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Microplastics in the Deep: Comparing Dietary and Plastic Ingestion Data between Two Mediterranean Bathyal Opportunistic Feeder Species, *Galeus melastomus*, Rafinesque, 1810 and *Coelorinchus caelorhincus* (Risso, 1810), through Stomach Content Analysis

Umberto Scacco [1,2,*], Emanuele Mancini [3,4], Federica Marcucci [2] and Francesco Tiralongo [3,5]

1 National Centre of Laboratories, Biology (CN-LAB-BIO), Italian Institute for Environmental Protection and Research (ISPRA), 00128 Rome, Italy
2 Department of Marine Eco-Biology (DEBM), University of Tuscia, 01100 Viterbo, Italy; federicamarcucci93@gmail.com
3 Ente Fauna Marina Mediterranea, Scientific Organization for Research and Conservation of Marine Biodiversity, 96012 Avola, Italy; e.mancini@unitus.it (E.M.); francesco.tiralongo@unict.it (F.T.)
4 Italian Fishery Research and Studies Center, 00184 Rome, Italy
5 Department of Biological, Geological and Environmental Sciences, University of Catania, 95129 Catania, Italy
* Correspondence: umberto.scacco@isprambiente.it

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Abstract: Marine plastic pollution is currently an issue of mounting concern around the world. Stomach content of marine fish has been increasingly used as a valid proxy for detecting the presence of such a pollutant in marine biota, both for coastal and deep-water environments. Although ingestion of microplastics has been reported in an increasing number of species, the patterns of ingestion still remain unclear, depending closely on the interaction between the species and types of microplastics involved. In this context, we analysed and compared the stomach contents of two bathyal dwelling opportunistic feeder species namely *Galeus melastomus* and *Coelorinchus caelorhincus*. In particular, we analysed microplastic items according to their dimension, morphology and colour, and diet's variation with size obtained through prey identification. Both species showed a higher frequency of occurrence of the blue filament-like middle-sized microplastics (1.01–4.75 mm) compared with the other categories, although this pattern was much more marked in *C. caelorhincus* than in *G. melastomus*. The latter conversely showed a larger array of ingested plastic items in terms of shape and colour. Matching plastic ingestion with dietary data suggested potential predator confusion occurring in *C. caelorhincus* through active mis-selection of a defined type of microplastic instead of some particular family of polychaetes, which resemble in shape, size, and color to that type. Otherwise, *G. melastomus* appeared more prone to a random ingestion of a larger array of microplastic items because of a more generalistic and less selective feeding strategy. Although further validation is needed, stomach contents of the two species showed evidence strong enough to be considered as potential bioindicator species of microplastic pollution, as required by the Marine Strategy Framework Directive for monitoring this pollutant in the marine environment.

Keywords: *Coelorinchus caelorhincus*; *Galeus melastomus*; microplastics; bio-indicator; feeding strategy; Mediterranean

1. Introduction

As a proof of their ubiquity, microplastics, and their fine fragmentation stadium (nanoplastics), were found even in human tissues and fluids [1]. Such a pollutant is nowadays bioavailable to vascular plants [2] and seriously affects aquatic ecosystems [3,4]. Marine environment's contamination is even worst, as microplastics tend to accumulate right there. In fact, they are introduced directly, through shipping and fishing activities, or indirectly, through fluvial and aeolian systems from the terrestrial environment [5].

Microplastics can be classified as primary or secondary. In the first case, they are either manufactured (e.g., blasting media, microbeads in cosmetics, plastic fibers). On the other hand, secondary microplastics are derived from the fragmentation of larger plastic products [6,7]. Plastic waste was first documented in the marine environment in 1970s and microplastics were instead recorded in 1972 on the surface of the Sargasso Sea [8]. Since then, it has become increasingly clear how plastic interaction also produces a deleterious effect on marine wildlife, particularly turtles and marine mammals [9–11].

Deepened and detailed descriptions on the negative consequences of the interaction between macro and microplastics and marine life have been surprisingly increasing in recent years. Between plastic debris, the microplastics represent the fractions potentially more dangerous for marine ecosystems [12,13]. In fact, due to their dimensional characteristics, microplastics are bio-available for several marine species [14–20], traversing all the marine food webs [21–23]. Microplastics can be confused with food by marine organisms and their ingestion has negative impacts on the biological functions and physiology of species [12,19,24–26]. For instance, direct ingestion can produce gastro-intestinal blockages and inflammation [27] and deteriorate body condition [28] in several different marine species. Furthermore, microplastics are preferential sites for the adhesion of organic and inorganic pollutants [29], and their ingestion can release toxic compounds [30–33]. Such small plastic fragments can be also colonized by microbial pathogens [34] such that they can be a carrier of biological agents, which showed cytotoxic effects in vitro exposure [35].

The models currently recognized as potential mechanisms for microplastic ingestion include random ingestion, predator confusion, bio-magnification [24,36] and/or indirect ingestion through gill openings [37]. For example, detritivorous and predatory lobsters and shrimps presumably ingest microplastics passively with prey or sediment, deposit-feeding burrowing polychaete lugworms likely ingest microplastics within the sediment [38–40], whereas filter-feeding bivalves and polychaetes likely ingest microplastics that are suspended in the water [41,42]. As a matter of fact, benthic organisms are dramatically exposed to microplastics'ingestion such that they play an important role in the trophic transfer of this pollutant throughout the food chain [42–44]. In fish, mechanisms of ingestion could be related to the opportunistic feeding strategies of some species or to the accidental ingestion during predation of specialist feeder [45]. Other ways are represented by bio-magnification in predator fish species [46] and indirect ingestion through gill openings via respiration [37,47,48].

Although a close link clearly exists between species-specific feeding habits and microplastic ingestion patterns, the latter can act simultaneously even within the same species. This extremely complicates the understanding of mechanisms of ingestion of microplastics in marine organisms, which is far from being complete [49].

Pollution levels (microplastics included) are considered one of the most worrying problems for marine ecosystems at the global scale [21,50]. The Mediterranean Sea has been described as one of the most affected areas by marine litter in the world [3,51] especially within Italian seas [52–65]. Plastic, which is the main litter component, has now become ubiquitous and may comprise up to 95% of waste [66–68] accumulated on shorelines, the ocean surface, and sea floor [51,69].

Ideally, the best informative assessment of microplastics' ingestion in fish would require obtaining two intricately related and problematic pieces of information [70]. On the one hand, direct bio-ecological and behavioural observations of the species under study should be collected in the natural environment. On the other, a simultaneous evaluation of the concentration of plastic debris' types in such an environment is necessary to obtain a valid proxy of their bioavailability to species. While both the conditions are often logistically impractical, particularly for those species that live in the deep-sea grounds, the second one can be affected largely by variability of hydrological conditions, in particular in the water column [28]. Deep-sea environments are accumulation sites for microplastics [71,72] such that their concentration exhibits a much lesser variability in time and space compared with the sea water column [73]. Consequently, species inhabiting the deep-sea grounds, and having similar feeding strategies, are likely to face a similar microplastic exposure [74,75]. In this context, indirect methods that compare the composition of microplastics (in terms of shape, color, and size) with dietary data can be useful for understanding the different patterns determining plastic ingestion in fishes.

In this research, we compared the ingestion of microplastics between two deep-water species with a slightly different feeding strategy, such as *Galeus melastomus* Rafinesque, 1810 and *Coelorinchus caelorhincus* (Risso, 1810) sampled in the bathyal plane of the eastern Central Tyrrhenian Sea.

Galeus melastomus is an oviparous benthopelagic predator [76], with a generalistic-opportunistic feeding strategy, as extensively described by several works during the last two decades [76–79] in the Mediterranean Sea. Such a strategy can fit a nutrient-poor environment as the Mediterranean, as being a mean of energy transfer between the pelagic and benthic environments, thanks to the ability of the species of feeding both along the water column and on the bottom [80]. This fish is widely distributed in the Mediterranean Sea and in the north-eastern Atlantic, where it is commonly caught as bycatch of the trawl fishery. It is commonly found between 300 and 800 m depth, although it is also caught in shallower and deeper waters [78,81].

