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SHORT COMMUNICATION

Development of a method for the detection of polystyrene microplastics in paraffin-embedded histological sections

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Abstract The concerns about the presence of microplastics (MPs) in marine ecosystems have widely increased in the past years. This is reflected in a growing number of studies addressing the effects of exposure to these materials in indigenous, farmed and even laboratory marine animals subjected to toxicity-oriented bioassays. There have been, however, many constraints in the detection of MPs in biological tissues, as routine histological techniques tend to degrade these materials, which are especially sensitive to organic solvents. This issue hinders the application of standard histopathological procedures based on convenient paraffin wax-embedding protocols, with consequences for biomonitoring and bioassay procedures. The method described here was developed and validated for the detection of polystyrene microplastics in biological tissue processed for paraffin-based histology. The strategy was developed and tested from whole-soft body sections of marine mussels that internalised the MPs following dedicated bioassays. The protocol is based on the replacement of xylenes with isopropanol for the purpose of intermediate infiltration and deparaffinization. Special modifications for staining, mounting and archiving are needed and are detailed as well. The protocol is shown to be a highly cost- and time-effective procedure compatible with formalin-based fixatives plus standard sectioning and staining, yielding complete preservation of MPs and optimal tissue conditioning. The method also produced excellent results with pre-stained MPs, with fluorochromes included, altogether providing excellent localisation of polystyrene MPs in paraffin-processed biological tissue.

Keywords Histopathology · Plastic · Microparticles · Isopropyl alcohol · Paraffin sections

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Introduction

The ubiquity of plastic debris in marine ecosystems has been leading to a growing awareness towards these emerging potential pollutants in the past years, with emphasis on their potential internalisation by aquatic organisms. As a consequence, the number of studies aiming at determining the exposure and effects of microplastics (i.e. particle size less than 5 mm) has been increasing (see for instance, Duis and Coors 2016; Wang et al. 2016; Wright et al. 2013). Despite the wide variety of plastics being released into the marine environment, polystyrene is considered to be one of the most important polymers (Hidalgo-Ruz et al. 2012; Moore 2008).

Detecting microplastics (MPs) eventually ingested by organisms is a key component of both biomonitoring and testing procedures. Histopathology, in particular, is, as for other contaminants, a critically important tool that permits inferring the presence of MPs in tissues and organs and eventual adverse effects altogether. However, standard histological processing has been shown to be incompatible with MPs, polystyrene and similar polymers in particular, as strong organic solvents tend to dissolve the materials or render them too brittle for proper sectioning. Previous experiments conducted by the authors of the present work showed indeed that solvents such as xylenes or similarly chloroform and even alcohols damage, deform or completely dissolve

polystyrene MPs. On the other hand, fresh preparations of biological tissue are not compatible with histopathological assessment and cryotomy is far more challenging and expensive than paraffin embedding (or similar wax), which still provides the most effective method for routine processing of animal samples.

In face of the abovementioned constraints, the present work aimed at developing an adequate, simple and efficient technique for the processing of paraffin-based histological samples that is able to preserve polystyrene or similar MPs while safeguarding tissue quality for histopathological assessment. For the purpose, mussel (*Mytilus* sp.) was taken as a biological model, since these organisms are one of the most surveyed targets for addressing ingestion and potential effects of microplastics, due to being filter-feeding bivalves (Browne et al. 2008; Farrell and Nelson 2013; Van Cauwenberghe et al. 2015; Wegner et al. 2012).

Materials and methods

Experimental exposure and sampling

Mussels, *Mytilus* sp. (40–50 mm shell length; 15–20 mm shell width), were collected from the Portuguese west coast, between April and September 2016. After a short acclimatisation period, mussels were exposed laboratorially for 20 min to the nominal concentration of 1000 particles·mL^{-1} (aqueous suspension) of two polystyrene MPs with different diameters: 6.0 µm (Alfa Aesar) and 10.0 µm (Sigma-Aldrich). The smaller MPs were yellow-green fluorescent (excitation-emission range of 441 and 486 nm, respectively). At the end of the experiment, three mussels from each treatment plus a control (exposed to clean seawater only) were collected for histological processing.

