Environmental Pollution
August 2016, Volume 215 Pages 223-233
http://dx.doi.org/10.1016/j.envpol.2016.05.018
http://archimer.ifremer.fr/doc/00335/44582/

Microplastics in seafood: Benchmark protocol for their extraction and characterization

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Abstract :

Pollution of the oceans by microplastics (<5 mm) represents a major environmental problem. To date, a limited number of studies have investigated the level of contamination of marine organisms collected *in situ*. For extraction and characterization of microplastics in biological samples, the crucial step is the identification of solvent(s) or chemical(s) that efficiently dissolve organic matter without degrading plastic polymers for their identification in a time and cost effective way. Most published papers, as well as OSPAR recommendations for the development of a common monitoring protocol for plastic particles in fish and shellfish at the European level, use protocols containing nitric acid to digest the biological tissues, despite reports of polyamide degradation with this chemical. In the present study, six existing approaches were tested and their effects were compared on up to 15 different plastic polymers, as well as their efficiency in digesting biological matrices. Plastic integrity was evaluated through microscopic inspection, weighing, pyrolysis coupled with gas chromatography and mass spectrometry, and Raman spectrometry before and after digestion. Tissues from mussels, crabs and fish were digested before being filtered on glass fibre filters. Digestion efficiency was evaluated through microscopical inspection of the filters and determination of the relative removal of organic matter content after digestion. Five out of the six tested protocols led to significant degradation of plastic particles and/or insufficient tissue digestion. The protocol using a KOH 10% solution and incubation at 60 °C during a 24 h period led to an efficient digestion of biological tissues with no significant degradation on all tested polymers, except for cellulose acetate. This protocol appeared to be the best compromise for extraction and later identification of microplastics in biological samples and should be implemented in further monitoring studies to ensure relevance and comparison of environmental and seafood product quality studies.

Graphical abstract :

Highlights

► Integrity of 15 plastics were tested using six protocols of digestion. ► Protocols using HNO_3 led to significant polyamide degradation. ► KOH 10% solution does not affect the integrity of all tested plastics except for CA. ► KOH 10% provides effective digestion of mussel, crab & fish tissues. ► KOH 10% is the best compromise for extraction and identification of microplastics.

Abbreviations

- CA, Cellulose Acetate;
- ePS, expanded Polystyrene;
- GC, Gas chromatography;
- HCl, Hydrochloric acid;
- HDPE, High Density Polyethylene;
- LDPE, Low Density Polyethylene;
- MS, Mass Spectrometry;
- PA, Polyamide;
- PA-12, Polylauryllactam;
- PA-6, Polycaprolactam;
- PC, Polycarbonate;
- PE, Polyethylene;
- PET, Polyethylene terephthalate;
- PMMA, Poly(methyl-methacrylate);
- PP, Polypropylene;
- PS, Polystyrene;
- PTFE, Polytetrafluoroethylene;
- PUR, Polyurethane;
- Pyr, Pyrolysis;
- PSXL, crosslinked Polystyrene;
- uPVC, unplasticized Polyvinyl Chloride

Keywords : Microplastics, Digestion, Method, Seafood products, Tissue, Plastic integrity

1. Introduction

Worldwide annual production of plastics has been steadily increasing since 1950 and was estimated at 311 million tons in 2014 (PlasticsEurope, 2015). Plastics include more than twenty families of polymers among which six are referred to as the "big six": polypropylene (PP), high- and low-density polyethylene (HDPE & LDPE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET) and polystyrene (PS) and correspond to 80% of the plastic production in Europe (PlasticsEurope, 2015). Very little plastic is recycled and it fragments or degrades at a very slow rate, thus accumulating in all environments. The first observations of microplastic pollution in marine ecosystems were recorded in 1972 (Carpenter *et al.*, 1972). More recently, it has been estimated that 10% of plastics produced end up in oceans (Thompson, 2006), comprising 60% to 80% of the marine litter (Laist, 1987; Moore, 2008). From the surface to the ocean floor, studies have described between 7000 tons and 250,000 tons of plastics floating at the surface of seawater (Cozar *et al.*, 2014; Eriksen *et al.*, 2014), in the water column (Lattin *et al.*, 2004) and in seabed sediments (Fischer *et al.*, 2015; Fries *et al.*, 2013; Van Cauwenberghe *et al.*, 2013). Plastics in oceans are encountered in macro- (>25 mm), meso- (5-25 mm) and microplastic forms (<5 mm) (Arthur *et al.*, 2009; Shim and Thompson, 2015; Thompson, 2004). Primary microplastics are referred to as microparticles produced as such, *i.e.* plastic pellets, exfoliating cosmetics or synthetic clothing fibres (Chang, 2015; Napper *et al.*, 2015; Mato *et al.*, 2001; van Wezel *et al.*, 2015), while secondary microplastics derive from the breakdown of larger plastic debris (Browne *et al.*, 2007; Cole *et al.*, 2011; Shah *et al.*, 2008; Thompson, 2004).

Ingestion of microplastics has been shown in laboratory and field studies for numerous marine organisms including zooplankton, worms, bivalves, crustaceans, demersal and pelagic fish, seabirds, reptiles and mammals (Codina-García *et al.*, 2013; Cole *et al.*, 2013, 2014; De Witte *et al.*, 2014; Lusher, 2015; Lusher *et al.*, 2013, 2015; Moore *et al.*, 2001; Sussarellu *et al.*, 2016; Tourinho *et al.*, 2010; Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe *et al.*, 2015; Watts *et al.*, 2014). Studies of fish have found between 2% to 40% of individuals to be contaminated, with a mean number of particles from 1 to 7.2 per individual (Boerger *et al.*, 2010; Foekema *et al.*, 2013; Lusher *et al.*, 2013).

For molluscs and especially *Mytilus edulis*, this microplastic load was reported to vary from 0.2 to 0.5 plastic particles per gram of tissue, leading to an average number being around one particle per individual (De Witte *et al*., 2014; Van Cauwenberghe and Janssen, 2014). It is noteworthy that all types of fibres could not be doubtlessly assessed in these latter studies due to the use of nitric acid. Microplastic accumulation through trophic levels is suspected to occur, as has already been reported at the lower levels of the food web (Farrell and Nelson, 2013; Setälä *et al*., 2014).

There is a great concern surrounding microplastics in numerous environmental and food science fields of research, as these pollutants can be deleterious for biota; first by their nature as a mechanical hazard (Cole *et al*., 2011), and most importantly as they are suspected to transfer microorganisms or pollutants adsorbed on their surface, additives, and other toxic chemicals into the guts of organisms, which may affect physiological functions (Browne *et al*., 2013; Teuten *et al*., 2009; Thompson, 2004; Van Cauwenberghe and Janssen, 2014; Zettler *et al*., 2013). Microplastics might also represent a threat for human consumers through the consumption of seafood products; although potential risks for human health posed by plastics as such, or as carriers of pollutants remains undocumented (Van Cauwenberghe and Janssen, 2014). If they wrote that adsorbed pollutants should not be a threat considering the "low significance of this transport from microplastics", a greater concern arose from intrinsic additives, active at low concentrations such as phthalates, bisphenol A or flame retardants.

Numerous methods have been developed to extract microplastics from sediment and biological tissues. They can be classified as acidic (Claessens *et al*., 2013; De Witte *et al*., 2014), alkaline (Cole *et al*., 2014; Foekema *et al*., 2013), oxidizing (Nuelle *et al*., 2014; Avio *et al*., 2015) and enzymatic methods (Cole *et al*., 2014). However, most of these methods are not adapted for large scale monitoring as they are either time consuming or too expensive to be implemented on a large scale. As a consequence, these methods do not accurately meet, to date, the call of some international bodies such as OSPAR or the Marine Strategy Framework Directive to provide standard, effective and cost efficient analytical methods (European Commission, 2010; OSPAR, 2015). Indeed, the relatively low percentage of animals exhibiting plastics in their tissue reported so far in the literature (Lusher, 2015) implies that a high

number of animals should be analysed for routine plastic monitoring as advised by OSPAR (OSPAR, 2015). More worrying is the often insufficient efficiency in digesting biological tissues and the damage that can potentially be observed in plastics after the digestion and extraction processes. For instance, most published papers, as well as OSPAR recommendations, are using protocols containing nitric acid to digest biological tissues, despite reports of substantial degradation of some polyamide types (Avio *et al.*, 2015; Cole *et al.*, 2014; OSPAR, 2015). This might lead to underestimations of microplastic loads and misinterpretations of the actual levels of contamination in marine organisms. This is especially true as polyamide (PA) is among the 10 most produced plastics, this polymer is therefore likely to be released and found in the environment. Finally, the range of tested families of plastics is often small in published studies, with little concern for the impact of the digestion procedure on plastic identification by either Raman spectroscopy, Fourier transform infrared spectroscopy, or Pyrolysis-GC/MS (Pyr-GC/MS) (Cole *et al.*, 2014; Foekema *et al.*, 2013; Fries *et al.*, 2013; Lusher *et al.*, 2014; Nuelle *et al.*, 2014; Van Cauwenberghe *et al.*, 2015).

The present study aims to identify a protocol to digest organic matter from biological samples that conforms to the technological limitations for large scale monitoring of microplastic contamination in fish and shellfish consumed by humans. To do so, existing and adapted methodologies were reviewed, and their limits were defined by testing: (i) polymer integrity in up to 15 plastic families through microscopic inspection and weighing; (ii) polymer identification using Pyr-GC/MS and Raman micro-spectrometry analyses prior to and after digestion; and (iii) efficiency in digesting biological tissues of molluscs, crustaceans and fish, achieved by filtration, visual inspection and evaluation of the remaining organic matter content on the filters. A first experiment (experiment 1) was conducted on 5 polymer types (including polyamide) to quickly rule out non-suitable digestion protocols, *i.e.* protocols leading to substantial degradation of polyamide and potentially other polymers. Subsequently, a more thorough experiment (experiment 2) was performed in order to test the suitable selected digestion protocols on a wider range of polymer types. Finally, the protocols identified in experiment 2 were tested on biological matrices (experiment 3).

2. Materials and Methods

Out of the fifteen types of plastics tested in this study, fourteen were supplied by Goodfellow Cambridge Ltd (Lille, France) (Supplemental Table 1). These included cellulose acetate (CA), high and low density polyethylene (HDPE & LDPE), polylauryllactam (PA-12), polycaprolactam (PA-6), polycarbonate (PC), polyethylene terephthalate (PET), poly(methyl-methacrylate) (PMMA), polypropylene (PP), polystyrene (PS), crosslinked polystyrene (PSXL), polytetrafluoroethylene (PTFE), polyurethane (PUR) and unplasticized polyvinyl chloride (uPVC). Expanded polystyrene (ePS) was collected on our own.

2.1 Experiment 1: elimination of the non-suitable protocols

For this preliminary study, five uncoloured, granule-like, plastics particles (*ca.* 3 mm diameter): LDPE, HDPE, PP, PA-12 and PS were tested using the six digestion protocols described below. Three particles of each of the plastic families were weighed five times on a 1 mg sensitivity analytical balance (Sartorius CP224S, Dourdan, France). Each sample was also photographed using an Olympus SZ61 binocular microscope mounted with a DP21 camera (Rungis, France) and 2650K light. Weighing and image capture were performed before and after application of the digestion protocols to determine their potential deleterious effects.