Coelorinchus caelorhincus is known as a benthic feeder on a large array of macro-zoobenthic preys [82,83]. The species, in fact, has a mouth positioned inferiorly and can feed on prey in slow motion with the snout oriented towards the substrate [84,85]. This species is commonly caught as discard with trawls, mainly between 400 and 600 m depth, and, similarly to *G. melastomus*, is widely distributed in the Mediterranean Sea and in the north-eastern Atlantic. Among the Mediterranean macrourid fish, it was reported to be the most common species in terms of abundance and biomass [86].

In this study, investigating the relationships between plastic ingestion and dietary data highlighted potential predator confusion and random ingestion of microplastics occurring in the hollowsnout grenadier (*C. caelorhincus*) and the black mouth catshark (*G. melastomus*), respectively.

Species-specific biological traits can influence microplastics ingestion pattern, whose understanding is an extremely important issue in the assessment of the impact of this pollutant in the various fish species. Therefore, the comparative study of the stomach contents of fish species assumes great importance, above all in order to identify species that represent good environmental descriptors for this type of widespread pollutant. In this regard, the European Union, through the Marine Strategy Framework Directive (MSFD), with the indicator 10.2.1, provides for the control of the "trend in the quantity and composition of waste ingested by marine animals (for example by means of stomach analysis)". Published studies [74,87,88] already suggested *G. melastomus* as a model species for descriptor 10.2.1 due to both its abundance and widespread distribution, despite overfishing, and generalist feeding behavior, in the Mediterranean. Dealing with species-specific feeding strategies, present data also propose *C. caelorhincus* as a potential bio-indicator for a particular type of microplastics (filaments), which are firstly detected in this species.

2. Materials and Methods

2.1. Sample Collection

We obtained samples as by-catch of a professional fishing otter trawler within the winter-autumn period during 2017, at the depths ranging between 250 m and 415 m on the fishing grounds comprised between Civitavecchia and Tarquinia (Figure 1; SI.4: Table S1). Fourteen-hour long fishing trips (3:00 a.m. to 5 p.m.) were carried out in four occasions. 6 h long fishing hauls were performed on each sampling day with "volantina" bottom trawl nets. The gear is characterized by cod-end mesh size of 50 mm diamond and a vertical opening of 4 m [89–91], and the main towing speed was approximately 3 nautical miles per hour. We checked mesh type and cod-end integrity prior to each sampling to assess the raw likelihood of a potential ingestion of net fragments (mesh strands) that might form during capture. We obtained a total of 75 *C. caelorhincus* and 200 *G. melastomus*, respectively, for the entire period. Samples were measured for their total (TL) or pre-anal length (PAL) and weighted for their total body mass at the nearest mm and decigram, respectively. We performed classical stomach content analysis of preys under Olympus SZX 16 stereomicroscope. All the organisms were sorted and identified to the finest taxonomic level possible depending on the digestion's degree of the prey. Dichotomical taxonomical keys for prey identification and common dietary indexes (F% and N%) were used for diet description [92]. Data were grouped by three length classes based on PAL and TL for *C. caoelorhincus* and *G. melastomus* (20–35 mm, 36–50 mm and 51–86 mm; 104–200 mm, 201–290 mm and 291–380 mm), respectively. Simultaneous sampling of the bottom sediment to determine microplastics abundance was not possible due to the opportunistic sampling design.

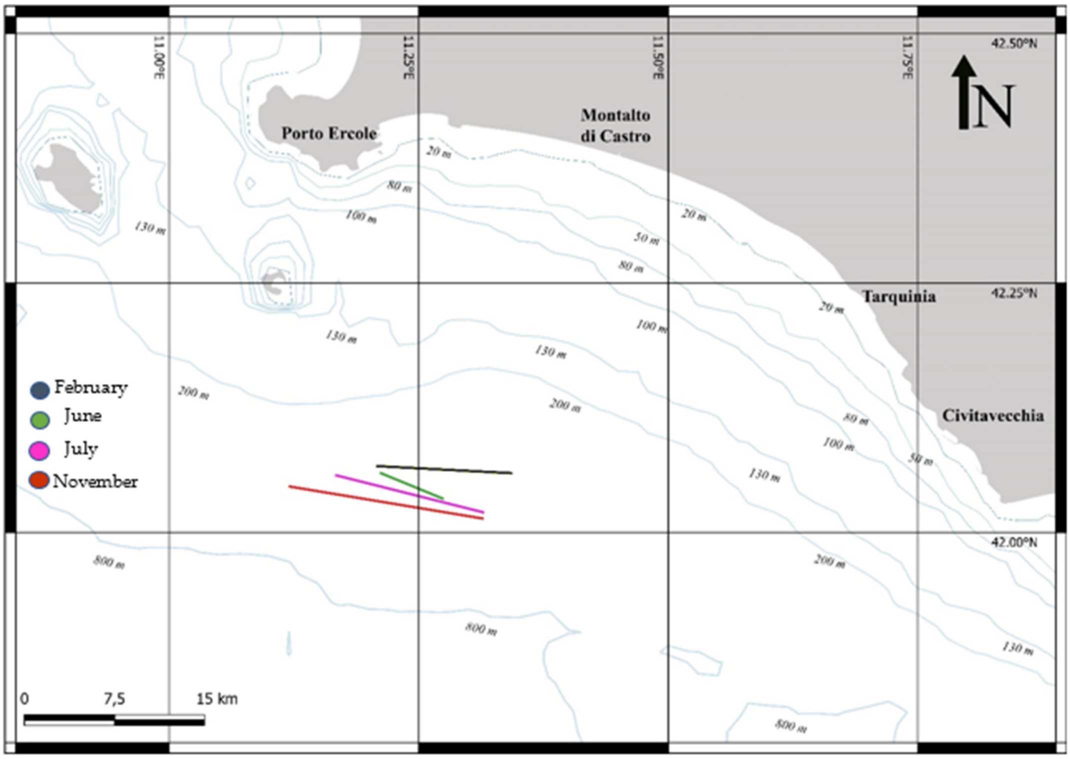

Figure 1. Study area with indications of haul routes carried out during 2017, bathymetry and main fishing harbours in the eastern central Tyrrhenian Sea (Italy).

2.2. Microplastics' Identification and Validation

As fibres were not considered in the analyses, we isolated potential plastic items through serial dilutions of each fresh or de-refrigerated stomach content. Dilutions were obtained by adding MilliQ ultrapure water to each stomach content, then progressively dividing it into groups with a reduced number of elements (comprising prey and non-prey remains). This optimized items' separation and identification within separate Petri dishes. Additionally, it limited items' dispersal and/or damage potentially connected to the use of flushed water through a sieve of 0.3 mm in mesh size. We performed the whole process directly under the stereo microscope (Olympus SZX 16, Hamburg, Germany).

After separating food remains form litter (organic terrestrial, metallic, plastic, other), each litter element was isolated and observed separately in a new solution of MilliQ ultrapure water. Thanks to light and the stereo microscope, the assessments on the plastic nature of each item were based on MEDSEALITTER protocol (MEDSEALITTER deliverable 4.6.1 "Final common monitoring protocol for marine litter"). Identification criteria were based on both the presence/absence of any animal or plant structure (presence of a cellular matrix and other biological microstructures) and the material's reaction to physical inspections (tensile elasticity, both typical distorted shapes and curved edges and uniform thickness, texture upon contact with tweezers, and heat resistance) according to [93]. In case of microplastic items, we also assessed the shape (filaments, fragments and laminas), size, and color (transparent, blue, light blue, green, red, beige, white and dark) of the item according to [94]. Microplastics' size assessments were done by using a millimeter-sized grid (5 mm $\times$ 2 mm) consisting of ten 1 mm $\times$ 1 mm squares, with each square composed of 16 sectors. Then, we divided microplastics into four dimensional classes [95]: macroplastics (>200 mm); mesoplastics (4.76–200 mm); medium microplastics (1.01–4.75 mm); smaller microplastics (0.33–1.00 mm). We considered the latter three-dimensional classes for the analyses.

