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Coupling Gastro-Intestinal Tract Analysis With an Airborne Contamination Control Method to Estimate Litter Ingestion in Demersal Elasmobranchs

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This study aims to assess the litter ingestion in some demersal elasmobranchs, combining a classical gastro-intestinal tract (GIT) analysis with a procedure methodology to reduce airborne fibers contamination. In order to prevent the overestimation of litter ingestion, we applied severe mitigation measures to avoid airborne contamination during the analyses, integrating a new approach for the correction of estimates of fibers abundance using control procedure. In this study, we assessed the anthropogenic litter ingestion in four elasmobranch species from the southern Tyrrhenian Sea: *Scyliorhinus canicula* (n = 27), *Etmopterus spinax* (n = 16), *Galeus melastomus* (n = 12), and *Raja clavata* (n = 6). The GIT of each specimen was analyzed by visual sorting and the polymers identified by Fourier transform infrared spectroscopy technique. Overall, 19 litter particles were found in the GIT of 13 demersal elasmobranchs (%O = 21) and for the first time, evidence of litter ingestion by *R. clavata* in Mediterranean waters was also reported. In *G. melastomus* and *R. clavata* all anthropogenic particles were plastics, whereas in *S. canicula* other litter categories were also found. No litter ingestion was instead observed in *E. spinax.* More than 50% of litter particles belonged to microlitter category (<5 mm). Polyamide was the only polymer typology found in all examined species. We described the procedures to control the airborne contamination applied at each step of laboratory analysis and, thanks to the application of our control method, it was possible to exclude the 95% of fibers found in samples from the assessment. Moreover, we compared fibers abundances observed in samples and controls. This study, combining an approach for minimizing the bias associated to airborne fiber contamination, provided a reliable assessment of marine litter ingestion in demersal elasmobranchs.

Keywords: selachians, GIT analysis, microplastics, FT-IR spectroscopy, polymers, airborne contamination control, Mediterranean Sea

INTRODUCTION

In the last decades, the marine litter (ML) pollution has generated many concerns about the potential global implications on marine environment and organisms (Laist, 1997; Kühn et al., 2015; Rochman et al., 2016). The increasing development of both coastal and maritime human activities is one of the main reasons of the ML ubiquity in all marine habitats, from the beaches to the open ocean and seafloor (Galgani et al., 2015).

Ingestion of anthropogenic debris represents one of the main threats for marine fauna (Galgani et al., 2013a; Fossi et al., 2018) and, in particular, plastics are the most common litter found in the stomach contents of marine organisms (Anastasopoulou and Fortibuoni, 2019). Organisms can intentionally ingest ML, because debris particles are mistaken or confused as prey, or accidentally eat debris during foraging activity (e.g., filter feeders) (Kühn et al., 2015; Romeo et al., 2015, 2016). It is also known that secondary ingestion occurs in the marine environment during the predator–prey interaction, when predators consume ML contaminated prey (Chagnon et al., 2018; Nelms et al., 2018; Welden et al., 2018). Ingested ML may cause physical/mechanical damages in tissues and induce toxicological harm in marine species, affecting several levels of the trophic web (Rochman et al., 2013, 2014; Pedà et al., 2016; Fossi et al., 2018). The semi-enclosed basin of the Mediterranean Sea is largely affected and threatened by ML pollution and several monitoring programs and mitigation actions have been launched in order to reduce its impacts. In this context, the Marine Strategy Framework Directive (MSFD; EC 2017/848) aims to achieve the Good Environmental Status (GES) in European waters, i.e., to ensure that "*the amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned*" (Descriptor 10, criterion D10C3). Moreover, Member States are required to follow standardized methods for monitoring and assessment the amount and composition of litter and micro-litter ingestion in the following groups: birds, mammals, reptiles, fish, or invertebrates.

In the Mediterranean basin, ML ingestion in marine organisms has been documented in both invertebrates (Fossi et al., 2014; Alomar et al., 2016; Digka et al., 2018a) and vertebrates (Anastasopoulou et al., 2013a; Romeo et al., 2015, 2016; Battaglia et al., 2016; Giani et al., 2019; Schirinzi et al., 2020), including endangered species (Campani et al., 2013; Fossi et al., 2014), and a large number of these studies regarded fish, including cartilaginous species (Deudero and Alomar, 2015; Fossi et al., 2018; Anastasopoulou and Fortibuoni, 2019).

So far, several methods have been used for the extraction of ML from fish [e.g., visual sorting and chemical digestion protocols of gastro-intestinal tract (GIT)] as well as for ML quantification, categorization and polymer identification (e.g., visual identification, infrared or Raman spectrometry) (Romeo et al., 2015, 2016; Alomar and Deudero, 2017; Digka et al., 2018a; Giani et al., 2019; Rios-Fuster et al., 2019; Capillo et al., 2020; Schirinzi et al., 2020). However, despite the growing number of scientific publications, data on the occurrence, amount and categorization of litter ingested by fish are often not comparable due to the lack of harmonized protocols (Hermsen et al., 2017; Bray et al., 2019; Giani et al., 2019). In addition, another crucial issue concerns the need for the application of standardized methodologies to ensure quality assurance and quality control for the ML analysis in biota (Torre et al., 2016; Hermsen et al., 2018; Kühn et al., 2020). Some authors have shown that contamination by airborne fibers may represent a serious problem in studies on ML ingestion in marine fauna (Torre et al., 2016; Hermsen et al., 2017, 2018; Kühn et al., 2018, 2020), leading to the overestimation of ML pollution and also to potential erroneous conclusions (Torre et al., 2016). Contamination prevention measures have only recently been introduced in ML studies (Lusher et al., 2013; Romeo et al., 2016; Hermsen et al., 2017; Digka et al., 2018a; Giani et al., 2019; Capillo et al., 2020; Schirinzi et al., 2020). However, these procedures are not often applied during all steps of laboratory analysis or they are poorly described or reported (Hermsen et al., 2017; Kühn et al., 2020).

For these reasons, this study aims to couple a standard GIT analysis with an airborne contamination control method, in order to estimate ML ingestion in some demersal elasmobranchs from the southern Tyrrhenian Sea (GFCM Geographical Sub-Area – GSA 10): *Scyliorhinus canicula* (Linnaeus, 1758), *Etmopterus spinax* (Linnaeus, 1758), *Galeus melastomus* Rafinesque 1810, and *Raja clavata* Linnaeus, 1758. This methodology is applied in order to reduce ML overestimation originated from airborne fiber contamination.

