Identification and quantification of potential microplastics in shellfish harvested in Sardinia (Italy) by using transillumination stereomicroscopy

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Abstract

Plastics are non-biodegradable polymers made up of different groups of petrochemical materials. Several biotic and abiotic factors can change the density of plastic fragmenting it and originating microplastics (MPs). MPs have been defined as small pieces of plastic less than 5 mm in size. Due to their small size, they are an emerging concern in the marine environment since they can be ingested by aquatic organisms, especially filter-feeding organisms, such as bivalve mollusks. Impacts of MPs exposure have been shown at various levels of biological organization, from cellular to tissue to individual and population levels. For example, oxidative stress and inflammation have been observed in copepods and mussels, obstruction and physical damage of the digestive tract were found in fish and swimming behavior alterations, disruption of foraging and feeding behavior and overall reduced fitness and survival were observed in fish and oysters. In addition, MPs can act as a vector for the transfer of chemicals to marine biota. The aim of the present study was the identification and quantification of potential MPs in shellfish harvested in Sardinia (Italy) by using transillumination stereomicroscopy. Bivalves were collected from 4 of the main production areas located along the Sardinian coast and selected according to the principles of the risk assessment. The results of the present study demonstrated the presence of potential MPs in 70% of the analyzed samples: the presence of MPs in bivalve mollusks may pose a threat to food safety, and there is an urgent need to evaluate the potential risks of MPs to human health.

Introduction

Plastic debris is a generic name used to indicate most of the synthetic organic polymers ubiquitously present in the world's seas and oceans and exhibit the property of plastic. The world plastic production has increased exponentially, and the trend is still growing, passing from 335 million tonnes (Mt) in 2016 to 367 Mt in 2020 (Plastic Europe, 2021). Despite their advantages (low-cost production, water, and corrosion resistant, chemically inert), due to the large-scale use of plastic products and poor management by consumers, approximately 50% of the produced plastics are thrown after only one use and 10% of the plastics end up in oceans (Thompson *et al.*, 2009). Plastics have permeated all marine environments. More than 5 trillion plastic items (equivalent to ca. 250,000 tons) are currently estimated to be afloat at sea (Carbery *et al.*, 2018). However, their measured abundance in surface layers seems two orders of magnitude lower than that expected from conservative models (Isobe *et al.*,2019). Plastics require several centuries, or even thousands of years, to degrade (Barnes *et al.*, 2009). Moreover, many biotic and abiotic factors, such as microorganisms, erosion, abrasion, ultraviolet radiation, wave action, and photo-oxidation, can change the density of plastic (Kowalski *et al.*, 2016), degrading it and originating microplastics (MPs). MPs have been defined as small pieces of plastic, with size ranging from 1 μm to 5 mm, which may further degrade to nanoplastics (NP) (<1 μm) (Materić *et al.*, 2022). The widespread use and persistent nature of plastic have made MPs ubiquitous in the water column and offshore and deep-sea sediments near stormwater outfalls or other sources (Bergmann *et al.*, 2017). They are found in almost all marine environments (Phuong *et al.*, 2016) including the Antarctic and at the bottom of the deep sea. It has been estimated that between 15 and 51 trillion plastic particles, weighing up to 236,000 tons have accumulated globally in marine systems. These microparticles have received significant attention as an emerging contaminant of concern in the world (Bosker *et al.*, 2017). Several studies have been done to determine the quantities of MPs in the different environmental compartments such as seawaters (Lucia *et al.*, 2014), sand/sediments and marine organisms (Desforges *et al.*, 2015). MPs are presently dispersed throughout the water column based on the specific gravity of the polymer type (Avio *et al.*, 2017). Polymers denser than seawater (1.025 g cm3) (like PVC and PS) will sink, while those with lower density (*e.g.* PE and PP) will tend to float in the water column (Avio *et al.*, 2017). MPs can be ingested by several marine organisms with their different life stages, including species of fish (Pozo *et al.*, 2019), crustaceans and bivalve mollusks (Teng *et al.*, 2019) used for human consumption. Bivalve mollusks filter a large volume of seawater while feeding and, thus, accumulate MPs in their tissues from seawater. Filter-feeding organisms are particularly susceptible to MPs because the plastic particles are similar in size to their planktonic food (Browne *et al.*, 2008). Once ingested, MPs can be passed through the organism and rejected as feces, accumulate in the gut, or translocated through the intestinal wall to the circulatory system of the exposed mollusks (Browne *et al.*, 2008). MPs <10 μm have been shown to translocate from the digestive gland lining of the mussel *Mytilus edulis* (Browne *et al.*, 2008) and Pacific oyster *Magallana gigas* (Van Cauwenberghe and Janssen, 2014) into the circulatory system including the hemolymph (Browne *et al.*, 2008). Once ingested, the toxicity of MPs largely