Fixation

Davidson's was taken as fixative of choice, being one of the most common alternatives for marine invertebrates (9–10% formalin, 10% acetic acid and 10% ethanol, in distilled or pure water). The fixation procedure follows Costa (2018). In brief, the fixative solution was applied through intravalvar injection coupled with immersion o/n (overnight) at room temperature. After this period, the soft body was removed from the shell, sectioned dorsoventrally and allowed to fixate up to 24 h total in fresh fixative at room temperature as well.

Embedding

The tissue was processed by adapting the protocol of Doxtader (1948), who described a method that uses isopropanol (isopropyl alcohol) for intermediate infiltration as a faster alternative to xylenes, potentially leading to better preservation of tissue. This alcohol, being miscible with water and molten paraffin, replaces ethanol during dehydration. Briefly, after fixation samples were washed with MilliQ-grade (16.2 MΩ cm) water (4 × 15 min) and then subjected to a series of progressive (aqueous) isopropanol: 70% (1 × 30 min); 95% (3 × 15 min) and 100% (3 × 1 h). Low-pressure vacuum was applied at every step to assure complete infiltration. Afterwards, samples were immersed in molten paraffin wax (Paraplast grade, melting temperature 56–58 °C, Merck) and allowed to embed o/n. After that, samples were subjected to low-pressure vacuum for 5–10 min (with heat) and allowed to infiltrate for a further 24 h to optimise embedding. At least one extra paraffin change was encouraged for larger specimens to assure removal of isopropanol.

Staining and mounting

Histological sections (5 µm thick) were obtained using a Jung RM 2035 rotary microtome (Leica Microsystems). Deparaffinization and rehydration were done using isopropanol as well. However, as isopropanol is a weaker remover of solidified paraffin compared to xylenes or similar chemicals, slides were heated to 65°–70° (to melt paraffin without damaging tissue) before immersing in 100% isopropanol, which was repeated until complete removal of wax. Slides were then immersed in 95% v/v aqueous isopropyl alcohol (2 × 1 min), followed by 70% v/v aqueous isopropyl alcohol (30 s) and finally brought to MilliQ-grade water (6 min). Slides were stained with haematoxylin (Harris') and counterstained with alcoholic Eosin Y (working concentration 0.2% m/v), followed by a quick rinse in water (H&E staining). Blueing of haematoxylin was done with tap water. Partial dehydration before eosin dyeing was done by immersion in 70% v/v aqueous ethanol for 3 min, which did not cause significant damage to plastics. As resinous mounting media contain strong solvents (commonly xylenes), aqueous agents are required. In the present case, slides were mounted with 50% v/v aqueous glycerol immediately after staining (preparations should not be allowed to dry) and sealed with nail polish. Other agents, such as gum arabic, may also give satisfactory results. Finalised slides can be stored in the dark at room temperature for an indefinite period of time without damage to tissue or dye fading. Analyses were done with a DMLB model microscope adapted for epifluorescence

with an EL6000 light source for mercury short-arc reflector lamps and equipped with a DFC480 digital camera (all from Leica Microsystems).

Results and discussion

Whereas xylenes dissolve polystyrene MPs (Fig. 1b), isopropanol does not affect MP structure (Fig. 1c), at least in a timescale compatible with tissue processing. Particle fluorescence was not affected by this solvent, showing that the protocol was compatible with chromogens and fluorochromes embedded in plastic, which are handy modifications for localising MPs (Fig. 1d). As demonstrated, the replacement of ethanol and xylene with isopropanol maintained the shape of polystyrene and retained tissue integrity for histological and histopathological analyses. In this case, MPs were unambiguously located in the mussels' midgut region

