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Tsveteslava Ignatova-Ivanova *, Stephany Toschkova, Sevginar Ibryamova, Darina Bachvarova, Teodora Koynova, Elitca Stanachkova, Radoslav Ivanov, Nikolay Natchev, Georgi Kolev

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The Assessment of the Bioaccumulation of Microplastics in Key Fish Species from the Bulgarian Aquatory of the Black Sea

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Abstract: One of the main problems of the world's oceans, reported by many scientific studies, is microplastic pollution. The Black Sea is one of the main sources of pollution, which is mainly caused by the anthropogenic factor.The present study demonstrated detailed MPs contamination of five fish species important for the commercial fishing (Garfish, Mullet, Knout goby, Pontic shad and Mediterranean horse mackerel) collected from the Sozopol area on the Bulgarian Black Sea coast. Within each microplastic morphological group, three size classes were recognized: 100-200 μm, 25-100 μm, and ≤ 25 μm. Microplastics were found in all parts of the fish, but in varying proportions of pellets, fibers and fragments. Pellets were most frequently isolated, followed by irregularly shaped fragments, and fibers were the least numerous. The bulk of insulated plastics are made of polyethylene (PE) and polyethylene terephthalate (PET). Our results pointed out serious pollution with plastic particles in the Bulgarian Black Sea aquatory, which in the future may seriously affect the health of the fish population and also human health.

Keywords: ocean; pollution; anthropogenic pressure; bivalves; food resources; sea water

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Introduction

The pollution of the world oceans by plastic waste has been reported in many scientific papers in recent years. There ape scientific evidencec of plastic particles detected even in the polar regions [1]. Plastics, as water-insoluble solid polymers, are widely distributed and used in a wide range of industries due to many advantages - e.g., flexibility, rigidity, temperature resistance, and chemical stability [2]. It is reported that the annual share of plastic production has reached 280 million tons. A number of scientific papers have reported the amount of microplastic particles (MPs, <5 mm) in seawater and sediments to be around 4.8 and 12.7 million tonnes [3,4]. These particles impact life in the seas and oceans. Microplastics (MPs) have recently been defined as " a synthetic solid particle or polymer matrix of industrial origin, not soluble in water, of regular or irregular shape and measuring between 1 μm and 5 mm " [5]. Plastics are categorized into three groups (macro (>100 mm), meso (1–10 mm), and micro (1–1000 mm). MPs can be produced as microparticles for personal care and cleaning products or occure due to the fragmentation of macroplastics in aquatic ecosystems by environmental factors such as UV radiation and wave abrasion [6]. The main sources of microplastic particles are the plastic bags, bottles and straws that are widely used. They are not biodegradable and remain in the environment for hundreds of years [7]. For this reason, the fight against microplastic pollution is one of the main environmental priorities of the United Nations Environment Program [8]. Microplastic particles have been reported in the bodies of mammals, shellfish, fish, birds and decapods, particularly in the Northern Hemisphere. Entering the organisms of bivalves and fish through the food, microplastics cause disturbances and damage - from oxidative stress to behavioral disturbances. Other negative impacts, such as decline in the swimming capacity in fish, reduction of reproduction, inhibition of growth and others were reported [9,10,11]. Once inside living organisms, microplastic particles have the ability to absorb and release toxic chemicals/organic or inorganic additives such as bisphenol A, PCBs and DDT, which creates additional potential risks to human health. On the other hand, microbial biofilms can form on microplastic particles, which contain microbial pathogens and have an adverse effect on animals and humans [12,13,14]. The pollution of the Black Sea, known as one of the most degraded marine ecosystems according to BSC (2007)[15], makes this semi-enclosed sea more vulnerable to microplastic pollution [16]. On the other hand, the estuaries of the Danube, Dnieper, Dniester and Don rivers further pollute the Black Sea with plastic waste (BSC, 2009)[17]. In their work, Lechner et al. (2014) [18] report that 4.2 tonnes of plastic/day reach the Black Sea via the Danube River. The Black Sea is surrounded by several industrialized countries and is an important places for both small-scale and large-scale fisheries (BSC,2007)[15]. There are several studies in the region on macroscale plastics and they showed a large amount of macroplastic pollution along the Black Sea coasts [19,20,21,22,23,24]. So far, more than 890 fish species have been found to ingest MPs, and the abundance of MPs has been reported to be highly variable among different fish species [25]. In addition, out of a total of 323 fish species found to ingest MPs in different regions of the world, 262 were reported as commercial species. In the modern world, people are increasingly interested in a healthy lifestyle and healthy eating. Fish has been one of the staple foods for humans for decades. In many cases, it is recommended as a healthy food and source of very useful omega fatty acids, vitamins and minerals. On the other hand, for many people living on the Black Sea coast, fishing is the main way of livelihood. There is data on the impact that microplastic particles have on the health of marine life, but there is no data on what microplastics would do to the human body and how this would affect human health and the health of our children in the future. Popular science films have recently emerged that clearly show that people who eat seafood have circulating microplastics in their blood.There is no data on studies from the Bulgarian Black Sea water area regarding the condition and amount of microplastic particles in fish. This is the first pilot study on the composition of microplastic particles in different parts of key fish species from the Bulgarian Black Sea water area.

