

# **Title: Evaluation of existing methods to extract microplastics from bivalve tissue: adapted KOH digestion protocol improves filtration at single-digit pore size**

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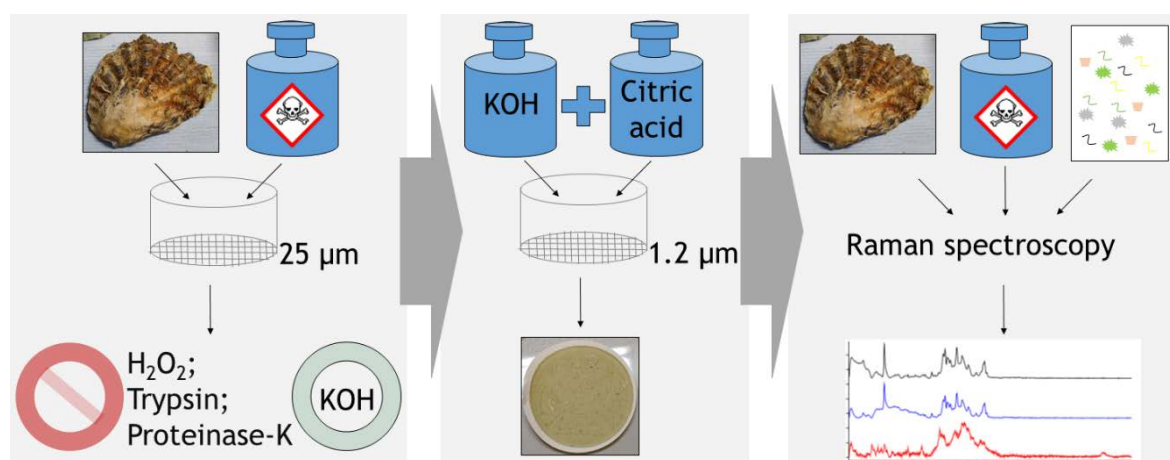
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## Abstract

Methods standardisation in microplastics research is needed. Apart from reagent-dependent effects on microplastics, varying target particle sizes can hinder result comparison between studies. Human health concerns warrant recovery of small microplastics. We compared existing techniques using Hydrogen peroxide, Proteinase-K, Trypsin and Potassium hydroxide to digest bivalve tissue. Filterability, digestion efficacy, recoverability of microplastics and subsequent polymer identification using Raman spectroscopy and a matching software were assessed. Only KOH allowed filtration at  $\leq 25\ \mu\text{m}$ . When adding a neutralisation step prior to filtration, KOH digestates were filterable using  $1.2\text{-}\mu\text{m}$  filters. Digestion efficacies were  $>95.0\%$  for oysters, but lower for clams. KOH destroyed rayon at  $60^\circ\text{C}$  but not at  $40^\circ\text{C}$ . Acrylic fibre identification was affected due to changes in Raman spectra peaks. Despite those effects, we recommend KOH as the most viable extraction method for exposure risk studies, due to microplastics recovery from bivalve tissues of single-digit micrometre size.

## Graphical abstract



## Keywords

Microplastics; digestion; improved filterability; Raman spectroscopy; small particles; bivalves

## Introduction

Research into microplastic pollution is a relatively recent topic. Microplastics are generally defined as particles of 0.1 to 5,000  $\mu\text{m}$  (Alexander et al., 2016; Arthur et al., 2009) and are a diverse group of particles in terms of shape and type (Hidalgo-Ruz et al., 2012). Plastics is an umbrella term for synthetic and semi-synthetic materials, such as polypropylene, polyethylene and acrylic. Small plastics emerged as a contaminant in the marine environment in the early 1970s (Buchanan, 1971) and can be present in the environment as fragments, microfibrils, microsheets and sphere-like particles (Hidalgo-Ruz et al., 2012). Thompson *et al.* (2004) coined the term 'microplastics' in an analysis of microplastic concentrations in the marine environment. Since then, microplastics have become a globally recognised contaminant (Andrady, 2011) and research output has increased greatly. With this, a variety of methodologies has been used to extract microplastic particles from marine-based samples. These methodologies vary according to the matrix of interest, such as water, sediment or biota but even within the same matrix (Hidalgo-Ruz et al., 2012; Lusher et al., 2017). There is a need to standardise methodologies to obtain comparable results, if this type of work is to be policy-relevant.

One matrix of interest is marine organisms. Demand for fish and shellfish is constantly rising; global per capita consumption was at 19.7 kg in 2013 (FAO, 2016), making microplastics in seafood a potential concern for human health. Within marine fisheries, bivalves deserve a special focus due to their value but also their exposure risk. Almost 13.6 million tonnes of bivalves were cultured globally in 2014 (FAO, 2016). As filter-feeders, many bivalves are at a greater exposure risk to microplastics (Wright et al., 2013). Mean concentrations of microplastics in such organisms have been reported as 2 particles per gram of the wet weight of the organism ( $\text{g}^{-1}$ ) (e.g. De Witte et al., 2014; Li et al., 2015; Vandermeersch et al., 2015). While the microplastic load in fish is thought to be concentrated in their gastrointestinal tract (GIT) (Foekema et al., 2013; Kühn et al., 2017), which is often removed prior to consumption, bivalves are consumed whole presenting a potential pathway to exposure for humans (Alexander et al., 2016).

The presence of microplastics  $>500 \mu\text{m}$  in organisms is usually assessed via digestion of biological tissues and subsequent analysis of filtrates (Hidalgo-Ruz et al., 2012; Lusher et al., 2017). Digestion techniques need to be able to deal with a number of different tissues, such as carapaces, scales and bony materials (Cole et al., 2014; Karami et al., 2017; Murray and Cowie, 2011). In addition, an

organism's GIT contains its own digestive tissues but also materials ingested for alimentation that have not been broken down by digestive fluids nor excreted yet (Kühn et al., 2017). The focus of this study is on bivalve tissues.

Digestions of bivalve tissue are commonly performed with a range of acids (Van Cauwenberghe and Janssen, 2014), enzymes (Catarino et al., 2017), alkalis (Rochman et al., 2015) and oxidising agents, such as  $H_2O_2$ , the latter relying on the additional step of density separation (Li et al., 2015) (see examples in Table 1). Some digestion protocols apply numerous steps, which may increase the possibility of airborne contamination entering the sample. For example, Collard et al. (2015) performed various extraction steps on filtered gastrointestinal tracts of fish species after visual inspection. Reagents included nitric acid solution (Collard et al., 2015). Acid solutions accelerate natural breakdown of soft tissues, but also partly dissolve bones (Karami et al., 2017). However, acids are the least suitable reagents due to the reported damages to microplastics. Such damages include fusing of PS, PP, PET, LDPE and HDPE, total loss of PA and colour changes to most polymers (Avio et al., 2015; Catarino et al., 2017; Claessens et al., 2013). Alkaline solutions and enzymes also break down soft tissue by dissolving fats and proteins (Masse et al., 2001; Undeland et al., 2002). Limited, or no impacts on polymers have been reported for such reagents at generally applied concentrations (Catarino et al., 2017; Dehaut et al., 2016; Karami et al., 2017). Extensive information regarding visual damage to microplastics by individual reagents exists (Table 1). Damage to microplastics may also arise on a molecular level and can be assessed using techniques such as Raman and IR spectroscopy or pyrolysis-gas chromatography (Dehaut et al., 2016; Kaeppler et al., 2015; Loeder et al., 2015).

Standardisation of methods should be achieved by comparing existing techniques for their suitability in extracting microplastics. Suitability can be determined by assessing the digestion efficacy, the degradation impact on microplastics and recoverability of microplastics from dosed environmental samples (Table 1; Catarino et al., 2017; Cole et al., 2014; Courtene-Jones et al., 2017; Karami et al., 2017).



