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A novel approach based on multiple fish species and water column compartments in assessing vertical microlitter distribution and composition

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Abstract

The assessment of the distribution and composition of microlitter in the sea is a great challenge. Biological indicators can be an irreplaceable tool since they measure microlitter levels in their environments in a way that is virtually impossible to replicate by direct physical measurements. Furthermore, trends can provide policymakers with statistically robust analysis. We looked into the capacity of multiple fish species to describe the distribution and composition of microlitter vertically across different compartments of the water column. A total of 502 individuals from six selected species (*Scomber scombrus, Oblada melanura, Spicara smaris, Boops boops, Merluccius merluccius and Mullus barbatus*) were collected on the western side of Sardinia island and allocated to three compartments: surface, mid-water and bottom. The species of the surface exhibited a higher frequency of occurrence (41.89%) of microlitter ingestion, compared to those of the mid-water and bottom (19.60%; 22.58%). A significant difference in the average number of ingested microlitter was found between the surface and the bottom compartment. All the microlitter fragments found were analysed through Fourier Transform Infrared Spectroscopy (FTIR). The comparison of the expected buoyancies of the polymers identified puth faith in the allocation of the species to the respective compartments. Therefore, considering the Marine Strategy Framework Directive objective, this approach could be useful in assessing microlitter distribution and composition vertically across the water column.

Keywords

Microplastics, fish, compartments, Mediterranean Sea, bioindicators

1. INTRODUCTION

Marine litter is “any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment” (UNEP, 2009). Various studies have shown that it consists primarily of plastics, mainly due to their continu ously increasing global production (PlasticsEurope, 2015) and the fact that it is virtually immune to environmental degradation (Barnes et al., 2009). Plastics are generally subdivided according to. their size into macroplastics (>25 mm), mesoplastics ($5 < x < 25$ mm) and microplastics (<5 mm) (Thompson et al., 2004; Arthur et al., 2009). Microplastics are further divided into “primary microplastics” (Cole et al., 2011) when they have been purposely manufactured of size less than 5 mm (i.e. microbeads fromcosmetics, hand cleaners and air blast cleaning media; Fendall and Sewell, 2009; Napper et al., 2015) or when they enter the environment already in micrometric size (i.e. microplastics from tire wear and tear, from the washing and wearing of synthetic textiles) (De Falco et al., 2018a, 2020). In contrast, “secondary microplastics” are the result of a progressive fragmentation once introduced into the environment, mainly due to chemical, physical and biological action (Andrady, 2011; Browne et al., 2007; Barnes et al., 2009). Most of the plastics produced have a lower density thanseawater(PlasticEurope, 2015); thus wewouldexpecttofinda prevalence of floating plastics in marine environments that mix withthesurfaceboundarylayer(Kukulkaetal.,2012).Manystudies have noted a great amount of low-density polymers and their persistence in surface waters (Ryan et al., 2009; Goldstein et al., 2013; Eriksen et al., 2014). Nevertheless, the density of virgin plastics can be modified by a plethora of natural processes once they are introduced into marine environments. For example, the density of polymers that reside for a long time at the surface can be altered by solar UV photodegradation reactions, thermal reaction (thermal oxidation), hydrolysis of the polymer and microbial degradationthat causeleachingofadditives(Andrady,1996;Barnes et al., 2009; Browne et al., 2010; Derraik, 2002; Thompson et al., 2004; Kooi et al., 2017) or by biofouling (Harrison et al., 2011; Moret-Ferguson et al., 2010). Moreover, microplastics density, together with particle size and shape, can strongly influence the advection velocity and hence the ability of the particle to reside for a long time at different depths in the water column (Ballent et al., 2012; Enders et al., 2015).

The abundance of plastics in marine environments has been shown to be inversely related with the particle’s size, and micro plastics have been found to be ubiquitous (Thompson et al., 2004; Bergman and Klages, 2012; Galgani et al., 2015). The monitoring of abundance and composition of smaller particles poses quite a lot of challenges since there are a whole variety of sources and pathways that affect their distribution (Browne, 2015). Moreover, currents and wind forces make them migrate over long distances and have been observed to accumulate in large convergence zones (Law et al., 2010; Moret-Ferguson et al., 2010; Lebreton et al., 2012). The five gyres (Moore et al., 2001; Davison and Asch, 2011; Eriksen et al., 2013) are examples of accumulation spots, as is the Medi terranean

Sea, which is a semi-enclosed basin, with an average concentration of 243,854 plastics/km2 in its surface waters, of which 83% are microplastics (Cozar et al., 2015). Different factors govern accumulation on the surface and in the sediments, and we do not always find accumulation of litter in both compartments over the same areas. The sampling tools used to study microlitter include manta trawls and bongo nets for the sea surface and mid water (Doyle et al., 2011; Eriksen et al., 2013; Colton et al., 1974; Moret-Ferguson et al., 2010), while Van Veen, Ekman grabs and various corers have been used to investigate sediment samples (Van Cauwenberghe et al., 2013; Vianello et al., 2013; Pagter et al., 2018; Palatinus et al., 2019). It is important to keep in mind that, although these can be powerful analytical tools, these instruments sample different sections of the water column and microplastics concentrations are presented in relation to the area, volume or length covered. These types of measurements must be considered local and time-dependent (Waldschl€ ager et al., 2020).