This paper also provides useful information on the typology and features of ML ingested by these Mediterranean demersal elasmobranchs, providing data which could be used for the assessment of the ML impact on these predators.

MATERIALS AND METHODS

Study Area and Fish Collection

A total of 61 individuals of demersal elasmobranchs (27 *S. canicula*, 16 *E. spinax*, 12 *G. melastomus*, and 6 *R. clavata*) were collected from bycatch in trawl and longline fisheries in the western Mediterranean Sea (southern Tyrrhenian Sea, GSA 10; **Figure 1**) during 2015. *R. clavata, G. melastomus*, and *E. spinax* were caught by bottom trawl on a seafloor ranging from 570 to 680 m, whereas *S. canicula* was caught by bottom longline at 340 m depth.

Elasmobranch species were identified according to taxonomic features reported by Compagno (2001); Serena et al. (2010) and individuals were measured to the nearest 0.1 cm (total length, TL) and weighed to the nearest 0.1 g (total weight, TW; Tab.1). Then, they were stored at −20°C, before the laboratory analyses.

Visual Sorting and Litter Quantification

In the laboratory, the GIT of each specimen was removed, transferred to a glass petri dish and analyzed by visual sorting, performed under a stereomicroscope Zeiss Discovery V.8. Ingested ML items were separated and categorized following the Litter Categories for marine Biota, reported in the Marine Strategy Framework Directive (MSFD) protocol (MSFD Technical Subgroup on Marine Litter, 2013). Then, ML items were counted, weighed (in grams to the nearest 0.0001 g),

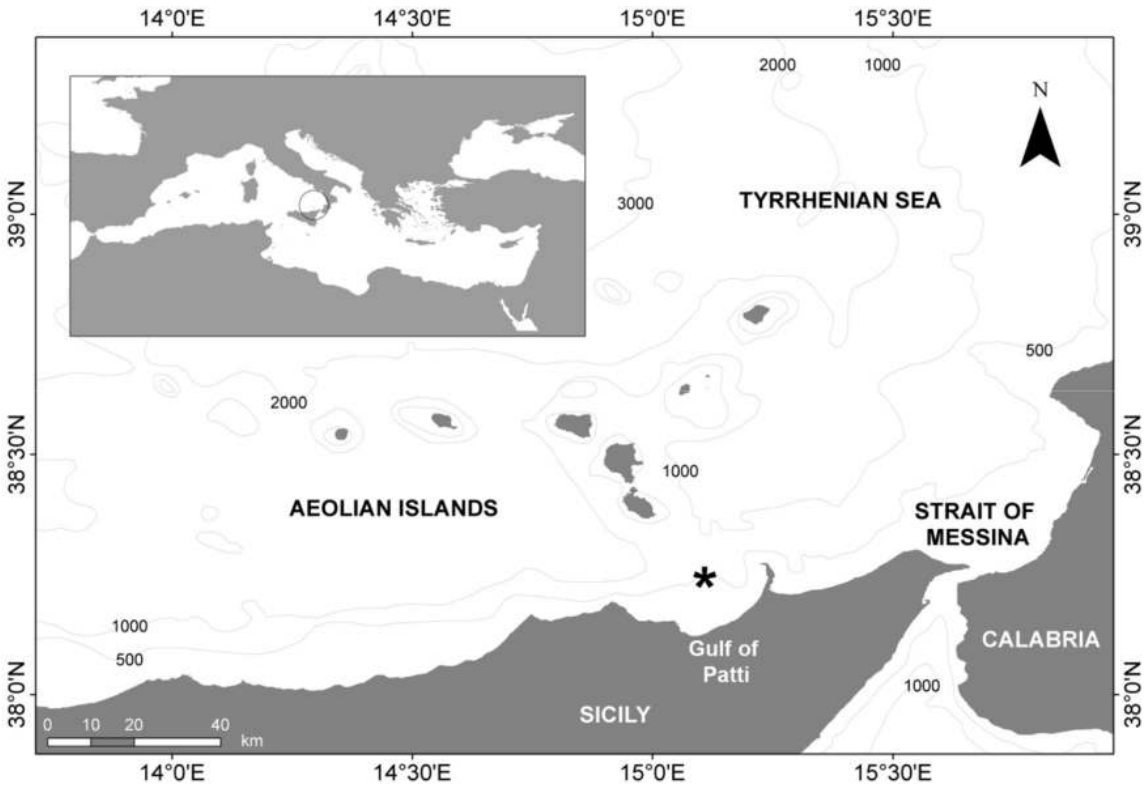

FIGURE 1 | Map of the study area in the western Mediterranean Sea (southern Tyrrhenian Sea), showing the sampling location (*).

measured (length and width in mm) and photographed using a stereomicroscope Zeiss Discovery V.8. coupled with AxioVs40 version 4.8.2.0 digital image processing software.

Marine Litter Identification and Classification

Marine litter items were analyzed by Fourier transform infrared (FT-IR) spectroscopy technique to identify their polymer nature. Fourier transform infrared polymer analysis was carried out using the Agilent Cary 630 spectrophotometer supplied with specific polymer libraries (Agilent Polymer Handheld ATR Library, Agilent Elastomer Oring and Seal Handheld ATR Library, Agilent Polymer, POLY_D, ATR Demo Library). According to Fossi et al. (2017) and Bernardini et al. (2018), three repeated measures were performed for each ingested ML item, setting up to 80% the level of similarity, in order to compare the sample spectra with ones contained in the software database. Only polymers matching reference spectra for more than 80% were accepted. Identified plastics were classified based on their color, shape (sheetlike, threadlike, foam, fragment, and other typologies), and size range (micro: <5 mm; meso: 5–25 mm; macro: >25 mm), according to literature (Galgani et al., 2013b; MSFD Technical Subgroup on Marine Litter, 2013; Romeo et al., 2015; Schirinzi et al., 2020). Marine litter abundance indices were calculated for each species as follows:

1. Litter and plastic percentage of occurrence (O%) was estimated as the proportion, on the total sample, of the individuals which ingested litter and plastics: (%O = N. individuals which ingested litter and plastics/N. total samples × 100);
2. Average number of plastic items found in the GITs, calculated on the total number of individuals (N. plastic items/N. all examined individuals);
3. Average number of plastic items found in the GITs, calculated on the total number of individuals which ingested plastics (N. plastic items/N. individuals which ingested plastics).

