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Microplastics in the Digestive System of the Atlantic Sharpnose Shark (*Rhizoprionodon terraenovae*) in Winyah Bay, SC

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Microplastics in the Digestive System of the Atlantic Sharpnose Shark
(*Rhizoprionodon terraenovae*) in Winyah Bay, SC

By Elise Pullen

Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in
Coastal Marine and Wetland Studies in the School of Coastal and Marine Systems Science

Coastal Carolina University 2019

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DEDICATION

I would like to dedicate this thesis to my loved ones, especially to my parents Pete and Gini, and my brother Rob, for fostering my academic drive, instilling in me a deep appreciation of nature, and teaching me how to take everything in stride.

ABSTRACT

The digestive tracts and livers of adult male Atlantic sharpnose sharks (N=16), *Rhizoprionodon terraenovae*, from Winyah Bay, South Carolina were examined for ingested microplastics. *R. terraenovae* is a small, locally abundant, coastal mesopredatory elasmobranch belonging to the family Carcharhinidae. Microfibers comprised the largest categories of plastics (94% of the total), and were found in 100% of sharks examined. The number of micro- and other plastics ranged from 34 to 75 per individual and totaled 927. The majority of plastics (40%) were blue in coloration, and 55% were <1 mm in length. Microplastics were observed on both the interior and exterior of the organs examined, and three microfibers were embedded within the stomach lining, an observation not previously reported in marine vertebrates and one which represents a potential pathway for the translocation of ingested microplastics.

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INTRODUCTION

OVERVIEW OF PLASTICS

Plastics directly impact a wide range of organisms and their environments, with many products composed of plastic being discarded after a single use (Cole *et al.*, 2013). Shipping, illegal dumping, and discarded fishing gear also add a substantial amount of plastics directly to the sea (Gregory, 2009). Land-based sources including landfills, littering, and outfalls from industry further contribute to marine plastic pollution. Eriksen *et al.* (2014) estimates that 5.25 trillion plastic particles, weighing approximately 268,940 tons, are currently in the ocean. The most abundant types of plastics in the environment include polyethylene, polypropylene, and polystyrene (Mato *et al.*, 2001). Plastic pollution has resulted in major environmental consequences, including an increase in entanglement (e.g., the phenomenon of *ghost fishing*, abandoned but still functional nets), transportation of invasive species, and ingestion of plastics by marine species (Gregory, 2009).

INGESTION OF PLASTICS

Upon consumption, hard plastics can cause damage by punctures and tears, whereas other plastic materials (i.e., plastic bags) can lead to digestive tract blockages (Lusher *et al.*, 2013). Blockages may reduce the uptake of nutrients and prevent normal digestive function, and in doing so may cause malnourishment and death. Davison *et al.* (2011) estimated the amount of plastic debris consumed by mesopelagic fishes in the North Pacific Subtropical Gyre ranges between 12,000 to 24,000 tons per year (Carson, 2013). In the Eastern Ionian Sea, Anastasopoulou *et al.* (2013) found that 86.5% of foreign material ingested by fishes was plastic. This included an assortment of plastic from both land- and marine-based sources, including hard materials, plastic bag fragments, fishing gear, and fibers from textiles. Although the types of

plastics consumed are dependent on particle size and location, Choy and Drazen *et al.* (2013) found that pelagic predatory fish consume fishing line more than any other type of debris.

Prior to research focused on isolating and enumerating plastics from digestive systems, plastic items were more frequently reported as *miscellaneous items* during diet studies. Bass *et al.* (1973) described spherical 1 mm plastic beads and 30 mm fine plastic sheets ingested by carcharhinid sharks on the east coast of Southern Africa, whereas Cliff and Dudley (1991) reported bull sharks (*Carcharhinus leucas*) as having ingested plastic, without providing any sizes or additional descriptors (Cliff and Dudley, 1991). In a 23-year study between 1978 and 2000 of stomach contents from 15,666 sharks caught in gillnets off of the coast of South Africa, Cliff *et al.* (2002) found 60 individuals as having ingested plastic, with 48% classified as plastic bag materials. Sampaio *et al.* (2018) reported that a stranded juvenile whale shark (*Rhincodon typus*) in Northeast Brazil was found to have ingested plastic packing materials, a fragmented cotton swab, and additional plastic debris.

CHARACTERIZATION OF MICROPLASTICS

Plastics are classified by size as *macroplastics* (> 200 mm), followed by *mesoplastics* (5 – 200 mm), *microplastics* (0.001 – 4.99 mm), and *nanoplastics* (< .001 mm) (Arthur *et al.*, 2009; Eriksen *et al.*, 2014, Gigault *et al.*, 2018). Microplastics are further subdivided into *primary* and *secondary* types. Primary microplastics are those intentionally manufactured to < 5 mm, such as microbeads in cosmetic and personal care products. Secondary microplastics, often collectively referred to as *fragments*, result from larger plastic objects breaking apart (due to physical processes such as biofouling and weathering), increasing the number of plastic particles even if inputs ceased (Cole, 2011). Microplastics can be further characterized based on shape and color, either at time of production or once having fragmented in the environment.

Shape and Color of Microplastics

The shape of microplastics can be classified into four categories, including *films*, *fragments*, *fibers*, or *pellets*, as identified in Figure 1. Plastics can also be distinguished based on a combination of a unique appearance, texture, and reaction to heat (Ladewig *et al.*, 2018). For example, films and fibers are flexible compared to fragments and pellets, which probing reveal as hard. In further categorizing fragments, Hidalgo-Ruz *et al.*, (2012) attributed fragment shape (e.g., round vs angular) as evidence of how recently the particle had broken off from its original source (i.e., rounder fragments would have likely been exposed to additional environmental degradation causing smoothing). Microfibers from textiles (i.e., plastic strands dislodging from clothing during washing cycles) appear elongated and uniform in length, although fraying may occur (Mato *et al.*, 2001; Ladewig *et al.*, 2018).

Color identification of ingested microplastics can aid in determining whether the plastic was intentionally or unintentionally ingested. Specific colors may lead to intentional targeting by an organism where plastics share similar sizes and colors of prey. Choy and Drazen *et al.* (2013) found that the longnose lancetfish, *Alepisaurus ferox*, favored white and clear plastic pieces. Since the diet of *A. ferox* includes hyperiid amphipods, salps, and siphonophores, which are white and translucent, similarly-colored plastics may have been mistakenly ingested (Bowman *et al.*, 2000). In a similar case, 79% of microplastics ingested by the omnivorous fish *Girella laevifrons* were red in coloration, which was attributed to it likely targeting one of its major food sources, red algae (Mizraji *et al.*, 2017). However, nontargeted ingestion also may occur while not actively foraging, particularly in areas with increased industrial development (Desforges *et al.*, 2015). For example, Dantas *et al.* (2012) examined the ingestion of nylon fragments in estuarine drums, *Stellifer brasiliensis* and *Stellifer stellifer*, detecting only blue fragments. These

findings were attributed to the blue polyfilament nylon ropes used by fisheries near the study site.

Distribution and Toxicity of Microplastics

Carpenter *et al.* (1972) discussed the presence of microplastics in the western Sargasso Sea as brittle pellets in masses of 3,500 pieces per km². In the last decade, the concern and attention dedicated to microplastics found in organisms and the environment has greatly increased (Jamieson *et al.*, 2019). Microplastics have now been recorded in extreme environments such as the Arctic Basin and the Mariana Trench (Kanhai *et al.*, 2018; Jamieson *et al.*, 2019).

Research on microplastic consumption has been conducted on a range of organisms including marine mammals, sea birds, fishes, and invertebrates (Fossi *et al.*, 2014; Tanaka *et al.*, 2013; Dantas *et al.*, 2012; Jang *et al.*, 2016). Farrell and Nelson (2013) demonstrated the trophic transfer of microplastics from mussels to crabs, and Gutow *et al.* (2015) found that microplastics adhering to the surface of seaweed were consumed by marine snails. This phenomenon of trophic level transfer has the potential to occur throughout the food web (Figure 2). The consequences of this trophic level transfer include that plastic particles can be physically transferred between individuals, and also that microplastics may serve to transport toxins (Farrell and Nelson, 2013; Fossi *et al.*, 2014).

The toxicity of microplastics can be attributed to two different causes. The first is toxic additives that are part of the production process of plastics (Teuten *et al.*, 2009). The second occurs when nonpolar chemicals in pollutants are adsorbed onto plastics due to the affinity of the chemicals for the nonpolar surface of the plastics. For example, Mato *et al.* (2001) demonstrated that plastic resin pellets adsorb polychlorinated biphenyls (PCBs) and dichloro-diphenyl-

dichloroethylene (DDE) from surrounding seawater, acting as a mechanism to absorb toxins. DDE is a major degradant of dichloro-diphenyl-trichloroethane (DDT) and is considered a probable human carcinogen by the Environmental Protection Agency (EPA) (National Toxicology Program, 2016). DDE and PCBs leach into the tissues of an organism if consumed, as both are readily fat-soluble (Fossi *et al.*, 2014).

The toxic effect of chemical exposure via microplastics has been shown in invertebrates, birds, fish, and marine mammals (Jang *et al.*, 2016; Tanaka *et al.*, 2013; Fossi *et al.*, 2014). Jang *et al.* (2016) identified the presence of expanded polystyrene (styrofoam) particles in mussels and the associated levels of a brominated flame retardant, hexabromocyclododecanes (HBCDs) in their tissues. The main concern with HBCDs in mussel tissues is that they are a persistent organic pollutant (POP) with a tendency to bioaccumulate (Jang *et al.*, 2016). Tanaka *et al.*, (2013) also found polybrominated diphenyl ethers (PBDEs) in the system of the short-tailed shearwaters, *Puffinus tenuirostris*, in addition to microplastics in their digestive systems. Fossi *et al.* (2014) found organochlorine compounds and phthalates (chemicals used to soften rigid plastics) had leached from microplastics in the basking shark, *Cetorhinus maximus*, and the Mediterranean fin whale, *Balaenoptera physalus*. Phthalates leach from plastics, the most abundant being di-(2-ethylhexyl) phthalate (DEHP).

INGESTION OF MICROPLASTICS IN SHARKS

Currently, nine species of sharks have been documented as having consumed microplastics. In *R. typus*, skin biopsies showed chemical evidence of microplastic ingestion (Fossi *et al.*, 2017). Blue sharks (*Prionace glauca*), spiny dogfish, blackmouth catshark (*Galeus melastomus*), Portuguese dogfish (*Centroscymnus coelolepis*), velvet belly lanternshark (*Etmopterus spinax*), and longnose spurdog (*Squalus blainville*) have also been reported to have

consumed microplastics (Bernardini *et al.*, 2018; Avio *et al.*, 2015; Alomar and Deudero, 2017; Cartes *et al.*, 2016; Anastasopoulou *et al.*, 2013).

