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Time-course distribution of fluorescent microplastics in target tissues of mussels and polychaetes

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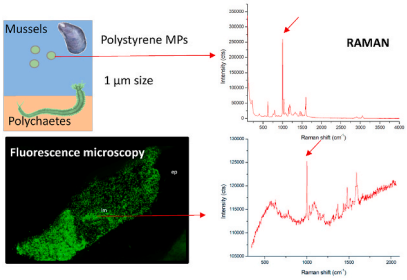
HIGHLIGHTS

- Mussels and polychaetes accumulate MPs spiked in water column (microcosm).
- Time-course quantification of MPs was carried out in mussels and polychaetes.
- MPs were localised in the lumen of different digestive structures of mussels.

GRAPHICAL ABSTRACT



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ABSTRACT

The majority of the plastic produced in the last century is accumulated in the environment, leading to an exacerbated contamination of marine environments due to transport from land to the ocean. In the ocean, mechanical abrasion, oxidation, and photodegradation degrade large plastics into microplastics (MPs) - 0.1 µm to 5 mm (EFSA, 2016) which are transported through water currents reaching the water surface, water column, and sediments. Further, they can be accumulated by aquatic and benthic species, entering the trophic chain and becoming a potential threat to humans. In the present research, we aimed to decipher the accumulation and distribution time-courses between different organs or target tissues of organisms inhabiting coastal areas such as mussels *Mytilus galloprovincialis* and polychaetes *Hediste diversicolor*. Both were exposed in microcosm experiments to fluorescent polystyrene MPs (1 µm) which were spiked at two doses (10^3 and 10^5 particles/mL) for 1, 4, 24, and 72 h. Mussels and polychaetes were digested with 10% KOH and filtered to quantify the number of MPs incorporated. Different anatomical parts of the body were selected and processed for cryosectioning and posterior microscopic localisation of MPs. Both species accumulate MPs spiked in water column, mainly after exposure to the highest dose. In mussels, particles were found in distinct parts of the digestive tract (stomach, digestive diverticula, ducts) and gills. Even if the majority of MPs were localised in the lumen of the digestive tract, in some cases, were inside the digestive epithelium. The identification of MPs and their internalization in the digestive system was studied using Raman spectroscopy. A decreasing trend with time regarding MPs number

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in the digestive tract (stomach) of mussels was observed while the opposite was recorded for polychaetes and sediments. The combination of microscopical observations of frozen sections and Raman, appeared to be accurate methodologies to address MPs abundances and to reveal their localisation in different organs. This work has enabled to understand the distribution and fate of MPs in different environmental compartments and it could contribute to gain knowledge about their impact after ingestion by coastal organisms.

1. Introduction

The deficient management of plastic waste has led to its entry into complex abiotic and biotic ecosystem compartments (Bank and Hansson, 2019). The migratory wildlife, rivers, wind, (Carson et al., 2013; Jambeck et al., 2015), and surface waters are some of the main vectors that allow plastics to be transported to different ecosystems and strongly influence the flux mechanisms and sink dynamics of plastics (Bank and Hansson, 2019). Plastic waste is present at a global scale, especially in marine environments including lakes and tropical beds, pristine locations such as remote Antarctic Island shores, and abyssal zones, thus, it has been internationally recognized as ubiquitous pollutants (Barnes et al., 2009).

Small fragments generated by gradual breakdown are recognized as microplastics (MPs) (UNEP, 2018). However, there is no internationally agreed definition of the size below which a small piece of plastic should be called a microplastic (GESAMP, 2015). Despite the lack of consensus on the categorisation of plastic debris, many researchers, based on the definition established by the US National Oceanographic and Atmospheric Agency (NOAA), consider size as main differentiator. Thus, plastic particles with different shapes, smaller than 5 mm in at least one of their dimensions, are categorised as MPs (Hartmann et al., 2019).

MPs are classified as primary when are manufactured to be microscopic or secondary derived from the fragmentation of macroplastics litter (Lehtiniemi et al., 2018). MPs have a broad spectrum of chemical nature and may be constituted with monomers of high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) among others (Lambert et al., 2017). In addition, they can be degraded and generate plastics with smaller dimensions, the nanoplastics (NPs, 1–100 nm) (EFSA, 2016), which have largely unknown fates and toxicological properties (Kögel et al., 2019).

The high molecular weight and lack of natural analogues are characteristics that allow plastics to persist in the marine environment (Worm et al., 2017). Once in the ocean, these polymers are usually found floating on the surface along shorelines, however, more than the half sink and accumulate in sediments due to their density exceeding the density of seawater (>1.02 g/cm^3) (Hammer et al., 2012; Van Cauwenberghe et al., 2015). Buoyant pieces will also eventually sink being incorporated into the sediment, due to an increase in weight caused by biomass that accumulates and/or aggregates to the plastic due to biofouling (Andrady, 2011; Lebreton et al., 2019).

Occupying the same size fraction as sediments and some planktonic organisms, MPs present a potential bioavailability to a broad scope of organisms which includes filter and deposit feeders, detritivores, and planktivorous (von Moos et al., 2012; Wright et al., 2013b; Setälä et al., 2016). MPs, once ingested, can accumulate inside the organism, resulting in physical damage such as abrasions and internal blockages, feeding deficiencies, and causing cellular and tissue damage (Wright et al., 2013a). It is important to notice that the physiological effects and the implications of MPs ingestion along food chains are poorly understood and require further research (Collignon et al., 2012). Besides the above mentioned adverse physiological effects, MPs can pose an additional chemical hazard in the marine environment (Hammer et al., 2012; Gallo et al., 2018) since they are able to adsorb, transport, and release compounds. The toxicological profiles of the additives and chemical compounds added to plastics to obtain specific properties (e.g. durability, flexibility, UV resistance) are, in general, very well known. However, the main problem remains in the inability to predict how these compounds are transferred from plastics into organisms, whether this occurs and how it will result in a significant impact on the organism (UNEP, 2008). Once ingested by animals, the compounds may start to leach out (UNEP, 2008) damaging cell and tissue structures.

Mussels *Mytilus galloprovincialis*, as a filter-feeding bivalve, are considered a sentinel organism and has historically been used as optimal biological predictor for monitoring anthropogenic coastal pollution due to their wide range of attributes (Beyer et al., 2017; von Moos et al., 2012). Seawater, through synchronized movements of cilia on the surface of the gills, is constantly pumped through mussels pallial cavity, where the gill mucus captures, and traps suspended particles and mediate their transport to mussels mouth and digestive system (von Moos et al., 2012; Ward and Kach, 2009). Resuspension events can occur and represent one way in which filter feeders are repeatedly exposed to previously deposited MPs (Martin et al., 2017). Furthermore, Taylor et al., 2016 found that deposit feeders can ingest greater amounts of MPs than suspension feeders. From this perspective, species inhabiting estuarine sediments, such as polychaetes (e.g. *Hediste diversicolor*), a predominant group of organisms in benthic communities (Dorgham et al., 2014), can be an important tool that allows an assessment of the effect of contaminants, including emerging contaminants of concern such as MPs (Lewis and Watson, 2012; Revel et al., 2020). This ragworm species, features different ways to trap and capture food, according to the feeding models.

