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# Optimisation of enzymatic digestion and validation of specimen preservation methods for the analysis of ingested microplastics

Winnie Courtene-Jones<sup>1\*</sup>; Brian Quinn<sup>2</sup>; Fionn Murphy<sup>2</sup>; Stefan F. Gary<sup>1</sup>; Bhavani E. Narayanaswamy<sup>1</sup>

## Addresses:

<sup>1</sup> Scottish Association for Marine Science, Scottish Marine Institute, Oban, Argyll, PA37 1QA, Scotland

<sup>2</sup> Institute of Biomedical and Environmental Health Research (IBEHR), School of Science & Sport, University of the West of Scotland, Paisley, PA1 2BE, Scotland.

## \*Corresponding author:

Email: winnie.courtene-jones@sams.ac.uk

## Abstract

Microplastics are considered to be a widespread environmental contaminant. Due to their small size microplastics have the potential to be ingested by a range of aquatic organisms which mistake them for a food source and can suffer adverse impacts as a result. Development of standardised methods is imperative to provide reliable and meaningful data when analysing microplastic ingestion by marine fauna. A range of proteolytic digestive enzymes (trypsin, papain and collagenase) were tested to establish optimum digestion efficacy of biological samples and assess the effects of enzymes on microplastics; additionally the applicability of freezing and formaldehyde followed by ethanol as specimen preservation techniques for microplastic research was investigated. Of the enzymes investigated, trypsin yielded the greatest digestive efficacy based on weight reduction ( $88\% \pm 2.52$  S.D.) at the lowest concentration (0.3125 %) with no observed impacts on microplastics. Enumeration of microplastics from wild collected *Mytilus edulis* revealed mean numbers of  $1.05 \pm 0.66$  S.D. (minimum) to  $4.44 \pm 3.03$  S.D. (maximum) microplastic particles  $g^{-1}$  wet weight mussel tissue depending on location. There was no significant difference based on preservation method on the quantification of ingested microplastics and no detrimental impacts were observed on the microplastics directly. Enzymatic digestion using trypsin therefore provides a suitable, time and cost effective method to extract microplastics from *M. edulis*. Furthermore the preservation methods did not have detrimental effects on microplastics, serving to highlight the suitability of biological samples preserved either way for future inquiries into ingested microplastics.

## Keywords:

Microplastic, Enzymes, Trypsin, Dissociation, Preservation, Mussels

## 1. Introduction

Worldwide plastic production has increased rapidly since the mid-twentieth century<sup>1</sup>. Around 30 % of the plastic items made have single-use application and are discarded within a year of manufacture<sup>2</sup>. The durable nature of plastics coupled with a throw-away culture has led to escalating plastic waste management issues and the global accumulation of this pollutant. Of particular concern due to their ubiquity and persistence in the environment are microplastics: plastics fragments, fibres and beads between 0.5 mm to 100 nm in diameter<sup>3</sup>, produced by the degradation of larger plastic items or manufactured purposely to be of small size<sup>4</sup>. Microplastics are potentially bioavailable to a wide range of organisms being of a size similar to prey items or sediment grains<sup>5,6</sup> and pose a severe threat to aquatic life<sup>7</sup>. Microplastic ingestion has been documented in a range of vertebrate (e.g. fish) and invertebrate (e.g. zooplankton, mussels) species in both laboratory and field conditions<sup>8-13</sup> with detrimental effects observed.

Microplastic research is a developing field and as a result a number of fundamental research questions remain. However, progress and data comparability is hampered by a lack of methodological uniformity. To effectively monitor the temporal and spatial trends of microplastics it is imperative to establish standard operation protocols (SOPs) which deal with a range of samples, e.g. sediment, water and fauna. Currently the most widely used techniques to extract microplastics from fauna are based on wet digestion using strong bases, or more commonly acids<sup>14</sup>. While acid and alkaline dissociation are effective in dissolving organic material they degrade or even destroy some pH-sensitive polymers<sup>14-16</sup>. Dissociation enzymes offer an alternative method due to their high digestive specificity, meaning proteinaceous material is acted upon leaving synthetic materials intact and unaffected<sup>17</sup>, thus providing a more appropriate way to extract microplastics from organic material. The development of an enzymatic method using proteinase-K has been utilised to extract ingested microplastics from zooplankton samples<sup>15</sup>. It is necessary to consider a range of proteinaceous enzymes and their digestive efficiencies on other species to develop an alternative enzyme extraction method and provide a cost effective and rapid protocol for widespread application across environmental monitoring sectors.

While efforts are being made to develop and standardise extraction methods it is also important to consider the treatment of samples prior to analysis, and evaluate what effects methods of sample storage may have on microplastics, to ensure comparability between all stages. Biological samples are usually stored for a period of time between collection and processing in one of two ways, either by using formaldehyde followed by ethanol, or by freezing. Fixation of tissues using dilute concentrations of buffered formaldehyde followed by storage in ethanol is a widely used technique especially for morphological species identification and community analyses and microplastics have been extracted from specimens preserved using this technique<sup>18-22</sup>. Samples treated with formaldehyde and ethanol can be stored for long periods of time in cool, dark conditions. More recently freezing at -20°C has been suggested as a preferred non-destructive preservation method for specimens specifically for the study of microplastics<sup>23,24</sup>. While fauna preserved using both techniques are utilised to assess ingested microplastics, there is no investigation as to whether the techniques produce comparable results. Both techniques can cause changes in the cellular stability and size of specimen tissues<sup>25,26</sup>, but what effect these techniques may have on ingested microplastics is unclear. Elucidating methodological differences and establishing best practise is imperative to provide comparable and meaningful data and develop the field of microplastics research.