Airborne Fiber Contamination Control Method

Rigorous contamination mitigation measures were adopted during the laboratory analysis in order to reduce the risk to overestimate fibers (**Figure 2**). Air currents were reduced through closing of windows, doors, and air conditioners and all researchers wore 100% cotton lab coats. During the study, only glassware and metal equipment were used and all laboratory instruments, including dissection tools such as tweezers, scalpels and scissors, were cleaned with filtered water whenever samples were examined. Specimens were washed using filtered water (0.45 μm) before GIT removal and each step of analysis (from the GIT dissection to the GIT content separation) was performed

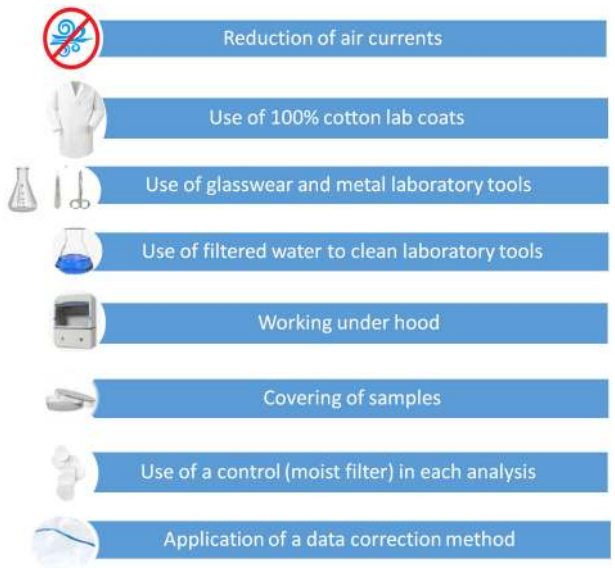

FIGURE 2 | An overview of adopted mitigation measures to reduce airborne contamination in samples.

under the fume hood. In order to limit airborne contamination, Petri dishes containing samples were kept covered using a clean glass cover, while moving from the fume hood to the stereomicroscope.

In parallel to the analysis of each sample, a control procedure was used for the assessment of airborne fiber contamination: a cleaned filter, moistened with filtered water (to simulate the same conditions of the wet GIT content), was put in a control glass Petri dish and maintained near the Petri containing the sample, during all operations under the fume hood and during visual sorting at the stereomicroscope. The number of fibers observed in both samples and control Petri dishes was recorded (and here named as observed fibers and control fibers).

The airborne fiber contamination control followed the procedures reported in **Figure 3**. In order to exclude the airborne fiber contamination, for each species, we statistically tested the difference in fiber abundance between samples and controls, using the Wilcoxon rank sum test. When p-value is <0.05, the comparison indicates that there are significant differences between observed and control fibers; then, they may have different origin and observed fibers should be considered in the ML assessment, without control fiber detraction. Otherwise, when p-value is > 0.05 the output indicates that control and observed fibers are probably coming from the same source, suggesting a potential contamination. Therefore, the correlation test (we used Kendall's rank based on the nature of our data) was conducted to establish the best method to detract the number of control fibers from the observed values. If the results of test Kendall's rank test do not show significant correlation, the mean number of fibers in the control sample should be detracted from observed fibers, according to Kühn et al. (2018). In contrast, if significant correlation is observed, the total number of control fibers should be detracted from observed fibers in samples. In addition, as last step, to further reduce the potential bias associate to the entire detraction of fibers, according to Hermsen et al. (2017) and Schirinzi et al. (2020), only the control fibers having similar features (i.e., structure, color) to observed fibers in GITs are detracted (**Figure 3**).

This statistical approach allows to exclude the potential airborne fiber contamination from the results of ML assessment, achieving a more reliable assessment of ML ingestion. Statistical analyses were performed using R and R-Studio software (R Core Team, 2019; RStudio Team, 2019).

RESULTS

Marine Litter Ingestion

A total of 61 specimens of demersal elasmobranchs were examined. **Table 1** shows the size and weight ranges of individuals and the corresponding mean values for each species. Overall, 19 ML particles were found in the GITs of 13 individuals (%O = 21.3): 9 items in *S. canicula* (%O = 22.2), 6 items in *G. melastomus* (%O = 33.3), and 4 items in *R. clavata* (%O = 50), whereas no litter ingestion was observed in *E. spinax*. In *G. melastomus* and *R. clavata*, the 100% of anthropogenic particles found in the GIT were identified as plastics, while in *S. canicula* only the 56% of them belonged to this category and the remaining 44% were chicken remains and polyacrylamide particles, classified as other rubbish (%O = 11.1) and pollutants waste (%O = 33.3), respectively (**Table 2**).

A total of 15 plastics items (0.25 items/specimen; range: 0–2 items per specimen) have been ingested by 11 elasmobranch specimens (%O = 18) (**Table 2**). In **Table 2**, the length, width and weight ranges of ingested plastics for each species are also reported. The average length and width of all plastics recovered from fish were 19.42 ± 50.44 and 1.42 ± 1.37 with a range from 1.28 to 200 mm and from 0.01 to 3.91 mm, respectively. The average weight was 0.01 ± 0.02 g, varying between <0.0001 and 0.0738 g (**Table 2**).

Characteristics and Polymers Typology of Plastics

Based on their size, plastic items found in the GITs of demersal elasmobranchs mainly belonged to microplastics category (53.4%), but also mesoplastics (33.3%) and macroplastics (13.3%) were found (**Figure 4A**).

The shape of plastic items (**Figure 4B**) was quite variable between species; threadlike was the most common plastics in stomachs *G. melastomus* (50%) and foam was the most abundant shape category in *S. canicula* (40%). The proportion of each of the following shape categories in GITs of in *R. clavata* was 25%: sheetlike, threadlike, fragment, and other plastic typologies (dense rubber).

White and transparent were the most frequent colors of the ingested plastics, but also blue, red, and brown items were found (**Figure 4C**).