Filter and deposit feeders are especially vulnerable to the ingestion of MPs as they can feed directly on MPs and may even selectively ingest them (Graham and Thompson, 2009). Previous studies described that the size of the ingested particles plays an important role in the uptake (Woods et al., 2018). In fact, smaller particles can result in a greater threat than larger particles due to their ability to interact with organisms cells (Fernández and Albentosa, 2019; González-Soto et al., 2019).

Considering the increase of MPs in the ocean and potential negative consequences due to its accumulation in individuals and the food chain, it has become necessary to deepen and develop research regarding the lack of knowledge related to the uptake, transport, and distribution of plastic by marine species in different environmental compartments (e.g. sediment, water column). MPs present in the environment can be incorporated by different uptake routes by mussels together with suspension materials in the water column transported along the gills and digestive tract, passing through digestive processes and finally can finish into sediment together with faeces. Therefore, the aim of the present work was to decipher the uptake, accumulation and localisation of MPs in target tissues of coastal organisms (*M. galloprovinciallis* mussel and *H. diversicolor* polychaetes) exposed to different concentrations of MPs along a time-course (1 h, 4 h, 24 h, and 72 h).

2. Materials and methods

2.1. Experimental species

Mussels (*M. galloprovincialis*) with 3.5–4.5 mm of shell length were collected in a clean rocky intertidal area in January 2021, in Butroe estuary (Plentzia, 43°24′32.9″N 2°56′51.0″W). Mussels were transported to the laboratory and were placed in acclimation tanks with naturally filtered seawater (sand filtration in the uptake wells aided with a pump and gas balance in the Station and then passes through a decantation/inertial tank). No food was administered during the

acclimation process (5 d prior experiments). Seawater had a constant flow with 33 psμ salinity and persistent aeration, controlled temperature (18 °C) and a photoperiod of 12/12 h/h.

H. diversicolor polychaetes were purchased in December 2020 being organisms received a day after manual collections in the Galician coast. Polychaetes were readily placed in acclimation tanks (30 L), filled with a layer of 6 cm of sediments from Plentzia and seawater directly taken from the Plentzia Bay with a constant flow, a salinity of around 33 psμ and persistent aeration. Ragworms were kept in acclimation tanks, being fed with commercial fish food (Vipagram granular, 41.9% protein and, 8.7% fat) twice a week. The acclimation in the laboratory followed the same conditions maintained for mussels.

2.2. Microplastics characterization

A fluorescent polystyrene (PS) microsphere suspension, with particles of 1 μm size, was used as microplastic source (Fluoro-MaxTM Green Fluorescent Polymer Microspheres, Thermoscientific). Microspheres had green fluorescence (maximum excitation of 468 nm and maximum emission of 508 nm) incorporated into the polymeric matrix. They were packaged in aqueous suspension at 1% solid by weight with a refractive index of 1.59 and a density of 1.06 g/cm^3.

2.3. Time-course exposure (1–72 h)

Experimental tanks (20 L, Polyethylene) were filled with a sediment layer (6 cm) and water column (3 L of 0.2 μm filtered seawater) and were left stabilising for 24 h to separate both phases. Mussels were kept in nets in the water column and polychaetes were introduced into the sediments and were exposed to two different concentrations of MPs by spiking 10^3 and 10^5 particles/mL into the water column. In addition, a MPs free control treatment was prepared. Tanks were divided into four parts in which each compartment corresponded to a time of exposure (1, 4, 24 and 72 h), the division allowed to avoid disturbing the environment when removing animals at each time point. For each exposure dose and time, six organisms were used. Exposures were carried out with the absence of food, persistent aeration with a high porosity airstone (Tetra AS25), a controlled temperature of 18 °C, and a 12/12 h photoperiod.

After each exposure time, mussels (n = 6) and polychaetes (n = 6) were removed from their tank compartments and left in 0.2 μm-filtered seawater to avoid MPs attached to the surface of the organisms. In mussels, a cross-section duplicate, including the digestive gland and gills, and individualized digestive gland and gills were dissected out and cryofixed for cryostat sectioning. For the polychaetes, three different anatomical parts of the body were selected and processed for microscopical observations: foregut (with around 20 segments), midgut and hindgut. Remaining organisms (n = 3) were frozen whole for subsequent alkaline digestion, filtration and quantification of MPs under a fluorescence microscope. Alkaline digestion was performed in mussels of all times of exposure and in polychaetes exposed for 1 and 72 h.

Water and sediment samples were collected in each experimental group at the beginning (1 h) and at the end (72 h) of the exposure assay, and frozen at - 40 °C for semi-quantification of MPs.

2.4. MPs quantification and localisation in target tissues

In order to dissolve all the organic part of the samples and retain only inorganic elements, specifically the MPs incorporated by experimental organisms, an alkaline digestion of whole organisms was carried out with 10% potassium hydroxide (KOH). For that, mussels and polychaetes were placed in glass vials and a volume of 10% KOH equal to three times the volume of the samples was added. The samples were then placed in an oven where digestion took place for a total of 48 h at a temperature of 40 °C. To ensure complete digestion of all organic matter, a shaking process was carried out after 24 h. Finally, the digested samples were filtered in a filtration column connected to a vacuum pump, using glass fibre filters of 1 μm pore size (WhatmanTM GF/F 47 mm diameter).

Table 1
Quantification of MPs (n° particles/individual) in *M. galloprovinciallis* mussels and *H. diversicolor* polychaetes exposed to 10^3 and 10^5 particles/mL concentrations along a time-course (1 h, 4 h, 24 h, and 72 h). Values are represented as means ± standard deviations. n.m, *Not measured*.

Exposure time	1 h	4 h	24 h	72 h
M. galloprovincialis				
Control.	0.00	0.00	0.00	0.00
Low dose (10^3 particles/mL) Mean (±SD)	0.44 10^5 (±0.61 10^5)	0.92 10^5 (±1.18 10^5)	0.05 10^5 (±0.01 10^5)	0.05 10^4 (±0.04 10^4)
High dose (10^5 particles/mL)	16.53 10^5 (±4.80 10^5)	9.71 10^5 (±4.13 10^5)	1.12 10^5 (±1.31 10^5)	0.72 10^5 (±0.14 10^5)
H. diversicolor.				
Control	0.00	n.m	n.m	0.00
Low dose (10^3 particles/mL)	0.00			0.00
High dose (10^5 particles/mL)	0.00			116.61 (±177.32)

Dissected and cryofixed specimens were sectioned through cryostat Leica CM 3050 S at a chamber temperature of - 20 °C with a thickness of 10 μm and stored again at - 40 °C until being observed under the microscope. Some samples were counterstained with a rapid metachromatic stain with a mixture of toluidine, azure II and borax and mounted immediately with Kaiser's glycerol gelatine. During the sectioning, extreme care was taken to avoid cross-contamination between the different exposure treatments. That is, a cleaning protocol was applied after sectioning each sample consisting of careful cleaning with acetone 99.5% 3 times, in all the materials that were in direct contact with the samples, preferentially the anti-roll and the knife blade holder. Moreover, to minimize possible contaminations, all blades and anti-rolls were changed after each exposure treatment.

