Preliminary survey on the occurrence of microplastics in bivalve mollusks marketed in Apulian fish markets

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Abstract

Microplastics (MPs) are a relevant threat to food safety because they are ingested by humans through various foods. Bivalves are at high risk of microplastic contamination due to their filter-feeding mechanism and pose a risk to consumers as they are ingested whole. In this work, microplastics were detected, quantified, identified, and classified in samples of mussels (*Mytilus galloprovincialis*) and oysters (*Crassostrea gigas*) marketed in the Apulia region. The total number of plastic debris was 789 particles in the mussel samples and 270 particles in the oyster samples, with size ranging from 10 to 7350 µm. Fragments with size within the category of 5-500 µm were the predominant findings in both species, with blue as the predominant color in mussels and transparent in oysters; most of the debris was polyamide and nylon polymers in the mussels and chlorinated polypropylene in the oysters. These results show that mussel and oyster samples purchased at fish markets are contaminated with microplastics. The sources may be diverse and further studies are needed to assess the impact of the marketing stage on microplastic contamination in bivalves to better define the human risk assessment associated with microplastic exposure from bivalves consumption.

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Introduction

Plastics were first introduced in the 1950s. They are used in many industries and contribute to food safety by extending the shelf-life of food, thus reducing food waste. Since then, the flip side of the coin has become apparent: plastic waste accumulates in the environment.

Due to mechanical, physical, and microbiological mechanisms, plastic debris (meso and macroplastics) fragments in the aquatic environment resulting in the formation of microplastics (<5mm). These particles are usually referred to as secondary microplastics, while primary microplastics (MPs) come from intentional production, such as virgin plastic pellets, scrubbers, and microbeads used in personal care (Prata *et al.*, 2021).

Due to their small size and high persistence in the environment, MPs can spread in both terrestrial and marine ecosystems and thus be ingested by a wide range of marine organisms, including fish and crustaceans associated with seafood intended for human consumption, which may mistake plastic particles for their prey (Van Cauwenberghe and Janssen, 2014; Wang D *et al.*, 2021).

Bivalves deserve a particular focus since they filter high volumes of seawater through the filter-feeding mechanism, accumulating MPs in the organism (Ward *et al.*, 2019). Therefore, they represent a route of exposure to MPs for humans, considering that they are consumed without gut removal and their consumption is high worldwide (Li *et al.*, 2018, Cho *et al.*, 2019).

Several studies documented the occurrence of MPs in a variety of bivalve species from Asia to the Americas, such as the US, where polyethylene terephthalate (PET), acrylic, and cellophane microfibers were detected in oysters (*Crassostrea gigas*) and razor clams (*Siliqua patula*) (Baechler *et al.*, 2019); in China PET, polypropylene (PP), polyethylene, polyamide (PA) and polystyrene fibers have been detected in scallops (*Chlamys nobilis*), mussels (*Perna viridis*), oysters (*Crassostrea hongkongensis*) and clams (*Paphia undulata*, *Ruditapes philippinarum, Meretrix meretrix*), with higher abundance in clams (Li *et al.*, 2022). In Thailand, Ta *et al.* (2022) studied blood cockles (*Tegillarca granosa*) and green mussels (*Perna viridis*), both of which showed contamination with plastic fragments and fibers. In Europe, according to the literature, there are only a few species studied so far, but it has been observed that contamination of bivalves with MPs shows a site-related rather than a species-related influence (Hermabessiere *et al.*, 2019). MPs thus enter the food chain and, together with airborne plastic particles inhaled, pose a threat to public health; recently, their occurrence has been detected in human blood, pla-

centa, and lungs. Nevertheless, very little data on human exposure to MPs exist to date (Barboza *et al.*, 2018; Amato-Lourenço *et al.*, 2021; Ragusa *et al.*, 2021; Leslie *et al.*, 2022). To assess the risk associated with the consumption of bivalves, a worldwide estimation of the quantity and quality of MPs in bivalves purchased in fish markets is very important, as they are the main source of these products for consumers (Bom and Sà, 2022).

