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Plastic ingestion by commercial fish of contrasting ecology off the Azores region

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Executive Summary

Plastic production continues to increase annually and consequently plastic debris continues to accumulate in marine systems all over the globe. Once in the marine system this debris is present in sizes that ranged from nanometres to metres. Accidently or by mistake plastic items are ingested from species across all trophic levels, including fish. In the present study 157 individuals of three species collected through commercial fisheries operating in Azores region were dissected and its stomatal content analysed for plastic ingestion. 55 individuals of Blackbelly rosefish (*Helicolenus dactylopterus*), 52 of Blue jack mackerel (*Trachurus picturatus)* and 50 of Skipjack tuna (*Katsuwonus pelamis*). All individuals were sampled under a fixed length range and with a minimum of sampling size of 50 as required by EU directives. The stomatal analysis was divided in: Visual analysis, KOH digestion and filter analysis. Prior to digestion in 10% KOH, all individual stomachs were visual analysed for presence of plastic debris. After visual analysis, stomachs were digested and after a minimum period of 48 hours filtered through a 50 µm and 1 µm mesh filter. All filters were later on analysed under a Leica binocular MZ16FA with a MC 190 Leica camera attached. Potential particles were photographed and counted. As microfiber contamination control, water, airborne and material sources were tested to evaluation of it degree of contamination through the protocol. Overall 38.71% of individuals over all species were found to ingest potential plastic debris with an abundance of 0.58 ± 0.95 items and intensity of 1.5 ± 0.98 items. Frequency of ingestion of potential plastic debris per species were particularly higher compared to studies with the same species in other geographical areas. The frequency of ingestion was higher for Skipjack tuna (44%), followed by Blue jack mackerel (40,38%) and Blackbelly rosefish (32.08%). Blackbelly rosefish was the species that was found to ingest more potential plastic particles with an intensity of 2.12±0.34 items. All potential particles found still have to be checked with FI-IR method for certainty of its type. Microfiber found in quality control tests was used as double check comparison with the microfibers found in the stomachs and correct the results. According to quality control measures were found that use water filtered through two processes (resins and 20 µm mesh filter) and use a glass bottle between filtration reduce microfiber contamination during filtration. This improvement in the protocol increased the confidence in the results obtained.

Abstract

Plastic in its diverse sizes (micro to macro) is ubiquitous in all marine systems and known to be ingested intentional or accidently marine organisms of all trophic levels, including fish. In this study, it was analysed the stomatal contents of 157 individuals of three commercial species of contrasting ecology collected in the Azores region. 55 individuals of Blackbelly rosefish (*Helicolenus dactylopterus*), 52 of Blue jack mackerel (*Trachurus picturatus)* and 50 of Skipjack tuna (*Katsuwonus pelamis*). 38.71% of individuals over all species were found to ingest potential plastic debris with an abundance of 0.58 ± 0.95 items and intensity of 1.5 ± 0.98 items. Frequency of ingestion of potential plastic items was 32.08% for Blackbelly rosefish, 40.38% for Blue jack mackerel and 44% for Skipjack tuna. Results of this study showed a frequency of ingestion per species higher than previously reported in studies carried out in other geographic areas. Despite that all potential plastic debris found must be confirmed with FT-IR method. Improvements in the stomatal analysis of fish for plastic ingestion protocol reached with this study minimized microfiber contamination during analysis.

Keywords

Marine debris; Microplastics; Blackbelly rosefish; Skipjack tuna; Blue jack mackerel; Azores;

Introduction & Aims

Marine litter, particularly plastic, has been identified as one of the major environmental problems that oceans are currently facing. Plastic has become a ubiquitous material in today's world and a life without it, seems unimaginable. Since its invention in the early 1950´s, demand for plastic has sharply increased due to its applications in diverse sectors, such packaging, building and construction, automotive, electrical, and electronic, agriculture, textile, and medical (PlasticsEurope, 2017). Between 1950 and 2016, around 8200 million metric tons (Mt) of resins and fibres have been produced and most of it since 2000 (Geyer *et al*., 2017; PlasticsEurope, 2017). China alone is the biggest producer of plastic, both resins and fibres (Geyer *et al*., 2017), followed by Europe and NAFTA (PlasticsEurope, 2017). Most of plastics have short-term use yet long life duration in nature. Geyer *et al*. (2017) estimates that 30% of plastics produced between 1950 and 2015 are currently in use, while the remaining 70% become waste. Plastic waste can be treated, recycled or incinerated, however the majority have been discarded and are accumulating in landfills or in natural environments (Geyer *et al*., 2017). While the quantity of waste sent to landfills has been decreasing and energy recovery (through incineration), and recycling rate have been increasing (PlasticsEurope, 2017), today almost 10% of annual plastic production (Avio *et al*., 2017) still ends up accumulating in marine (Ryan and Moloney, 1993; Derraik, 2002; Thompson *et al*., 2004; Jambeck *et al*., 2015 ; Avio *et al*., 2017) and freshwater environments (Dris *et al*., 2014; Wagner *et al*., 2014).

In the oceans, plastic items are found from coastal areas (Tanaka and Takada, 2016) down to the deep-sea floor (Pham *et al*., 2014; Chiba *et al*., 2018). Plastic reaches the marine environment through inland sources (e.g. wastewater treatment plants, beach litter, urban litter, harbours, and industrial activities residual waters) and sea-based sources (e.g. fisheries and cargo shipping) (Derraik, 2002; Browne *et al*., 2011; Wagner *et al*., 2014). Once in the oceans, their high durability coupled with their general low density impels that plastics can get carried by surface currents and travel long distances and accumulate in some areas in the open ocean, such as oceanic gyres (Eriksen *et al*., 2013; Eriksen *et al*., 2014). On the other hand, some plastics that are denser than water or that gets heavily fouled will sink and accumulate in the sediment (Van Cauwenberghe *et al*., 2015).

One of the major issues of plastic pollution in the marine environment lies in the durable characteristics of plastic items, making them highly persistent. Although plastics are incapable of being completely degraded through natural processes, some chemical degradation can weaken plastic items, promoting its degradation and fragmentation (Barnes *et al*. 2009). Degradation happens when the mechanical integrity of a polymer and its molecular weight changes due to a chemical reaction, that can be promoted by photo and thermo-oxidation (e.g. solar radiation and temperature), hydrolysis and microbial activity (Singh and Sharma, 2008). The rate of degradation for every plastic is dependent upon its fabrication process and environmental conditions, such as temperature, solar radiation, and oxygen (Andrady, 2011;

Fotopoulo and Karapanagioti, 2017). Fragmentation on other hand, is the result of physical and mechanical processes, such wave action, sand abrasion or animal contact (Corcoran *et al*., 2009). Fragmentation rate depend on environmental conditions (e.g. wind speed, curling and current speed) and polymer type (Andrady, 2017). Degradation also have a strong effect on fragmentation, it weakens mechanical stability of plastic and combined with physical forces results in higher fragmentation (Andrady *et al*., 2011; Andrady, 2017). Fragmentation is a continuous process that leads to an increasing amount of plastic debris of small sizes ranges compared to the initial quantities entering the marine environment. Therefore, plastic items in the oceans are present in a wide different size range, from nanoparticles up to large items. The Marine Strategy Framework Directive (MSFD) Technical Subgroup on Marine Litter has proposed the use of standardized terminology for defining plastic items based on size. Macroplastics includes all particles larger than 25 mm, while mesoplastics includes items between 25 and 5 mm. Microplastics includes all particles ranging between 5 mm and 1 µm while all particles smaller than 1 µm are termed nanoplastics MSFD GES Technical Subgroup on Marine Litter, 2013).