Filters and cryostat sections were observed with brightfield and fluorescence (excitation at 482/35 nm) illumination provided by an epi-illumination mercury light source (Nikon INTENSILIGHT C- HGFI) in a Nikon ECLIPSE Ni (Nikon, Tokyo, Japan) microscope. For the photographic record, an imaging software NIS-Elements F 3.2 (Nikon) was used. The quantification of MPs in filters was performed with the aid of a millimetric grid. In cryostat sections the abundance of MPs along the digestive tract were semi-quantified using the following scale: 0: no MPs; 1: between 1 and 20% of the area of the lumen of the respective organs in transversal section; 2: 20–40%; 3: 40–60%; 4: 60–80%; 5: 80–100%. The abundance of MPs in the gills were semi-quantified following the same scale but taking into account only the MPs attached to the epithelium (longitudinal axis). Similarly, defrosted samples of water and sediments from both exposure groups were observed under the fluorescence microscope to estimate the abundance of MPs using the same semi-quantitative scale.

2.5. Qualitative analyses by Raman spectroscopy

In order to ensure that detected MPs in mussel and polychaetes tissues exhibited the same chemical nature of the spiked ones, Raman spectroscopy was performed in cryostat sections of a selection of samples. A Renishaw inVia confocal Raman microscope (Renishaw, Gloucestershire, UK), was used for Raman point by point analysis. Samples were analysed using a 50x objective lens with a slit aperture of 65 μm, with an integration time of 5–10 s, 3 accumulations, a laser power of 5% of the total nominal power and a scanning spatial resolution of 1 μm/pixel. The measurements were carried out with the 532 nm excitation laser. When necessary, baseline correction, smoothing and cosmic ray removal of the acquired spectra were performed with the Renishaw WiRE 5.2 software. Raman spectra were obtained from the experimental

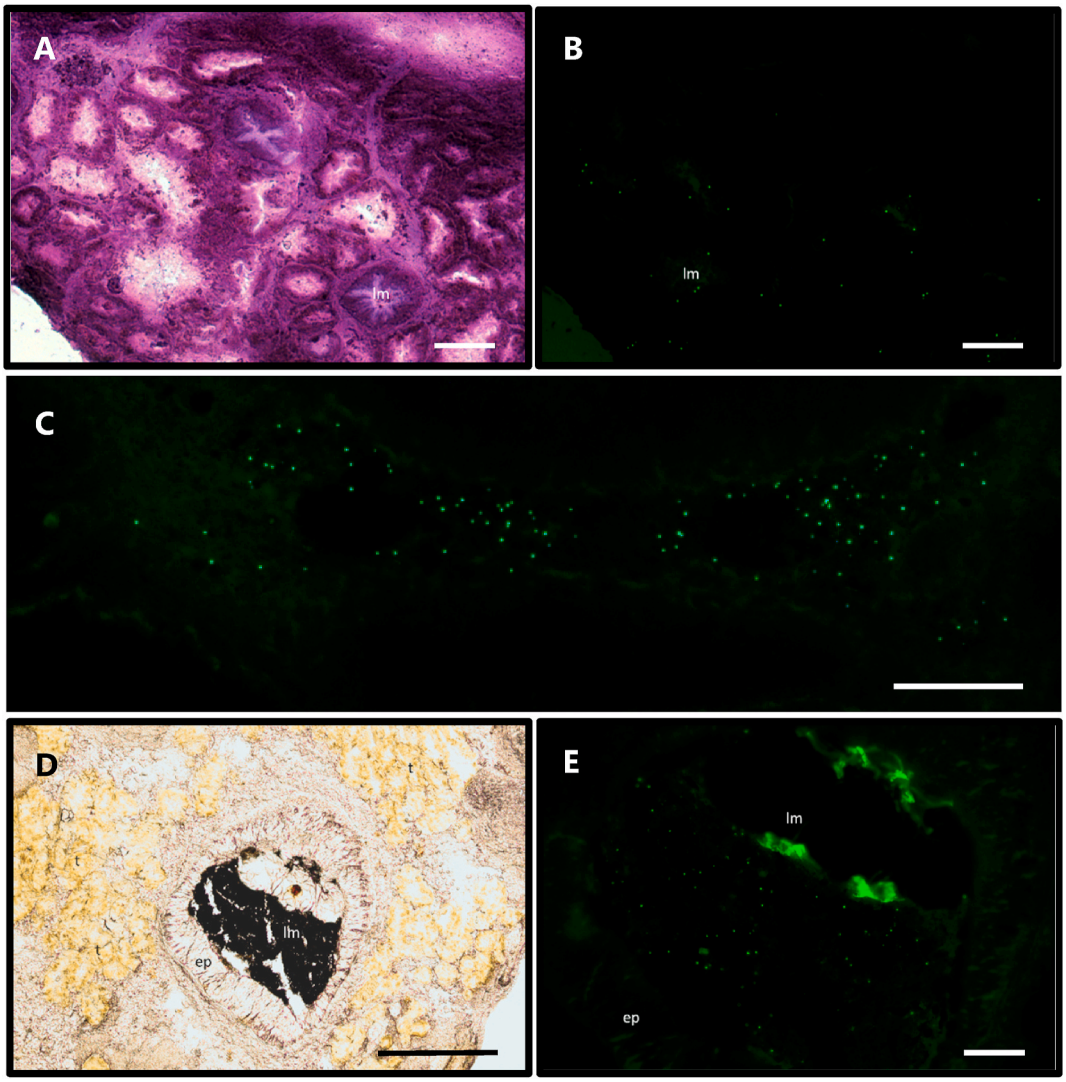

Fig. 1. Micrographs of frozen sections of different digestive gland (A–D) of mussels exposed for 1 h to the low dose (10^3 particles/ml) of MPs. **A**: Digestive gland stained with H/E showing the digestive tubules and ducts with their respective lumens (lm). **B**: Fluorescence micrograph of the same area shown in A revealing the presence of fluorescent MPs in the lumen (lm). **C**: Longitudinal section of the digestive tract with MPs in the lumen; **D**: Brightfield non-stained cross-section of the digestive gland showing the gastro-intestinal segment with the lumen (lm) surrounded by a thick epithelium (ep) full of dark-brown material from digestion. **E**: Detail of the presence of MPs in the lumen of the gastro-intestinal segment mixed with digestion matter. Scale bars: (A, B, C, E): 100 μm. (D): 500 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fluorescent polystyrene microbeads and from MPs present in stomach and tissues of mussels exposed to different concentrations and times.