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depends on their size, with smaller particles able to travel further into marine organisms (Browne *et al.*, 2008). MPs can damage or reduce the functioning of marine organisms by mostly mechanical means including laceration, inflammation, and even starvation (Carbery *et al.*, 2018). MPs are also known to act as vectors, transporting toxic chemicals adhered to the plastic's surface (Hartmann *et al.*, 2017). Concerns are growing worldwide about the human ingestion of MPs: it has been estimated that humans ingest on average 39,000 to 52,000 microplastic particles per year with contaminated seafood contributing significantly to this intake. Among seafood, the consumption of bivalve mollusks represents one pathway for human MPs exposure (Teng *et al.*, 2019) because they are consumed whole, without gut removal, transferring MPs into the human food chain (Carbery *et al.*, 2018). Previous studies reported that top European consumers can ingest up to 11,000 MPs per year through shellfish consumption while minor mollusk consumers had a dietary exposure to 1,500 MPs per year (EFSA, 2011). By now, it is generally accepted that MPs in bivalves may pose a potential health risk to humans although no direct evidence was detected (Santillo *et al.*, 2017). The long-term impact of MPs on human health remains largely unknown as most studies have been limited to the ingestion of MPs by marine organisms and the impact of MPs on marine life (Kontrick, 2018). Humans are exposed to MPs via the consumption of bivalve mollusks, but so far, the consequences and potential risks have not yet been quantified. At present, much of the evidence from field studies have demonstrated a high abundance of MPs ingestion in bivalves, particularly in commercially important mussels, clams, and oysters (Vandermeersch *et al.*, 2015) grew up both in natural beds and farming areas, from different European and Asian countries (Davidson and Dudas, 2016). The Mediterranean Sea was defined as one of the areas most contaminated by plastic in the world (Suaria *et al.*, 2016). For this reason, the Mediterranean Sea represents an excellent area to investigate the fate of MPs in marine environments and their availability to bivalve mollusks. Being a semi-enclosed basin with a limited outflow of surface waters, densely populated coastlines, and intensive sea-based activities, it is estimated to retain between 21% and 54% as several global MPs particles (3.2-28.2 x 1012 particles), equivalent to 5-10% of the global plastic mass (4.8-30.3 x 103 tons) in the oceans (Suaria *et al.*, 2016). In the Western and Central Mediterranean Sea, MPs analyses in biota are still missing and have been identified as a significant data gap (Abidli *et al.*, 2018). Sardinia island is located at the junction of the western and central basins of the Mediterranean Sea with a coastline over 1,849 km long. Only 178 and 188 km separate Sardinia from the North African and Italian coasts respectively. In Italy, bivalve mollusks are the most important farmed seafood resource, representing more than half of the total national aquaculture production (Sferlazzo *et al.*, 2018) and mainly represented by *Ruditapes philippinarum*, *Mytilus galloprovincialis* and *Crassostea gigas.* Italy is the leading European producer of *R. philippinarum* and the second in the world after China (Esposito *et al.,* 2020). Moreover, it is the third-largest worldwide producer of *M. galloprovincialis*, after China and Spain (FAO, 2020). *R. philippinarum* is mainly farmed in the regions of Veneto and Emilia Romagna, while the production of *M. galloprovincialis* is typical in Emilia Romagna, Veneto, Sardinia, and Puglia (Sferlazzo *et al.*, 2018). In Sardinia, the regional shellfish sector is well consolidated: the annual production accounts for 83% of the aquaculture species, and it almost exclusively rests on two of the most valuable shellfish species, *M. galloprovincialis* and *C. gigas*, (Sardegna Agricoltura/Laore, 2013). The aim of the present study was the identification and quantification of MPs in shellfish harvested in Sardinia (Italy) by using transillumination stereomicroscopy.