The Marine Strategy Framework Directive (MSFD/2008/56/EC), which set out the major contaminant issues related to the marine environment and prioritises the topics to be investigated in order to achieve Good Environmental Status (GES), has made the assessment of plastic ingestion in marine species a research priority. Many species are impacted by marine litter, mainly due to entanglement or ingestion, and the number reported is constantly growing (Gall and Thompson, 2015). A few bioindicators for mac roplastic ingestion have already been adopted and recognised as invaluable tools to measure the amount of litter in their environments and trends can provide policymakers with statistically robust analysis (van Franeker, 1985; van Franeker et al., 2011; Matiddi et al., 2017). Moreover, the search for indicators for microplastic ingestion is still ongoing and various efforts have been made to cover different ecological and biological aspects (Galimany et al., 2009; Fossi et al., 2014; Vandermeersch et al., 2015). In addition to giving crucial information on the distribution, composition and trends, indicators for microplastic ingestion could pro vide guidance on which species to perform further investigations on toxicity (Rochman et al., 2013), chemical transfer (Oliveira et al., 2013; Bakir et al., 2016), biomagnification (Rochman et al., 2013; Lusher, 2015) and bioaccumulation (Besseling et al., 2013; Browne et al., 2013). Microlitter ingestion is currently being assessed in various organisms ranging from invertebrates to vertebrates (Wright et al., 2013; Werner et al., 2016). Just recently fish have started to be investigated for microlitter ingestion and have been recognised as potential indicators for specific aquatic compartments and/or regions (Galgani et al., 2013; UNEP/MAP SPA/RAC, 2018; Bray et al., 2019). To date studies on fish have mainly selected representative species of pelagic and dermesal habitats (Rummel et al., 2016; Guven et al., 2017) and *Sardina pilchardus*, *Platichthys flesus*, *Gadus morhua*, *Scomber scombrus*, *Clupea harengus* are some examples of common species that have been investigated. Therefore, it is probable that once appropriate suitable sentinel species are selected, fish will start to be mandatorily monitored (Fossi et al., 2018), also because they could warn of potential threats to human health (Barboza et al., 2018; Wright and Kelly, 2017).

Recent studies on microlitter ingestion in fish species have been trying to understand how to best investigate and interpret the data in order to help assess microlitter abundance, distribution, composition, fate and impacts. Some studies have compared the ingestion of microlitter with fish feeding behaviour or diet (Peters and Bratton, 2016; Vendel et al., 2017; Mizraji et al., 2017). Others have taken into account the overall habitat use, while most studies have divided the species into demersal (Avio et al., 2015; Bellas et al., 2016; Torre et al., 2016; Güven et al., 2017), mesopelagic (Boerger et al., 2010; Davison and Asch, 2011; Lusher et al., 2016) and pelagic (Deudero and Alomar, 2015; Romeo et al., 2015). Geographical distribution has also been taken into account, for example by considering the species proximity to coastal environments(Nadaletal., 2016; Neves etal., 2015; Battaglia et al., 2016)or by comparing small vs. large scale (Medsealitter project: https:// medsealitter.interreg-med.eu). Recently, the microlitter frequency of ingestion, among multiple fish species, has been proposed as a good proxy to highlight differences between areas (Anastasopulou et al., 2018; Avio et al., 2020). Although the frequency of ingestion for multiple fish species seems to be a good parameter to evaluate abundances of microlitter across areas, much remains to be done to assess the different accumulation patterns across compartments of the water column.

Thus, the objective of the present study is to evaluate the capacity of multiple indicator fish species to describe the distribution and composition of microlitter vertically across the water column. In order to do so, the species fidelity to three compartments (sur face, mid-water and bottom) was taken into consideration. This study was conducted with the intention of further supporting the planned actions for implementing the MSFD.

2. MATERIAL AND METHODS

2.1. *Study area*

This study was carried out in Sardinian waters (Western Mediterranean Sea) which are part of the Geographical Sub-Area11(GSA 11) identified by the FAO's General Fisheries Commission for the Mediterranean (GFCM) and in the middle of the Western Medi terranean Sea sub-region (MSFD). Fish samples were collected over a period of 3 years (2017e2019) from local fisherman and fishing in f irst-hand in an area comprised between the Gulf of Oristano (central west of Sardinia) and the Gulf of Cagliari (south of 2 Sardinia). The area presents heterogeneous fishing grounds (Sabatinietal.,2013) and is characterised by a 25 km wide continental shelf (De Falco et al., 2015), where the main water mass present in the area is the Modified Atlantic Water (MAW).

2.2. Sample collection in relation to the ecology and assignment to compartments

The choice of the species was based on a first evaluation of the most common and easily available fish species (landings) in the area. Among these, we chose the species that had an appropriate spatial coverage of the area, and on which we had good amount of information on the ecology and biology of the species. Moreover, importance was given to species where microplastics ingestion had been observed in the past, *Boops boops,* for example, has already been proposed by Tsangaris et al (2020) to assess microplastic ingestion in the Mediterranean Sea. Only one species, *Oblada melanura*, was selected following personal observations and preliminary analysis on microplastic ingestion, even if, to our knowledge, this is the first record for this species.

Literature research was done in order to understand the feeding behaviour and deduce when was best to collect the species in order to be representative of the compartment they exploited. The mackerel *Scomber scombrus* is a pelagic-neritic planktivorous species that was collected during summer when it came closer to the coast. Jansen et al. (2019) showed, by comparing the zooplankton distribution and composition, that there was no evidence during this time of the year that they fed below the mixed layer since they simply shifted between different types of prey as they become progressively available in the mixed surface layer. The seabream *Oblada melanura* is a benthopelagic omnivorous species which feeds mainly on copepods (Pallaoro et al. 2003) and was considered representative of surface coastal environments (Bauchot and Hureau 1986) because of its opportunistic predator behaviour (Pallaoro et al. 2003), that takes place at the surface during daytime in the summer season (Pers. Obs.). The bogue *Boops boops* is a demersal omnivore fish that feeds on benthic (Crustacea, Mollusca, Anellida, Sipuncula, Plantae) and pelagic preys (Siphonophorae, Copepoda, eggs) (Derbal & Kara 2008). This gregarious can be found on the continental shelf in the summer, and it feeds across the water column, generally ascending to the surface (El-maremie & El-mor 2015). The picarel *Spicara smaris* is a pelagic-neritic species and is generally observed in open waters feeding on copepods, other crustaceans, fish eggs and larvae during summer (Vidalis, 1994; Karachle & Stergiou 2014) and is therefore considered a good species to represent the middle of the water column. The European hake *Merluccius merluccius* is a demersal predator species, and the young typically feed on crustaceans and small fish close to the sea bottom during daytime (Alheit and Pitcher, 1995; Buchholz et al., 1995; Carpentieri et al., 2005). We therefore collected only juveniles <25 cm in length (Ungaro et al 1993). The red mullet *Mullus barbatus* is a demersal species that feeds typically on zoobenthos such as crustaceans, worms and molluscs (Mahmoud et al., 2017). All the individuals collected that presented signs of stomach eversion or of net feeding were discarded. Additional information for each species is presented in Table1.