3. Results

Fluorescent MPs were quantified in both experimental species and in all exposure times, with the exception in controls and polychaetes after 1 h and 72 h exposure to 10^3 MP/mL, where particles were not visualized (Table 1). In mussels, higher abundances were recorded after 1 and 4 h of exposure, mainly at the highest dose, with 16.53 10^5 ± 4.80 10^5 and 9.71 10^5± 4.13 10^5 MPs per individual, respectively. In the low dose the same time trend was obtained but mussels contained less MPs (≤0.92 10^5 ± 1.18 10^5) than in the high dose exposure (Table 1). After 24 and 72 h of exposure the number of MPs in mussel bodies decreased considerably. No MPs were quantified in polychaetes after exposure to the low dose at 1 h and 72 h (Table 1). In the case of the high dose, the abundance of MPs per polychaete increased up to 116.61 ± 177.32 particles after 72 h of exposure.

After observation of cryostat tissue sections, fluorescent particles

Table 2
Semi-quantitative analysis of MPs in target tissues of *M. galloprovinciallis* mussel and *H. diversicolor* polychaetes, water and sediments after exposure to different concentrations of MPs along a time-course. MPs abundanced are given following the scale: 0: no MPs; 1: between 1 and 20% of the area of the lumen of the respective organs in transversal section (attachment to epithelium in the case of gills); 2: 20–40%; 3: 40–60%; 4: 60–80%; 5: 80–100%. n.m, *Not measured.*

	Low dose (10^3 particles/ml)				High dose (10^5 particles/ml)			
	1 h	4 h	24 h	72 h	1 h	4 h	24 h	72 h
M. galloprovincialis								
Stomach	3	2	1	1	5	5	1	2
Digestive diverticula	2	1	0	0	3	3	0	1
Ducts	2	1	0	0	4	4	0	0
Gills	0	0	0	0	5	1	1	0
H. diversicolor	0	n.m		0	0	n.m		0
Water	3			1	3			1
Sediments	1			0	1			3

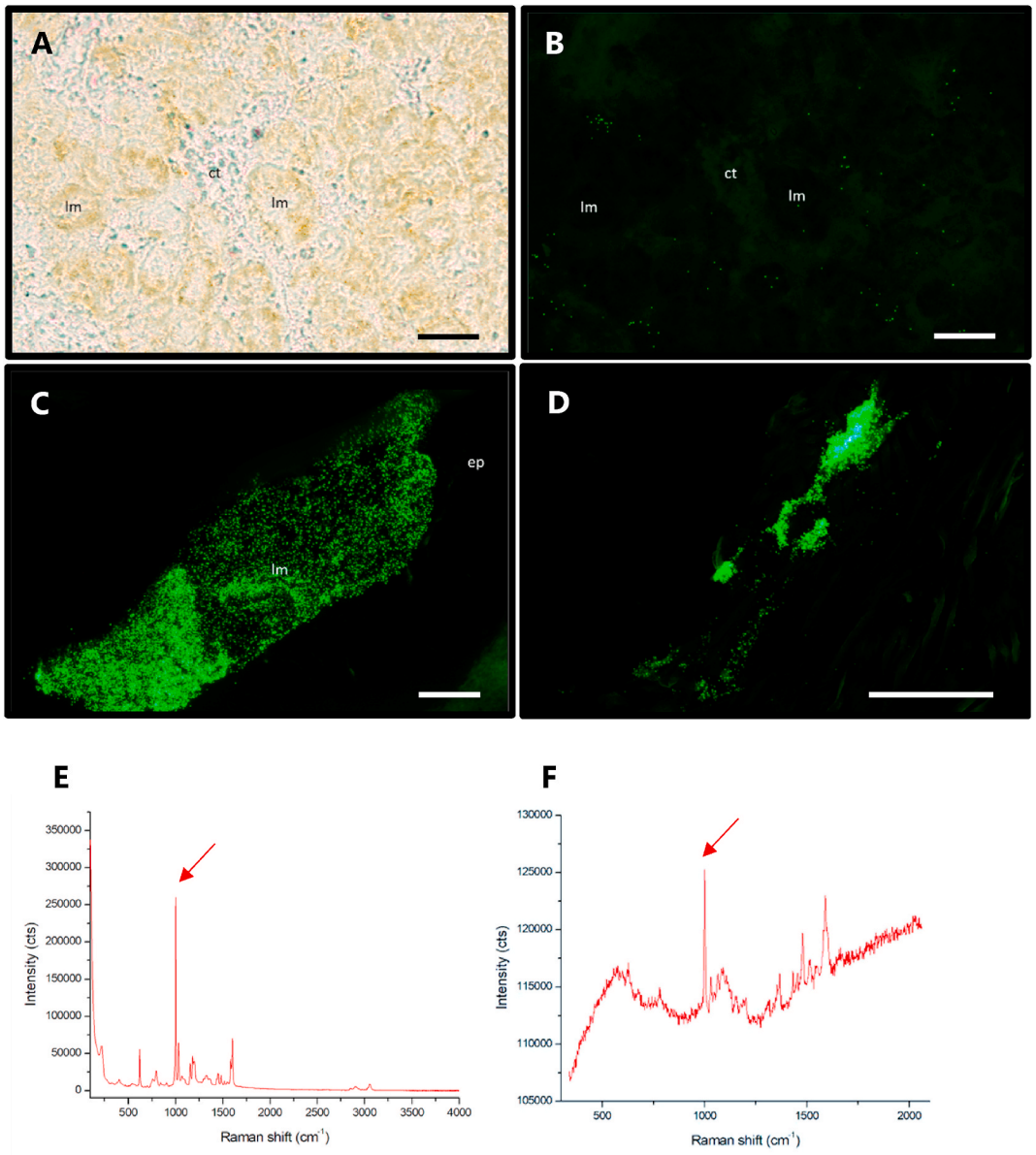

Fig. 2. Micrographs of frozen sections of the digestive gland (A–C) and gills (D) of mussels exposed for 1 h to the high dose (10^5 particles/ml) of MPs. **A**: Brightfield non-stained micrograph of the digestive gland tissue with digestive diverticula (dv) and ducts and their lumen (lm), and connective tissue (ct). **B**: Fluorescence micrograph of the same field shown in A revealing the presence of fluorescent MPs in the lumen (lm). **C:** Fluorescence micrograph of the digestive gland showing the stomach lumen (lm) full of MPs. **D**: Fluorescence micrograph of the gill full of MPs. Scale bars: (A, B, C): 100 μm. (D): 500 μm. **E**: Raman spectra from the experimental fluorescent polystyrene microbeads. **F**: Raman spectra of a fluorescent polystyrene MP (PS-MPs) present in the gut lumen of mussels from Figure C. The arrow indicates the main peak at 1000 cm $^{-1}$. Experimental conditions in the text.

were not visualized in digestive gland or gills sections from control mussels at different exposure times. After 1 h of exposure to 10^3 particles/mL, fluorescent MPs were detected in distinct areas of the digestive system in mussels. Fluorescent MPs occupied the 40–60% of the lumen area of the stomach while only the 20–40% of the lumen of digestive diverticula and ducts were occupied after exposure to 10^3 MP/mL (Fig. 1A-C; Table 2). MPs were mainly observed in the lumen of the stomach and ducts associated and mixed with digestive matter (e.g. algae), while no trace of MPs was observed in the digestive epithelium of the tract (Fig. 1D and E) neither in gills (results not shown). MPs were found occupying nearly the whole area of the lumen of the stomach (80–100%, Table 2) possibly associated to the presence of crystalline sac (Fig. 2C) in mussels exposed to 10^5 particles/mL for 1 h. MPs were also located in the digestive diverticula (Fig. 2A–B) and attached to the outer surface of the gills (Fig. 2D; Table 2).