Over the past decade, microplastics have brought a particular concern among the scientific community (Galloway and Lewis, 2016).

Their small size means that microplastics are bioavailable to ingestion by a variety of taxa including zooplankton, marine invertebrates, fish, turtles and marine mammals (reviewed by Gall and Thompson, 2015). Microplastic particles are generally subdivided into primary and secondary according to its origin (Barnes *et al*., 2009). They are considered primary when its original shape is maintained, examples of that are micron-sized particles present in exfoliants, toothpastes and clothes (Avio *et al*., 2017). Secondary microplastics result of the progressive process of fragmentation of large items (Gregory and Andrady, 2003).

The abundance of microplastic particles floating at the surface of the world's ocean has been grossly estimated by various authors (Cózar *et al*. 2014; Eriksen *et al*., 2014). However, these models suggest that a large proportion of the plastic is found elsewhere, either on the seafloor (Woodall *et al*., 2013). or in the food web. Ingestion of plastic by fish has been reported for a wide variety of species. Yet, the intake rate is variable according to geographic location and its proximity to high populated urban areas or hotspots of marine litter accumulation. The plastic ingestion rate in Mediterranean Sea was reported as 68% near Balearic Islands (Nadal *et al*., 2016), 58% in Turskish territorial waters (Guven *et al*., 2017), 28% in Adriatic Sea (Avio *et al*., 2015), 24.3% (Battaglia *et al*., 2016) and 18.2% (Romeo *et al*., 2015) in Strait of Messina, 18.8% in Mediterranean coast of Spain (Bellas *et al*., 2016) and 1.9% in deep water species in Ionian Sea (Anastasopoulou *et al*., 2013). In North Sea rate of ingestion reported was 2.6-3% (Foekema *et al*., 2013; Brate *et al*., 2016) and in both North and Baltic Sea was 5.5% (Rummel *et al*., 2013). In North East Atlantic reported values were 11% (Lusher *et al*., 2017), 47.7% in costal and 2.4% in off-shore areas around Scotland (Murphy *et al*., 2017), 36.5% in English Channel (Lusher *et al*., 2013), 32% in Atlantic coast of Spain (Bellas *et al*., 2016) and 19.8% in Portuguese coast of Lisbon region (Neves *et al*., 2015). Also, 73% of ingestion was reported in mesopelagic fish sampled between Galway (Ireland) and St. John (Canada) (Wieczorek *et al*., 2018). In North West Atlantic ingestion rate reported was 2.4% in cod fish fished in east coast of Canada (Liboiron *et al*., 2016) and 42.4% in Texas Gulf coast (Peters *et al*., 2017). In South Atlantic it was reported in South coast of Brazil 0.7% of ingestion of cigarette (Di Beneditto *et al*., 2014), 18-32% of ingestion of plastic in North coast of Brazil (Possatto *et al*., 2011; Miranda *et al*., 2016) and 73%

in costal South Africa (Naidoo *et al*., 2016). In North Pacific was reported an ingestion rate of 25% in West coast of US (Rochman *et al*, 2015), 24.5% in North Pacific Subtropical Frontal zone (Jantz *et al*., 2013), 9.2% in the North Pacific Subtropical gyre (Davison and Asch, 2011), 77% in coastal Tokyo (Tanaka and Takada, 2016) and 7%-40% in Mariana Islands (Van Noord *et al*., 2013). While in South Pacific was reported 2.1% in West Coast of South America (Ory *et al*., 2018), 28% in Indonesia (Rochman *et al*., 2015). 0.3% ingestion rate was reported in Australian Ocean (Cannon *et al*., 2016). Over 150 species has been analysed, yet in some cases the minimum sampling size was only 1 individual. Atlantic cod (*Gadus morhua*), European pilchard (*Sardina pilchardus*) and Atlantic horse mackerel (*Trachurus trachurus*) are the some of the most studied species.

The minimum size particles of interest between studies were also variable: smaller than 1 mm (Avio *et al*., 2015; Neves *et al*., 2015; Bellas *et al*., 2016; Lusher *et al*., 2016; Rummel *et al*., 2016; Tanaka and Takada, 2016; Guven *et al*., 2017), between 1 – 3 mm (Battaglia *et al*., 2016; Ory *et al*., 2018) or bigger than 3 mm (Anastasopoulou *et al*., 2013; Jantz *et al*., 2013).

In individuals that were found to ingest plastic the mean of particles per individual was reported in most studies to range between 1.2 and 1.9 (Lusher *et al*., 2013; Neves *et al*., 2015; Bellas *et al*., 2016). However, in some the mean of particles were bigger than 2 particles per individual, being 3.75 the highest average numbers of particles reported (Nadal *et al*., 2016). Many studies have revealed that sites located in the coastal zones, close to urban areas have higher proportion of presence of fibres compared to non-urban areas (Rochman *et al*., 2015; Bellas *et al*., 2016; Guven *et al*., 2017; Peters *et al*., 2017; Murphy *et al*., 2017). Although fibres are highly abundant, fibres are not considered in the results of many studies (Ory *et al*., 2018), most probably due to possibility of airborne contamination by microfibers.

The Azorean archipelago is in the middle of the North Atlantic Ocean, between 36.5°–40° N and 24.5°–31.5° W. It is a low populated region and geographically isolated. However, the archipelago is found at the vicinity of the North Atlantic Subtropical Gyre which is a plastic accumulation hotspot (Law *et al*., 2011). Due to its proximity, marine currents end up transporting plastic particles from southern waters up to Azores region. Fisheries in the Azores are an important economic resource and its fishery methods are mostly traditional. The asset of the Azorean fish in the international market, is its sustainability but most importantly its high quality since it originates from a so-called “pristine” and remote area. Therefore, it is important to obtain knowledge on plastic ingestion by commercial fish species in the Azores.

In the present study, 157 stomachs of three different fish species were opened to quantify and characterize potential debris items. All species have economic value and are important resources for human consumption. Blackbelly rosefish, *Helicolenus dactylopterus*, is a bathy demersal carnivorous species that feeds on both pelagic and benthic organisms (Hureau and Litvinenko, 1986). Species with high economic and gastronomic value in Azores. Blue jack mackerel, *Trachurus trachurus*, is a benthopelagic species that feeds mostly on crustaceans and its depth range is between 305-370 m (Smith-Vaniz, 1986). Skipjack tuna, *Katsuwonus pelamis*, is pelagic-oceanic carnivorous species with a common length of 80 cm (Collette and Nauen, 1983).

This study aims to develop an efficient methodology for microplastic identification in stomatal contents of fish considering both visual methods and pre-filtration analysis of stomachs digested with KOH 10%. In addition, different measures of microfiber contamination control were tested to ensure accurate distinction between microfiber contamination to the microfibers

present in the digestive tract. Microfibers are known to be one of the most abundant microplastic type in the ocean, having accurate results of its real abundance in gastrointestinal content is therefore essential.