In Europe, the three countries with the highest mussel consumption were Spain, France, and Italy. The last one recorded a household consumption of mussels of 42,750 tons, while about 4400 tons of oysters were consumed per year (EUMOFA, 2019; Cormac Coughlan, 2019). In the Apulia region, the average annual consumption of bivalve mollusks, especially mussels, clams, and oysters was 21,691, 236, 956 tons, respectively, in 2013; however, since 2016, it has been increasing in parallel with the domestic trend (Miedico *et al.*, 2013; EUMOFA, 2019). These products are mainly purchased from local fish markets that are widely spread all across the region, considering the high demand for fish and seafood. This is due to the fact that seafood and, in particular, bivalve mollusks are regularly used as main ingredients in a wide range of traditional recipes and their price is lower compared to northern Italian regions.

Although they are recurrent in the Apulian diet, data regarding MPs contamination of mussels and oysters supply in the aforementioned region are scarce to date. Therefore, this study aims to assess the occurrence of MPs in bivalve mollusks purchased in local fish markets in Bari and its neighboring, providing data that will help define the risk assessment related to bivalve mollusk consumption. In particular, samples of *Mytilus galloprovincialis* and *Crassostrea gigas* were analyzed, determining the number, size, shape, and color of the debris detected in each sample.

Characterization of the polymers of the potential plastic particles was performed using Fourier transform infrared spectroscopy in attenuated total reflectance mode (FTIR-ATR).

Materials and Methods

Sample collection and preparation

Three samples of mussels (*M. galloprovincialis*) (M1, M2, M3) and three samples of oysters (*C. gigas*) (O1, O2, O3) were collected from January to May 2022 from different fish markets in Bari and neighboring towns (Apulia region, Italy). Each sample from the same batch consisted of 60 individuals for mussels and 6 individuals for oysters. Each mussel sample was divided into three aliquots of 20 individuals and each oyster sample into three aliquots of two individuals. Analyses were repeated on each aliquot for a total of three replicates per sample. At the time of sampling, mussels and oysters were alive, refrigerated and labeled in net bags or unpacked. Information on the declared species, the fishing location of wild bivalve mollusks, and the origin of farmed bivalves were obtained from the labels provided.

To avoid microplastic contamination caused by transportation or other environmental factors, the collected samples were packed in aluminum foils, delivered to the laboratory in a refrigerated box at 4°C, and immediately analyzed. Each individual was rinsed 3 times with pre-filtered distilled water to remove sediment and debris. The length of the shells and the weight of the extracted soft tissue (wet weight) were recorded for each individual.

Quality control of the analysis

In order to avoid microplastic contamination, all liquids (distilled water, saline solution, and hydrogen peroxide) were filtered with a 1 µm pore size, 47 mm diameter cellulose nitrate filter membranes (Axiva Sichem Biotech, India) before use. All the equipment, containers, and beakers were rinsed three times with filtered distilled water and covered with aluminum foils before and after use to reduce contamination of the samples by airborne microplastics.

One blank extraction sample without tissue was performed simultaneously to identify and correct the potential procedural contamination.

Hydrogen peroxide treatment

Hydrogen peroxide 30% (1:20 p/v) was used to digest 10 g of soft tissue for each sample, in 500 mL flasks covered with aluminum foils. In this study, bivalve mollusk sample flasks were placed in an incubator at 65°C and oscillated at 80 rpm for 24 hours. Next, the digestion of the organic matter continued at room temperature for other 24-48 hours, depending on the status of the soft tissue (Li *et al.*, 2016). When no organic particles were visible in the solution, the digestion was considered completed.

Floatation and filtration

After the digestion of the oysters' soft tissue, the mixed solution was filtered directly through a cellulose nitrate membrane filter with a pore size of 5µm and a diameter of 47 mm (Axiva Sichem Biotech, India) using a vacuum pump (KNF Flodos AG, Svizzera) (Teng *et al.*, 2019). For mussels, a solution supersaturated with NaCl (1.2 g mL^{-1}) was used to separate microplastics from the mixed solution by flotation (Li *et al.*, 2016). Approximately 800 mL of filtered NaCl solution was added to each bottle, mixed, and incubated at room temperature overnight. The overlying water was filtered over 5 µm pore size, 47 mm diameter cellulose nitrate membrane filters (Axiva Sichem Biotech, INDIA) with Membrane-Laborpunpe (KNF Flodos AG, Schweiz) vacuum system. The filters were placed into glass Petri dishes, covered, and dried at room temperature. The filters were observed under a stereomicroscope (Nikon, Italy) to analyze the presence of debris, and images were taken with a digital camera (Nikon X_Entry, Italy). The number, size, shape, and color of the potential plastic particles on the filters were recorded (Li *et al.*, 2016). The length of the detected particles was determined, and every particle was assigned to one of four distinct size classes: 5-500µm, 501-1000µm, 1001-5000µm, and >5001µm. Nikon's software for imaging analysis was applied to the litter dimensional measurements (Nikon X_Entry, Italy).