Materials and Methods

Sampling Place and duration of the study:

The study was conducted at the Department of Biology, University of Shumen, Bulgaria. Probe from different anatomical structures were sampeled in Garfish (*Belone belone*), Mullet (*Mugil cephalus*), Knout goby (*Mesogobius batrachocephalus*), Pontic shad (*Alosa immaculata*) and Mediterranean horse mackerel (*Trachurus mediterraneus*) catched in the region of Sozopol in February 2022.

Collection of the samples

After catching, the fish was immediately transported to the laboratory at 4°C, where it was dissected. Samples were taken from the skin, musculature, gills, intestinal tract and caviar and these probes were subjected to analysis. The probes were obtained from three specimens of each fish species, which were simmilar in size.

Tissue digestion and microscopic inspection

Tissues obtained from fish were minced according to [26]. To prevent contamination with microplastics, work only with glass and metal tools. All tools used are rinsed with bidistilled water before use. Similarly, the reagents used in the analysis are tested for the presence of microplastics using the black sample method. After cutting each sample, five ml of 1M NaOH was added to it and heated to 50°C for 15 minutes. The temperature is controlled at all times. 17.5 ml of HNO3 (49%) and 2.5 ml of ultrapure water were added to the NaOH-treated sample. This was followed by reheating to 50°C for 15 minutes, with the temperature again controlled. The whole sample is heated for 15 minutes, but already at the temperature up to 80°C, which aims to remove the remaining suspended solids from the sample. The next step is filtration, after which the samples are diluted 1:2 (v:v) with ultrapure water heated to 80°C. Filtration was performed through a cellulose nitrate filter (Ø 47 mm, pore size 8 µm, Sartorius Stedim Biotech, Goettingen, Germany). The glassware used in the experiment is also filtered and washed. Nitrocellulose filters were dried for 24 h at 37°C and then analyzed for MPs. For microplastics identification, the filters were visually observed under a stereomicroscope SZM-D (OPTIKA Italy, 1000x magnification) coupled with Dino-Eye AM4023X eyepiece camera (Dino-Lite ANMO Electronics, Taiwan). The digital images were examined by using "DinoCapture 2.0" software and the plastic particles were quantified by size (≤ 25 µm, 25-100 µm, and 100-200 ìm) based on their largest cross section and shape (pellets, fibers and fragments).

Polymer identification

To identify the polymer we used FTIR spectroscopy according to [27]. All results were done in triplicate.

Results

A total of three specimen from each species were analyzed for the presence of microplastics. In all tables averaged results from the three samples were plotted. The presence of microplastics was detected in 100% of the specimens. The microplastic particles ranged from 10 to 40 particles per individual (Figure 1 and Tables 1–4).