Raman spectroscopy was performed in experimental fluorescent polystyrene microbeads and in a fluorescent particle present in the gut lumen of mussels obtaining in both cases the same main peak at 1000 cm $^{-1}$ suggesting the equal chemical nature of both particles (Fig. 2E–F).

After 4 h exposure to low dose, MPs occupied around the 20–40% of the lumen of stomach, and less than 20% of the lumen of ducts and diverticula (Fig. 3A, Table 2). Exposure to the highest dose rendered the same amount of MPs quantified after 1 h of exposure, some of them having been observed presumably in the epithelium of the stomach (). In gills very few particles were observed after 4 h of exposure to both exposure doses ().

Further, after 24 h to the lower dose only a low amount of MPs was observed in the lumen of the stomach (0–20%; Table 2; Fig. 4A–B). In the higher dose, MPs were observed in the lumen and epithelium of the stomach (Fig. 4D,F) while MPs were not observed in the ducts and

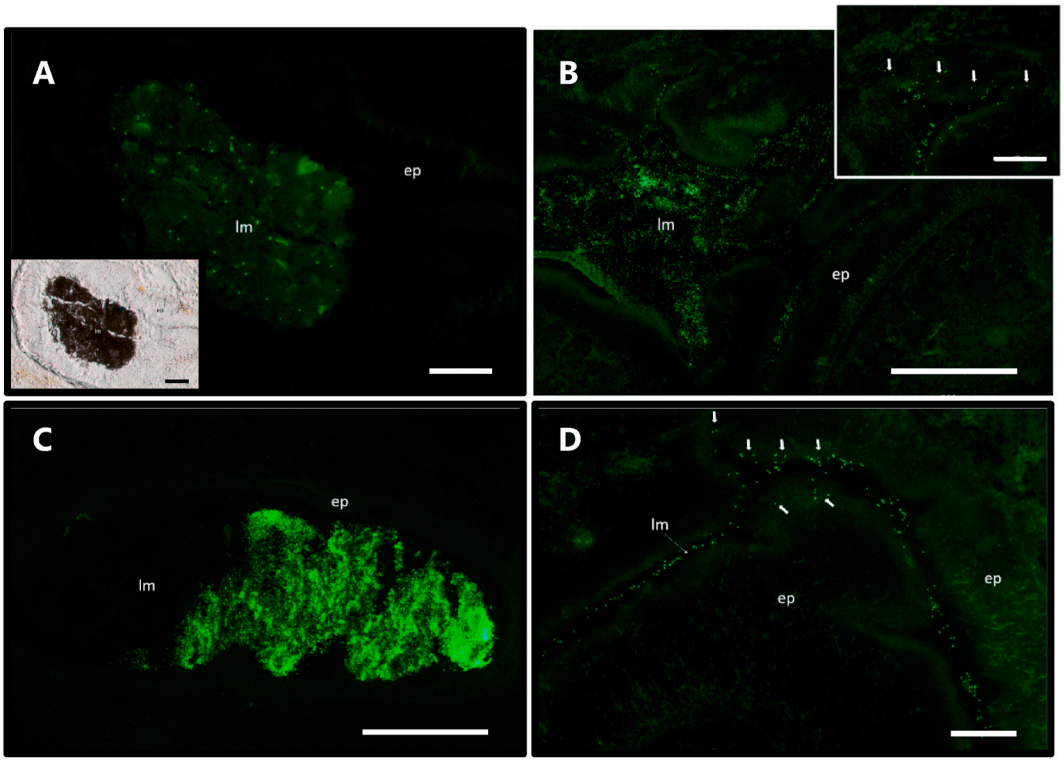

Fig. 3. Micrographs of frozen sections of the digestive gland of mussels exposed for 4 h to the low dose (A) and to the high dose (B–D) of MPs. **A**: Fluorescent micrograph of the duct with MPs; inset: Brightfield non-stained micrograph of the gastro-intestinal segment filled with digestive matter. **B**: Stomach section with MPs in the lumen (lm) and epithelium (ep); inset: White arrows shown MPs in the epithelium. **C**: Fluorescence micrograph of the stomach with MPs in the lumen. **D**: Fluorescence micrograph of the stomach (longitudinal) with MPs in the lumen and epithelium. White arrows: MPs in epithelium. Scale bars: (A, inset A, D, inset B): 100 μm. (B, C): 500 μm.

digestive diverticula (Fig. 4E; Table 2). Gills were nearly devoid of MPs after exposure to both doses (Fig. 4C, Table 2).

After 72 h of MPs exposure to the low dose, the lumen of stomach exhibited few MPs (Fig. 5B) while the lumen of ducts and diverticula were devoid of MPs (Fig. 5A; Table 2). The exposure to the high dose followed the same decreasing pattern (Table 2) although the lumen of the stomach showed an occupation of 20–40% (Fig. 5C, D). MPs were not present in gills.

In polychaetes fluorescent MPs were not detected in cryostat samples in any of the anatomical sections of the digestive tract (Fig. 6).

MPs were found in water samples at the beginning of the experiment following a decreasing pattern with time for both exposure doses (Table 2). In the case of sediments, fewer MPs than in water were found after 1 h. Exposure to the high dose after 72 h rendered more MPs in sediment than in water (Table 2). MPs were absent in sediments after 72 h of exposure to the low dose (Table 2).

4. Discussion

Presently, the accumulation and presence of 1 μm sized PS MPs in organs and tissues of two coastal species with different feeding habits was undoubtedly demonstrated at along a time-course microcosm experiment. The tracking of the uptake of MPs from the seawater and their distribution in different compartments was aided by microscopical observations onto digested organism (filters) and cryostat sections (non-fixed tissues). Moreover, Raman spectroscopy proved that the fluorescent MPs were the same spiked into experimental tanks. Cross-contamination along the histological procedure at the cryostat and filtration procedure was totally minimized through the cleaning protocol. Therefore, it can be concluded that both the experimental design and methodological approaches were adequate and can be used in assessing MPs interaction with biota, including toxicopathic effects.