BIOLOGY OF THE ATLANTIC SHARPNOSE SHARK

R. terraenovae is small carcharhinid shark found along the east coast of North America and in the Gulf of Mexico, and is currently a species of least concern according to the International Union for Conservation of Nature and Natural Resources (IUCN) (Cortés, 2009). It is consumed by humans, and NOAA currently lists United States wild-caught Atlantic sharpnose shark as a *smart seafood choice* due to its life history characteristics (including its high reproductive potential) and its sustainable management (NOAA Fisheries, 2017). Females reach a maximum total length of 107 cm, maturing between 2.8 to 3.9 years old at 85-90 cm. Males tend to reach a maximum total length of 105 cm, maturing between 2.4 to 3.5 years at a length between 80-85 cm (Parsons, 1985). In northwestern Atlantic sharpnose shark populations, 64% of the diet consisted of teleosts, followed by 34% crustaceans (Gelsleichter *et al.*, 1999). In samples collected near Cape Hatteras, North Carolina, Bowman *et al.* (2000) found 80% of the *R. terraenovae* diet to be fish, followed by 9.7% crustaceans. In coastal Florida waters, mature *R. terraenovae* consumed 71.4% sciaenids (Bethea *et al.*, 2006).

R. terraenovae was selected as the study species because it is a locally abundant mesopredatory shark of least concern and, as such, it serves as a model organism in Winyah Bay, SC in providing a baseline for ingestion of microplastics for other tertiary and quaternary consumers. This holds importance given that Winyah Bay provides habitat for endangered species including the shortnose sturgeon (*Acipenser brevirostrum*) and vulnerable species such as the sandbar shark (*Carcharhinus plumbeus*) (Kynard *et al.*, 2016; Collatos, 2018; Musick *et al.*, 2009). Furthermore, three studies have examined the presence of microplastics in Winyah

Bay, including ongoing research by Drs. George Boneillo and Jane Guentzel at Coastal Carolina University, a comparative study of Charleston Harbor and Winyah Bay by Gray *et al.* (2018), and an examination of microplastics in the water column and sediments of Winyah Bay by Ladewig *et al.* (2018).

The primary objective of this study was to isolate, identify, quantify, and characterize microplastics in the digestive systems of *R. terraenovae*, and compare these to published values for other sharks.

METHODS

SAMPLE COLLECTION

Sixteen mature male *R. terraenovae* were obtained during May and July 2018 from experimental longlines using 16/0 and 18/0 circle hooks targeting sandbar sharks, lemon sharks, and bull sharks for Coastal Carolina University's Shark Project (Abel *et al.*, 2007). Longlines baited with Boston mackerel and ladyfish were set for one hour. *R. terraenovae* specimens that were moribund or dead on retrieval were initially placed on ice for transport and kept frozen until the time of dissection (Avio *et al.*, 2015).

CONTAMINATION PROTOCOL

During each step of the procedure, working surfaces and materials were cleaned with alcohol prior to introduction of samples. Additionally, 100% cotton laboratory coats were worn to prevent microfiber contamination (Bellas *et al.*, 2016). Hypersaline and hydrogen peroxide solutions were filtered using a 0.45 µm filter to prevent contamination by microplastics already present (Avio *et al.*, 2015). All filtering was completed under a vacuum hood to prevent additional contamination. In order to detect any atmospheric contamination within the hood, a

borosilicate glass petri dish with 3 ml of distilled water was placed next to the isolation sites and examined for presence of plastics upon completing the protocol (Bellas *et al.*, 2016).

INITIAL ASSESSMENT AND DISSECTION

Prior to dissection, each shark was thawed to room temperature, and sex, total length, weight, and maturity stage (based on length of the animal and clasper development) were recorded. A ventral lengthwise incision was made from the cloacal opening to posterior of the coracoid bar to expose the abdominal cavity (Figure 3). The intact liver was removed first, weighed, and examined under a binocular dissecting microscope to note and photograph any surface abnormalities. Following removal of the liver, the intestinal tract and stomach were removed from the esophagus posterior to the mesentery located anterior of the rectal gland. After removal, the digestive tracts from each specimen were stored in a foil tray with a foil cover and labeled RTM1 through RTM16. Stomach fullness (excluding any consumed bait) was categorized by following an empirical five stage scale, as; (1) empty stomach, (2) low content, (3) middle amount, (4) high content, and (5) full stomach (Anastasopoulou *et al.*, 2013). The surfaces of the cardiac stomach, pyloric stomach and intestine were then examined for abnormalities and preliminary evidence of microplastics using a binocular dissecting microscope at a magnification of 40x. Once the surface tissues had been examined, a lengthwise incision was made to open the stomach to examine the contents. The intestine in this species is a scroll type as seen in Figure 3 and was unrolled to reveal contents (Bianchi, 1999). Any visible whole prey items were photographed and classified to the lowest possible taxon (Anastasopoulou *et al.*, 2013).

FILTRATION AND PARTIAL DIGESTION OF THE DIGESTIVE SYSTEM

Our protocol followed that of Avio *et al.* (2015), the most efficient of six published protocols for extracting plastics from fish stomachs with an estimated plastic recovery yield of over 90%. For each of the sixteen samples, the stomach, stomach contents, and intestinal tract were combined in a single foil container with a 200 ml NaCl hypersaline solution (1.2 g/ml). Samples were stirred for ten minutes and decanted. This initial process was performed twice prior to moving remaining solid material to 15% H₂O₂ solution in borosilicate glass petri dishes. Samples were then dried in an oven at 50°C for eight hours (Avio *et al.*, 2015). This process bleached and dried the remaining tissues and allowed for better visibility of fibers during subsequent examination. The liquid separated during the decanting process was vacuum-filtered using six 47 mm, gridded, cellulose-nitrate filters with a 0.45 µm pore size (GF/B, Whatman, USA). Filters were next dried in order to perform the hot needle test to confirm the material as plastic (Barrows *et al.*, 2017; Devriese *et al.*, 2015). The hot needle test uses a heated dissection needle to distinguish between microplastics, which tend to curl or melt in the presence of heat, and biological material, which does not.

CATEGORIZATION OF PLASTICS

A binocular dissecting microscope at a magnification of 40x was used to view the filters and the remaining tissues for presence of any plastic materials (Miranda *et al.*, 2016). A length estimate, as well as the shape, and the color were recorded for each plastic. Measurements were taken by photographing each microplastic, then digitally scaling each using FIJI Image J software to provide an accurate measurement. The shape of each plastic was classified as a film, fiber, pellet, or fragment (Figure 1), and the color of each plastic was categorized under the standard Red-Yellow-Blue color model. Images and data were then analyzed for duplicates found in individuals between the *in situ* visual analysis (performed on intact digestive tract prior

to the decantation) and the filtration analysis (performed on separated liquids and tissues) by comparing and matching photographs based on shape, length, and color.

DATA ANALYSIS

Hepatosomatic Indices (HSI)

The hepatosomatic index of each specimen was calculated using a ratio of the liver weight to the total weight of the individual (Hussey, 2009). In Equation 1, MTL represents the total body mass of the lobes of the liver and MTB represents the total body mass of each individual.

$$HSI = [MTL \text{ (kg)} / MTB \text{ (kg)}] \times 100 \quad (1)$$

Condition Factor (CF)

Condition factor serves as a general health indicator as the total body weight as a function of total body length of the individual (Hussey, 2009). In Equation 2, MTB represents the total body mass of each individual, and PCL represents the precaudal length

$$CF = [MTB \text{ (kg)} / PCL \text{ (cm)}^3] \times 10^5 \quad (2)$$

Statistical Analyses

Using IBM SPSS Statistics Version 25, ANOVA single factor tests were run to test for significant differences within the thirteen different color groupings, within the four different shapes, and among the six different size classes. When significant, the ANOVA followed by post-hoc analysis Tukey test (α -level = 0.05) was used to determine which groups differed significantly.

RESULTS

MICROPLASTIC ABUNDANCE

Microplastics were detected in 100% of samples (n=16), with the frequency ranging from 34 to 75 particles per individual (Table 1). Of the 997 particles isolated from *in situ* visual and filtration analyses, on the basis of the hot needle test 17 were deemed sediment or biological material (e.g., plant material, fish scales, and fish lenses), and 53 were identified as duplicates. The duplicates, sediment, and biological material were then subtracted from the total count, leaving 927 microplastics among the sixteen individuals. Atmospheric contamination in the vacuum fume hood was minimal, with 2 to 6 particles per dish for the 10 petri dishes examined, which were not subtracted from the final count.

DISTRIBUTION OF MICROPLASTICS BY SHAPE

The most common shapes of microplastics in *R. terraenovae* were fibers (93.6%), and fragments (5.7%), followed by films (0.5%) and pellets (0.1%) (Figure 4). For every individual, fibers were also dominant (Figure 5). There were statistical differences in microplastic shape (ANOVA; $R^2 = 0.948$; $df = 3, 60$; $F = 367.66$; $P < 0.001$) with differences between the subgroup of pellets, films, and fragments, and the subgroup of fibers (Tukey post-hoc test; Table 2).

DISTRIBUTION OF MICROPLASTICS BY SIZE

The range of microplastic length was 0.024 to 17.260 mm, with a mean length of 1.211 ± 1.358 mm (SD). The majority (55%) of the particles were in the smallest size class (< 1.0 mm) (Figures 6 & 7). There were statistical differences in microplastic size classes (ANOVA; $R^2 = 0.846$; $df = 5, 90$; $F = 98.76$; $P < 0.001$) with subgroups of size classes less than 1 mm, 1 to 2

mm, and together one subgroup including classes 3 to 4 mm, 4 to 5 mm, and greater than 5 mm (Tukey post-hoc test; Table 3).

DISTRIBUTION OF MICROPLASTICS BY COLOR

The predominant color detected was blue (41%), followed by clear (22%), black (15%), and gray (9%), with the other nine colors forming a combined 13% of the total microplastics (Figures 8 & 9). There were statistical differences in color (ANOVA; $R^2=0.799$; $df= 12, 195$; $F= 64.79$; $P < 0.001$) with statistically significant subgroups (Tukey post-hoc test; Table 4). The *in situ* visual analysis yielded 40% blue, 29% black and 14% gray particles, with 17% of the plastics comprising the ten other color groups (Figure 10). In contrast, post-filtration analysis yielded 46% blue particles, 30% clear, 7% gray and 7% black, with seven colors making up the other 10% of the color distribution (as no brown or white particles were found during the post-filtration analysis. (Figure 10).

HEALTH INDICATORS

Hepatosomatic indices ranged from 2.53 to 6.56, with a mean index of 3.9 ± 1.1 (SD), with no correlation between the number of microplastic particles and the HSI ($R^2 = 0.025$). Condition factor ranged from 0.52 to 0.85, with a mean index of 0.61 ± 0.08 (SD), with no correlation between the number of microplastic particles and the CF ($R^2 = 0.004$).