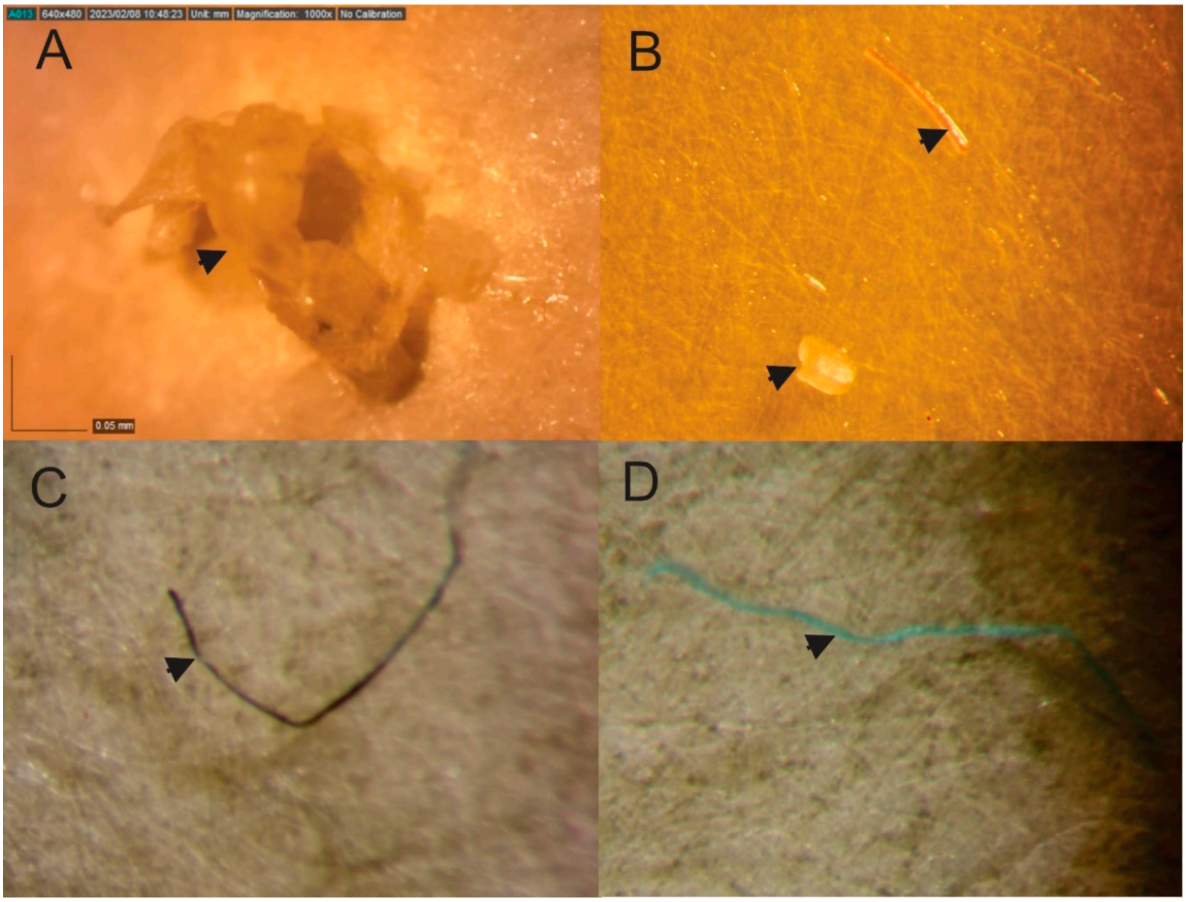

Figure 1. Stereomicroscope picture of morphological types of microplastics (arrowheads) recognized in the studied specied from: A) *B. belone*; B) *M. batrachocephalus* C) *A. immaculata* and D) *T. m. ponticus*.

Table 1. Number of microplastic particles in samples from *B. belone.*

species				*Belone belone*					
form of MPs		pellets			fibers			Irregular form	
Specimen	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm
skin	0	0	0	0	0	0	9	2	0
meat	3	0	0	0	0	0	2	0	0
gills	23	0	0	21	0	0	2	0	0
GI tract	0	0	0	0	0	0	6	0	0
caviar	10	0	0	0	0	0	2	0	0

Table 2. Number of microplastic particles in samples from *M. cephalus.*

species				*Mugil cephalus*					
form of MPs		pellets			fibers			Irregular form	
Specimen	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm
skin	5	0	0	1	0	0	1	0	0
meat	0	0	1	1	0	0	9	2	0
gills	8	0	2	0	0	0	2	0	0
GI tract	30	0	2	1	0	0	3	0	0
caviar	0	0	0	0	0	0	0	0	0

Table 3. Number of microplastic particles in samples from *M. batrachocephalus.*

species				*Mesogobius batrachocephalus*					
form of MPs		pellets			fibers			Irregular form	
Specimen	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm
skin	9	0	0	1	0	0	5	1	0
meat	2	0	0	3	0	0	5	0	0
gills	3	0	0	0	1	0	2	1	0
GI tract	4	0	0	0	0	0	1	0	0
caviar	0	0	0	0	0	0	0	0	0

Table 4. Number of microplastic particles in samples from *A. immaculata.*

species				*Alosa immaculata*					
form of MPs		pellets			fibers			Irregular form	
Specimen	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm
skin	2	0	0	0	3	0	0	0	0
meat	9	0	0	0	0	0	5	1	0
gills	2	0	0	20	1	0	0	0	0
GI tract	0	0	0	0	0	0	0	0	0
caviar	0	0	0	0	0	0	0	0	0

The microscopic pictures in Figure 2 demostrate the types of MP particles were isolated - irregularly shaped (Figure 2A), pellets (Figure 2B) and fibres (Figure 2C, D), and also different color, which is probably due of the various impurities and trace elements used by the manufacturer for the specific type of microplastic.

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The data in Table 1 showed that in the Garfish, the pellets with sizes ≤ 25 μm isolated from the gills, muscles and skin of the fish were predominant. Fibers of the same size ≤ 25 μm were isolated from the gills only. Irregular formed MPs were isolated in all parts of the fish, most notably from the skin and gastrointestinal tract. As in the skin, particles with larger sizes (25-100 μm) were also found (Figure 1 A). MPs with sizes of 100–200 μm were not isolated.

In the Mullet (Table 2), prevailed the pellets with sizes ≤ 25 μm. They were isolated mostly from in the gastrointestinal tract, gills and skin. Pellets 100-200 μm in size were detected in the muscles, gills and the gastrointestinal tract. Fibers ≤ 25 μm in size were isolated from skin, the muscles and the gastrointestinal tract. Irregular formed MPs were isolated from all parts of the fish eccept the chaviar, but were most abundant in the muscles and the gastrointestinal tract. As In the muscles were detected particles with size of 25-100 μm.

From *M. batrachocephalus*, pellets with sizes ≤ 25 μm were isolated from the skin, gastrointestinal tract, gills and flesh of the fish prevailed (Table 3). Fibers ≤ 25 μm in size were isolated from muscles and the skin. Fibers 100-200 μm in size were also detected in the gills. Irregular formed MPs were isolated in all parts of the fish eccept the caviar, most abundantly in the muscles and the skin. In the skin and gills, larger particles of 25-100 μm were also found (Figure 1 C).

In *A. immaculata* predominated the pellets with sizes ≤ 25 μm and they were isolated from the skin and the gills of the fishes. Fibers ≤ 25 μm in size were isolated from the gills (Figure 1C). Fibers 25-100 μm in size were also isolated from the skin and gills. Irregularly shaped MPs were isolated from the muscles. Particles of larger size (25-100 μm) were also found

In *T. m. ponticus* prevailed pellets with sizes ≤ 25 μm. They were isolated from the gastrointestinal tract, the gills, the muscles and the skin of the fish. Fibers ≤ 25 μm and 25-100 μm in size were isolated from the gastrointestinal tract (Figure 1). In the gills and in the skin, larger particles of 25-100 μm were also found.

FTIR spectral analysis was performed to determine the nature of the isolated MPs. The obtained results are represented in Appendix A.