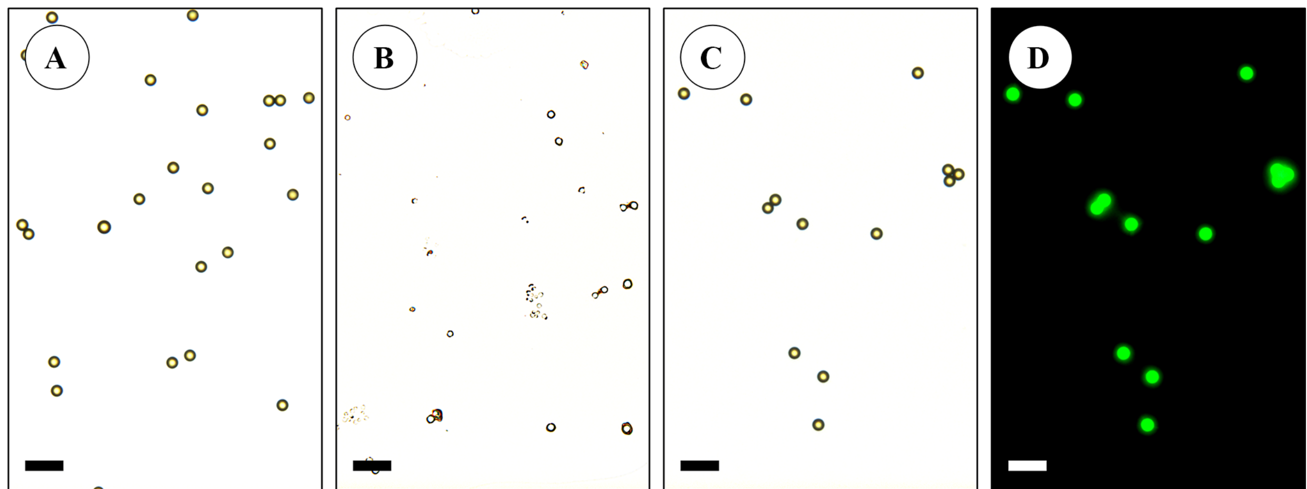

Fig. 1 Photomicrographs representing the comparison of 6 µm polystyrene microparticles exposed to different solvents. Scale bars: 20 µm. **a** A representation of MPs in aqueous medium, without adding organic solvents. **b** MPs submerged in xylene for 30 s, demonstrating clear and rapid degradation of polystyrene materials. **c** MPs submerged in isopropanol for 30 s. There is no evidence of any alteration in physical characteristics compared to **a**. **d** After 30 s of immersion in isopropanol, the MPs retain integrity and the YG fluorescence is not affected. Images were obtained using an N2.1 filter, 560/590 excitation/emission, respectively (Leica Microsystems)