TABLE 1 | Fish species, compartments, numbers, length, weight and relative abundances of litter in gut contents

Species	Compartment	No. fish dissected	Average length (cm) (± SE)	Average weight (g) (± SE)	No. with litter	% with litter
Scomber scombrus	Surface	65	27.22±1.98	213.13 ± 48.38	32	49.23
Oblada melanura	Surface	83	20±3.22	136.82 ± 62.42	30	36.14
Spicara smaris	Mid-Water	89	14.19±1.41	29.93 ± 5.28	7	7.87
Boops boops	Mid-Water	110	17.78±1.98	54.42 ± 0.91	32	29.09
Merluccius merluccius	Bottom	66	22.25±2.01	112.23 ± 21	15	22.73
Mullus barbatus	Bottom	89	14.44±1.30	40.31 ± 1.53	20	22.47
Total		502			136	27.29

2.3. Laboratory analysis

All procedures were developed following the indications of the "Harmonized protocol for monitoring microplastics in biota" (BASEMAN Project). Fish were collected once landed and frozen at -20°C. In the laboratory, each fish was individually measured (fork length; cm), weighed (second decimal point; g) and finally dissected on a metal tray in order to extract the entire gastrointestinal tract (Claessens et al 2013; Lusher et al 2013; Rocha-Santos and Duarte 2015; Bessa et al., 2018). At this stage, also the sex and the weight (g) of the gastrointestinal tract (GIT) were recorded. The extraction of microplastics from biological matrixes with H_2O_2 is one of the most widely employed methods (Renner et al 2018), it is efficient for the successful extraction of most polymers (Hamm et al 2018) and is fast and cost-effective (Collard et al 2015; Tagg et al 2017). Therefore, individual GIT were placed into glass beakers (500 ml) and 15% H_2O_2 1:20 (w/v) was added in order to digest the organic matter (Nuelle et al 2013; Mathalon & Hill 2014; Avio et al 2015), keeping them at room temperature (~25-30 °C) for maximum five days. If the exothermic reaction ended before all the organic matter was digested, an extra 1-2 ml of 15% H_2O_2 was topped up. It is important to point out that the digestion method adopted works well with small GIT tracts, such as in the case of the selected species, which weighed up to 20 g max. Once the organic material had been removed, the solution was filtered onto 100 µm sieve (Giuliani steel sieves). This size of mesh was considered to be an appropriate detection limit for identifying microplastics (down to 100 µm) with confidence (Markic et al 2018). Moreover, it allowed easy handling, further analysis processing (optical microscopy, FTIR spectroscopy) and is a meaningful size to make comparisons with most of the literature. Small aliquots of digested matter were positioned onto multiple sieves (all of 100 µm mesh) and covered immediately with a petri dish in order to be observed under a stereoscopic microscope (Carl Zeiss Micro-imaging GmbH) equipped with image analysis system (AxioCam ERc5s and Zen 2014 Blue edition software) (Lusher et al 2013; Goldstein & Goodwin, 2013 and Murray & Cowie, 2011). While observing at the stereoscopic microscope, an item was considered to be a microplastics if no cellular or organic structure was visible and it was homogeneously coloured (Hidalgo-Ruzetal.,2012;Primpkeetal.,2020). Fine-tipped tweezers were used to position the detected microplastics (> 0.1 mm) into individual glass Petri dishes (MSFD-TSGML, 2013; Lusher et al 2013; Rocha-Santos and Duarte, 2015). All suspected microlitter items were photographed, and the maximum length was measured by means of image analysis. In the case of fibres that presented bendings, the length was estimated when possible. Colours (white, grey, black, red, orange, pink, purple, blue, light blue, green, transparent, multi-colour) and shapes (circular, angular, spherical, flat, irregular and cylindrical) were recorded. Finally, the items were subdivided into typologies, according to Hidalgo-Ruz et al (2012) as fragment, film, sphere, rope/filament, sponge/foam and fibre. Pellets and microspheres have been grouped into the category "spheres" since rarely the two aforementioned categories have been found in the gut of fish.

Airborne contamination has been recognised as an important parameter to monitor while performing any study involving synthetic microlitters. Therefore, laboratory atmospheric deposition was monitored to obtain an estimation of the level of potential airborne contamination. Aside from this initial evaluation, other precautions were also taken during dissection, extraction, sorting and visual identification such as: wearing a cotton laboratory coat, cleaning all surfaces and material with alcohol, covering the samples at all times during analysis and clean filters were positioned while analysing samples to collect eventual atmospheric microplastics created during laboratory procedures. Although we found very few fibres in our blanks, we excluded all the fibres from the samples when the relative control presented them.

2.4. FTIR analysis

Fragments isolated during the visual examination, by optical microscopy, were analysed by using FTIR spectroscopy. In detail, 60 fragments out of the total 70 were chemically characterised through FTIR while the remaining fragments were too small or lost during manipulation.

FTIR spectra of fragments were recorded at room temperature by means of a Perkin Elmer Spectrum Frontier spectrometer (Waltham, MA, USA), equipped with an attenuated total reflectance accessory (ATR), over the range 4000–650 cm^{-1}, at a resolution of 4 cm^{-1} and 4 scans were averaged for each sample. The spectra obtained were compared to multiple spectral databases, both commercial (i.e.Hummel Polymer and additives, Aldrich Polymers, and others) and custom-built (BASEMAN project; siMPle, 2019) (Meyns et al.,2019;Rist et al.,2020).

2.5. Optical microscopy analysis

Regarding fibres,43 out of the overall 147 fibres, recovered as reported above, were analysed using a Leica M205 FA light microscope (Leica Microsystem, Wetzlar, Germany). The morphological features of fibres allow their discrimination between synthetic and natural or artificial ones. In fact, cotton fibres present convolutions with the typical twisted ribbon form; wool fibres present cuticular scale patterns; the artificial fibre rayon is smooth and straight but marked by striations; synthetic fibres present uniform and regular thick with a smooth surface and a shape similar to a long thin cylinder (Cook, 2001; Houck, 2009). According to these characteristics, the morphological features of the observed fibres were used to classify them in synthetic or natural based fibres, including in this last case fibres having a morphology typical of natural or artificial fibres.