Six existing protocols were tested. **Protocol 1** was adapted from Foekema *et al.* (2013): plastics were incubated for 3 weeks at room temperature (21 ± 2 °C) with 20 mL of 10% (w/w) potassium hydroxide (KOH) solution (Fisher Labosi, Elancourt, France). **Protocol 2** was based on the study of Karl *et al.*, (2014). Briefly a solution of 0.5% (w/v) pepsin was prepared in 0.063M hydrochloric acid (HCl) (VWR, Fontenay-sous-bois, France) from a 2,000 FIP-U/g pepsin solution (Panreac, Lyon, France). Plastics were incubated for 2 h at 35 °C in 20 mL of pepsin solution in a beaker placed on a VELP Scientifica AREX heating bench (Usmate, Italy) equipped with a VTF temperature sensor to maintain the specified temperature. **Protocol 3** was adapted from Van Cauwenberghe *et al.* (2015) & Van Cauwenberghe and Janssen (2014). Plastics were placed in 20 mL of a 65% nitric acid (HNO_3) solution (Merk, Fontenay-sous-Bois, France) and maintained at room temperature overnight prior to being heated at 60 °C for 2 h and diluted with warm (80 °C) distilled water. **Protocol 4** was adapted from the method used by De

153 Witte *et al.*, (2014) recommended by the Convention for the Protection of the Marine Environment of
154 the North-East Atlantic (OSPAR, 2015). Plastics were added in 20 mL of solution containing a 4:1 (v/v)
155 mix of 65% HNO_3 and 65% perchloric acid ($HClO_4$) solutions (VWR) respectively. Tubes were left at
156 room temperature, overnight, and were boiled for 10 min before being diluted with warm distilled water
157 (80 °C). **Protocol 5** was adapted from the work of Cole *et al.*, (2014): plastics were incubated at 60 °C
158 during 24 h with 20 mL of 10M sodium hydroxide (NaOH) (Sigma-Aldrich, Saint-Quentin-Fallavier,
159 France). **Protocol 6** was developed based on the method reported by Maher *et al.* (2002) using an
160 oxidizing solution prepared extemporaneously, composed of 0.27M peroxodisulfate potassium ($K_2S_2O_8$)
161 (Sigma-Aldrich) and 0.24M NaOH. Plastics in oxidizing solution were incubated at 65 °C for 24 h. All
162 experiments were performed in clean glass tubes, except protocol 2 that required a clean beaker.

163 ## 2.2 Experiment 2: impact of the three selected digestion protocols on the integrity of 15
164 plastic families

165 Only methods that exhibited acceptable results in experiment 1, *i.e.* no degradation of plastic polymers,
166 and that met Marine Strategy Framework Directive and OSPAR recommendations (time effective and
167 low cost), were kept for a more thorough study on a wider range of plastics.

168 ### *2.2.1 Protocols*

169 Based on the results of the preliminary study, only protocols 5 and 6 demonstrated suitable outcomes,
170 *i.e.* short duration of digestion and no degradation of plastics; thus they were selected for a thorough
171 study on a wider range of plastics. An altered protocol (**Protocol 1b**) was also selected after
172 modification of protocol 1, reducing the 3 weeks at room temperature step to a 24 h incubation step at
173 60 °C.

174 ### *2.2.2 Processing of plastic samples*

175 The range of plastic polymers tested was expanded from 5 to 15 families. Analyses were performed on 3
176 replicate samples per plastic family. Samples of plastic were cut with a scalpel as thin as possible to
177 obtain small fragments (1-5 mm) and put into glass Petri dishes. To test the impact of the digestion

protocols, 10 mL of each tested solution was poured into a Petri dish with plastic fragments and incubated without agitation. Before and after application of digestion protocols, fragments were rinsed with bi-distilled water and dried for 2.5 h at 50 °C. A series of weighings (n=5 per replicate) were performed for each plastic sample on a 0.1 mg precision analytical balance (Sartorius Genius, Dourdan, France). A photograph of each fragment was taken as described in 2.1. One replicate sample of each plastic was analysed by Raman micro-spectrometry and another one by Pyr-GC/MS in order to evaluate whether digestion protocols interfere with the identification of plastic families. All analyses (photographs, weighings, Raman micro-spectrometry and Pyr-GC/MS) were performed before and after application of digestion protocols.

2.2.3 Plastic identification

For Raman analysis, each particle was placed on a gold coated microscope slide and analysed using a combination of static image analysis of particles with a HORIBA Scientific LabRam HR800 Raman micro-spectrometer (Villeneuve d'Ascq, France) with laser wavelength set at 785 nm and a 10x Olympus objective. The analysis of particles was carried out using a combination of static image analysis of particles and automated Raman micro-spectroscopy, allowing the analysis of number, size, shape and chemical composition of a large number of particles. Polymers were identified using spectroscopy software (KnowItAll, Bio-Rad) with queries against our own database containing pre-established polymer spectra.

For Pyr-GC/MS analysis, a small piece (< 0.5 mm^3) of each sample was placed in a pyrolysis cup on the AS-1020E autosampler of a Frontier Lab EGA/PY – 3030D (Fukushima, Japan), before being pyrolysed at 600 °C. Pyrolysis products were directly injected, with a split of 20, on a coupled Shimadzu GC-2010 device (Noisiel, France) and separated on a Restek RXi-5ms ® column (Lisses, France). Helium was used as carrier gas with a linear velocity of 40 cm/s. The oven program was set as follows: 5 min at 40 °C increasing to 320 °C at 20 °C/min, maintained for 14 min. Mass spectra were obtained by a Shimadzu QP2010-Plus mass spectrometer coupled to the GC. Interface temperature was fixed at 300 °C to prevent re-condensation, ionization voltage was set at 70 eV and a mass range extending from 33

204 to 500 m/z was analysed with a 2000 Hz scan speed. Samples were identified using F-Search software
205 4.3, querying pyrograms against Frontier Lab's database and our own database containing pre-
206 established pyrograms with plastic samples. Identification was established based on the similarity
207 percentage between average mass spectra. As advised by the Pyr-GC/MS supplier, a minimal value of
208 80% was necessary to certify the proper identification.

209 **2.3 Experiment 3: application of selected digestion protocols on seafood products**

210 *2.3.1 Protocols*

211 Two out of the three previous protocols (1b and 6), were selected to be tested for digestion of biological
212 tissues. A sequential approach was used: they were first tested on mussels, then on crabs and fish, and
213 whenever a protocol was not considered as efficient on a seafood product, it was ruled out for the next
214 product. While incubation time and temperature remained unchanged, digestion of tissues and cartilage
215 for crabs from seafood products were carried out on a Labomoderne AG610 multi-positions magnetic
216 hot plate stirrer (Paris, France) at 300 rpm.

217 *2.3.2 Seafood products*

218 Tissues from three different marine animals were analysed: mussels (*Mytilus edulis*), velvet crabs
219 (*Necora puber*) and black seabreams (*Spondyliosoma cantharus*). All tools and glassware used for the
220 digestion tests were carefully rinsed with distilled water filtered through 90 mm GF/A 1.6 µm glass fibre
221 filters (Whatman, Velizy-Villacoublay, France). Water used to rinse all seafood products was
222 systematically distilled and filtered before use. All analyses were performed on 3 individuals per species
223 and digestion protocols. All animals were collected in the Bay of Brest (France) and stored at -20 °C
224 prior to analysis. Mussels (5.4 ± 1.3 g, mean ± standard deviation) were shelled, weighed and rinsed
225 before being placed in 250 mL of digestion solution. Crabs were rinsed and carefully shelled. Tissues
226 and cartilages were gathered with pliers and scalpels and weighed (5-10 g) before being placed in 250
227 mL of digestion solution. Black seabream were first sized and weighed before being rinsed, carefully
228 gutted to extract the whole alimentary tract and filleted. Fillet (150.7 ± 37.3 g) and whole alimentary

tract (34.9 ± 12.8 g) were weighed and respectively placed in 500 mL and 250 mL of digestion solution. After applications of protocols, digestates were filtered through GF/A 1.6 µm glass fibre filters. In the case of the presence of debris in the digestate, especially for whole alimentary tract, a density-based separation step using sodium tungstate was subsequently added. Briefly, a 70% (w/w) sodium tungstate (Acros Organics, Geel, Belgium) solution (d = 1.5g/cm^3) was added to the digestate (2:1, v/v) right after the 24 h digestion. The mixture was then thoroughly stirred for 10 minutes before being left to settle for 1 h. The supernatant, containing the floating plastic particles (Corcoran *et al*., 2009), was subsequently collected and filtered, as previously described.

Concerning the spiking approach described in 2.3.4, triplicate samples of cod (*Gadus morhua*) fillets (23.6 ± 3.2 g), saithe (*Pollachius virens*) whole alimentary tract (9.7 ± 0.6 g) and mussels (4.0 ± 0.9 g) were studied.

2.3.3 Assessment of digestion efficiencies

The propensity of the selected protocols to properly digest flesh was assessed through microscopic inspection of filters using a Zeiss Stemi 2000-C binocular microscope (Marly-le-Roi, France). This device was mounted with a Canon EOS 600D camera and illuminated with a 2500K light. A digestion was qualified as efficient in the absence of debris, organic matters, shell or cartilage, which can hinder microplastic detection on the filter. Also, dry weight of each filter, obtained by placing filters at 60 °C for 24 h, was measured before and after digestion to assess the proportion of remaining organic matter on each filter after digestion. Digestion efficiencies (*%De*) were calculated as follows, where *%De* corresponds to the digestion efficiency, DW_f and DW_{fad} correspond respectively to the dry weights (n=5) of the "clean" filter before filtration and the filter covered by organic matter and debris after digestion. Finally, T_w corresponds to the average weight of tissues subjected to digestion (n=50).

$$\%De = 100 - \left(\frac{DW_{fad} - DW_f}{T_w} \times 100\right)$$

252 *2.3.4 Spiking tests*

253 A spiking approach was also performed to endorse protocol 1b. A commercial yellow fluorescent fine
254 fishing line was cut into small particles of *ca.* 500 µm. Its polymer composition was identified thanks to
255 Pyr-GC/MS as PA-6 (94% similarity). Ten yellow PA-6 particles were spiked in the samples of tissue
256 with a thin sewing needle. Samples were digested for 24h at 60 °C and 300 rpm with 250 mL of 10%
257 (w/w) KOH. A recovery percentage was calculated with a ratio of the count of yellow particles lying on
258 filters after filtration and the number of initially spiked particles. Finally, nylon particles recovered after
259 digestion were analysed by Pyr-GC/MS.

260 **2.1 Statistics**

261 Results of weight measurements were represented on bar charts as means of the 5 replicates. Error bars
262 on charts represent the expanded weighing uncertainty of the results (U_{eb}), evaluated based on the
263 recommendations of the International organization for standardization (ISO/IEC Guide 98-3, 2008).
264 This parameter provides a fine determination of uncertainty compared to the value given by the
265 maximum permissible error and takes into account uncertainty related to reproducibility and resolution
266 of the analytical balance.

267 U_{eb} was estimated as follows, with d being the resolution of analytical balance and e the standard
268 deviation obtained after evaluation of the reproducibility by consecutive weighing (n=50). Mean values
269 with more than 0.1 mg difference were considered as significantly different.

270 $$U_{eb} = 2 \times \sqrt{\left[\left(\frac{d}{2\times\sqrt{3}}\right)^2 + \left(\frac{e}{\sqrt{3}}\right)^2\right]}$$

271 Size evolution was assessed using the histogram tool on GIMP 2 software (2.8.16). Briefly, each
272 fragment area was selected and the number of pixels was recorded. A loss percentage was evaluated as a
273 ratio of pixel numbers before and after treatment by each protocol.

3. Results and Discussion

3.1 Experiment 1: elimination of the non-suitable protocols

This experiment aimed to test the six protocols on a small subsample of polymers, as degradation of polymers such as polyamide allows the exclusion of some of the tested protocols. Protocols 1 and 2 did not lead to any weight change (Supplemental Table 2) or plastic degradation, as confirmed by the absence of visual change. However protocol 1 could not be kept as such, due to the long extraction duration (3 weeks), which was considered to be too constraining to be performed routinely and on a large scale. Although having proved to be effective for research of parasites in fish fillets (Karl *et al.*, 2014), and having no adverse effect on the tested polymers, protocol 2 was not conserved. Indeed, debris and organic matter were already observed after the digestion step (Llarena-Reino *et al*., 2013) and could have been problematic for experiment 3, particularly with clogging issues. Protocol 2 was consequently not retained for further investigation. Protocols 3 & 4 both used nitric acid and led to poor results regarding plastic integrity. The main observations that stand out after digestion with protocol 3 were: (i) the degradation of PA-12 (**Fig. 1a, b**), accompanied by a decrease in particle weight; and (ii) marked sample yellowing (**Fig. 1c, d**) observed for all polymers. Identical modifications were also observed for plastics subjected to protocol 4, though to a lesser extent, probably due to the dilution of HNO_3 by one volume of $HClO_4$. Similarly, Claessens *et al*. (2013) observed a critical melting of PS particles directly exposed to HNO_3, although this was not observed when particles were embedded in tissues, suggesting the importance of the amount of acid directly in contact with plastics. The present study confirms previous findings of polyamide degradation (Claessens *et al*., 2013; OSPAR, 2015). Such a result is a major concern, as this plastic family represents a significant volume of production worldwide, and may thus be found in the environment with an increased likelihood (Dantas *et al*., 2012; Lusher *et al*., 2013; Rochman *et al*., 2015). Therefore, the use of HNO_3 to digest seafood products for microplastic surveys should be proscribed.