Materials and methods

Identification of the study sites

Collection, identification, and quantification of potential MPs were carried out in n. 76 shellfish samples belonging to the species *M. galloprovincialis*, *C. gigas* e *R. decussatus*. Shellfish samples were collected from 4 of the main production areas located along the Sardinian coast and selected according to the principles of the risk assessment. Three main risk factors have been taken into account to evaluate the possible exposition of bivalve mollusks to potential MPs: 1) presence of plastics industrial settlements; 2) presence of large urban settlements; 3) presence of seaports which are recipients of a large number of plastic discharges through ballast water, ship traffic, and other commercial activities. The following production areas have been selected based on the presence of at least two of the three above mentioned risk factors: the Olbia gulf (S1, north-eastern Sardinia); the Tortolì lagoon (S2, eastern Sardinia), the Santa Gilla lagoon (S3) and Oristano gulf (S4, western Sardinia) (Figure 1). S1 and S3 were characterized by the presence of large urban settlements and commercial/industrial seaports while S2 is located close to industrial settlements and commercial/industrial seaports. S4 is the main Sardinian brackish environment and is characterized by the presence of large urban settlements, commercial/industrial seaports, and industrial settlements.

Sampling

Samples of *M. galloprovincialis* (n. 36), *C. gigas* (n. 18) and *R. decussatus* (n. 22) were collected from November 2020 to April 2021. Bivalve mollusks were collected from breeding areas before purification. Samples were collected from S1 (n. 25); S2 (n. 21); S3 (n. 18) and S4 (n.12) by the local veterinary services of the National Health System (Table 1) and were shipped refrigerated to the laboratories of the Veterinary Public Health Institute of Sardinia in Sassari.

Sample preparation and extraction

Once arrived in the laboratory, the mollusks were frozen at -20 °C before analysis. To avoid potential MPs contamination, all the equipment used in the protocol was rinsed three times with demineralized water

Table 1. Samples of bivalve mollusks included in the present study

Study site	Species of bivalve mollusks			Total
	M. galloprovincialis	*C. gigas*	*R. decussatus*	
S1	9	9	7	25
S2	9	9	3	21
S3	9	0	9	18
S4	9	0	3	12
Total	36	18	22	76

(PURLAB, Option R-7/15) before utilization. Moreover, for prevention of contamination, lab coats and gloves in nitrile were worn all the time, and all sample processing was performed in a clean laminar flow hood. Shellfish samples were taken out of the freezer just before preparation for analysis. Samples were analyzed for potential MPs identification and quantification according to Dehaut *et al.* (2016) and Phuong *et al.* (2018). Briefly, after thawing for 1 h, all the samples were washed, measured, and weighed before to be processed. Subsequently, their byssus was cutted and the whole soft tissues were removed from the shells. For each analysis, soft tissues of six clams, three mussels or two oysters were pooled and settled in specific flasks. KOH 10% (Potassium Hydroxide Pellets, Carlo Erba, Milano, Italy) was added in each flask with mollusks soft tissues. The KOH 10% amount was determined based on the weight difference of the flask with muscle tissue minus the weight from empty flask and the value obtained was multiplied by 3. Bottles were capped with glass caps, placed in a shaker (HeidolphTM Agitatore Rotamax 120, Thermo Fischer Scientific, USA) at 80 rpm and incubated (Cooled Incubator MIR-154 PE Panasonic, Kadoma-Japan/Analytical Control De Mori, Milano-Italy) at 40°C for 48 h. The digested solutions were filtered by using 5 µm and 47-50 mm diameter nitrocellulose filters (Merck TM SSWP04700) and a vacuum pump (Thermo Savant VLP200 Valu Pump, Thermo Fischer Scientific, USA). After filtration, filters were gently removed with the aid of steel tweezers and were placed inside glass petri dishes (Zetalab s.r.l Padova, Italy). The glass Petri dishes were capped with their glass cap and the filters were dried at room temperature for 1-3 days before reading at the stereomicroscope. In each sampling, a procedural blank (a sample with KOH 10% without bivalve mollusks) was processed as the mollusk's samples. No positive controls were included in this study: the recovery of our method is based on the instruments used and practices performed. The total number of potential MPs observed in procedurals blanks has been subtracted from the total number of potential MPs in samples (González-Pleiter *et al.*, 2020).