2.6. *Frequency, rate, and statistical analysis*

The particles found in species for each individual fish allowed for “frequency of occurrence” (number of fish that ingested microlitter/total number of fish dissected) and the “encounter rate” (total number of microlitter particles ingested/number of fish dissected) to be calculated. Univariate two-way permutational analysis of variance (Permanova) was used to check for any significant difference in the average number of microlitter ingested, among the factors compartment and species, for all samples (individuals). For all the analysis performed, the compartment was considered a fixed factor, while the species were random and nested in compartment. The same analysis was performed by considering only the fish that presented microlitter ingestion. A multivariate two-way Permanova was used to check for differences for the typologies identified among compartments and the species in compartments, considering only individuals with microplastics. Finally, a univariate two-way Permanova was used to test for differences by considering only fibres. These tests are based on Euclidean distance for univariate, and Bray Curtis for multivariate and each term is analysed through 9999 random permutations and associated with a Monte Carlo test (Andersonetal.,2008).

3. RESULTS

A total of 502 individuals (six species) were collected between 2017-2019, 148 individuals were assigned to the surface, 199 to mid-water and 155 to bottom (Table 1). The overall frequency of occurrence (FO) of microliter for the six species was 27.29 %. While, if we consider the single compartments, the surface had a FO of 41.89%, mid-water 19.60% and bottom 22.58%. The highest FO was found for *S. scombrus* (49.23 %) and the lowest for *S. smaris* (7.87 %; Table 1). The highest encounter rate was found in the “surface” compartment where *S. scombrus* and *O. melanura* displayed 1.06 and 0.93 (Table 2).

TABLE 2 | Species, sex, GIT weight, number of litter particles, size and encounter rate for the species analysed

Species	% males	% females	GIT weight (g) (± SE)	No. litter particles	Size range (mm)	Encounter rate
Scomber scombrus	33.33	66.67	12.40 ± 5.33	69	0.345 - 19	1.06
Oblada melanura	49.25	50.75	5.56 ± 2.83	77	0.208 - 7.537	0.93
Spicara smaris	45.95	54.05	1.18 ± 0.71	9	0.175 - 3.664	0.10
Boops boops	37.27	62.73	2.45 ± 0.91	48	0.257 - 5.176	0.44
Merluccius merluccius	45	55	5.75 ± 3.09	21	0.396 - 2.946	0.32
Mullus barbatus	59.46	40.54	2.76 ± 1.53	36	0.102 - 14.742	0.40
Total				260		

A significant difference in the average number of ingested microplastics (considering all individuals) was found between compartments (p=0.0473), as well as for the species contained within compartments (p=0.004) (Fig. 1; Table 3). The resultant pairwise test showed a significant difference between surface and bottom compartment (p=0.0067) and among *S. smaris* and *B. boops* in the mid-water compartment (p=0.0002) (Table 3).

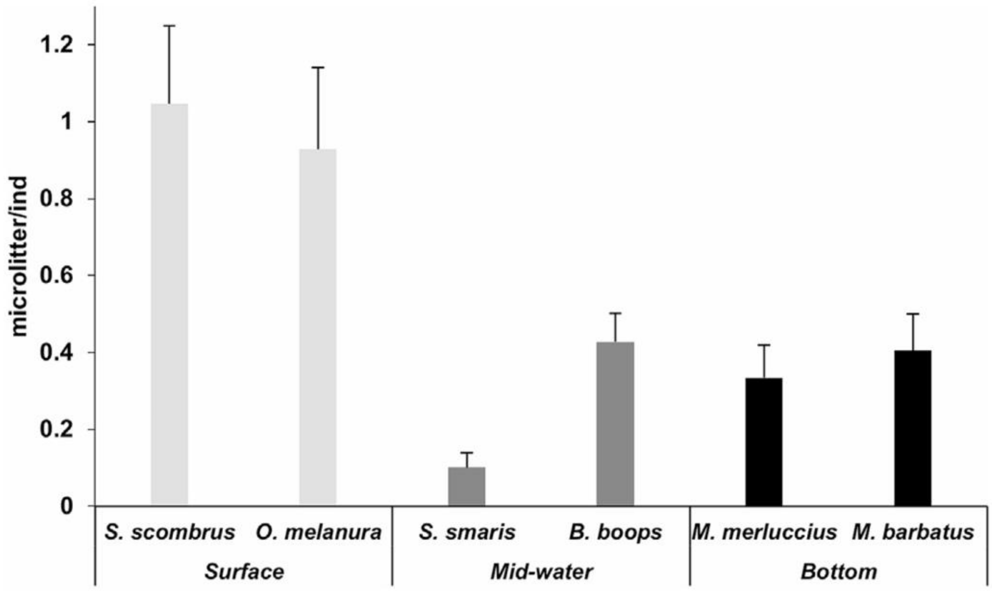

Fig 1. Average number of litter particles (±SE) found in species divided into compartments

TABLE 3 | Statistical analysis performed

Analysis and variables	Source of variation	df	SS	MS	Pseudo-F	p(MC)	Pairwise test
Univariate two-way PERMANOVA	Compartments	2	5.10*E^10	2.5119*E^10	3.2883	**0.0473**	Surface ≠ Bottom p<0.01
N of MP considering all individuals	Species	3	2.34*E^10	7.812*E^9	3.2561	**0.004**	*S. smaris ≠ B. boops* p<0.001
	Res	496	1.19*E^12	2.40*E^9			
	Total	501	1.26*E^12				
			Transform	Fourth root			
Univariate two-way PERMANOVA	Compartments	2	7.18*E^7	3.5905*E^7	3.0228	0.0601	
N of MP considering only individuals with MP	Species	3	3.51*E^7	1.169.8*E^7	0.84181	0.5375	
	Res	131	1.82*E^9	1.39*E^7			
	Total	136	193*E^9				
			Transform	Square root			
Multivariate two-way PERMANOVA	Compartments	2	994*E^7	4.9697*E^7	0.68169	0.6669	*S. scombrus ≠ O. melanura* p<0.0001
Typologies considering only individuals with MP	Species	3	235*E^8	7.8292*E^7	6.3163	**0.0001**	
	Res	131	1.62*E^9	1.24*E^7			
	Total	136	1.96*E^9				
			Transform	Square root			
Univariate two-way PERMANOVA	Compartments	2	6.85*E^7	3.43*E^7	0.44146	0.7947	*S. scombrus ≠ O. melanura* p<0.0001
Fibres considering all individuals	Species	3	2.50*E^8	8.34*E^7	6.7346	**0.0001**	
	Res	131	1.62*E^9	1.24*E^7			
	Total	136	1.93*E^9				
			Transform	Square root			