From the isolated microplastic particles, the following types were identified: LDPE – low-density polyethylene used in the production of plastic cups; PA – polyamide used in the production of cords.; PET – polyethylene terephthalate used in the production of bottles for soft drinks; PP – polypropylene used in the production of a shampoo bottle; PC – polystyrene/polystyrene used in the production of a CD case; EPS - expanded polystyrene used in the production of packaging and PVC - plasticized polyvinyl chloride.

Discussion

This study presents a detailed assessment of MPs contamination in five key fish species, caught in the southern part of the Bulgarian Black Sea water area. Every examined species was found to be contaminated with MPs. A study by [28] report on contamination with 95 MPs in three commercial fish species - *Engraulis encrasicolus*, *Merlangius merlangus* and *Mullus barbatus* from the Turkish Black Sea coast. The most contaminated with MPs was found the red mullet and the MPs were mainly in form of fibers. However, these authors did not dissect the fish into separate parts and it is not clear exactly in which part of the fish the respective microplastics were isolated. Polyethylene and polypropylene were the most dominant type of polymer. This is in correlation with our results, as we also isolated mostly polyethylene. In our study, mainly pellets were isolated, and fibers were in second place in quantity. We found the highest amount of MPs (80 particles) in *T. m. ponticus*. Neves et al. (2015) [29] found, that along the Portuguese coast the number of MPs per fish was higher, compared to the study of Eryaşar et.al. (2022) [28]. Compared to our results, however these are relativelly low numbers. Also lowerer number of MPs was reported in the analysis of the gastrointestinal tract in fishes from the Turkish Marmara, Aegean and Mediterranean coasts [30,31], the Spanish coast [32] and Portuguese coasts [33]. In our study, the most MPs were reported in the gastrointestinal tract of *M. cephalus* - 36 MPs particles. Differences in the number of MPs can be due, on the one hand, to differences in research methods that are used in scientific research, and on the other hand, to different degrees of seawater pollution [29,32,34,35]. In general, benthic fish species reported higher MPs content, which could be related to differences in fish feeding and behavior. In general, MPs in the seabed is quite high, due to the sedimentation and deposition of these particles there [36,37,38]. There are evidences that bottom dwelling fish species can ingest sediment in the process of feeding on their prey [39]. One of the main pathways for MPs to enter the gastrointestinal tract of fish, which may lead to higher levels of MPs uptake by benthic species, is the presence of MPs on the seabed [35,40]. In our study, however, a higher percentage of MPs was reported in pelagic fish species – Garfish and Mediterranean horse mackerel (Tables 1 and 5).

Table 5. Number of microplastic particles in samples from *T. m. ponticus.*

species			*Trachurus mediterraneus ponticus*						
form of MPs		pellets			fibers			Irregular form	
Specimen	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm	≤ 25 µm	25-100 µm	100-200 µm
skin	1	0	0	0	0	0	4	1	0
meat	8	0	0	0	0	0	3	0	0
gills	15	0	0	0	0	0	10	2	0
GI tract	20	0	0	14	2	0	5	0	0
caviar	0	0	0	0	0	0	0	0	0

Some of the isolated microplastic particles were shown to be polyamide (nylon fibers) (Appendix AB). They are characterized by spectra that are characterized by strong amide I-amide II spectral bands at 1650 and 1543 cm-1 and polyethylene terephthalate (polyester, PET) (Appendix AC) showing C= O with bands at 1725 cm-1, C–O with bands at 1250 and 1100 cm-1 (Veerasingam et al., 2020). Two of the analyzed fibers have a spectrum with a peak at 1729 cm-1 (Appendix AE, G). This peak is consistent with the C=O stretching mode of the acrylates. The absence of a nitrile band (2237 cm-1) allowed us to reject polyacrylonitrile as a component of the polymer matrix. The plain translucent fiber presents a spectrum similar to that of siloxanes, characterized by peaks at 1030–1065 cm-1 (Si-O-Si stretch) and 1280 cm-1 (Si-CH3 strain) (Appendix AC). This type of material is commonly used to make domestic and marine sealants.The majority of isolated microplastic particles mainly contain polyethylene (PE) and polyethylene terephthalate. Polyethylene is used and is included in the composition of plastic bottles, cups, stirrers and plastic bags. This polymer is very light and floats on the surface of the sea because its density is lower than that of water. Polyethylene terephthalate, on the other hand, is denser than water and is more likely to sink and accumulate in it and benthic organisms.These polymers are widely used in fabrics, in nets, ropes and strings used for fishing - one of the main economic activities of the Black Sea. The predominant types of polymers - the PE, corresponds to the content of manufactured plastics everywhere in Europe, as almost half of the plastics produced in Europe are reported as PE [7]. The sinking of plastics and sedimentation is related to the fact that the upper layer of the Black Sea is less dense than other seas. On the other hand, the weight of these particles increases due to the accumulation of marine plants and nutrients on them, and this can affect the distribution of plastics and their sedimentation on the seabed [41]. In studies on the black mussel was reporeted, that these polymers are dominant in the mussels [42].