|                                   |               |   |            |                                  |                      |   |  |                            |  |
|-----------------------------------|---------------|---|------------|----------------------------------|----------------------|---|--|----------------------------|--|
| KOH (60° C for 24 hours)          | 10 % (v/w)    | 3 | 1.6        | 99.6 - 99.8                      | 3 (***)              | LDPE, HDPE, PA6, PA12, PC, PMMA, PP, ePS, PS, PTFE, PUR, PSXL, PVC<br>CA<br>PET | none reported<br>degraded<br>change in shape   | 3 (10 fibres 100 of nylon) | Dehaut et al. (2016)   |
| KOH (25, 40 & 50° C for 96 hours) | 10 % (v/w)    | 3 | 8          | 97.1 - 98.9****                  | 1 (2 - 3 items each) | LDPE, HDPE, PA6, PET, PP, PS, PVC<br>PA66                                       | none reported<br>at 50° C yellowing  | 3 (0.5 g /polymer)         | 84.7 - 105.2****<br>Karami et al. (2017)   |
| KOH (60° C for 96 hours)          | 10 % (v/w)    | 3 | 8          | 97.61 (±0.17)                    | 1 (2 - 3 items each) | PP<br>LDPE<br>HDPE<br>PS<br>PA6<br>PA66<br>PET<br>PVC                           | none reported<br>none reported<br>none reported<br>none reported<br>none reported<br>yellowing<br>none reported<br>none reported | 3 (0.5 g /polymer)         | 102.0 (4.31)<br>103.6 (5.12)<br>103.8 (2.08)<br>101.3 (8.51)<br>99.4 (2.61)<br>99.7 (1.35)<br>78.0 (2.49)<br>85.0 (2.51)<br>Karami et al. (2017) |
| Collagenase (frozen)              | 0.31%         | 3 | 80         | median c.78                      |                      |   | not assessed   | not assessed               | Courtene-Jones et al. (2017)   |
| Corolase 7089                     | 0.5 ml /100ml | 2 | 0.8 or 1.6 | 100, but <0.01 g tissue remained | 1 (***)              | HDPE, PA, PET   | none reported  | 3 (30 per polymer)         | 94 (±10.0 SD)<br>Catarino et al. (2017)  |
| Papain (frozen)                   | 0.31%         | 3 | 80         | median c.75                      |                      |   | not assessed   | not assessed               | Courtene-Jones et al. (2017)   |
| Proteinase-K                      | 500 µg/ml     | 4 | 50         | >97 (not reported)               | 1 (***)              | PA, PE, PS, PVC, PL   | none reported  | not assessed               | Cole et al. (2014)   |
| Trypsin (formaldehyde)            | 0.31%         | 3 | 80         | 78 (±9.45 SD)                    |                      |   | not assessed   | not assessed               | Courtene-Jones et al. (2017)   |

|                     |       |   |    |                   |                         |                          |               |              |                                     |
|---------------------|-------|---|----|-------------------|-------------------------|--------------------------|---------------|--------------|-------------------------------------|
| Trypsin (fresh)     | 0.31% | 3 | 80 | 86<br>(±13.39 SD) | 3 (10<br>items<br>each) | PA, PE, PET, PP, PS, PVC | none reported | not assessed | Courtene-<br>Jones et al.<br>(2017) |
| Trypsin<br>(frozen) | 0.31% | 3 | 80 | 88<br>(±2.52 SD)  |                         |                          | not assessed  | not assessed | Courtene-<br>Jones et al.<br>(2017) |

7

8 Evaluation of methods should go beyond the extraction capacity of techniques. Costs, potential  
9 hazards associated with the reagents and how much time a researcher will have to spend extracting  
10 microplastics from samples are also important considerations. The EU Marine Strategy Framework  
11 Directive (2008/56/EC) and other regionally organised commissions such as OSPAR may require their  
12 partners to perform long-term microplastics monitoring projects (Hanke et al., 2013). Currently,  
13 biota is excluded from such programmes due to lack of knowledge about suitable indicator species  
14 (Hanke et al., 2013). Monitoring programmes to influence policy-making rely on comparable data  
15 using standardised methods. OSPAR published a guidance protocol suggesting tissue digestions with  
16 an acid technique (Dehaut et al., 2016). As described above, such techniques may grossly  
17 underestimate certain microplastics. For this reason, it is important to highlight advantages of other  
18 digestion techniques.

19

20 The aim of this study was to establish the most suitable technique to digest bivalve tissues and  
21 recover microplastics based on a comparison of methods employed in recent studies: Trypsin,  
22 Proteinase-K, KOH and H<sub>2</sub>O<sub>2</sub>. Digestion protocols based on acids were excluded from this study due  
23 to the established damage to microplastics caused by those reagents (see Cole et al., 2014; Karami  
24 et al., 2016; and further references listed above). Protocols with numerous steps were also excluded  
25 to reduce the potential of introducing airborne contamination (such as Avio et al., 2015; Collard et  
26 al., 2015). Density separation techniques were also excluded due to the potential of underreporting  
27 of small microplastics (Haggett, 2017). Techniques were evaluated based on their digestion  
28 performance using three bivalve species, cost, expenditure of time and potential health risk of the  
29 reagents used. In addition, the most suitable technique was assessed for recoverability of dosed  
30 microplastics from oyster tissue. Recovery trials are often performed with microplastics of a single  
31 size and shape (Dehaut et al., 2016; Imhof et al., 2012). Since microplastics in the marine  
32 environment are variable in sizes and shapes (Hidalgo-Ruz et al., 2012), dosing material should  
33 reflect this. Damage to microplastics was assessed through analysis of their Raman spectra.

34



## Materials and methods

### Sampling and sample preparation

*Magallana gigas* (n = 33, tissue wet weight (w.w.)  $14.31 \pm 5.84$  g), *Ostrea edulis* (n = 12,  $7.15 \pm 4.63$  g w.w.) and *Ruditapes philippinarum* (n = 4,  $2.46 \pm 0.14$  g w.w.) were collected from coastal locations in southern England and stored at  $-20^{\circ}\text{C}$  (Oaten et al., 2015). Defrosted soft tissues were removed from shells, placed in glass containers and re-frozen to  $-20^{\circ}\text{C}$ .

Contamination control measures were 100 % cotton clothing, use of glassware and samples covered with aluminium foil. Whenever possible, work was performed in a clean air cabinet (Bassaire 03VB, BS EN ISO14644, class 5, with additional cover). Background contamination enumeration was deemed unnecessary as the microplastic enumeration that took place was of materials of known conspicuous appearance.

### Optimisation and efficacy of digestion protocols

Four digestion protocols were tested:  $\text{H}_2\text{O}_2$ , KOH and enzymes Trypsin and Proteinase-K (Table SI.1). Adaptations were made as follows: consistent with the Proteinase-K protocol (Cole et al., 2014), tissues were freeze-dried prior to digestion. Additionally, the cost and hazardousness of reagents and time expenditure of each method were compared to establish the most suitable digestion technique (see supplementary information 'Costs, time needed and hazardousness of reagents').

**Filters used for digestions.** Initially, cellulose filters (20-25  $\mu\text{m}$ , Whatman grade 4) were used for all digestion protocols. This size was chosen to be the largest acceptable filter size to assess human exposure risks. Filtration through 25  $\mu\text{m}$  was unsuccessful for enzymatic digestions. Due to the cost of Proteinase-K, this technique was abandoned. Filtration of trypsin, on the other hand, due to its lower cost and hazardousness as well as time needed (see supplementary information 'Costs, time needed and hazardousness of reagents') was attempted with 63  $\mu\text{m}$  carbon fibre mesh. Optimisation of filtration to achieve filtering over smaller pore sizes was attempted for the remaining digestion protocols. For this, cellulose nitrate (5  $\mu\text{m}$ , Whatman) and glass fibre (1.2  $\mu\text{m}$ , Whatman GF/C) filters were used.

64

65 **H<sub>2</sub>O<sub>2</sub>.** Li et al. (2015) digest various bivalve species with 30 % H<sub>2</sub>O<sub>2</sub> in conjunction with a NaCl  
66 density separation based on a protocol for salt (Yang et al., 2015). For that matrix, digestate filtration  
67 is possible without a density separation step (Haggett, 2017), which was also attempted here. For  
68 this, 30 % H<sub>2</sub>O<sub>2</sub> (Fisher Scientific, UK) was added to individual oysters at 6x the tissue volume and left  
69 to stand for 24 hours in an oven at 60° C.

70

71 **Proteinase-K.** Karlsson et al. (2017) digested 0.2 g tissue of *Mytilus edulis* based on Cole et al. (2014).  
72 Adaptations were made as follows: 15 ml of homogenisation solution (prepared to 450 ml in pure  
73 water with 31.52 g Trizma-HCl (to 400 mM, Sigma Aldrich), 9.767 g EDTA (to 60 mM, Fisher  
74 Scientific), 4.383 g NaCl (150 mM, Fisher Scientific) and 5.0 g SDS (1 %, Sigma Aldrich), adjusted to pH  
75 8.0) was added, samples were homogenised and subsequently incubated in an oven at 50° C for 15  
76 minutes. Proteinase-K (8 mg) was added and samples further incubated at 50° C for 2 hours. Sodium  
77 perchlorate (375 µl) was added. Jars were placed on a shaking table for 20 minutes and then further  
78 incubated at 50° C for 20 minutes. Karlsson et al. (2017) filtered over 1.2 µm borosilicate filters.  
79 Since common filter size for trials was set to 25 µm, volumes were not adjusted for the increase in  
80 tissue size.

81

82 **Trypsin.** Courtene-Jones et al. (2017) treated *M. edulis* with trypsin (0.3125 % v/v), filtering the  
83 digestate using 50-µm mesh. Based on initial trials, maximum optimisation was achieved using 4x  
84 tissue volume of trypsin (0.3125 % v/v, ThermoFisher) and samples placed on magnetic stirrer at 38  
85 – 42 °C at 250 turns per minute for four hours.