In total 260 litter particles were found in 136 individuals. Considering compartments, 146 items were found in the surface, 57 in mid-water and 57 in bottom. The number of particles found per species are shown in Table 2. If we consider only the fish that ingested microplastics, the average number of microplastics per specie was: (mean ± SE) 2.16±0.32 *S. scombrus,* 2.57±0.45 *O. melanura,* 1.47±0.13 *B. boops,* 1.28±0.17 *S. smaris,* 1.4±0.2 *M. merluccius* and 1.8±0.24 *M. barbatus*. No significant difference was shown, by comparing these values, among compartments (p=0.06; Tab 3) and the species (p=0.53; Table 3). The size range of the litter particles found went from a minimum of 102 µm (*M. barbatus*) to a maximum of 19 mm (*S. scombrus*; Table 2). Particles were subdivided into size ranges (0.5 bins in size distribution) and the two most frequent size ranges found were the 1-1.5 and the 1.5-2 mm (Fig. 2).

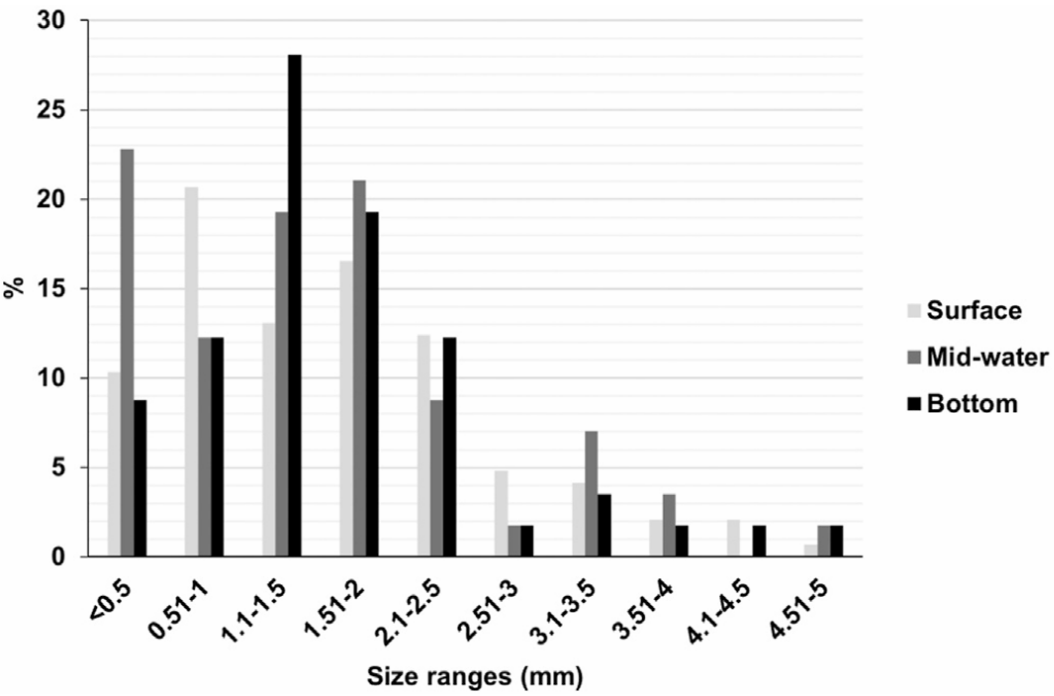

Fig. 2. Size frequency distribution of microlitter per compartment (surface, mid-water and bottom).

The typologies found, overall, distributed as follows: 147 fibres (56.53%), 71 fragments (27.31%), 26 filaments (10%), 9 films (3.46%), 4 sponges (1.54%) and 3 spheres (1.15%). In the surface we found: 71 fibres (48.63%), 45 fragments (30.82 %), 15 filaments (10.27 %), 9 films (6.16 %), 4 sponges (2.74 %) and 3 spheres (1.37). In mid-water: 33 fibres (57.89 %), 21 fragments (36.84 %), and 3 filaments (5.26 %). While in the bottom: 43 fibres (75.44 %), 8 filaments (14.03 %), 5 fragments (8.77 %), and 1 sphere (1.75 %). The average number of typologies found per species are shown in figure 3. No significant difference was observed for typologies distribution among compartments (p=0.70), although a significant difference was observed between species within the same compartment (p=0.0001), specifically between *S. scombrus* and *O. melanura* (p=0.0001) in the "surface" compartment (Fig. 3, Tab. 3). By considering only fibres no significant difference was observed between compartments (p=0.80; Tab 3), while a significant difference was noted for species within compartments (p=0.0001; Tab 3). We conducted the pairwise test and found that *S. scombrus* was different from *O. melanura* (p=0.0001; Tab 3).

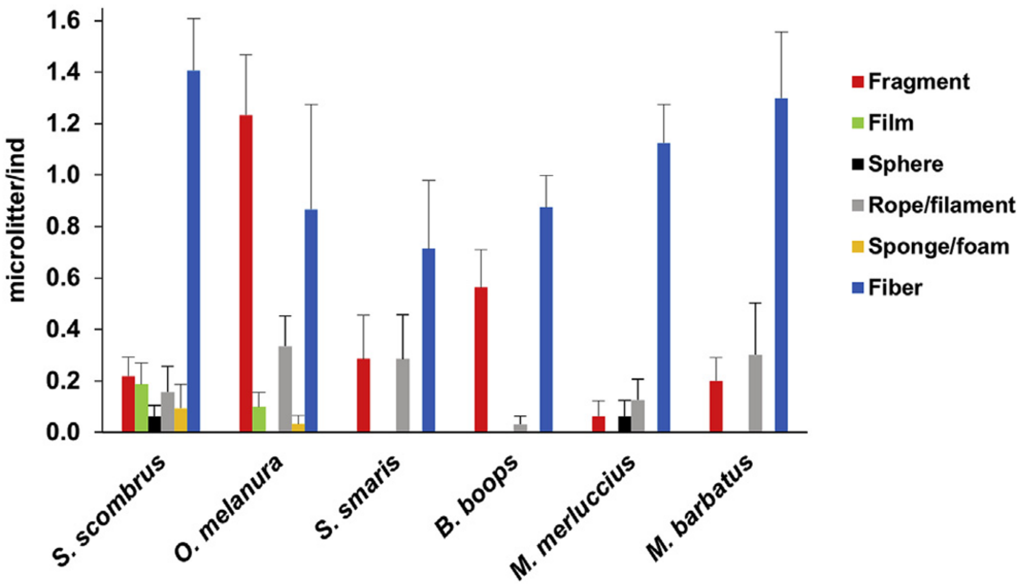

Fig. 3. Average number of microlitter (±SE) found in species divided into typologies.