STOMACH FULLNESS

The range of stomach fullness was from 1 (no contents) to 5 (full stomach) with a mean fullness of 2.25 ± 1.34 (SD). A weak positive linear relationship ($R^2 = 0.248$) occurred between increasing particle number and increasing stomach fullness.

SUBSURFACE MICROFIBERS

During the *in situ* visual analysis, microplastics were found below the stomach serosa (outermost layer of the stomach lining) in the muscularis propria in 19% (n=3) of sharks (Figure 11). Of the total 927 particles isolated, 3 were embedded into this stomach lining.

DISCUSSION

This study provides the first documentation and categorization of microplastics in the Atlantic sharpnose shark *Rhizoprionodon terraenovae* in a southeast United States estuary, and adds to the growing list of sharks in which microplastics have been found (Bernardini *et al.*, 2018; Avio *et al.*, 2015; Alomar and Deudero, 2017; Cartes *et al.*, 2016; Anastasopoulou *et al.*, 2013; Fossi *et al.*, 2014). Major findings include quantification of microplastics, categorization by shape, size, and color, and the first evidence of a potential pathway for translocation of microplastics from the stomach contents to the external surface of the stomach.

MICROPLASTICS ABUNDANCE

The mean number of microplastic particles found in this study per individual was 57.93 ± 11.71 (SD), with a maximum of 75 particles per shark. This is the highest reported average of particles and frequency of microplastic ingestion among existing literature on microplastics ingested by sharks (Table 1). However, of these only Avio *et al.* (2015) performed the same protocol as we used, that is, initial visual analysis and vacuum filtration followed by a partial digestion of remaining tissues. Avio *et al.* (2015) reported 44% of 9 examined *S. acanthias* in the Adriatic Sea with an average of 1.25 ± 0.5 ingested microplastics per individual, although fibers were not counted (to eliminate those contributed from atmospheric contamination). As such, variation in protocols and the exclusion of fibers may explain the high microplastic abundance in

this study compared to other shark studies (Fossi *et al.*, 2018, Bernardini *et al.*, 2018). These studies examined only the stomach or the stomach contents as opposed to the entirety of the digestive tract, and used different means to isolate plastics. For example, Miranda *et al.* (2016) reported a maximum of 3 plastic pellets per individual in Brazilian sharpnose sharks, however the protocol varied from our study in that it only included a visual analysis examining the stomach contents. Bernardini *et al.* (2018) found 25.26% of 95 blue sharks consumed microplastics in the North Western Mediterranean Sea, with a range of 1 to 30 plastic items ingested per individual.

Whether the microplastics we found in *R. terraenovae* were derived from local estuarine or more distant pelagic waters cannot be discerned, since the species is highly migratory and is not a year-long resident of Winyah Bay.

Small teleost fishes are the main prey of *R. terraenovae*, therefore biomagnification of plastics could occur via their consumption (Gelsleichter *et al.*, 1999, Ferreira *et al.*, 2019). In Charleston Harbor, SC, seven species of teleosts were found to have ingested an average of 13 microplastics per individual, as identified through the use of fluorescence and bright-field microscopy in (Payton, 2016). This average is higher than those found in the Northeast Atlantic, where among seven species, 73% of 233 teleosts had consumed microplastics, with an average of 1.8 ingested particles per individual (Wieczorek *et al.*, 2018). In comparison to other estuarine systems, in the Mondego estuary in Portugal, three teleosts had consumed an average of 1.67 ± 0.27 (SD) microplastics per individual (Bessa *et al.*, 2018). In the Goiana Estuary in Brazil, the common snook, *Centropomus undecimalis*, consumed a maximum of 3.66 ± 1.20 ingested particles per individual (Ferreira *et al.*, 2019).

As stated above, the geographical source of the microplastics found in our study species cannot be known with certainty. However, the dominant shape (fiber), size class (< 1 mm), and color (blue) of plastics found in the digestive tracts of *R. terraenovae* are consistent with their proportional presence reported by Ladewig *et al.* 2018 in Winyah Bay. Although Ladewig *et al.* (2018) found fibers and blue particles dominant, this contrasts with the findings of Gray *et al.* (2018) in which the dominant color found in Winyah Bay was black, and the dominant shapes were fragments. Ladewig *et al.* (2018) attributed this disparity to the black fragments (likely from tires) possibly having a lower density than the surface microlayer (the top 1 mm boundary layer interacting with the atmosphere) which was sampled in the study by Gray *et al.* (2018).

DISTRIBUTION OF MICROPLASTICS BY SHAPE AND COLOR

The results of microfibers as the dominant shape are also in agreement with Desforges *et al.* (2014) and Desforges *et al.* (2015) in which zooplankton located in inshore regions had consumed more fibers than zooplankton had consumed in offshore regions. Desforges *et al.* (2014) attributed this fiber distribution to fishing, recreational boating, and wastewater effluent. However, these findings are also consistent with research in the Atlantic Ocean, the Mariana Trench, and in the Arctic Basin, suggesting that a higher proportion of fibers is not restricted to nearshore regions (Kanhai *et al.*, 2017; Jamieson *et al.*, 2019; Kanhai *et al.*, 2018). In previous shark studies, sheet-like particles (classified as *film* in our study) comprised 72.4% of plastic particles consumed by blue sharks, with only 3.8% of particles classified as threadlike (categorized as *fibers* in our study) (Bernardini *et al.*, 2018). In spiny dogfish, 57% of particles found were fragments, however fibers were not included in this study (Avio *et al.*, 2015). Color also varied between previous shark studies, such as in the blue shark, in which transparent and white were the most common colors found (Bernardini *et al.*, 2018).

SUBSURFACE MICROFIBERS

Microfibers have not been previously reported as being embedded within the layers of the stomach lining in fish. This finding is significant in that it suggests that the exterior surfaces of the organs be systematically and microscopically examined and provides evidence for a pathway for translocation of ingested fibers and microplastics from the lumen of the digestive tract to the external surface. Our finding carries implications for future studies where the entirety of the digestive system is chemically digested prior to examination, or only the stomach contents are examined. In either case, critical information regarding a plastic's location within an organism's system could be overlooked. Embedded microfibers cause a longer duration of plastic (and potentially chemical) exposure to an organism's system in contrast to the shorter duration of microplastics passing through the digestive tract.

The fate of ingested plastics is either elimination in feces, stomach or intestinal eversions, or retention either adhered to the linings or translocated (Christie, 2012, Brunnschweiler, 2005; Avio *et al.*, 2015). The deposition of microplastics in fecal matter has been demonstrated in laboratory setting in the copepod *Centropages typicus*, isopod *Idotea emarginata*, and the periwinkle *Littorina littorea* (Hämer *et al.*, 2014; Gutow *et al.*, 2015). Though possible, field studies of fish fecal content present more challenges than those performed in a laboratory setting such as faster dispersion in the water column (Wetherbee and Gruber, 1990; Saba *et al.*, 2012). Determining the amount of microplastics retained in the tissues of an animal versus the amount entirely passing through the system will be critical in determining the range of potential impact of microplastics, particularly in regards to toxicity.

In consuming non-digestible items, sharks have been observed performing stomach and intestinal eversions that remove inedible objects from their systems (Christie, 2012; Brunnschweiler, 2005). In one unique case, a lemon shark was observed over the course of a

year with a metal fish stringer expelled from within the body cavity through the body wall (Kessel *et al.*, 2017), supporting the possibility of other mechanisms for sharks to expel foreign objects from their stomachs.

Three possible mechanisms for translocation of microplastics include (i) stomach expansion and contraction, (ii) pressure on the coelom, and (iii) movement through existing pathways.

(i) *Stomach Expansion and Contraction*: During feeding, the stomach of vertebrates, including sharks, expands to accommodate larger meals, a phenomenon called receptive relaxation (Holmgren and Nilsson, 1999). The first stages of digestion occur in the stomach, principally the release of concentrated hydrochloric acid, which activates the enzyme pepsin for digestion of proteins (Papastamatiou and Lowe, 2005). To ensure that sufficient mixing of these digestive chemicals occurs, the smooth muscle of the stomach wall rhythmically alternate between inactivity and strong contractions (Holmgren and Nilsson, 1999). Microfibers may contact the stomach wall at any time during their presence in the stomach, but it is during this mixing process that microfibers have a higher likelihood of penetrating into the tissues, either pushed by harder stomach contents (e.g., whole fish, crab shells) or by the contact between rugae folds. Repeated contraction could lead to these microfibers becoming more deeply embedded in the stomach lining and possibly translocating to the exterior surface of the stomach.

ii.) *Pressure on Coelom*: Pressure on the abdominal cavity during normal swimming activity could create a similar situation of compression of the stomach leading to the lodging of microfibers in the stomach lining. Natural changes in pressure on the coelom could occur particularly in sharks with anguilliform and carangiform swimming types, due to the contraction of locomotory muscles and resulting tightening of skin (Wainwright *et al.*, 1978).

iii.) *Movement Through Existing Pathways*: Small tears and punctures may occur within the stomach lining due to both natural and unnatural stomach contents, and possibly through other existing channels, such as those created by parasites. Sharks stomachs are known to have a variety of parasites including nematodes, trematodes and cestodes (Heupel and Bennett, 1998; Fyfe, 1953; Dailey and Vogelbein, 1982). During this study, nematodes were observed in the same sub-serosal layer as the microfibers. Although the uptake of nanoplastics have been shown in the nematode *Caenorhabditis elegans*, the possibility of parasitic transport of microfibers is still unknown (Kim *et al.*, 2019). However, the channels created by their movement through the stomach lining could provide a pathway for a microfiber to become lodged into the tissues.

HEALTH INDICATORS

There were no significant correlations between the number of microplastics and either of the shark health indicators tested. HSI is considered to be a more accurate health indicator for short term effects whereas CF is typically considered for long term health (Hussey, 2009). Mizraji *et al.*, (2017) found a decline in condition factor with increased plastic consumption by the omnivorous fish *Girella laevis*. Foekema *et al.* (2013) also found a negative relationship between condition factor and the presence of microplastics in the haddock, *Melanogrammus aeglefinus*, however this only represented one out of five species examined in the North Sea, (the other four had no significant relationship between plastic ingestion and condition factor). Regarding the hepatosomatic index, Lu *et al.* (2016) showed that microplastic exposure was correlated to liver inflammation and accumulation of lipids in the livers of zebrafish, *Danio rerio*. Although our study did not show a strong correlation between plastic accumulation and either of the health indices analyzed, negative impacts from microplastics could be potentially detected through analyzing toxins.