Fourier transform infrared spectroscopy in attenuated total reflectance polymer identification

The characterization of plastic polymers was performed using FTIR-ATR. The FTIR spectra of the samples were obtained with Spectrum Two PE instrument (PerkinElmer) equipped with the universal attenuated total reflectance accessory (ATR) (UATR, Single Reflection Diamond/ZnSe) accessory, and spectra were acquired in the range 400 to 4000 cm^{-1}. The measurement resolution was set at 4 cm^{-1} with 16 scans. ATR crystal was rinsed with ethanol and a background scan was performed before each sample analysis. Several areas of the filter were considered until a consistent result was obtained between the points examined. The particles were identified by comparing the FT-IR absorbance spectra of the analyzed MPs to those in a polymers reference library. An index of at least 70% match was considered acceptable (Joint Research Centre, 2014).

Statistical analysis

Data were analyzed with Microsoft Excel software (Windows, 2010). The concentration of MPs was expressed in items/g and items/individual. Mean values and standard deviations were calculated for each replicate of each sample, including the blank. One-way analysis of variance ($P<0.05$), was performed to detect significant differences between the number of MPs of the samples and between the three repetitions. Furthermore, to assess significant differences between the total number of microplastics in the different samples and the total number of microplastics in the procedural blanks (negative controls), a t-test ($P<0.001$) was also performed.

Results

A total of 180 individuals of *M. galloprovincialis* and 18 individuals of *C. gigas* were analyzed. The average values of the shell lengths were 6.3±0.85 cm and 12.59±0,17 cm respectively.

The average values of the shell lengths and the wet weight of the extracted soft tissue for each sample are in Table 1.

All samples tested positive for the presence of microplastics. In detail, 789 MPs were detected in mussel samples and 270 MPs in oyster samples. Statistical analysis revealed that the correlation between the data for the different samples and between replicates was not significant ($P>0.05$).

MPs contamination in the procedural blank was low in relation to the average values detected in the analyzed samples (1.25±0.65 MP/g and 4.53±2.1 MP/individual for mussel samples, and 0.56±0.2 MP/g and 15±3.5 MP/individual for oyster samples) with an average value of 0.44±0.4 MP/mL. In both species of bivalve mollusks, the higher number of plastic debris was statistically significant compared to the blank procedural samples ($P<0.05$).

In mussels, the average MP values ranged between 0.6±0.07 and 1.9±0.18 MP/g tissue (wet weight) and between 2.5±0.3 and 6.6±0.62 MP/individual. In oyster samples, the average MPs values ranged between 0.41±0.12 and 0.71±0.16 MP/g and between 12±2.65 and 18.83±2.57 MP/individual (Table 1).

The MPs detected in all samples were fibers, fragments, films, and spherical granules. Fragments were the predominant MPs in both oysters and mussels, accounting for 36.7 and 60.96%, respectively. Fibers were 28.52% in oyster samples and 37.01% in mussel samples. Plastic films were 30.74% in oyster samples and 0.38% in mussel samples. Spherical granules were 2.3% in both bivalves.

The most represented microplastic size category was 5-500 µm with an incidence of 70.74% in oysters and 69.08% in mussels. The size of plastic debris in both samples ranged between 10 and 7350 µm. The most common colors of MPs found in mussel samples were blue (40.43%), transparent (18.76%), and yellow (11.41%). In addition, grey, brown, black, red, green, and white MPs were present with an occurrence ranging between 7.73 and 0.63%.

MPs found in oysters showed higher incidences for transparent (44.82%) and blue color (26.67%). The values of other colors ranged between 9.63 and 2.22%

The most detected polymer in oyster samples was chlorinated PP, whereas PA was the most isolated polymer in mussels; nylon 6/6 and polyurethane were identified in a smaller percentage (Table 1).

Discussion and Conclusions

Marine microplastic debris is an important issue for food security, food safety, and human health and it is a very important field of research. They have been found in different coastal and marine ecosystems, in the marine biome and in sites where marine organisms are fished and farmed (GESAMP, 2015; Abidli *et al.*, 2018; Thushari and Senevirathna, 2020; Li *et al.*, 2022). They are widespread in water and sediments with concentrations ranging from 0.001 to 140 particles/m^3 in water and from 0.2 to 8766 particles/m^3 in sediments (Thushari and Senevirathna, 2020).