86

87 **KOH.** Similar to Rochman et al. (2015), samples were treated with KOH (10 % w/v, 3x tissue volume,  
88 Acros Organics), but incubated without agitation at 60 °C for 48 hours to increase filtration capacity.  
89 Since filtration over 25-µm filters was achieved, optimisation trials were performed to enable  
90 filtration through smaller pore sizes. Filtration issues when using cellulose nitrate (5 µm) and  
91 borosilicate glass filters (1.2 µm) were overcome by neutralising the digestate with 1 M citric acid  
92 solution (see supplementary information 'Effect on filter papers' for the in-depth assessment of  
93 reagent effects on filters). After slowly adding the citric acid (VWR Chemicals) to achieve a neutral  
94 pH, the solution was gently swirled once and then immediately filtered. This step allowed for

filtration to 1.2 µm. Efficacy was further tested with this adaptation incubating samples with 5 % and 10 % KOH at 40 °C for 48 hours similar to Kühn et al. (2017).

## Digestion efficacy

Two digestion methods were excluded from further experiments: H<sub>2</sub>O<sub>2</sub> due to excessive foaming and therefore possible sample losses and Proteinase-K due to its cost. The trypsin protocol could not be optimised to 25-µm filtration. However, its efficacy was assessed on *M. gigas*, filtering over 63-µm mesh. This was done because of the negligible damage of enzymes to microplastics in the digestion process (see references in Table 1). Tissue digestions were performed in triplicate. Reagent controls were run alongside to establish effects on filters. The KOH protocol was applied to *M. gigas*, *O. edulis* and *R. philippinarum*. Digestates were filtered over pre-weighed filters (Table 2), rinsed with ultrapure water, dried overnight at 60° C and re-weighed at room temperature. Efficacy was calculated by comparing the relative removal of organic material:

$$Efficacy\ in\ \% = 100 - \frac{Dry\ weight\ on\ filter\ paper\ (g) * 100}{Wet\ weight\ entire\ organism's\ tissue\ (g)}$$

Based on Karami et al. (2017), a method was deemed suitable when a digestion efficacy of ≥95.0 % was achieved. The effect of the reagents was calculated by establishing the relative weight change of dry filters:

$$Reagent\ effect\ in\ \% = \frac{Dry\ weight\ of\ filter\ after\ filtration\ (g) * 100}{Dry\ weight\ of\ filter\ prior\ to\ filtration\ (g)}$$

## Quality assurance of digestion method through recovery rate assessment

Microplastics were created from ten post-consumer items (Table SI.3). Fragments were produced with an electrical coffee bean grinder and dry-sieved through 63 and 600 µm stainless steel sieves. Particles <600 µm were retained. Fibres were obtained by plugging and cutting. Film/sheet was also cut. Thirty particles of each material were added to *M. gigas* tissue. Containers used for storing those particles prior to spiking were subsequently inspected for potentially left behind items. Spiked samples were re-frozen and treated like efficacy experiment specimens. Since small pore size for filtration is paramount when assessing human exposure rates (Wright and Kelly, 2017), only the performance of the KOH digestion (10 %, 60° C) was tested (n = 4).

123

124 Handling controls were prepared by dosing ultrapure water (60 ml, n = 4) and kept at room  
125 temperature for 48 hours. This was done to assess potential losses unrelated to the reagent,  
126 temperature or interaction with biological tissue. An additional water blank was run after filtering  
127 the samples to assess if any particles from previous samples could have contaminated subsequent  
128 ones. Samples were vacuum filtered (25-µm filters) and rinsed with 20 ml of ultrapure water. This  
129 amount was chosen because initial trials with tissue samples indicated that larger amounts of water  
130 were not filterable. During such trials, particles were observed stuck to the sample containers and  
131 filtration funnels. To enumerate potential losses, after sample filtration a new filter was placed in  
132 the unit and the sampling jars and funnel flushed with approximately 400 ml of ultrapure water.  
133 Filters were placed in lidded petri dishes and dried at 60° C overnight. Lids were secured with tape.  
134 Particle enumeration was conducted with a microscope (magnification 10x – 60x). See  
135 supplementary information for additional quantification of losses.

136

137 Rayon was affected by 10 % KOH at 60° C during the dosing experiment. Therefore, the effects of  
138 further treatments on rayon were investigated. Ten fibres (n = 4) were treated as follows: 5 % KOH,  
139 10 % KOH (both incubated at 40° C for 48 hours) and Trypsin (according to the optimised protocol).  
140 Samples were filtered over 11-µm and fibres counted.

141

#### 142 **Effect of KOH on Raman spectra**

143 To establish the effect of KOH on the Raman spectra, microplastics from the following treatments  
144 were compared: untreated, exposed to ultrapure water at room temperature and to 10 % KOH at  
145 60°C for 48 hours. Further spectra were obtained from rayon and acrylic fibres exposed to 10 % KOH  
146 at 40°C for 48 hours. Particles were transferred from filters onto quartz slides. Raman spectroscopy  
147 was performed using a 785 nm Renishaw inVia with Leica DM 2500 M microscope, 50x magnification  
148 lens. Particles were manually selected. Extended spectra were obtained at 1-5 % laser power, 10  
149 seconds exposure time and five accumulations. WiRE 4.1 software was used to process the data.  
150 BioRad KnowItAll was used to identify Raman spectra by individual and multi-components as well as  
151 agreement of spectral peaks.

152

## Statistical analysis

Data are reported as means with one standard deviation. Statistical analyses were conducted with RStudio 1.0.153 using an alpha value of 0.05. Independence of data was assumed. Normality was assessed with Shapiro-Wilk tests and homogeneity of variance with Levene's tests. Failing those tests, filter weight changes were assessed with Kruskal-Wallis tests. Recovery rates after KOH digestion were assessed with a two-way ANOVA (factors: polymer type and microplastic category, i.e. fragment, fibre or microsheet). A non-parametric  $\chi$ -squared test was performed to establish if recovery rates were significantly different from 100 %.

## Results

**Digestion efficacy and competitiveness.** Treatments with H<sub>2</sub>O<sub>2</sub> and Proteinase-K were abandoned due to excessive foaming and subsequent sample loss, and high cost respectively. Digestion efficacy for oysters treated with Trypsin and KOH were  $\geq 95.0$  % (Table 2). Trypsin digestates were only filterable using 63- $\mu$ m mesh. KOH digestates were filterable with 25-  $\mu$ m and smaller filters. The lowest variability was achieved using 25- $\mu$ m filter paper. Digestion efficacy of *Ostrea edulis* was comparable to *Magallana gigas* at 5- $\mu$ m pore size:  $97.0 \pm 2.7$  % and  $96.4 \pm 4.0$  % respectively. The slightly lower efficacy and increased variation for *M. gigas* tissue is likely to be due to the presence of small pearls. Further optimisation by neutralising the KOH digestate allowed for filtration over 1.2- $\mu$ m borosilicate filters (see Figure SI.1 for results on effects of KOH on filter papers). Reduced incubation temperature to 40° C did not affect the digestion efficacy but decreased variability for digestates of *M. gigas* tissue (Table 2). Digestion efficacy for the clam species *Ruditapes philippinarum* was  $91.2 \pm 0.5$  % at 1.2  $\mu$ m pore size (Table 2). In absolute terms, when treated with 10 % KOH at 40° C and subsequent neutralisation with citric acid,  $0.021 \pm 0.002$  g of *R. philippinarum* was not digested in compared to  $0.079 \pm 0.037$  g of *M. gigas*.

Table 2 – Digestion efficacies of bivalve tissue and effect of reagent on blank filters. KOH treatment of 10 % solution strength and incubation at 60° C unless otherwise stated; \* 5 % KOH, 40° C; \*\*10 % KOH, 40° C. Mean values based on triplicates, except for ^ which consisted of duplicates.

| Treatment | Species                 | Filter      | Efficacy %<br>(mean $\pm$ 1 SD) | Effect of reagent on blank<br>filters: change in weight in mg<br>(mean $\pm$ 1 SD) |
|-----------|-------------------------|-------------|---------------------------------|--|
| Trypsin   | <i>M. gigas</i>         | 63 $\mu$ m  | 95.8 ( $\pm$ 2.4)               | +0.4 ( $\pm$ 0.4)  |
| KOH       | <i>M. gigas</i>         | 25 $\mu$ m  | 98.2 ( $\pm$ 0.3)               | -0.5 ( $\pm$ 1.2)  |
| KOH       | <i>M. gigas</i>         | 5 $\mu$ m   | 96.4 ( $\pm$ 4.0)               | +3.7 ( $\pm$ 0.5)  |
| KOH       | <i>O. edulis</i>        | 5 $\mu$ m   | 97.0 ( $\pm$ 2.7)               | n/a  |
| KOH       | <i>M. gigas</i>         | 1.2 $\mu$ m | 96.8 ( $\pm$ 0.7)               | +3.0 ( $\pm$ 1.1)  |
| KOH* ^    | <i>M. gigas</i>         | 1.2 $\mu$ m | 96.9 ( $\pm$ 1.0)               | n/a  |
| KOH**     | <i>M. gigas</i>         | 1.2 $\mu$ m | 98.0 ( $\pm$ 0.5)               | n/a  |
| KOH**     | <i>R. philippinarum</i> | 1.2 $\mu$ m | 91.2 ( $\pm$ 0.5)               | n/a  |

182

183 The KOH protocol is the most economic digestion method at £0.55/sample (as of December 2017)  
184 out of the four methods tested (Table SI.2). In addition, together with H<sub>2</sub>O<sub>2</sub>, this digestion method  
185 took <120 minutes of researcher time over three and two days respectively (Table SI.2). Each  
186 digestion method scored once in health hazard category 1, except for Proteinase K, which scored  
187 twice (Table SI.2). Overall, trypsin did score once, the H<sub>2</sub>O<sub>2</sub> method twice and the other methods  
188 five to 13 times in the health hazard class.