The FT-IR spectroscopic analysis allowed to identify the following plastic polymers items: 2 polyethylene (PE), 2

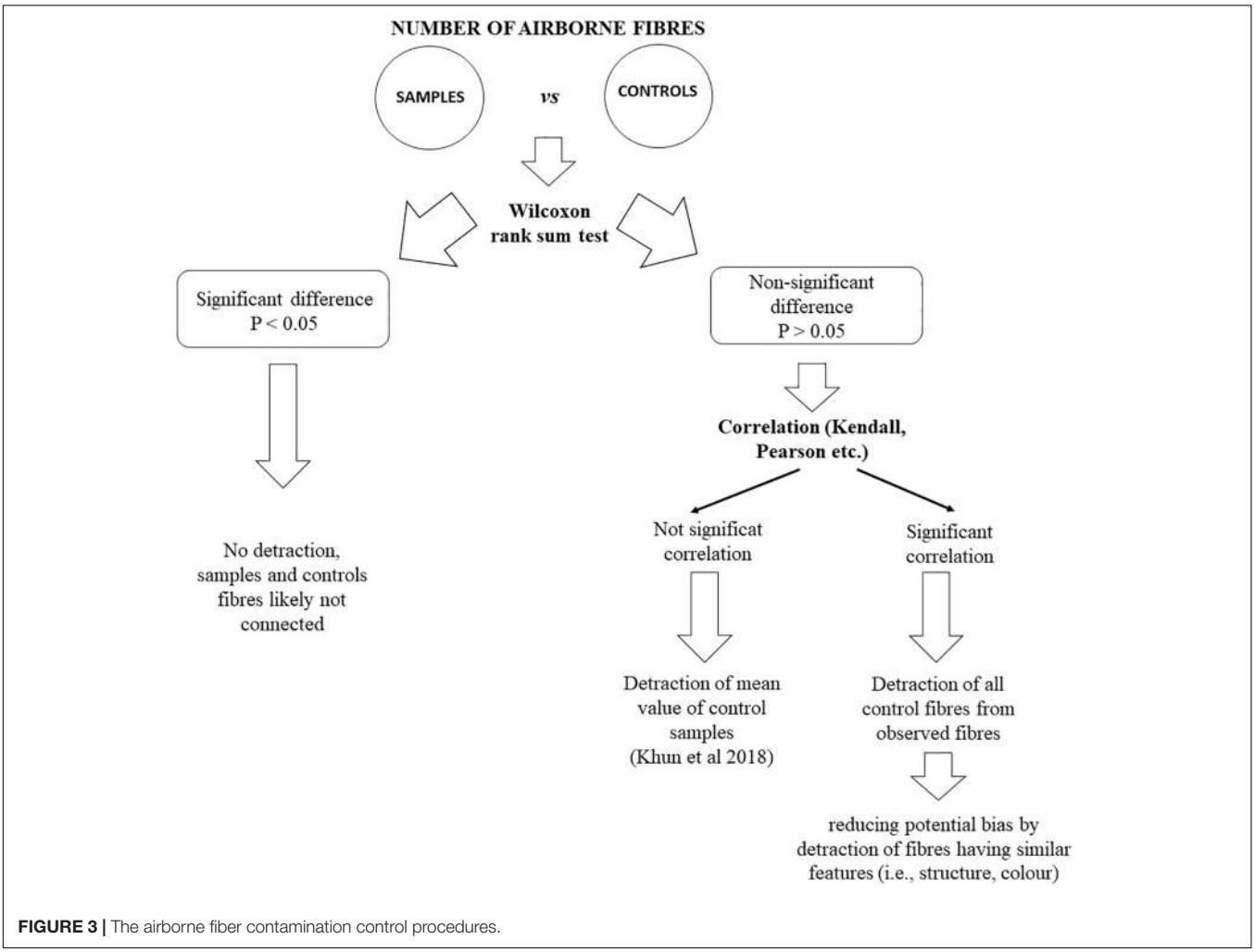

FIGURE 3 | The airborne fiber contamination control procedures.

TABLE 1 | Number of examined gastro-intestinal tracts (GITs), size (TL, cm), and weight (TW, g) ranges for each species.

Species	Number of GITs examined	Mean TL ± SD (cm)	TL range (cm)	Mean TW ± SD (g)	TW range (g)
SYC	27	40.5 ± 6.3	26.2–51.2	187.4 ± 96.3	32.5–378.5
ETX	16	21.2 ± 7.7	14.3–39.3	55.7 ± 58.5	11.6–197.2
SHO	12	20.7 ± 3.0	17.3–27.9	24.9 ± 10.8	12.7–51.1
RJC	6	65.8 ± 2.8	62.9–69.8	31.7 ± 1.3	30.0–34.0

SYC, Scyliorhinus canicula; ETX, Etmopterus spinax; SHO, Galeus melastomus; RJC, Raja clavata; SD, standard deviation.

polypropylene (PP), 2 polystyrene (PS), 5 polyamide (PA; including nylon and aromatic polyamides), 1 polyester (PL), 1 polyurethane (PUR), and 1 rubber (**Figure 4D**). Polyamide was the only polymer found in all species. In *S. canicula* 40% of plastics analyzed were identified as PS followed by PE, PP, and PA (all having 20%). Polyamide (50%) was the most frequent polymer in GITs of *G. melastomus,* while PE and PL were ingested to a lesser extent (16.7%).

In addition, one fiber (16.7%) was also recorded in the gut of this species, but it was impossible to analyze this sample by FT-IR because it was too thin (0.03 mm) and, then, it was considered as not determined (N.D.). Finally, PP, PA, PUR, and rubber had a frequency value of 25% in GITs of *R. clavata*. Images of some plastic samples found in the GITs of demersal elasmobranchs are reported in **Figure 5**, together with the corresponding FT-IR spectra.

Fiber Contamination in Samples

A total of 21 fibers were found in 25% of the demersal elasmobranchs GITs. **Table 3** shows the information on fibers abundance in the examined samples, with the comparison between the observed values in GITs (0.34 ± 0.66 items/individual) and controls (0.61 ± 0.99 items/individual). The number of fibers per fish GIT ranged between 0 and 2, and in controls between 0 and 4. According to the results of Wilcoxon rank sum test, no significant differences were found between

TABLE 2 | Results on the occurrence, abundance and size of marine litter ingested by demersal elasmobranchs (*Scyliorhinus canicula* = SYC, *Galeus melastomus* = SHO, *Raja clavata* = RJC).

