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

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Does sample preservation and enzymatic digestion adversely impact detection of microplastics in fauna?

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Abstract

Microplastics are considered to be one of the greatest emerging environmental contaminants. 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 validated 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 (88 % ± 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 ± 0.66 S.D. (minimum) to 4.44 ± 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 biological samples. 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[1]. Around 30 % of the plastic items made have single-use application and are discarded within a year of manufacture[2]. 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 <5 mm in diameter[3], produced by the degradation of larger plastic items or manufactured purposely to be of small size[4]. Microplastics are potentially bioavailable to a wide range of organisms being of a size similar to prey items or sediment grains[5,6] and pose a severe threat to aquatic life[7]. 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[8–13] 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[14]. While acid and alkaline dissociation are effective in dissolving organic material they degrade or even destroy some pH-sensitive polymers[14–16]. Dissociation enzymes offer an alternative method due to their high digestive specificity, meaning proteinaceous material is acted upon leaving synthetic materials intact and unaffected[17]. Enzymes therefore provide a more accurate way to extract and quantify ingested microplastics from tissues.

While efforts are being made to develop and standardise extraction methods it is also important to consider the treatment of samples prior to analysis 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[18–22]. 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[23,24]. 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 size of the specimens tissues[25,26], but what effect these techniques may have on ingested microplastics is unclear. Elucidating methodological differences and establishing best practise is critical 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[8,12], and mussels act as bioindicators for aquatic

contaminants due to the large volumes of water drawn across the gills when filter feeding[27]. 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[28]. 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[29] was used to test for surface microfibres. After the benches had been cleaned, a 5 cm^2 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.

2.2.2. Efficiency of enzymatic digestion

Mussels were removed from the freezer and allowed to defrost for one hour. The length and width of each mussel was recorded using dial calipers. 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 tissue mass 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 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 (Pre weight – Post weight / Pre weight x 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.

2.2.3. Validation of enzymatic digestion

Polyethylene microplastic beads were extracted from a facial scrub by passing the scrub through a clean 400 µm mesh stainless steel sieve. Beads retained on the sieve were washed three times with 70 % ethanol followed by washing three times with distilled water and allowed to dry fully before being transferred to a clean sealed glass container. Polyethylene fibres were generated by cutting orange monofilament line into small pieces (< 1 mm) using scissors. The polymer identities were confirmed using Fourier Transformation infrared spectroscopy to confirm. The length and width measurements were recorded for 30 microplastics of each shape using the ocular scale of a Novax Holland dissecting microscope. Six frozen mussels were removed from the freezer and allowed to defrost for one hour. These were then opened and the tissue mass dissected away from the shell in separate clean glass petri dishes. A small incision was made into the tissue of the mussels and each were spiked by placing either ten polyethylene beads (n = 3) or ten polyethylene fibres (n = 3) into each mussel. Mussels were then enzymatically digested using 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. Following digestion the contents of the beakers were filtered using 80 µm mesh gauze. The microplastics were recovered, measured and their physical properties examined under a dissecting microscope and surface characteristics were 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.

2.3. Differing specimen preservation techniques

2.3.1 Preservation effects on microplastics

A collection of microplastics was established by collecting plastics of known polymer types from everyday items, as identified by the resin identification code[30] 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)). Small pieces of each were obtained using a coffee bean grinder and 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. Ten microplastics of each polymer type listed above, along with 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[31] 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 calipers. 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 cm^{-1} with a spectral resolution of 4.00 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[28]. 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. Efficiency of enzymatic digestion

The lowest concentration of trypsin with the highest efficiency was 0.3125 %, with a mean value of 88 % ±2.52 S.D. of mussel tissue dissociated after 30 minute enzyme exposure (Figure 2a). Papain and collagenase both yielded lower digestive efficiencies than trypsin at a concentration of 0.3125 % (Figure 2b), therefore trypsin was selected to be used for further experimental procedures.

3.3. Effect of enzymatic digestion on microplastics

There were no changes in overall particle shape, colour or size for either the beads or fibres after exposure to enzymatic digestion (Figure 3). SEM surface observations varied between particles (Figure 4), the surface texture of one of the beads sampled appeared rougher with more pitting after enzyme exposure with the partial loss of the outer layer from the bead; however this was not the case for the other microbead imaged. The surface structure of fibres appeared to have very little difference 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). 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.

