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Evidence of internalized microplastics in mussel tissues detected by volumetric Raman imaging

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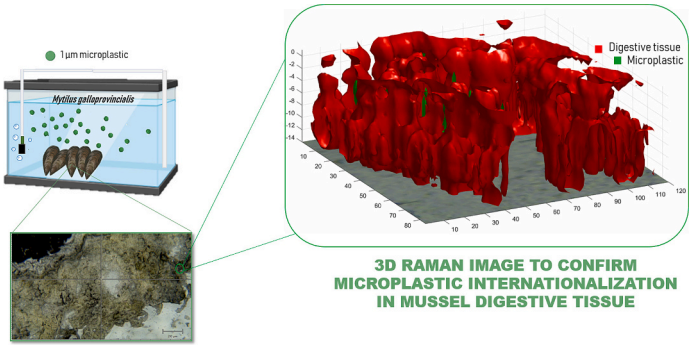
HIGHLIGHTS

- Microplastics analysis is challenging due to the risk of sample contamination.
- Analytical approach to confirm the internalization of MPs in mussel tissues
- 3D confocal Raman microscopy with chemometrics detected MPs of 1 μm.
- First evidence of MPs internalized in the mussels digestive epithelial tissue

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics are a global ecological concern due to their potential risk to wildlife and human health. Animals ingest microplastics, which can enter the trophic chain and ultimately impact human well-being. The ingestion of microplastics can cause physical and chemical damage to the animals' digestive systems, affecting their health. To estimate the risk to ecosystems and human health, it is crucial to understand the accumulation and localization of ingested microplastics within the cells and tissues of living organisms. However, analyzing this issue is challenging due to the risk of sample contamination, given the ubiquity of microplastics. Here, an analytical approach is employed to confirm the internalization of microplastics in cryogenic cross-sections of mussel tissue. Using 3D Raman confocal microscopy in combination with chemometrics, microplastics measuring 1 μm in size were detected. The results were further validated using optical and fluorescence microscopy. The findings revealed evidence of microplastics being internalized in the digestive epithelial tissues of exposed mussels *(Mytilus galloprovincialis)*, specifically within the digestive cells forming digestive alveoli. This study highlights the need to investigate the internalization of microplastics in organisms like mussels, as it helps us understand

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the potential risks they pose to aquatic biota and ultimately to human health. By employing advanced imaging techniques, challenges associated with sample contamination can be overcome and valuable insights into the impact of microplastics on marine ecosystems and human consumers are provided.

1. Introduction

As widely demonstrated, microplastics (MPs) can enter the environment through different pathways (Allen et al., 2022a). Moreover, they are present in freshwater (Koelmans et al., 2019), groundwater (Samandra et al., 2022), wastewaters (Yurtsever and Yurtsever, 2019), snow (Bergmann et al., 2023), ice (Kelly et al., 2020), soil (Wahl et al., 2021), sediment (Martin et al., 2022), biota (Büks et al., 2020), air (Brahney et al., 2020) and even ocean spray (Campos da Rocha et al., 2021).

Besides, MPs can be transported by wind and water, and can be dispersed over long distances from their origin and sources. A prominent example is given by the first evidence of MPs in fresh snow in Antarctica (Aves et al., 2022) and in the atmosphere (Marina-Montes et al., 2022), indicating potential long-range transportation of up to 6000 km. In addition, MPs can accumulate in the environment, particularly in areas where currents and other environmental conditions cause them to concentrate, such as in ocean gyres (Ter Halle et al., 2017). Therefore, they can be found in many different places in the environment, and can be difficult to remediate.

MPs have been widely demonstrated to be a global environmental problem, posing a threat to wildlife (Allen et al., 2022b; Malafaia et al., 2022a; Schrank et al., 2019a) and human health (Leslie and Depledge, 2020; Kutralam-Muniasamy et al., 2023; Malafaia and Barceló, 2023). One of the main concerns is that MPs can be ingested by marine animals (Koongolla et al., 2020; Lusher et al., 2013; Neves et al., 2015), birds (Carlin et al., 2020; de Souza et al., 2022; Monclús et al., 2022), and other organisms, and can enter the food chain (Elizalde-Velázquez et al., 2020; da Costa Araújo and Malafaia, 2021; da Costa Araújo et al., 2020a; Manríquez-Guzmán et al., 2023; Rose et al., 2023). Ingestion of MPs can occur either via primary ingestion, i.e., MPs are ingested directly, usually by mistaken for food or accidental ingestion, or by secondary digestion when prey that already contains plastic is ingested (Wootton et al., 2021a). The latter is referred to as trophic transfer (Hu et al., 2022) and can lead to bioaccumulation and biomagnifications (Wootton et al., 2021b).

