



Combination of Microscopic and Spectroscopic Techniques to Study the Presence and the Effects of Microplastics in Mussels

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Published: 4 December 2015

Abstract: The growing concern due to the presence of plastics, especially micro and nanoplastics, in environmental aquatic media requires the development of new methodologies to study the distribution of these particles and the effects that might cause in many organisms. In this work we have performed experiments using synthetic polystyrene microplastics (6-90 μm diameter) and mussels (*Mytilus galloprovincialis*) and we have studied the distribution of these particles by different techniques including FTIR and Raman spectroscopy, light and polarized light microscopy after being exposed for different periods of time (1-72 h). As a result of this work we were able to fine tune the preparation of the samples, from conservation to image and spectra analysis, and it was concluded that it was better to freeze the samples and to prepare the cryosections instead of embedding in paraffin. Regarding the light microscopy darkfield illumination offered less background signals than polarized one and therefore it was more suitable for small size particles. Finally, Raman spectroscopy allowed the characterization of the polystyrene particles better than FTIR allowing the development of image analysis techniques.

Keywords: microplastics; mussels; uptake, accumulation and distribution; method development; microscopy; Raman spectroscopy, FTIR spectroscopy

1. Introduction

There are few features comparable to the evolution and deep impact of polymeric materials and plastics in modern way of life. According to the information provided by the manufacturers, the global production is above 300 million of tons (1). Part of this production is discarded in uncontrolled plastic debris that are physically fragmented in smaller pieces and end up in river and oceans. In addition to the coarse plastic materials, the production and use of microplastics (MPs) in cosmetics and personal care products shows a specific interest due to the higher distribution rate in many environmental compartments. In any case, the impact of the presence of these MPs in aquatic organisms and in food is still being studied (2-4).

2. Results and Discussion

Differences in MPs distribution were observed in mussels according to the size of the MPs. Big MPs were mainly detected in the lumen of the stomach and also in the digestive conducts, but not in the digestive tubule epithelium. Additionally, smaller MPs (6 and 10 μm diameter) were observed in the connective tissue surrounding the stomach and digestive gland and also in a lesser extent in the lumen of the digestive tubules and inside the digestive epithelium.

Significant differences were observed according to the technique for the MP visualization. Both polarized light and darkfield illumination were able to detect MPs more relevantly than brightfiels illumination. However, darkfield illumination presented more signal to noise ration (Figure 1). Finally, it is noteworthy that in paraffin embedded samples no

The analysis of the microplastics in the physical aquatic media has been described in the most recent literature (2, 5) but the analysis in organisms still requires a deeper methodological development. In any case, the methodological and instrumental approaches depend among others on the size ranges of the particles that are analysed and the sort of effects that are looking for (6).

In this context, the main aims of this work were to develop the methodology to study the accumulation of polystyrene microplastics in controlled exposure experiments to support further studies and to get some insights about the accumulation and distribution of these microplastics in the different tissues.

microplastic were detected but mechanical damage related to big microplastics was observed (Figure 1). In fact, during the sample preparation of paraffin embedded samples the MPs were dissolved.

The Raman results acquired at the same time that we made the histological study helped us to assure that we looked at the microscope was undoubtedly polystyrene MPs. In the Raman spectra of the figure 1 we can observe the main band of the polystyrene at 1000 cm^{-1} . We found the Raman measurements necessary since we got some noise problems or even false positives with the microscopic techniques. It is true that in this study there are few confounding factors since the shape of the MPs that we used for this experiments were identical. But, in the real world, with real samples, the MPs present a huge variety of shapes and colours. Therefore we

found useful to combine microscopy results with Raman results.

The FTIR images provided us biochemical information as well as the distribution of the

MPs. Anyway, to get the chemical information we were looking for it was necessary to use chemometric tools such as MCR and PCA.