Here, we aim to develop and validate a non-destructive method to extract microplastics from fauna preserved using different techniques. The model organism *Mytilus edulis* is utilised as microplastic ingestion has been widely reported in this species<sup>8,12</sup>, and mussels act as bioindicators for aquatic contaminants due to the large volumes of water drawn across the gills when filter feeding<sup>27</sup>. Enzymatic digestion using a range of dissociation enzymes and concentrations were performed on wild mussels to optimise digestive efficacy and establish the effects on microplastics. This study additionally signifies the first efforts to examine the effect of biological specimen preservation on microplastics directly and those present in fauna. A simple, rapid and effective enzyme digestion extraction method is developed to accurately assess microplastics in biological samples, considering extraction from differently preserved specimens and working to establish an accessible and efficient operation protocol.

## 2. Method

### 2.1. Contamination mitigation protocol

Given the ubiquity of microplastic fibres in the environment a number of steps were employed to monitor and reduce potential routes and sources of contamination. Samples were prepared and analysed in a separate small laboratory to minimise the number of people coming into contact with samples. Air vents were covered to mitigate against air borne contamination and the door remained closed for the duration of the experiment. The benches were cleaned with 70 % ethanol on non-shredding paper and allowed to dry fully; this was repeated three times prior to commencing work. Standard non-plastic equipment i.e. glass and metal, were used wherever possible and consumables were used directly from sterile packaging. All apparatus was washed with deionised water prior to use and equipment was inspected under a dissecting microscope to ensure they were free from contaminants. The samples were covered wherever possible to minimize exposure risk. Personnel working on the samples wore natural fibre clothes under a clean 100 % cotton laboratory coat.

Two further methods were applied to take into account any potential background contaminants based on Murphy et al<sup>28</sup>. Dampened filter paper (30 mm diameter, Whatman No. 1) was placed in a clean petri dish to be used to collect any air borne contaminants, this was present throughout the sampling process before being sealed and labelled for further analysis. Tape lift screening (TLS), a common procedure in forensic laboratories<sup>29</sup> was used to test for surface microfibrils. After the benches had been cleaned, a 5 cm<sup>2</sup> piece of adhesive tape was cut and placed on the bench surface in three random locations before being placed on an acetate sheet and examined under a microscope, this process was carried out before and after the laboratory procedures.

### 2.2. Comparison and validation of enzymatic digestion methods

#### 2.2.1. *Mytilus edulis* collection

Mussels were collected from the Clyde estuary (Figure 1), individually wrapped in aluminium foil and placed in lidded buckets, these were frozen at -20°C upon return to the laboratory.



**Figure 1. Map of UK and Ireland with sampling locations on the West coast of Scotland, blue box indicates location of the Clyde estuary and red box highlights the Oban area, with the detailed section illustrating the location of sample sites (Maps from GEBCO\_2014 and Google Earth V.7.1.5.1557)**

### **2.2.2. Digestion efficacies**

The three proteinaceous digestive enzymes trypsin, collagenase and papain were investigated; these have specificity for peptide bonds therefore cause dissociation of tissues, and are of a lower cost than proteinase-K<sup>15</sup>. Mussels were removed from the freezer and allowed to defrost for one hour. The length and width of each mussel was recorded using dial callipers. A dilution series of the digestive enzyme trypsin was prepared by diluting with deionised water to achieve 2.5 %, 1.25 %, 0.625 %, 0.3125 %, 0.15 % and 0.08 % concentrations. Six mussels were dissected to remove the soft tissue in separate clean glass petri dish and weighed using an Ohaus Pioneer electronic balance. Each mussel was quartered and added to a beaker containing 20 ml of each of the six concentrations of trypsin and were then placed on heated magnetic stirrers to stir gently at 38-42°C for 30 minutes. Six pieces of 80 µm mesh gauze were weighed and labelled before filtering the contents of each beaker through individual pieces of gauze. The gauze was then weighed again to determine the amount of mussel soft tissue left undigested; this process was carried out in triplicate. The initial weights of gauze were taken into account before being used in an equation ( $\text{Pre weight} - \text{Post weight} / \text{Pre weight} \times 100$ ) to determine the digestive efficiency of each concentration of trypsin enzyme. Once the optimum concentration which produced maximum digestive efficacy for the lowest concentration of trypsin was calculated, the protocol was repeated at this concentration in triplicate for the dissociation enzymes papain and collagenase to provide comparative digestive efficiencies. Additionally, the effects of specimen preservation method were investigated by digesting freshly collected mussels and those preserved in formaldehyde for three days followed by ethanol for 7 days with the optimum concentration of trypsin, these were each carried out in triplicate. Digestion efficacies were compared using a Kruskal-Wallis test with a Nemenyi posthoc test using the package PMCMR<sup>30</sup> in R Studio V 0.99.892.