Overall, the items presented the following morphology percentages: cylindrical 68.85%, irregular 29.23%, circular 0.77%, angular 0.77% and spherical 0.38%. In total 12 colour types were identified (white, grey, black, red, orange, pink, purple, blue, light blue, green, transparent and multi-colour): 11 in the surface compartment, 7 in mid-water and 8 in bottom (Fig. 4). Of these *S. scombrus* presented 10 (all colours apart from grey), *O. melanura* 10 (all colours apart from grey), *S. smaris* 3 (blue, green and transparent), *B. boops* 7 (white, black, red, blue, light blue, green and transparent), *M. merluccius* 4 (black, blue, green and transparent) and *M. barbatus* 8 (white, grey, black, red, blue, light blue, green and transparent).

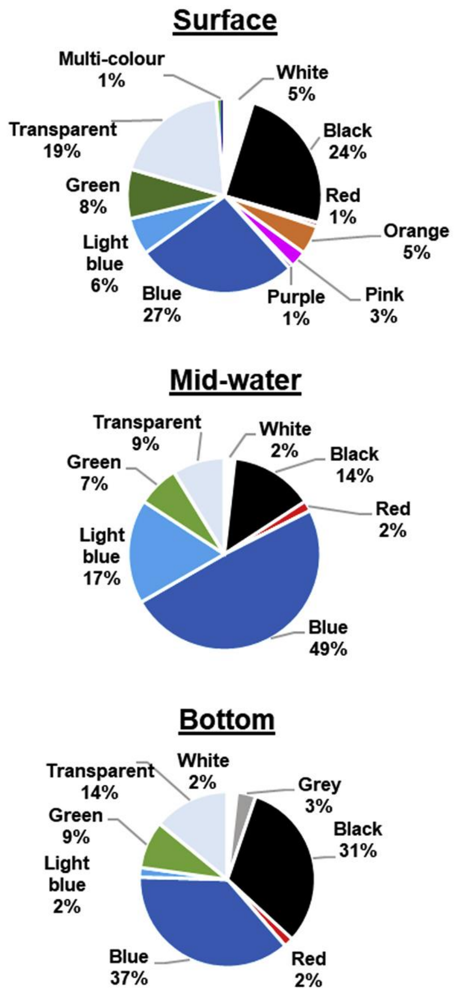

Fig. 4. Colour categories found in gastrointestinal tract of fish according to the compartment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.

The FTIR analysis allowed the identification of different types of synthetic polymer such as: polypropylene (PP), polyethylene (PE), polyurethane (PUR), polyester (PES). Some examples are reported in figure 5.