3.5. Comparison and effects of preservation techniques

For all polymers investigated there was no difference in visual appearance (discolourisation, cracking, cavities) or any significant differences in the length of the microplastic after either preservation technique for any of the polymers tested ($V = 765.5$, $p = 0.1946$).

While the quantities of microplastics varied between individual mussels and between sites (mean values of 1.05 ± 0.66 S.D. g^{-1} at Site 1 to 4.44 ± 3.03 S.D. g^{-1} at Site 3) (Figure 6); 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 g^{-1} detected ($V = 237$, $p = 0.1348$) between mussels, or on the mean number of each type of microplastic g^{-1} between sites (Figure 7) (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.

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 efficacy 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 efficiencies did not differ greatly between the three digestive enzymes trialled in this study; trypsin yielded digestive efficiencies of 88 % ± 2.52 % S.D. at a concentration of 0.3125 %; 12 % and 16 % greater than collagenase and papain respectively at the same concentration, thus trypsin was preferentially selected. While the digestive efficacy attained with trypsin was lower than that reported by Cole et al[15] using the proteolytic enzyme Proteinase-K, the associated costs and experimental time are greatly reduced with trypsin. SEM images revealed a slight increase in surface pitting in some polyethylene particles exposed to enzyme digestion and the outer surface of one microbead appeared to be fragmenting, however it is not possible to ascertain whether this was due to enzyme exposure or simply due to the high variability in surface structure between microplastics, even in those termed 'pristine'. The method developed and optimised here is shown to produce reliable data and presents a balance between cost, time efficiency 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[8,32]. 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.

The Oban mussels were 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 ± 0.66 S.D. to a maximum of 4.44 ± 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 ± 0.07 S.D. microplastics g^{-1},[12] whilst the numbers of microfibres (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}.[33] 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[34] 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[12], De Witte et al[33] and Li et al[34] 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 [14,15,23] 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[35,36]. 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. Fibres are predominantly reported from a range of environmental samples such as sediment, water and ice[14,37–40] and are ubiquitously found in the everyday environment[41–44], 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,[29,45,46] 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[15,47–49].

Previous studies have quantified microplastic ingestion by fauna preserved by freezing[23,50] and those preserved in formaldehyde and ethanol[18,22,51], 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[24] 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[24], 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[52], 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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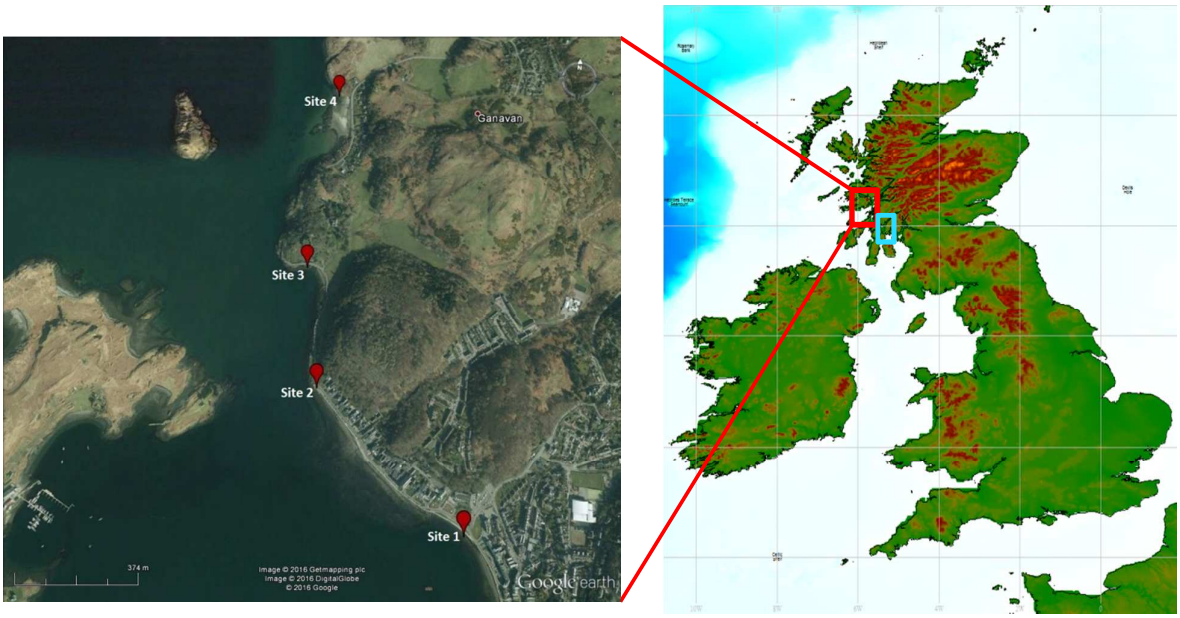