STOMACH FULLNESS

Although Alomar and Deudero (2017) found a positive correlation between stomach fullness and the amount of microplastics consumed, there was no strong correlation between these factors in *R. terraenovae*. This could be attributed to a smaller sample size of *R. terraenovae* N=16 as opposed to the N=125 of the blackmouth catshark (Alomar and Deudero, 2017). However, in seven species of teleosts in the Northwest Atlantic, Wieczorek *et al.*, 2018 found no significance between the amount of ingested microplastics and stomach fullness. It is also important to note that obtaining samples via baited longlines likely contributed to the mean stomach fullness of 2.25, (low content), as sharks with fuller stomachs may be less likely to target bait.

LIMITATIONS AND FUTURE DIRECTIONS

This study provides an initial baseline of microplastic ingestion by adult, male *R. terraenovae*, though future studies of microplastic ingestion by *R. terraenovae* in Winyah Bay could include an examination of changes undergone by ingested plastics, examining polymer type, estimations of microplastic consumption by other species of Winyah Bay, and examining additional *R. terraenovae* samples.

The effect stomach acid on various types of plastic may cause alterations to the composition of the plastic (Haetrakul *et al.*, 2009). As the stomach acid in sharks is extremely variable between species and ranges widely within an individual based on foraging activity, the exact effect of stomach acid on plastics within the digestive tract is still unknown (Holmgren and Nilsson 1999). In an actively foraging species, empty stomachs of the leopard shark (*Triakis semifasciata*) had a pH of 1.5 ± 1.4 (SD) and increased to 3.1 ± 0.7 (SD) after feeding (Papastamatiou and Lowe, 2004). A less active species, the nurse shark (*Ginglymostoma*

cirratum) had a stomach acid pH range of 0.4 immediately after feeding to 8.7 three days after feeding (Papastamatiou and Lowe, 2005). Gastric acid exposure should be considered as a factor impacting the composition of ingested plastics, as demonstrated in a 2009 study in which a plastic straw became fatal when consumed by a whale shark by causing lacerations and hemorrhaging in the stomach (Haetrakul *et al.*, 2009). The straw which had been produced as flexible and clear had undergone a physicochemical change in the stomach and had become hardened and opaque. This physical change into a hard structure is what ultimately caused the internal lacerations. This phenomenon should be considered on the scale of microplastics as well, which may undergo similar changes when encountering acids. The implications of this reaction apply to both changes occurring due to stomach acid exposure, and changes due to intentional chemical exposure during laboratory procedures.

Future studies could also be improved by using Fourier transform infrared spectroscopy (FTIR), which is currently considered one of the most optimal methods for identifying polymer type (Jung *et al.*, 2018). As the initial polymer structure will impact the transfer of chemical pollutants to an organism due to different degrees of adsorption, this could be a critical step in determining the impact of plastic consumption.

In order to gain a more comprehensive understanding of microplastic distribution within organisms in Winyah Bay, a future study could encompass examining organisms at other trophic levels, including prey items of *R. terraenovae* as well as predators. Sample prey items of *R. terraenovae* found within Winyah Bay include the Atlantic menhaden *Brevoortia tyrannus*, the broad striped anchovy *Anchoa hepsetus*, and the Atlantic silverside *Menidia menidia*. Predators of *R. terraenovae* in Winyah Bay include apex predators such as the lemon shark *Negaprion brevirostris*, and the bull shark *Carcharhinus leucas*. Information on the microplastic distribution in these species as well as other residents of Winyah Bay would help to expand the current

knowledge of the scope of microplastic ingestion in an estuarine environment. However, measuring the degree of trophic level transfer of plastics in a field setting remains difficult due to factors such as unintentional ingestion of microplastics from the water column (Dantas, 2012).

Another consideration is that this study included only males, which can be attributed to sexual segregation of the sharpnose sharks. Sexual segregation due to foraging or social reasons in sharks is not infrequent, and has been demonstrated in at least 38 species of sharks (Mucientes *et al.*, 2009; Sims, 2005). In the north central Gulf of Mexico, Parsons (2005) caught only 9 female Atlantic sharpnose sharks in comparison to 718 adult males, postulating that it is uncommon for females to enter shallow waters after maturation. Although foraging and habitat differences would likely be the driving factors in varying microplastic consumption frequencies, no significant differences were found between female and male consumption of microplastics in the blue shark (Bernardini, *et al.*, 2018). A comparison between male and female consumption of microplastics in *R. terraenovae* could support whether or not this is the case in other sharks.

Additionally, only adult *R. terraenovae* were examined in this study, but samples at various life stages would allow for a better understanding of the magnitude of microplastics being introduced throughout their life span. Plastic ingestion studies on short-tailed shearwaters, harbor seals, Franciscana dolphins, four species of sea turtles, and blue sharks have supported that juveniles tend to consume more plastic items than adults (Acampora *et al.*, 2014; Rebolledo *et al.*, 2013; Denuncio *et al.*, 2011; Plotkin and Amos 1990). In blue sharks, Bernardini *et al.* (2018) attributed a higher frequency of plastic ingestion to the opportunistic feeding style of juveniles, whereas Plotkin and Amos (1990) attributed this disparity to juvenile sea turtles foraging mainly on drift lines (which tend to have higher amounts of debris). Both differences in habitats and foraging techniques could lead to a higher consumption rate of plastics by juveniles. A total count of plastics accompanied by the utilization of a nonlethal biomarker would enable

the formulation of a baseline correlation between the presence of microplastics and the subsequent leaching of toxins in sharks.

CONCLUSION

Plastic in the ocean is expansive issue and has a spectrum of low to severe consequences for organisms and their habitats. The small size of microplastics allows for biomagnification and their high degree of adsorption furthers the bioaccumulation of toxins. This project adds to the growing field of microplastic research by considering a mesopredator in an estuary with published data on microplastic occurrence in the water column and sediment (Ladewig *et al.*, 2018). As this species is consumed by humans, concern may rise due to the absorption of probable carcinogens into the systems of other mammals (Fossi *et al.*, 2014). Future studies could improve current methods by identifying the presence of microplastics in a non-lethal manner such as blood tests, particularly with species that have populations that are endangered.

This study is the first to describe microfibers within the layers of the stomach lining, and suggests potential mechanisms for transport of microplastics from the stomach towards the coelom, a phenomenon that could potentially increase an organism's length of exposure to toxins carried by the microplastics.

TABLES

Table 1. Comparison of the frequency of microplastic ingestion in sharks out of the total number of individuals sampled (N) within existing literature, grouped by species. The inclusion of a filtration analysis in the procedures and the inclusion of microfibers in the total count of microplastics within each study are indicated. Adapted from Bernardini *et al.*, 2018.

<i>Species</i>	<i>N</i>	<i>Frequency (%)</i>	<i>Filtration Analysis</i>	<i>Fibers Included</i>	<i>Bibliography</i>
<i>Centroscymnus coelolepis</i>	11	9%	No	Yes	Cartes <i>et al.</i> , 2016
<i>Centrophorus granulosus</i>	5	0%	No	Yes	Anastasopoulou <i>et al.</i> , 2013
<i>Etmopterus spinax</i>	16	6%	No	Yes	Anastasopoulou <i>et al.</i> , 2013
<i>Etmopterus spinax</i>	323	6%	No	Yes	Deudero and Alomar, 2015
<i>Etmopterus spinax</i>	9	11%	No	Yes	Cartes <i>et al.</i> , 2016
<i>Galeus melastomus</i>	741	3%	No	Yes	Anastasopoulou <i>et al.</i> , 2013
<i>Galeus melastomus</i>	125	16%	No	Yes	Alomar and Deudero, 2017
<i>Galeus melastomus</i>	125	15%	No	Yes	Cartes <i>et al.</i> , 2016
<i>Prionace glauca</i>	95	25%	Yes	Yes	Bernardini <i>et al.</i> , 2018
<i>Rhizoprionodon lalandii</i>	6	33%	No	No	Miranda <i>et al.</i> , 2016
<i>Rhizoprionodon terraenovae</i>	16	100%	Yes	Yes	Present Study
<i>Scyliorhinus canicula</i>	1	0%	No	Yes	Anastasopoulou <i>et al.</i> , 2013
<i>Squalus acanthias</i>	16	6%	Yes	No	Avio <i>et al.</i> , 2015
<i>Squalus acanthias</i>	323	6%	No	Yes	Anastasopoulou <i>et al.</i> , 2013
<i>Squalus blainville</i>	9	11%	No	Yes	Anastasopoulou <i>et al.</i> , 2013

Table 2. Microplastic abundance (N), mean particle length, and health indicators for each sample.

<i>Sample ID</i>	<i>N</i>	<i>Mean Particle Length (mm) (SD)</i>	<i>HSI</i>	<i>CF</i>
<i>RTM1</i>	34	1.28 (2.25)	5.81	0.56
<i>RTM2</i>	67	1.18 (1.15)	6.57	0.64
<i>RTM3</i>	45	1.51 (1.79)	3.09	0.52
<i>RTM4</i>	45	1.57 (1.25)	3.55	0.85
<i>RTM5</i>	43	2.58 (3.27)	4.86	0.55
<i>RTM6</i>	70	1.08 (1.06)	3.74	0.53
<i>RTM7</i>	59	1.00 (1.08)	4.29	0.65
<i>RTM8</i>	54	1.14 (1.29)	3.21	0.61
<i>RTM9</i>	67	1.13 (0.88)	4.32	0.54
<i>RTM10</i>	49	1.18 (1.11)	3.46	0.58
<i>RTM11</i>	60	0.92 (0.81)	2.54	0.65
<i>RTM12</i>	67	1.24 (0.98)	3.03	0.58
<i>RTM13</i>	75	0.99 (1.02)	3.44	0.61
<i>RTM14</i>	60	1.25 (1.19)	2.66	0.58
<i>RTM15</i>	64	1.10 (1.29)	2.96	0.68
<i>RTM16</i>	68	1.11 (0.90)	4.62	0.61

Table 3. Post-hoc analysis (Tukey test) comparing particle shape present among individuals. (α -level = 0.05).

<i>Shape</i>	<i>N</i>	<i>Mean (SD)</i>	<i>Frequency (out of n=16)</i>	<i>Group</i>
<i>Fiber</i>	868	54.25 (10.94)	16	a
<i>Fragment</i>	53	3.31 (1.66)	15	b
<i>Film</i>	5	0.31 (1.48)	6	b
<i>Pellet</i>	1	0.06 (.25)	4	b

Table 4. Post-hoc analysis (Tukey test) comparing particle size present among individuals. (α -level = 0.05).

<i>Size Class (mm)</i>	<i>N</i>	<i>Mean (SD)</i>	<i>Frequency (out of n=16)</i>	<i>Group</i>
<1	508	31.75 (9.93)	16	a
1 to 2	233	14.56 (5.16)	16	b
2 to 3	108	6.75 (6.75)	16	c
3 to 4	39	2.44 (1.71)	15	cd
4 to 5	20	1.25 (.86)	13	d
>5	19	1.19 (1.23)	10	d

Table 5. Post-hoc analysis (Tukey test) comparing color present among individuals. (α -level = 0.05).