Direct or indirect uptake of MPs could potentially affect the health of these organisms (Malafaia et al., 2022b). When animals ingest MPs, they can suffer from physical damage of their digestive systems (Wright et al., 2013) and can also absorb and release chemicals from plastic, including toxic substances, which can harm their health (Schrank et al., 2019b). In addition, MPs can interfere with the normal functioning of the animals' bodies, such as their ability to digest food and absorb nutrients, which can lead to a range of side effects (Barboza et al., 2020; da Costa Araújo et al., 2020b), including reduced growth and reproductive success, and in some cases, death. In a recent study (Lu et al., 2022), authors demonstrate that high-density polyethylene (HDPE) ranging from 100 to 125 μm in feed can be evacuated by yellow perch with no impact on growth. However, dietary exposure to HDPE reduced whole fish nutrition, altered nutrient metabolism, and caused intestinal pathologies through changes in the microbiota community of yellow perch. The results indicated that extended exposure may pose a risk to fish health and jeopardize the nutrition quality of aquaculture end products.

However, even though MPs intestinal absorption is considered limited due to their size (Sánchez et al., 2022), there is evidence of size alteration of MPs ingested by zooplankton, Antarctic krill, and potentially other species, which opens a new research field in the biogeochemical cycling and fate of plastics (Dawson, n.d.). This means that large MPs could be fragmented into much smaller MPs that are small enough to allow translocation. This issue is controversially discussed in fish and seafood-related literature. While some studies have found MPs in digestive tracts and gills, assuming that MPs are ingested and excreted from the animals, other studies pointed out the potential internalization of MPs in animal cells and tissues. Thus, some animals seem to have the capability to depurate and excrete MPs from their digestive system (Calmão et al., 2023a), demonstrating that MPs can pass through the whole digesting system with a soft interaction. On the contrary, some authors have found MPs in fish muscle tissues (Makhdoumi et al., 2021) and even brain tissues (Atamanalp et al., 2021), suggesting they may potentially affect human health (Thiele et al., 2021). In a recent study on European sea bass *Dicentrarchus labrax (*Zeytin et al., 2020), authors estimate the retention/translocation rate at approximately 1 MP particle reaching the potentially consumed fillet for every 1.87×10^7 MPs ingested. Special attention has been focused on water filter animals that are part of the human diet, specifically seafood (Smith et al., 2018) and mussels (Li et al., 2018a; Vinay Kumar et al., 2021). Besides, mussels are an internationally accepted sentinel species of early warning for marine pollution monitoring (Von Moos et al., 2012a).

Regarding the effects of MPs in humans, there is evidence for the presence of MPs in human tissues and body fluids, such as the studies on MPs in human lung tissue (Jenner et al., 2022), MPs detected in cirrhotic liver tissue (Horvatits et al., 2022a), or human breast milk (Richaud et al., 2022). However, as in other animals, it is still unclear up to what size MP particles can migrate through the intestinal wall, or whether the presence of MPs in blood or stool also indicates abundance in peripheral organs (Horvatits et al., 2022b). In fact, a recent study has found MPs in human blood (Leslie et al., 2022a), but some authors are questioning the methodology used during the analysis (Kuhlman, 2022; Leslie et al., 2022b).

According to the literature, there exist a variety of methods that can be used to detect and measure MPs in biological samples, such as tissue and fluid samples. As with any other sample, the best way to prepare samples of animal tissue for MPs analysis depends on the specific research question and objectives, as well as on the techniques to be used for analysis. In general, the samples are subjected to chemical digestion/extraction, filtration, and further analytical detection (Abbasi et al., 2018; Li et al., 2018b; Wootton et al., 2021c). While this approach is suitable for determining whether or not MPs are present in the sample of interest, because the structure of the sample is lost, the study of important aspects such as the internalization of MPs into cells or tissues of the digestive tract is not possible. Besides, contamination of the sample is likely taking into account that MPs are present everywhere, which makes the use of procedural blanks, clean rooms, lab and equipment cleaning, labware and lab clothes control, etc. extremely important (Horvatits et al., 2022a; Richaud et al., 2022; Horvatits et al., 2022b; Prata et al., 2021; Noventa et al., 2021).

To avoid the drawbacks of extraction methods, cryogenic cross-sections can be used for MPs analysis in animal tissues (von Moos et al., 2012b; Irazola Duñabeitia et al., 2015; Benito-Kaesbach et al., 2023). In this process, tissue samples are frozen and then cut into thin slices or sections using a cryostat microtome. Then, sample sections are collected on a glass slide and analyzed by microscopy or vibrational spectroscopy. As this technique preserves the structural and chemical integrity of the tissue, it can allow for the observation of the distribution and localization of MPs ingested by living organisms within the tissue, as well as the translocation of MPs to other organ tissues (Collard et al., 2017). For example, Irazola et al (Irazola Duñabeitia et al., 2015). used cryosection and paraffin-embedded sections to study the accumulation and distribution of polyestirene MPs in different tissues (digestive gland and gills) of *Mytilus galloprovincialis*, concluding that frozen tissue allows

for better detection of MPs than paraffin-embedded tissue in mussels.