Figure 1. Map of UK and Ireland with sampling locations on the West coast of Scotland, blue box indicates location of the Clyde estuary and red box highlights the Oban area, with the detailed section illustrating the location of sample sites (Maps from GEBCO_2014 and Google Earth V.7.1.5.1557)

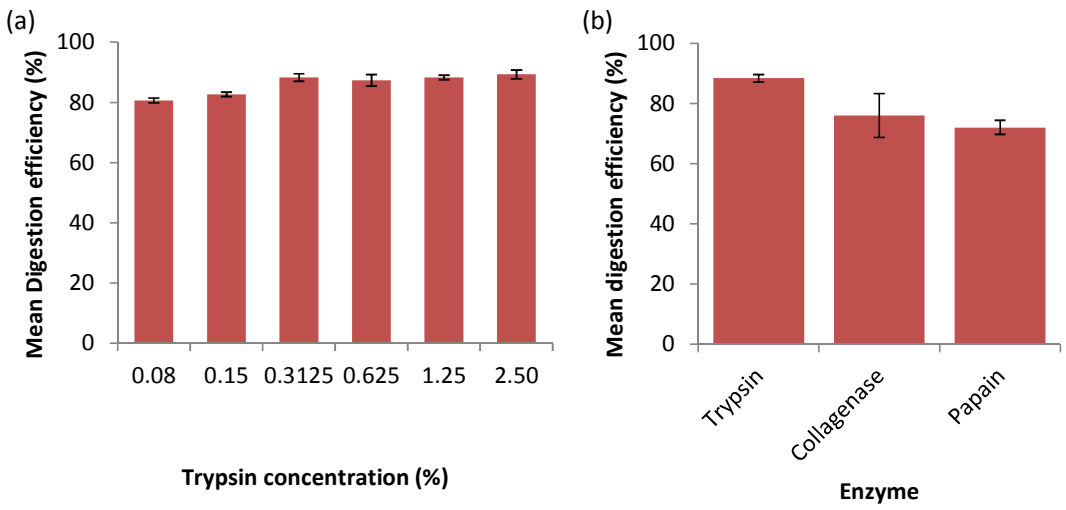

Figure 2. (a) Mean digestive efficiency of the enzyme trypsin at a range of concentrations, (b) Mean digestive efficiency of the three dissociation enzymes, trypsin, collagenase and papain at 0.3125% concentration. Error bars denote standard deviation.