Material and Methods

Sample Collection

Fish samples were obtained from fishing activity in waters of the Azores archipelago. Each species was caught with a different fishing method. Blackbelly rosefish, *Helicolenus dactylopterus*, was caught with longline fishing and handlines. Blue jack mackerel, *Trachurus picturatus* was captured with nets while the traditional pole-and-line fishing gear was used to catch Skipjack tuna, *Katsuwonus pelamis*. In the laboratory, each individual was measured and weighted the lowest 0.1 mm and 0.01 g. Total, fork, and standard length was measured for Blue jack mackerel and Skipjack tuna. While for the Blackbelly rosefish only total and standard length was registered. Length measurements were taken according to Miller and Lea (1972). Total length was measured as the straight distance from the tip of the longest jaw with mouth closed to the tip of the longest caudal lobe pinched together. Fork and standard length were taken as the length from the tip of the longest jaw (with the mouth closed) to the middle point of fork and to the end of hypural bone, respectively. After measurements fish was dissected and stomach, liver, gonad was separated and weighted. Gutted weight and sex were also registered. Each stomach was kept individually in a zip-lock plastic bag or plastic bag stapled in the opening part. Plastic bags were labelled (by species name, storage date, and individual number) and frozen at -20Cº until further analysis. An database with all information related with capture (fishing boat, capture date and fishing method) as well as lab measurements, including samplers name and code of each stomach sample was built.

Sample selection

A minimum sample size of 50 individuals per species was established according to the EU guidelines for litter ingestion by fish (MSFD GES Technical Subgroup on Marine Litter, 2013). In order to reduce variability associated with fish size, we selected a tight size range for each species. Each range was selected according to the total length interval with more individuals, considering the minimum sampling size of 50. The length range selected was 32-36, 40-47, and 45.5-57.5 cm for Blackbelly rosefish, Blue jack mackerel and Skipjack tuna, respectively. As measure of randomization the sample order within each species was chosen with the “sample function” of R 3.3.1. in order to increase the degree of blindness in selection. With this method, a total of 55 Blackbelly rosefish 52 Blue jack mackerel and 50 Skipjack tuna were sampled.

Stomach opening procedure and visual analysis

Each sample was left thawing at room temperature for a minimum of 18 hours prior to analysis. After being thawed, each sample was weighted in a digital analytical scale (to the nearest 0.001g). In order to limit the potential of microfiber contamination before being frozen, every stomach was thoroughly washed with 20 µm pre-filtered tap water. Then they were kept individually in petri dishes, previously washed with filtered water, and dried. The work surface was cleaned with 95% alcohol and decontaminated tweezers and scalpel blade were used to open the stomachs in a straight line starting in the aperture that connected the oesophagus to

the stomach. Degree of fullness was registered according to the ethogram in Table 1 and stomach contents were analysed under a dissection microscope for the presence of plastic particles. The duration of this procedure was also registered for each stomach. For the control of airborne contamination during the exposure to the air, a Whatman Grade GF/A with 47mm diameter was left in a 50 mm diameter sterile petri dish open to the air. In this phase every stomach had an individual control that was analysed right after the end of each procedures.

Scale	Definition
0	Stomach is completely empty.
1	Stomach is nearly empty. Residual evidence of digestion.
2	Quarter-full stomach. With exception for some hard digestion structure, all contents are digested and in the last stage of digestion.
3	Stomach is half full. Contents are digested, with exception for scales and spines.
4	Stomach is three-quarters-full. Some contents are already semi-digested.
5	Stomach is completely full of non-digested food. Evidence of recent feeding activity.

Table 1 - Ethogram of fullness degree in fish stomachs.

To further limit the contamination, a white lab coat and blue nitrile gloves were used during the entire process. Petri dishes and tweezers were checked under the dissecting microscope for the presence of microfibers before starting the work. In between stomachs, the scalpel blade and tweezers were cleaned and checked under the microscope for the presence of microfibres, avoiding contamination between samples.

KOH digestion

After visual analysis, stomachs were digested with a 10% KOH solution, that was prepared by dissolving 100 g of KOH in 900 ml of water filtered at 20 µm membrane and reserved in a transparent glass bottle with a blue cap at 5 Cº. Open stomachs were stores into a white plastic bottle (with a white plastic cover and black cap), previously washed with 20 µm filtered water to avoid fibre contamination. Afterwards approximately 3 times the volume of the stomach of 10% KOH was added to the stomachs and left for 72 hours at 40 Cº for digestion of organic material, following the recommendation of Karami *et al*. (2017) for reducing plastic degradation.

1 and 50 µm Filtration

The filtration procedure was divided in two steps. At first, digestion solution was filtered with a 50 µm membrane filter under vacuum and then re-filtered through a 1 µm pore Whatman Grade GF/B. The filtration was divided into two size fractions to avoid clogging the filter and facilitate encountering the plastic particles. For the skipjack tuna, it was necessary to add an extra step and first filter the digested solution through a 1 mm sieve to separate hard biologic structures (e.g. spines and scales). This preliminary separation with 1 mm sieve net reduced efficiently the accumulation of biological structures in the 50 µm and 1 µm membrane filters. Between step 1 and 2 filtration, the digested solution was returned to the initial container that was used to digest the stomach in KOH. Due to exceptionally high biological loads in some samples, it was necessary to divide the solution into more than one 1 µm filter.

After both filtration step, all filters were kept individually in 50 mm sterile petri dishes and dried at 40 Cº for one day in the oven. Afterwards, each petri dish (with a lid) were stored in a cabinet

drawer until further analysis. The remaining solution from both filtrations were saved in a 10 L container for recycling.

As control for airborne contamination, a blank, similarly to the procedure of stomach dissection was left open to air, and analysed under a dissection microscope every 5 samples filtered. All filtration material was washed with 20 µm pre-filtered tap water multiples times before starting the filtration procedure. Furthermore, all work surfaces were cleaned with alcohol prior starting the work. Between filtrations, the filtration Erlenmeyer was also washed with 20 µm filtered water to avoid contamination between samples.

Filters analysis

Filters were analysed under Leica binocular MZ16FA with a MC 190 Leica camera attached for identifying the presence of microplastics. Every potential plastic particle was counted and photographed. Measurements of approximate length for fibres and length of the largest distance between sides of beads and fragments were obtained with Leica LAS V4.12 software. Resolution, colour, and type of plastic was registered with the measurements, as well as photograph label. Position of every particle found in filter was also registered in paper sample file. Potential plastic particles were allocated to the following type: fragments, fibres and microbeads.

As previously, controls were left open to air during the entire time when the samples were exposed to air. Entries and exists in the laboratory were conditioned to avoid suspension of microfibers in the air. Before starting the analysis, work surfaces were cleaned with 95% alcohol and petri dishes were handled from distance. Focus and zoom were controlled from computer and a PVC plate structure were used to move the petri dish.

Contamination control

Despite of the use of controls for airborne contamination in all the phases of sample exposure to air, additional tests were conducted to assess degree of microfiber in the air that could contaminate samples. A glass microfiber filter GF/B, diameter 47mm was left in a small petri dish open to air for 5 hours in both Wet and Optic lab to assess degree of microfiber precipitation in work area. Degree of contamination by dress and undress lab coat was also evaluated by practicing this movement near a blank filter. For testing possibility of extreme airborne contamination, a white glass microfiber filter GF/B was waved in the air in several areas outside the working lab where higher levels of microfiber contamination were expected.