### **2.2.3. Validation of enzymatic digestion**

A collection of microplastics was established by collecting plastics of known polymer types from everyday items, as identified by the resin identification code<sup>31</sup> branded onto the plastic in production (polymer category 1: Polyethylene Terephthalate (PET), category 2: High-Density Polyethylene (HDPE), category 3: Polyvinyl chloride (PVC), category 5: Polypropylene (PP) and category 6: Polystyrene (PS)), along with polyamide (PA). Small pieces of each were obtained using a coffee bean grinder, plastic was milled for several minutes until a small particle size was produced. The fragments were placed on a 0.5 mm mesh sieve to divide them into two classes: < 0.5 mm and 0.5 - 5.0 mm. The length of 30 microplastics of each polymer were recorded using the ocular scale of a Wild M5 dissecting microscope. 10 microplastic fragments were added to a glass beaker containing 20 ml of 0.3125 % concentration of trypsin enzyme and placed on a heated magnetic stirrer for 30 minutes set to stir gently and maintain a temperature between 38-42°C. This was repeated for each polymer type in triplicate. Following enzyme exposure the contents of the beakers were filtered using separate 80 µm mesh gauze. The microplastics were recovered, measured and their physical properties examined under a dissecting microscope; surface appearance was investigated by Hitachi S4100 Scanning Electron Microscope (SEM) with a 10 kV accelerating voltage. Microplastic samples were lightly coated with conductive gold prior to SEM imaging to prevent sample charging. Size differences were compared using a rank-sign paired Wilcoxon test in RStudio V 0.99.892.

## **2.3. Differing specimen preservation techniques**

### **2.3.1 Preservation effects on microplastics**

Ten microplastic fragments of the polymers PET, HDPE, PVC, PP, PS and PA from the reference collection detailed in Section 2.2.3, along with monofilament fibres obtained from orange, green and blue nets stranded as beach debris, were visually characterised and photographed using a Zeiss photomicroscope with Axiovision V 4.8.2.0 software. These were then transferred to individual durum tubes and were subjected to different preservation techniques. Five samples were frozen at -20°C for ten days and the remaining five samples were preserved in 4 % formaldehyde diluted in seawater and buffered to pH 7.5 with borax<sup>32</sup> three days before being transferred into 70 % ethanol for a further seven days. Exposure to formaldehyde for three days was chosen as this time period appropriately reflects contact times for specimen preservation. After this time microplastics were re-photographed using the Zeiss photomicroscope and examined for any visual decolouration, cracks, fragmentation, embrittlement and any other changes. Measurements of plastic length taken before and after the treatment were compared statistically for size changes using a rank-sign paired Wilcoxon test in RStudio V 0.99.892.

### **2.3.2. *Mytilus edulis* specimen collection and preservation**

*M. edulis* were collected at four locations on the West coast of Scotland in December 2015 and February 2016 (Figure 1). Specimens were wrapped tightly in aluminium foil, labelled clearly and placed into separate sealable freezer bags for each location. On return to the laboratory preservation techniques varied between the collections. For December 2015 samples mussels were frozen at -20°C, while mussels collected in February 2016 underwent a widely used specimen preservation technique involving formaldehyde and ethanol. *M. edulis* were covered with buffered formaldehyde for three days before being transferred to 70 % ethanol and stored in screw top containers for a further 7 days.

### **2.3.3. Preservation effects on microplastics ingested by *Mytilus edulis***

Mussels were removed from the freezer and allowed to defrost for one hour; specimens preserved in formaldehyde and ethanol were rinsed well using deionised water. The length and width of each mussel was recorded using metal dial callipers. The entire mussel was dissected from the shell in a glass petri dish to contain all parts of the tissue and the tissue was then weighed using a Sartorius electronic balance. The mussel was quartered and placed into a beaker containing 25 ml of 0.3125 % concentration trypsin solution. Beakers were placed on a heated magnetic stirrer set to stir gently at 250 turns per minutes at 38-42°C and left to digest for 30 minutes. The mixture was poured through a 52 µm mesh gauze before being placed into a covered glass petri dish. The gauze was thoroughly examined under a dissecting microscope and any non-prey items were removed from the gauze and placed into a small petri dish containing 30 mm filter paper. Petri dishes were kept covered when not in use to reduce the potential for contamination exposure. Small petri dishes were sealed with black electrical tape, labelled and stored for further analysis using attenuated total reflectance Fourier transformation infrared spectroscopy (ATR-FTIR). Microplastic quantities were standardised by the number of particles per gram of wet weight (w.w.) of mussel tissue, taking into account site-specific size differences and paired rank-sign Wilcoxon tests were performed in RStudio V 0.99.892 to identify any preservation technique effects.

### **2.3. ATR-FTIR spectroscopy**

Microplastics were identified using a Perkin-Elmer Spectrum 100 Fourier Transformation Infrared spectroscope coupled with a universal Attenuated Total Reflectance accessory (ATR-FTIR) equipped with a diamond detector. The spectra were recorded as the average from four high resolution scans in the range of 600 - 4000  $\text{cm}^{-1}$  with a spectral resolution of 4.00  $\text{cm}^{-1}$  in the software Spectrum V 6.3.4.0164 (Perkin-Elmer). The principle of ATR-FTIR is that infrared radiation excites various bonds on the surface of the material being sampled so producing a percentage transmittance spectrum. Examination of the transmittance spectra can facilitate in determining the material. Spectra obtained were visualised in OMNIC 9.2.98 (Thermo Fisher Scientific Inc.) using the inbuilt reference library collection to assist with the analysis and characterisation of percentage transmittance spectra.