Marine Litter	SYC	SHO	RJC	Total
Number of GITs with ML	6	4	3	13
Number of ML items	9	6	4	19
Percentage of occurrence (%O) of ML items	22.2	33.3	50	21.3*
Number of GITs with plastics	4	4	3	11
Number of plastic items	5	6	4	15
Percentage of occurrence (%O) of plastic items	15	33.3	50	18*
Plastics' abundance:				
(i) N. plastic items/N. all examined individuals (average ± SD; range)	0.19 ± 0.48 (0–2)	0.50 ± 0.80 (0–2)	0.67 ± 0.82 (0–2)	0.25 ± 0.57* (0–2)
(ii) N. plastic items/N. individuals which ingested plastics (average ± SD; range)	1.25 ± 0.50 (1–2)	1.5 ± 0.58 (1–2)	1.33 ± 0.58 (1–2)	1.36 ± 0.50 (1–2)
Plastics' length (average ± SD; range; mm)	8.64 ± 10.62 (1.28–27.29)	38.25 ± 79.45 (1.46–200)	4.64 ± 2.59 (2.31–8.29)	19.42 ± 50.44 (1.28–200)
Plastics' width (average ± SD; range; mm)	2.11 ± 1.45 (0.34–3.91)	0.42 ± 0.43 (0.01–0.94)	2.07 ± 1.51 (0.14–3.83)	1.42 ± 1.37 (0.01–3.91)
Plastics' weight (average ± SD; range; g)	0.02 ± 0.01 (0.0002–0.0738)	0.001 ± 0.001 (<0.0001–0.0016)	0.01 ± 0.01 (0.0004–0.014)	0.01 ± 0.02 (<0.0001–0.0738)

*No litter ingestion was observed in E. spinax = ETX. *Data calculated on 61 examined stomachs (i.e., also including E. spinax samples).*

samples and controls ($p > 0.05$) in each species, indicating that likely fibers in samples may be due to airborne contamination (**Table 4**). According to the results of Kendall correlation test, the correlation was significant for all species (**Table 5**). Based on these results, the number of observed fibers was corrected by detracting the total number of control fibers. The last step of our control method consisted in the comparison of structure and color of observed and control fiber, in order to confirm the exclusion of those fibers having the same features (**Figure 6**). According to our approach, only in *G. melastomus* the presence of one ingested fiber can be confirmed.

DISCUSSION

Marine Litter Ingestion in Demersal Elasmobranch Species

The present study provided information on the ML ingestion in some elasmobranch species (*S. canicula, G. melastomus*, and *R. clavata*) collected as bycatch in trawl and longline fisheries in the southern Tyrrhenian Sea (GSA 10). ML ingestion has been observed in 21.3% of individuals, although one of the investigated species (*E. spinax*) did not show the presence of ML.

The present study reports, for the first time, data on the litter ingestion by *R. clavata* in Mediterranean waters. Although only six specimens were analyzed, half of them resulted affected by ML ingestion. Previous investigations on *R. clavata*, collected off Cephalonia Island (Greece, Eastern Ionian Sea) and central Tyrrhenian Sea, did not show any sign of ML pollution (Anastasopoulou et al., 2013a; Valente et al., 2020). However, recently, in a close-related species (*Raja miraletus*), Capillo et al. (2020) observed ML debris in GIT of just one individual, caught in the southern Tyrrhenian Sea. So far, the impact of ML on species belonging to the family Rajidae has been poorly studied and this aspect is worth of further investigations.

The occurrence of ML (22.2%) in GITs of *S. canicula* in the study area was lower than data previously observed on the same species from the Tyrrhenian Sea; Valente et al. (2019) and Capillo et al. (2020) reported %O values of 66.7 and 33%, respectively. The highest level of plastic ingestion in *S. canicula* was observed by Mancia et al. (2020) in the Strait of Sicily (about 80% of occurrence).

Among the analyzed species, *G. melastomus* is the most investigated demersal elasmobranch for ML ingestion in the Mediterranean Sea. In the present study, the ingestion of ML (%O = 33.3) was higher than values previously observed by Capillo et al. (2020) in the same study area (%O = 8), but lower than the one (%O = 78.1) reported by Valente et al. (2019) in the central Tyrrhenian Sea. Data on ML ingestion in *G. melastomus,* from studies carried out in other Mediterranean areas, revealed a lower interaction with this species, reporting %O values ranging from 3.2 to 12.5% in the eastern Mediterranean Sea (Anastasopoulou et al., 2013a,b; Madurell, 2003) and from 6.3 to 16.8% in the western Mediterranean Sea (Carrassón et al., 1992; Cartes et al., 2016; Alomar and Deudero, 2017).

In this study, no litter particles were found in the GITs of *E. spinax,* although other authors had already observed ML ingestion in this elasmobranch. Indeed, in the western