To assess the possibility of contamination during filtration from other source than airborne microfibres, blank filtration tests were made. Each test addresses a different variable that could contribute to contamination during the procedure. At first two preliminary tests using water filtered once through a 20 µm mesh net. In the first (Fw) test the water was filtered under vacuum through a 1 µm Whatman Grade GF/B, and then placed in the same plastic bottles used to digest the stomachs before being filtered again, using the same filter. In the second preliminary test (FwC) a volumetric cup was used between filtrations to assess any possible contribution of microfiber due to the recipient used between filtrations. The number of replicates for preliminary tests corresponded to 9 for Fw and 5 for FwC. Despite the tests mimic the real filtration conditions, the use of the same filter in both step filtrations didn't allow to

figure in each filtration contamination would happen. So, a test following the exact same protocol but using a filter per filtration was made. This test was divided in phases F-1 and F-2 corresponding to first step filtration and second step filtration, respectively. Each step filtration is affected by different factors that could be source of contamination. In F-1 phase water type was the variable considered, three types of water were used: 20 µm pre-filtered water, Milli-Q water and water filtered through resins and 20 µm membrane. F-2 phase assessed variability due to the type of recipient used to store the water between filtrations, for that plastic and glass bottles were used. Total of 12 replicates crossing variables were made. A test to presence of microplastics in tap water, replicated 5 times, was also made (NFw). Tap water reserved previously in a wash bottle was filtered through a 1 µm Whatman Grade GF/B under vacuum. An efficiency test (CF) to the filtration process using water filtered through two processes (resins and 20 µm membrane) and glass digestion bottles was repeated twice. It followed the filtration protocol used to filtrate the digested stomachs.

Any microfiber found in either in air or water controls was considered a contamination positive. Every positive was counted and photographed. The methodology used to test the water and recipient with null or nearly positives found was considered the most efficient method to be used in filtration. As measure of correction of the results, every potential plastic particle was compared to the positives found in the controls. Any particle identical to a positive was no considered in the results.

Statistical analysis

Data analysis was performed in R 3.3.1 and the graphs were plotted with ggplot2 function. Spearman's Correlation was used to assess the relation between fullness degree and the weight of stomach. The abundance ingestion rate was computed by dividing the total of potential plastic items counted by the total number individuals, including those who ingested and did not ingested plastic. The intensity ingestion rate was calculated dividing the total of potential plastic items counted by the total number of individuals that ingested plastic. Calculations were made for all individuals regardless of species and per species. Definition of abundance and intensity used was described by Provencher *et al*. (2017). Ingestion rates (abundance and intensity) were also computed according to the potential plastic type (fibre, fragment or microbead). Statistically significant differences in ingestion rate (abundance and intensity) between species was calculated with Kruskal-Wallis test and two-tail Mann-Whitney. Differences between potential plastic type within species were also tested. The average of the size of items consider in the results by its type classification was also computed.

Results

Sample size and weight

Stomachs sampled were chosen according to individual total length recorded before dissection procedure. Length class histograms with all the individuals available per species (Blackbelly rosefish n=193, Blue jack mackerel n=75 and Skipjack tuna n=64) were plotted and sampled range was sorted around length class with more individuals (Figure 1). Range of selection was to aim minimum sampling size of 50 individuals and its breadth was according to total individual size of each species. Out of the 332 existent individuals within the three species, 157 were sampled (Blackbelly rosefish n=55, Blue jack mackerel n=52 and Skipjack tuna n=50).

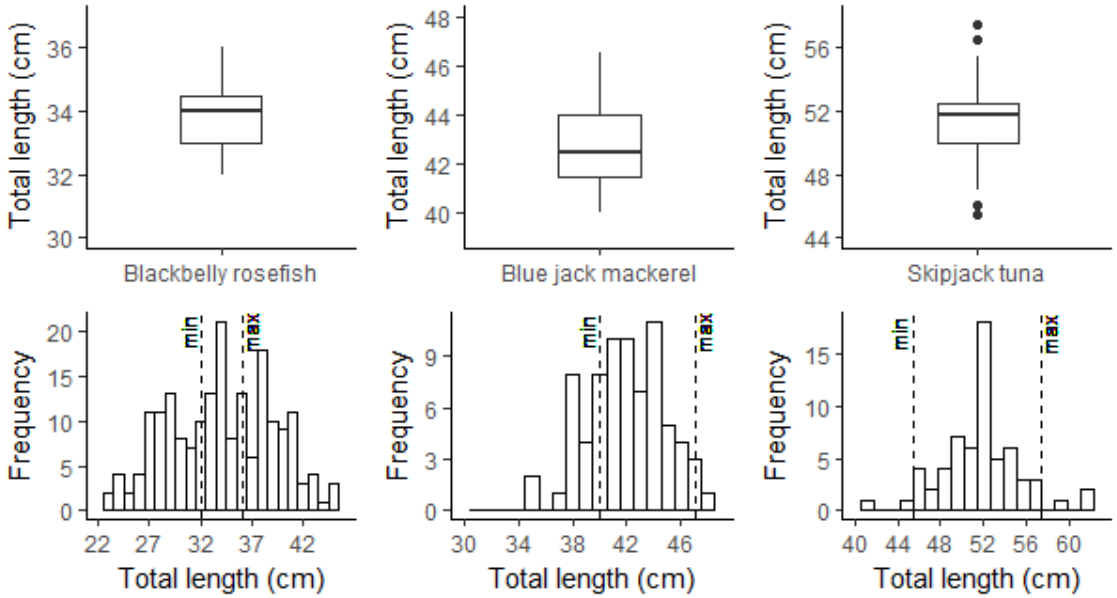

Figure 1 - Histogram of length class distribution of individuals of each species stored (in the bottom) with its length selection range marked with dashed lines. Boxplot of length class distribution of the sampled individuals of each species (in the top), including only individuals used in the analysis.

Yet only 155 were used in the results, 2 stomachs of Blackbelly rosefish sampled were partially opened and its contents was lost before visual analysis. The mean length size for Blackbelly rosefish was 34.01 ± 1.16 cm, 42.66 ± 1.74 cm and 51.48 ± 2.43 cm for Blue jack mackerel and Skipjack tuna, respectively (Figure 1). Normality of length class distribution of sampled individuals for each species was maintained.

The weight distribution of the stomachs prior to visual analysis were not normally distributed, except for Skipjack tuna (p-value=0.29). For Skipjack tuna the mean weight was 85.24 ± 36.47 g which was clearly higher than for the other species, 11.58 ± 4.43 g for Blackbelly rosefish and 13.54 ± 3.05 g for Blue Jack mackerel (Figure 2).

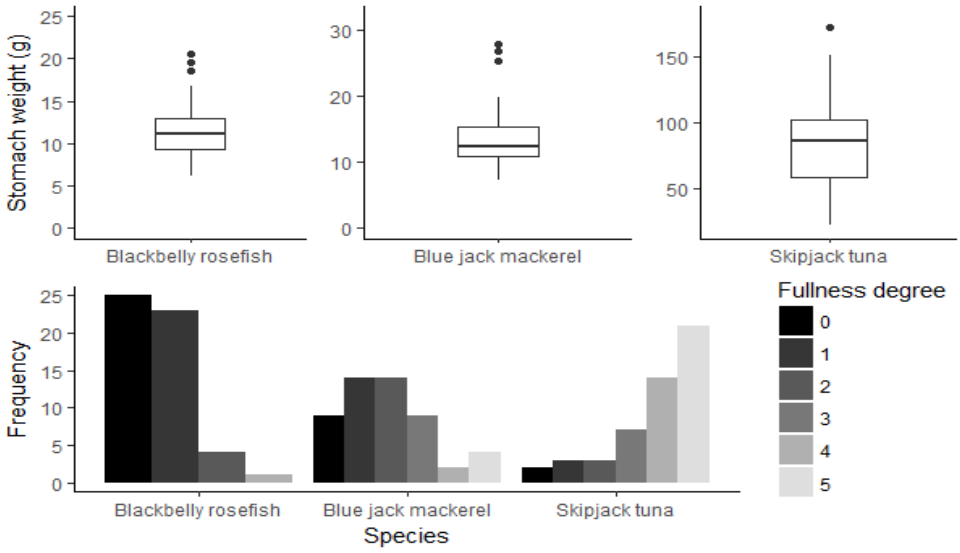

Figure 2 - Boxplot of stomach weight distribution per species (in the top). Frequency of stomachs classified according to each fullness degree index, ranging from 0-5 (in the bottom).