## **3. Results**

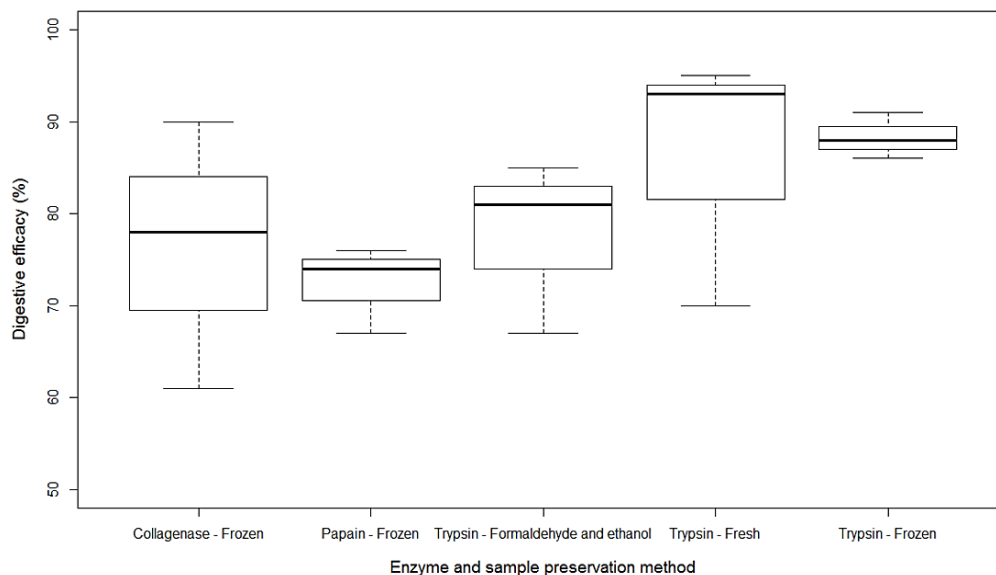
### **3.1. Contamination**

Close inspection of the contamination mitigation procedures revealed that fibres found on TLS and atmospheric controls were different to those in the mussels. The median number of fibres on the tape samples was 19, with ranges of between 1 - 18 found before work was undertaken and 3 - 26 after laboratory experiment had been conducted. The lengths of fibres ranged from 0.45 - 5.56 mm and were red and blue in colour, reflecting the colour of the natural fibre clothes worn during laboratory work. Close visual examination of all fibres revealed them to be cotton, clearly characterised by the flat, spirally twisted, ribbon-like appearance of the fibres; characteristics absent in man-made fibres; furthermore, a sub-sample of these were analysed using ATR-FTIR to confirm the material was cotton; similar results were found by Murphy et al<sup>28</sup>. The number of fibres were much lower on atmospheric

controls with a median number of 0 (range 0 - 3) being recorded. Lengths varied from 0.67 - 1.78 mm and all fibres were also identified as cotton.

### 3.2. Enzymatic digestion efficacy

The lowest concentration of trypsin with the highest efficiency was 0.3125 %, with a mean value of 88 %  $\pm$  2.52 S.D. of mussel tissue dissociated for frozen specimens after 30 minutes enzyme exposure. Papain and collagenase both yielded lower digestive efficacies than trypsin at a concentration of 0.3125 % (Figure 2), therefore trypsin was selected to be used for further experimental procedures. By comparison, mean digestive efficacy using 0.3125 % concentration trypsin was 86 %  $\pm$  13.89 S. D. and 78 %  $\pm$  9.45 S.D. for fresh mussels and those preserved by formaldehyde followed by storage in ethanol respectively. There was no statistical difference in digestive efficacy between the different enzymes or specimen preservation prior to digestion ( $H = 5.4681$ ,  $df = 4$ ,  $p = 0.2425$ ). Trypsin was effective at reducing the mussel soft tissue for all preservation methods, the majority of the residue which was left undigested consisted of a transparent film of gelatinous material, in which microplastics and fragments of other materials (such as shell and sand grains) were visible. In some samples the adductor muscles and mantle skirt were only partially digested, these were examined carefully to remove any microplastics from the surface.