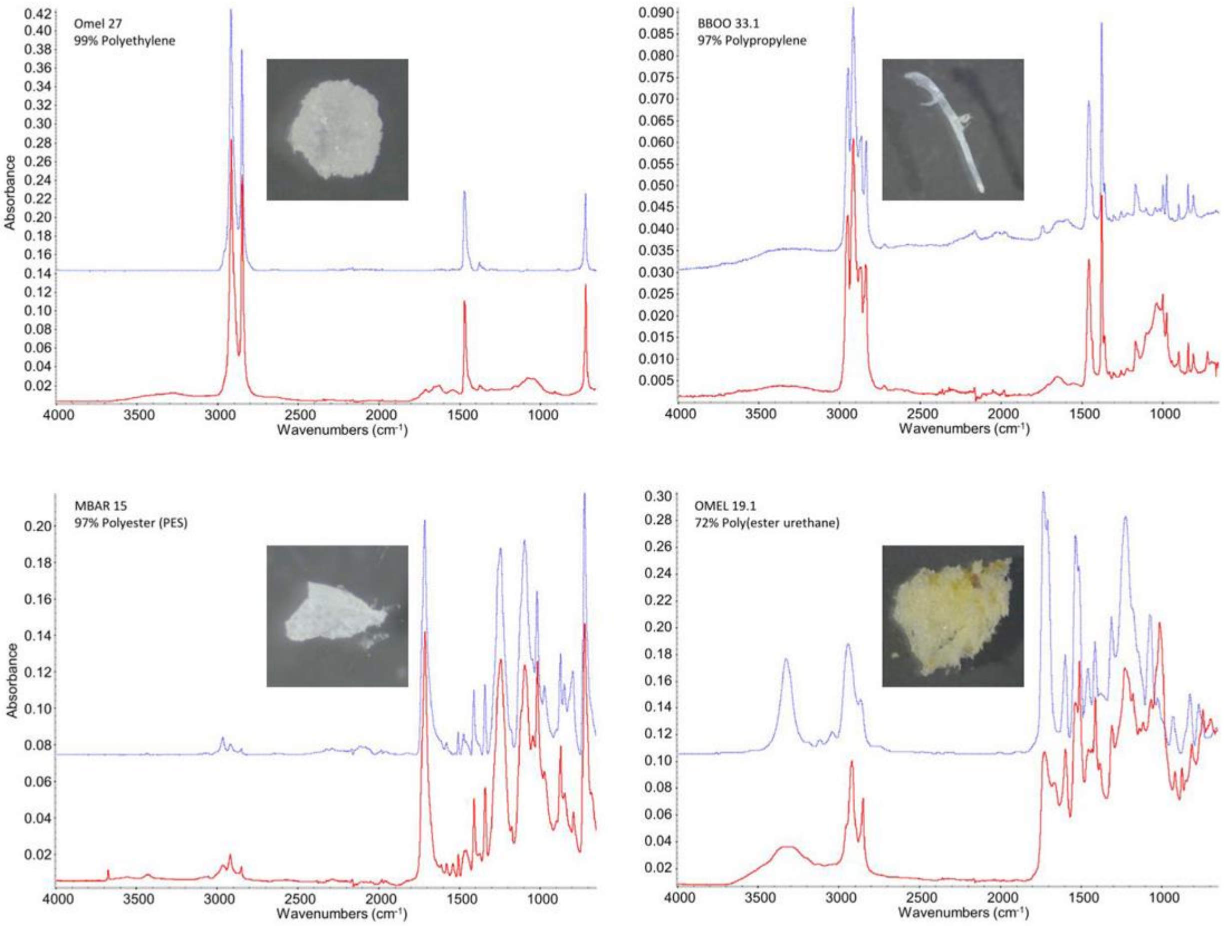

Fig. 5. FTIR spectra (red line) of some fragments recovered from fish, and reference spectra highlighting the best match used for identification (blue dashed line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In addition, other polymeric fragments were detected, but since their chemical composition was not clearly identified, they were classified as "others" for the pourpose of the article. Morphological analysis by optical microscopy allowed the detection of synthetic fibres and natural based fibers. This last were mainly constituted by cellulose based fibres, of natural or artificial origin.

In the surface compartment we found 4 polymers (PP, PE, PUR, PES), 2 polymeric fragments classified as others, 4 synthetic and 1 cellulose based fibres. In mid-water 2 polymers (PP, PE), 1 other , 2 synthetic and 7 cellulose based fibres. Finally in the bottom compartment we encountered 1 polymer (PES), 11 synthetic and 18 cellulose based fibres. Polymer fragments, synthetic and celluloe based fibres are presented in Fig. 6.

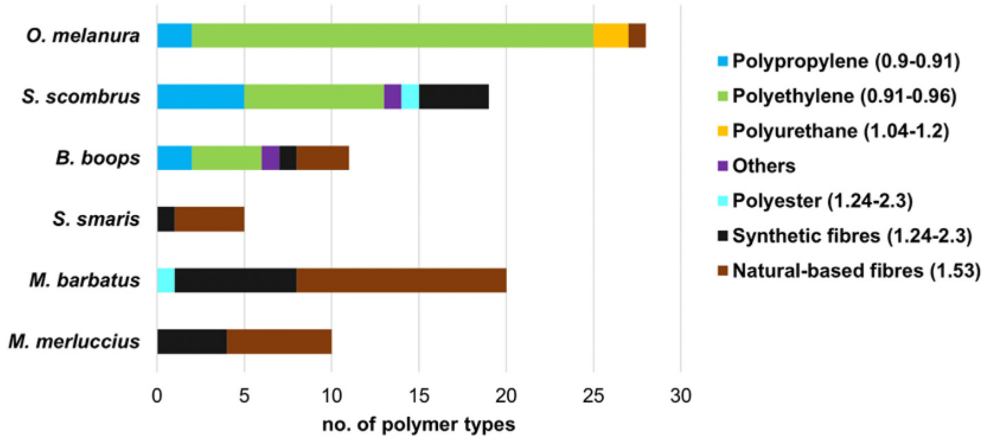

Fig. 6. Number of items per polymer types and species analysed through FTIR spectroscopy

From the figure, it is evident that the species from the surface compartment presented a wider range of polymers compared to the other two compartments. Moreover, by looking into the polymer densities (Enders et al 2015; Li et al 2016; Andrady et al 2017) of the items found in our fish and comparing them with the sea-water density (1.02-1.07 g/cm^3), we noticed that 82 % in the surface, 37.5 % in mid-water and none in bottom of the items considered were positively buoyant. In contrast, 18 % at the surface, 62.5 % in mid-water and 100 % in the bottom were negatively buoyant.

4. DISCUSSION

Our results show that by selecting multiple fish species and considering their biology and ecology, we can assign them to compartments of the water column and therefore assess microlitter distribution and composition, providing a more detailed and comprehensive picture of the potential threats posed to the marine communities that inhabit different compartments. Microlitter and particularly microplastics distribution in a vertical sense through the water column firstly depends on intrinsic factors, such as the particle's density, size and shape, which all together influence its advection velocity (Ballent et al 2012; Enders et al 2015). Secondly, it is influenced by extrinsic processes like wind-induced turbulence, which can cause, for example, breaking surface waves (Kukulka et al 2012). The distribution of microplastics throughout the water column has commonly been studied by taking water samples, generally pointing out high concentrations in the upper layers and an exponential decrease with depth (Goldstein et al 2013; Reisser et al 2013; Rios-Fuster et al 2019). Regarding the spatial distribution of microplastics on the surface, gyres have been recognised as zones of convergence and accumulation (Law et al 2010; Eriksen et al 2013; Goldstein et al 2013), as well as closed bays, gulfs, watersheds and seas surrounded by densely populated coastlines such as in the case of the Mediterranean Sea (Reisser et al 2013; Collignon et al 2012).

The seafloor also concentrates microplastics in great amounts, so much that has been proposed to be a major sink for microplastics debris (Woodall et al 2014). This idea is likely to be true if we consider processes such as weathering (Hidalgo-Ruz et al. 2012; Andrady 2015), biofouling (Holmström, 1975; Ye and Andrady, 1991), entanglement with planktonic aggregates (Long et al 2015) and transfer via plankton faecal pellets (Cole et al 2013) that make even the buoyant particles sink to the bottom. As for the surface, also on the seafloor accumulation happens due to external processes and generally seems to happen when debris becomes entrapped in areas of low circulation where sediments are already accumulating (Galgani et al 1996; Schlining et al 2013; Pham et al 2014). Therefore, given this extreme complexity in the distribution of microplastics both vertically and horizontally across the water column, it can be an irreplaceable tool to use multiple fish species to monitor compartments of the water column in space and time. Another advantage presented by using multiple fish species is that the average number of microlitter items ingested per individual is very constant in the literature of fish species, generally presenting between 1-2 items/individual (Davison & Asch 2011; Lusher et al 2013; Avio et al 2015; Bellas et al 2016; Hemsen et al 2017; Avio et al 2020). Even our study showed that the fraction of fish that ingest microlitter seems to uptake particles at a rather constant rate, that transcends compartments and species.