Stomach fullness

Around 90% of Blackbelly rosefish stomachs were empty or nearly (fullness degree 0 and 1). Stomachs of Blue jack mackerel were in its majority less than half-full, with fullness degree index of 1 and 2 representing approximately 50% (Figure 2). 70% of the Skipjack tuna sampled stomachs were three-quarter or fuller.

There is a positive Spearman's correlation between the weight of the stomach and its fullness degree for Skipjack tuna (ρ= 0.67) and Blue jack mackerel (ρ= 0.40), yet no correlation was found for stomachs of Blackbelly rosefish.

Quality control

During visual analysis, microfibre positives were identified in 5 of the blanks for the Blackbelly rosefish stomachs: 2 fibres in one of the blanks and 1 fibre in each of the three other contaminated controls. No contamination was found during visual analysis of the other species. During the filtration procedure, fibres were found in 4 of the blanks used for Blackbelly rosefish samples and in 1 the blank for the other species. Each positive had only one fibre. No contamination was found in any of the blanks used during filters analysis procedure.

The additional tests for airborne contamination, did not reveal contamination. No contamination was found in the filters left open to the air for 5 hours in any of the laboratories (Wet and Optic). However, 3 fibres were found in the in the blank used to test the degree of contamination related to dressing and undressing the laboratory coat. Similar results were found in the blank that was transported in areas of expected higher airborne contamination, with a total of 2 fibres and a film fragment recovered.

The quality control tests of the non-filtered tap water (NFw) revealed contamination in 3 out of the 5 filters analysed, represented by 2 fragments and 1 fibre. However, the filtered 20 µm water (Fw) test it was found fibres in 78% of the analysed filters (n= 9) and a total number of fibres per filter ranging between 1 and 7 (Table 2). For the FwC test, 40% of the filters were contaminated of the FwC test, 2 fibres per contaminated filter. While testing the 20 µm pre-filtered used in to clean material in simulation of the filtration procedure done with KOH digested samples it was found contamination in 4 out of 5 filtrations using a plastic container between step filtrations. The number of fibres found per contaminated analysed filters ranged between 1 and 4 fibres (Table 2). While using water filtered through two processes (resins and 20 µm mesh filter) and a glass container between step filtrations, no contamination was found. Same results were found in the test of efficiency of the filtration procedure using water filtered through two processes and glass cup between filtrations.

Code	Date	Filtered water (20 µm)	Type of container	Steps	Positives	Notes
Fw1	28/03/2018	Yes	Plastic	2	5 fibres	3 blue fibres [b] and 2 black fibres.
Fw2	02/04/2018	Yes	Plastic	2	5 fibres	3 black fibres [a], 1 red fibre [c] and 1 blue fibre [b].
Fw3	02/04/2018	Yes	Plastic	2	7 fibres	2 yellow fibres, 3 black fibres [e], 1 red fibre [f] and 1 blue fibre [b].
Fw4	02/04/2018	Yes	Plastic	2	NA	
Fw5	02/04/2018	Yes	Plastic	2	1 fibre	1 blue fibre [b].

Fw6	02/04/2018	Yes	Plastic	2	1 fibre	1 blue fibre [b].
Fw7	04/04/2018	Yes	Plastic	2	NA	
Fw8	04/04/2018	Yes	Plastic	2	2 fibres	1 red fibre [c] and 1 blue fibre [b].
Fw9	04/04/2018	Yes	Plastic	2	1 fibre	1 blue fibre [b].
F1-1	02/04/2018	Yes	Plastic	1	2 fibres	Possible contamination before filtration. 1 red fibre [c] and 1 blue fragment [g].
F1-2	02/04/2018	Yes	Plastic	1	NA	
F2-1	02/04/2018	Yes	Plastic	1	1 fibre	1 black fibre [e].
F2-2	02/04/2018	Yes	Plastic	1	NA	
F3-1	03/04/2018	Yes	Plastic	1	NA	
F3-2	03/04/2018	Yes	Plastic	1	1 fibre	1 black fibre [e].
F4-1	03/04/2018	Yes	Plastic	1	NA	
F4-2	03/04/2018	Yes	Plastic	1	NA	
F5-1	03/04/2018	Yes	Plastic	1	NA	
F5-2	03/04/2018	Yes	Plastic	1	4 fibres	2 blue fibres [b], black fibre [a] and 1 white fibre [d].
F6-1	06/04/2018	Yes	Glass	1	NA	Milli-Q water used.
F6-2	06/04/2018	Yes	Glass	1	NA	Milli-Q water used.
F7-1	06/04/2018	Yes	Glass	1	NA	Milli-Q water used.
F7-2	06/04/2018	Yes	Glass	1	NA	Milli-Q water used.
F8-1	06/04/2018	Yes	Glass	1	NA	Milli-Q water used.
F8-2	06/04/2018	Yes	Glass	1	NA	Milli-Q water used.
F9-1	06/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F9-2	06/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F10-1	09/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F10-2	09/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F11-1	09/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F11-2	09/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F12-1	09/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
F12-2	09/04/2018	Yes	Glass	1	NA	Water filtered through 2 processes, resins and 20 µm filter.
FwC1	02/04/2018	Yes	Glass	2	NA	
FwC2	02/04/2018	Yes	Glass	2	2 fibres	2 blue fibres [b].
FwC3	04/04/2018	Yes	Glass	2	NA	
FwC4	04/04/2018	Yes	Glass	2	2 fibres	1 red fibre [f] and 1 blue fibre [b].
FwC5	04/04/2018	Yes	Glass	2	NA	

CF1	09/04/2018	Yes	Glass	1	NA	
CF2	09/04/2018	Yes	Glass	1	NA	
NFw1	02/04/2018	No	Plastic	1	1 fragment	1 blue fragment [g].
NFw2	02/04/2018	No	Plastic	1	1 fragment	1 blue fragment [g].
NFw3	03/04/2018	No	Plastic	1	NA	
NFw4	03/04/2018	No	Plastic	1	1 fibre	1 blue fibre [b].
NFw5	03/04/2018	No	Plastic	1	NA	

Table 2 – Results for each water contamination test made regarding each variable (type of water and type of container). Each positive was divided to its type (fragment or fibre) and colour (colours in figure 3).

When compared the plastic particles found in the water quality control, same kind of fibres or fragments were found in most cases and classified in 7 categories (Figure 4).

