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Novel methodology to isolate microplastics from vegetal-rich samples

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ABSTRACT

Microplastics are small plastic particles, globally distributed throughout the oceans. To properly study them, all the methodologies for their sampling, extraction, and measurement should be standardized. For heterogeneous samples containing sediments, animal tissues and zooplankton, several procedures have been described. However, definitive methodologies for samples, rich in algae and plant material, have not yet been developed. The aim of this study was to find the best extraction protocol for vegetal-rich samples by comparing the efficacies of five previously described digestion methods, and a novel density separation method. A protocol using 96% ethanol for density separation was better than the five digestion methods tested, even better than using H_2O_2 digestion. As it was the most efficient, simple, safe and inexpensive method for isolating microplastics from vegetal rich samples, we recommend it as a standard separation method.

1. Introduction

Plastics are synthetic organic polymers with features, such as durability and low price, that make them perfect for many applications. Unfortunately, the same characteristics that make plastic the perfect material cause it to become a serious pollution problem. Recent studies report that 4.8 to 12.7 million metric tons of plastic were disposed to the ocean in 2010 (Jambeck et al., 2015). At present, plastic marine pollution is one of the major concerns of the scientific community and organizations responsible for environmental policies at the global level (Andrady, 2010, 2011; European Parliament, 2008; Galgani et al., 2010, 2013; Scientific and Technical Advisory Panel, 2011).

Plastic particles smaller than 5 mm are classified as microplastics (Arthur et al., 2009). Secondary microplastics are the product of degradation and fragmentation of larger plastics, while primary microplastics are manufactured with size < 5 mm, mainly for use in cosmetics, cleaning products or as raw material for the production of plastic products (pre-production pellets). Due their small size, microplastics can impact marine organisms including zooplankton. They can be ingested directly or indirectly through the food web (Barnes et al., 2009; Setälä et al., 2014). Their consumption is likely to constitute a chemical, physical, and biological hazard (Browne et al., 2008; Setälä et al., 2014; Teuten et al., 2009; Von Moos et al., 2012; Wright et al., 2013; Zettler et al., 2013).

To obtain reliable and reproducible data on microplastic contamination and to investigate its effects on marine biota and the environment, it would be beneficial to first harmonize and standardize the sampling, extraction, and quantification methods that are being used by the scientific community (MSDF Technical Subgroup on Marine Litter, 2013; Rochman et al., 2017). Sampling techniques, and analytical techniques to isolate and quantify microplastic samples from different environments, have been reviewed extensively (Besley et al., 2017; Hanvey et al., 2017; Hidalgo-Ruz et al., 2012; Lusher et al., 2017; Miller et al., 2017; Van Cauwenberghe et al., 2015). For microplastics extraction, most techniques are based on density separation via flotation (Claessens et al., 2013; Cole et al., 2015; Coppock et al., 2017; Imhof et al., 2012; Thompson et al., 2004). Density separation requires highly dense solutions, such as sodium chloride (NaCl, 1.2 g/cm^3), sodium iodide (NaI, 1.6 g/cm^3) and zinc chloride ($ZnCl_2$, 1.6–1.7 g/cm^3) because the specific densities of the most common plastics in environmental samples range from 0.01 g/cm^3 to 1.60 g/cm^3 (Table 1). Other separation strategies for microplastics include evaporation, filtration, sieving, and visual sorting (Crawford and Quinn, 2017; Hidalgo-Ruz et al., 2012; Masura et al., 2015; Song et al., 2014; Yamashita and Tanimura, 2007). These techniques are useful for isolating microplastics from sediments, but isolating them from biological material requires a different treatment. The density of the biological material (leaves, seeds, wood, etc.) is, in most cases, lower than the density of the solutions used in the separation process, and therefore they float together with microplastics. Another problem is that microplastics are

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Table 1
Density ranges of common plastic polymers (modified from Crawford and Quinn (2017)) and 96% ethanol.

Plastic polymers	Abbreviation	Density in g/cm^3
Polystyrene (expanded foam)	EPS	0.01–0.05
Polystyrene (extruded foam)	XPS	0.03–0.05
Polypropylene	PP	0.88–0.91
Low-density polyethylene	LDPE	0.92–0.94
High-density polyethylene	HDPE	0.94–0.97
Nylon 6.6	PA	1.05–1.10
Polyvinyl chloride	PVC	1.45–1.70
Polyethylene terephthalate	PET	1.40–1.60
Polystyrene	PS	1.04–1.05
Polystyrene (30% glass fibers)	PS	1.40–1.50
Polyurethane	PUR	1.20–1.40
Polyurethane (foam)	PUR	0.03–0.80
Ethanol 96%	EtOH	0.805–0.812

imbedded in the organic material and cannot be isolated by density only.