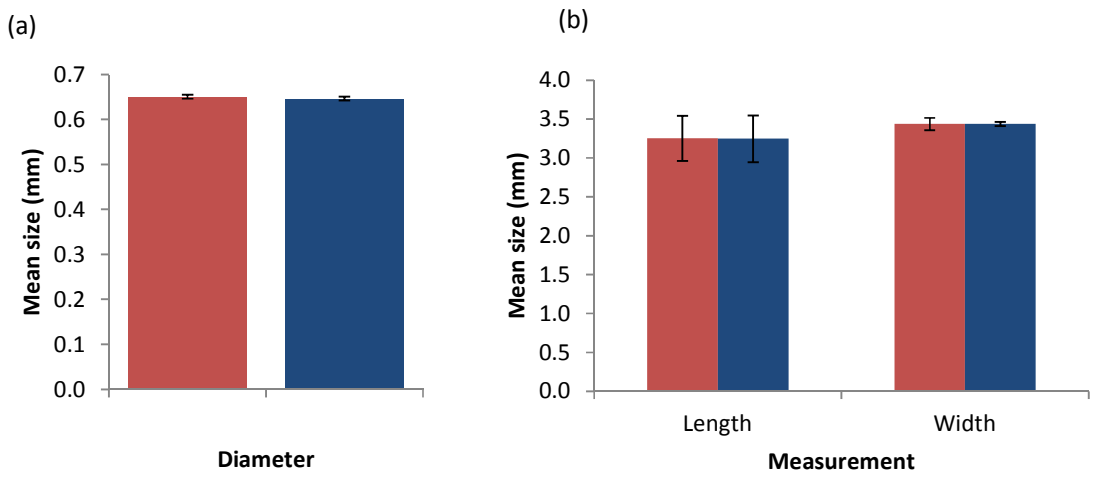

Figure 3. Mean (a) blue PE bead diameter and (b) orange PE fibre length and width before (red) and after trypsin enzymatic (blue) digestion; there are no observed changes in size. Error bars show standard deviation.