298 Protocols 5 and 6 did not affect the plastic polymers tested here. No modification of weight and shape
299 was observed. Furthermore, these protocols have the additional advantages of short incubation duration
300 (24 h) and use relatively low cost chemicals.

301 Based on these results, it was decided to keep protocols 5 and 6 as such, and to revise protocol 1 as
302 suggested by Rochman *et al*. (2015), by reducing the incubation time to 24 h and increasing the
303 incubation temperature to 60 °C (Protocol 1b).

304 **3.2 Experiment 2: impact of the three selected digestion protocols on the integrity of 15**
305 **plastic families**

306 Protocols 1b, 5 and 6 were tested on a wide range of 15 plastic families (1-5 mm), using microscopic
307 observation, precision weighings, Raman micro-spectrometry and Pyr-GC/MS identifications.

308 *3.2.1 Change in weights*

309 The majority of plastics appeared not to be affected by the application of protocols 1b, 5 and 6, with no
310 significant changes in weight for the three replicates of respectively 10, 11 and 10 families (**Table 1**;
311 Supplemental Fig. 1, 2 & 3). Regardless of the tested protocol, ePS masses were too close to the
312 quantification limit of the analytical balance, with weights lower than 0.1 mg. This was not surprising
313 considering that expanded polystyrene mainly contains air. Thus, weighing was not conclusive for ePS,
314 and impacts of the protocols on sample integrities were exclusively evaluated by microscopic
315 observations, Pyr-GC/MS and Raman spectroscopy analyses.

316 Regarding protocols 1b and 6, incoherent weight increase/decrease were observed for one out of the
317 three replicates of respectively HDPE, PC and PP and with PA-6, PMMA and PTFE, as illustrated for
318 PC in Fig. 2. Microscopic inspections of fragments did not show any variation suggesting these results
319 could not be considered as representative of the overall trend for each of these families. This emphasized
320 the need of a strict control by microscopy, as performed in this study, when assessing impact of
321 protocols on microplastics integrity.

A single family of plastic, CA, was substantially degraded by each of the three protocols (**Fig. 2**). The mass reduction was close to 50% with protocol 1b (**Fig. 2a**), and was even more pronounced with protocol 5 (**Fig. 2b**) and 6 (**Fig. 2c**). For the latter, the degradation of the plastic was almost total with a drop in weight to below the limit of quantification by the analytical balance.

Protocol 5 led to the degradation of two additional plastics: PC and PET (**Fig. 2b**). The case of PC is especially obvious with a complete dissolution of the plastic during the post-protocol flushing. The decrease recorded for PET was less drastic but still large, around 50%.

3.2.2 Microscopic inspection

All protocols led to a marked modification of CA fragment sizes and shapes (**Fig. 3**). Decrease in size was particularly important and comprised between 64 and 67% for protocol 5 and between 69 and 95% for protocol 6. A noticeable opacification of CA was observed with protocol 1 and apparitions of streaks were observed with protocols 5 and 6 (**Fig. 3**).

The weight changes described for PET and PC after application of protocol 5 were confirmed by visual inspection (**Fig. 3**), where a marked decrease of fragment size (53-60%) was observed together with the apparition of streaks on PET, and a complete dissolution of PC during the post-protocol flushing (**Fig. 3**). Before this decomposition, the colour of PC was yellow/brown.

No substantial modification of plastic shapes and sizes was recorded with protocol 1b, 5 and 6 for all the other plastic families, corresponding respectively to 14, 12 and 14 types of the 15 tested polymers.

3.2.3 Plastic identification

Regardless of which protocol was applied, it is noteworthy to mention that the Pyr-GC/MS and Raman micro-spectrometry methods allowed the identification of the native molecule for PE and PS, but it remained difficult to establish differences between the subtype of polymers, *i.e.* LDPE *vs*. HDPE or ePS *vs*. PS *vs.* PSXL.

For all protocols a large majority of plastic were correctly identified by Pyr-GC/MS after application of digestion procedures with pyrogram similarities higher than 80%. This is the case for 14 families with protocol 1b, 13 with protocol 5, and 14 families with protocol 6 (**Table 1**). CA identification was problematic after application of protocols 1b and 5. Inaccurate identification and similarity to the pyrogram for "wood powder" were probably due to the digestion of CA. Indeed, wood powder is rich in cellulose that is also a major compound of CA. However, despite being clearly degraded by protocol 6, CA was well identified by Pyr-GC/MS suggesting that the mass decrease had no incidence on the molecule structure. Similarly, despite a clear degradation of PET by protocol 5, identification by Pyr-GC/MS was not affected. Finally, PC and PUR respectively treated with protocol 5 and 6 were not accurately identified. These results could be explained respectively by the fact that PC was completely dissolved by 10M NaOH, and by an adverse impact of peroxodisulfate on PUR identification.

Results of the Raman micro-spectroscopy analysis showed modifications of spectra intensities for all treatments compared with untreated reference materials (**Fig. 4**). These variations of intensity are mainly due to the location of the impact point of the laser on the surface of the analysed polymer despite a focus adjustment before each acquisition. The heterogeneity of the particle in terms of morphology, roughness or orientation and the move of particles before and after chemical treatments conduct to intensity fluctuations (Lenz *et al*., 2015). It is however not excluded that these modifications of intensity could also be due to polymer molecular alteration after the chemical treatments (Collard *et al*., 2015). But, in this case, band shifts would also have been expected. On contrary, chemical treatments did not affect polymer successful identification (**Table 1**), except for CA where the spectral fingerprints changed after treatment by NaOH and KOH, suggesting a modification of the molecular structure. Moreover, the spectrum quality of CA was impacted by a strong fluorescence with the KOH treatment, affecting its identification. No effect was observed on the spectra of any polymer families after digestion with peroxodisulfate.

3.2.4 *Acquired knowledge*

Protocol 1b (10% KOH, 24h, 60°C) appeared to be the most promising protocol in our experiments in terms of absence of substantial degradation of the plastic polymers tested here. It is noteworthy that the impacts of this protocol on the 15 plastic polymer families were further assessed under agitation (300 rpm), to match the optimal conditions for biological tissue digestion (see below), and similar results were obtained, *i.e.* no detrimental effect on plastics (except for CA). While resistance of plastic particles subjected to KOH 10% was mentioned as "unpublished data" in Foekema *et al*. (2013), no detailed information was available in the literature to confirm the absence of detrimental effects on a wide range of plastic polymers and on their identification. Only CA was degraded by protocol 1b, yet in a lesser extent than by protocol 5 and 6, without this being detrimental. Indeed, if CA is the main macroplastic found on coastlines (Andrady, 2015), it is a polymer derived from cellulose, a natural polymer. This property confers a good potential for environmental degradation (Puls *et al*., 2011) which would explain the fact that it is not recovered in marine organisms or environmental studies. The use of cellulose acetate filter should be proscribed with this method, to avoid contamination as already reported with other methods (Collard *et al*., 2015).

Protocol 5 (10M NaOH 60°C, 24h) appears to be less promising than protocol 1b, since three plastics were degraded by the use of 10M NaOH. Contrary to previous observations (Cole *et al*., 2014) neither degradation of nylon (PA-6 and PA-12), even when the protocol was tested directly on nylon fibres (Supplemental Fig. 4), nor uPVC yellowing was reported in the present study. Yet worryingly, protocol 5 degraded PET, which is one of the main components of plastic beverage bottles often recovered in the marine environment (Andrady, 2015), and which ranks among the "big six", with a production estimated at more than 3 million tons in 2014 (PlasticsEurope, 2015). A decrease in peak intensity in Raman microscopy was also observed, leading to poor identification compared with protocol 1b. As a consequence, protocol 5 was dismissed due to the degradation of three types of polymers.

Protocol 6 ($K_2S_2O_8$/ NaOH 65 °C, 24h) seems less promising than protocol 1b, due to CA intense degradation, along with some technical concerns related to the difficulty to prevent crystallisation of the

395 peroxodisulfate in solution, and its relative cost. However, in order to evaluate its efficiency in
396 successfully digesting the flesh of seafood, this protocol was retained for experiment 3.

397 **3.3 Experiment 3: application of selected digestion protocols on seafood products**

398 Protocol 1b using KOH led to a good digestion of mussel tissues, *i.e.* no remaining particles were visible
399 in the digestate, which consequently allowed a good filtration on the GF/A 1.6 µm fibre glass filters
400 (**Table 2**; Supplemental Fig. 5). A close observation using a binocular microscope revealed a negligible
401 amount of debris, allowing good observation and detection of microplastics (Supplemental Fig. 5).
402 Conversely, following protocol 6, rough fragments were still observed to be present; mussel
403 hepatopancreas remained completely undamaged (**Table 2**; Supplemental Fig. 5). These observations on
404 filters were confirmed by evaluations of digestion efficiencies (**Fig. 5**). Efficiency was higher for
405 protocol 1b with *%De* ranging from 99.6 – 99.8 % compared to *%De* with protocol 6 ranging between
406 98.2 and 99.7 %. Consequently, filtration of the digestate subjected to protocol 6 on GF/A 1.6 µm glass
407 fibre filters was difficult and led to a rapid clogging of the filters that inevitably ended up heavily
408 loaded. The presence of rough organic or inorganic debris, tissues and even organs (hepatopancreas)
409 recovered after digestion prevents the accurate detection of plastic debris in bivalves. As a consequence,
410 protocol 6 was considered unsuitable for microplastic extraction from biological matrices, and was thus
411 excluded from further analyses with crustacean and fish. It should also be noted that further experiments
412 demonstrated that *%De* was not affected after reduction of both volume of digestion solution (100 mL)
413 and stirring speed (200 rpm) when using the protocol 1b (Supplemental Fig. 6).

414 For crab tissues, fish fillets and whole alimentary tract, the only tested method was protocol 1b. It led to
415 a very efficient digestion of crab tissues; and easy filtration and detailed observations of the filters for
416 microplastic detection (**Table 2**; Supplemental Fig. 7). The presence of pieces of cartilage was not
417 problematic as a careful rinse was performed. Fish filet were efficiently digested and the filtration on
418 one to two GF/A 1.6 µm glass fibre filters was successful. However, the fish bones must be carefully
419 removed prior to digestion to prevent their partial dissolution leading to a high quantity of bone
420 fragments ending up on the filters (Supplemental Fig. 8). Several filters may be needed due to the higher

mass of tissues digested and the presence of a thin layer of grease observed on the edges of the Petri dish that may lead to some difficulties in accurately detecting microplastics if they are located below this layer (Supplemental Fig. 8). Direct filtration of the whole alimentary tract digestate was impossible because of the presence of inorganic debris present in black bream alimentary tracts collected in the Bay of Brest (France) that remained intact after digestion (data not shown). These debris consisted mainly of mærl and sand, present in large quantities in the Bay of Brest (Potin *et al*., 1990); therefore a slightly modified protocol using a sodium tungstate solution (1.5 g/cm^3) was tested. This allows the recovery of all plastic particles, including the densest of the "big six": PVC (1.38 g/cm^3) or PET (1.37 g/cm^3) (Andrady, 2015); without filtration of the high load of inorganic particles. It is noteworthy to mention that this density-based separation step should be suitable for sediment dwelling molluscs and crustaceans that may exhibit high coarse sediment contents in their digestive tracts. Due to this additional step, the filtration and the filter observation were much easier (Supplemental Fig. 8), allowing a good detection of microplastic-like particles.

Finally an integrated approach was performed using protocol 1b, the digestion of the different spiked samples of seafood tissues led to excellent recovery and no impact of protocol 1b was observed on the integrity of particles. Recovery percentages were of 100% for cod fillets, saithe whole alimentary tracts and mussels. It should also be mentioned that digestion process did not affect identification of the nylon particles spiked in fillets, whose pyrograms displayed 93% of similarity with the one of PA-6.

4. Conclusion

In conclusion, the protocol using KOH 10% solution with incubation at 60 °C for 24 h overcame current methodological barriers and was proposed as a good compromise for extraction and characterization of microplastics from seafood tissues. This protocol appears to be the best compromise and should be implemented for further studies to assure the relevance and comparison of environmental studies, notably following OSPAR and Marine Strategy Framework Directive recommendation, as well as for

seafood product quality. Mollusc, crustacean, and fish were tested, suggesting the broad usefulness of this protocol on aquatic species.