**Figure**

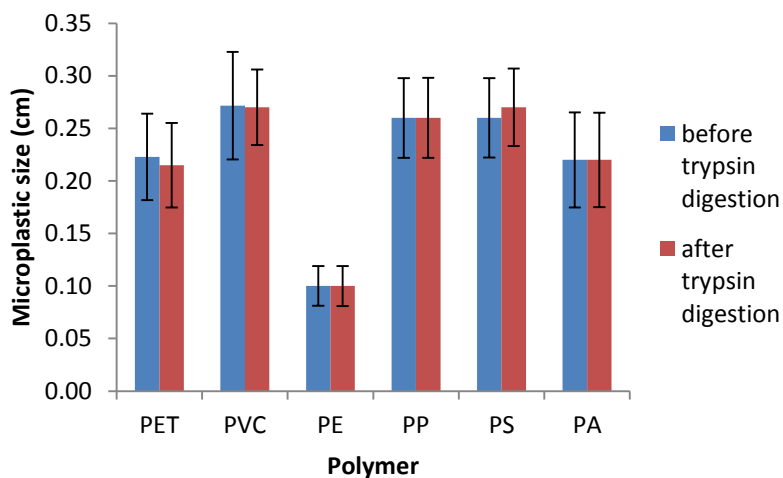
**2. Digestive efficacy of the three dissociation enzymes trypsin, collagenase and papain at 0.3125 % concentration on differently preserved mussel specimens. Digestive efficacy for frozen mussels are shown for all enzymes, and additional comparisons with fresh and formaldehyde and ethanol preserved mussels are provided for trypsin.** Thick black lines represent median values, boxes depict the first and third quartiles and the whiskers illustrate the interquartile range. No significant difference was found between the different enzymes or preservation methods.

### 3.3. Effect of enzymatic digestion on microplastics

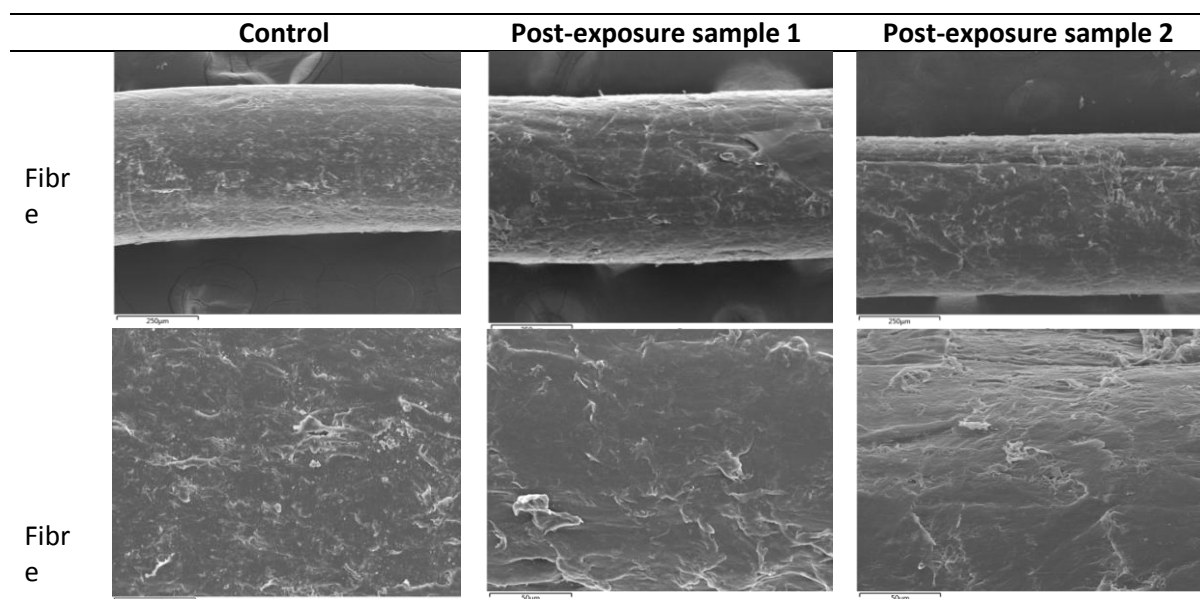
There were no changes in overall particle shape, colour or significant differences in size for any of the polymers investigated (PET:  $V = 62.5$ ,  $p = 0.0709$ ; PE:  $V = 12.5$ ,  $p = 0.7498$ ; PP:  $V = 47$ ,  $p = 0.5531$ ; PS:  $V = 12$ ,  $p = 0.7963$ ; PVC:  $V = 48$ ,  $p = 0.0368$ ; PA:  $V = 41$ ,  $p = 0.9043$ ) following enzymatic digestion



(Figure 3). SEM surface images of microplastics showed little differences in surface appearance after enzyme digestion with 0.3125 % trypsin (Figure 4).



**Figure 3. Mean microplastic size for a range of polymers before and after enzymatic digestion with 0.3125% trypsin.** Error bars denote standard deviation. No significant size differences were observed following enzyme digestion for any of the polymers investigated.