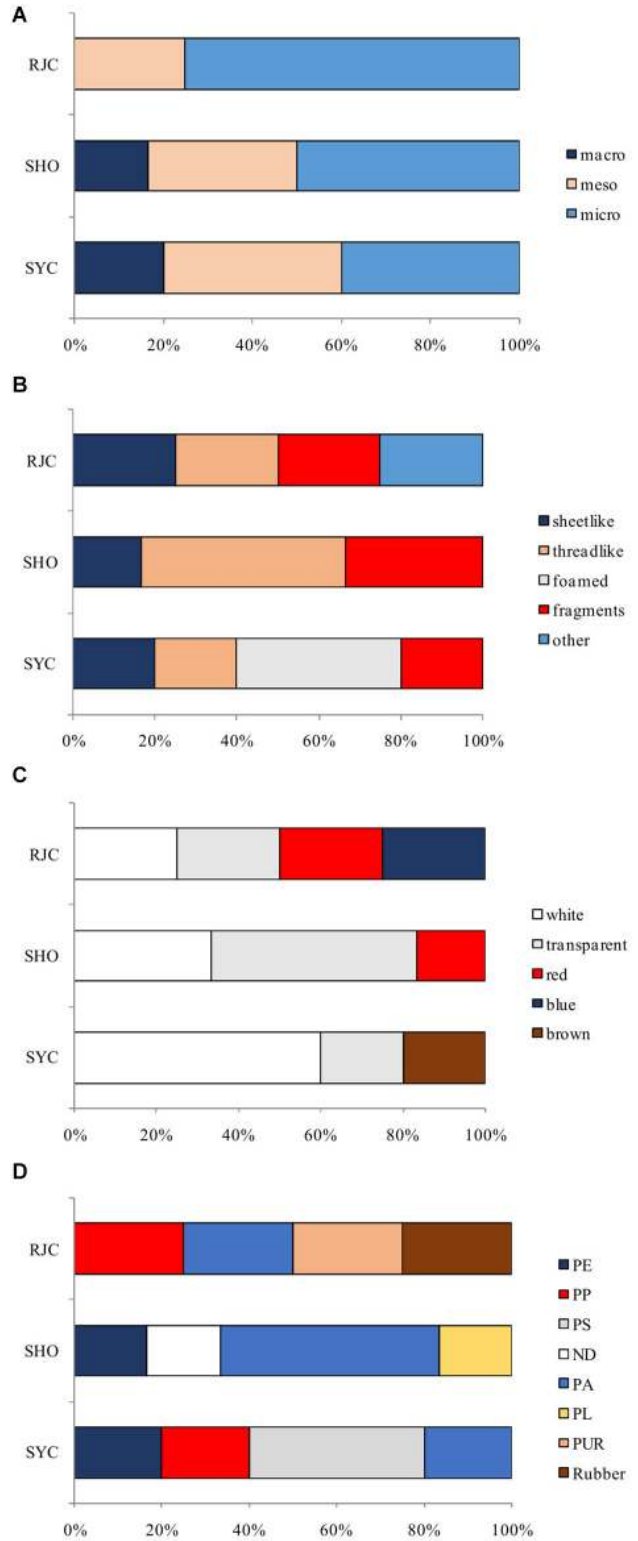

FIGURE 4 | Classification by size, shape, color and polymer type of plastics detected (n = 15) in demersal elasmobranchs (*Scyliorhinus canicula* = SYC, n = 5; *Galeus melastomus* = SHO, n = 6; *Raja clavata* = RJC; n = 4). **(A)** Percentage of size classes; **(B)** Percentage of shapes; **(C)** Percentage of colors; and **(D)** Percentage of polymer types.

Mediterranean Sea, Cartes et al. (2016); Alomar and Deudero (2017) and Valente et al. (2019) reported %O values of 7.8, 50, and 61.8%, respectively, while ML ingestion in the eastern Mediterranean Sea resulted around 7% (Anastasopoulou et al., 2013a; Madurell, 2003).

From the analysis of these results and bibliographic information, it is possible to observe that ML ingestion in Mediterranean demersal elasmobranchs shows high variability in terms of occurrence. These differences may be related to the mutability of environmental factors and features (i.e., river inputs, convergence currents, etc.) and anthropic pressure in the study areas, but also, more probably, to the different methods used to assess ML ingestion. The main source of variability is due to the use of different plastic extraction methods and contamination control procedures. For instance, Anastasopoulou et al. (2013a; 2013b), Alomar and Deudero (2017), and Capillo et al. (2020) analyzed the stomach contents by visual sorting, whereas Valente et al. (2019) and Mancia et al. (2020) digested stomach contents by chemical digestion protocols. Sometimes, data on ML ingestion are not the main focus of a research program and information are additionally collected during studies on trophic ecology of different marine species (Madurell, 2003; Anastasopoulou et al., 2013b) and, for this reason, they may lack of airborne contamination control procedures or quality assurance measures. Moreover, the polymer identification by IR or Raman spectrometry is a procedure only recently used in ML studies.

In addition, the diverse sample sizes considered in Mediterranean studies is another important limit to data comparability.

The finding of ML particles in the GITs of demersal elasmobranchs is certainly related to their feeding behavior and their strong relationship with the seafloor, as also suggested by other authors (Alomar and Deudero, 2017; Fossi et al., 2018; Valente et al., 2019; Capillo et al., 2020). Indeed, these species live in direct contact with the seafloor (Fanelli et al., 2009; Valls et al., 2011), are characterized by a generalist feeding behavior and mainly feed on the bottom invertebrates and benthic fish (Fanelli et al., 2009; Valls et al., 2011; Šantić et al., 2012; Anastasopoulou et al., 2013b). Furthermore, *R. clavata* is able to find its food by excavating in soft sediments were prey is usually buried (Gray et al., 1997; Saglam et al., 2010; Šantić et al., 2012); this feeding behavior may determine an increase of the risk to ingest ML, which could be accumulated inside the sediments. Indeed, the seafloor represents an important sink for the accumulation of ML and their density is often greater in deep waters along the continental shelf edge than in shallow waters (Galgani et al., 1995, 2000; Barnes et al., 2009). The density of ML in the seafloor of the study area may be also affected by the absence of bottom trawl fishing up to a bathymetry of about 500 m, due to access restrictions established since 1990 (Battaglia et al., 2017).

Debris particles may be accidentally ingested during feeding activity or confused with their prey, but secondary ingestion cannot be excluded. For instance, it is known that *G. melastomus* is able to feed also on lanternfish (Fanelli et al., 2009; Valls et al., 2011), which have been reported as species affected by ML ingestion (Romeo et al., 2016). Elasmobranchs are also known