Regarding fish health, it has been reported that plastics <1000 µm in size can reach the digestive tract or the gills, and in turn can cause adverse effects such as a weak immune response and reduced fertility [43,44,45]. No particles larger than 100 µm were found in our study. Considering the wide variety of types of MPs detected in the digestive tract, we assumed, that the fish regularly ingest the MP during feeding. Some researchers reported, that the main mode of MPs ingestion is not by misidentifying these particles as prey, but fishes passively consume MPs during the feeding process [35, 46]. Many nutrients are also adsorbed on the plastic particles which fool the fish that it is food, it can be assumed that the fish do not recognize the microplastic but identify it as food. Bowen et al. (2021)[25] showed that fish ingest MPs inadvertently rather than intentionally. The authors found that fishes did not actively ingest microfibers and instead, the MPs were passively absorbed by breathing. Fish have also been reported to exhibit fiber rejection behavior by expelling them out when

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mixed with mucus [25]. MPs can accumulate in the predatory fish species, unfortunatly very limited research was performed on bioaccumulation and biomagnification in food webs [38], therefore more studies are needed to reach this conclusion.

MPs enter food chains and threaten aquatic ecosystems through their ability to transport pollutants, pathogenic microorganisms and alien invasive species. They have the ability to absorb and release toxic chemicals of organic and inorganic origin such as bisphenol A, PCBs and DDT, creating an additional potential risk to human health. Humans are exposed to BPA in the environment they live in from the air we breathe, the food and drinks we consume, etc. So, even if BPA intake is below some accepted limits, this does not guarantee that the additive will not accumulate and cause more pronounced effects and chronic toxicity in the food chain, given the tendency to accumulate. As a result of the results obtained and the amount and type of polymer obtained in the study and the literature, the source of contamination in our opinion can be mainly attributed to the domestic wastewater discharges coming from the washing of synthetic fabrics. However, detailed studies are needed to prove this. In Bulgaria, wastewater is discharged directly into marine ecosystems, as is the case in other neighboring countries along the Black Sea coast. Given that the number of discharges would increase following population growth, the abundance of MP in the Black Sea is expected to increase proportionally. For this reason, all countries along the Black Sea coast must use modern wastewater treatment systems. There is also a need to take preventive and remedial measures internationally and locally to assess the toxicity of available polymers, by recycling and reducing plastics, using alternative materials and using sustainable practices to better manage plastic pollution and waste. Our results show a wide variety of micropollutants of MPs originating from the commonly used plastic cups, stirrers, bags, soft drink bottles, fishing nets, packaging of hygiene and personal hygiene preparations and others that have systematically entered the Black sea and are degraded into microplastic particles. The present study demonstrated the MPs contamination of five commercial fish species from Black Sea. A higher abundance of MPs was found in pelagic species. This may reflect the presence of more MPs in the surface layers of the Black Sea and the feeding behavior of fish. It is important future research to determine the toxicological side effects of plastic ingestion for fish.

The United Nations Environment Program (UNEP) has found relatively high levels of the substance, (SVHCs), which gives rise to serious concern. It has been found in plastics through research and is contained in some plastic staples such as textiles, clothing and magazines. Therefore, in addition to the direct physical effect of microplastics, they can cause the accumulation of intermediate and persistent chemicals and the bioaccumulation of toxic substances in organisms. On the other hand, biofilms accumulated on microplastics can be sources of microbial pathogens, affecting the health of fish and, respectively, humans as consumers. The entanglement and ingestion of various types of microplastics by aquatic organisms, especially fish, can block the gastrointestinal tract, and other pollutants can also enter their bodies. According to a report on the relationship between biological impacts and marine litter, more than 80% of the litter available is plastic, of which 11% is microplastic. More than 250 marine species are exposed to plastic in aquatic systems. Microplastics are the same size as sediment and planktonic organisms, so they can be used for food by many aquatic organisms. As a result, organisms can accumulate them in their body and this can lead to physical effects, such as toxic effects from pollutants in microplastic components. Due to the small size of the microplastic, it is not possible to remove it them from habitats. However, even if you stop introducing plastics into water system, the number of microplastics can continue increase due to the breakdown of larger plastics in environment, so fundamental measurement and subsequently reducing the plastic input is the best method.

This study shows the need to carry out further studies and characterization of microplastics using different types of microscopic and spectral analysis. Even though microplastics may not pose a risk to humans who consume fish, these contaminants pose a potential risk to marine food webs and endangered species. We found particles of different sizes, types and colors in different fish species. We believe that the variability of polymer species in fish can reveal/indicate the polymer species in water to some extent. Our results show that fish are important as ecological bioindicators and serve as a basis for future studies on microplastic pollution in tourist sandy beaches.

Author Contributions: Ts. I-I and N.N. conceived and designed the study. N.N., G.K. and R.I. obtained the samples. T.K. performed the fish dissection. Ts. I-I and N.N supervised the data analysis and wrote the manuscript. S.T., S.I., E.S., and D.B. performed the testing and contributed to data analyzes and summaries. All authors have read and agreed to the published version of the manuscript.