The cryostat microtome uses a sharp blade to cut the tissue samples into thin slices. However, it can happen that during the sectioning, the MPs located in the gastric cavity or lumens of mussels are dragged to other epithelial areas, which could be interpreted as internalization into tissue (Benito-Kaesbach et al., 2023). In addition, random contamination of any analyzed sample is always possible due to the ubiquity of MPs by their simple deposition on the surface of the sample. These problems could lead to misinterpretation of data.

Considering this, the confirmation of the internalization of MPs in tissues of real samples is very difficult to determine. Even when all quality assurance and cleaning protocols are taken into account, the possibility of sample contamination is always high due to the ubiquity of MPs. Moreover, the small size of the MPs that are of interest and concern for ecosystems and human health, represents another serious challenge. Independent of the objective of the MPs analysis in tissues, the presence of MPs can lead to controversy, as analytical procedures can be questioned if any step of the analytical approach is susceptible to contamination. Even very well-established and accepted methodologies, such as FTIR and Raman imaging, must be considered with care and precaution regarding their sample preparation protocols that can imply contamination (Benito-Kaesbach et al., 2023).

Taking into account that the presence of MPs in animal tissues due to translocation from the digestive cavity is an important social, economic, and health issue, and that the sample may be contaminated at any time, we propose volumetric Raman imaging analysis (3D analysis) of potential target tissues in the digestive system. Detection of polyestirene MPs by surface-only Raman imaging analysis (2D analysis) of the tissue could be interpreted as the presence of MPs due to ambient contamination (spontaneous deposition of MPs on the surface of the tissue) or incorrect sample preparation (translocation of MPs from lumens to tissue surface by the blade of the cryotome). The hypothesis to be demonstrated is the following: If we were able to find MPs completely embedded in tissue, we would prove the unequivocal internalization of MPs into cells or tissues.

Mussels (*Mytilus* sp.), as sessile filter feeders, are ideal marine animals for examining the intake and accumulation of MPs, being considered as bioindicators of coastal MP pollution (Gonçalves et al., 2019a). Moreover, MPs fate in mussels tissues (digestive gland potentially) need to be assessed to understand the mechanisms of action of MPs and their possible biological impacts. Mussels are involved in the transfer of MPs along the trophic chain, which is of great importance given their active consumption by humans.

In this study, we present an analytical approach for the detection of MPs in mussel tissues (cryogenic cross sections) that avoids misinterpretation of data due to contamination of the sample as well as translocation of MPs by the microtome blade, taking advantage of the confocality of Raman microscopy. We have focused on MPs of 1 μm diameter due to their potential ecotoxicological effects on marine biota and their impacts on ecosystems health by disrupting food chain. Besides, alterations in the physical size of ingested MPs in some animals appeared to increase the chances of translocation of MPs into human tissue through the food chain. To our knowledge, this is the first time that such small particles are detected in situ in animal tissue, discarding any possibility of sample contamination or data misinterpretation.

2. Material and methods

2.1. Mussels sampling and tissue preparation for fluorescence and Raman microscopy analysis

Mytilus galloprovincialis mussels with a shell length of 3.5–4.5 cm were collected in a clean rocky intertidal area in Butroe estuary (Plentzia, 43°24′ 32.9″N 2°56′51.0″ W), in January 2021. Once in the aquaria facilities, mussels were placed in acclimation tanks for 5 days under controlled conditions (Múgica et al., 2015): salinity 33 g/Kg, 18 °C temperature, constant flow of filtered seawater and aeration, 12:12 h photoperiod, and with deprivation of food.

The MPs used were commercial particles of 1 μm in size, made of polystyrene (PS) and internally dyed with green fluorescence (FluoroMax™ Green Fluorescent Polymer Microspheres G0100B, Thermo Fisher Scientific). These particles emit green fluorescence allowing an easy detectability and tracing in mussel tissues under an epifluorescence microscope.

For the exposure, two concentrations of MPs (10^3 and 10^5 MPs/mL) and a MPs free control treatment were prepared. The concentrations used in the present work were high in order to allow the objective of the work, to assess the internalization of MPs in target tissues of mussels. Selected concentrations were in line with those used in similar works exposing marine organisms to PS MPs of 1 μm, ranging 42–2000 particles/mL (Paul-Pont et al., 2016). Nevertheless, marine plastic litter is expected to increase over the next decades, leading to an increasing global abundance of MPs in oceans. Six mussels were placed in 3 L tanks containing seawater (0.2 μm filtered). For each treatment four tanks were used, corresponding each one to a different time of exposure (1, 4, 24 and 72 h). Exposures were carried out at similar conditions as in the acclimation, maintaining mussels deprived of food in a static system. After the exposure, at each time, the soft tissue of the mussels was removed from the shell, washed in filtered seawater to avoid MPs attaching to the surface, and then cross-sectioned. The obtained sections from the digestive gland were frozen in liquid nitrogen and stored at −40 °C for further sample processing.

Dissected and cryofixed cross-sections were cut with the Leica CM 3050 S cryostat at a chamber temperature of −25 °C, resulting in 20 μm thick slices, and stored again at −40 °C before microscopy imaging. During the sectioning, cross-contamination between the different exposure treatments was avoided following the cleaning protocol used in Calmao et al., 2023 (Calmão et al., 2023b), consisting in a thorough cleaning with absolute acetone of all the material in direct contact with the samples.

