 112.7; min–max: 32.3–377.8 items/kg d.w. in Telašćica) were lower than 1445.2 items/kg recorded by Vianello et al. (2013) in Venice lagoon, and values reported by Liebezeit and Dubaish (2012) but comparable to levels reported by Claessens et al. (2011), Laglbauer et al. (2014), Nor and Obbard (2014). Nylon fibres represented a large percentage in both Silba and Telašćica sediments suggesting the possible presence of pollution originated by domestic effluents containing fibres from the washing of synthetic fabrics (Corami et al., 2020). Fibres represented, also, main microparticles records in holothurians' stomach contents suggesting a direct relation to sediments. Concerning other types, percentages recorded in sediments are different compared to those recorded in holothurians. Fragments recorded in sediments ranged within 17.5–55.6% (Silba) and 0–7.4% (Telašćica) while in holothurians were 35.3–40.2% and 34.3–53.9% respectively. Films ranged within 0.2–29.7% (Silba) and 1.4–33.5% (Telašćica) in sediments while in holothurians were 29.5–31.6% and 0–4.4% respectively. These data suggest enrichments

Table 4
ANOSIM test one-way performed on factors affecting chemical fingerprints in sediments and holothurians. Notes: ANOSIM test performed on the factor matrix (two levels, fixed) showed high significance values (Global $R = 0.124$, $p = 0.0008$) and for this reason, statistics on sediment and holothurians databases were run separately. Part I) ANOSIM test results and significance levels on tested factors. Part II) Pairwise test performed between couples of considered levels of tested factors is reported. In this part of the Table, only significance relationships are reported. In bold are highlight significance relations, ** = high significance; * = slight significance.

Part I)	Matrix ($n = 2$)	Sediments		Holothurians	
	Tested levels	Global R	Signif. level (%)	Global R	Signif. level (%)
Island group	2	0.545	**0.01****	0.419	**0.05****
Level of protection	3	0.674	**0.02****	0.384	**0.5****
Morphology	3	−0.110	78.1	0.186	8.0
HP_BC	5	0.102	16.5	0.176	6.3
Orientation	7	0.135	19.4	0.343	**1.7***

Part II)	Matrix (n = 2)		Sediments		Holothurians	
Factor	Levels	Couples	Global R	Signif. level (%)	Global R	Signif. level (%)
Level of protection	3	Touristic-MPA	0.673	**0.03****	0.428	**0.5****
		d_MPA-MPA	0.987	**1.5***	0.550	**3.0***
Morphology	3	SEB-OAS	–	–	0.284	**3.0***
HP_BC	5	0– > 50	–	–	0.306	**1.3***

of fragments and films shapes in holothurians. These data are supported by previous literature reporting active ingestion of plastic fragments by holothurians under in vitro (Graham and Thompson, 2009) and in vivo conditions (Renzi et al., 2018a; Renzi et al., 2020). The observed greater diversity of microplastic shapes in holothurians compared to sediments was, also, recorded concerning chemical types. In fact, our study shows a different chemical fingerprint of microparticles recorded in sediments compared to holothurians supporting the hypothesis of active ingestion of plastic fragments.

4.2. Ecological meaning of observed results

Marine Protected Areas (MPA) are of large ecological and economical value (Sanchirico et al., 2002). The institution of MPA shows an increasing trend to target protection of overexploited species in Mediterranean Sea (Lubchenco et al., 2003) representing a buffer zone and a source of migrating species for the near not protected areas (Kelleher, 1999). Nevertheless, MPA proofed to be ineffective to protect from some impacts such as biological invasion by alien species, indirect chemical pollution (Allison et al., 1998), long-range run-off depositions (Lohmann et al., 2007), and coastline (Mille et al., 2007) or shipping (Renzi et al., 2010) derived impacts. No-take, no-access zones (Zone A) showed chemical pollution of sediments (Renzi et al., 2010; Perra et al., 2011; Perra et al., 2013).