189

190 **Recovery rates.** The recovery rates (RR) of 10 types of microplastic (Table SI.3) were established for  
191 oyster tissue digestions using 10 % KOH (Figure 1). Rayon was not recovered after incubation at 60°  
192 C. Removing this outlier, overall recovery was 86.2  $\pm$ 7.9 % from dosed tissue samples. Individual  
193 recoveries ranged 77.5 to 91.6 %. Mean RR from water controls was 78.2  $\pm$ 12.4 %. Without rayon,  
194 there is no significant main effect of polymer type on recovery, ANOVA F(1, 32) = 0.102, p = 0.751.  
195 There is no significant main effect of microplastic category, F(1,32) = 0.557, p = 0.461. There is no  
196 significant interaction effect between polymer and microplastic category, F(1,32) = 0.971, p = 0.332.  
197 The recovery rates of individual types of microplastic are significantly different from 100 % ( $\chi^2$  (15) =  
198 68.110, p < 0.001).

199

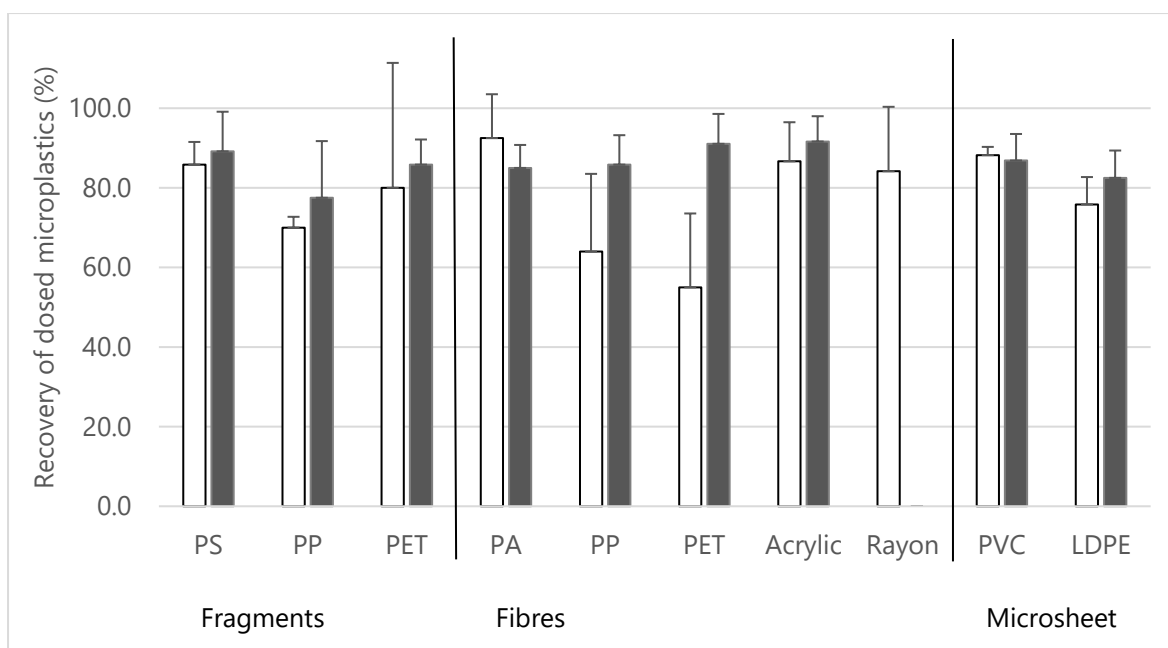


Figure 1– Recovery rate of microplastic particles from oyster tissue exposed at 60° C for 48 hours. Dosed ultrapure water controls (light bars) and oyster tissue digested with 10 % KOH (dark bars). Data are mean values  $\pm$  1 SD , n=4. Recovery rates adjusted for particles that were not transferred into the samples and particles recovered through additional rinsing (see Table SI.3). Key for polymers: PS: polystyrene, PP: polypropylene, PET: polyethylene terephthalate, PA: polyamide, PVC: polyvinyl chloride, LDPE: low-density polyethylene. Note that rayon was recovered when exposed to 10 % at 40° C (see paragraph 3 in 'Recovery rates')

Potential losses of microplastics during the experiment were quantified. Some microplastics were observed in storage containers after dosing (see supplementary information 'Quantification of microplastic particle losses during the spiking experiment'). Recovery rate results were adjusted for those losses. Some microplastics stuck to glass sampling containers and funnel system were only recovered through additional rinsing. On average, 4.1 % of particles were recovered through additional rinsing from filtering spiked bivalve digestates and 17.0 % from spiked water controls. The highest potential losses from the water control were particles and fibres of low density (PS and PP), remaining types had lower loss rates. Recovery rate results were adjusted for these losses. Potential particle loss could also have arisen through accidental transfer of spiked microplastics from one sample to the next. However, filters of water blanks were empty suggesting negligible cross contamination between samples.

221

222 The effect of KOH and Trypsin on rayon fibres at 40° C was assessed. This reduction in incubation  
223 temperature from 60° led to a recovery of rayon similar to the other microplastic types (Figure 1).  
224 Treating rayon fibres (10 fibres per treatment, n = 4) with 5 % KOH for 48 hours led to a recovery  
225 rate of  $87.5 \pm 37.7$  %. Recovery was  $80.0 \pm 8.2$  % for exposure to 10 % KOH for 48 hours. Exposing  
226 rayon to trypsin (4 hours, without using a magnetic stirrer) led to a recovery of  $82.5 \pm 23.6$  %. The  
227 increased variation for the trypsin exposure can be traced to a recovery of 50 % for one of the  
228 samples, the remaining samples yielded 80 – 100 % recovery. There is no significant difference in  
229 recovery between treatments (Kruskal-Wallis,  $H(2) = 0.128$ ,  $p = 0.938$ ).

230

231 **Comparison of Raman spectra of microplastics after KOH exposure.** Raman spectra were obtained  
232 from the microplastics used in this study prior to exposure and after 48-hour exposure to ultrapure  
233 water at room temperature and to 10 % KOH at 60° C (Figure 2). In general, fluorescence and  
234 subsequent baseline adjustments hindered comparison below a Raman shift of  $700\text{ cm}^{-1}$ . Spectra of  
235 polypropylene fibres (A) and fragments (B) exhibited a decreased intensity at peak locations of  $840$   
236 and  $1150\text{ cm}^{-1}$  after exposure to water and KOH. At  $997\text{ cm}^{-1}$ , peak intensity increased with water  
237 and KOH exposure for fibres but was diminished for those treatments in PP fragments. No change in  
238 number of peaks or peak intensity was observed for PS fragments (C) across treatments. Changes  
239 were observed in spectra of acrylic fibres (D). Fluorescence hindered comparison of spectra between  
240 treatments, especially  $<1500\text{ cm}^{-1}$ . The number of peaks and their intensities varied between  
241 treatments. The peak at  $1000\text{ cm}^{-1}$ , was most pronounced for untreated acrylic, reduced for the  
242 water-exposed and absent for the KOH-exposed fibres. Peaks observed at  $1603\text{ cm}^{-1}$  in untreated  
243 and KOH-exposed acrylic shifted slightly to  $1590\text{ cm}^{-1}$ . Furthermore, a peak observed at  $2240\text{ cm}^{-1}$  for  
244 KOH-exposed fibres was not present in water-exposed and untreated acrylic. PET fibres were not  
245 evaluated due to issues with fluorescence and the small fibre diameter. Spectral acquisition of PET  
246 fragments was also hampered by fluorescence, but the number of peaks and their intensity was  
247 similar across treatments (E). A similar number of peaks was observed for LDPE microsheets across  
248 treatments (F). Peak intensities were reduced in untreated compared to water and KOH-exposed  
249 LDPE between  $1250\text{--}1480$  and  $2830\text{--}2900\text{ cm}^{-1}$ . Conversely, at  $1094\text{ cm}^{-1}$ , the peak was most  
250 pronounced for untreated LDPE. The intensity of peaks for PA fibre were generally similar (G) with  
251 the exception of the peak at  $994\text{ cm}^{-1}$ . Here, a peak was observed for the untreated fibre, which was  
252 reduced for the KOH-treated and absent for the water-treated fibre. PVC sheet (H) only exhibited a



difference at  $1003\text{ cm}^{-1}$ ; a peak was observed in the untreated but not in the two treated  
microsheets. The intensity and location of peaks for rayon fibres (I) were generally similar.