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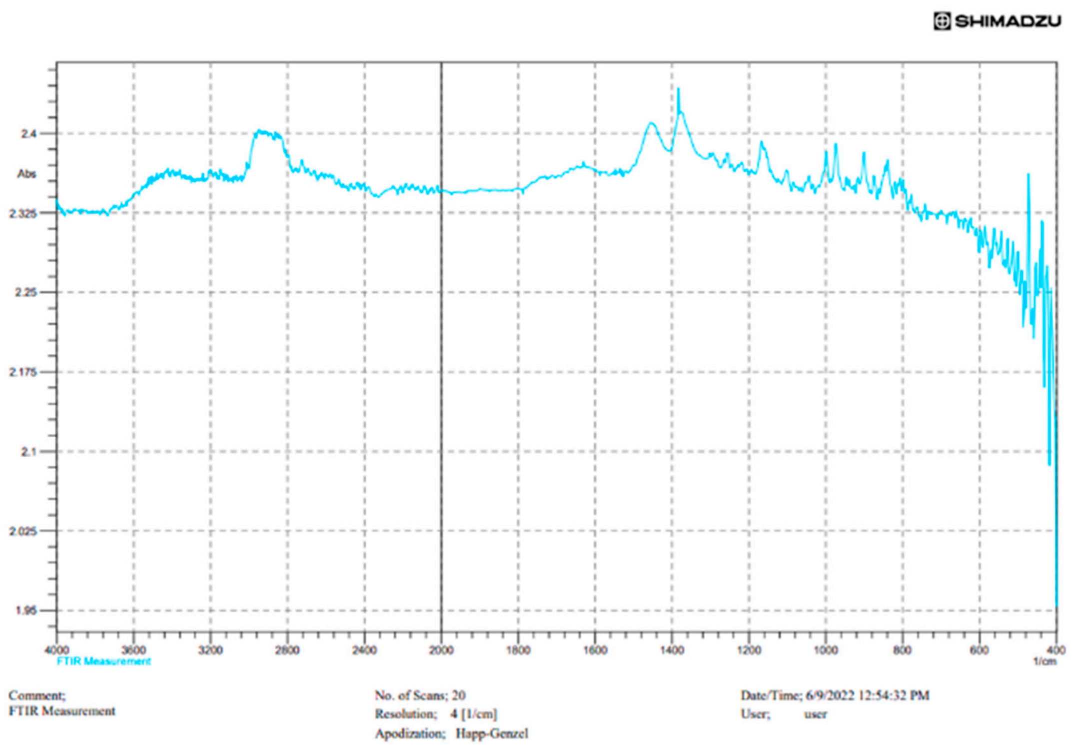
Conflicts of Interest: The authors declare that they have no competing interests.

Appendix A

FTIR spectrum of the microplastic particles isolated from the Black mussel: A) **LDPE** – low density polyethylene; B) PA – polyamide.; C) PET – polyethylene terephthalate; D) PP – polypropylene; E/ PC – polystyrene/polystyrene; F) EPS - expanded polystyrene; G) PVC - plasticized polyvinyl chloride spectrum.