Based on the present work, the use of nitric acid is not recommended for the study of microplastics because of its degrading action on polyamide and its tendency to yellow plastics. An approach using enzymatic digestion was discarded because of the difficulty in its implementation and digestion efficacy issues. The use of oxidizing solution was promising, with almost no deleterious consequence on plastics, but application to seafood products led to incomplete digestion of mussel tissues. The NaOH solution resulted in adverse destructive effects on integrity of three plastics (CA, PC and PET), thus its use is not recommended here.

5. Acknowledgments

Authors warmly thank Mélanie Gay and Régine Quenu for their valued help with metrology issues, Marie-Ange Huchin and Mr. Bourdin for their kind technical assistance. This work was funded by ANSES (French Agency for Food, Environmental and Occupational Health & Safety) through a "C*onvention de Recherche et Développement*". Laura Frère was supported by a Université de Bretagne Occidentale and a Brest Métropole PhD grant. Ludovic Hermabessiere is thankful to Région Nord-Pas-de-Calais and ANSES for the financial support of his PhD. Authors also thank Dr. E. Harney for his helpful revision of the English and his comments on the manuscript.

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612

613 **Figure legends**

614 **Figure 1.** Microscopic observations of plastics treated with protocol 3 (65% HNO_3 at room temperature
615 overnight, then 60 °C for 2 h). On the upper half: pictures of polylauryllactam (PA-12) before (a) and
616 after (b) application of protocol. On the lower half: pictures of low density polyethylene (LDPE) before
617 (c) and after (d) application of the protocol.

618 **Figure 2.** Bar chart representing evolution of weight (mg) for cellulose acetate (CA), polycarbonate
619 (PC) and polyethylene terephthalate (PET) before and after application of protocol 1b, 10% KOH, 24 h,
620 60°C (a), protocol 5, 10M NaOH 60°C, 24 h (b) and protocol 6 $K_2S_2O_8$/ NaOH 65 °C, 24 h (c). White
621 and dark grey bars represent the mean of 5 repeated weight measures of three plastic samples (S1, S2 &
622 S3) respectively before and after application of protocols. Error bars on charts represent the expanded
623 weighing uncertainty of the results U_{eb} (see 2.1).

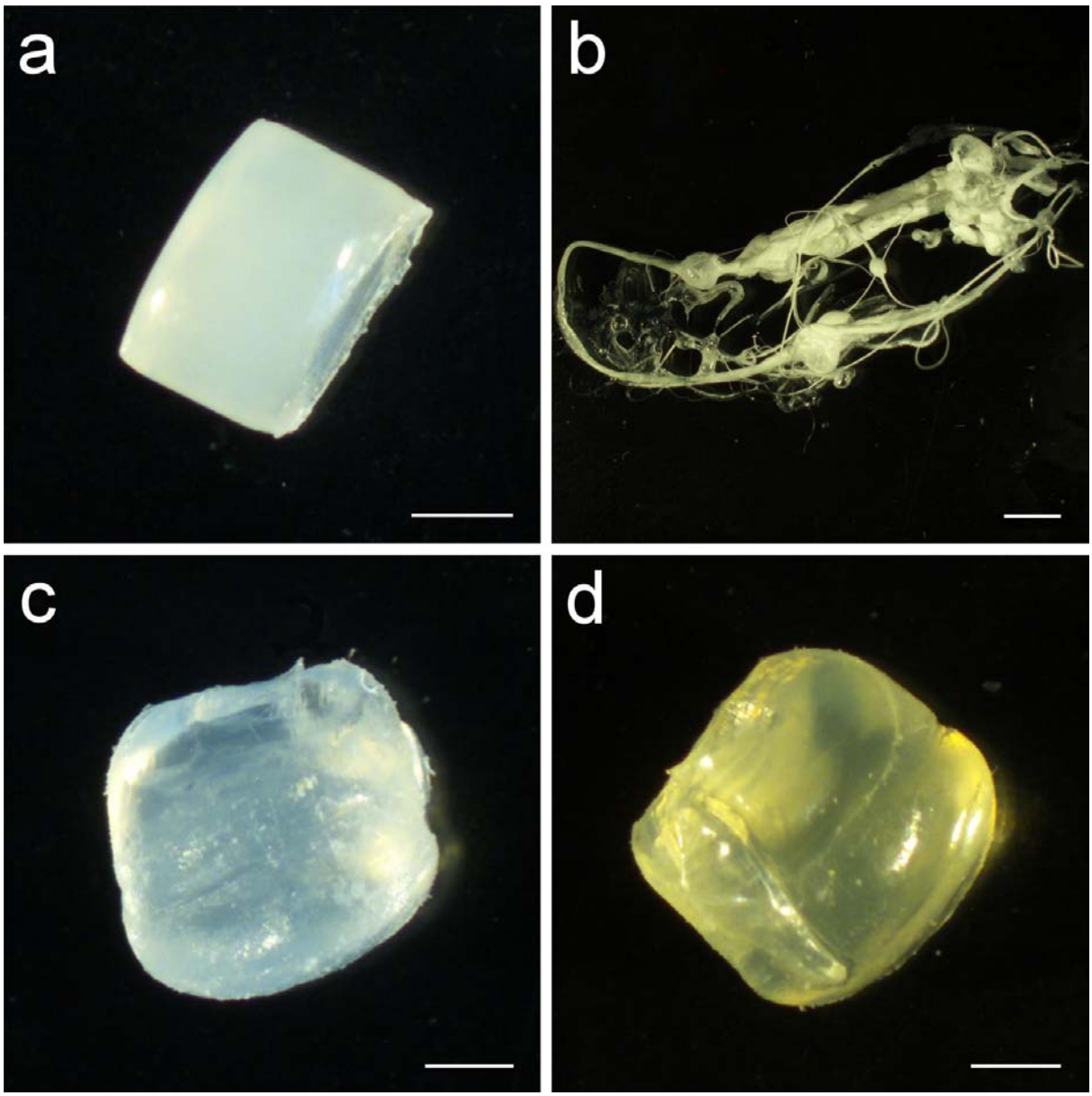
624 **Figure 3.** Pictures of the three types of polymers; cellulose acetate (CA), polycarbonate (PC),
625 polyethylene terephthalate (PET) mostly affected by protocols 1b (10% KOH, 24h, 60°C), 5 (10M
626 NaOH 60°C, 24h) and 6 ($K_2S_2O_8$/ NaOH 65 °C, 24h). For each protocol the picture of a single sample is
627 presented before and after application of the protocol. White bars correspond to 1 mm.

628 **Figure 4.** Raman spectra of the 15 polymers prior and after treatment by protocol 1b (10% KOH, 24h,
629 60 °C), 5 (10M NaOH 60°C, 24h) and 6 ($K_2S_2O_8$/ NaOH 65 °C, 24h). All spectra were normed excepted
630 for CA spectrum treated with protocol 5, for which a simple decrease in intensity was applied. A normed
631 spectrum is obtained by dividing peak areas by that of the major peak. (CA: cellulose acetate; ePS:
632 expanded polystyrene; HDPE: high density polyethylene; LDPE: low density polyethylene; PA-12:
633 polylauryllactam; PA-6: polycaprolactam; PC: polycarbonate; PET: polyethylene terephthalate; PMMA:
634 poly(methyl-methacrylate); PP: polypropylene; PS: polystyrene; PTFE: polytetrafluoroethylene; PUR:
635 polyurethane; PSXL: crosslinked polystyrene; uPVC: unplasticized polyvinyl chloride).

636 **Figure 5.** Bar chart representing individual digestion efficiencies (*% De*) (n=5) on three mussels, M1 to
637 M3 for protocol 1b (KOH 10%, 24h, 60°C) in black and M4 to M6 for protocol 6 ($K_2S_2O_8$/ NaOH 65

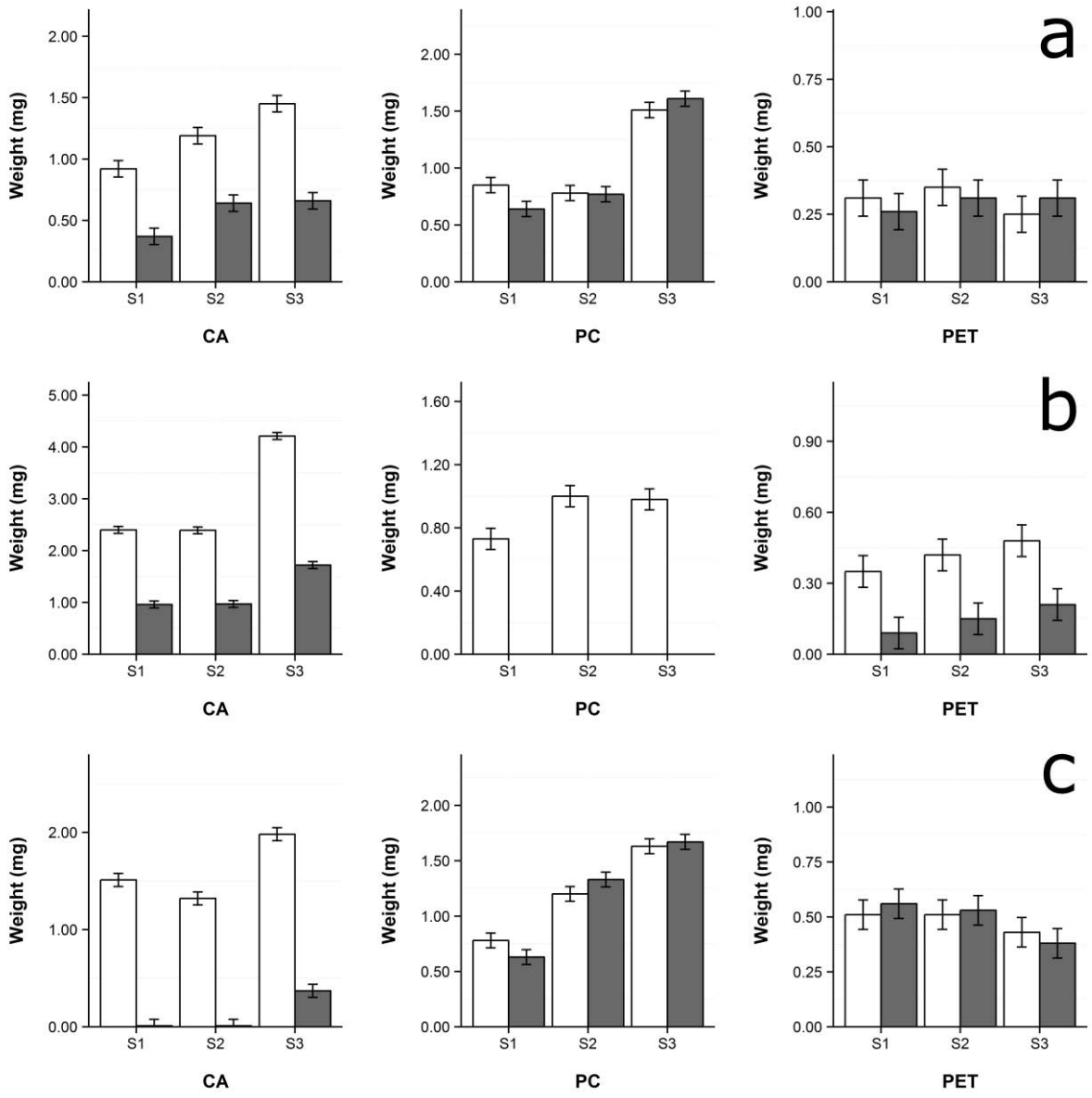
638 °C, 24h) in white. The very low standard deviations (< 0.01%) do not appear on bar diagrams as they
639 could not be distinctively traced.