All the assessments (matter, size, shape and color) were based on multiple independent observations by three observers and photo-recording for each potential microplastic item.

The unavailability of an infrared spectrophotometer impeded validation for polymer type in present sample. Nevertheless, the meticulous and multiple analysis of each potential microplastic partially reduced the overestimation bias usually associated with the optical and physical inspections of microplastics [96], as used in this study. Additionally, traditional infrared spectroscopy is usually used for a reduced (and not always representative) number of particles only, out of the total microplastics retrieved.

2.3. Secondary Contamination

Laboratory instruments and tools were washed with ultrapure MilliQ water and checked every time, to prevent cross-contamination. To prevent airborne contamination in the laboratory, microlitter exposure to air was kept as short as possible. Working environment was monitored through procedural control blanks. All steps were performed under a sterile laminar flow cabinet previously cleaned with 100% ethanol and the personnel involved wore white disposable Tyvek® (USA) protective suits. Secondary contamination of laboratory water was considered negligible because of the use of ultrapure MilliQ-water in all diluting steps. Additionally, fibres, which are the main responsible of secondary contamination [97] were not considered in this study. Despite our attention during all the laboratory processes (Figure 2a–j), the possibility of environmental contamination is not erased (Figure 2j). In case of doubt between fibres and filaments, we re-inspected the object in water solution with tweezers. We categorized as fibres ($\geq$0.33 mm in length) those objects that tended to distort uniformly several times on themselves with a negligible thickness. Otherwise, the filaments remained straight, and the thickness was distinguishable (Figure 2j).

2.4. Statistical Analyses

We interpreted the dietary indexes through the Costello's graphical interpretation [98,99] thus obtaining information on feeding strategies displayed by the two species towards the preys found in their stomachs, microplastic items included. We checked the statistical significance of the differences in the corresponding Prey Importance Index (PII = F% $\times$ N%) of prey categories by the non-parametric Friedman ANOVA. Tests were run on prey data aggregated by macro and micro-taxa level for the three size classes of the two studied species, separately.

We used the same analysis, together with the Kendall's concordance coefficient, to check for: (1) significant differences on concordance in microplastics ingestion between the two species. As input for the analysis, we used frequency of occurrence (F%) in this case, according to the various categories of the microplastic items found (and potentially present; 72 combinations overall in the two species). Categories having simultaneously zero F values in both species were excluded from the analysis. (2) Significant differences and concordance in PII of microplastics, total polychaetes and related families along the three size classes of the hollow-snout grenadier.

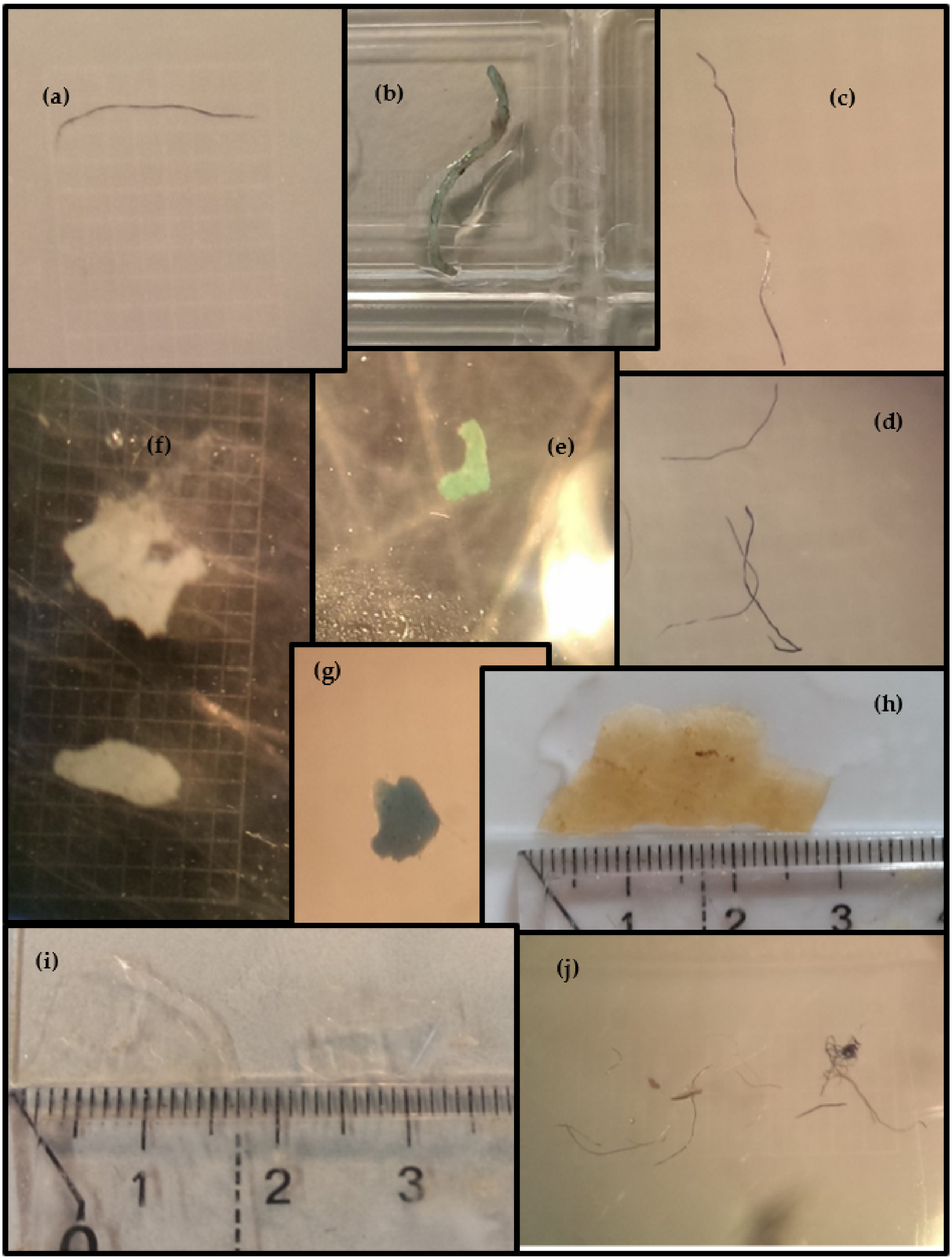

Figure 2. Images of microplastics particles found in the stomach contents of two opportunistic deep–water predators *Coelorinchus caelorhinchus* and *Galeus melastomus* from the bathyal plane of the Central Thyrrhenian Sea. Filaments (**a**–**d**) are distinguished by having both lengths much higher than height and thickness detectable by a needle pit. Fragments (**e**–**h**) are irregularly shaped with different height and length and distinct thickness. Laminas (**i**) are smoothly shaped with different height and length and negligible thickness. Fibres and filaments compared (**j**), on the left and right side of the image, respectively.

We used correspondence analysis to illustrate the gradients of variation describing the dietary shift with increasing size class considering the two species at once, each divided by three length classes. The analysis was run on values of PII aggregated at the macro and micro-taxa levels. We finally used the non-parametric gamma-correlation to check for the relationships that exist between microplastics and polychaetes worms ingested by the hollow-snout grenadier. Using counts by individual as input values, we compared "Filament Blue Medium Sized Microplastics" to Onuphidae, Lumbrineridae, Eunicidae and Polychaeta in general, sorted by predator's size classes.

The Friedman test was used as a nonparametric alternative to a one-way analysis of variance for repeated measures to compare dependent samples due to nature of data. The Kendall concordance coefficient, on the other hand, is generally used as a test for the verification of the concordance hypothesis between two or more ranking categories. Correspondence analysis was chosen as a powerful descriptive analysis, based on the chi-square test and its associated p-value. All analyses were performed by STATISTICA 7.0, [100].