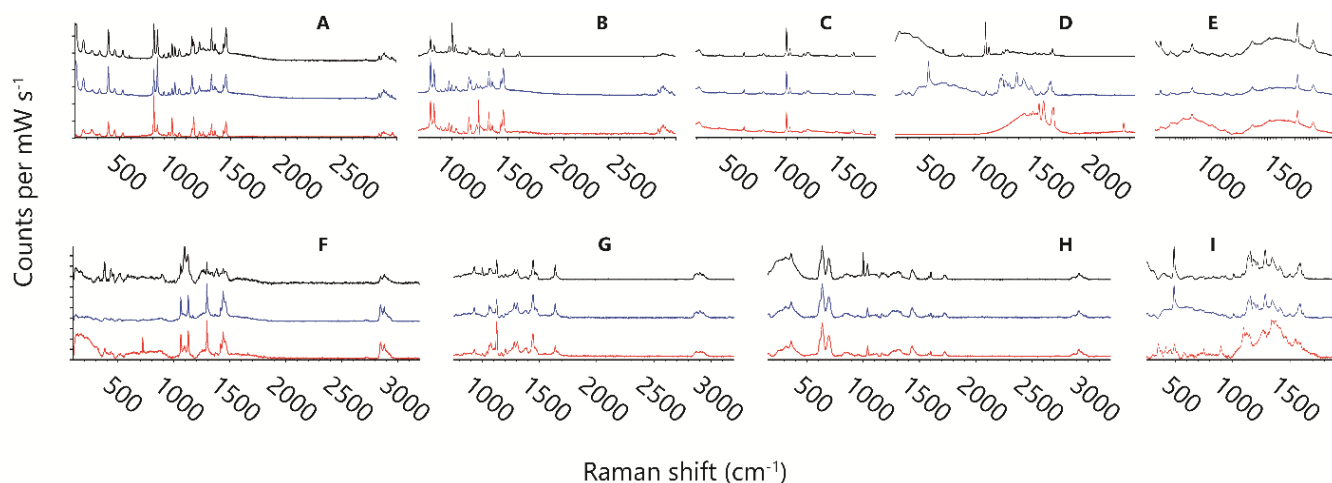


Figure 2 – Raman spectra of microplastics from recovery rate experiment: polypropylene fibres (A), polypropylene fragments (B), polystyrene fragments (C), acrylic fibres (D), polyethylene terephthalate fragments (E), low-density polyethylene film (F), polyamide fibres (G), polyvinyl chloride film (H) and rayon fibres (I). Spectra were obtained using a 785 nm laser at 1-5 % power using a 50x magnification lens. Key for spectral colours: black (top) untreated, blue (middle) exposed to ultrapure water at room temperature for 48 hours and red (bottom) exposed to 10 % KOH at  $60^{\circ}\text{C}$  for 48 hours, except for rayon fibre where top fibres were exposed to 10 % KOH at  $40^{\circ}\text{C}$  instead.

Using a matching software to compare microplastics of unknown composition to a spectral library, most particles exposed to KOH were correctly identified (Table 3). Acrylic could circumstantially be identified as an anthropogenic fibre due to the identification of dye. A number of untreated and water-exposed microplastics were misidentified by the software. Rayon was only identified when exposed to KOH at  $40^{\circ}\text{C}$ .

Table 3 – Results of using a matching software to identify polymer types using Raman spectra.

Spectral peak locations as well as individual and multi-component results were taken into account. "Likely to be" signifies that a choice had to be made by the researcher.

\*Identification after exposure to KOH at  $40^{\circ}\text{C}$ .

| Microplastic       | Treatment of particles  |                            |  |
|--------------------|---|----------------------------|--|
|                    | Untreated   | Water-exposed              | KOH-exposed  |
| LDPE microfilm     | Likely to be rayon  | Polyethylene               | Polyethylene   |
| PA microfibre      | Likely to be nylon  | Likely to be nylon         | Nylon  |
| Acrylic microfibre | Identified as plastic (polyester, computer disk or polystyrene) | Likely to be nylon         | Likely to be identified as some sort of anthropogenic/plastic fibre due to dye |
| PET microfibre     | Polyester   | Polyethylene terephthalate | Not assessed   |
| PET fragment       | Polypropylene   | Likely to be polyester     | Likely to be Polyethylene terephthalate  |
| PP microfibre      | Polypropylene   | Polypropylene              | Polypropylene  |
| PP fragment        | Could be polypropylene or polystyrene                           | Polypropylene              | Polypropylene  |
| PS fragment        | Likely to be polystyrene  | Polystyrene                | Polystyrene  |
| PVC microfilm      | Polyvinyl chloride  | Polyvinyl chloride         | Polyvinyl chloride   |
| Rayon microfibre   | Not identifiable  | Not identifiable           | Lyocell*   |

276

## 277 Discussion

278 A number of studies already set out to compare different digestion methods (Table 1). However,  
279 target methods are often evaluated against protocols not used in microplastics research. NaOH was  
280 compared to other reagents by four studies (Catarino et al., 2017; Cole et al., 2014; Karami et al.,  
281 2017; Phuong et al., 2018). This reagent has not been applied to environmental samples on its own  
282 to our knowledge, but only in conjunction with HNO<sub>3</sub> or sodium dodecyl sulfate (SDS) with the  
283 recommendation of further adding HCl to digest the GIT of fish (Budimir et al., 2018; Roch and  
284 Brinker, 2017). Reagent concentrations of 10 M (NaOH) (Cole et al., 2014; Dehaut et al., 2016) or 100  
285 % (KOH) (Enders et al., 2017) are unlikely to yield satisfactory results, but were used for comparison  
286 studies. Here, four published methods were tested for their suitability to digest bivalve tissue in an  
287 attempt to derive a standardised method. Two of these were aborted during the trials. An  
288 optimisation attempt of an existing H<sub>2</sub>O<sub>2</sub> technique resulted in unacceptable foaming. This would  
289 explain why previous studies had to rely on additional steps to extract microplastics from tissue  
290 using this technique. Karlsson et al. (2017) pre-treated their samples with enzymes. Such approach is  
291 likely to increase the cost of an already costly technique (Table SI.2). Li et al. (2015) added a NaCl  
292 flotation step. However, this approach is likely not to recover a large portion of microplastics  
293 (Haggett, 2017). Avio et al. (2015), using NaCl flotation followed by H<sub>2</sub>O<sub>2</sub> digestion, successfully  
294 extracted microplastics from fish GITs, obtaining recovery rates of around 80 to 90 % depending on

particle size classes. However, it should be noted that they dosed samples with lower density polymers polyethylene and polystyrene only (Avio et al., 2015). Optimisation of an existing Proteinase-K approach would have resulted in unacceptable costs. Since previous studies digested only 0.2 g of tissue with Proteinase-K (Cole et al., 2014; Karlsson et al., 2017), the cost of digesting tissue of entire oysters would likely have increased fourfold or more (Table SI.2). Adjusting the tissue sample amount to suit the existing protocol is not advisable, as this would lead to sample heterogeneity. So far, most studies on European bivalves reported <1 microplastic/gram w.w. (De Witte et al., 2014; Van Cauwenberghe et al., 2015; Van Cauwenberghe and Janssen, 2014; Vandermeersch et al., 2015).

For continuing trials, efficacy of oyster tissue digestion efficacies (*M. gigas* and *O. edulis*) were above the set threshold of 95 % adopted from Karami et al. (2017). Trypsin digestions, while meeting the threshold (95.8 %), were only filterable using 63-µm mesh. This seems an improvement compared to digesting *M. edulis*. Courtene-Jones et al. (2017) obtained a digestion efficacy of 88 % when using 80- µm mesh. Depending on the research aim, such minimum particle size may be suitable for some studies, especially due to its relatively low cost (Table SI.2). An enzymatic digestion is usually preferred over other reagents due to the negligible effect on plastic polymers (Catarino et al., 2017; Cole et al., 2014). However, it should be noted that some enzyme digestion techniques also rely on oxidising reagents to be added, e.g. sodium perchlorate or hydrogen peroxide (Cole et al., 2014; Karlsson et al., 2017). Catarino et al. (2017) successfully tested Corolase 7089 (AB Enzymes) for bivalve digestion; however, this enzyme is not commercially available any more. Here, we find that an adapted KOH protocol yielded the best results in terms of filtration ability, extraction costs, hazardousness of reagents and approximate time needed for digestions. It is worth noting that a recent study by Wagner et al. (2017) conceived a protocol to extract microplastics from fish GITs without digestion using H<sub>2</sub>O and pulsed ultrasonication. While this extraction method has a short sample turnaround time and does not involve hazardous chemicals, Caron et al. (2018) suggest that ultrasonication may lead to fragmentation of microplastics into smaller particles. In addition, it remains to be tested if ultrasonication would work for entire specimens of bivalves compared to relatively loose tissues and other aggregates in digestive tracts.