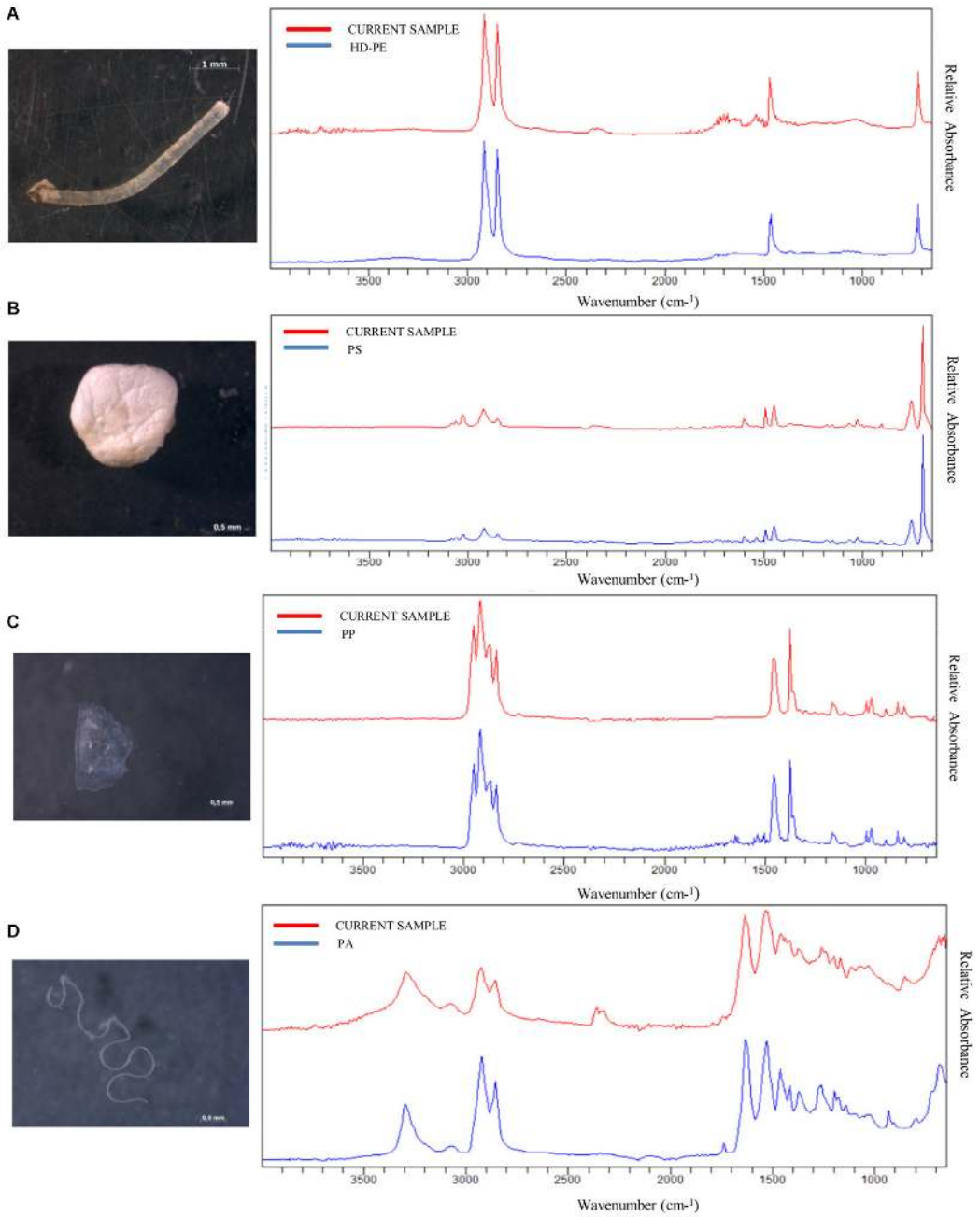

FIGURE 5 | Images of some plastics samples found in the GITs of demersal elasmobranchs (*Scyliorhinus canicula* = SYC, *Galeus melastomus* = SHO, *Raja clavata* = RJC) with the respective FT-IR spectra. **(A)** HD-PE threadlike in SYC; **(B)** PS foam in SYC; **(C)** PP sheet in RJC; and **(D)** PA threadlike in SHO.

as scavengers, feeding opportunistically on carrions and preying on dying, dead or decomposing individuals (Olaso et al., 1998), which may have potentially ingested ML in upper waters and that sink toward seafloor (Olaso et al., 1998; Valente et al., 2019).

Plastic Characterization

Plastic represented the main litter category found in the GIT of examined demersal elasmobranchs, as also observed in previous studies on Mediterranean demersal sharks (Anastasopoulou et al., 2013a; Kühn et al., 2020; Cartes et al., 2016; Alomar and Deudero, 2017; Valente et al., 2019; Capillo et al., 2020; Mancia et al., 2020) and on a wide range of marine organisms (Campani et al., 2013; Romeo et al., 2015, 2016; Battaglia et al., 2016; Bernardini et al., 2018; Digka et al., 2018a; Bottari et al., 2019; Savoca et al., 2019, 2020; Schirinzi et al., 2020). *G. melastomus* and *R. clavata* ingested only plastics while *S. canicula* ate also chicken remains (probably used as bait by local artisanal fishermen targeting common octopus) and polyacrylamide

TABLE 3 | Information on the presence of fibers in all examined species (*Scyliorhinus canicula* = SYC, *Etmopterus spinax* = ETX, *Galeus melastomus* = SHO, *Raja clavata* = RJC).

Species	n fish	Fiber in controls			Fiber in samples (observed)			Fiber in samples (corrected)		
		n fiber (range/specimen)	Average number ± SD	%O	n fiber (range/specimen)	average number ± SD	%O	n fiber (range/specimen)	Average number ± SD	%O
SYC	27	16 (0–3)	0.593 ± 0.931	33.3	11 (0–2)	0.407 ± 0.747	25.9	0	0.000 ± 0.000	0.0
ETX	16	8 (0–4)	0.500 ± 1.095	25	3 (0–2)	0.188 ± 0.544	12.5	0	0.000 ± 0.000	0.0
SHO	12	8 (0–3)	0.667 ± 0.985	41.7	5 (0–2)	0.417 ± 0.669	33.3	1 (0–1)	0.083 ± 0.289	8.3
RJC	6	5 (0–3)	0.833 ± 1.329	33.3	2 (0–1)	0.333 ± 0.516	33.3	0	0.000 ± 0.000	0.0
Total	61	37 (0–4)	0.607 ± 0.988	34.4	21 (0–2)	0.344 ± 0.655	24.6	1 (0–1)	0.016 ± 0.128	1.6

The abundance (average number ± standard deviation, SD) and percentage of occurrence (%O) of fibers observed in samples and controls are provided together with the corrected values after application of control procedures.

TABLE 4 | Wilcoxon rank sum test between samples and controls.

Species	Parameters (W)	*p*-value
SYC	332.5	0.4977
SHO	64	0.6149
ETX	111.5	0.3755
RJC	16	0.7745

TABLE 5 | Kendall's rank correlation Tau of each species between samples and controls.

Species	*z*	Tau	*p*-value
SYC	4.72	0.86	2.4e-06
SHO	2.16	0.60	0.03061
ETX	2.81	0.67	0.004912
RJC	2.19	0.94	0.02846

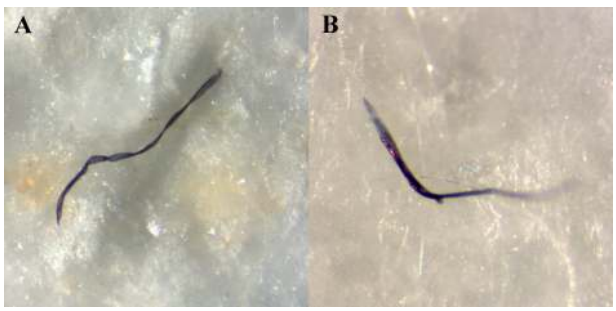

FIGURE 6 | Example of fibers having similar characteristics, found in samples **(A)** and in the respective control filter **(B)**.

particles. Polyacrylamide (a high molecular weight polymer) are widely used in industrial processes to aid flocculation and complexation and in oil production processes (Hansen et al., 2019). The potential effects of polyacrylamide on marine organisms are still not well investigated (Hansen et al., 2019).