3. Results

3.1. Dietary Data

3.1.1. The Hollow-Snout Grenadier

Thanks to Costello's interpretation, dietary data indicated that the two species share an opportunistic-generalistic feeding habit, yet with some important differences (Figures 3 and 4). The hollow-snout grenadier fed on a large array of preys (SI.1: Figures S1–S11). Most of the species were more (several crustacean families) or less (Onuphids for the small and intermediate sized individuals) rare. Others showed either a slightly dominant importance (total crustaceans) or a high within-phenotype component (total polychaetes and amphipods for the larger size classes) in the fish diet (Figure 2). A clear ontogenic pattern was observed for microplastics increasing with fish size; as opposed, cephalopods decreased with size, above all as relative abundance (Figure 2). Considering the corresponding PII calculated upon products F% $\times$ N% from Costello's diagrams, the differences observed between the different prey categories were at borderline p-level of significance and significant at the macro-taxa (Friedman Anova: $X^2 = 9.33$, $n = 3$, D.F = 4, $p = 0.053$) and at the micro-taxa levels (Friedman Anova: $X^2 = 18.64$, $n = 3$, D.F = 10, $p < 0.05$), respectively.

3.1.2. The Black Mouth Catshark

In comparison, the black mouth catshark ingested a lesser assortment of preys (SI.2: Figures S12–S17). Among crustaceans, several families resulted very rare (Mysids, Amphipods and Isopods), rare (Euphasids) or moderately abundant (Decapods) (Figure 4). The latter increased as frequency of occurrence along the predator's size classes, as well as cephalopods, which increased also as relative abundance (Figure 4). Both fish and total crustaceans displayed a high within-phenotype component, decreasing in abundance and increasing in frequency of occurrence with increasing fish size (Figure 4). Microplastics were rare in the diet of this shark species, though such a pollutant showed a little increase with fish size (Figure 4). Based on corresponding PII, these results were at borderline p-level of significance and significant at the macro-taxa (Friedman Anova: $X^2 = 6.60$, $n = 3$, D.F = 3, $p = 0.08$) and at the micro-taxa levels (Friedman Anova: $X^2 = 10.88$, $n = 3$, D.F = 4, $p < 0.05$), respectively.

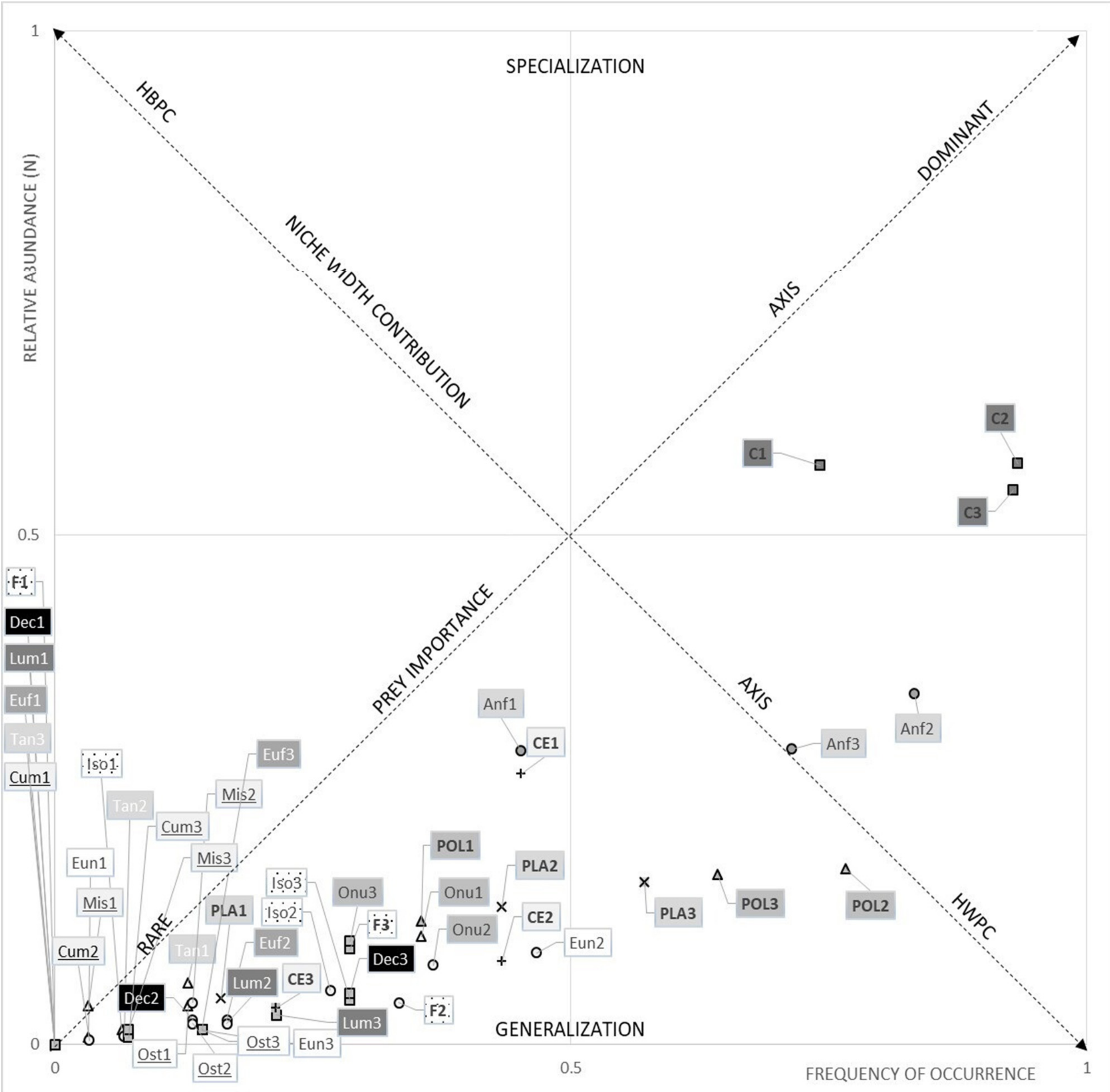

Figure 3. Costello's diagram applied to prey composition found in the stomachs of a sample of the hollow-snout grenadier *Caelorinchus coelorhincus* from the bathyal plane of the central Tyrrhenian Sea 1, 2 and 3 inside the data labels represent small, intermediate and large size classes and same labels 'background denotes same prey category. C: total crustaceans, dark grey squares; POL: total polychaetes, dark grey triangles; F: bony fishes, light grey circles; CE: cephalopods; crosses; MP: microplastics, asterisks; Dec: Decapoa; light grey squares; Euf: Euphasiacea, intermediate grey circles; Tan: Tanaidacea, white triangles; Ost: Ostracoda, intermediate grey squares; Anf: Anfipoda, dark grey circles; Mis: Misydacea, withe circles; Iso: Isopoda, very light grey circles; Cum: Cumacea, white diamonds; Onu: Onuphidae, light grey circles; Eun: Eunicidae, light grey circles; Lum: Lumbrinereidae, dark grey squares.