A



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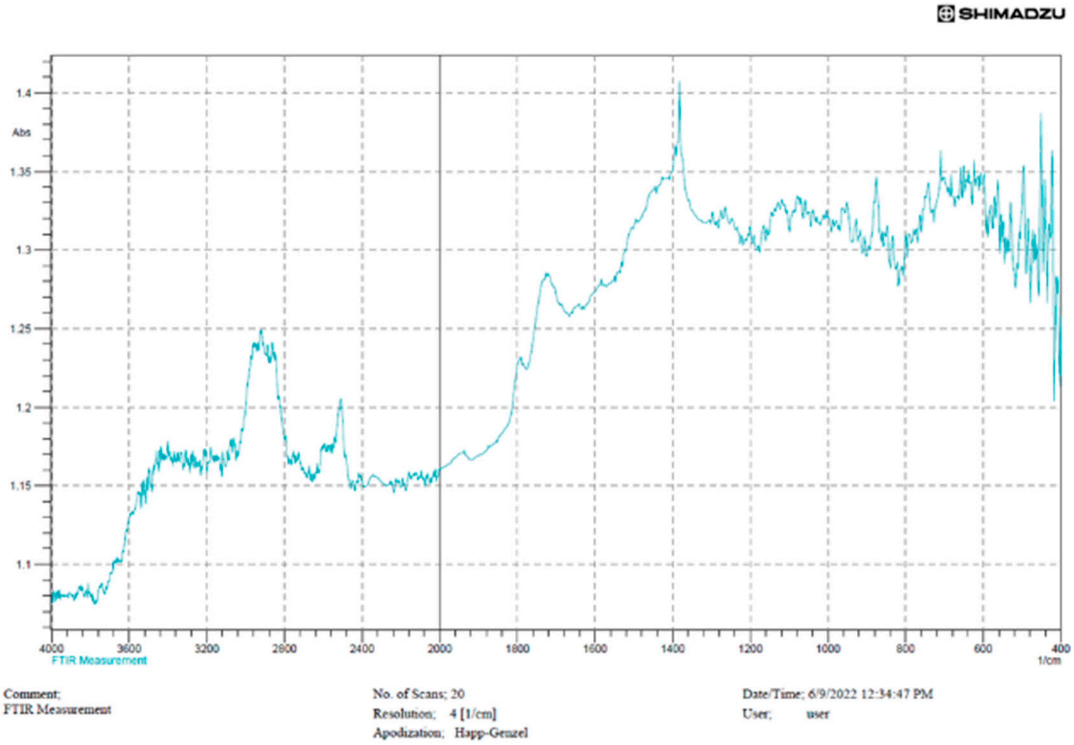
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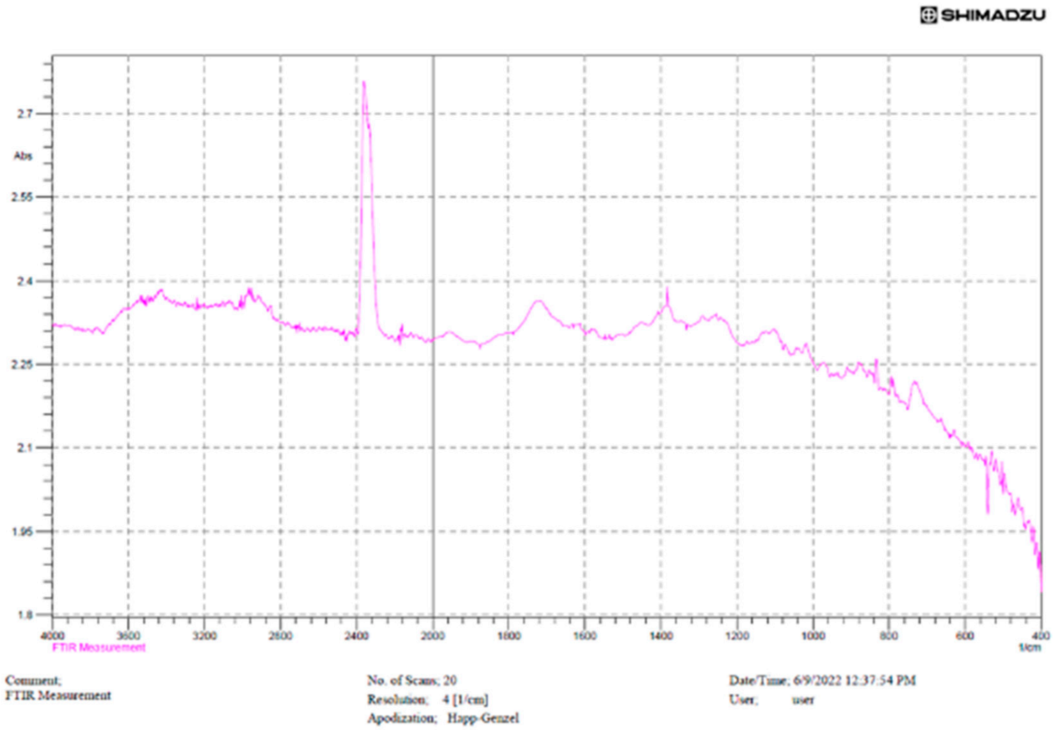
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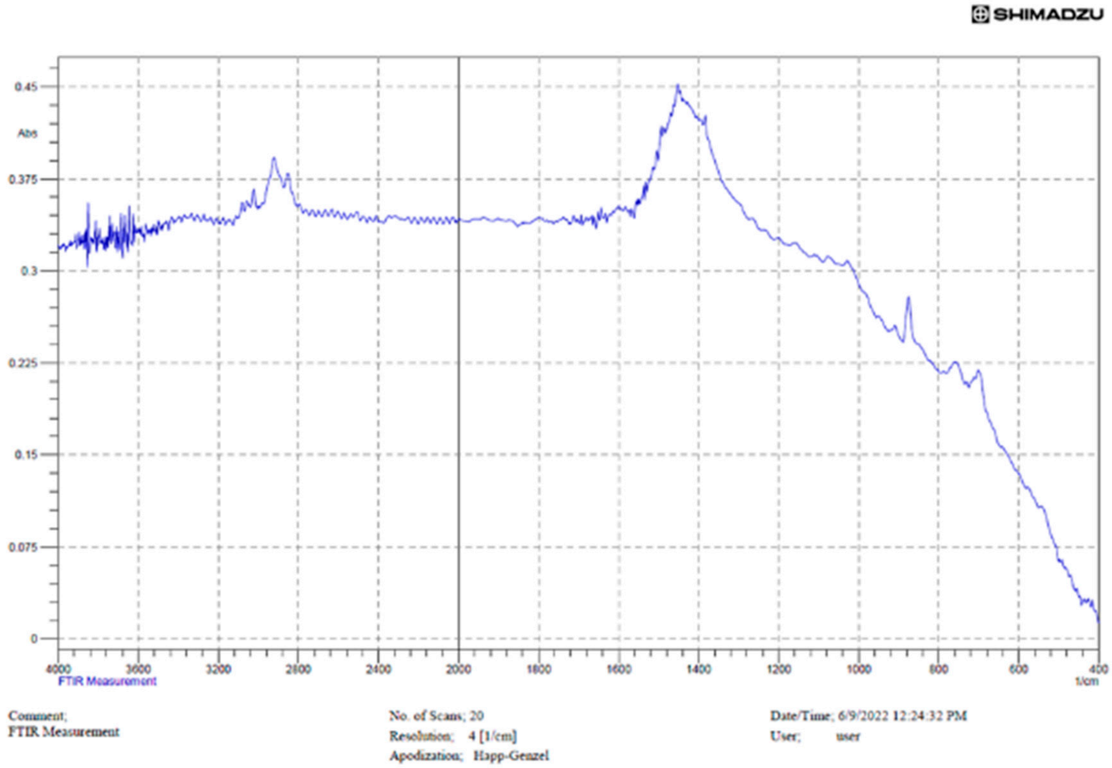