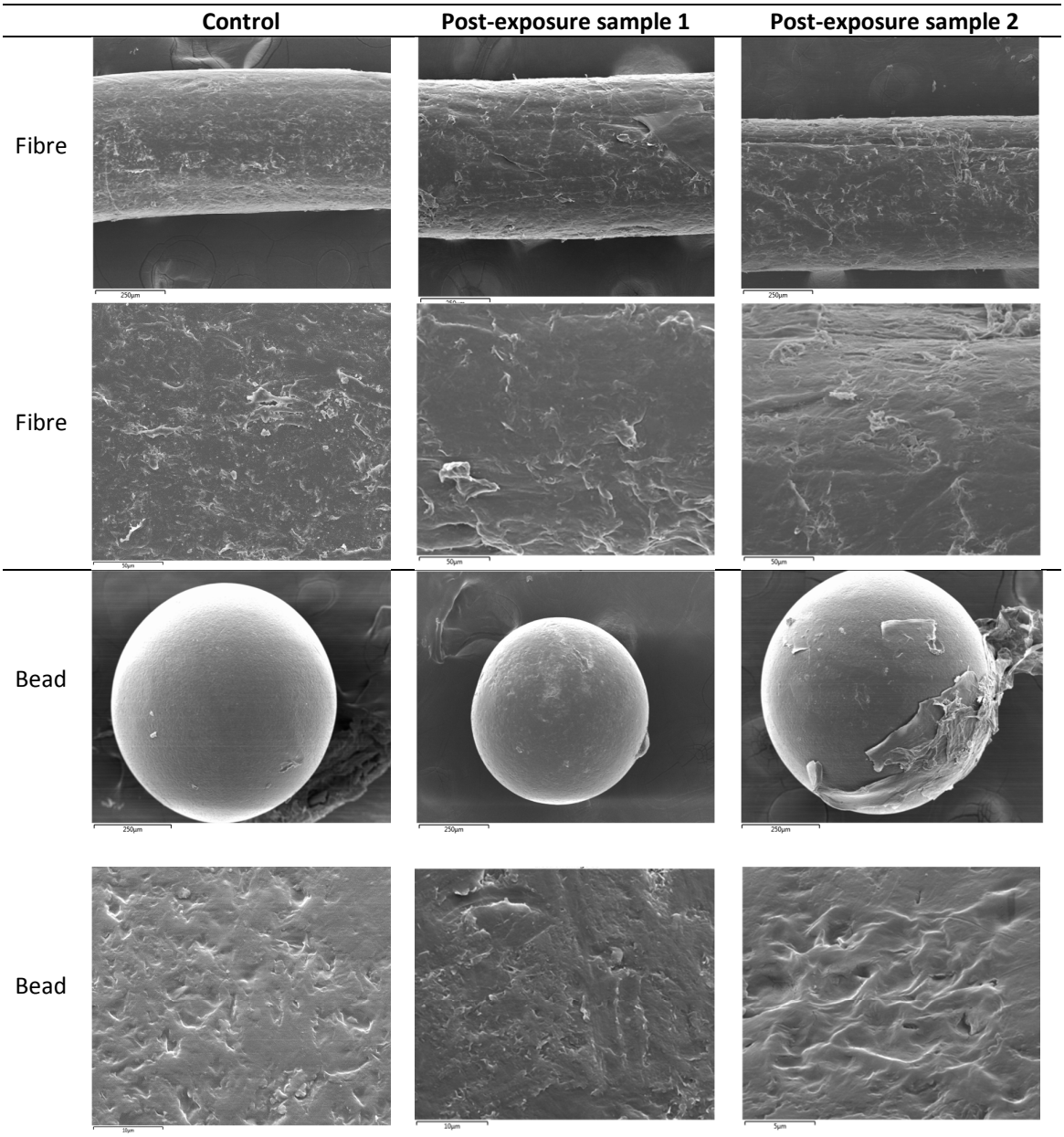

Figure 4. SEM images of polyethylene fibres and beads exposed and not exposed to enzymatic digestion with 0.3125% trypsin. Fibre magnification top row: x100, bottom row: x500 at 60° tilt; bead magnification top row: x100, bottom row: x 2500 at 60° tilt.

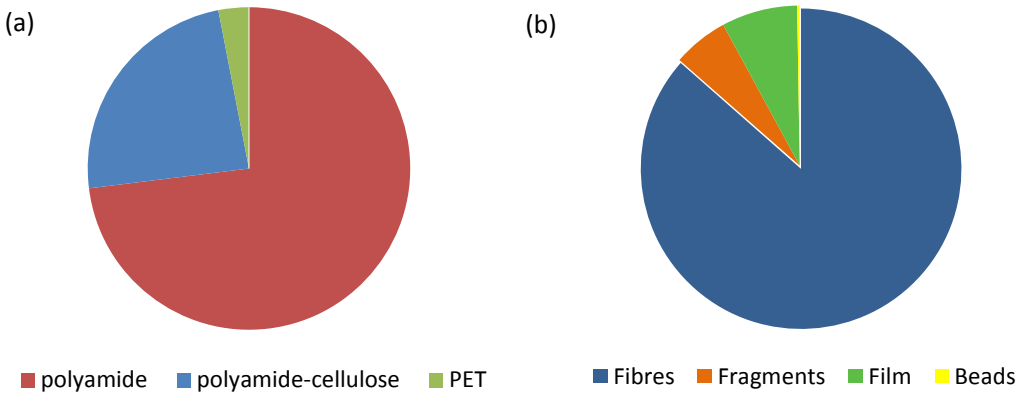

Figure 5. (a) Constituent polymers and (b) microplastic shapes ingested by mussels at four sites identified using ATR-FTIR spectrometry.

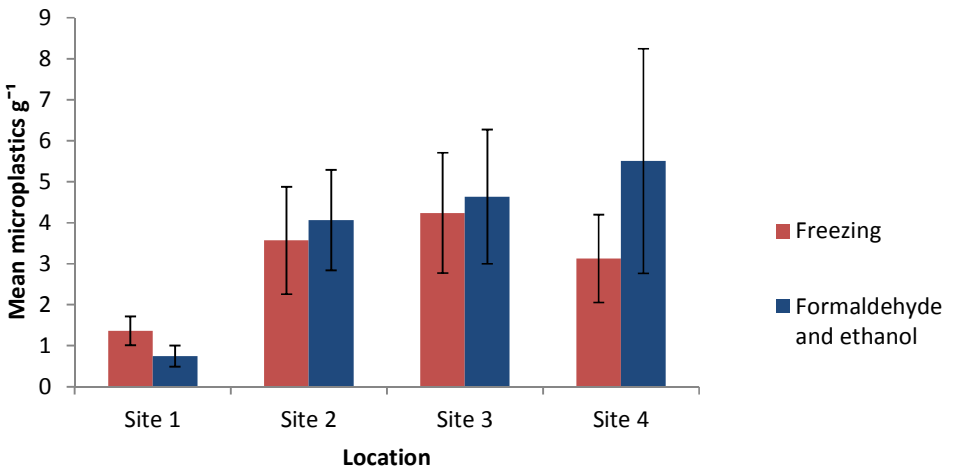

Figure 6. Mean number of microplastic particles ingested per gram of w. w. mussel tissue across sampling locations, error bars denote standard deviation. No significant difference was found in microplastic quantities based on preservation technique for each site.