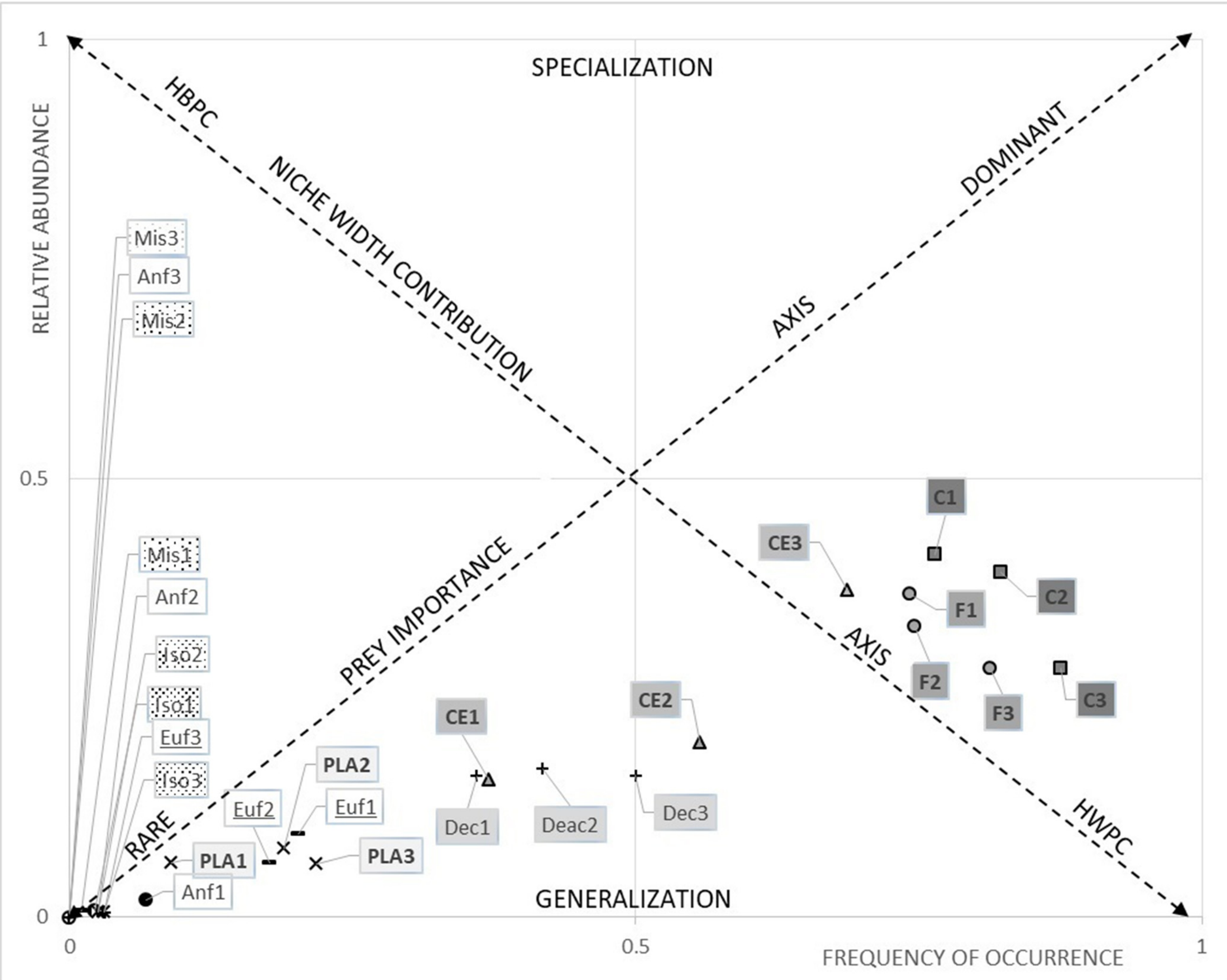

Figure 4. Costello's diagram applied to prey composition found in the stomachs of a sample of the black mouth catshark *Galeus melastomus* from the bathyal plane of the central Tyrrhenian Sea. 1, 2 and 3 inside the data labels represent small, intermediate and large size classes and same labels 'background denotes same prey category. C: total crustaceans, dark grey squares; F: bony fishes, dark grey circles; CE: cephalopods, dark grey triangles; MP: microplastics, asterisks. Dec: Decapoda, crosses; Euf: Euphasiacea, dashes; Anf: Anfipoda, dark squares; Mis: Misydacea, empty circles; Iso: Isopoda, empty triangles.

3.1.3. Diet Comparison between Species

Correspondence analysis clearly separated the diet of two studied species when compared each other for PII (X^2 = 29,184.0, D.F. = 75, $p < 0.001$). Prey importance of total polychaetes, and related families, and six out eight crustacean families were strongly associated the hollow-snout grenadier, with three crustacean families and polychaetes being exclusive of the species (SI.5: Figure S30). As opposed, fish, cephalopods, decapods and euphausids were associated with the black mouth catshark the most (SI.5: Figure S30). Total crustaceans appeared to be shared prey between the two species. Importance of microplatstics resulted strongly associated with the hollow-snout grenadier (SI.5: Figure S30). Consequently, we interpreted dimension 1 as defining a gradient of exclusivity vs. sharing of prey categories and dimension 2 as describing an ontogenic gradient in the diet of the two predators, both in terms of prey importance. For instance, prey importance of euphasids, fish and decapods, and cephalopods resulted associated with the smallest, intermediate and largest size classes, respectively, in the black mouth catshark (Figure 3). This result confirmed data from the Costello's interpretations, which described clear ontogenic patterns for these prey categories (Figures 2 and 3). It is worth to note the inverse ordination of the predators' size classes along dimension 1 ((SI.5: Figure S30).

3.2. Microplastics Ingestion

3.2.1. Comparison between Species

Microplastics were found in the stomachs of 26 over 75 and 30 out of 200 individuals in *C. caelorhincus* (F% = 35%) and *G. melastomus* (F% = 15%), respectively. The two frequencies were significantly different (χ^2 = 4.40; D.F. = 1; $p < 0.05$). The collected data showed that 61 and 58 microplastics items were found (Figure 4) in the stomachs of *C. caelorhincus* (SI.3.1: Figures S18–S22) and *G. melastomus* (SI.3.2: Figures S23–S29), respectively. The breakdown of categories according to shape, color and size indicated the categories 'medium sized', 'blue' and 'filaments' to be the most represented as number of items (Table 1).

Table 1. Macro and subcategories of microplastics as number of items found in the stomachs of two bottom dwelling opportunistic feeder species from the bathyal plane of the eastern central Tyrrhenian Sea.

Categories		*C. caelorhincus*	*G. melastomus*
Shape	Filament	56	42
	Fragment	5	11
	Lamina	0	5
Color	Red	3	4
	Trasparent	5	7
	Blue	46	35
	Black	2	2
	White	5	0
	Light Blue	0	7
	Green	0	1
	Beige	0	2
Dimension	Smaller microplastics (0.33–1.00 mm)	18	21
	Medium microplastics (1.01–4.75 mm)	39	22
	Mesoplastics (4.76–200 mm)	4	15

Comparing F values by each of the 72 combinations between the two species, a marked prevalence of the small-medium sized blue filaments was noted in the stomachs of hollow-snout grenadier (Table 2). This category had F values one or two orders of magnitude higher than the other categories. Small and medium, and additionally large, blue filaments attained the highest F values also for the black mouth catshark (Figure 5). The other categories were only one order of magnitude lower than the overall blue filaments.

Notwithstanding the fact that results from Friedman's Anova (X^2 = 17.74, n = 2, D.F. = 21, p = 0.66) indicated ingestion of microplastics was generally similar as far as it concerns shape, colour and dimension between the two species, they showed concordance for little more than the half (concordance coefficient = 0.58) of the considered categories. The remaining 42% represented categories that differed between the two species. The much of dissimilarity was observed primarily in the category medium sized blue filaments, and secondarily in the small sized blue filaments (FILMB, FILPB, respectively, Figure 5). In both cases, the hollow-snout grenadier showed much higher F values than the black mouth catshark. In turn, the latter ingested a larger array of microplastics compared with the former species, though at very low values of F (Figure 5).

Table 2. Number and corresponding (%) frequency of occurrence (on the left and right, respectively, separated by semicolons) of the different microplastic items found (and potentially present) in the stomachs of two bottom dwelling opportunistic feeder species *Galeus melastomus* (GM) and *Caelorhinchus coelorhinchus* (CC) from the bathyal plane of the eastern central Tyrrhenian Sea.