<i>Color</i>	<i>N</i>	<i>Mean (SD)</i>	<i>Frequency (out of n=16)</i>	<i>Group</i>
<i>Yellow</i>	3	0.19 (0.75)	1	a
<i>Brown</i>	4	0.25 (1.00)	1	a
<i>White</i>	5	0.31 (.70)	3	a
<i>Orange</i>	6	0.38 (0.72)	4	a
<i>Multi</i>	9	0.56 (0.89)	6	a
<i>Green</i>	10	0.63 (0.72)	8	a
<i>Purple</i>	21	1.31 (1.25)	11	ab
<i>Red</i>	27	1.69 (1.62)	14	ab
<i>Pink</i>	37	2.31 (1.82)	15	ab
<i>Gray</i>	85	5.31 (2.94)	16	bc
<i>Black</i>	137	8.56 (3.37)	16	c
<i>Clear</i>	208	13.00 (8.08)	16	d
<i>Blue</i>	375	23.44 (7.53)	16	e

FIGURES

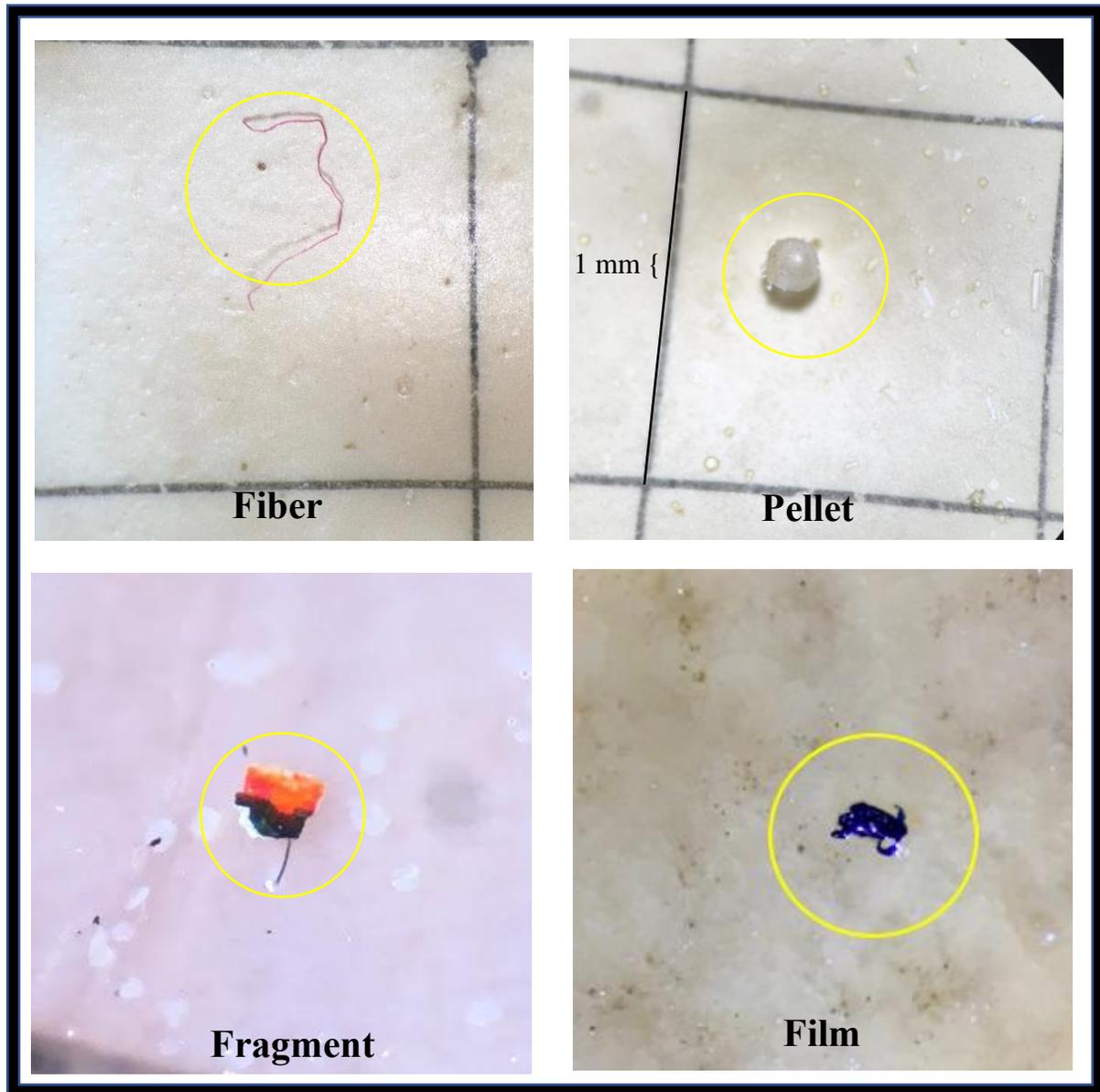


Figure 1. Four distinct shapes of microplastics, where the scale bar represents 1 mm.

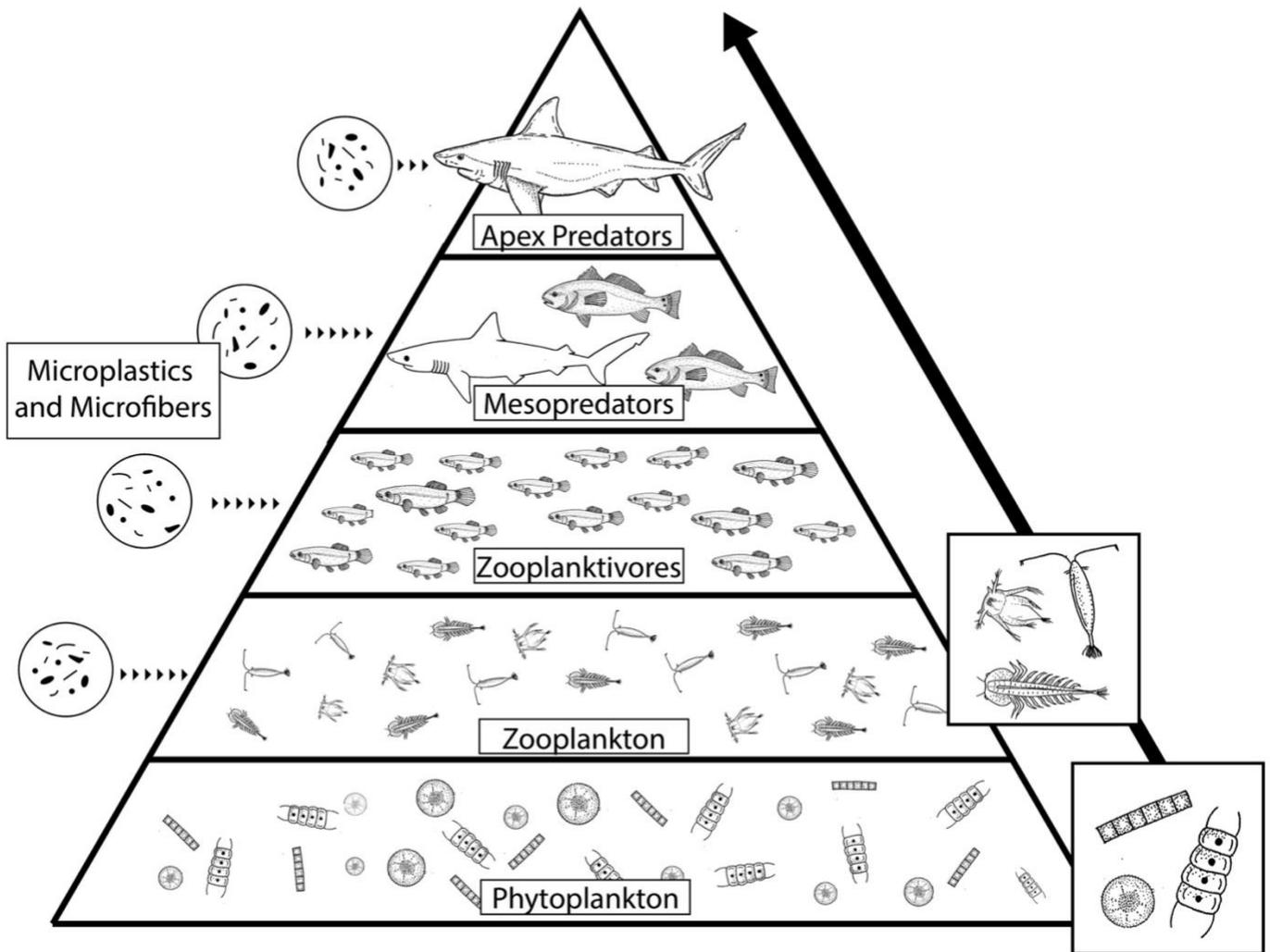


Figure 2. The mechanisms of direct ingestion and the transfer via the food chain of plastics into the systems of organisms. The insets on the left represent microplastic ingestion at each trophic level, and the insets on the right are enlargements of the phytoplankton (bottom) and zooplankton (top).