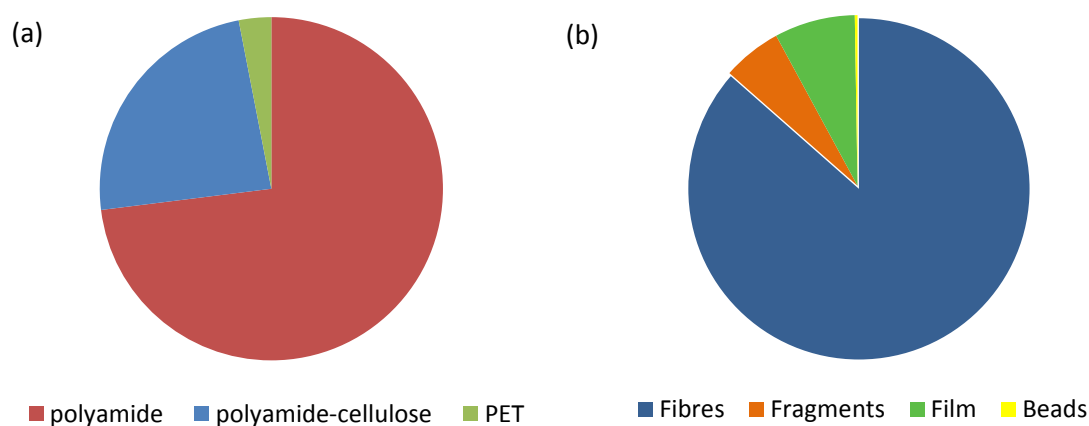


**Figure 4. SEM images of polyethylene fibres exposed and not exposed to enzymatic digestion with 0.3125% trypsin.** Magnification of top row: x100, bottom row: x500 at 60° tilt; Polyethylene is pictured here as an example; there were no observed differences in appearance for any of the polymers investigated after enzyme digestion.

### 3.4. Identification of microplastics ingested by *Mytilus edulis*

A total of 634 potential microplastic samples were collected and analysed using ATR-FTIR. Of these 392 were identified as being synthetic from their percentage transmittance spectra, 122 were identified as organic material (sand, calcium carbonate, cellulose etc.), 12 items were not able to be

identified based on their percentage transmittance spectra and the remaining items did not give a usable spectral reading. Of the items that were positively identified as being microplastic, the dominant polymer was polyamide (n = 285), other polymers present were a co-polymer of polyamide and cellulose (n = 94) and PET (n = 12) (Figure 5a). 97 % of the mussels investigated were identified to have ingested microplastics. The majority of microplastics were blue and red fibres (n = 339), accounting for 86 % of the total microplastics identified; fragments (n = 22), films (n = 30) and beads (n = 1) were also recorded in much lower abundances (Figure 5b). The median length of microplastic ingested by mussels was 1.22 mm, however lengths ranged from 0.2 – 10.67 mm.



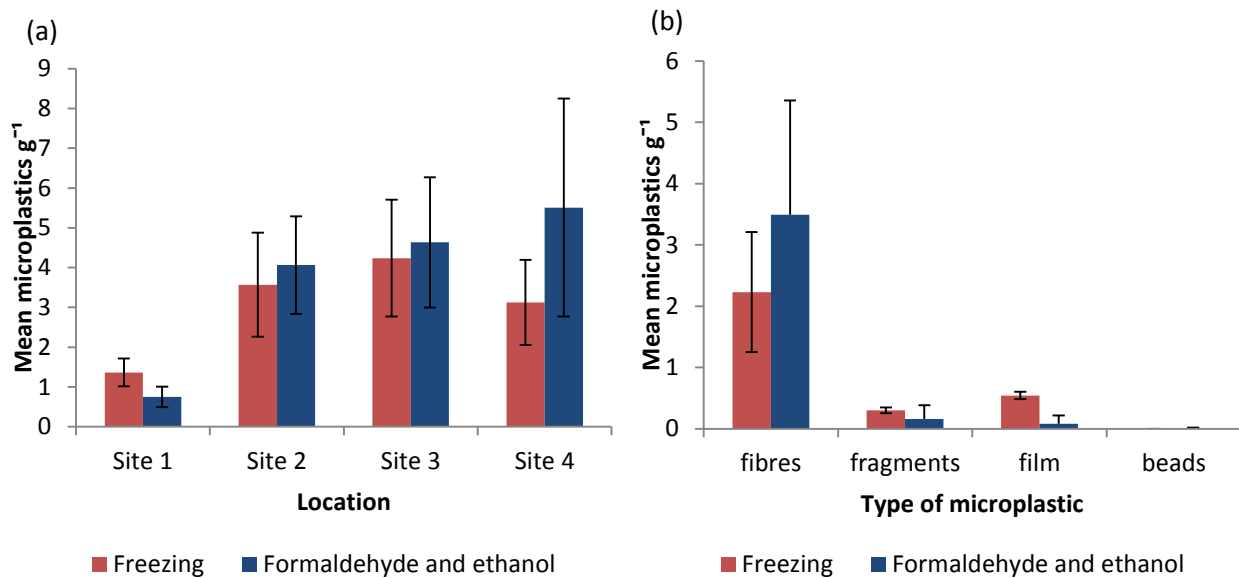
**Figure 5. (a) Constituent polymers and (b) microplastic shapes ingested by mussels at four sites identified using ATR-FTIR spectrometry.** Polyamide was identified to be the dominant polymer accounting for 72 % of ingested microplastics; fibres represent the majority of microplastic types (86 %) extracted from mussels.

### 3.5. Comparison and effects of preservation techniques

For all polymers investigated there was no difference in visual appearance (discolourisation, cracking, cavities, embrittlement) or any significant differences in the length of the microplastic after either preservation technique for any of the polymers tested ( $V = 510$ ,  $p = 0.3073$ ).