Color	*Red*		*Transparent*		*Blue*		*Black*		*Light Blue*		*White*		*Green*		*Beige*	
Species	*GM*	*CC*	*GM*	*CC*	*GM*	*CC*	*GM*	*CC*	*GM*	*CC*	*GM*	*CC*	*GM*	*CC*	*GM*	*CC*
Shape and Dimension																
Small Filament	0; 0	0; 0	2; 0.01	0; 0	9; 0.045	16; 0.21	0; 0	1; 0.013	4; 0.02	0; 0	0; 0	0; 0	0; 0	0; 0	1; 0.005	0; 0
Medium Filament	1; 0.005	3; 0.04	1; 0.005	5; 0.066	12; 0.06	30; 0.35	1; 0.005	1; 0.013	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0
Large Filament	0; 0	0; 0	0;0	0; 0	11; 0.055	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0
Small Fragment	2; 0.01	0; 0	0;0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	1; 0.013	1; 0.005	0; 0	1; 0.005	0; 0
Medium Fragment	0; 0	0; 0	2; 0.01	0; 0	2; 0.01	0; 0	0; 0	0; 0	3; 0.015	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0
Large Fragment	0; 0	0; 0	0;0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	4; 0.053	0; 0	0; 0	0; 0	0; 0
Small Lamina	1; 0.005	0; 0	0;0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0
Medium Lamina	0; 0	0; 0	0;0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0
Large Lamina	0; 0	0; 0	2; 0.01	0; 0	1; 0.005	0; 0	1; 0.005	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0	0; 0

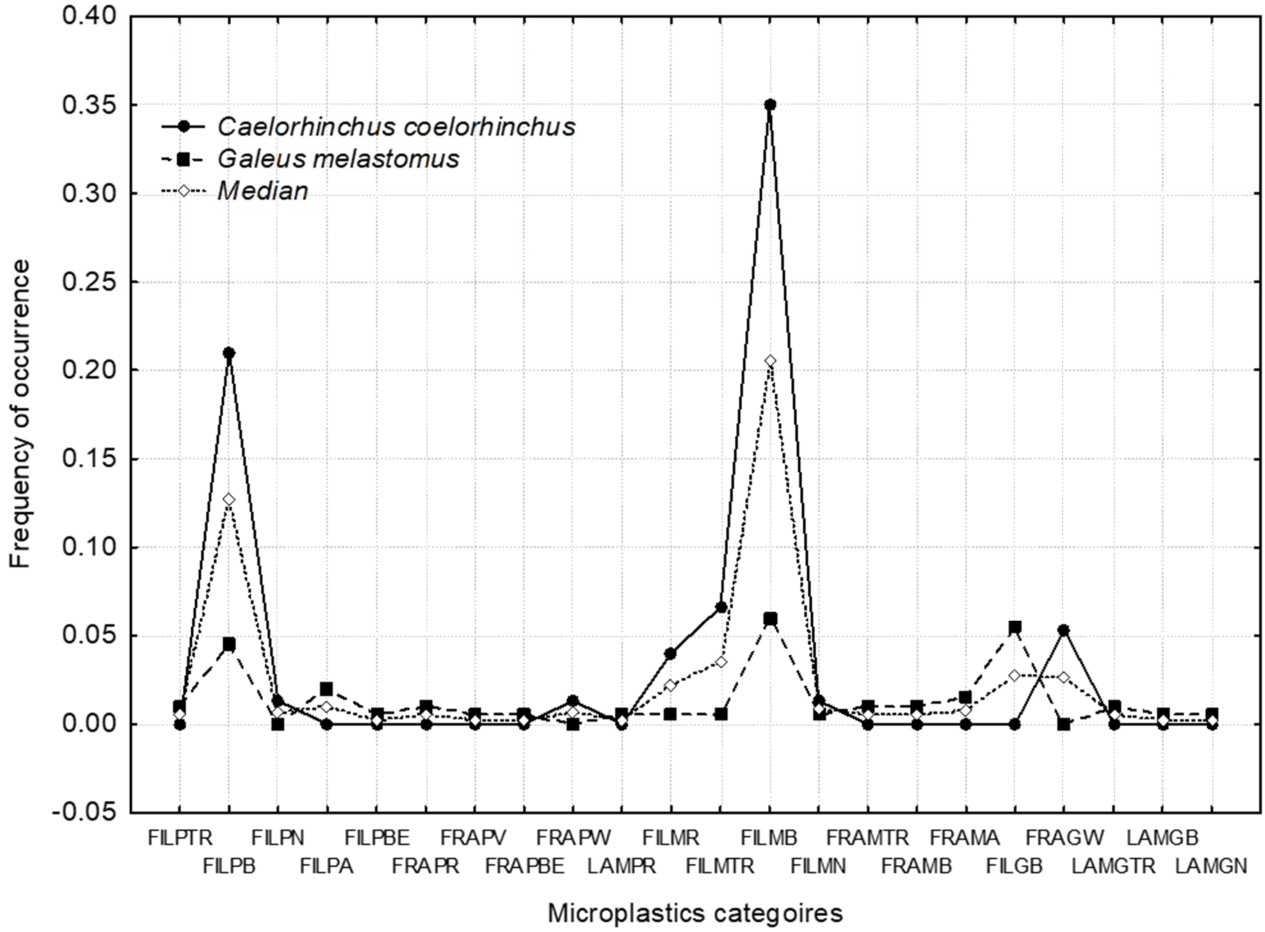

Figure 5. Comparison between values of frequency of occurrence for microplastic categories found in the stomachs of two generalistic and opportunistic feeder species from the bathyal plane of the central Tyrrhenian Sea. FIL: films; FRA: fragment; LAM: lamina; P: small sized; M: medium sized; G: large size; TR: transparent; B: blue; N: dark; A: light blue; BE: beige; W: white; R: red V: green.

3.2.2. Relationships between Microplastic Filaments and Polychaetes

Gamma non-parametric correlations between ingested preys, microplastics included, had significant results ($p < 0.05$) in the pairwise comparisons of Polychaetes vs. Cephalopods and of the latter vs. Fish at the macro taxa level (Table 3). It is worth to note the inverse relationship between the number of ingested polychaetes and microplastics (SI.5: Figure S31a; Table 3) by stomach, though at a borderline *p*-level ($p = 0.06$) of significance. This inverse relationship resulted much stronger and significant ($p < 0.05$; Table 4) when calculating gamma correlation between ingested worms of Eunicidae and Onuphidae families and microplastics (r = -1, SI.5: Figure S31b and r = − 0.69, SI.5: Figure S31c, respectively). Friedman's ANOVA indicated significant (Chi-squared = 9.87, $n = 3$, d.f. = 4, $p < 0.05$) differences between PII values of microplastics, total polychaetes and related families, with a high overall concordance (Kendall's coefficient = 0.82, Mean rank = 0.73) between size classes. The smallest difference in PII was noted between total polychaetes and medium-sized microplastic blue filaments, whereas the highest between total polychaetes and Lumbrinereidae (Table 5). PII of microplastics, total polychaetes and Lumbrinereidae had similar trends, generally increasing with predator size compared with Onuphidae and Eunicidae. The former fluctuated around at PII =300 in all size classes and the latter peaked in the intermediate size class (CC2) (Figure 6).

Table 3. Gamma non–parametric correlations between the five prey categories (microplastics included) at the macro taxa level found as number of items per stomach from a sample of the hollow–snout grenadier from the bathyal plane of the eastern central Tyrrhenian Sea. Bold values are significant $p < 0.05$ and n.m. stands for no match between data series concerned.

	Fish	Crustaceans	Polychaetes	Cephalopods	Microplastics
Fish	1.00	n.m.	n.m.	**−1.00**	n.m.
Crustaceans	n.m.	1.00	0.20	0.04	−0.09
Polychaetes	n.m.	0.20	1.00	**−0.51**	−0.31
Cephalopods	**−1.00**	0.04	**−0.51**	1.00	0.11
Microplastics	1.00	−0.09	–0.31	0.11	1.00

Table 4. Gamma non–parametric correlations between microplastics and polychaetes with related families found as number of items per stomach from a sample of the hollow–snout grenadier from the bathyal plane of the eastern central Tyrrhenian Sea. Bold values are significant $p < 0.05$ and n.m. stands for no match between data series concerned.