2.2. Microscopic localization of MPs

To get a first overview of the presence of MPs in the mussel tissues, the fluorescent properties of the PS particles were utilized. Prior to Raman analysis, cryostat cross-sections of mussels were observed by fluorescence microscopy (Nikon ECLIPSE Ni) to affirm the uptake of MPs. In this work, 488 nm and 515 nm were used as excitation and emission wavelengths, respectively. Areas of interest, both in the lumen and in epithelia of the digestive structures, where internalization of MPs is likely to occur, were localized. For improved visualization, some sections were counterstained with a rapid metachromatic stain with a mixture of toluidine, azure II, and borax, and observed by brightfield microscopy. In this way, a pre-selection of areas of interest for the subsequent Raman analysis was made.

2.3. Volumetric Raman imaging

A Renishaw inVia confocal Raman microscope (Renishaw, Gloucestershire, UK), was used for Raman imaging analysis. The measurements were carried out with a 532 nm excitation laser (Renishaw, Gloucestershire, UK, RL532C50) with a nominal output power of 300 mW. The system has a CCD detector cooled by Peltier effect (−70 °C) and is coupled to a Leica DMLM microscope (Bradford, UK) that allows the analysis of the cross-sections by using 5× N PLAN (0.12 NA), 20× N PLAN EPI (0.40 NA) and 50× N PLAN (0.75 NA) objectives. The nominal power of the source goes from 0.05 % to 100 % of the total power. To avoid thermo-decomposition of the sample, the nominal power of the laser was adjusted to preserve the sample. The InVia spectrometer was calibrated daily, setting the 520.5 cm^{-1} silicon line. The system works under Renishaw WiRE 4.2 software.

For Raman image mapping, the samples were scanned using a 50×

objective (theoretical laser spot size diameter on the focus plane at 532 nm is 0.87 μm) with an integration time of 1.5 s, a scanning spatial resolution (motor step of the stage) of 1 μm/pixel for all axes XYZ and only 1 scan at an operating spectral resolution of $\leq$1 cm^{-1} according to the manufacturer. Different confocalities were tested (standard and high) by opening or closing the slits. In the standard confocality configuration, the slit has a 65 μm aperture and the CCD is in 10 pixels mode. Whereas in in high confocality configuration, the slit has 20 μm of aperture and the CCD in 2 pixels mode. Finally, all measurements were performed in high confocality mode to allow a more detailed analysis of the Z planes.

To obtain the Raman images and to perform the data analysis, Matlab software (MathWorks, Massachusetts, USA) was used (see below).

2.4. Data analysis

For each sample, Raman spectra were recorded with 1015 wavenumbers in 16 slices, resulting in a data cube of 120x84x16 pixels (XYZ). The MP Raman signal is expected to be strongly mixed with Raman signals from a large variety of chemical compounds in each layer and also to contain uncontrolled background fluctuations due to fluorescence as well as superimposed cosmic rays. All these artifacts have to be considered in data preprocessing. A pre-screening method was performed based on preprocessing the spectra with standard normal variate (Rinnan et al., 2009) and further sample factorization using principal component analysis (PCA) (Wold et al., 1987). This step targeted the pixels in all layers that could contain signatures of the MP. As a pre-screening method, the surface areas in each layer that showed traces of MP were identified and further analyzed individually using a spectral unmixing strategy. This spectral unmixing strategy, applied to each relevant surface region, consisted of a baseline removing preliminary step (using the Asymmetric Least Squares approach proposed by Eilers et al. (Eilers and Boelens, 2005) followed by multivariate curve resolution-alternating least squares (de Juan, 2019), imposing a non-negativity constraint in the spectral and concentration profiles. Moreover, after calculating the optimal model for each individual region, the concentration profile of the MP was isolated and scaled from 0 to 1 for better visualization (Fig. 1), and the spectral profile of the MP was validated by comparison with the corresponding standard spectrum (Fig. 2).

All data analysis was performed using HYPER-Tools (Mobaraki et al., 2018) (version 3.0. freely available at https://www.hypertools.org/-last accessed 1st June 2023) working under MATLAB environment (version 2022b. The Mathworks, Inc. Natick, MA, USA).

3. Results and discussion

The following results present two cases, first, MP found on top of digestive epithelia but not internalized into the tissue, and second, examples of MP incorporated into digestive structures, i.e., internalized into digestive cells/epithelia. We also discuss constraints in data acquisition and how some acquisition parameters affect the results.

3.1. Microplastics on top of digestive epithelia

As mentioned in the introduction, MPs could be displaced across the surface of the cryosection drawn from the microtome blade and interpreted as incorporated into the cells/tissues. Besides, due to the ubiquity of MPs, contamination of the analyzed sample by simple deposition of MPs on the sample surface is likely to occur unless strict cleaning conditions are assured. In both cases, MPs would be found on the surface and never embedded in the tissue. However, by just applying 2D Raman imaging, data misinterpretation can easily occur. For this reason, 3D imaging is absolutely essential.