Our adapted KOH digestion technique can cope with a large fraction of the entire microplastics range, therefore being suitable for a range of studies. Earlier studies of bivalve tissues achieved recovery with single-digit µm filters, but might have underestimated microplastic concentrations.

These studies either employed a combination of digestion and density separation (Li et al., 2015; Phuong et al., 2018), digested with acids (Van Cauwenberghe and Janssen, 2014) or digested only small amounts of tissue (Dehaut et al., 2016; Karlsson et al., 2017). Interestingly, while Catarino et al. (2017) achieved a digestion efficacy of 100 % for bivalve tissues using a similar reagent (1 M of NaOH) over 0.8 µm filters, Cole et al. (2014) obtained 90 % zooplankton tissue removal over 50 µm filters. This suggests that digestion techniques may not universally applicable between different phyla. We achieved digestate filtration over 1.2 µm. Capturing smaller particles still comes with further challenges as confirmation of chemical composition through spectro-fingerprinting (Raman and FTIR approaches) is limited at these sizes (Käppler et al., 2016; Loeder et al., 2015).

Target particle size will depend on the aim of individual studies. Aims may include potential health effects on individuals, their populations or consumers such as humans. Observation from the natural environment suggest that microplastics are ingested by bivalves beyond the size of their natural prey particles. Particle retention of *M. gigas* is highest between 4 and 12 µm and reported absent for particles of 15 µm (Ropert and Goulletquer, 2000). Van Cauwenberghe and Janssen (2014) found mainly microplastics <25 µm in *M. gigas*, but larger items were also present. Rochman et al. (2015) found fibres between 2,300 and 15,800 µm in length in *M. gigas*. Fibre length may not be crucial for microplastic size determination as the particle could be present compacted or knotted up (Thompson et al., 2004). However, diameters of those fibres were 20 to 50 µm (Rochman et al., 2015). In addition, Courten-Jones et al. (2017) found fragments and film >52 µm in *M. edulis*, a species with a particles retention range of 2 -12 µm (Cranford et al., 2016). In a laboratory setting, the ribbed mash mussel *Geukensia demissa* ingested microbeads of 250-300 µm in size, which is beyond their usual feeding range (Khan and Prezant, 2018). This shows that an extraction technique needs to be able to cope with a large size range. For studies relating to potential human health implications, authorities suggest <150 µm as a suitable target size (Alexander et al., 2016; Wright and Kelly, 2017). Translocation of microplastics from the digestive system to other body parts seems most likely for particles <500 µm (Browne et al., 2008; Collard et al., 2017). Most evidence, especially in mammalian and human observations, indicate that <130 µm could be the potential threshold for particle toxicology, with particles <10 µm potentially posing a greater risk (Browne et al., 2008; Papageorgiou et al., 2014; Volkheimer, 2001; Wright and Kelly, 2017). Potential effects on bivalves were assessed with <10 µm diameter microbeads in laboratory studies. Effects shown so far range from translocation of microbeads from the digestive to the circulatory system to changes in gene

expression to effects on offspring (Browne et al., 2008; Paul-Pont et al., 2016; Sussarellu et al., 2016).

KOH has previously been used for many types of tissue, including digestive tracts of fish and oyster tissue (e.g. Foekema et al., 2013; Rochman et al., 2015; Tanaka and Takada, 2016; Wesch et al., 2016) indicating its suitability for a range of tissues. However, digestion efficacy will depend on filter sizes. Rochman et al. (2015) recovered particles >500 µm from *M. gigas*. Here we show that digestion and filtration with 1.2-µm filters is possible for this species. The digestion efficacy of *R. philippinarum* was below 95 %. However, it should be noted that – being of smaller body size – the absolute quantity remaining on the filter was almost four times lower compared to *M. gigas*. Such lower particle load should allow for adequate visual inspection of the material.

For successful filtration of KOH digestates with 1.2 µm borosilicate filters, it was necessary to neutralise the digestate. Wagner et al. (2017) prevented damage to polycarbonate filters through neutralising KOH with 10 % HNO<sub>3</sub>. Dehaut et al. (2016) filtered over 1.6 µm borosilicate filters without neutralisation. However, their specimens of *M. edulis* were almost one third the size of *M. gigas* in this study (Dehaut et al., 2016). Filtration of such digestates with lower tissue load may be able to be performed quickly enough without affecting the filter matrix. Issues with filtering KOH solution without prior neutralisation using borosilicate filters may be related to the effect of KOH on the glass. The increase in filter weight may be explained by redistribution of borosilicate within the filters preventing complete filtration of samples and water afterwards. Previous studies have reported effects of KOH on borosilicate and other glass. Interestingly, Molchanov and Prikhid'ko (1958) and Kouassi et al. (2010) report reductions in glass weight after exposure to KOH. However, Kouassi et al. (2010) report the formation of precipitates. They quantified the glass loss using 40-µm filters (Kouassi et al., 2010). It may be that those precipitates were small enough to escape in their study, but - if formed during our experiment - be leading to precipitates decreasing filtration capacity by blocking the filter. This issue seems to be avoidable by neutralising the KOH solution prior to filtration. Short-term exposure to citric acid should not damage plastic polymers according to freely available on-line chemical resistance tables (e.g. Bürkle, 2017; TedPella, 1996-2019).

Overall recovery rate of spiked microplastics is similar to overall recovery rates reported elsewhere for alkaline reagents. Some individual recovery rates were lower while others were higher than

reported by Karami et al. (2017) for KOH digestions indicating general variability in microplastics recovery from environmental samples. While our recovery rate is lower than a desirable 100 %, we attempted to recover particles from a range of polymers, categories and sizes, which may be more in line with environmental samples (Hidalgo-Ruz et al., 2012). Spiking particle size has been shown to affect recovery rates. Dehaut et al. (2016) obtained a high recovery rate for PA fibres of 500  $\mu\text{m}$ ; however, Avio et al. (2015) noted significantly lower recovery rates of microplastics <100  $\mu\text{m}$ . In line with this, a study of microplastics recovery from sediments recovered 99.7 % of large microplastics (2,000 – 5,000  $\mu\text{m}$ ), but only 39.8 % of particles ranging 40 – 309  $\mu\text{m}$  (Imhof et al., 2012). The spiking particles used in our study potentially covered a large portion of the microplastics range ( $\geq 63 \mu\text{m}$ ). Some of the microplastics created for this experiment were prone to stick to jars and equipment despite copious rinsing. This issue was also observed by others with environmental samples (Haggett, 2017; Karlsson et al., 2017). Recoveries from spiked tissue samples and water controls were similar. This suggests that the handling process may still be the source of the reduced recovery rate and flushing of the equipment with >500 ml of water may be required. Furthermore, microplastics may have been electrically charged and therefore escaped during processing. This was previously suggested for polyamide (Catarino et al., 2017) and was observed for PET fibres and fragments and PS fragments during this study.

In addition to previous studies (Catarino et al., 2017; Karami et al., 2017), we found that 10 % KOH is also suitable for recovery of acrylic and rayon fibres, PP and PET in fibre-form and film of PVC and LDPE. The digestion temperature needs to be carefully chosen. At 60° C, rayon was not recovered. It seems that rayon was dissolved by the alkaline reagent, similar to the process of producing rayon where cellulose is dissolved in NaOH and fibres subsequently recovered from the solution (Jarvis, 2003). However, decreasing the incubation temperature to 40° C allows for recovery of rayon fibres in the same range as the other tested microplastic polymers. In line with previous research (Table 1), no effects were observed on most other polymers. No obvious visual impacts were observed on polymers during enumeration of spiked microplastics from the recovery experiment, suggesting negligible impact on those. Dehaut et al. (2016) reported damage to PC, with 50 % being physically degraded. This degradation was observed at 60° C. An incubation temperature of 40° C using 10 % KOH is therefore recommended for the recovery of all microplastics types assessed here.

The degree of polymer damage through KOH exposure seems acceptable as most obtained Raman spectra were correctly identified by a matching to a spectral database. Acrylic fibre was only identified circumstantially by the presence of the dye 'Eriochrom Blue' that is used in the textile industry (Ding and Freeman, 2017). Acrylic fibre may be affected on a molecular level as revealed by Raman spectroscopy. It appears that acrylic fibres are easily hydrolysed by alkalis. Gupta et al. (2004) observed similar changes in nitrogen, amide and carboxyl groups during acrylic exposure to NaOH. A reduction in digestion temperature to 40° C did not improve the identification. The effect of KOH on the Raman spectra of the remaining microplastics was limited. This is in line with previous studies (Dehaut et al., 2016; Karami et al., 2017). Some differences, however, do exist. For example, Karami et al. (2017) observed a reduced intensity at 1610 cm<sup>-1</sup> in PET when exposed to 10 % at 50 or 60° C for 96 hours. It could be possible that this effect may show when exposing the polymer for >48 hours as neither we (48 hours at 60° C) nor Dehaut et al. (2016) (24 hours at 60° C) observed this. Changes in spectra of LDPE observed here but not elsewhere (Dehaut et al., 2016) may also be related to differences in exposure time. Changes observed in PP and PVC were not reported elsewhere (Dehaut et al., 2016; Karami et al., 2017). It could therefore be possible that those were induced by a temperature >40° C and an exposure time exceeding 24 hours. In addition, we found that changes in Raman spectra of LDPE, PA, PP and PVC observed in this study may be due to the exposure to a liquid in general for 48 hours rather than KOH-specific. Since no study to our knowledge has assessed the effect of pure water on polymers, conclusions are difficult to draw.