640 **Figure 1**



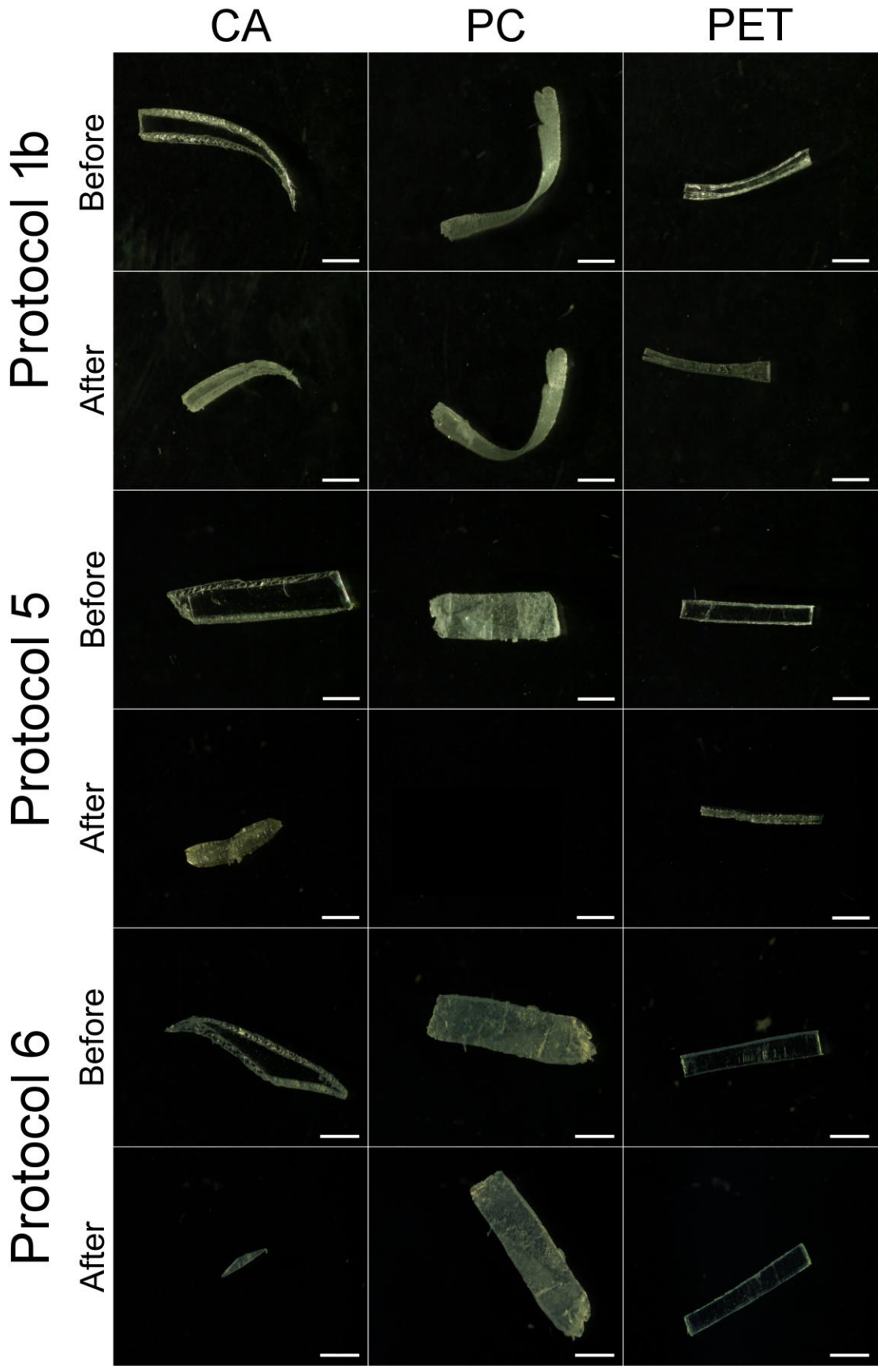
641

642 **Figure 2**



643

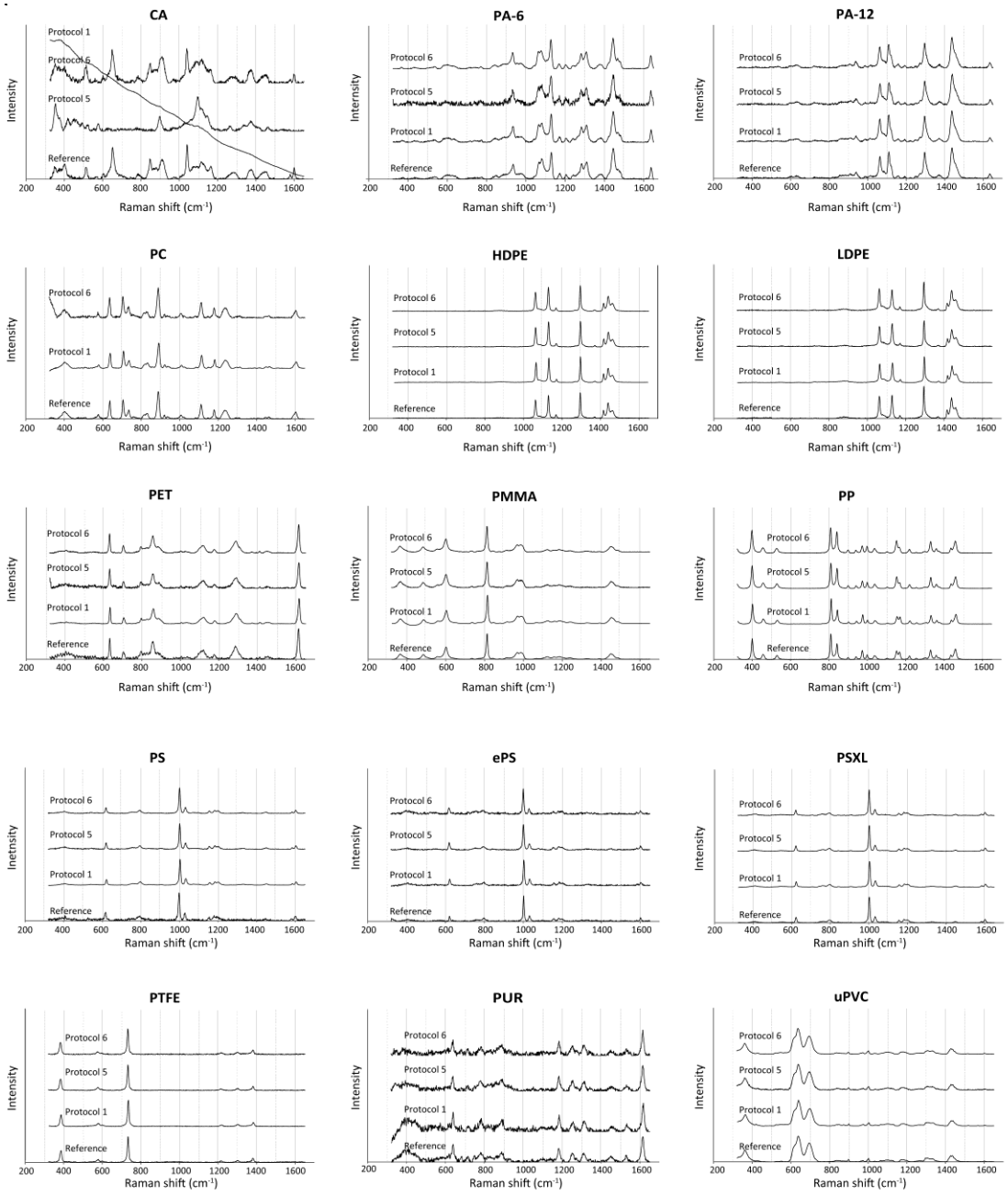
644 **Figure 3**



645

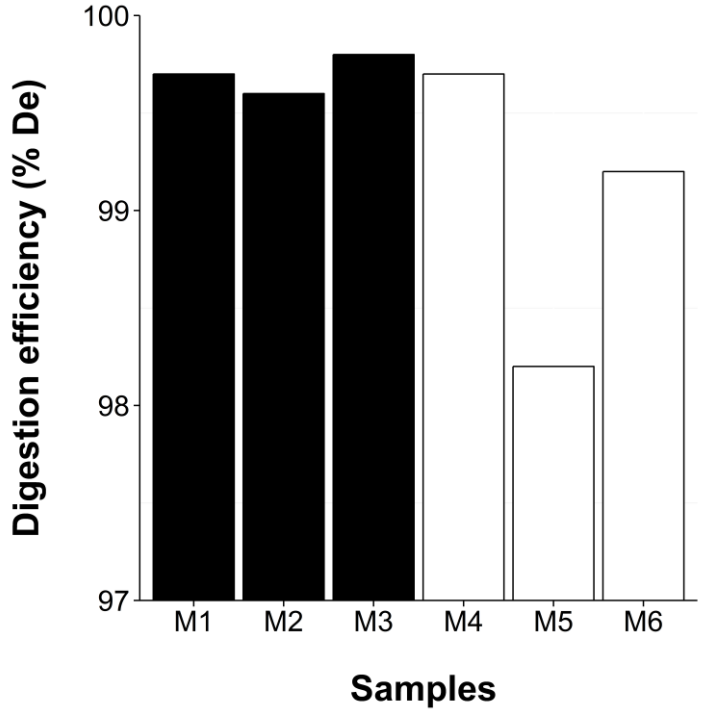
646 **Figure 4**

647



648

649 **Figure 5**



650

Tables

Table 1
Summary of passed/unpassed analyses by plastics in experiment 2.

Protocols	Analyses	CA	ePS	HDPE	LDPE	PA-12	PA-6	PC	PET	PMMA	PP	PS	PTFE	PUR	PSXL	uPVC
1b 10% KOH (*60°C, 24 h*)	Weighing	✗	NA	✓*	✓	✓	✓	✓*	✓	✓	✓*	✓	✓	✓	✓	✓
	Visual	Sh, Si	✓	✓	✓	✓	✓	✓	Sh	✓	✓	✓	✓	✓	✓	✓
	Pyr-GC/MS	✗	✓#	✓#	✓#	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓#	✓
	Raman	✗	✓#	✓#	✓#	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓#	✓
5 10 M NaOH (*60°C, 24 h*)	Weighing	✗	NA	✓	✓	✓	✓	✗	✗	✓	✓	✓	✓	✓	✓	✓
	Visual	Sh, Si	✓	✓	✓	✓	✓	Sh, Si	Sh, Si	✓	✓	✓	✓	✓	✓	✓
	Pyr-GC/MS	✗	✓#	✓#	✓#	✓	✓	NA	✓	✓	✓	✓	✓	✓	✓#	✓
	Raman	✓	✓#	✓#	✓#	✓	✓	NA	✓	✓	✓	✓	✓	✓	✓#	✓
6 0.27 M $K_2S_2O_8$/0.24 M NaOH (*65 °C, 24 h*)	Weighing	✗	NA	✓	✓	✓	✓*	✓	✓	✓*	✓	✓	✓*	✓	✓	✓
	Visual	Sh, Si	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pyr-GC/MS	✓	✓#	✓#	✓#	✓	✓	✓	✓	✓	✓	✓	✓	✗	✓#	✓
	Raman	✓	✓#	✓#	✓#	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓#	✓

(CA: cellulose acetate; ePS: expanded polystyrene; HDPE: high density polyethylene; LDPE: low density polyethylene; PA: polyamide; PA-12: polylauryllactam; PA-6: polycaprolactam; PC: polycarbonate; PE: polyethylene; PET: polyethylene terephthalate; PMMA: poly(methyl-methacrylate); PP: polypropylene; PS: polystyrene; PTFE: polytetrafluoroethylene; PUR: polyurethane; PSXL: crosslinked polystyrene; uPVC: unplasticized polyvinyl chloride).
✓: weighing, visual (absence of noticeable change) and identification analysis passed.
✗: degraded polymers or identification problem.
Sh: change in the shape.
Si: change in the size.
"*": no change on weighing analysis for 2 out of 3 analysis.
"#": only native molecule identified (not the type of polymerization).
NA: not analysed because of technical issues.

Table 2
Quality of the digestion, filtration and filter observations after digestion of mussels, crabs and fish tissues following protocols 1b (KOH 10%, 24 h, 60 °C) and 6 ($K_2S_2O_8$/NaOH 65 °C, 24 h). (NA: not analysed).