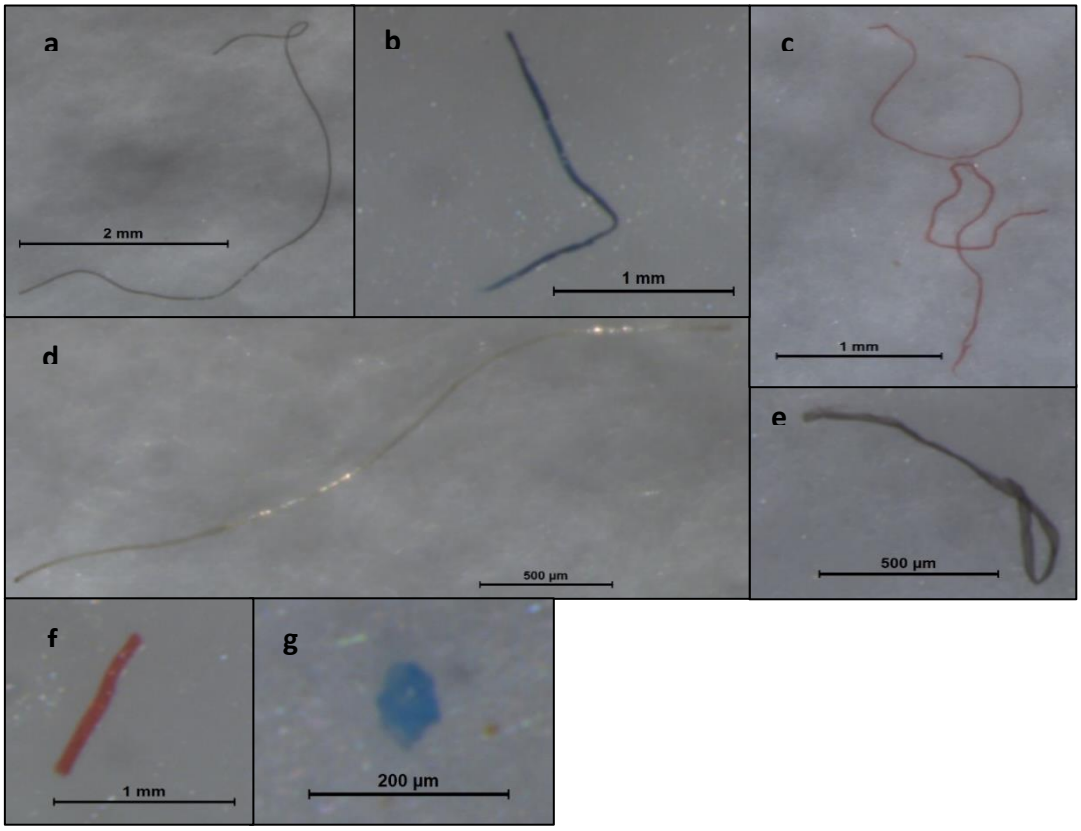

Figure 3 - Fibres found in filtration controls, photographed with Leica MC19 camera attached to Leica MZ16FA binocular. a) Black fibre; b) Blue fibre; c) Red fibres; d) White fibre; e) Black fibre; f) Red fibre; g) Blue fragment.

Incidence of plastic ingestion

90 particles of possible plastic polymers were found over the 155 fish analysed, corresponding to a 38.71% of individuals (n=60) that ingested plastic. Skipjack tuna was the species with more individuals (n=22) with potential plastic particles, corresponding to a 44% of the sampled individuals, followed by Blue jack mackerel with 21 individuals found with possible plastic polymers (40.8%). For Blackbelly rosefish only 17 individuals were found to ingest potential plastic particles (32,08%), yet it was the species with more particles ingested per individual. . In the present study case, species with higher abundance ingestion rate were also the species with higher intensity ingestion rate (Table 2).

Species	Ingestion rate (%)	Ingested pieces	$\bar{X} \pm se$ (Abundance of ingestion)	$\bar{X} \pm se$ (Intensity of ingestion)
Blackbelly rosefish	32.08%	36	0.68±0.17	2.12±0.34
Blue jack mackerel	40.38%	25	0.48±0.09	1.19±0.09
Skipjack tuna	44%	29	0.58±0.12	1.32±0.17

Table 3 – Table summarise the ingestion rate per sampled species in percentage, number of plastic pieces found, mean and standard error ($\bar{X} \pm se$) of abundance of plastic ingestion (including all individuals) and its mean and standard error ($\bar{X} \pm se$) intensity of ingestion (including only individuals that ingested plastic).

The abundance ingestion rate for the Blackbelly rosefish was 0.68±0.17 particles while the intensity ingestion rate was 2.12±0.34 particles. Skipjack tuna abundance ingestion rate was 0.58±0.12 and its intensity ingestion rate was 1.32±0.17 particles. Blue jack mackerel was the species with lower abundance and intensity ingestion rate, 0.48±0.09 and 1.19±0.09, respectively.

To evaluate the possibility of correlation between plastic particles ingested and the fullness degree of stomachs a Spearman's rank correlation coefficient was run for each species. No correlation was found between fullness degree and plastic ingestion for any species: Blackbelly rosefish (ρ= - 0.08; p-value= 0.55); Blue jack mackerel (ρ= -0.07; p-value= 0.61); Skipjack tuna (ρ= -0.07; p-value= 0.64). There was no statistically difference in plastic ingestion for both abundance and intensity rate of ingestion between species checked with two-tail Mann-Whitney test.

Plastic composition

Out of the total (n = 90) of possible anthropogenic plastic particles, 53 were fragments (58.8%), while 37 were clearly fibres (≅ 41.1%). All fibres were double checked and compared to airborne controls and water controls due possibility of contamination. For the Blackbelly rosefish sampled, 72% of the particles found were fragments (n=26), and only 28% were fibres (n=10). As for the two other species, the percentage of both plastic types was near 50%. Skipjack tuna was the only species with higher percentage of fibre than fragments (52%). Regarding

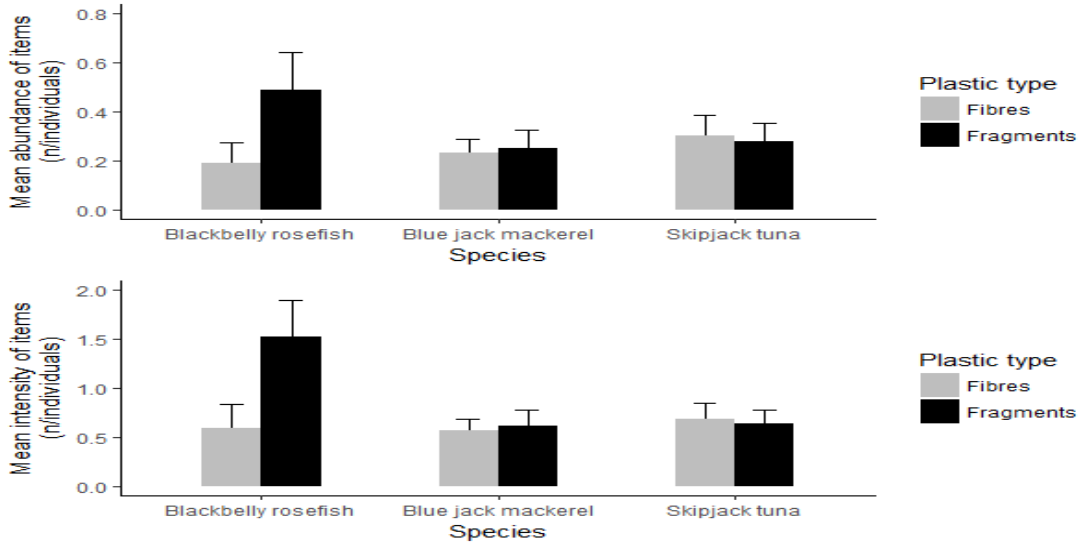

Figure 4 - Mean abundance of ingestion by plastic type for each species (in the top) and mean intensity of ingestion by plastic type for each species (in the bottom). Error bars represent standard error (SE).

abundance of ingestion and its intensity by plastic type (fibre or fragment), Blackbelly rosefish ingested fragments with higher intensity (1.52± 1.5 pieces) than any other species (Figure 3). A similar pattern was found for the abundance of fragments ingested.