Considering all the above, we decided to choose multiple fish species that complement each other (Bonanno & Orlando-Bonaca 2018) and have allocated them according to their prevalent use of 3 compartments (surface, mid-water, bottom) of the water column. In order to do so, various factors have been taken into account, such as the biology and ecology, as well as the time of year in which they were sampled. This has allowed us to create adequate pollution indicator fish groups that can be descriptive of the state of the different compartments in relation to the pollution by microlitter. According to the expected buoyancies (Enders et al 2015) of the polymers we found, it gives confidence to the choice of species and the following allocation to the respective compartments. The reason why we found negatively buoyant particles in the surface can be explained by the fact that most of these items were fibres. Since this typology of microlitter has already been proposed empirically to have a slower vertical advection velocity, therefore contributing to their longer residence time in the upper layers of the water column (Ballent et al 2012; Reisser et al 2013).

Our study area is located in the middle of the western side of the Mediterranean basin, which has been proposed to be among the most impacted regions of the world concerning microplastics pollution in surface waters (Cozar et al 2015; Faure et al 2015; Suaria et al 2016) and models predict some of the highest concentrations of floating plastics (Lebreton et al 2012). To our knowledge, there are still no studies on microlitter distribution in sediments on the western side of the island. Although, a recent study (Soto-Navarro et al 2020), based on the realistic distribution of marine litter sources, produced a 3D simulation on microplastics distribution and accumulation (making previsions based on the density: floating, neutral and sinking), where they found that sinking particles remained very close to where they were released. As a result, this area, having a low population density (1.639.591

ha; ISTAT 2018) and modest river outlets, did not present any sinking microplastics in the model outcome. Moreover, the same model (neutrally buoyant particles) and de Lucia (2018) measured the distribution of microplastics in levels below the surface and found medium to low values compared to the other areas they studied. Our multiple fish species divided into compartments collected on the western side of the island of Sardinia seem to confirm these expectations since we found a higher FO in the surface compartment. Moreover, the species from the surface compartment (*S. scombrus* and *O. melanura*) showed an average number of ingested microplastics, among all individuals, that was significantly higher compared to the species of the bottom compartment (*M. merluccius* and *M. barbatus*). Most studies that have sampled the surface of the sea directly, but when dividing the items into typologies, have decided to exclude fibres because of concern for airborne contamination (Suaria et al 2016; Faure et al 2015). This type of contamination can be limited by carrying out extraction and analysis in a laboratory such as in our case. By considering all the typologies of debris found and comparing them among the three compartments, they appeared to be distributed homogeneously throughout the water column, and fibres were the dominant type encountered (56.53%), as in many other studies on microlitter ingestion in fish (Avio et al 2015; Bellas et al 2016; Peters et al 2017; Ory et al 2018; Compa et al 2018). Browne et al (2011) suggested that washing machine's wastewater are the main source of fibres to marine environments. De Falco et al. 2018b found that polyester fabrics release, during a simulated washing test, is about 1733000 microfibres per kg of washed fabric. More recently, De Falco et al 2019 showed that microfibres released during washing range from 124 to 308 mg/Kg of washed fabric, that corresponds to 640.000-1.500.000 microfibres. Bearing that in mind, it is likely that the Mediterranean Sea, which it is a semi-enclosed basin with a slow water exchange (Millot & Taupier-Letage 2005), would present very high fibre-based pollution. Therefore, it is important to consider them when analysing microlitter ingestion in fish, so to assess their distribution in different areas, although they probably disguise and prevent a correct comparison of the other typologies among compartments.

Properties of microlitter such as density and typology seem to be critical factors to consider when looking for appropriate fish bioindicator species. Therefore, grouping multiple fish species according to their usage of defined compartments may be a good solution to describe the distribution of this type of pollutant and could become a valuable approach to integrate within the MSFD under Descriptor 10. Studies up to today have generally analysed the presence of microlitter in the gut of fish in relation to their ecology by selecting one species (Tanaka & Takada 2016; Alomar et al 2017; Pellini et al 2018; Cardozo et al 2018; Sbrana et al 2020) or by subdividing multiple species according to the habitat use: pelagic, mesopelagic, benthopelagic, demersal, benthic (Phillips & Bonner 2015; Rummel et al 2016; Guven et al 2017; Jabeen et al 2017; Murphy et al 2017; Lusher et al 2013; Neves et al 2015; Bellas et al 2016). This information is very useful, and by making appropriate considerations on the compartment's use and the time of year in which they were sampled, we may be able to evaluate microlitter pollution of compartments across different areas and further contribute to provide a more comprehensive mapping of microlitter distribution. Moreover, by extending the number of species per compartment, we may be able to increase our accuracy and add other valuable information. For example, we observed a significant difference of typologies ingested, in the surface compartment, between *S. scombrus* and *O. melanura*. This finding, for instance, can be explained by the different scale at which the two species uptake microlitter from the environment. *S. scombrus* is a fast-moving carnivorous predator that shows a prevalence of fibres, while *O. melanura* has a quite strong site fidelity and an opportunistic feeding behaviour thus ingesting more fragments. These assessments may be useful to show which items have a broader or a more local impact, thus supporting the development of necessary mitigation measures. For example, multiple fish subdivided into compartments could have a role in accelerating the development of solutions to stop the discharge of staggering amounts of microlitter to marine environments. This study was conducted over an area with a wide continental shelf. Therefore, in the future, it could be interesting to investigate if compartments together with multiple fish species can be a valuable approach to study distribution and composition of microlitter also in areas beyond the continental shelf, as well as other environments, such as lakes for example.

5. Conclusions

We found microlitter in all the six species and three compartments analysed. The surface exhibited the highest values for our area suggesting that, indeed, appropriately selected fish species grouped into compartments can be an extremely important tool, since they can continuously integrate microlitter levels in their environments in a way that is virtually impossible to replicate by direct physical measurements. Therefore, these pollution indicator fish groups can potentially give us a better understanding of distribution, characterisation, fate and accumulation of microlitter across compartments as well as infer on the possible ecological implications. This approach is well in line with the recent MSFD objectives and would enable the development of adequate mitigation strategies.

Declaration of interest

We confirm that there are no known conflicts of interest associated with this work, and there has been no significant financial support for this work that could have influenced its outcome. We confirmed that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. Due care has been taken to ensure the integrity of the work. No part of this paper has been published or submitted elsewhere.

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Compliance with ethical standards

The authors declare that they have no conflict of interest.

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