Tissues	Steps	Protocol 1b	Protocol 6
Mussels	*Digestion*	**Good** No particle visible to the naked eye in the digestate.	**Bad** Presence of small organic/inorganic fragments. Hepatopancreas not diges-ted
	Filtration	**Good** Use of a single GF/A 1.6 μm filter.	**Poor** One to two filters used depending on the individuals. Rapid clogging.
	Filter	**Good** Negligible amount of debris. Filter lightly loaded.	**Bad** High quantity of debris leading to inability to accurately detect micro-plastics.
Crabs	*Digestion*	**Good** No particle visible to the naked eye in the digestate. Pieces of cartilage rinsed with distilled water.	**NA**
	Filtration	**Good** Use of a single GF/A 1.6 μm filter.	**NA**
	Filter	**Good** Negligible amount of debris. Filter lightly loaded.	**NA**
Fish (fillet)	*Digestion*	**Good** No particle visible to the naked eye in the digestate. Pieces of fish bones rinsed with distilled water.	**NA**
	Filtration	**Good** Use of a single GF/A 1.6 μm filter.	**NA**
	Filter	**Good** Negligible amount of debris. Filter lightly loaded	**NA**
Fish (whole alimentary tract)	*Digestion*	**Poor** Particle visible to the naked eye in the digestate. Dense digestate (mærl, sand, etc.).	**NA**
	Filtration	**Bad** Impossible to filter due to clogging.	**NA**
	Filtration (after separation step)	**Good** The use to sodium tungstate facilitated the filtration step. Use of a single GF/A 1.6 μm filter.	**NA**
	Filter	**Good** Absence or negligible amount of debris. Filter lightly loaded.	**NA**

Microplastics in seafood: benchmark protocol for their extraction and characterization

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Supplementary files

Supplemental Table 1: Plastics and their common brand names

Polymers	Abbreviations	Common brand names
Cellulose acetate	CA	Clarifoil, Dexel, Tenite Acetate
High density polyethylene	HDPE	Hostalen HD, Lacqtene HD, Lupolen, Rigidex
Low density polyethylene	LDPE	Alkathene, Carlona, Lacqtene, Lupolen, Stamylan LD
Polylauryllactam	PA-12	Rilsan A, Vestamid
Polycaprolactam	PA-6	Akulon K and F, Capron, Maranyl B, Nylacast, Orgamid, Ultramid B
Polycarbonate	PC	Lexan, Makrofol, Makrolon
Polyethylene terephthalate	PET	Arnite, Dacron, Hostaphan, Impet, Melinar, Melinex, Mylar, Rynite, Terylene, Trevira
Poly(methyl-methacrylate)	PMMA	Diakon, Lucite, Oroglas, Perspex, Plexiglas
Polypropylene	PP	Appryl, Hostalen PP, Lacqtene, Novolen, Propathene
Polystyrene	PS	-
Crosslinked polystyrene	PSXL	Q.200.5, Rexolite
Polytetrafluoroethylene	PTFE	Fluon, Hostaflon TF
Polyurethane	PUR	-
unplasticized Polyvinyl chloride	uPVC	Corvic, Evipol, Geon, Hostalit, Lacovyl, Lucorex

Supplemental Table 2: Plastic weights before and after application of the protocols of the preliminary study

		HDPE		PP		LDPE		PS		PA-12	
		Before	***After***	***Before***	***After***	***Before***	***After***	***Before***	***After***	***Before***	***After***
P1	**S1**	2,3 ± 0,2	2,3 ± 0,0	25,6 ± 0,1	25,6 ± 0,1	3,6 ± 0,1	3,5 ± 0,0	15,5 ± 0,1	15,5 ± 0,0	15,5 ± 0,0	15,5 ± 0,0
	S2	2,6 ± 0,1	2,7 ± 0,1	22,1 ± 0,0	22,1 ± 0,1	3,3 ± 0,1	3,3 ± 0,1	20,0 ± 0,0	20,0 ± 0,0	15,8 ± 0,1	15,8 ± 0,1
	S3	2,5 ± 0,1	2,6 ± 0,1	21,9 ± 0,1	21,9 ± 0,1	3,5 ± 0,1	3,5 ± 0,1	14,3 ± 0,1	14,3 ± 0,1	16,1 ± 0,1	16,2 ± 0,1
P2	**S1**	2,1 ± 0,1	2,1 ± 0,1	19,1 ± 0,0	19,2 ± 0,1	2,5 ± 0,0	2,6 ± 0,1	16,3 ± 0,0	16,3 ± 0,1	16,7 ± 0,1	16,7 ± 0,1
	S2	2,3 ± 0,1	2,3 ± 0,1	19,4 ± 0,1	19,4 ± 0,1	2,8 ± 0,1	2,8 ± 0,1	3,0 ± 0,0	3,1 ± 0,1	16,2 ± 0,1	16,2 ± 0,1
	S3	2,5 ± 0,1	2,5 ± 0,0	23,8 ± 0,1	23,9 ± 0,0	1,6 ± 0,1	1,8 ± 0,1	16,2 ± 0,0	16,2 ± 0,1	7,4 ± 0,1	7,3 ± 0,1
P3	**S1**	2,9 ± 0,1	2,8 ± 0,1	21,0 ± 0,0	21,0 ± 0,1	4,9 ± 0,0	4,9 ± 0,1	14,5 ± 0,0	14,5 ± 0,1	9,8 ± 0,1	9,5 ± 0,1 *
	S2	2,6 ± 0,0	2,6 ± 0,0	19,8 ± 0,1	19,7 ± 0,1	3,6 ± 0,1	3,5 ± 0,1	16,6 ± 0,1	16,7 ± 0,1	13,1 ± 0,1	12,2 ± 0,1 *
	S3	3,6 ± 0,1	3,5 ± 0,1	20,9 ± 0,1	20,9 ± 0,1	2,3 ± 0,1	2,3 ± 0,1	17,4 ± 0,1	17,5 ± 0,1	15,9 ± 0	15,5 ± 0,1 *
P4	**S1**	2,0 ± 0,1	2,0 ± 0,1	24,6 ± 0,1	24,6 ± 0,1	1,1 ± 0,0	1,1 ± 0,0	18,3 ± 0,1	18,3 ± 0,1	19 ± 0,1	11,8 ± 0,1 *
	S2	1,6 ± 0,1	1,6 ± 0,1	22,3 ± 0,1	22,3 ± 0,1	4,3 ± 0,0	4,3 ± 0,0	16,1 ± 0,1	16,2 ± 0	15,2 ± 0,1	6 ± 0,1 *
	S3	2,3 ± 0,1	2,4 ± 0,1	22,7 ± 0,1	22,8 ± 0,1	2,1 ± 0,1	2,1 ± 0,1	16,9 ± 0,1	17 ± 0	9,7 ± 0	4,3 ± 0,1 *
P5	**S1**	2,6 ± 0,0	2,6 ± 0,1	7,7 ± 0,1	7,7 ± 0,1	2,3 ± 0	2,3 ± 0,1	14,1 ± 0,2	14,1 ± 0,1	11,3 ± 0,1	11,2 ± 0,1
	S2	3,6 ± 0,1	3,6 ± 0,1	4,7 ± 0,1	4,7 ± 0,1	2,9 ± 0,1	2,8 ± 0,1	18,5 ± 0,1	18,5 ± 0,1	15,7 ± 0,1	15,7 ± 0,1
	S3	1,5 ± 0,0	1,5 ± 0,0	5,3 ± 0,1	5,3 ± 0,1	1,0 ± 0,0	0,9 ± 0,1	11,7 ± 0,1	11,6 ± 0,1	7,4 ± 0,1	7,3 ± 0,1
P6	**S1**	22,0 ± 0,1	22,1 ± 0,1	25,0 ± 0,0	25,1 ± 0,1	25,0 ± 0,1	24,9 ± 0,1	16,3 ± 0,0	16,2 ± 0,1	15,9 ± 0	15,7 ± 0,1
	S2	19,4 ± 0,1	19,3 ± 0,1	18,3 ± 0,1	18,2 ± 0,1	22,1 ± 0,0	22,0 ± 0,1	17,0 ± 0,1	17 ± 0,1	19,5 ± 0,1	19,5 ± 0
	S3	16,5 ± 0,1	16,5 ± 0,0	21,9 ± 0,1	21,9 ± 0,1	24,5 ± 0,1	24,5 ± 0,0	15,3 ± 0,1	15,1 ± 0,1	14,8 ± 0	14,8 ± 0,1

(HDPE: high density polyethylene; LDPE: low density polyethylene; PA-12: polylauryllactam ; PP: polypropylene; PS: polystyrene)

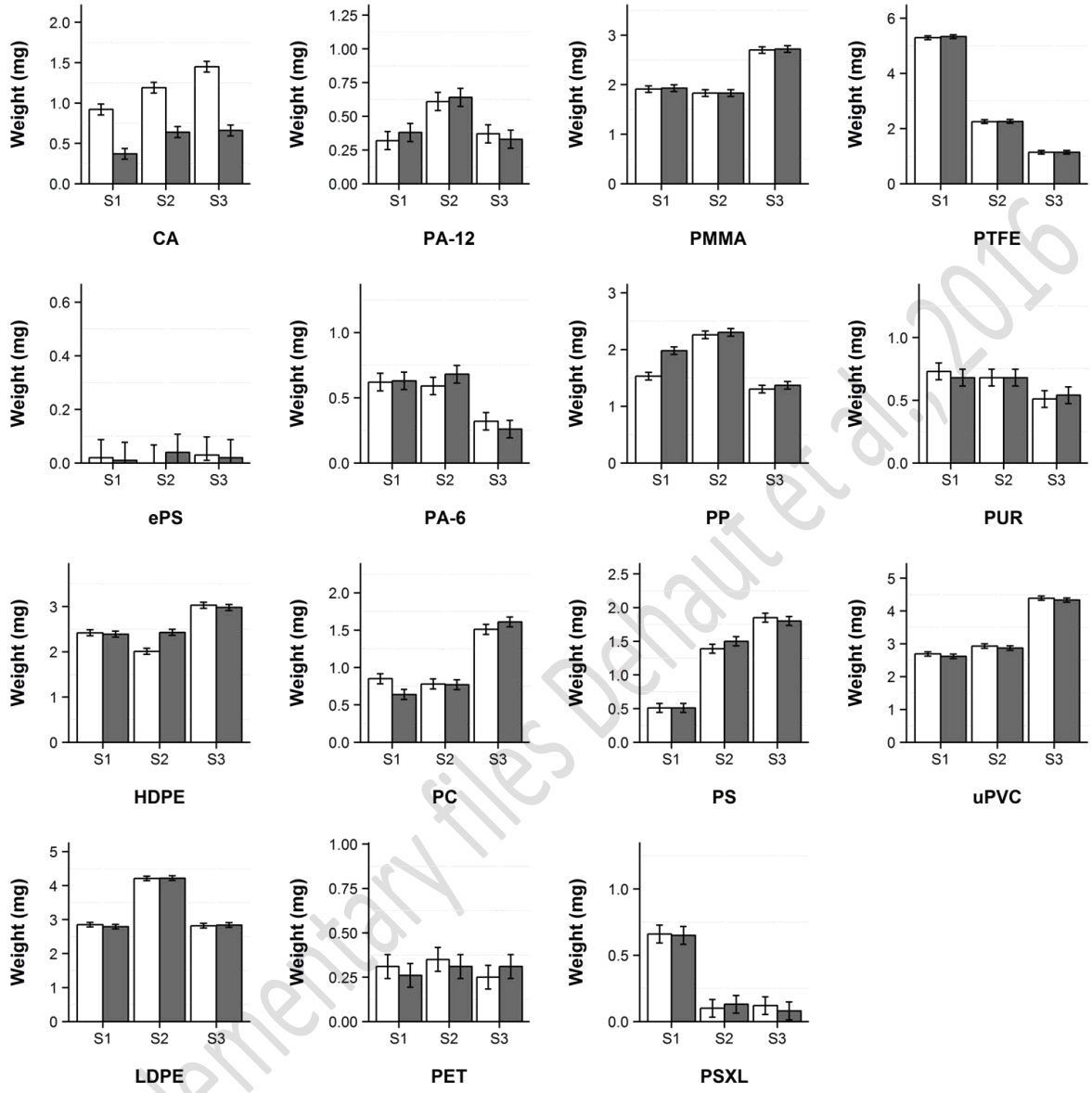
P1: Protocol 1 (10% KOH, 3 weeks, Room temperature); P2: Protocol 2 (0.5%Pepsin, 2h, 37 °C); P3: Protocol 3 (65% HNO_3 at room temperature overnight, then 60 °C for 2 h); P4: Protocol 4 (4:1 (v/v) mix of 65% HNO_3 and 65% $HClO_4$ at room temperature overnight, then boiled for 10 min); P5: Protocol 5 (10M NaOH, 60°C, 24h); P6: Protocol 6 ($K_2S_2O_8$/ NaOH, 65 °C, 24h)