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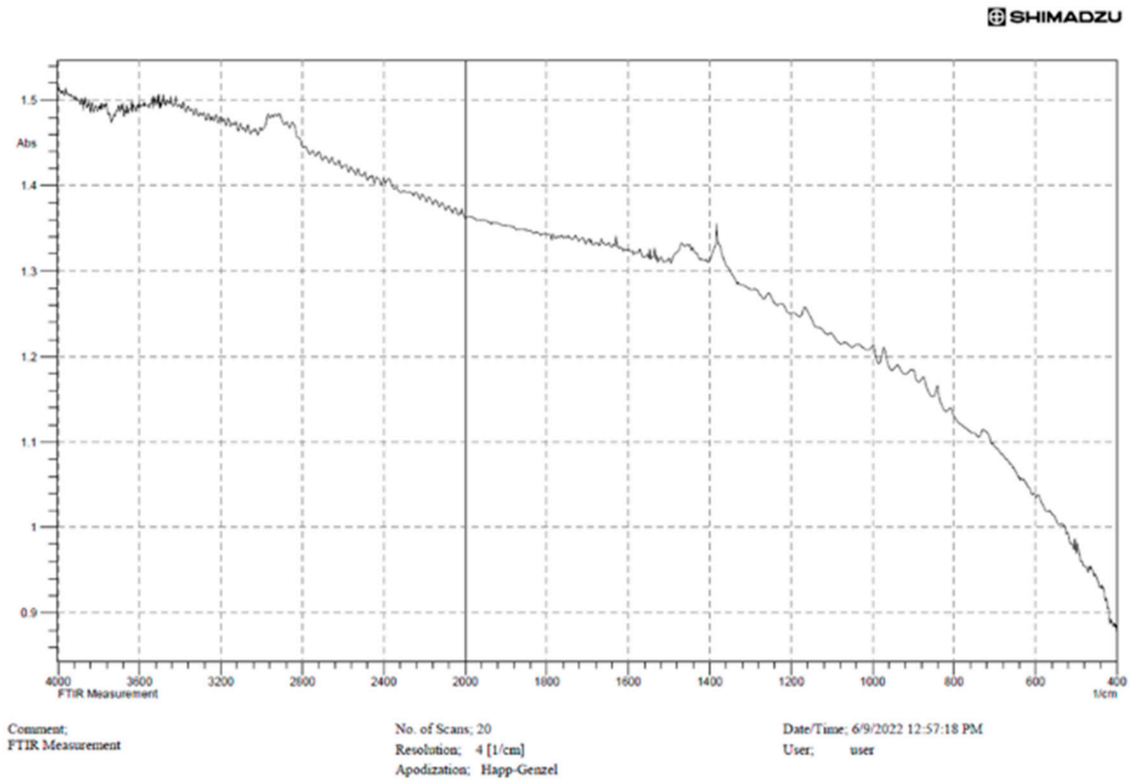
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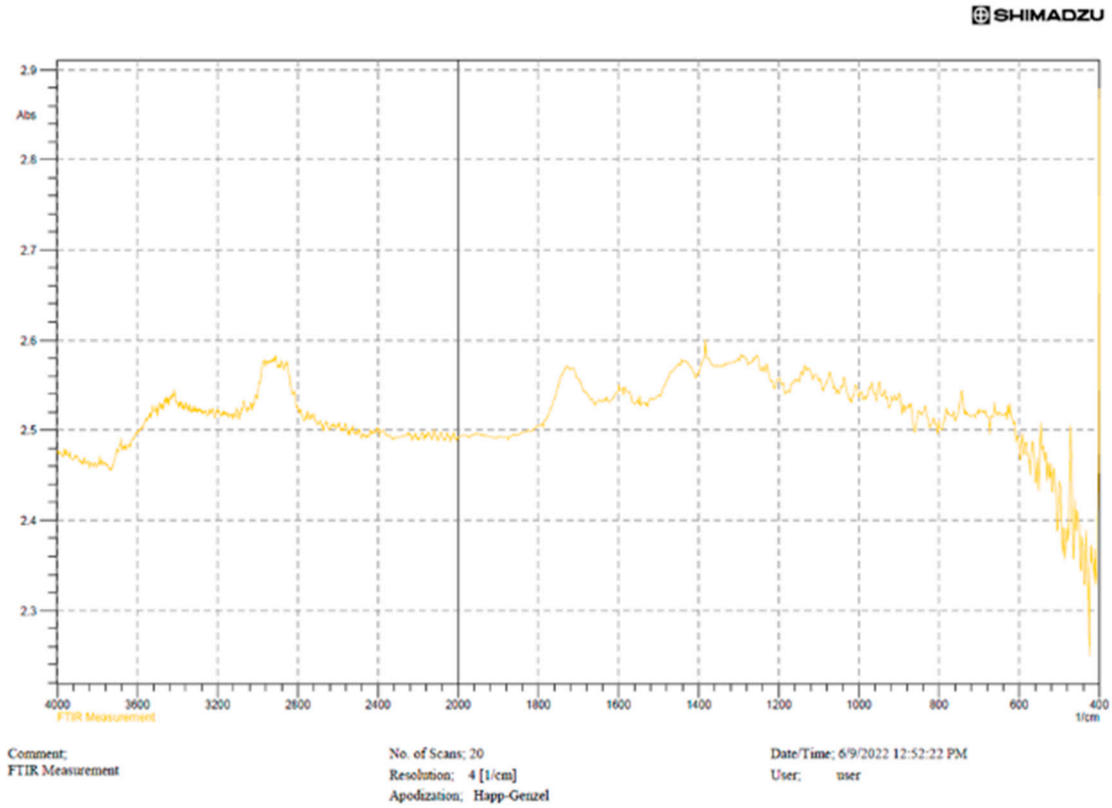

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