## Conclusion

Assessment of potential health risks to humans is likely to rely on quantification of microplastics on the lower end of their size range. We compared digestion techniques previously applied by other studies. Based on the evidence presented here, 10 % KOH is a suitable digestion technique for bivalve tissues, even when the target particles are of single-micrometre size. Filtration was achieved using filter papers of 1.2 µm when neutralising the digestate prior to filtration. The digestion temperature is important and effects on semi-synthetic polymers (e.g. rayon) can be minimised by choosing an incubation temperature of 40° C or lower. The lower temperature also delivered more consistent results. The use of a matching software to assess the Raman spectra of the microplastics used in this study showed that all types were identifiable after KOH exposure. KOH is also the most economical and least time-consuming method. Its hazardousness to human health is similar to or less than other reagents used for tissue digestions. Since KOH has also successfully been used for

other tissues, we suggest that this method is adapted as a standard method for biological tissue digestions to extract microplastics. Such a harmonised approach will allow for comparability between studies and make such results more policy-relevant.

## **Data availability**

Data supporting this study are openly available from the University of Southampton repository at <http://dx.doi.org/10.5258/SOTON/xxxxx>.

## **Statement of competing interest**

The authors declare no competing interest.

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## **Disclosure of previous publications**

A small selection of results from this manuscript were presented at EMCON 2018, Oslo, Norway, in June 2018 as a poster.

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482

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**Supplementary Information to ‘Evaluation of existing methods to extract microplastics from bivalve tissue: adapted KOH digestion protocol improves filtration at single-digit pore size’ by Thiele, Christina J.; Hudson, Malcolm D.; Russell, Andrea E.**

Content of file:

- Table SI.1 – Existing approaches of extracting microplastics from bivalve tissue with the exception of acid digestions.
- Section on ‘Costs, time needed and hazardousness of reagents’.
- Table SI.3 – Microplastics produced in-house from consumer products by polymer and used for a range of recovery experiments by our research group, including relative densities and approximate global production in 2015 in million metric tonnes.
- Section on ‘Effect on filter papers’.
- Section on ‘Quantification of microplastic particle losses during the spiking experiment’.

1 Table SI.1 – Existing approaches of extracting microplastics from bivalve tissue with the exception of acid digestions (method development studies only not included)

| Reagent & study  | Types of bivalves  | General approach  | Filter type & size   | Notes  |
|--|--|---|--|--|
| H <sub>2</sub> O <sub>2</sub> by Li et al. (2015)                  | <i>Scapharca subcrenata</i> , <i>Tegillarca granosa</i> , <i>Sinonovacula constricta</i> , <i>Ruditapes philippinarum</i> , <i>Meretrix lusoria</i> , <i>Cyclina sinensis</i> , <i>Patinopecten yessoensis</i> , <i>Alectryonella plicatula</i> , <i>Mytilus galloprovincialis</i> | 200 ml of 30 % H <sub>2</sub> O <sub>2</sub> to sample (1 – 5 specimens)<br>Incubation at 65° C for 24 hours, additional incubation at room temperature for 24 – 48 hours<br>Flotation with NaCl solution | Cellulose nitrate, 5 µm  | Optimisation attempt to cut out density separation step led to unacceptable levels of foaming.   |
| KOH by Rochman et al. (2015)                                       | <i>Magallana gigas</i>   | 3 times volume of tissue of 10 % KOH to sample (entire specimen), incubation at 60° C overnight   | n/a<br>(Microplastics of >500 µm were directly visualised with microscope) | Optimisation by increasing incubation time to 48 hours. Effects on filter papers and filtration efficiency removed by neutralisation of digestate prior to filtration. Decrease in incubation temperature and KOH strength possible (details in text).       |
| Trypsin by Courtene-Jones et al. (2017)                            | <i>Mytilus edulis</i>  | Entire specimen, 0.3165 % Trypsin, 20 ml per specimen, incubation at 38-42° C for 30 minutes  | 80 µm mesh gauze   | Optimisation achieved by adding 4 times volume of wet tissue to sample and incubate for 4 hours, filtration over 63-µm mesh.   |
| Proteinase-K by Karlsson et al. (2017) based on Cole et al. (2014) | <i>Mytilus edulis</i>  | sample: 0.2 g of tissue, additionally used Tris-HCl, SDS, CaCl <sub>2</sub> , H <sub>2</sub> O <sub>2</sub> , incubated at 60° C and filtered next day  | 1.2 µm GF/C  | Digestion of whole specimens (c.1.0-1.2 g DW) with additional Tris-HCl, EDTA, SDS, NaCl, sodium perchlorate similar to Cole et al. (2014). Quantities not adjusted, but filter size increased to 25 µm. Digestate was not filterable with vacuum filtration. |

### **Costs, time needed and hazardousness of reagents**

Costs of digestion per sample were based on a mean oyster tissue volume of 20 ml ( $11.8 \pm 6.4$  g w.w.). When optimisation was not possible, calculations were performed with the most promising protocol (e.g. observation of highest digestion rates). Prices were taken of the website from Fisher Scientific, or Sigma Aldrich if not available at the former, on 29<sup>th</sup> December 2017. The cost of ultrapure water- which will be laboratory-dependant - was estimated at £0.232/litre based on the following estimates:

- DI pack for ultrapure - £556.00 (replaced approximately every 9 months on average)
- UV lamp for ultrapure - £141.00 (replaced approximately every 18 months)
- Delivery speed of pump – 2L/min
- Estimated daily usage – 10 L
- Average month: 30 days
- Consumables that provide the water in the tank to get it to a certain grade to feed the ultrapure water are not included.

The length of time needed per digestion was recorded during optimisation and efficacy trials. H-phrases of the individual reagents were obtained from Material Safety Data Sheets from the two suppliers. Categories of health hazards associated with each method was used as a proxy for hazardousness. The results can be seen in Table SI.2.

Table SI.2 – Cost, expenditure of time and hazardousness of different digestion methods.

Reagent cost were calculated in December 2017 (website of Fisher Scientific, or Sigma Aldrich if not found at the former). Health hazard scores according to Material Safety Data Sheets from the same suppliers divided into categories C1 to C4.

| Digestion method  | Reagent costs per sample (£) | Type 1 water (£) cost | Cost per sample (£) | Approximate time needed for digestion (per batch)   | Health hazard category scores      | Notes  |
|---|------------------------------|-----------------------|---------------------|---|------------------------------------|--|
| 10 % KOH (with $C_6H_8O_7$ )                                | 0.52                         | 0.03                  | 0.55                | Day 1: block of <60 min, day 3: block of <60 min  | C1: 1;<br>C2: 3;<br>C4: 1          |  |
| 30 % $H_2O_2$   | 2.88                         | 0.05                  | 2.93                | Day 1: block of <60 min, day 2: block of <60 min  | C1: 1;<br>C4: 1                    |  |
| Trypsin   | 0.98                         | 0.02                  | 1.00                | <120 min over one day (approx. 4 hours of unoccupied time)  | C1: 1;                             | Cost could increase as method not optimised                                    |
| Proteinase K (with $NaClO_4$ , SDS, NaCl, EDTA, Trizma-HCl) | 2.98                         | >0.01                 | 2.98                | Day 1: c.120 min over ½ day (c.2 hours unoccupied time), day 2: block of <60 min; occasional preparation of reagents 60 min | C1: 2,<br>C2: 6,<br>C3: 2<br>C4: 3 | Method not optimised; amount for 0.2 g tissue, average oyster dry weight 1.5 g |

## Making of microplastics for spiking experiment

Table SI.3 – Microplastics produced in-house from consumer products by polymer and used for a range of recovery experiments by our research group, including relative densities and approximate global production in 2015 in million metric tonnes.