While the quantities of microplastics varied between individual mussels and between sites (mean values of  $1.05 \pm 0.66$  S.D.  $g^{-1}$  at Site 1 to  $4.44 \pm 3.03$  S.D.  $g^{-1}$  at Site 3) (Figure 6a); there was no significant effect of preservation technique on the overall number of ingested microplastics per gram of w. w. mussel tissue ( $V = 370$ ,  $p = 0.5707$ ) or for the mean number of microplastics  $g^{-1}$  of w.w. mussel for each site ( $V = 7$ ,  $p = 0.625$ ). Furthermore there was no significant difference between preservation techniques on the type of polymer (polyamide:  $V = 408$ ,  $p = 0.2451$ ; polyamide and cellulose copolymer:  $V = 199$ ,  $p = 0.3419$ ; PET:  $V = 20$ ,  $p = 0.2664$ ).

When considering the type of microplastics, there was no effect of preservation technique on the overall number of fibres  $\text{g}^{-1}$  detected ( $V = 237$ ,  $p = 0.1348$ ) between mussels, or on the mean number of each type of microplastic  $\text{g}^{-1}$  between sites (Figure 6b) (fibres:  $V = 2$ ,  $p = 0.375$ ; fragments:  $V = 8$ ,  $p = 0.375$ ; film:  $V = 6$ ,  $p = 0.1814$ ). Beads were insufficient in number to undertake the test.



**Figure 6. Mean number of ingested microplastics per gram of w. w. mussel tissue (a) across sampling locations, and (b) classified by shape for each of the two sample preservation techniques.** Error bars denote standard deviation. There was no significant difference between microplastic quantities or type based on preservation method.

#### 4. Discussion

This study addresses important gaps regarding the application of enzymatic digestion to extract microplastics from fauna and assess the validity of different preservation methods at a time when concerted efforts are being made to standardise microplastic operation protocols. Our results clearly present the first evidence that the use of freezing, or formaldehyde and ethanol as a specimen preservation technique does not cause significant differences in the enumeration of microplastics from faunal tissues or cause any degradation to microplastics of various polymers. Furthermore we found the dissociation enzyme trypsin to yield a high digestive efficiency of biological material while causing no severe detrimental effects to microplastics directly thus producing a suitable, time and cost effective method for microplastic extraction.

Digestion efficacies did not differ significantly between the three enzymes trialled in this study or between the preservation methods. Trypsin yielded a mean efficacy of  $88\% \pm 2.52\%$  S.D. for frozen mussels at a concentration of  $0.3125\%$ ;  $12\%$  and  $16\%$  greater than collagenase and papain respectively at the same concentration, thus trypsin was preferentially selected for further experimental procedures. Comparable mean digestive efficacy was produced from frozen and fresh mussels with  $0.3125\%$  trypsin, while a lower result was produced from mussels preserved by formaldehyde and ethanol. Trypsin was found to be more efficient at dissociating tissue for each

preservation method than either papain or collagenase was at dissociating frozen mussel samples. While the digestive efficacy attained with trypsin was lower than that reported by Cole et al<sup>15</sup> using the proteolytic enzyme Proteinase-K to digest zooplankton, it is likely that enzyme efficiency will vary between species and tissue structures and careful visual sorting is necessary to remove any potential microplastics from residue remaining following digestion. Mussels were utilised in this optimisation protocol as the adductor muscles and mantle skirt in particular are dense muscular structures, providing a thorough assessment of the effectiveness of enzyme digestion. While these were not completely digested in all cases, it is unlikely that these regions would accumulate microplastics internally; trypsin was effective at dissociating the majority of soft tissue including respiratory, digestive and reproductive structures. It is hypothesised that comparable digestive efficacies will be obtained for other similar marine species, however efficacy may vary between species and further work is required to consider this.

Close examination and SEM imaging of microplastic revealed that a concentration of 0.3125 % trypsin caused no detrimental impacts to a range of polymers; while it was not possible to test the susceptibility of all polymers to enzyme digestion, a range of polymer sensitivities have been considered, and from the results obtained it is unlikely that other polymers would be degraded by a 30 minute exposure to a low concentration of trypsin. The enzyme method developed and optimised here while not dissociating all soft tissue, is shown to produce reliable data and presents a balance between cost, duration of experimental procedure and digestive efficacy with the aim of its widespread use by research laboratories and by monitoring and regulatory bodies, where these factors play an important role in the selection of protocols.

Quantification of microplastic ingestion by wild blue mussels in the Oban area, West Scotland, show abundances to vary widely between individuals and sites even over a relatively small geographic location (less than a 2 km stretch of coastline); highlighting the heterogeneous distribution of microplastics in the marine environment. Blue mussels have been described previously to ingest microplastics in a laboratory setting<sup>8,33</sup>. While these studies serve to show the potential of organisms to ingest microplastics, they do not necessarily capture the variability of distribution and concentrations in the environment. Considering wild populations and validating laboratory trials with field studies produces more biologically relevant data.