In the examined cryosections (see Fig. 1), we found areas with multiple MPs on the surface. Precisely, the mussels exposed to 10^5 MPs/mL during 4 h showed the major abundances of MPs in the digestive system being, therefore, those chosen for subsequent Raman analysis. Fig. 1a shows the prismatic epithelium of the mussel stomach, with pixels in which a MP was identified highlighted in white. In all samples studied, the presence of MPs was identified utilizing at least four or five Raman bands (Fig. 2) of PS, of which the main band occurs at 1001 cm^{-1}, and secondary bands arise at 1583, 1602 and 1032 cm^{-1}. In some cases, further Raman bands of PS were distinguished at 1155 and 1450 cm^{-1}. Special care was taken concerning overlapping bands of PS and specific pigments, for example, carotenoids.

Fig. 1b shows the depth profiles of normalized Raman signal at 1001 cm-1 (main Raman band for PS) for each MP detected. There it can be observed that most of the particles are on the surface. The MPs in the water column should enter the exposed mussels through the mouth and passed through the gastrointestinal tract via the esophagus into the stomach (Eggermont et al., n.d.). The epithelium of the stomach is composed of a single layer of columnar ciliated cells that could enable the movement of MPs in the same way they push food particles. The transfer of MPs over the stomach epithelium was not expected so it reinforced the detection of 1 μm PS MPs on the surface of the cryosection. Previous works revealed 1 and 10 μm sized PS MPs within

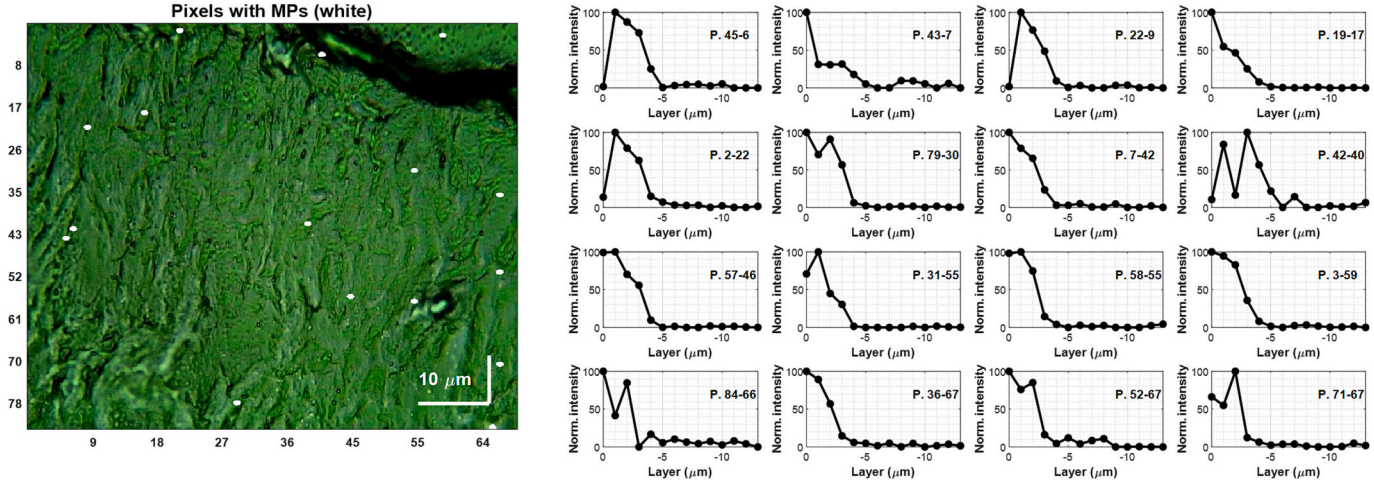

Fig. 1. a) Microscope image of the stomach epithelium of a mussel exposed to 10^5 MPs/mL for 4 h. The white spots represent detected MP particles. b) Normalized Raman intensities from the peak at 1001 cm^{-1} for the highlighted pixels on the left as a function of depth.