Microplastics, small plastic particles with a diameter within 1.0–5000 µm (Barnes et al., 2009), originated directly (Costa et al., 2010) or by fragmentation of larger pieces (JRC EU, 2013) and represent a new source of impact, also, for MPA. Microplastics reach marine environments by accidental releases, geomorphological processes (Wright et al., 2013), direct inputs from agriculture activities (Alomar et al., 2016; Guerranti et al., 2017), cosmetics dispersions (Guerranti and Renzi, 2016), industrial effluents (Maynard, 2006), municipal wastewater effluents (Fendall and Sewell, 2009; Mani et al., 2015; Eerkes-Medrano et al., 2015). Starting from Thompson et al. (2004), microplastics were recorded in from harbours (Reddy et al., 2006; Claessens et al., 2011; Romeo et al., 2015b), designated-MPAs (Fastelli et al., 2016; Renzi et al., 2018a; Renzi et al., 2020), and MPAs (Blašković et al., 2017). Detritivores and filter feeder species ingested microplastics (Graham and Thompson, 2009; Murray and Cowie, 2011; Fossi et al., 2014; Fossi et al., 2016; Renzi et al., 2018a; Renzi et al., 2020). Recent literature reported MPs presence at different levels of the trophic web in marine ecosystems (Setälä et al., 2014; Romeo et al., 2015a). MPs can both to release toxic plastic additives (i.e. phthalates and bisphenol-A) and to adsorb environmental pollutants on their surface (Rochman et al., 2013; Pedà et al., 2016). Blašković et al. (2017) reported that some environmental local factors as well as wind/current ratios and wide/length ratios could affect MPs distribution in sediments. Renzi et al. (2018b), reported the absence of statistical correlations between levels of plastic litter measured in sediments and the abundance of *B. lanceolatum* species or the presence/absence of Mäerl bed habitats. A recent research (Renzi et al., 2018a) showed that the chemical composition of microplastics recorded in sediments and holothurians are related to differences among habitat types tested suggesting possible relations among ecological features and the MPs assessment in marine ecosystems. In this study, tested islands showed a significance difference concerning chemical composition of both sediments and holothurians. The interesting aspect, is represented by the fact that the factor “level of protection” significantly affected chemical composition of both tested matrices. In particular, designated-MPA significantly differs by MPA sampling stations suggesting that the absence of local pollution sources could affect recorded chemical types. This study showed that habitat types affected chemical fingerprints of microparticles recorded in holothurians. In fact, sampling sites located in Open Adriatic Sea are significantly different from SEB (semi-enclosed bay or lagoons) and animals sampled from bottoms characterized by 0% coverage by *P. oceanica* are significantly different from animals collected from 50% of bottoms coverage. The occurrence of significance difference on chemical composition of microplastics related to morphology and bottom coverage by *P. oceanica*, it opens interesting scenario concerning researches on microplastic pollution in aquatic ecosystems suggesting complex dynamics involving benthic species and local habitats that could affect microplastic intakes and routes through the trophic web.

4.3. Methodological remarks

A detailed and standardized protocol for sampling should include the collection of some important environmental and ecological features to allow a better understanding of observed scenario on MPs distribution such as water depth, bottom type, habitat type, coverage by phanerogams, distribution of the oceanographic currents, occurrence and strong of winds, winds direction, human impacts, human use of the marine resource, etc.

Digestion of holothurians represents a critical methodological point to be accurately solved by scientific literature. This paper proposed a specific modification of the preliminary digestion reported by the recent literature on biological tissue of holothurians (Renzi et al., 2018a; Renzi et al., 2020) to better dissolve organic tissue. Nevertheless, used method shall further optimized, because the pre-treatment process is yet time consuming and complex to be performed for large number of samples. It is important to highlight that concerning microplastic, microscopy shall be used only to perform the sorting phases if pre-cleaning and extraction methods do not allow to by-pass this phase. Determinations of microplastics by stereomicroscope shall be avoided due to the impossibility to discriminate plastic by non-synthetic materials on the basis of eye-based analysis at small-levels scale (< 500 μm) as largely documented by recent literature (Wirnkor et al., 2019) showing identification mistakes ranging within 20%–70% (Hidalgo-Ruz et al., 2012; Eriksen et al., 2013). As reported by recent literature, plastic fibres are impossible to be distinguished by natural fibres such as cellulose only by stereomicroscope (Renzi et al., 2019b) and μFT-IR represent the most suitable technique to distinguish and determine chemical composition of microplastics (Wirnkor et al., 2019), and microfibers in particular (Corami et al., 2020). As reported in this study, the occurrence of cellulose and cellulose acetate in natural samples such as sediments and stomach contents is high (ranging within 0–67.9% and 0–33.3% of total items respectively) also after the extraction and pre-cleaning processes performed. The extraction and pre-cleaning method followed by this study shall better optimized to reduce if it will possible manual sorting of targets by stereomicroscope and to improve the cleaning process of recovered particles to reduce time wastes during μFT-IR chemical analyses. Statistical significance recorded in this study should be tested on a larger statistical population of data to assess on a more solid population of data significance of all tested levels for each considered factor.

5. Conclusions

Results obtained in this study concerning levels of plastic litter in sediments and holothurians are consistent to values recorded in Silba and Telašćica by previous studies (Blašković et al., 2017; Renzi et al., 2019a, 2019b). Holothurians are now protected species by the Italian Law n. 156 (27/02/2018, Ministry of Agriculture and Forestry) till 31/12/2019 to avoid human overexploitation and results reported in this study could represent an improvement of knowledge on a threatened species. Further researches focused to better understand factors that could affect microplastic distribution and levels in marine environments could be of significant usefulness to better size sampling protocols focusing on data that should be collected in field.

CRediT authorship contribution statement

Monia Renzi: Conceptualization, Investigation, Writing - review & editing, Supervision, Formal analysis, Funding acquisition. **Andrea Blašković:** Resources, Methodology, Investigation, Data curation, Writing - original draft.

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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