Enzyme digestion of wild collected mussels were filtered through 52 $\mu$ m mesh, while 80 $\mu$ m was used to calculate digestion efficacy, the smaller mesh size was chosen here to retain a greater number of ingested microplastics and ensure a wide size range was captured for analysis. 97% of the Oban mussels were found to contain microplastics; this is a slightly greater proportion than documented in the nearby location of the Clyde Sea where 84 % of *Nephrops norvegicus* individuals contained microplastics<sup>34</sup>. The Oban mussels were also found to contain a much greater number of microplastics per gram than has been reported in other areas with mean values ranging from a minimum of  $1.05 \pm 0.66$  S.D. to a maximum of  $4.44 \pm 3.03$  S.D. microplastics  $g^{-1}$  of mussel tissue depending on sample location. In Germany wild caught mussels were found to contain a mean number of  $0.36 \pm 0.07$  S.D. microplastics  $g^{-1}$ ,<sup>12</sup> whilst the numbers of microfibrils (other microplastics types were not considered) found in mussels along a stretch of the Belgian coastline ranged between 0.26 - 0.51 fibres  $g^{-1}$ .<sup>35</sup> Compared to other published works, these results are in the same order as those found in highly environmentally polluted areas of China, where Li et al<sup>36</sup> report 3.3 items  $g^{-1}$ . These data do not necessarily signify that Oban is a more polluted area, but may serve to highlight discrepancies within

the microplastic extraction methods employed. Prior to enzyme digestion mussels were quartered, the small size of microplastics make it extremely unlikely that individual fibres would be dissected which is not likely to affect the overall microplastic abundance for all mussels examined; therefore it is unlikely to be responsible for the higher reported quantities in Oban. It is more likely that these differences highlight discrepancies between acid and enzymatic dissociation methods. Van Cauwenberghe & Janssen<sup>12</sup>, De Witte et al<sup>35</sup> and Li et al<sup>36</sup> used acid digestion methods to dissociate microplastics from the faunal tissues (nitric acid, nitric and perchloric acid, and hydrogen peroxide respectively) which have been established to have detrimental impacts on certain plastic polymers<sup>14,15,23</sup> and therefore may result in the underestimation of microplastics in organisms.

The majority of the microplastics ingested by wild mussels in Oban were identified as polyamide fibres, this polymer has wide application including in the manufacture of netting and rope used by the maritime sector<sup>37,38</sup>. Oban is a busy maritime town, with passenger ferry terminals, sightseeing boat trips, a fishing industry operating from the town and marinas, mussel and fish farms and water treatment works all in close proximity and being potential sources of plastic pollution. Sample locations were all within a 2 km stretch of coastline, with Site 1 being located in the town of Oban, and Site 4 the furthest North. Mussels collected at Site 2 were located at an outflow pipe from a waste water treatment works and were expected to contain high levels of microplastics<sup>28</sup>; however similar quantities were extracted from mussels at Sites 2, 3 and 4. Mussels at Site 1 were found to have the lowest microplastic load, which is surprising as this location is central within the town and is closest to the harbour, therefore having a number of pollution input sources. This data serves to highlight the heterogeneous distribution of marine microplastics even over small geographic scales. Fibres are predominantly reported from a range of environmental samples such as sediment, water and ice<sup>14,39-42</sup> and are ubiquitously found in the everyday environment<sup>43-46</sup>, therefore care is needed to avoid contamination with external sources and accurately document this important fraction of microplastics. Both the atmospheric and tape lift screening used in this study are common place within forensic laboratories,<sup>29,47,48</sup> these methods are designed to be robust under the scrutiny of the criminal justice system and provide thorough controls for the purpose of microplastics research, the widespread adoption of contamination mitigation measures should be seen as good practise in this field<sup>15,49-51</sup>.

Previous studies have quantified microplastic ingestion by fauna preserved by freezing<sup>23,52</sup> and those preserved in formaldehyde and ethanol<sup>18,22,53</sup>, with no reasoning as to why one technique was used in favour of the other. While it is stated that formaldehyde is a 'plastic-friendly' fixative<sup>24</sup> no documentation to support this is available to the authors knowledge and no comparison has been made between the two preservation methods. The present study, using blue mussels as a model organism illustrates that comparable data is produced from both preservation methods and no impacts to a range of sizes, shapes and polymers of microplastics are observed; concluding neither preservation method has an advantage over the other in terms of producing accurate microplastic quantification.

Establishing the validity of these samples for analysis of microplastic ingestion has implications for future lines of inquiry. While more recently the freezing of specimens has been recommended<sup>24</sup>, this may not always be possible in some field situations. Additionally, for some soft bodied fauna (e.g. holothurians) or very small organisms (e.g. polychaetes) freezing and subsequent thawing may cause damage to tissues<sup>54</sup>, making identification of organisms to species level almost impossible in most

cases and therefore may render this method unsuitable. For most research facilities, freezing is not appropriate for preserving large collections spanning over long time scales, due to space requirements and the associated cost of keeping specimens frozen for any length of time. Most historical specimen collections therefore are preserved in ethanol after initially fixing tissues with formaldehyde. It is important to bear in mind the potential of contamination of historical specimens, as mitigation or control measures applied now would not have been enforced at the time of collection and processing. Nonetheless, archival collections present an important source of data to advance the knowledge of microplastic pollution and allow for the exploration of temporal changes of abundance and polymer composition over long-term time series.

## 5. Conclusion

The results presented above clearly demonstrate that using the digestive enzyme trypsin to extract microplastics from biological samples does not cause damage to ingested microplastics and provides a rapid, cost efficient and effective method. Comparable data are produced from wild mussels treated with widely used specimen preservation techniques, without any detrimental effects to microplastics. It is anticipated that these methodological developments will be applied to future research into ingested microplastics by fauna from both newly sampled organisms and archival collections by a range of interested groups.

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