Identification of potential MPs items

Each filter was placed on glass Petri dishes and observed with stereomicroscope (LEICA M205 C, Microsystems GmbH, Germany) noting the magnification used. Potential MPs were identified depending on the shape and type: a) industrial pellets; b) fragments; c) thin films; d) filaments or lines; e) foam and classified by color: a) black, glossy black, and gray; b) blue, deep blue, transparent blue; c) green; d) brown and light brown; e) orange; f) pink; g) red; h) transparent, opaque; i) white; l) yellow; i)

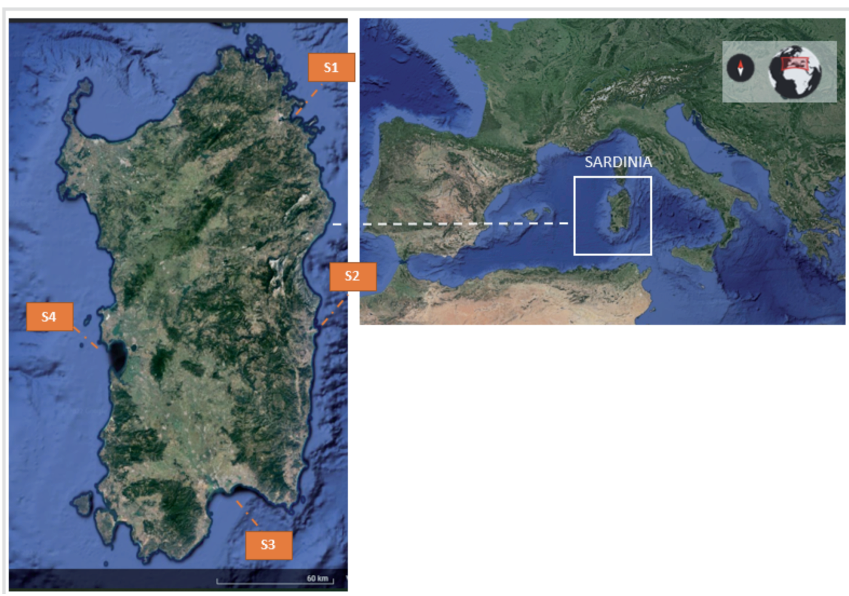

Figure 1. Study area and sampling sites of bivalve mollusks: Olbia gulf (S1), Tortolì lagoon (S2), Santa Gilla lagoon (S3) and Oristano gulf (S4).

Table 2. Tipology (%) and color (%) of MPs found in the production areas included in the present study.

Study site		S1	S2	S3	S4
Tipology of MPs (%)	Filaments	91	97	94	89
	Fibers	2	-	-	6
	Foam	7	-	6	5
	Fragments	-	3	-	-
Color of MPs (%)	Tranparent	40	35	37	39
	Blue	35	45	23	36
	Black	11	2	-	6
	Red	1	14	3	6
	Orange	1	-	-	-
	Green	3	2	3	3
	Yellow	1	-	-	2
	White	5	2	34	8

other colors (GESAMP 2019).

Several characteristics should be indicative of a non-biologic origin. Major plastic features are: the absence of repetitive structures indicative of biological origin, homogeneous colouring unless due to transparency, and in case of fibrous forms equal thickness and three-dimensional bending (Enders *et al.*, 2015).

Quantification of potential MPs

The quantification of potential MPs was expressed as several objects (n), identified by shape and color, for g of the edible part (n / g e. p.) of the bivalve mollusks. For each study site, the arithmetic means of the densities of potential MPs found in *C. gigas*, *R. decussatus*, and *M. galloprovincialis* was calculated as follows: the arithmetic mean (Ma) for numerical values XN, is the relation between the sum of the XN values and the number N of the values calculated by adding all the available values and dividing the result by the total amount of data