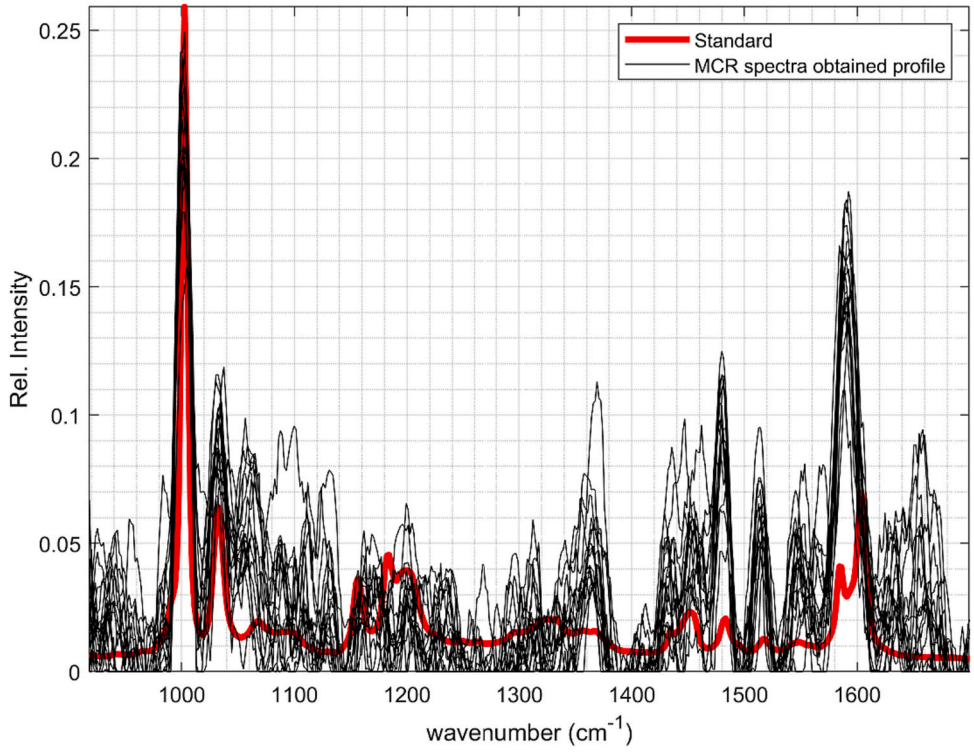

Fig. 2. Raman reference spectrum of PS in red and Raman spectra acquired during the analysis of mussel tissue.

different regions of the digestive tract of mussels, including the stomach epithelium, but concluded that most of the MPs in the lumen were rapidly cleaned by the digestive system (Gonçalves et al., 2019b).

In Fig. 1b, the particles appear to be larger than 1 μm, and some of them appear to be deeper than the focal plane Z = 0 (surface of the cryosection), i.e., they are partially embedded in the tissue and thus partially internalized. The optical resolution of the images is diffraction-limited with an Airy disc of 0.87 μm, which is smaller than the size of the MPs. One reason for the particles appearing larger than 1 μm may be the pixel size of the camera/detector, or that the observed microparticles are not single particles but aggregations of several particles. Another artifact can occur when a particle falls into two or more image pixels due to the spatial resolution of the scanning XYZ stages.

3.2. *Microplastics internalized in cells/tissues*

Microscopic observations of cryosections showed fluorescent particles located in the digestive gland of exposed mussels, especially after 4 h of exposure to 10^5 MPs/mL (Fig. 3). In fact, the food digestion in mussel has been demonstrated to last around 4 h according to the lysosomal activity of the digestive cells (Izagirre et al., 2009). From the stomach, MPs could reach the digestive gland, conformed by several tubules that end in alveoli where digestion takes place (Zaldibar et al., 2008). The interesting observation is that MPs were present in the lumens and epithelia of the digestive gland (Fig. 3).

Using the volumetric Raman technique, several particles were found embedded in the tissue (Figs. 4 and 5) in addition to the MPs found on the focal plane Z = 0 (surface of the cryosection), indicating internalization by digestive cells corresponding to the digestive vesicles. These cells have a well-developed endolysosomal system with a high capacity to perform cytosis processes and internalization of foreign particles (Zaldibar et al., 2008).

After analyzing several 3D Raman images, we found that in all cryosections the clearest examples of internalized MPs appeared in the

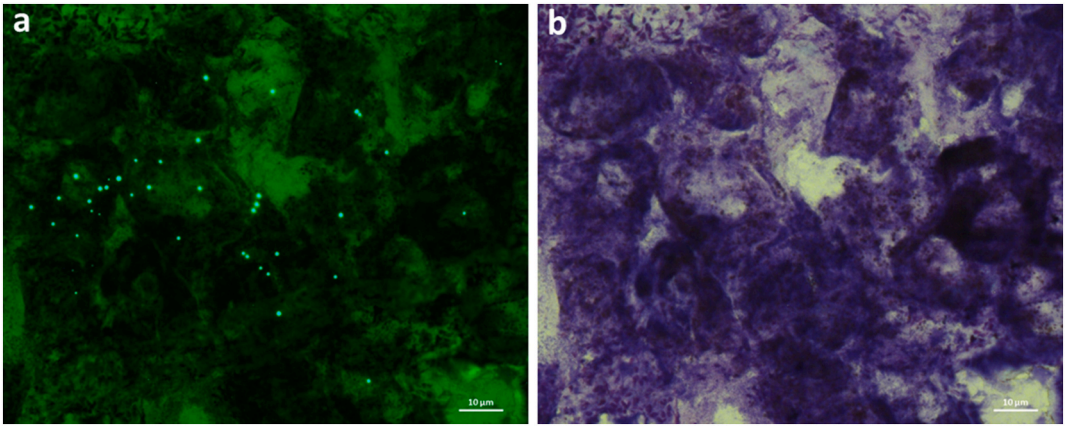

Fig. 3. a) Fluorescence and b) toluidine stained brightfield micrographs of the digestive alveoli of the digestive gland of mussels exposed to 10^5 MPs/mL for 4 h. Scale bar: 10 μm.