The occurrence of microplastics in fish species of commercial interest is a worldwide problem and humans are vulnerable to exposure to microplastics through the consumption of seafood and other foods. In particular, oysters and mussels are the bivalve mollusks most exposed to MPs contamination as they are bred for human consumption. They are mainly produced in intertidal coastal areas and they grow on ropes suspended from rafts or on structures built above the seabed, where they are exposed to any pollutants in the seawater, including MPs.

The occurrence of MPs was investigated in mussels and oysters collected from harvest and farming sites all around the world, with results ranging from 0.05 to 4.6 MP/g and 1.5 to 7.6 MP/individual for mussels, and 0.11 to 7.9 MP/g and 10.00 to 27.5 MP/individual for oysters (Li *et al.,* 2016; Bonello *et al.*, 2018; Wang D *et al.*, 2021).

In our survey, we evaluated the occurrence of MPs in mussels and oysters collected in different fish markets in Bari and neighboring towns (Apulia region, Italy), in order to assess the direct risk for consumers. All analyzed samples were contaminated by MPs and the average values in mussels were higher (1.25±0.65 MP/g) than in oysters (0.55±0.19 MP/g) when expressed in MP/g. In contrast, when expressed in MP/individual, the average values observed in oysters (15±3.5 MP/individual) were higher than those in mussels (4.53±2.1 MP/individual). This is probably due to the size of the

Table 1. Average values of microplastics in samples of mussels (*M. galloprovincialis*) and oysters (*C. gigas*).

Samples	Shell length (cm)	Soft tissue weight (g)	n. MP	MP/g	MP/individual	Polymers	
M1	7.2±0.7	255	150	0.6±0.07	2.5±0.3	Polyamide	33.33%
M2	6.3±0.3	204	396	1.9±0.18	6.6±0.62		
M3	5.5 ±1.4	195	243	1.25±0.09	4.5±0.27	Nylon 6/6 Polyurethane	8,33% 8.33%
O1	12.43±0.40	157	85	0.56±0.2	14,17±3,69	Polypropylene	50%
O2	12.77±0.25	161	113	0.71±0.16	18.83±2.57		
O3	12.57±0.90	180	72	0.41±0.12	12±2.65		

MP, microplastic; M, mussel; O, oyster.

single individuals and the different areas of origin (Table 1; Figures 1 and 2). Furthermore, the area of origin of the mussels is also very important: according to the information on the labels, mussels came from Italy and Greece (FAO area 37.2), and oysters from the Netherlands and France (FAO areas 27.4 and 27.7).

Due to its particular geography, the Mediterranean is one of the most affected in the world by marine litter (Fossi *et al.,* 2018). It is estimated that more than 62 million objects float on the surface of the Mediterranean sea. The highest densities of floating debris (>52 pieces/km^2) were found in the Adriatic sea and the Algerian basin, whereas the lowest densities (<6.3 pieces/km^2) were observed in the central Tyrrhenian sea and the Sicilian sea (Suaria and Aliani, 2014). In fact, the Mediterranean sea is characterized by closed areas, such as the Adriatic sea, or semi-closed areas such as the Tyrrhenian sea and the lower Mediterranean sea. This characteristic could influence the different circulation of MPs and consequently their distribution in the different areas of the abovementioned sea. Therefore, as a direct consequence, there would be a different concentration of MPs in bivalve mollusks, bred or fished, marketed in the different Italian regions.

In fact, in our study, samples of mussels reared in the lower Adriatic and Ionian seas had a lower incidence of MPs than the results of a study conducted in the upper Adriatic, where the number of MPs was higher (2 MPs/g and 12.4 MPs/individual) even compared to samples reared and marketed in open areas of the Mediterranean (Sardinia, La Spezia, and Talamone) (Renzi *et al.*, 2018). In a study on mussel samples (*M. galloprovincialis, M. edulis, M. chilensis*), collected from fish markets and large-scale retailers in Sicily (Italy), MPs contamination was still lower for *M. galloprovincialis* reared from FAO area 37, but higher (0.39±0.25 pieces/g) if compared to *M. edulis* and *M. chilensis* from other production areas (FAO area 27 and 87) (Nalbone *et al.*, 2021).