	Microplastics	Polychaetes	Onuphidae	Eunicidae	Lumbrinereidae
Microplastics	1.00	−0.31	**−0.69**	**−1.00**	n.m.
Polychaetes	−0.31	1.00	**0.86**	−0.22	1.00
Onuphidae	**−0.69**	**0.86**	1.00	n.m	n.m.
Eunicidae	**−1.00**	−0.22	n.m.	1.00	n.m
Lumbrinereidae	n.m.	1.00	n.m.	n.m.	1.00

Table 5. Friedman ANOVA results on the breakdown of the contribution by microplastics, total polychaeates and related families to the total variability in Prey Importance Index (PII) between three size classes from a sample of the hollow-snout grenadier from the bathyal plane of the eastern central Tyrrhenian Sea.

	Mean Rank	Sum of Ranks	Mean PII	s.d (±)
Medium–sized microplastic blue filaments	3.66	11.00	523.45	422.32
Polichaetes	5.00	15.00	940.40	459.03
Onuphidae	3.00	9.00	317.24	51.23
Eunicidae	2.00	6.00	147.97	236.42
Lumbrineridae	1.33	4.00	32.09	31.10

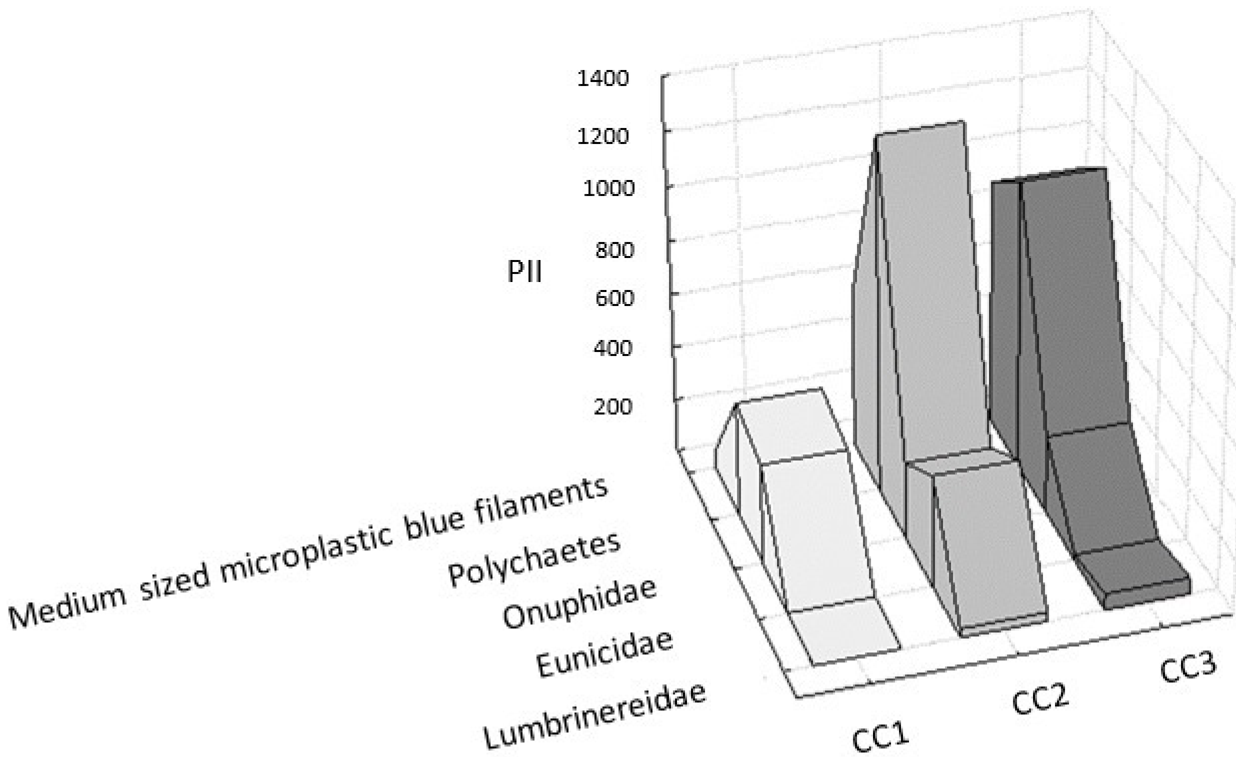

Figure 6. Trends of PII's values of microplastics, total polychaetes and related families according to three size classes (1: small; 2: intermediate; 3: large) of *Coelorinchus caelorhinchus* (CC) from a sample from the bathyal plane of the Central Thyrrhenian Sea.

3.2.3. Control for Secondary Contamination

The control checks made on the used fishing nets indicated the cod end's meshes were equipped with raw transparent filaments and the gear was in good conditions prior to each sampling occasion.

Laboratory secondary contamination by fibers was detected in three occasions in two stomachs (F% = 7.3×10^{-3} for the total sample) distributed one per species (F% = 1.3×10^{-2} and 5×10^{-3} for the hollow-snout grenadier and the black mouth catshark, respectively) with two items simultaneously present in a stomach of *G. melastomus*.

4. Discussion

4.1. Dietary Data

Dietary data of the two studied species illustrated a similar generalistic diet that is typical of opportunistic feeder species, as already described for other Mediterranean areas [78,79,84,101], though on slightly different prey species assemblages.

Despite their similarities, the two species exhibited somewhat but important differences in feeding strategies toward their preys. The hollow-snout grenadier exhibited a more articulated generalist feeding strategy on a larger array of prey categories compared with the black mouth catshark. In fact, the diet of the teleost fish species showed the presence of dominant, as well as rare or very rare, preys, some of them having an ontogenic shift toward a High Within Phenotype Component.

The diet of *G. melastomus* resulted less articulated, with a narrower number of ingested prey categories compared with the hollow-snout grenadier. All size classes exhibited similar preferences towards some preys and equally contributed to HWPC. Ontogenic shifts have also been found in this species, but for other prey categories, such as decapods and cephalopods.

4.2. Microplastics Ingestion

The pattern of microplastics ingestion exhibited a general resemblance between the two species. This can indicate that the several microplastics types retrieved in the stomachs may have a similar environmental bioavailability (see Section 4.3.) to both species Despite this, strong differences for some microplastics categories, as indicated by quantitative data, were detected between the two species. Such differences can be related to the different feeding strategies observed between *C. caelorhincus* and *G. melastomus*, even within a similar generalistic feeding habit [42,45].

On the one hand, the ingestion of microplastics resulted much more randomly ruled in the blackmouth catshark, as already found elsewhere in the Mediterranean [74,87] than in the hollow-snout grenadier. The higher number of microplastic categories, along with their similar low frequency, is related to a raw generalist predator with a poorly articulated diet. The data illustrated a generalist predator that feeds on a narrower range of prey categories, with all size classes showing low importance for microplastics, albeit with slightly increasing abundance as predator size increases. Regurgitation has also to be considered in order to explain the relatively lower occurrence of microplastics found in the black mouth catshark compared with the hollow-snout grenadier. Regurgitation was suggested for a demersal sharks' assemblage from a neighboring area [75].

On the other hand, medium to large specimens of the hollow-snout grenadier exhibited a high within-phenotype component toward polychaetes. It indicated these individuals are monomorphic generalist predators targeting in particular this prey. Their feeding niche breadth is large as it is primarily related to prey abundance in the natural environment [98,102]. Polychaetes, and related families, and blue medium-sized microplastic filaments showed a similar ontogenetic ingestion pattern, as demonstrated by both Costello's interpretation and the high overall concordance of their PII's values.

Although a random ingestion cannot be excluded for the hollow-snout grenadier [37], macrourids had evolved more articulated feeding strategies and sophisticated apparatus to target their preys, in response to a difficult environment such as deep compared with coastal grounds [103]. This is the case of the hollow-snout grenadier, which, in addition to having a sharp snout with a barbell on ventral side of the mouth, has a light organ anterior to the pelvic fins. Although the role of the light organ in fishes is uncertain, ranging from a preys' attraction to a vision's enhancing devices [104], it is interesting to note that the light frequency of the blue is one of the last absorbed by marine seawater [105,106], thus remaining visible in deep-water grounds typical of the species.