There appears to be a relationship between MPs contamination load within environmental compartments and MPs burden within biota (Su et al., 2018). In the present work, two exposure concentrations (above environmentally relevant predictions) were used to track the uptake and localisation of MPs in target tissues of coastal organisms. Selected concentrations were based on laboratory experiments with marine organisms exposed to PS MPs of 1 μm (van Cauwenberghe et al., 2015; Paul-Pont et al., 2016), which used concentrations in a range of 42–2000 particles/mL. The quantification of MPs in whole mussel's bodies showed a greater number of particles in organisms exposed to the highest dose (10^5 MP/mL), so the interactions between MPs and mussels vary according to concentration. Among aquatic species, bivalves are considered selective particle feeders with well-developed mechanisms for pre-ingestive selection to process efficiently complex mixtures of material that they encounter, allowing particle discrimination (Evan Ward and Shumway, 2004; Fernández and Albentosa, 2019). Although the information about those mechanisms is still scarce, it is known that these organisms select particles according to their availability, shape, size, and even surface properties (Cole et al., 2011; Wang et al., 2021). Additionally, the anatomical characteristics of the organism, especially their feeding structures determine capture efficiency of particles (Fernández and Albentosa, 2019).

The first site of MPs uptake by mussels is on the surface of the gills and as particle size increases above 100 μm, anatomical constraints could occur reducing the probability of ingestion (von Moos et al., 2012; Ward et al., 2019). In the gills, no MPs were detected at any exposure time at the low dose exposure, and only were observed after 1 h of exposure to the high dose. As mentioned before, gills are considered the first site of particles uptake (Browne et al., 2008; von Moos et al., 2012), however, given the concentration used in the low dose experimental group (10^3 particles/mL), interestingly no MPs were detected after 1 h. It seems conceivable that a high filtration rate might occur allowing

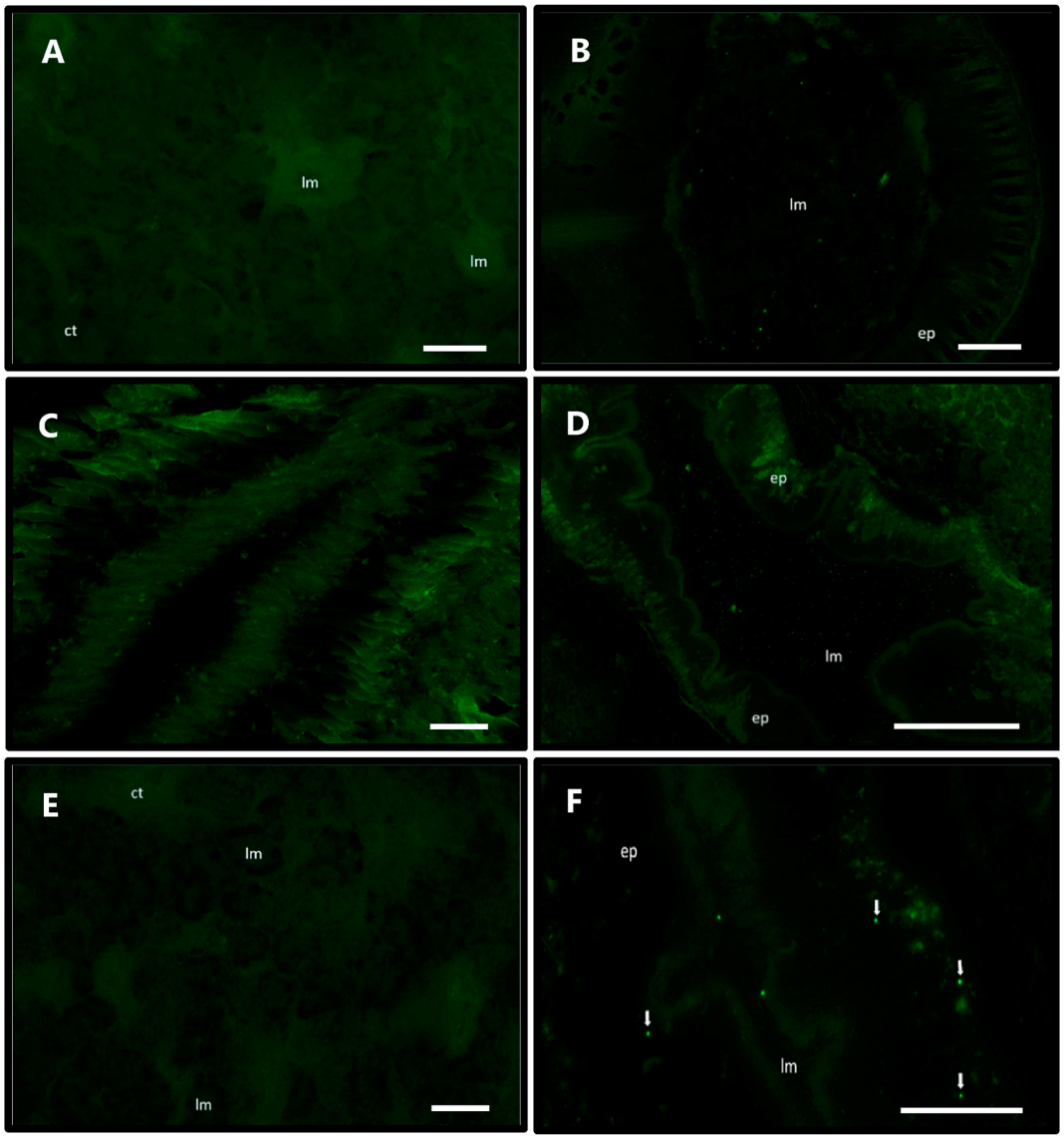

Fig. 4. Micrographs of frozen sections of digestive gland (A-B, D-F) and gills (C) of mussels exposed for 24 h of exposure to the low (A–C) and to the high (D–F) dose of MPs. **A**: Lack of MPs in digestive diverticula and ducts; lumen (lm); connective tissue (ct). **B**: MPs in the lumen of the stomach. **C**: Fluorescence micrograph of the gill with scarce MPs. **D.** Stomach section with MPs in the lumen (lm) and epithelium (ep). **E:** Lack of MPs in diverticula and ducts. **F**: Stomach with MPs in the epithelium (ep). Scale bars (A, B, C, E, F): 100 μm; (D): 500 μm.

them passing through the organ towards the digestive system. This is consistent with observations made by Winter (1973) where a low algal concentration is counterbalanced by a higher filtration rate. The influence of MPs availability in the medium on filtration rate could explain the variations observed in short-term (1 h) and long-term (72 h) exposure periods in the high dose. Considering MPs as food, their intense permanence in the gills at the first hours could then be explained by a decrease of filtration rate which would lead to a greater accumulation. With the decrease of particle concentration in the medium, due to their ingestion and subsequent excretion, the filtration rate increases leading to a decrease of visible particles in gills from 4 h on (likely decreasing of MPs availability). In fact, gills have been defined as a not typical tissue for a steady particle accumulation (Wang et al., 2021).