S1, S2, S3 correspond to the three sample of the triplicate analysis

Weighing (mg) are expressed as *mean ± s.d*

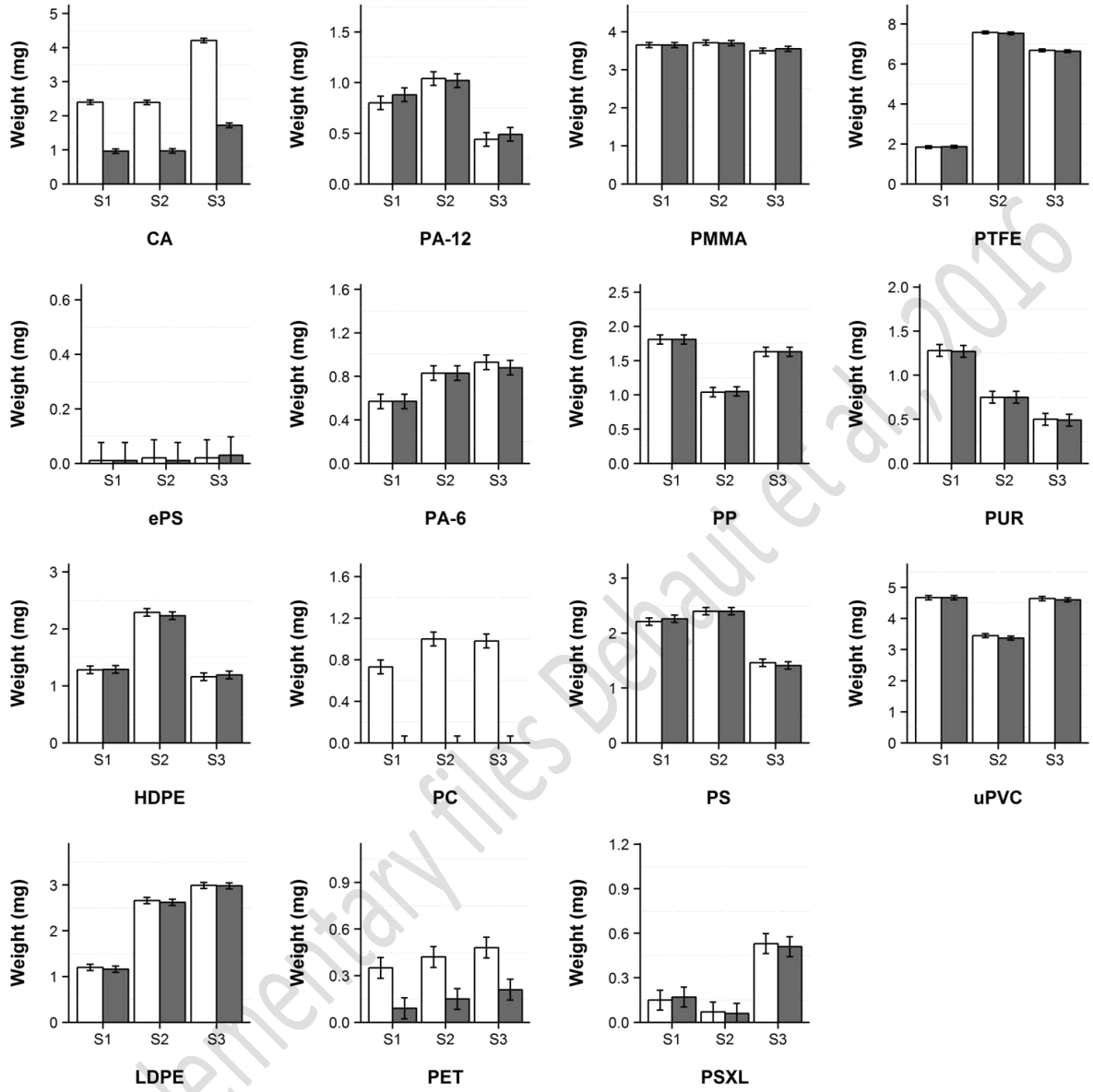
'*' Samples that melted during the application of digestion protocol

Supplemental Figure 1: Plastic weights before and after application of protocol 1b



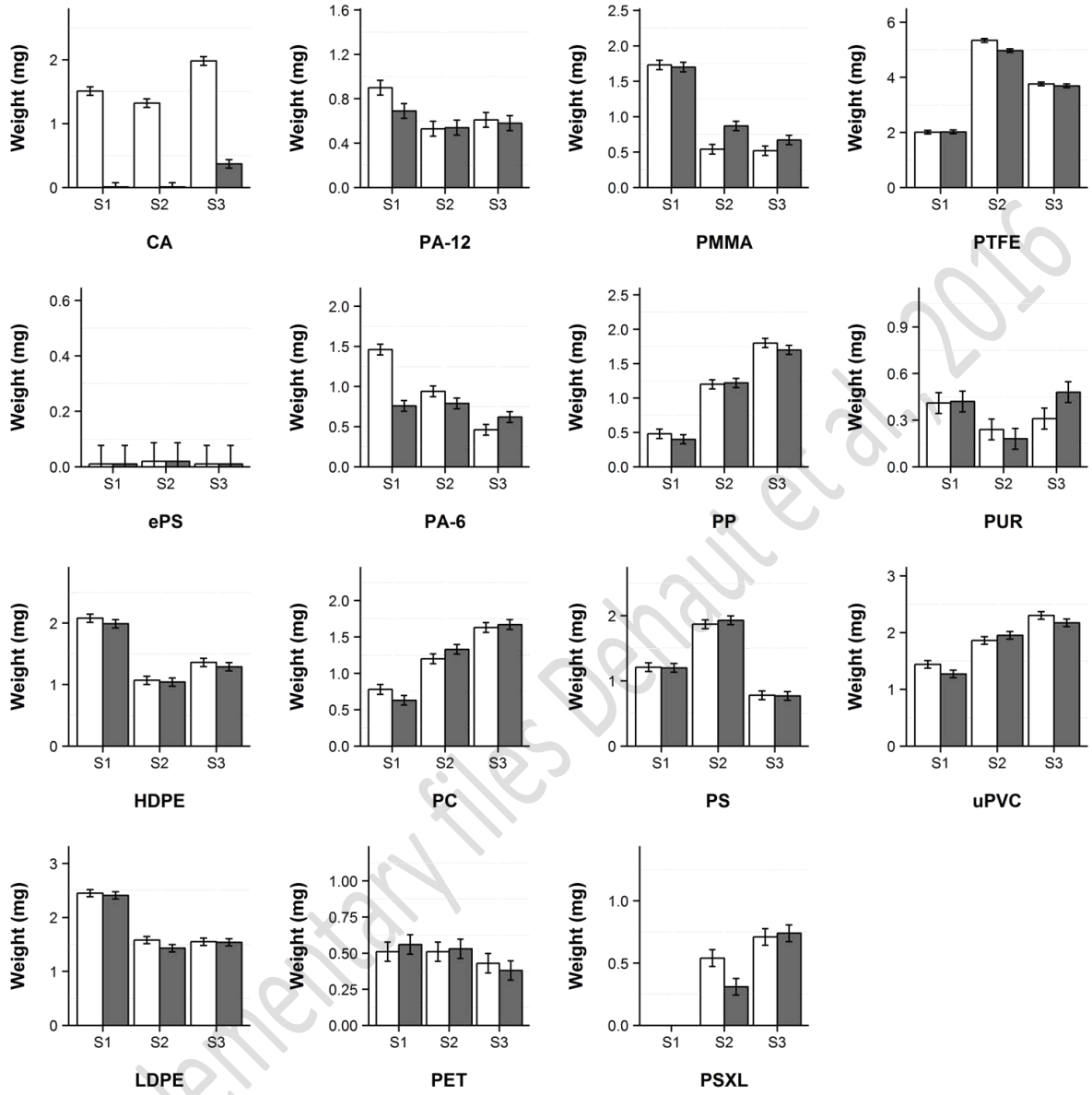
Bar chart representing change in weight (mg) for cellulose acetate (CA); expanded polystyrene (ePS); high density polyethylene (HDPE); low density polyethylene (LDPE); polylauryllactam (PA-12); polycaprolactam (PA-6); polycarbonate (PC); polyethylene terephthalate (PET); poly(methyl-methacrylate) (PMMA); polypropylene (PP); polystyrene (PS); polytetrafluoroethylene (PTFE); polyurethane (PUR); cross-linked polystyrene (PSXL); unplasticized polyvinyl chloride (uPVC); before and after application of protocol 1b, 10% KOH, 24h, 60°C. White and dark grey bars represent the mean of 5 repeated weight measures of three plastic samples (S1, S2 & S3) respectively before and after application of protocols. Error bars on charts represent the expanded weighing uncertainty of the results *Ueb* (see 2.4).

Supplemental Figure 2: Plastic weights before and after application of protocol 5



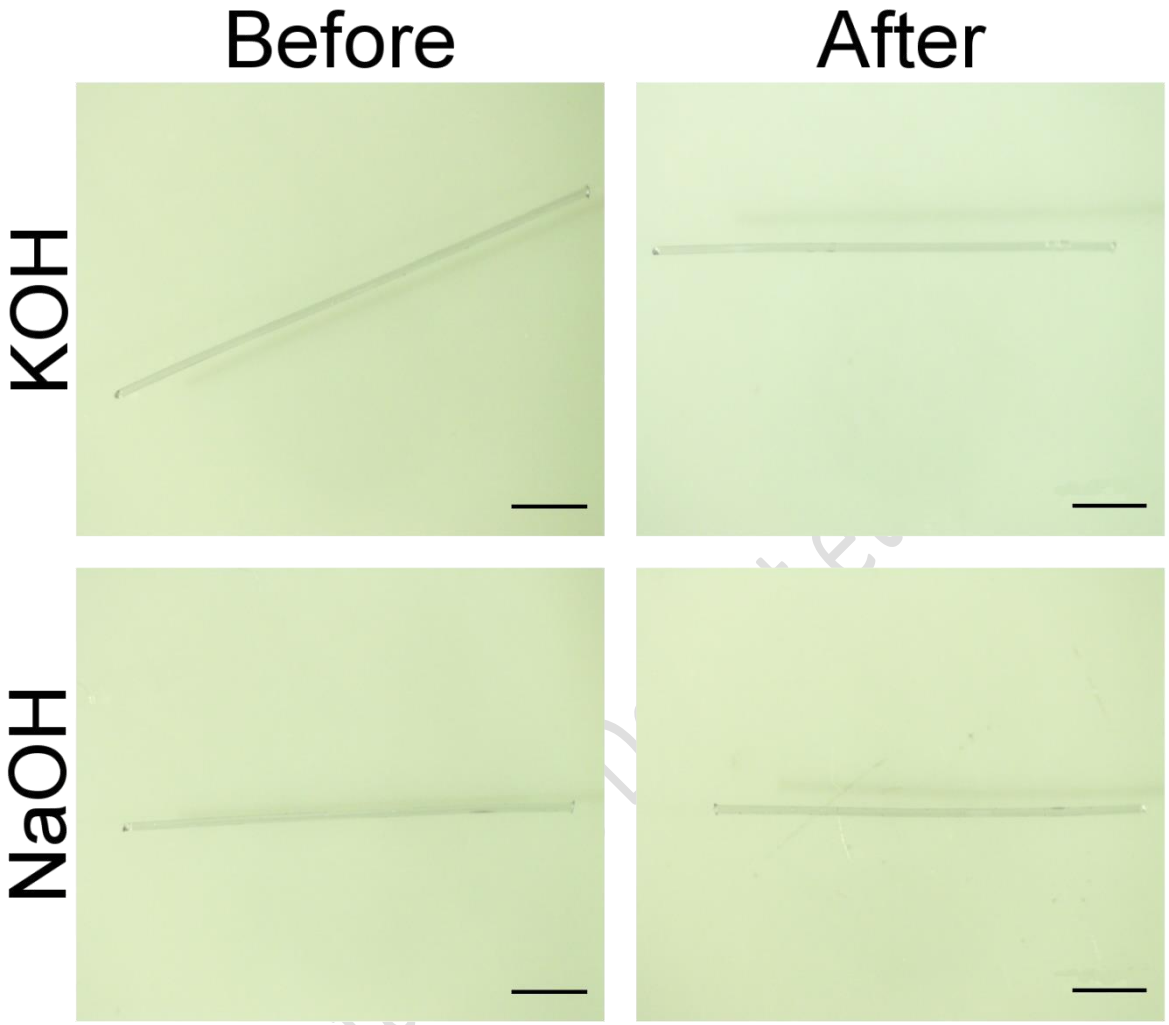
Bar chart representing change in weight (mg) for cellulose acetate (CA); expanded polystyrene (ePS); high density polyethylene (HDPE); low density polyethylene (LDPE); polylauryllactam (PA-12); polycaprolactam (PA-6); polycarbonate (PC); polyethylene terephthalate (PET); poly(methyl-methacrylate) (PMMA); polypropylene (PP); polystyrene (PS); polytetrafluoroethylene (PTFE); polyurethane (PUR); cross-linked polystyrene (PSXL); unplasticized polyvinyl chloride (uPVC); before and after application of protocol 5, 10M NaOH 60°C, 24h. White and dark grey bars represent the mean of 5 repeated weight measures of three plastic samples (S1, S2 & S3) respectively before and after application of protocols. Error bars on charts represent the expanded weighing uncertainty of the results Ueb (see 2.4).