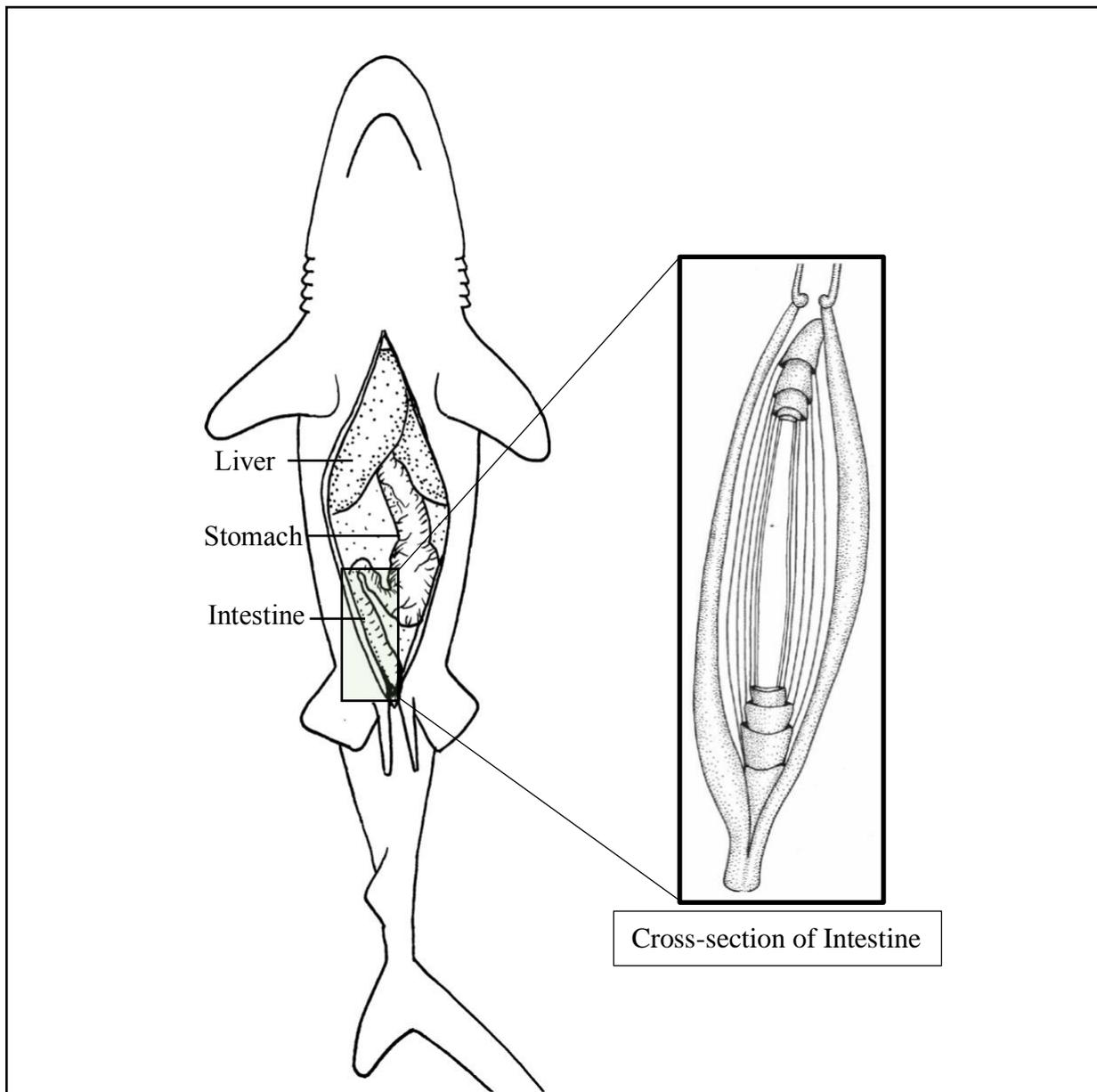


Figure 3. Generalized ventral dissection of the Atlantic sharpnose shark to expose the digestive tract, with an enlargement of a cross-section of the scroll intestine (right).

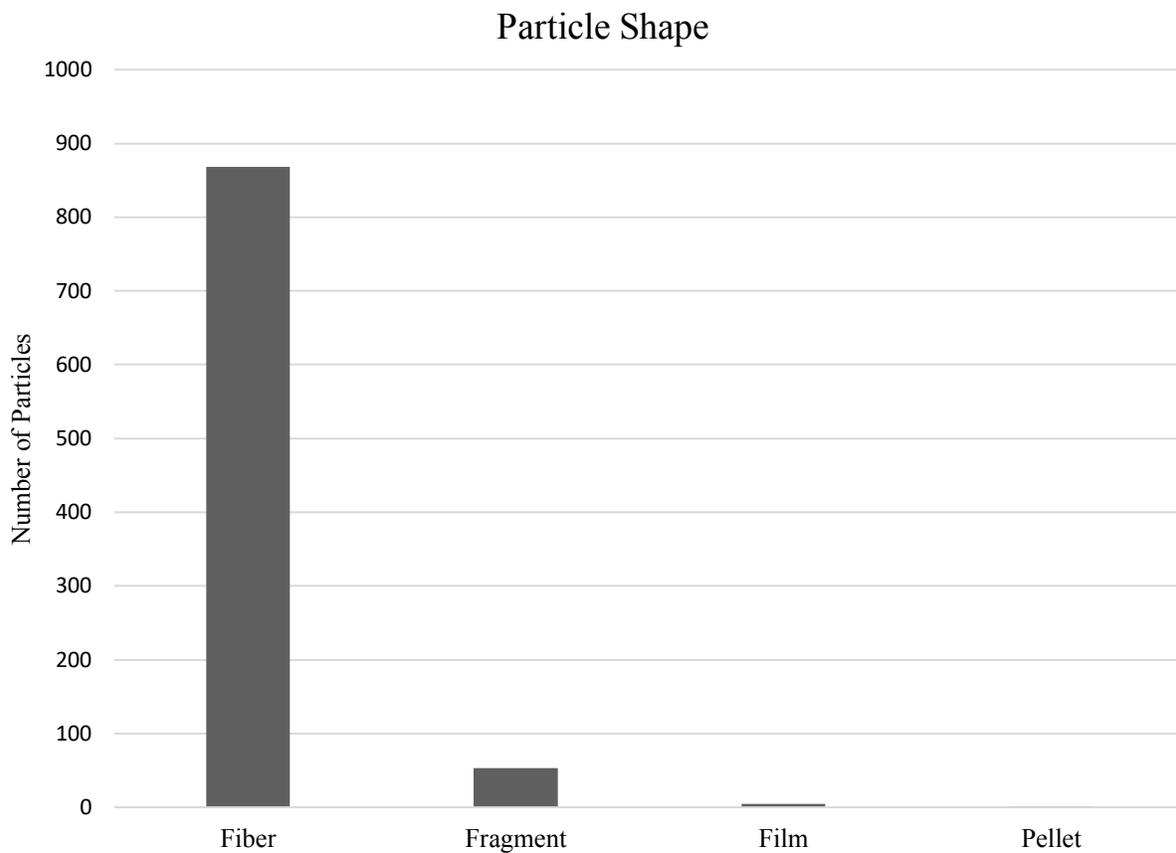


Figure 4. Distribution of microplastic shapes during *in situ* visual analysis and during the post-filtration process analyses, and corrected for biological material and duplicates.

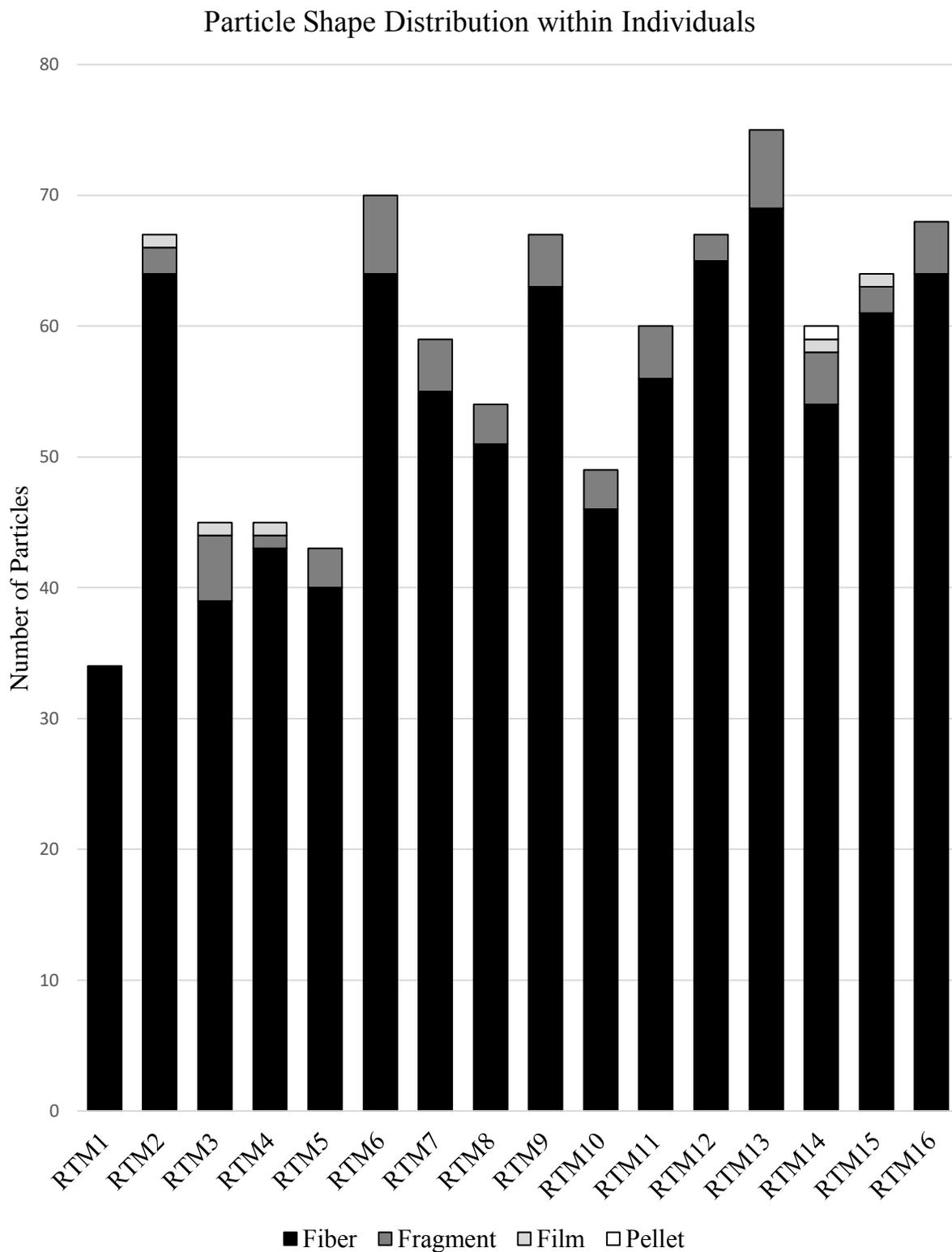


Figure 5. Distribution of microplastic shapes per individual after *in situ* visual analysis and post-filtration analysis, and corrected for biological material and duplicates.