The analysis of ML size revealed that the largest amount of plastics found in the stomachs belongs to microlitter category (53%). This result could be related to the morphological traits of the investigated elasmobranchs, which have a small mouth more suitable for the ingestion of small prey. Information on microplastics' levels in sediments of the study area is not available, but data from an adjacent zone (Aeolian Islands) indicates that this ML category is quite abundant on the seafloor (Fastelli et al., 2016; Martellini et al., 2018). On the other hand, macroplastics were only found in few stomachs of *S. canicula* and *G. melastomus*, in agreement with findings of Capillo et al. (2020) and Valente et al. (2020), which had previously observed low levels of macrolitter ingestion in such species. According to Valente et al. (2020), these demersal elasmobranchs may regurgitate macrolitter, being the intestinal spiral valve an obstacle to their transit toward their intestinal tract.

Threadlike and fragment were the most abundant plastic shape categories, as observed by other authors in different Mediterranean areas (Digka et al., 2018a,b; Capillo et al., 2020; Valente et al., 2020). However, these studies reported a

considerably higher abundance of fibers, whereas in our research, the threadlike category included almost exclusively filaments (most of them were remains of fishing lines) and only one fiber. This is probably due to the application of airborne contamination control method, which allowed to exclude fibers derived from secondary contamination.

The color of ingested plastics was mainly transparent or white, even though also red, blue and brown particles were found. The prevalence of clear color could mirror the real ML patterns in the marine environment, otherwise it could be related to their resemblance of elasmobranchs' potential prey. Indeed, according to Kühn et al. (2015), specific colors might attract predators which may confuse ML for their prey.

The characterization of plastic polymers through FT-IR analysis showed that PA (aliphatic and aromatic polyamides) was the most abundant plastic compound found in the stomachs. The PA polymers have a large number of applications. For instance, nylon is an aliphatic polyamide of high commercial importance (Aoki et al., 1979), highly used for fishery purposes. Aromatic polyamides (Nomex and Kevlar fiber) are utilized for firefighting and in fire-resistant clothing, automotive, nautical, planes, and space sectors but also employed in several sports equipment (Aoki et al., 1979; Baker, 2018). Although PA is not among the most used and produced plastic polymers at global level (Geyer et al., 2017), their high use in industrial production is probably the main cause of their presence in the marine environment and then in the GITs of examined species, and the occurrence in the study area could be related to the specific vocation toward recreational and professional fishing activities, as well as to the presence of touristic harbors and nautical activities, that are highly developed also for their closeness to the Aeolian Archipelago (Savoca et al., 2019).

The other most common polymers (polyethylene, polypropylene, and polystyrene), identified in the GITs, are quite abundant in the marine environment (Andrady, 2011; Cózar et al., 2014; Suaria et al., 2016; Geyer et al., 2017; Digka et al., 2018b). Although these polymers have positive buoyancy, after density modifications due to abiotic (e.g., currents and water circulation) and biotic (biofouling) factors they could sink in the sediments becoming potentially available for demersal marine organisms (Andrady, 2017).

Finally, the study area is characterized by an intense fishing activity (Battaglia et al., 2017), and then, PE, PP, and nylon particles may derive by the degradation of lost or abandoned fishing gears (Consoli et al., 2018).

Contamination Control Method

The airborne fiber contamination is a crucial and complex issue. Several studies suggest that the risk of contamination by airborne fibers, both natural or synthetic, is very high and unavoidable during the sampling and laboratory analysis (Torre et al., 2016; Hermsen et al., 2017, 2018; Kühn et al., 2018, 2020; Schirinzi et al., 2020). For instance, air currents, operators' clothing and a not accurate cleaning of laboratory tools can lead to samples contamination and, then, to overestimation of fibers ingestion in biota (Hidalgo-Ruz et al., 2012; Torre et al., 2016).

To date, some studies on ML ingestion in marine organisms have demonstrated that, if mitigation measures are correctly applied, the assessment of this phenomenon is lower thanks to the reduction of airborne fibers in samples and controls (Rummel et al., 2016; Hermsen et al., 2017, 2018; Kühn et al., 2018, 2020). Furthermore, although the quality assurance criteria are applied in almost all studies, details on fiber contamination and correction of estimates through the application of a control contamination method are rarely provided (Kühn et al., 2018, 2020).

For these reasons, our study applied severe mitigation measures to avoid airborne contamination during the analysis, integrating a methodology for the correction of estimates of fibers abundance using control procedures. In this regard, we also suggest in future studies to carry out a separated analysis for the estimation of fibers presence and to provide clear information on both fibers abundance observed in samples and controls. This will be useful for the procedure and data standardization and will help the comparison between different studies on ML ingestion.

The integrated method applied in the present study achieved a reliable assessment of ML ingestion in demersal elasmobranchs, having excluded potential airborne fiber contamination from the results. Indeed, the large part of observed fibers was excluded by our assessment, after the comparison with fibers found in the control filters, and only the presence of one fiber was confirmed as anthropogenic litter in the GIT of a *G. melastomus* individual. Other studies, using different methodologies, reported a consistent amount of fibers in GITs of elasmobranch species from some Mediterranean areas: *S. canicula* and *G. melastomus* from the southern Tyrrhenian Sea (Capillo et al., 2020); *S. canicula, G. melastomus* and *E. spinax* from the central Tyrrhenian Sea (Valente et al., 2019) and *G. melastomus* from the Balearic Island (Alomar and Deudero, 2017).

Although it is impossible to totally avoid airborne contamination, our control procedures aims to avoid the overestimation of ML, being this issue a crucial point in studies on ML ingestion.

Based on these results and considerations, it is possible to recommend in future studies to provide a detailed description of mitigation measures, applied at each step of laboratory analysis and aimed to tackle airborne contamination: reduction of air currents, use of 100% cotton lab coats, use of glassware and metal laboratory tools, use of filtered water to clean laboratory tools, working under the fume hood, covering of samples, use of a control (moist filter) in each analysis and application of a data correction method (**Figure 2**).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because we used dead fish collected from bycatch of local fisheries.

AUTHOR CONTRIBUTIONS

CP organized the database, performed the data analysis, and wrote the manuscript. PB contributed to the conception and design of the study, wrote, commented on, and edited the manuscript. MD'A performed the GITs analysis and revised the manuscript. FLa performed the GITs analysis. DM performed the statistical analysis and revised the manuscript. PC, TV, FA, TB, and SG contributed to the manuscript revision. FLo contributed to the sample collection. MG, MB, and MF performed the FTIR analysis and revised the manuscript. TR provided funds, contributed to conception and design of the study, commented on, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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