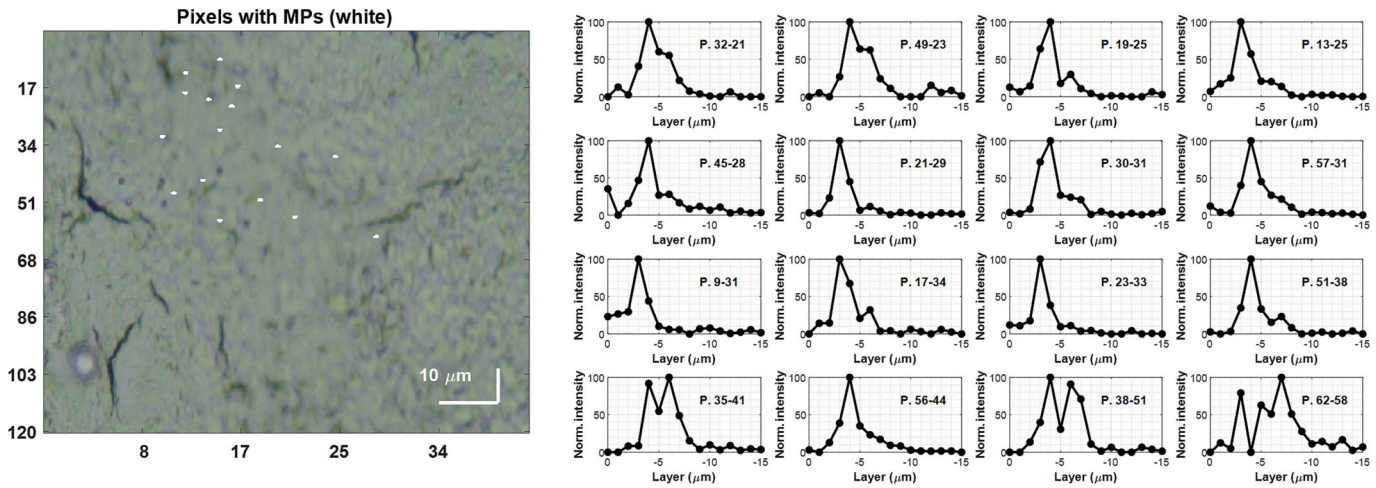

Fig. 4. a) Microscope image of the digestive gland of a mussel in the area of the digestive alveoli. The white spots represent detected MP particles. Scale bar: 10 μm. b) Normalized Raman intensities from the peak at 1001 cm-1 for the highlighted pixels on the left as a function of depth.

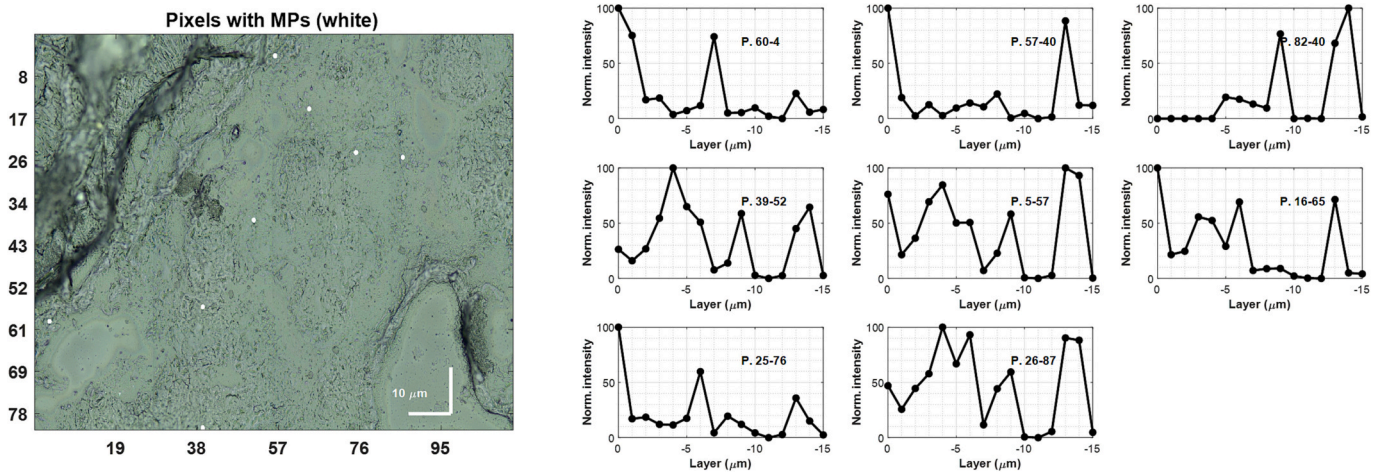

Fig. 5. a) Microscope image of the digestive gland of a mussel in the area of the digestive alveoli. The white spots represent detected MP particles. Scale bar: 10 μm. b) Normalized Raman intensities from the peak at 1001 cm^{-1} for the highlighted pixels on the left as a function of depth.