That said, data on microplastic ingestion strongly supported the hypothesis inferring a predator confusion occurring for the hollow-snout grenadier: In particular, the inverse relationships between polychaetes, and related families, and medium-sized blue microplastic filaments suggested a clear dietary relation between the preys and the pollutants. Microplastics and polychaetes may produce a similar stomach-filling effect, precisely because they are similarly targeted by *C. caelorhincus*. It is very likely that the species may unfortunately confuse medium-sized blue microplastic filaments with polychaetes, due to their similarity in shape and size, and high abundance in the continental slope. Worms such as those (Eunicidae and Onuphidae) retrieved in the stomachs typically display an iridescent chithinous tegument [107] and have high abundances in the bathyal slope of the western Mediterranean [108].

A similar phenomenon was described in estuarine fish preying on Nylon, while mistaking it for Polychaeta [109]. Furthermore, [110] noted that the species that most prey on Polychaeta are also those most contaminated by microplastics. In all cases, the general ingestion of indigestible items due to confusion with digestible items was suggested for fishes also in other studies [111,112].

Blue microplastics have been found in numerous fish species [109,113–116] and Ref. [117] highlighted that some fish show greater selectivity for blue microplastics as their colour is similar to prey (e.g., copepods). It has also been observed that in opportunistic predatory fish the ingestion of plastics can be done voluntarily [118] or that predators can temporarily bite prey for the purpose of examining them and likewise can attack plastic items [36]. It is probable that the movement (caused by currents and other factors) size and the color of the fragments, when similar to that of common prey, can confuse the predator [119,120]; as well as odour associated with plastic debris [121].

Some studies show, moreover, how amphipods and polychaetes accumulate microplastics themselves [38–40,122–126], so that a biomagnification process can be also hypothesized for *C. caelorhincus*.

Despite fibers were not considered in this study, incidental ingestion via respiration through gill openings [37] cannot be excluded. Strong bottom currents, such as turbidities [127], periodically stir up the soft deep water sediments of the continental slope [128], consequently re-suspending microplastic particles in the suprabenthic water environment the species are likely to breath [47,48]. Therefore, it remains an open question whether the presence/absence of gill opercula also contributed to the difference in microplastic filament ingestion rates found between the teleost and cartilaginous fish compared. Similar to feeding strategies, morpho-anatomical differences could also play a determining role in the accumulation of microplastics. For instance, gastro-intestinal retention time of microplastics is influenced by the presence of spiral valve, which is typical of the digestive apparatus of elasmobranchs [74,75].

4.3. Data Validation

Several studies have found that marine plastic contamination has been accelerating worryingly in the oceans and seas all over the world during recent years, causing interactions at species, community or ecosystem level [129–131].

The data here presented found support in the existing information on environmental contamination from microplastics, as well as on their interaction with fish species, both at the general and local level. For instance, feeding interactions are the stronger where environmental accumulation of microplastics on the sea ground are the greater [50]. The high F% and N% found for microplastics in both the species studied (and 15% for *C. caelorhincus* and 35% for *G. melastomus)* are likely an effect of the highly microplastics-polluted sampling area, the central eastern Tyrrhenian Sea [28,74,132]. In fact, comparing with other Mediterranean areas highlights dramatic differences. From the study by [88], carried out in the Eastern Ionian Sea, microplastics are present only in 3.2% of the individuals of *G. melastomus* they considered. On the other hand, in the study conducted by [87], in two different locations of the Balearic Islands, a percentage corresponding to 16% and 18% of sampled individuals had ingested microplastics. In all the considered studies [74,87,88], ingested microplastics belonged to several categories in terms of shape, size and color.

As we did not find any detailed information on microplastics ingestion in the hollowsnout grenadier, this work is the first to offer a thorough evidence of ingestion of this type of pollutant.

The relative abundance index N% obtained through stomach contents analysis is considered a good proxy of the availability a given prey (microplastics, in this case) in the environment, especially when it is patrolled by a generalist predator [92,98,99]. Therefore, it can provide a raw indication of the abundance of microplastics filaments in deep bathyal grounds between Civitavecchia and Tarquinia. These environments likely suffer from the heavily anthropized coastal region they are in front of [133–135]. This is reflected on a massive presence of anthropogenic litter and microplastics in sediments, in the water column and in some marine species [35,60,126,134]. For instance, [136] observed a concentration of MPs that oscillates between 591.5 ± 127.7 MP/kg d. w. and 992 ± 477.2 MP/kg d. w in the coastal sediments of Civitavecchia. Additionally, [35] identified microplastics in the hake *Merluccius merluccius* (Linnaeus, 1758) and the mullet *Mullus barbatus* Linnaeus, 1758 in the same fishing area. µFT-IR analysis highlighted that plastic particles (HDPE, polyamide and polypropylene) were present in 100% and 83.3% of analyzed hakes and mullets and 14.67 ± 4.10 and 5.50 ± 1.97 items/individual were retrieved from their stomachs, respectively. Recent investigations showed also that filaments concentration is the highest in the coastal shallow sediments [3,58,61,64] and also in coastal fish that prey on benthic fauna [109,110,114,137,138]. Actually, filaments appeared to be the most represented microplastics categories in both the species studied.

Filaments likely derived from degradation of lost and damaged fishing gear, such as lines and nets, which are an additional source responsible of plastic pollution in the world ocean [139]. Nevertheless, accidental ingestion of new formed net fragments that might originate during catch should be considered. While evaluating the extent of this potential contamination is very difficult, its likelihood can be evaluated ultimately through a laboratory comparison between ingested microplastics and a sample of the used net. However, the ingestion of blue filaments found in both species likely suggested an environmental origin of stomachs' load in microplastics. In fact, the color of the mesh's filaments was different, as observed during visual inspections on mesh integrity and type in the cod-end of the used gear. Nevertheless, a fishing net contamination, yet negligible, cannot be excluded. Despite being difficult, controlling all potential sources of secondary contamination, from field to laboratory equipment [96], is highly recommended to obtain an unbiased measure of microplastics load from environment by organisms [140], as required by MSFD [141–143].

Overall data in the context of available information highlight the potential of the two studied species as bioindicators for microplastics contamination in the deep-sea grounds of the Mediterranean Sea. This is not a novelty for *G. melastomus* [87] but it is indeed for *C. caelorhinchus*, due to the very high values of both occurrence and relative abundance of a specific type of microplastics (blue medium-sized filaments) firstly found in its stomach contents.

The comparative method applied allowed discriminating different microplastic ingestion patterns depending on slightly different feeding habits observed for two generalist species. However, μFT-IR analysis will be necessary for obtaining both a quali-quantitative validation of all items found and a negligible overestimation bias in the microplastics number's assessment. Parallel samplings of their abundance and distribution in deep water sediments will be also crucial to elicit species as bioindicators for microplastics ingestion, especially in the case of the hollow-snout grenadier.

5. Conclusions

In conclusion, the study of both mechanisms of ingestion and microplastics composition in the diet of marine organisms remain a key role in the monitoring and evaluation of this pollutant in the marine environment. The comparative approach adopted in this study allowed understanding the species-specific linkage that exists between microplastics ingestion and feeding habits. This is of value for the identification of good model species, as also pointed out by the MFSD, especially in one of the most impacted marine areas in the world by this pollutant, such as the Mediterranean Sea.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse10050624/s1, SI.1: Examples of preys found in the stomachs of *Coelorinchus caelorhinchus*; SI.2: Examples of preys and parasites found in the stomachs of *Galeus melastomus*. SI.3.1: Microplastics found in the stomachs of *Coelorinchus caelorhinchus.* SI.3.2: Microplastics found in the stomachs of *Galeus melastomus.* SI.4: Fishing data. SI.5: Dietary data.

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