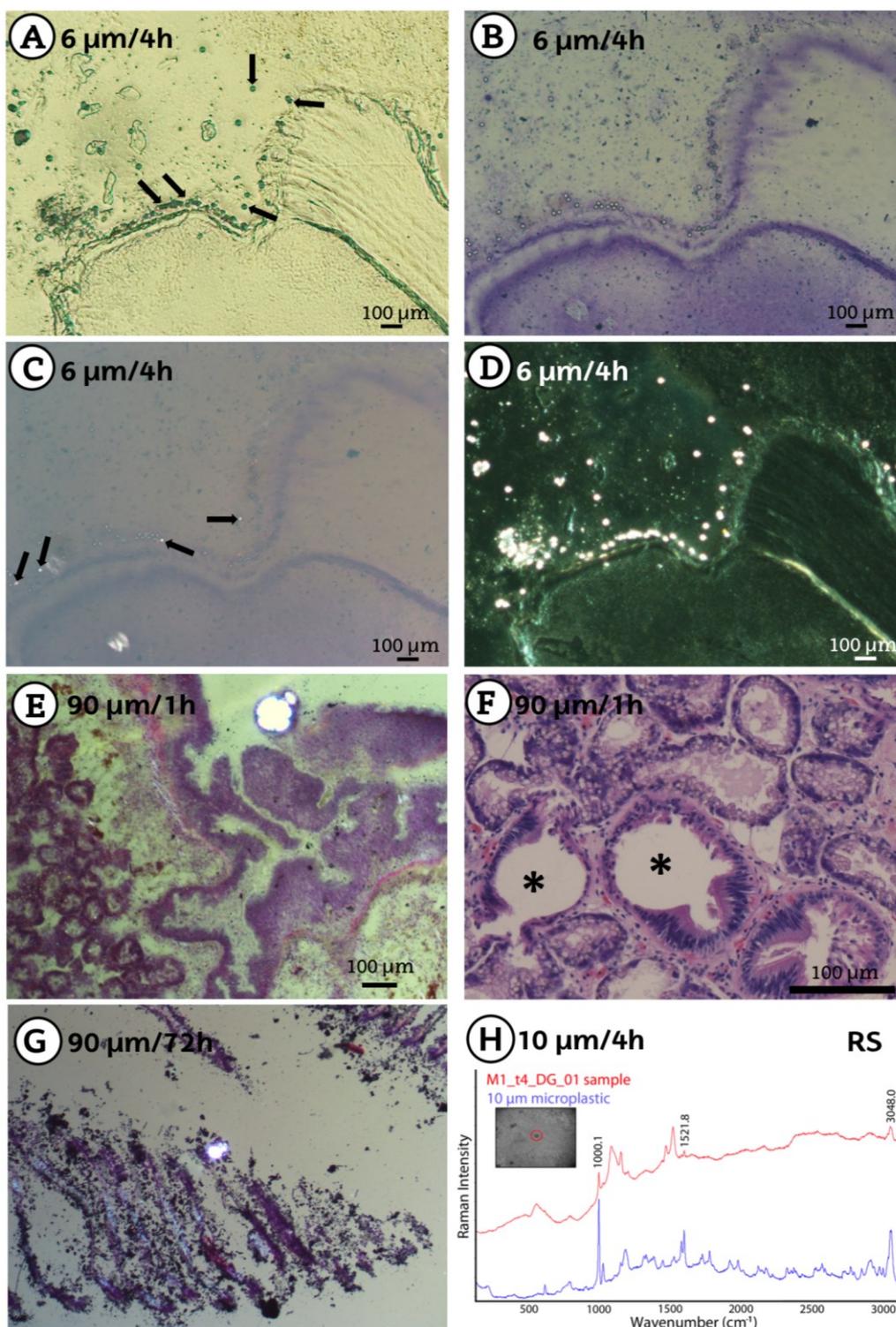


Figure 1. Microplastics (MPs) in the digestive gland of mussels with different illumination techniques (A-F). A-D; 6 µm diameter MPs after 4 h of exposure after brightfield (A and B), polarized (C) and darkfield illumination (D). E-F; 90 µm diameter MPs after 1 h of

exposure after polarized (E) illumination and brightfield illumination in paraffin embedded tissue (F). And asterisks indicate the mechanical damage induced by 90µm diameter polystyrene MPs in the secondary ducts. In the G picture can be observed 90 µm MPs in the gills after 72 h of exposition. Raman Spectroscopy (RS) was employed to check if we really are observing MPs. Arrows indicate the MPs.

3. Materials and Methods

I. Exposure experiments.

Mussels (*Mytilus galloprovincialis*) were collected in the estuary of the Butroe river (Bay of Biscay, Basque Country) and immediately transferred to the laboratory.

Mussels were exposed to three polystyrene (Alfa Aesar) microplastics of different sizes (6, 10 and 90 µm diameter). Mussels were collected after, 1, 4, 8 and 72 h of exposure. Then, they were dissected and some were formalin fixed and routinely processed for histological observation after paraffin embedding. Other mussels, were snap frozen in liquid nitrogen and stored at -80°C for further cryostat sectioning.

II. Microscopy study.

Both paraffin embedded, and cryostat frozen samples were observed under the microscope in different illumination conditions in order to detect the MPs. On the one hand, mussel tissue sections were observed with brightfield and darkfield illumination in a Nikon ECLIPSE TI-S (Nikon, Tokyo, Japan) microscope. On the other hand, samples were also observed under polarized light in a Olympus BH2 microscope (Olympus Corporation, Tokyo, Japan).

III. Spectroscopy study.

Raman spectra were acquired at the same time that we were performing the microscopy study to verify the presence of the MPs and to avoid false positives. InnoRaman™ portable spectrometer (B&WTEK_{INC}, Newark, USA) coupled to a microscope (20x and 50x magnification) and provided with 532 nm laser and CCD detector (Peltier cooled) was used for this issue.

The region of interest, previously selected in the microscopy study, was imaged on a Jasco IMV4000 FTIR imaging system equipped with a liquid nitrogen cooled 16 element linear array MCT detector. The cryosections were placed on a ZnSe sample holder and imaged directly. For each sample image, background was recorded using the same experimental parameters and on an empty region of the ZnSe sample holder. Once the FTIR images were acquired, principal component analysis (PCA) and multivariate curve resolution (MCR) analysis (MATLAB) were performed to get the spatial distribution of MPs .

4. Conclusions

Tissue distribution of MPs varies with their size. 90 µm diameter MPs were mainly limited to the stomach and ducts while 6 and 10 µm diameter MPs were found mainly in the connective and sometimes in the digestive epithelium as well.

One of the keys to obtain good results has been the sample preparation. Frozen tissue allows a better detection of MPs than paraffin embedded tissue in mussels.

Regarding to the microscopy study, polarized light and darkfield microscopy are useful tools to detect microplastics in mussel's tissue but they are not 100% reliable due to the fact that we found some false positives. We recommend verify the results with molecular spectroscopy, such as Raman and FTIR.

Acknowledgments

This work was supported by the Swedish Museum of Natural History, University of Stockholm, Sweden and by the University of the Basque Country/Basque Government through the grants to CBET and IBeA Consolidated Research Groups. Technical support in the FTIR imaging analysis provided by SGIker (UPV/EHU) is gratefully acknowledged. Mireia Irazola acknowledges to the UPV/EHU for the PhD grant.

Conflicts of Interest

The authors declare no conflict of interest.

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