range between the focal plane and 10 μm in depth (Figs. 4b and 5b). For the selected wavelength and laser power, the penetration depth is limited by the extinction, and Raman signals from deeper layers do not have a proper signal-to-noise ratio (SNR). To obtain better signals from deeper layers, higher laser power is required, but only at the cost of tissue damage (tissue is burned) and the risk of melting the MP particles. Increasing the integration time and the number of scans can possibly improve the SNR, but the whole measurement procedure will become unmanageable. It should be emphasized that under the selected conditions, the acquisition of 16 layers from the surface to −15 μm with an area of 80 × 100 μm^2 per layer required about 72 h.

3D representations of the Raman images allowed us to confirm the actual position of each detected MP and helped us determine whether the MPs were embedded in the digestive tissue or in the lumen. Supplementary material 1 represents the results obtained from the Raman analysis of a cryosection of a mussel in the digestive alveoli. MPs are shown in green color, and natural pigments (such as carotenoids) that are directly related to the tissue are shown in red color. Therefore, the zones with presence of pigments can be considered as the tissue structure. In this sense, it can be clearly seen that some MPs are completely embedded in the tissue at different depths. This finding is unequivocal evidence for the presence of MP inside the digestive epithelium. Further research is suggested to decipher whether MPs are placed within the cells after trespassing the cell membrane or crossing the epithelium through the intercellular space. According to the obtained results, Raman spectroscopy and especially 3D Raman imaging is becoming a self-sufficient technique for the complete detection and characterization of MP in complex matrices (Supplementary material 1. 3D representation of an analyzed mussel cryosection volume).

Nevertheless, our analytical approach cannot be considered a routine analysis, especially from the point of view of the total time required for the analysis. It is technically hard to find microparticles in large macroscopic areas in reasonable time by confocal scanning Raman spectroscopy. Nevertheless, by full-field imaging with fluorescence or polarized-light microscopy beforehand, identification of particles in target tissues can be faced in a valuable and accessible form. In this light, our approach would have difficulties to calculate the internalization rate or to be used for monitoring and epidemiological studies where a large number of samples have to be analyzed. However, we believe that our analytical approach is the best option to study very small MPs, to test whether internalization occurs, and to study other parameters related to the mechanisms. In addition, the proposed method completely avoids problems, misinterpretations, or doubts that could arise due to accidental impurities on the surface of the samples.It was not our intention to calculate the internalization rate or the minimum size at which the integration can occur, but the qualitative analysis of the samples

analyzed seems to indicate that the internalization rate is rather low when comparing the MP dose at which the mussels were exposed to the small number of MPs detected. In fact, features of the MPs such as the plastic nature (e.g. softness, smoothness), the size and shape, together with the exposure dose, could interfere with the internalization rate in the gut of bivalves (Sendra et al., 2021).

The size of MPs likely plays an important role in their ability to penetrate biological barriers, as noted by some authors. This fact is controversial, as several studies in the literature show that large MPs have been incorporated into tissues, even in fish muscles. However, volumetric Raman imaging was not used in all of this work. Instead, 2D Raman imaging was applied.

4. Conclusions

We have presented evidence for the internalization of MPs into digestive epithelial tissue (digestive cells that form the digestive alveoli) in mussels exposed to MPs using volumetric Raman imaging and chemometrics. In our analytical approach, we avoid doubts that might arise due to accidental sample contamination by looking for MPs that are completely embedded by the tissue and whose presence in such a situation can only be due to the MP internalization. However, it should be highlighted that the proposed methodologies present some limitations. For example, at low concentration of MPs exposure we are not able to discern if the MPs are not internalized or the technique is not sensible enough to detect them in the tissue. Moreover, the 3D Raman images suppose long acquisition periods and provide heavy files which treatment is not fast-forward. In contrast, this methodology is one of the easiest, cheapest, non-destructive, co-localized which allows to confirm without doubt the internationalization of MPs in an accurate position of an organism cryosection.

Regardless the limitations, the present work is extremely relevant as the worry about human health arises from the internalization of tiny MPs in mussel tissues and other species consumed by humans. If animals accumulate MPs, there is a concern that they could be passed on to humans through the consumption of contaminated food. The potential effects of MP are not fully understood, but multiple studies have described adverse effects and highlighted the possibility of MPs acting as carriers for hazardous or emerging contaminants, including the release of heavy metals.

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CRediT authorship contribution statement

Julene Aramendia: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Nerea García-Velasco:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Jose Manuel Amigo:** Data curation, Formal analysis, Software, Validation, Visualization, Writing – review & editing. **Urtzi Izagirre:** Conceptualization, Investigation, Methodology, Resources, Supervision, Writing – review & editing. **Andreas Seifert:** Funding acquisition, Resources, Supervision, Writing – review & editing, Investigation, Project administration, Validation, Visualization. **Manu Soto:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing. **Kepa Castro:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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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