Key for polymers: PS: polystyrene, PP: polypropylene, PET: polyethylene terephthalate, PA: polyamide, PVC: polyvinyl chloride, LDPE: low-density polyethylene. Relative densities were obtained from Andrady (2011), Gooch (2017), Taiseisha (1970) cited in

<http://www.pvc.org/en/p/specific-gravity-density>, [https://www.natureworkslc.com/~media/Files/NatureWorks/Technical-Documents/Fact-Sheets/Fibers/FactSheet\\_Fabrics\\_Fiber\\_FabricProperties.pdf.pdf?la=en&la=en](https://www.natureworkslc.com/~media/Files/NatureWorks/Technical-Documents/Fact-Sheets/Fibers/FactSheet_Fabrics_Fiber_FabricProperties.pdf.pdf?la=en&la=en), <http://www.dotmar.com.au/propertytables.php>;

Global production values were obtained from Geyer et al. (2017) estimates of fibre production from <https://www.cirfs.org/statistics/key-statistics/world-production-fibre>,

\* including other polyester fibres, \*\* including other cellulosic fibres

| Polymer type | Microplastic category | Colour | Origin                          | Relative density of virgin polymer | Approximate global production in 2015 (in million tonnes) |
|--------------|-----------------------|--------|---------------------------------|------------------------------------|---|
| PS           | Fragment              | White  | Lid of disposable coffee cup    | 1.04-1.05                          | 24  |
| PP           | Fragment              | Clear  | Single-use ready meal container | 0.57-0.91                          | 68  |
| PET          | Fragment              | Green  | Olive oil bottle                | 1.29-1.44                          | 32  |
| PP           | Fibre                 | Cream  | Carpet                          | 1.04-1.05                          | 4   |
| PA           | Fibre                 | Yellow | Tutu fabric                     | 1.11-1.29                          | 4   |
| PET          | Fibre                 | White  | Fleece blanket                  | 1.29-1.44                          | 50*   |
| acrylic      | Fibre                 | Blue   | Fleece fabric                   | 1.2                                | 1.7   |
| Rayon        | Fibre                 | Black  | Garment fabric                  | 1.52                               | 5**   |
| PVC          | Microsheet            | Clear  | Table cloth                     | 1.25-1.47                          | 36  |
| LDPE         | Microsheet            | White  | Lining of disposable coffee cup | 0.91-0.92                          | 62  |

## Additional references used

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Gooch, J. W. *Encyclopedic Dictionary of Polymers*. Springer-Verlag New York (2017).

**Effect on filter papers.** Initial trials revealed that KOH affected the filtering capacity of filter papers, e.g. the longer the digestate took to filter the more difficult it was to flush the filter with pure water afterwards. This effect was tested by subjecting pre-weighed cellulose (Whatman Grade 4), cellulose nitrate (5.0  $\mu$ m Whatman) and borosilicate (Whatman GF/C) filters to three different neutralisation treatments in triplicate. Samples of 25 ml of 10 % KOH solution were heated to 60° C. The following treatments were used: 1) no neutralisation, 2) part-neutralisation with 15 ml of 1 M citric acid and 3) complete neutralisation with 22 ml of 1 M citric acid. Mixtures were transferred immediately to the vacuum filtration unit. To mimic slow filtration, the pump was not switched on for 90 seconds, during which time part of the mixture passed through the filter gravitationally. The remainder (c.10 ml) was filtered using the vacuum. Filters were rinsed with 20 ml ultrapure water, transferred to petri dishes, dried at 60° C overnight and re-weighed at room temperature. The pH of the each mixture was established using paper indicator strips. The relative weight change of the filters was used as a measure of effect (Equation 4, supplement information).

The effect of the solution on the filter papers can be seen in Figure 3 (main text). Cellulose nitrate filters seem to become heavier with neutralised solutions (Kruskal-Wallis,  $H(1) = 3.86$ ,  $p = 0.05$ ). It is worth noting that cellulose nitrate filters exhibited only a small change in weight using the original solution despite one of the filters partly disintegrating. In addition, two filters split and all filters turned yellow. The weight of cellulose filters using original and neutralised solution was similar,  $4.36 \pm 3.32$  and  $4.03 \pm 1.78$  % respectively (Kruskal-Wallis,  $H(1) = 0.05$ ,  $p = 0.83$ ). Conversely, borosilicate filter weights increased by  $15.78 \pm 5.21$  % using the original solution, but only by  $3.56 \pm 1.27$  % when filtering neutralised KOH solution (Kruskal-Wallis,  $H(1) = 3.86$ ,  $p = 0.05$ ). No visual damage was observed for cellulose and glass filters.

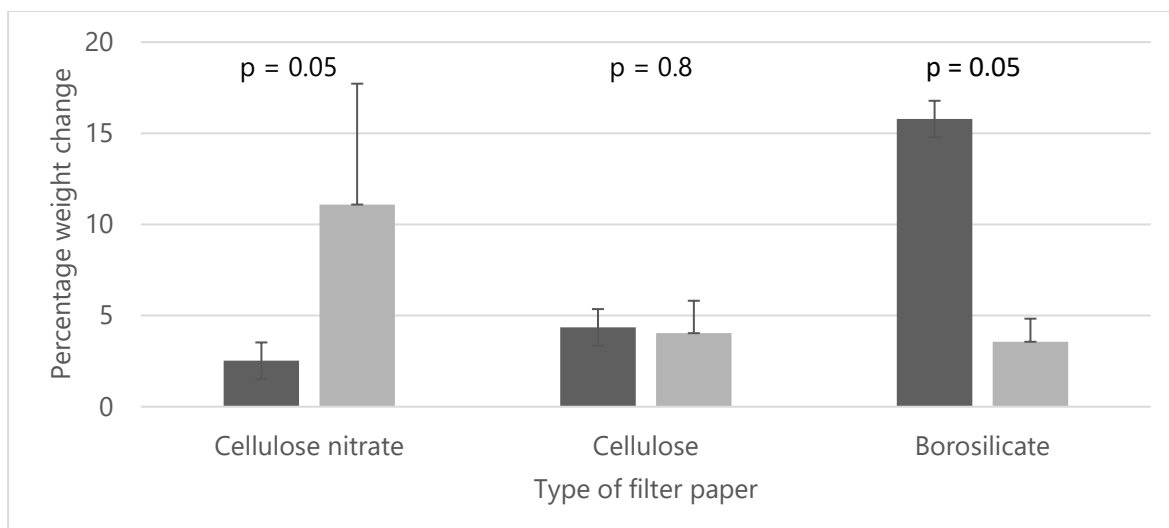


Figure SI.1 – Effect of neutralised (light grey bars) and not neutralised 10 % KOH solution (dark grey bars) on mean weight of filter papers of cellulose nitrate, cellulose and borosilicate glass (n = 3). Filter papers were subjected to the solution mixture for 90 seconds prior to flushing. Difference in weight changes were tested using Kruskal-Wallis.

## **Quantification of microplastic particle losses during the spiking experiment.**

Incorrectly counted out microplastics in preparation for the spiking experiment. Prior to spiking, known amounts of microplastics were placed into glass petri dishes. Spot checks of closed dishes showed that quantities were often below 30 particles per type of microplastic. In four containers, between six and all ten reference material types were recounted. All fragments and microsheets were counted. Estimation of number of fibres proved difficult when fibres were present in agglomerations. Dishes were not opened during this process to prevent additional losses through particles jumping or sticking to tools. In no case were there more than 30 particles. The minimum number of fragments was 24 for PP, 27 for LDPE microsheet and 28 PP fibres. Based on these findings, it can be assumed that a mean of  $28.75 \pm 1.91$  fragments,  $29.00 \pm 0.76$  fibres and  $29.25 \pm 1.04$  microsheets was prepared for each sample. Since only a fraction of prepared reference materials was recounted, results were not adjusted for those estimated losses. Otherwise, recovery rates would have increased between 2.1 and 3.9 %, except for rayon.

### Lack of transfer of counted out reference materials from their storage container to the sample.

Counted out microplastics were transferred from petri dishes to samples. Petri dishes were sealed with tape and inspected for non-transferred particles. In 16 out of 21 containers some particles remained. Mean particle content in those 16 was  $5.4 \pm 6.1$ . The maximum number of particles that remained in one dish was 21. Out of the microplastics created, PS and PET fragments, PA fibres and LDPE sheet all transferred to the samples. Between two and five particles were not transferred for PP fragments, PP, rayon and acrylic fibres respectively. Forty-four pieces of PVC microsheet and 29 PET fibres did not transfer overall. The results shown in Figure 1 (main text) were adjusted for those losses.

### Microplastics recovered through additional rinsing

As mentioned in the results section, Figure 1 was adjusted for microplastics that were only recovered through additional rinsing. Those additional recovery rates can be found in Table SI.4.



Table SI.4 - Recovered microplastics after thorough rinsing of the filtration unit from spiked oyster tissue. Data are percentage mean values ( $\pm$ SD) based on four replicates.

| Sample ID | Fragments |    |     | Fibres |    |     |         |       | Sheet |      |
|-----------|-----------|----|-----|--------|----|-----|---------|-------|-------|------|
|           | PS        | PP | PET | PA     | PP | PET | Acrylic | Rayon | PVC   | LDPE |
| RR6       | 0         | 1  | 0   | 0      | 1  | 0   | 0       | 0     | 0     | 0    |
| RR8       | 1         | 3  | 1   | 2      | 0  | 0   | 1       | 0     | 0     | 1    |
| RR10      | 3         | 0  | 4   | 3      | 2  | 0   | 2       | 0     | 1     | 0    |
| RR12      | 3         | 4  | 1   | 0      | 4  | 0   | 3       | 0     | 0     | 6    |