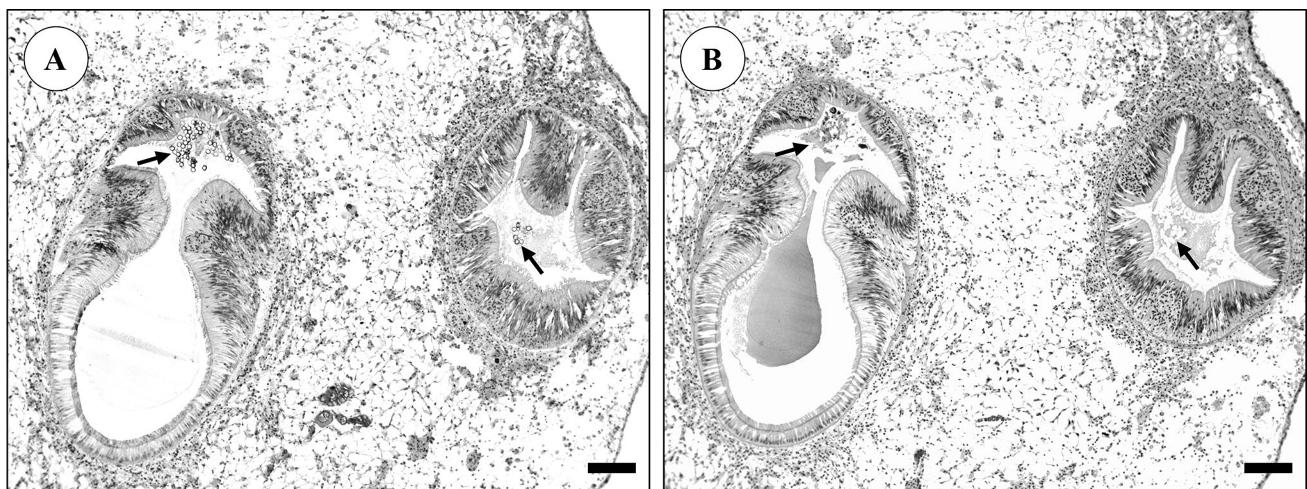

Fig. 2 Comparison of the same histological section of *Mytilus* sp. digestive tract, containing polystyrene microparticles, after exposure to two different solvents for tissue processing. Scale bars: 200 µm. **a** Section of tissue processed with isopropanol. Note that MPs (arrows) were visibly located in different sections of the midgut region and the tissue integrity was not compromised. **b** Section of tissue after being infiltrated with isopropanol, but deparaffinized with xylenes, to enable a direct comparison with the preceding panel. In this case, MPs were evidently dissolved by xylenes, hindering their detection in the tissue (arrows)

Table 1 Proposed protocols for the histological processing of biological samples for the detection of polystyrene microparticles

Step		Duration
Fixation	Davidson's	24 h total, with at least one change to fresh fixative for large samples
Wash	MQ water	4 × 15 min
Dehydration	70% v/v aqueous isopropanol	1 × 30 min
	95% v/v aqueous isopropanol	3 × 15 min
	100% isopropanol	3 × 1 h, with vacuum
Embedding	Paraffin wax	After 24 h, submit to vacuum. With larger samples, change to fresh paraffin to assure complete removal of isopropanol (at least once)
Staining (H&E)		
Deparaffinization	Alternate heating of slides (65°–70°) and 100% isopropanol	Until complete paraffin removal
Rehydration	95% v/v aqueous isopropanol	2 × 1 min
	70% v/v aqueous isopropanol	30 s
	MQ water	6 min
Stain	Haematoxylin and eosin	Traditionally 2 min (haematoxylin) + 1 min (eosin, 0.2% m/v). For partial dehydration 70% v/v aqueous ethanol (3 min) was employed, without adverse consequences
Mounting agent	Aqueous mounting medium (e.g. 50% v/v aqueous glycerol)	–

(Fig. 2a). Contrarily, as shown in Fig. 2b, which represents a consecutive section but deparaffinized with xylenes, polystyrene MPs were dissolved by the solvent. Preliminary trials had already shown that standard embedding processes, involving xylenes or similar chemicals, affected polystyrene MPs as well. Typically, the interactions between organic solvents and plastic may cause changes in the chemical properties of these materials (Lampman 2003). In the case of polystyrene, the effects of its interaction with a solvent containing either aromatic groups (i.e. xylene) or strongly polar groups (as in ethanol) can result in polymer dissolution.

Some authors highlighted that isopropanol is an inexpensive and easy-to-use solvent that, albeit with little or no use in current histological practice, may simplify and accelerate the procedure by eliminating intermediate embedding without generating artefacts (Viktorov and Proshin 2003). Also, this solvent is significantly less toxic comparatively to xylenes and assists in the preservation of animal tissue (Doxtader 1948; Hauser 1952). Although it is less efficient to remove solid paraffin, Paraplast or similar waxes by itself, the conjugation of heat with isopropanol proved to be suitable for deparaffinization with little extra time. In addition to H&E, slides were also tested for other fluorescence staining methods, namely using Acridine Orange (AO) and DAPI, and the results were equally satisfactory (not shown).

The results also showed that formalin-based fixatives are compatible with the procedure, even those that, such as Davidson's, contain a minor percentage of ethanol. On the contrary, anhydrous fixatives based on organic solvents only (such as Carnoy's) yielded complete dissolution of polystyrene MPs (not shown), despite the advantages of faster tissue infiltration and, in the particular case of marine invertebrates, better preservation of sugar-rich connective tissue. The detailed protocol is summarised in Table 1.

Conclusions

The proposed methodology is suitable and expeditious for studies involving the search for microplastics within biological tissues. The use of an alternative solvent, isopropanol, which did not react with polystyrene during tissue processing, ensured not only the integrity of plastic but also the perfect suitability of the tissue for histopathological practice. The method yielded good details and at least no more artefacts, sample hardening or tissue distortion than standard protocols. Furthermore, the procedure is compatible with common staining methods for bright field and fluorescent microscopy.

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Compliance with ethical standards

Conflicts interest The authors declare that there are no issues regarding ethics, conflicts of interest or animal testing to be declared.

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