Results and discussion

Potential plastic debris smaller than 5 mm was found in 90% of the analyzed samples. The filaments showed a higher prevalence: 91% in S1, 97% in S2, 94% in S3 and 89% in S4. Fibers, foam, and fragments were in found in small numbers (Table 2). The presence of filaments suggests that potential MPs are likely derived from the breakdown of larger plastic items from marine activity or fishing equipment. The presence of fibers can result from the domestic washing of fabrics and the subsequent release of drains into the urban sewer system (Browne, 2015). The presence of different forms of ingested or entangled MPs can cause harmful physical and chemical effects on bivalve mollusks, in terms of the blockage of the appendages and clogging of the digestive systems associated with false satiety and transport of chemical contaminants (Browne *et al.*, 2007). The color analysis (Table 2) of potential MPs showed high prevalence of blue (45% in S2), transparent (40% in S1 and 39% in S4 respectively), and white (34% in S3). Other colors (black, red, pearly, green, and yellow) showed lower percentages (Table 2). The concentration of objects of different colors observed in the tissues of bivalve mollusks may indicate the availability of these elements in the surrounding marine environment. The color can influence the consumption of debris by marine fauna: shades of color can induce predators to confuse marine debris with the prey of similar color. In a previous study (Boerger *et al.*, 2010) in the North Pacific Central Gyre, the most encountered plastic colors were white, clear, and blue. The authors associated these colors with those of the plankton present in that area and explained that this similarity could induce planktonophagous organisms to ingest debris instead of plankton (Tourinho *et al.* 2010). The highest average density of potential MPs (0.60 n/g ep) was found in the *R.decussatus* species in S1 (Figure 2) while the lowest average density of potential MPs (0.00 n/g ep) was found in the species *C. gigas* in S3. The particle sizes found in S4 were included in a dimensional range between 0.11 and 5.0 mm. In S1 were between 0.24 and 4.8 mm while in S2 were between 0.24 and 3 mm. In S3, the particle sizes were included in a dimensional range between 0.21 and 2.8 mm.

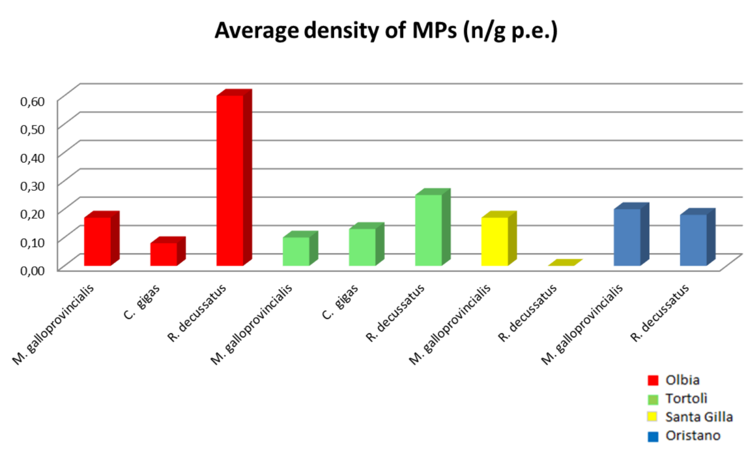

Figure 2. Average density of MPs (n / g p.e) based on the analyzed species and the study sites: Olbia gulf (S1), Tortolì lagoon (S2), Santa Gilla lagoon (S3) and Oristano gulf (S4).

Conclusions

Sardinia is one of the nationally relevant Italian shellfish production areas: the annual production accounts for 83% of the regional aquaculture. Specific literature on the risk assessment of MPs in the Sardinian coastal waters is limited and to the best of our knowledge, this should be the first field study investigating the occurrence and the accumulation of potential MPs in bivalve mollusks harvested in Sardinia. The results of the present study demonstrated the presence of potential plastic debris smaller than 5 mm in 70% of the analyzed samples. These characteristics, especially color and size, can affect the ingestion of particles by marine biota, being accidentally ingested and confused with phytoplankton or prey. This study confirmed that potential microplastic pollution in the coastal marine waters of Sardinia is present and constitutes an important socio-environmental problem. However, it is necessary to carry out further investigations about the nature of the polymers of the recovered items, in order to have a clear idea of the source of pollution. The problem must be addressed with the best management and monitoring strategies of the marine environment, for which the acquisition of further scientific data is required. To have a clear distribution of MPs in the Sardinian coastal marine waters and an evaluation of the different accumulation capacity of MPs by the species of bivalve mollusks it is necessary to expand the study area considering other sites of productive interest. In this way, it could be possible not only the evaluation of microplastics pollution sources into the sea but also the identification of possible areas of accumulation linked to an eventual seasonal and annual variability.

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