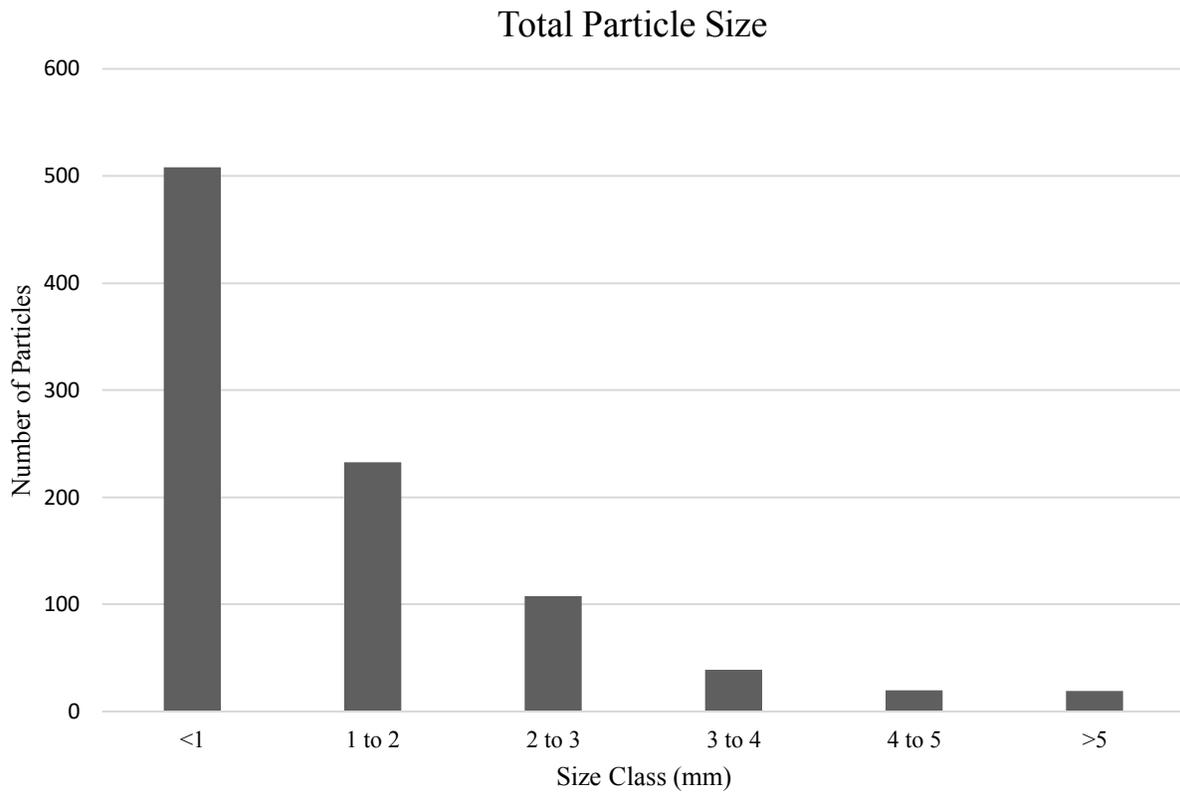


Figure 6. Distribution of microplastics by size class after *in situ* visual and post-filtration analyses, and corrected for biological material and duplicates.

Particle Size Distribution within Individuals

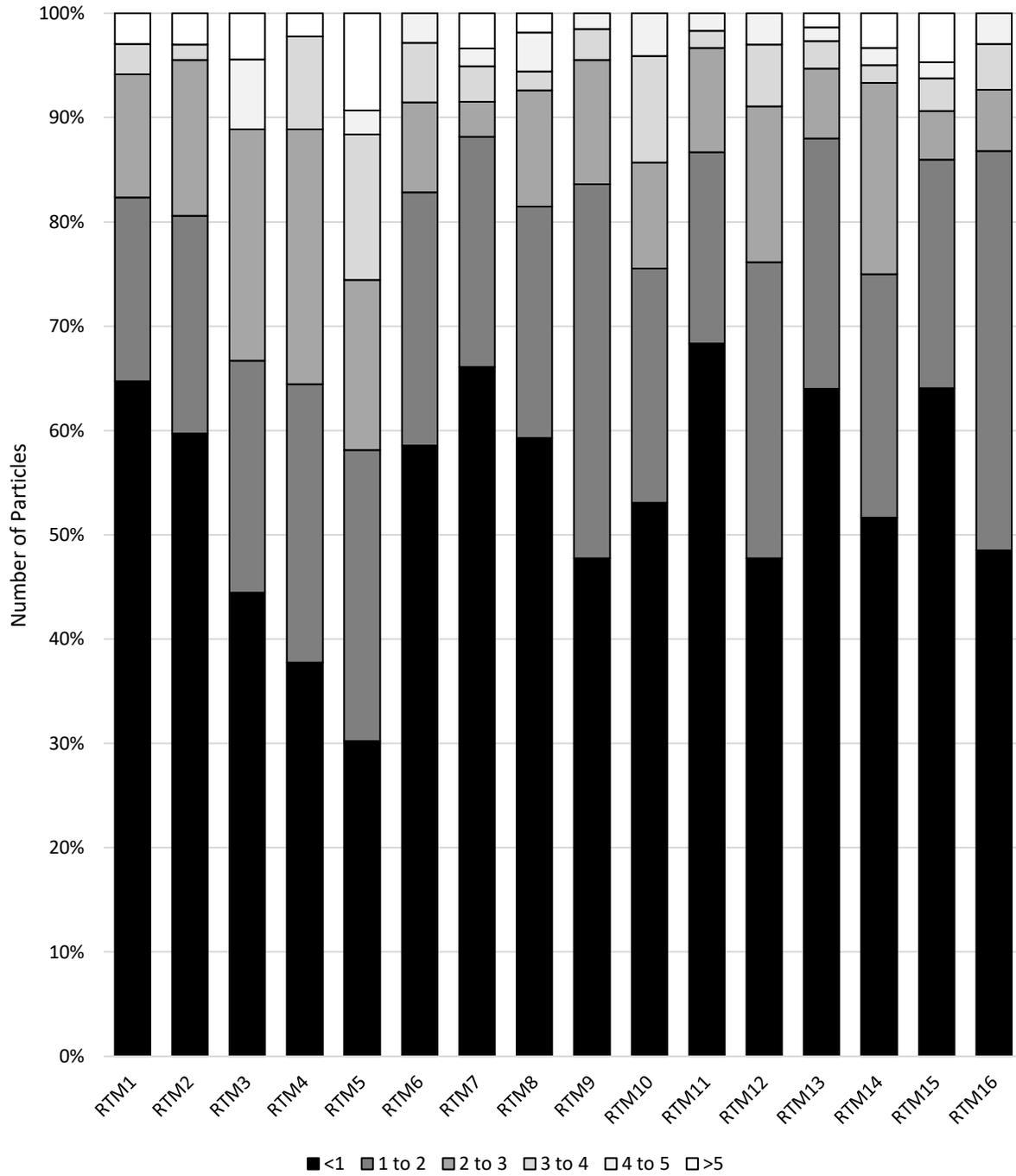


Figure 7. Distribution of microplastics lengths by individual, with size classes ranging from less than 1mm to greater than 5 mm, after *in situ* visual and post-filtration analyses, and corrected for biological material and duplicates.

Total Particle Color Distribution

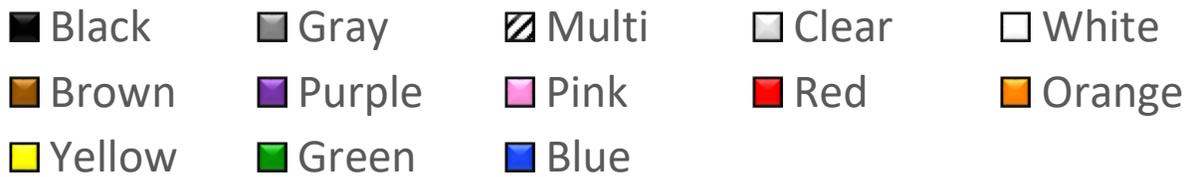
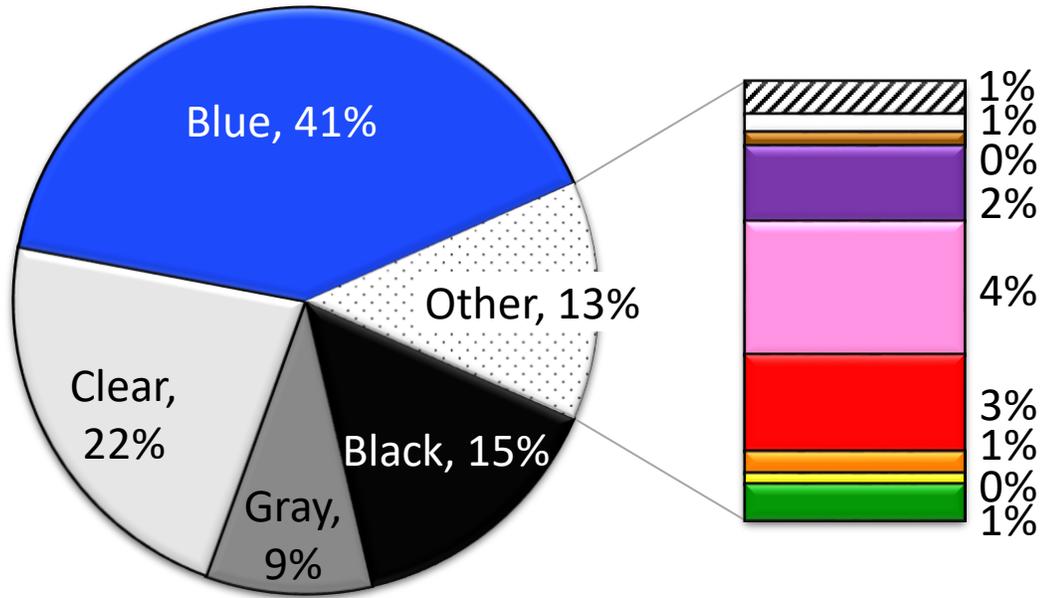


Figure 8. Distribution of total microplastics as percentages after *in situ* visual and post-filtration analyses, and corrected for biological material and duplicates.