Ingested particles were found in distinct parts of the digestive tract (stomach, digestive diverticula and ducts) of mussels. Considering the analysed organs as independent variables the stomach is the unique digestive structure that has MPs after 72 h of exposure to the both doses when compared with the digestive diverticula, ducts and gills. Bayne et al. (1989) demonstrated that a higher concentration of food in the medium leads to an increase of the ingestion rate and therefore a decrease of retention of food in the gut. Deeming that the MPs ingested with organic matter were recognized as food since they were not eliminated through pseudo-faeces, this could be the explanation for founding MPs after 1 h of exposure in all the digestive structures, and after 72 h, with less MPs available in the water column, only in the stomach. After longer exposure times (72 h) the retention rate of MPs might increase leading to a decrease of the elimination rate (Fernández and Albentosa, 2019). Similarly, the digestive diverticula and ducts showed a decrease in the amount of MPs over time, which is not gradual since after 4 h of exposure to the low dose an abrupt decrease occurred. These results suggest, as previously stated by Langton (1975), that the digestive diverticula and ducts of mussels present a lack of synchrony within the diverticulum since MPs can be found in some but not in other digestive units of the digestive gland. The same pattern could be observed for the high dose although the amount of accumulated MPs was seemingly larger.

The combination of histological observations of non-fixed tissues and Raman spectroscopy allowed to suggest that MPs were not only in transit

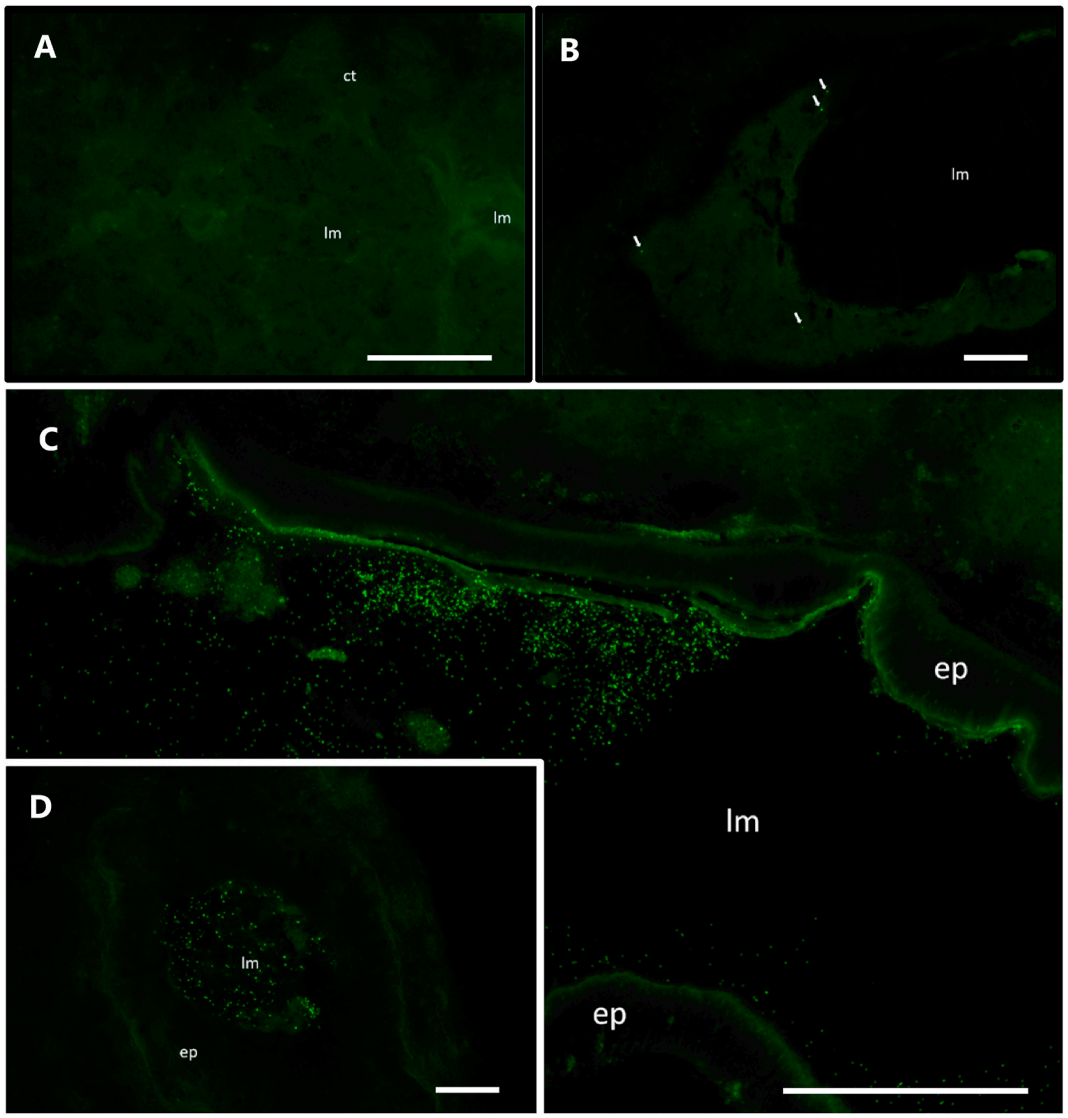

Fig. 5. Micrographs of frozen sections of different organs of the mussel *Mytilus galloprovincialis* after 72 h of exposure to the low (A, B) and high dose (C, D) of MPs. **A**: Fluorescence micrograph of the digestive gland without MPs. **B**: The lumen (lm) of stomach showed very few MPs. **C**: Stomach revealing the existence of MPs in the lumen (lm). **D**: Detail of the fluorescent MPs present in the lumen and surrounding tissue of the stomach. White arrows mark fluorescent MPs. Scale bar: (A, B, D): 100 μm; (C): 500 μm.