Supplemental Figure 3: Plastic weights before and after application of protocol 6



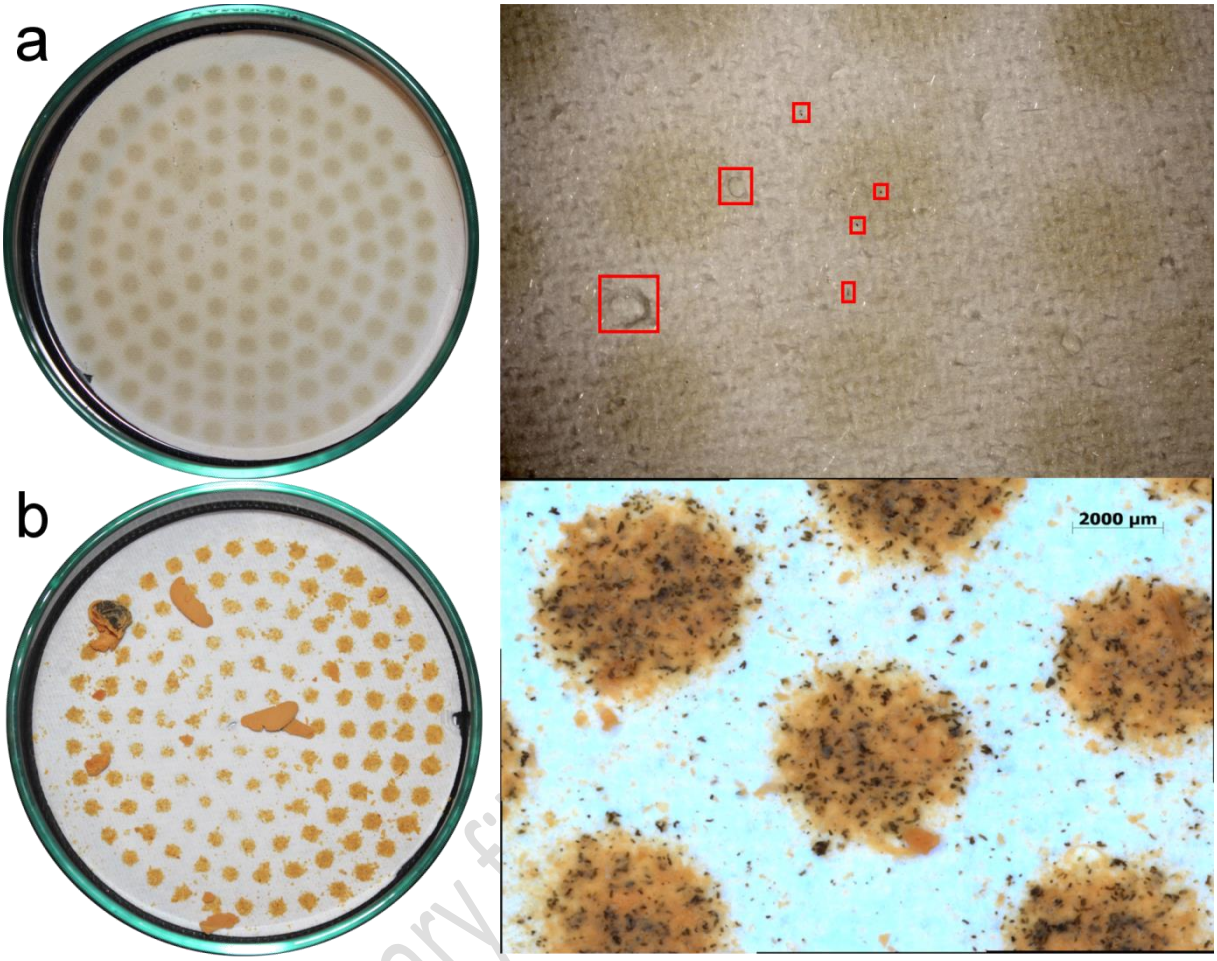
Bar chart representing change in weight (mg) for cellulose acetate (CA); expanded polystyrene (ePS); high density polyethylene (HDPE); low density polyethylene (LDPE); polylauryllactam (PA-12); polycaprolactam (PA-6); polycarbonate (PC); polyethylene terephthalate (PET); poly(methyl-methacrylate) (PMMA); polypropylene (PP); polystyrene (PS); polytetrafluoroethylene (PTFE); polyurethane (PUR); cross-linked polystyrene (PSXL); unplasticized polyvinyl chloride (uPVC); before and after application of protocol 6, $K_2S_2O_8$/ NaOH 65 °C, 24h. One sample of PSXL was lost during the second weighing. White and dark grey bars represent the mean of 5 repeated weight measures of three plastic samples (S1, S2 & S3) respectively before and after application of protocols. Error bars on charts represent the expanded weighing uncertainty of the results *Ueb* (see 2.4).

Supplemental Figure 4: Test of Protocols 5 and 6 on PA-6 fibres



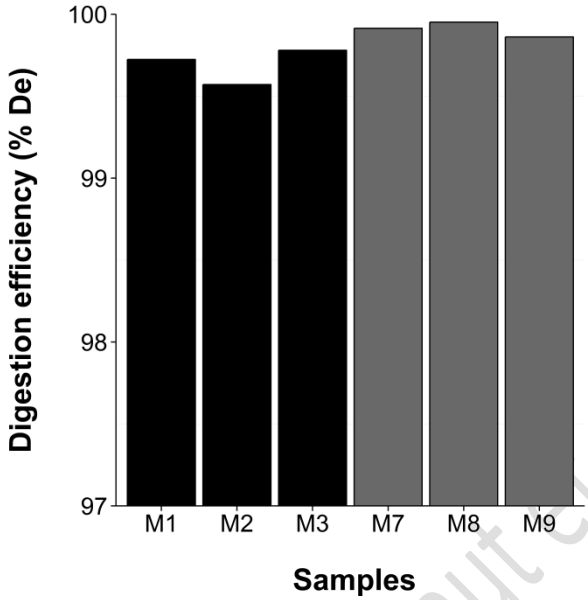
Visual inspection of nylon fibres (PA-6) before and after application of protocol 5 (10M NaOH 60°C, 24h) and 6 ($K_2S_2O_8$/ NaOH 65 °C, 24h). No significant change was observed and identification by Pyr-GC/MS led to good identification with respective similarities of 96 and 99%.

Supplemental Figure 5: Filters corresponding to digestion of mussel tissues with protocols 1b and 6



Filters corresponding to digestion of mussel tissues with protocols 1b (10% KOH, 24h, 60 °C) (a) and 6 ($K_2S_2O_8$/ NaOH 65 °C, 24h) (b). Areas highlighted by red squares correspond to fragments suspected to be microplastics.

Supplemental Figure 6: Comparison of *%De* for mussels treated by protocol 1b with different conditions (200 mL at 300 rpm *vs*. 100 mL at 200 rpm).

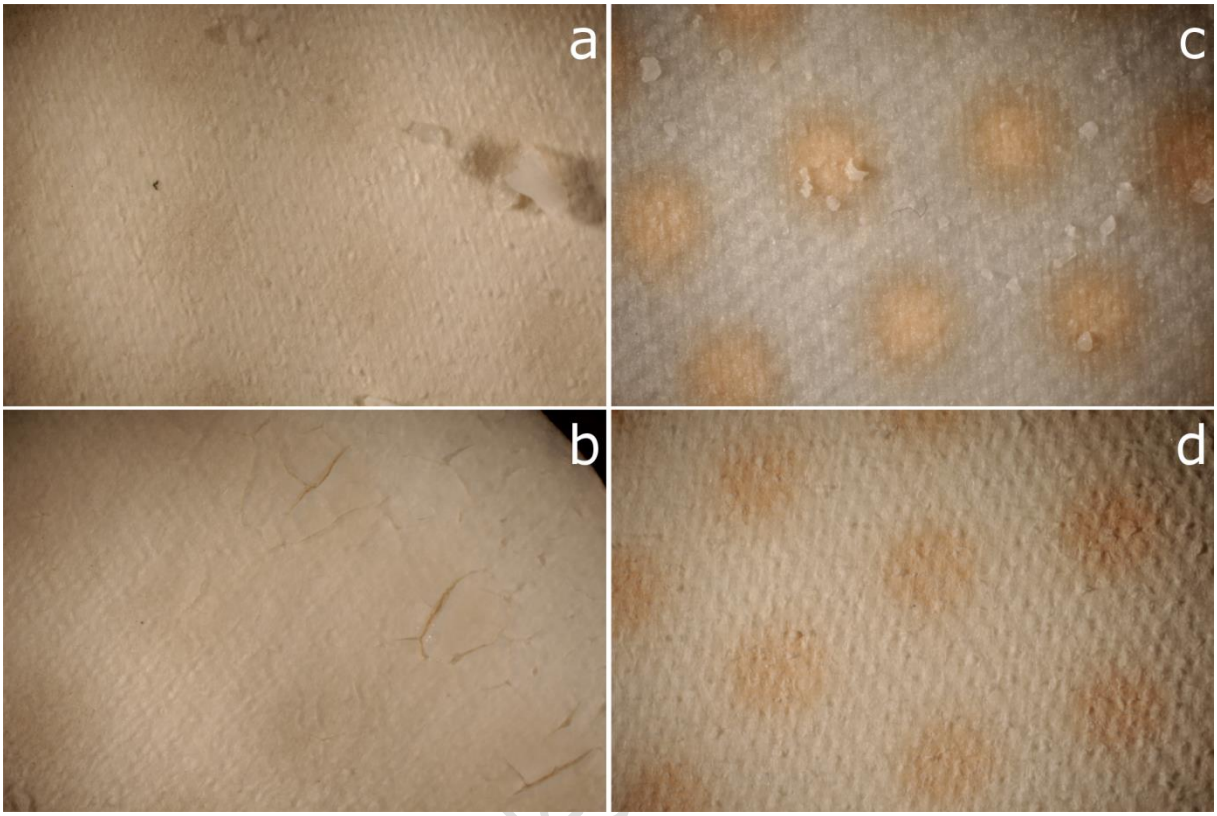


Bar chart representing individual digestion efficiencies (*% De*) on three mussels, M1 to M3 for protocol 1b (200 mL of 10% KOH, 300 rpm, 24h, 60°C) in black, and M7 to M9 for protocol 1b (100 mL of 10% KOH, 200 rpm, 24h, 60°C) in grey. The very low standard deviations (< 0.01%) do not appear on bar diagrams as they could not be distinctively traced.

Supplemental Figure 7: Filters corresponding to digestion of crab tissues with protocol 1b.

Filter corresponding to digestion of crab tissues with protocol 1b (10% KOH, 24h, 60 °C). Areas highlighted by red squares correspond to suspected microplastic fragments.

Supplemental Figure 8: Filters corresponding to digestion of fish tissues with protocol 1b.



Filters corresponding to digestion of fish tissues (fillets and whole alimentary tract) with protocol 1b (10% KOH, 24h, 60 °C). Concerning fillet (a & b), filters contain some fragments of bones partially digested (a). A fatty layer was noticed on the edge of filter (b). For whole alimentary tract (c & d), a few fragments from the stomach content, such as cartilage or starfish remainings, were reported (c). Generally, filters were clear, lightly loaded and easily observable (d).