On the other hand, Blackbelly rosefish was the species with lower abundance of ingestion of fibres, 0.19±0.62 pieces in average contrasting with values of 0.23 ± 0.43 and 0.3 ± 0.61 for the other species. Mean abundance of fragments for Skipjack tuna and Blue jack mackerel were 0.28 ± 0.08 and 0.25 ± 0.08, respectively. According to intensity the ingestion of fibres between species was around 0.59 ± 1.00 for Blackbelly rosefish, 0.57 ± 0.51 for Blue jack mackerel and 0.68 ± 0.78 for Skipjack tuna. For intensity rate of ingestion of fragments were found for Blue jack mackerel 0.62 ± 0.74 and Skipjack tuna 0.64 ± 0.66.

Size and colour

The majority (96.7%) of the recovered items where microplastics, with a maximum length <5mm. Only 3 macroplastic particles (> 5mm) were found, in Skipjack tuna, ranging between 1.2 cm and 11 cm.

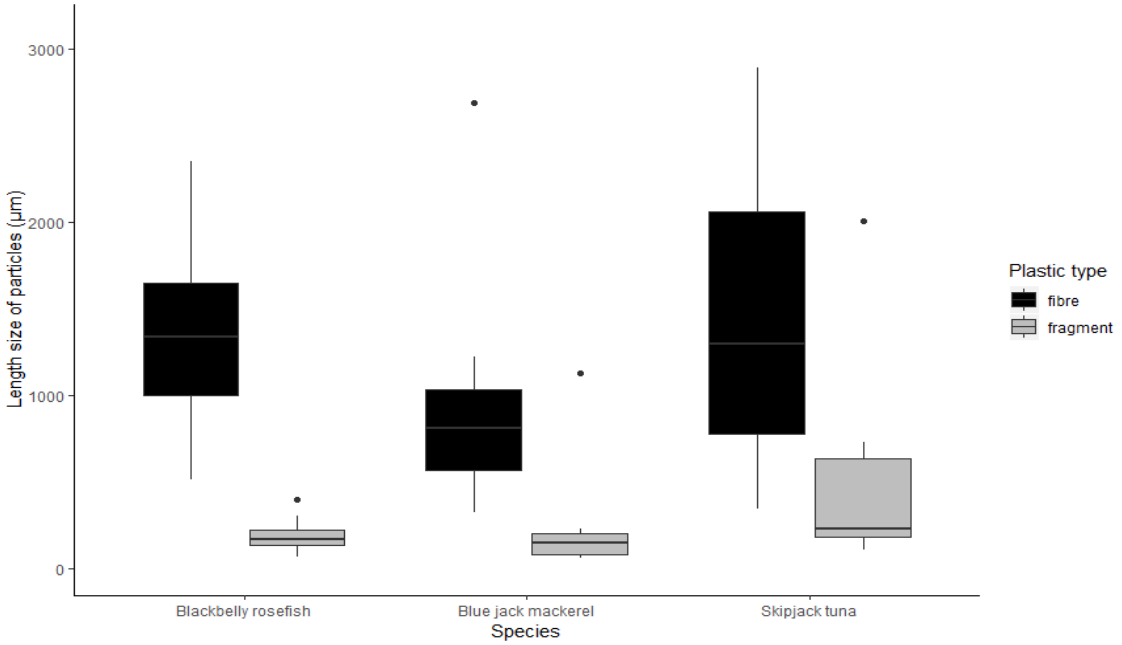

Figure 5 - Histogram of length size of particles by plastic type found within each species stomach. Measurements of length were recorded in the largest side of piece found.

Potential microplastic fragments were in general smaller than potential microplastic fibres found in each species (figure 2). The mean length of fibres found in Blackbelly rosefish was 1.41 ± 0.59 mm while the mean size of fragments was 0.19 ± 0.08 mm. As for Blue jack mackerel mean fibre size were 0.93 ± 0.61 mm and fragment mean were 0.22 ± 0.28 mm. Ignoring the macroparticles found in Skipjack tuna, the mean of fibre size was 1.26 ± 0.77 mm and fragments was 0.43 ± 0.52 mm.

Microplastic recovered from the blackbelly rosefish were mostly green (53%) and blue (28%). For Skipjack tuna the most common colours of ingested particles were blue (31%), black (28%), green (17%) and white (10%). The colour of fragments ingested by Blue jack mackerel were mainly black (28%) and green (28%), followed by blue (12%) and grey (12%).

Figure 6 - Examples of some potential plastic particles found in the stomatal contents of Blackbelly rosefish, Blue jack mackerel and Skipjack tuna. Photos taken under Leica binocular MZ16FA with a MC 190 Leica camera attached.

Discussion

This is the first study investigating plastic ingestion in commercial fish in the Azores region. The results revealed that all three species of contrasting trophic ecology and commercial interest ingested particles that are potentially artificial polymers. The frequency of occurrence of potential plastic particles were 38.71% over all individuals and 32.08% for Blackbelly rosefish, 40.38% for Blue jack mackerel and 44% for Skipjack tuna. It was also registered for the first-time ingestion of potential plastic particles in Skipjack tuna and Blackbelly rosefish, and higher frequency of potential plastic items ingestion by Blue jack mackerel than previously recorded by Neves *et al*. (2015) in Lisbon region. Due to controlled variability of size within individuals of same species and very careful measures regarding contamination in the results, there are confidence in the quality of the results hereby presented. Potential plastic items identity still must need to be confirmed with the FR-IR method because organic fibres are commonly found in marine environments (Browne *et al*., 2011). FT-IR method is also recommended by the EU guidelines for microparticles (MSFD GES Technical Subgroup on Marine Litter, 2013).

The frequency of occurrence found in the present study (38.71%) is relatively high for a remote area, such as the Azores. Yet, its proximity to the North Atlantic Subtropical Gyre (NASG), an area of accumulation of microplastics, may explain such elevated frequency of ingestion as already been reported in turtles (Pham *et al*. 2017). Similar results were found by Boerger *et al*. (2010) in the North Pacific Subtropical Gyre (NPSG), that reported 35% of ingestion in mesopelagic fish. However, these results are contrasting to another study in NPSG that reported a frequency of ingestion of 9.2% (Davidson and Ash, 2011). Both studies reported ingestion in mesopelagic fish and the difference in results may accrue from longer tow time that result in net feeding and consequently higher frequency of ingestion (Davidson and Ash, 2011). Since none of the fish sampled in this study was captured using tow net, the frequency of ingestion reported is not due net feeding event. In the present study 1 µm mesh filters used permitted to identify all ingested particles larger than 1 µm and consequently increased the number of particles detected. This may explain the difference to the results of Lusher *et al*. (2016) that reported 11% of ingestion in North Atlantic mesopelagic fish using 250 µm mesh filter. In the North Atlantic, Wieczorek *et al*. (2018) reported 73% of frequency of ingestion in mesopelagic fish. However, the targeting species and sampling area was different from the present study and that may explain the frequency of ingestion. Some studies in coastal areas of East North Atlantic also reported an ingestion rate near to 38% (Lusher *et al*., 2013; Bellas *et al*., 2016).