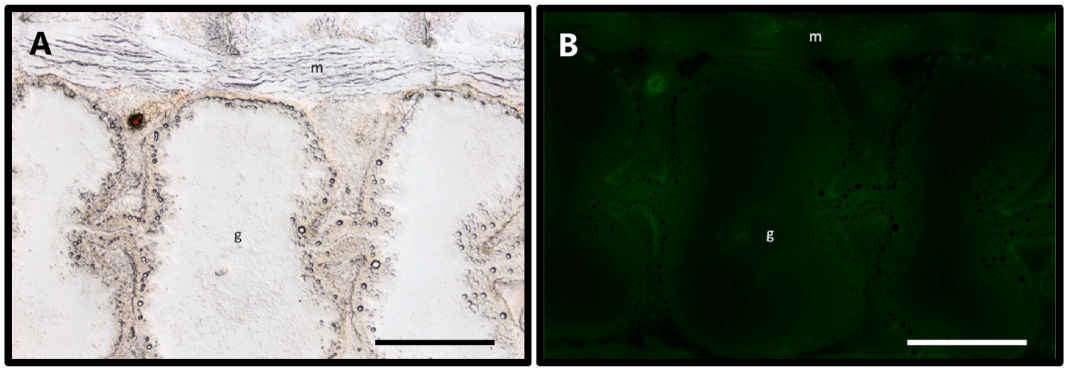

Fig. 6. Micrographs of frozen sections of the *Hediste diversicolor* digestive tract after 72 h of exposure to the high dose of MPs. **A** and **B**: Brightfield and fluorescence micrograph, respectively, that shows the foregut of *H diversicolor*. revealing a presence of muscle (m) layers along to the gut (g). No MPs were detected. Scale bar: 500 μm.

along the digestive tract (in the lumen), but also incorporated within the epithelium of the stomach. These preliminary findings are consistent with previous results (von Moos et al., 2012; Kinjo et al., 2019) that reported the internalization of high-density polyethylene (HDPE) MPs, ranging from 0 to 80 μm in size, in endo/phagocytotic vacuoles of digestive epithelial cells of invertebrates. The apparently low incorporation of MPs by digestive epithelia may suggest that mussels *M. galloprovincialis* as filter-feeding bivalves are well-adapted to cope with the filtration of suspended MPs, at least in the case of commercial PS microbeads of 1 μm. Overall, most of the MPs were ingested, passed throughout the digestive tract (stomach, ducts and digestive diverticula) and egested through faeces that subsequently might be accumulated in sediments (biodeposits). Nevertheless, further research is needed regarding the cellular and molecular mechanisms associated with the uptake of MPs (commercial and collected from the environment), which can lead to deleterious effects on cells and tissues.

Both, MPs quantification and histological observations of frozen sections of the entire digestive gland of mussels showed that there was a gradual decrease in MPs over time in all tested organs. This decrease can be related with a lower availability of MPs in water over time due to the filtration process, consequent ingestion, and excretion process carried out by mussels, which could lead to potential deposition in sediments. Likely, MPs tend to create a biofilm at sea, which increases the specific density of particles and causes them to sink (Mendoza et al., 2020). Accordingly, a decrease of MPs in water column and an increase in sediment occurred after 72 h for the high dose, probably increasing their availability polychaetes. The common ragworm *H. diversicolor* is a suspension feeder that does not select particles by their size but ingests sediment in order to feed on the associated organic matter and algae (Riisgard, 1991). As benthic feeders, this polychaetes, are likely to have a high potential to come into contact and interact with MP debris that sinks on the seafloor. In fact, MPs ingestion by polychaetes has been reported in field studies and during laboratory-based exposure experiments with sediments spiked with plastic particles (Van Cauwenberghe et al., 2015; Gomiero et al., 2018). In the case of Revel et al. (2020), MPs were observed in the tissues of *H. diversicolor* after 96 h exposure through sediments spiked with different concentrations of MPs (10 and 15 mg MP/kg sediment), being the amount of MPs higher at the highest concentration used in this study. Like in other studies (Van Cauwenberghe et al., 2015), very few particles seemed to have been incorporated and accumulated in polychaetes tissues. In fact, MPs after exposure through water were just recorded in filters containing digested material and no MPs were found in any of the body sections (foregut, midgut and hindgut) examined under the light microscope. Therefore, under present exposure conditions it seems that MPs spiked in the water column were not fully available for polychaetes, only at long exposure periods and at the highest concentrations (72 h, 10^5 particles/ml). This was coincident with a decreased amount of MPs in the water column. This might suggest the deposition of MPs released from mussels through faeces between 24 and 72 h becoming available for deposit feeders at that time point. Further experiments spiking the sediment are needed to clarify the possible translocation of MPs between both compartments and time-course accumulation in polychaetes tissues. Moreover, it cannot be discarded that the burrowing or bioturbation activity of polychaetes can increase the release of contaminants in sediments to the water column (Scaps, 2002; Martinez-Garcia et al., 2015).

One of the major concerns about plastics in the marine environment together with the uptake of MP, is their resultant consequences for the aquatic biota, such as internal damaging of cells and tissues and disruption of chemical and biological processes (Derraik, 2002; von Moos et al., 2012; Nelms et al., 2018; Sendra et al., 2021). It is known that all living species have defence and excretion mechanisms against xenobiotics (Ramsay and Kelley, 2020), including MPs, however, in high concentration and chronic exposures these mechanisms may not be efficient enough leading to deleterious effects (González-Soto et al., 2019). Previous works have described feeding deficiencies together with gut blockages and inflammations in marine worms (Wright et al., 2013a), but used MPs of polyvinylchloride that were much bigger (130 μm, mimicking the size and shape of sediment). Hypothetically, smaller microplastics could posed biological effects in sentinel organisms. Presently, MPs of 1 μm were dosed, but, even if addressing histopathological impairments was not the objective of present work, the stained cryostat sections were carefully checked under the light microscope and evident alterations were not recorded in any of the tissues analysed, neither in molluscs nor in polychaetes. In any case, further specific studies to assess possible effects at different levels of biological organization using biomarkers will be needed.

5. Conclusions

M. galloprovincialis mussels and *H. diversicolor* polychaetes accumulate fluorescent PS MPs of 1 μm spiked in water column, mainly after exposure to the highest dose. In mussels, particles were found in distinct parts of the digestive tract (stomach, digestive diverticula, ducts), and in gills at the beginning of the exposure. Even if the majority of MPs were localised in the lumen of the digestive tract, a few were also detected inside the digestive epithelium of the stomach. The identification of MPs and their internalization in the digestive system was studied using Raman spectroscopy. Obtained data reveal a trend of decrease in MPs number in the digestive tract of mussels with time, while the opposite trend was recorded for polychaetes and sediments. The interaction between MPs and the different environmental compartments could be explained by the filtration process, consequent ingestion and excretion process carried out by mussels, which could lead to potential deposition of MPs in sediments, increasing their availability to polychaetes. The combination of microscopical observations in filters and frozen sections together with Raman microscopy, appeared to be accurate methodologies to address MPs abundances in organisms with different feeding habits and to reveal their localisation in different organs. This work has enabled to understand the distribution and fate of MPs in different environmental compartments and it could contribute to gain knowledge about their impact after ingestion by coastal organisms.

Authorship contribution statement

Mariana Calmão: Methodology, Investigation, Writing – original draft. Nerea Garcia Velasco: Validation, Investigation, Writing – review & editing. Alba Benito: Methodology, Investigation. Rhea Thoppil. Methodology, Investigation. Imanol Torre Fernandez. Methodology, Investigation. Kepa Castro: Methodology, Validation, Writing – review & editing. Urtzi Izagirre: Conceptualization, Methodology, Validation, Writing – review & editing, Supervision. Manu Soto: Conceptualization, Methodology, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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