C. gigas samples analyzed were from farms along the French and Dutch Atlantic coasts. The amounts of MPs found in oysters were consistent with those reported in other surveys from Germany and France (Van Cauwenberghe and Janssen, 2014). The low concentration of MPs in Atlantic oysters could be due to low anthropogenic, oceanic, and terrigenous pressure at their sites of production. In fact, contamination of the place where they are bred is of great importance, as MPs occurring in mollusks reflect, for number, shape, and size, the type of MPs present in the surrounding waters during growth.

Based on morphological analysis, identified microplastics were primarily of four types. 97.73% were fibers, fragments, and plastic films with the highest percentage for fragments (54.77%) (Figure 3 a-d). These can be classified as MPs of secondary origin resulting from the fragmentation or degradation of larger plastic debris through mechanical forces and photochemical oxidation in the environment. The fourth type was spherical granules (2.3%) classifiable as primary MPs derived from scrubbers used in the mechanical and cosmetic industries (GESAMP, 2015).

Fragments and fibers are the most isolated plastics from bivalve mollusks worldwide (Li *et al.*, 2018; Renzi *et al.*, 2018; Cho *et al.*, 2019; Bom and Sà, 2022). The high incidence of fragments and fibers in bivalve mollusks could be due to the nature of the waste and to management strategies adopted in the waters where mollusks are farmed. The waste present may originate from land-based anthropogenic activities (plastic bags, packaging materials, or industry waste) or anthropogenic activities at sea such as fishing and shellfish farming (ropes and nets). Afterward, due to prolonged exposure to ultraviolet rays and physical abrasion, they fragment leading to MPs production. Important features of MPs that contribute to their bioavailability are their wide range of colors and their small size. In particular, colors increase the chance of ingestion by aquatic organisms as they are confused with prey. This way, MPs enter the trophic chain of aquatic biota and, consequently, of humans. Furthermore, the small size of plastic debris helps them to be absorbed by mollusks, which through filter-feeding, apply a more or less limited selectivity between the particles, by retaining anything that has the appropriate size (Wright *et al.*, 2013).

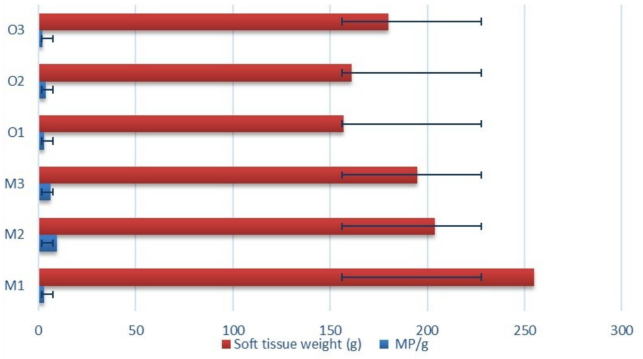

Figure 1. Correlation between microplastic/g in mussel and oyster samples and soft tissue weights (g). MP, microplastic.

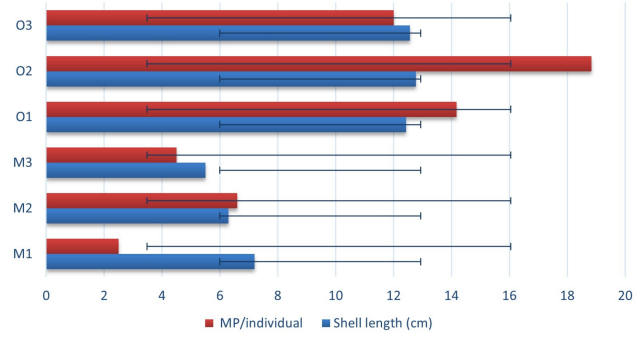

Figure 2. Correlation between microplastic/individual in mussel and oyster samples and shell length (cm). MP, microplastic.