Particle Color Distribution Among Individuals

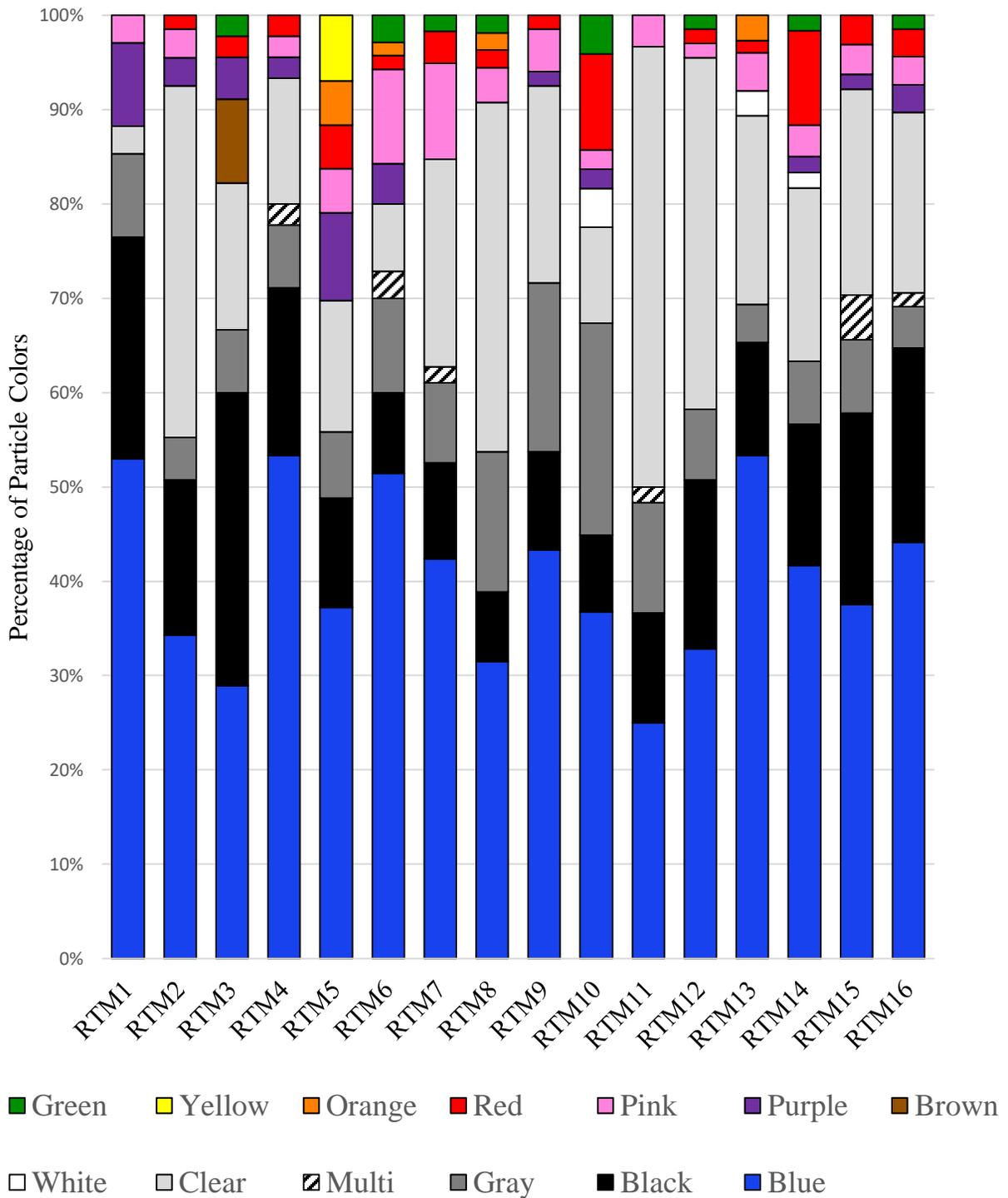
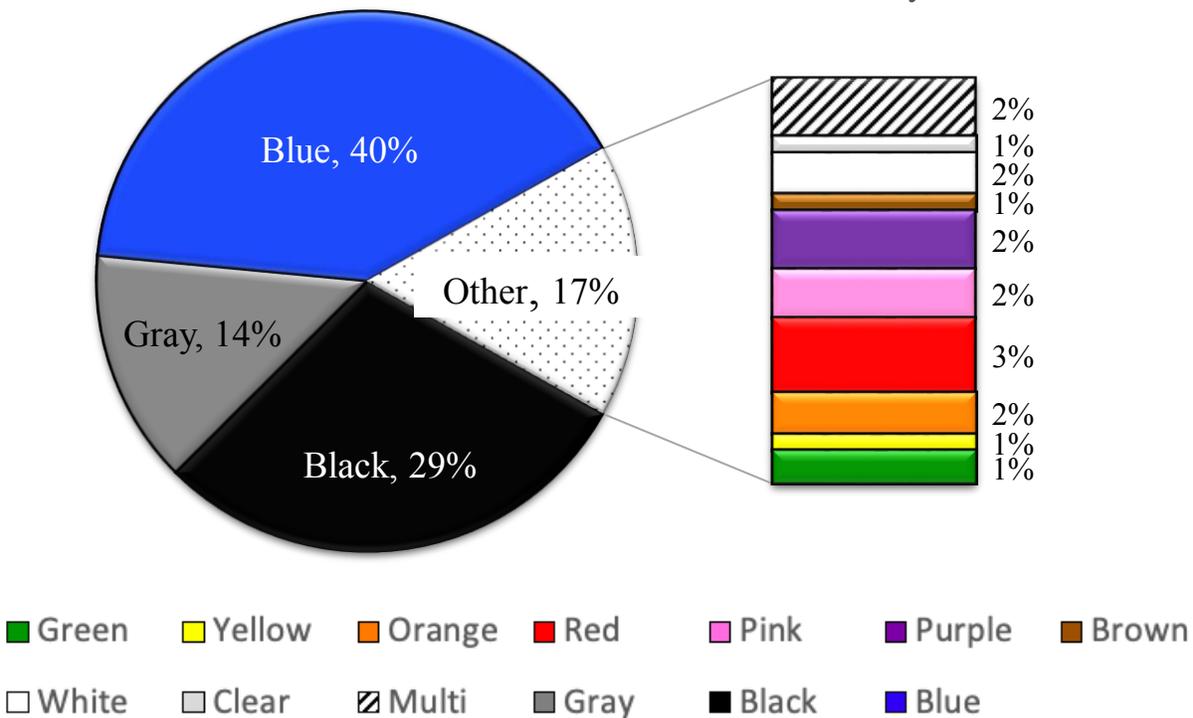


Figure 9. Distribution of total microplastics per individual after *in situ* visual and post-filtration analyses, and corrected for biological material and duplicates.

Color Distribution, Initial In Situ Visual Analysis



Color Distribution, Post-Filtration Analysis

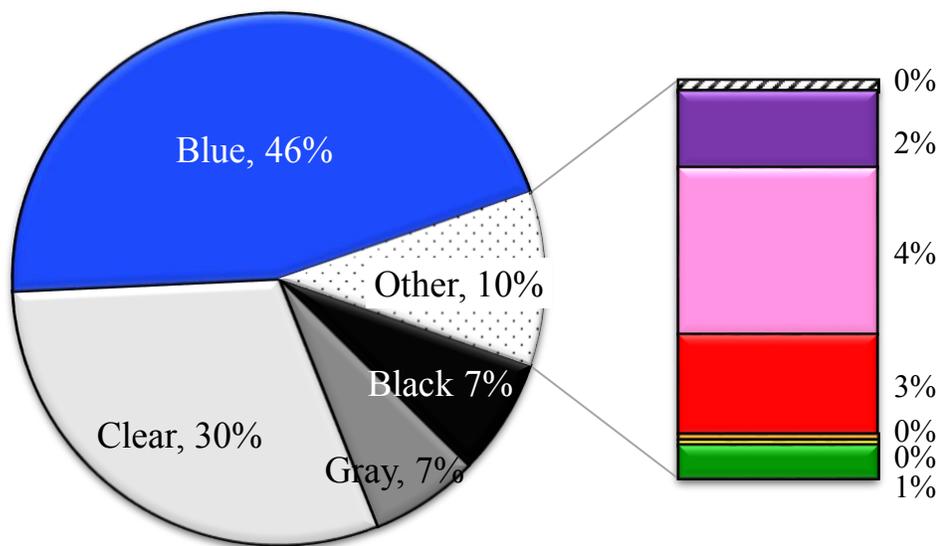


Figure 10. Distribution of microplastics as percentages by particle color, with *in situ* visual analysis (top) and post-filtration analysis (bottom).

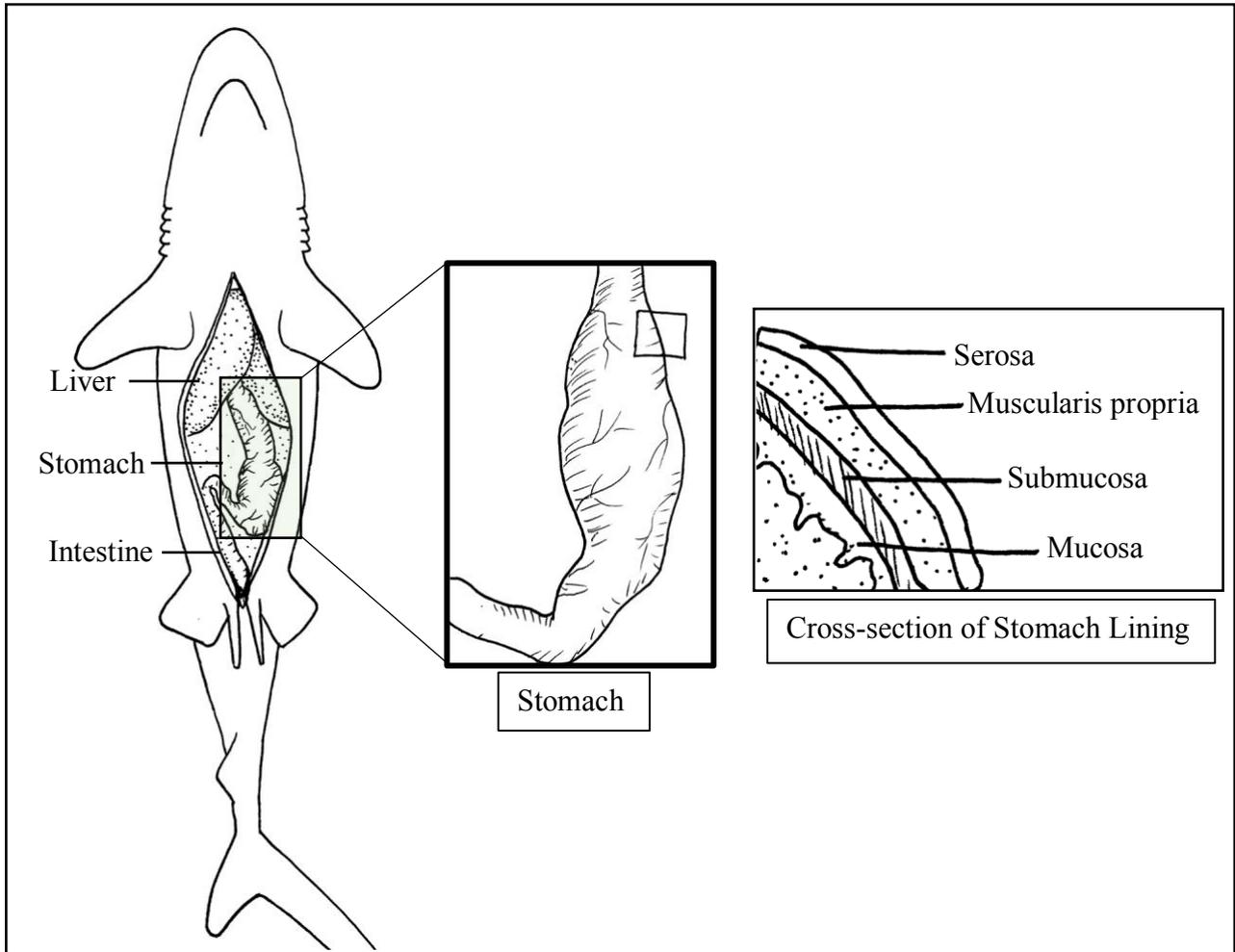


Figure 11. Stomach layers of a shark, including the mucosa, submucosa, muscularis propria, and serosa.

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