The three species analysed in the study are rather ecological different. Blackbelly rosefish is bathy demersal species that feed in both pelagic and benthic organisms. Skipjack tuna is a larger pelagic predator that feed on other pelagic fish. While Blue jack mackerel is benthopelagic species that feeds mostly on copepods (Deudero, 2001), this species is at the base of the trophic net in the Azores being one of the most important commercial species in both Azores and Portugal mainland. The presence of debris in Skipjack tuna and Blackbelly rosefish is the first register of plastic ingestion in these species. Frequency of occurrence for both species was elevated: Skipjack tuna (44%) and Blackbelly rosefish (32.08%). Although, most of the items are needed to be confirmed through FT-IR, we have little doubts for the larger fragments encountered in the skipjack tuna. Ingestion of plastic was previously investigated for the Blackbelly rosefish in the Mediterranean Sea (Anastasopoulou *et al*., 2013) and in Lisbon costal region (Neves *et al*., 2015) yet no plastic was found. In the Mediterranean Sea over 300 individuals were analysed (Anastasopoulou *et al*., 2013), against only 1 individual sampled in Lisbon region (Neves *et al*., 2015). The difference between the frequency of ingestion found in this study to the preview studies of plastic ingestion in Blackbelly rosefish may be due to the area itself. The proximity of Azores to NASG, area with high accumulation of microplastics may be the factor that cause these differences between results preview studies with the frequency of ingestion found in this study. Skipjack tuna has been analysed before in the South West Pacific also without any trace of plastic ingestion (Rochman *et al*., 2015; Cannon *et al*., 2016). However, for this species the sampling size was lower than 10 individuals which can explain the absence of detection in the other studies. While for Blue jack mackerel the 40% of frequency of ingestion was much higher than the 3% previously reported by Neves *et al*. (2016) in Lisbon region for a total sample of 29 individuals.

Overall, the abundance rate of ingestion was 0.58 ± 0.95 pieces per individual yet the intensity rate of ingestion was 1.5 ± 0.98 pieces per individual. While the abundance reports the quantity of ingested particle by all individuals, including those that have not ingested plastic, the

intensity of ingestion reports how many pieces are ingested in average by those who ingested plastic. Intensity is commonly used as comparison between studies of plastic ingestion in fish. Most of studies of plastic ingestion by fish reported values of intensity of ingestion between 1.2 and 1.9 (Lusher *et al*., 2013; Neves *et al*., 2015; Rochman *et al*., 2015; Bellas *et al*., 2016; Lusher *et al*., 2016). Blackbelly rosefish had a statistically significant higher intensity rate of ingestion than the other species. This means that individuals of Blackbelly rosefish in Azores region do not ingest plastic more frequently than the other species, but when ingesting, they ingest more pieces.

Out of the 90 pieces encountered within the fishes, around 60% were classified as fragments and 40% as fibres. However, to confirm if the particles are in fact synthetic polymers is essential to proceed to the FT-IR method. This method essential to confirm and identify the type of polymer in microplastics (Lusher *et al*., 2013; Cannon *et al*., 2016).

Despite the slightly higher percentage of ingestion of fragments, the difference between ingestion of fibres and fragments were not statistically significant, and consequently no preference in ingestion according to type for any species. Blackbelly rosefish ingested with more abundance fragments than the other species and also than fibres. Same results were found for intensity of ingestion. Despite the clear difference in ingestion of fragments than fibres, it was not significant and consequently no preference of ingestion was found. This higher number of fragments found in Blackbelly rosefish may be due to their depth range, which is deeper than the other species, and the consequent sedimentation of plastic fragments over time (Woodall *et al*., 2014). To confirm this hypothesis is necessary to have water samples of the fishing ground where these individuals were caught. Only comparing the water samples with the stomatal contents is possible to confirm if the higher frequency of plastic ingestion is due to its abundance in at that are or it comes from another source such as secondary ingestion from prey. As for the other species the abundance and intensity of ingestion was similar for both fragments and fibres and between them. Blue jack mackerel and Skipjack tuna are ingesting plastic with relatively same intensity independently of its type.

In general fragments found within the three species were smaller size, being its majority smaller than 0.2 mm. Fibres were in general between 1 and 2 mm. All microplastic particles are smaller than natural prey of these species, so it is unlikely that any of these species mistake plastic as food source. Studies also reported that processes of bioaccumulation and biomagnification of plastic are not the main routes of ingestion (Güven *et al*., 2017; Peters *et al*., 2017). Despite the size of particles ingested, the colours of the particles were more commonly green, black and blue. These colours evidence that its ingestion was probably due to accident than other cause. Skipjack tuna was the species that ingested more particles with larger size, despite the mean size of particles ingested being very similar to the other species. This species was also the only to be found with macroplastics. This fact may due to its ecological feeding depth being situated at more superficial water which are known to accumulate large size polymer particles (Eriksen *et al*., 2013; Eriksen *et al*., 2014).

The sampling size per species used in this study followed EU guidelines for plastic ingestion in fish established that minimum sample size per species must be 50 individuals (MSFD GES Technical Subgroup on Marine Litter, 2013). Respecting the minimum sampling size, results obtained must representative of the sampled population in the region, factor that contributes

to the confidence in the quality of the results obtained. Adding to the minimum sample size, reducing the variability in plastic ingestion due to length size was accomplished with a pre-selected length range for sampling. Size is related to age classes and diet within a species differ according to age class in some cases, which may cause variability in results of plastic ingestion that can be minimized reducing the length range (Peter *et al*., 2017). However, the difference between lower and higher sampling length limit size was dependent on the total number of individuals existing on the database. Nevertheless, the overall differences in length are likely not to be relevant since differences in size within individuals were minimized and for the length size selected was guaranteed no overlap in life stages (juveniles with adults). Taking in account the length ranged used in the present study it is believed that the potential variability caused by length differences was overcome.

All particles suspected to be polymers were doubled checked with the polymers found in the blank controls and not consider in the analysis if some similarity was found. This measure may underestimate the real frequency of ingestion but at least all the particles consider were with high certainty not contamination. It was considered preferable to underestimate the real quantity than count particles that came from airborne or water source during the analysis.

Airborne contamination was not the major factor of contamination during all steps of laboratory work. Yet water tests revealed high quantity of fibres, even after a pre-filtration with a 20 µm mesh. An evaluation to the recipient used between filtrations also was revealed to be a potential source of fibre contamination. The only system of filtration that guaranteed quality and absence of presence of fibres as contamination was filtering the water twice through a 20 µm mesh and use between filtrations a glass recipient washed previously with the twice filtered water.

Conclusion

The present study reports preliminary results on fish ingestion in the Azores region and a frequency of ingestion of 38,71%. However, this necessary to sample more fish species to establish a more accurate index of fish ingestion in Azores region. It was the first study aiming to report plastic ingestion in commercial species operating in this region and the first study reporting plastic ingestion in Blackbelly rosefish and Skipjack tuna. The frequency of ingestion by Blue jack mackerel (40%) was much higher than the 3% previously reported by Neves *et al*. (2016).

Plastic ingestion by these species is likely to be accidental which is explain the small size of particles found, most were smaller than 1 mm, and equal preference according to its type. It is essential in future studies to try to understand with more precision how fish ingest plastic and its physiologic consequences.

The present study also aimed to improve the analysis protocol, testing the efficacy of each step of analyses and reduce as much as possible any possible sources of contamination. Future studies must follow a protocol with the improvements obtain in this study.

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