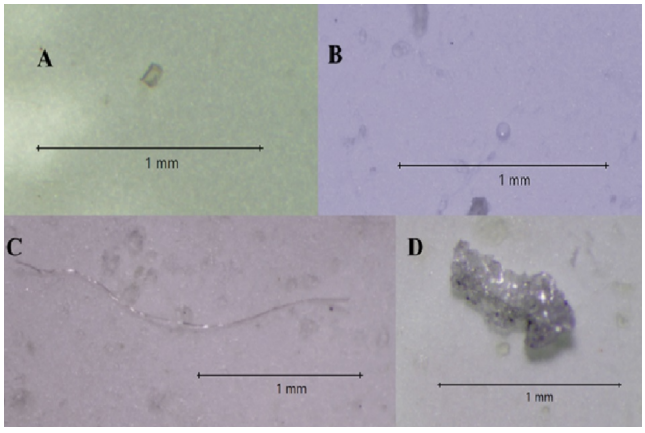

Figure 3. Type of microplastics detected in mussel and oyster samples. A) Fragments; B) Spherical granules; C) Fibers; D) Films.

The plastic debris isolated from mussel and oyster samples belong to four polymers. Chlorinated polypropylene was identified in 50% of samples, PA was identified in 33.33% of samples, and nylon 6/6 and polyurethane were identified in a smaller percentage (Table 1).

Polypropylene, occurring in all the oyster samples examined, is one of the most widely used plastic materials in the world. This polymer is characterized by low density (0.85-0.9 g/cm3) which allows it to float in the upper layers of the water column, available to mussels and oysters generally reared at depths of 3 and 8 m (Cho *et al.*, 2019).

Based on the chemical identification of the polymers, it can be hypothesized that one of the sources of microplastics found in the analyzed samples is the equipment used in aquacultures such as collectors, ropes, nets, and tubes used throughout the growth phases of mollusks. In addition, PA could result from the synthetic textile fibers used for the production of ropes employed in aquaculture plants for mussel stringing and buoy production. Furthermore, during mussel farming, tubular nets are often not replaced and, since they remain in contact with mussels, they represent an important source of MPs contamination.

These results show that farming methods (equipment used in aquaculture, water depth, and environmental conditions in the sea) and physicochemical properties of microplastics (specific density and shape) directly correlate with the contamination detected in fish products (Cho *et al.*, 2019). However, PP is also used for the production of food packaging, candy and snack wrappers, and microwave containers (Imasha and Babel, 2021).

A very important aspect of the detection of microplastics in food is the lack of standardized protocols. This could be the reason for inconsistent results in different studies and the possible underestimation. In particular, the chemical digestion of the organic matrix with acids, oxides, and peroxides may give different results. For example, using KOH or HNO_3 to digest the same sample may give completely different results. Furthermore, the use of overly aggressive reagents may degrade the surface, color, and size of some MPs, reducing extraction efficiency and leading to an underestimation of MPs presence (Bom and Sà, 2022).

In our study, H_2O_2 used in the digestion phase of the organic matrix worked well. It resulted in the complete dissolution of the samples and gave results which are consistent with those of other authors. Furthermore, for the correct identification of MPs, microscopic observation of the samples must always be corroborated by identification using Raman or FT-IR spectroscopy to avoid overestimating the presence of MPs in foods.

The results show that MPs contaminate the marine environment and consequently fish species of commercial interest, such as bivalve mollusks, representing a risk for the consumer. In Europe, to be marketed, bivalve mollusks must meet the microbiological criteria of Reg. CE n. 2073/2005 (European Commission, 2005) and, if necessary, undergo a depuration period. Several authors have hypothesized that a longer depuration step could result in the expulsion of larger microplastics, whereas smaller particles may be transferred to other tissues via the gastrointestinal tract and the circulatory system. However, for fresh and processed bivalve mollusks, commercialization could be an important source of contamination. Indeed, during this phase, mollusks are often reintroduced into the water or shelled at the consumer's request and may come into contact with plastic packaging.

Several authors have estimated the daily intake of MPs through shellfish consumption based on the amount of shellfish consumed and MPs concentrations found in different species, and they have concluded that shellfish consumption is an important route of exposure to MPs for consumers (Cho *et al.*, 2019). Although the potential risks for human health are unclear to date, the human health hazard related to MPs intake should not be overlooked as it is known that on their surface they can adsorb drugs, and chemical contaminants and provide a suitable habitat for many microbial communities (Zettler *et al.,* 2013). Furthermore, these microbial communities can produce biofilms that become reservoirs for pathogenic bacteria, fecal indicator organisms, and algal species. In this microcosm, antibiotic resistance can be transferred between different microorganisms (Wang Z *et al.,* 2021). Further investigations on all stages of bivalve mollusk chain, from production to commercialization, are needed to better understand when MPs contamination of bivalve mollusks occurs.

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