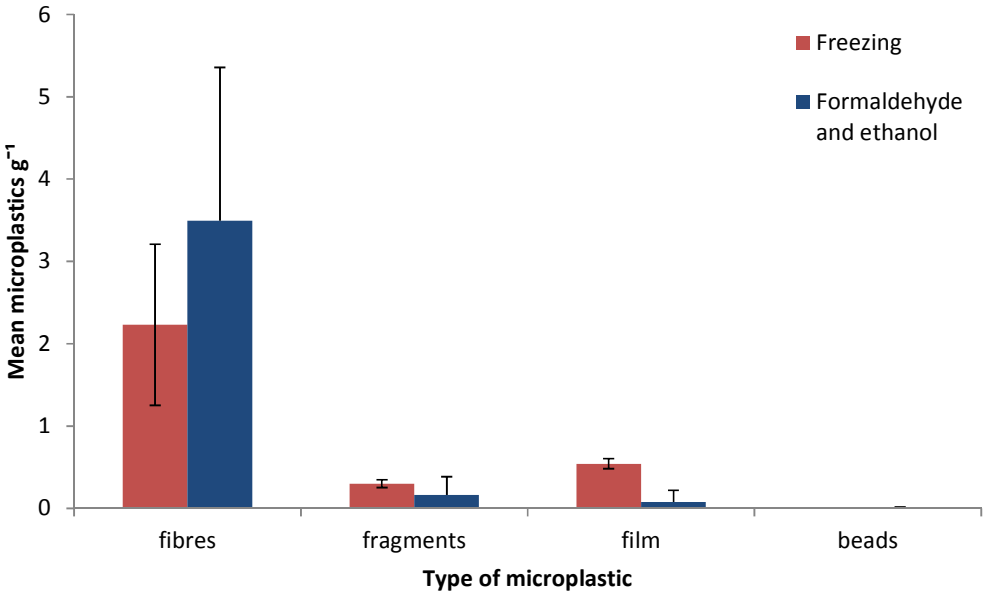

Figure 7. Mean number of ingested microplastics per gram of mussel tissue characterised by shape, for each of the two preservation techniques. Error bars denote standard deviation. No significant difference was found between the between type of microplastics based on the specimens preservation technique

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18th August 2016

Analytical Methods
Dear Prof. Regan, Dr. Rochman and Prof. Thompson

On behalf of my co-authors I am pleased to submit an original full article entitled 'Does sample preservation and enzymatic digestion adversely impact detection of microplastics in fauna?' for consideration in the 'Microplastics in the environment' themed collection of *Analytical Methods*

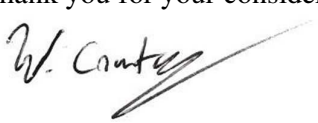
This paper develops and outlines a rapid, effective and non-destructive technique for the extraction of microplastics from fauna building on work by Cole et al (2014), and considers the effect of specimen preservation method for microplastics analysis for the first time.

The field of microplastics research suffers from a lack of standardisation and uniformity, as such data is not comparable and the true scale and impacts of microplastic pollution cannot be ascertained. This novel study optimises and validates an enzymatic digestion method using the proteolytic enzyme trypsin and demonstrates effective dissociation of organic material with no detrimental effects to microplastics. This provides a simple, low cost and time effective method which can be utilised by researchers and regulatory bodies alike to achieve effective and standardised monitoring. Furthermore, the effects of specimen preservation are investigated as archival collections present a valuable time-series over which to examine changes in microplastics abundances. Analysis of mussels preserved using formaldehyde followed by ethanol and by freezing demonstrated no impacts on microplastic quantification or degradation, substantiating the application of fauna treated either way and of historical specimen collections for the investigation of microplastic ingestion.

Our manuscript addresses the scope of *Analytical Methods*, providing key advances in the detection and analysis of microplastics. Reliable quantification of microplastics will elucidate the scale and environmental impacts, allowing for evidence-based focused management of plastic pollution. The multi-disciplinary approach, combining forensic science techniques and analytical chemistry to answer biological questions makes this research pertinent to a wide audience. The methodological developments detailed here are anticipated to be implemented by a broad range of interested groups, and provide an appropriate protocol for rapid monitoring by regulatory bodies.

The authors declare no conflict of interest and that the manuscript is of original work and has not been submitted anywhere else.

Thank you for your consideration



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