Several digestion techniques for the removal of the organic material in microplastic samples have been described (Catarino et al., 2017; Claessens et al., 2013; Cole et al., 2014; Dehaut et al., 2016). Many of them were specifically designed to be effective in extracting microplastics from animal tissue or zooplankton. However, techniques for digesting the algal and plant component of sediment samples have not been developed (Hanvey et al., 2017). This type of biological material is abundant in beach samples, and can even retain microplastics on its surface (Gutow et al., 2015). Finding a way to separate microplastics from this vegetal material is thus important to assess the extent of microplastic pollution in the aquatic environment. A recent study suggested that dried algae and seagrasses, among other residues present in the microplastic samples, could be removed by visual sorting or sieving, using the naked eyed or a microscope (Crawford and Quinn, 2017; Hidalgo-Ruz et al., 2012). These procedures may be acceptable for the biggest fragments, for large pieces of algae and leaves, and for a small number of samples. However, for the smaller particles and for a large number of samples, these procedures are time consuming and are likely to lead to underestimating the extent of microplastics pollution.

The objective of the present work was to find an efficient method to remove algae and plant material from microplastics samples. In order to achieve this, five existing digestion protocols based on HCl, NaOH, KOH and H_2O_2 treatments, and a novel density separation procedure using 96% ethanol (EtOH), were tested, and their separation efficacies were calculated and compared. In addition, the integrity of six types of plastic polymers (polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET; polyester fibers), and polystyrene (PS)) subjected to the different methodologies was studied in order to confirm that these methods do not damage plastic particles.

2. Materials and methods

2.1. Sampling collection and preparation

A one-liter sample was collected along the high tide line near the dunes at Famara beach, Lanzarote, Spain (N 29°6.941, W 13°33.461), on January 29th, 2016 (Fig. 1a). The sample was placed in a 5 l plastic container and mixed for 1 min with 3 l of sea water from the same beach. The supernatant fluid was then filtered through a 1 mm aperture mesh. No measures to prevent contamination were taken during sampling, because we did not have to determine the exact concentration of microplastics, but only had to obtain a representative sample. After separation of the samples in the laboratory, measures were taken to avoid contamination. All the procedures were done inside a fume hood. All personnel wore cotton laboratory coats. In addition, all the materials used, as well as the workplace, were cleaned with ultrapure water. The sample was always well protected to avoid contamination in the laboratory. However, to evaluate contamination, should it occur, two clean filters were exposed during the digestion procedures and density separation. They were then examined immediately after each procedure under a microscope. No contamination was found on any of them.

The sample was composed of organic matter (mainly vegetal debris) at a concentration (w/w) of 1/6 and of microplastics, 5/6 (Fig. 1b). In order to avoid differences in the separation efficiencies due to the different amounts of organic material present in the samples, we homogenized the sub-samples. To accomplish this, the microplastics and organic matter were manually separated. Then, replicate sub-samples of 6 g each, composed of 1 g of biological material and 5 g of microplastics, were taken (Fig. 2). Before being subjected to each of the protocols, the sub-samples were oven-dried at 60 °C and weighed on a high precision balance (0.1 mg). When we were able to confirm that the treatment used was safe for plastics, we were certain that any "weight loss" was due to digestion or separation of organic matter.

2.2. Separation efficacy

Five existing protocols to digest organic matter were tested for vegetal rich samples: 3% HCl, 40% NaOH, 4% NaOH + SDS, 10% KOH and catalytic 30% H_2O_2 (chemical solutions information in Table 2). In addition, density separation by 96% EtOH (16.44 M) was tested (Table 2). Triplicates of sub-samples composed of 1 g of biological material and 5 g of microplastics were processed with each protocol.

Protocol 1

Protocol 1 corresponded to the acid digestion method tested by Cole et al. (2014). The sample was previously oven-dried at 60 °C, then 40 ml of 3% HCl (1 M) were added to sub-samples, they were stirred for a minute, and finally, maintained at room temperature (20 °C) for 24 h.

Protocol 2

Protocol 2 was based on the alkaline digestion method tested by Cole et al. (2014). As above, the sample was previously oven-dried at 60 °C, then 40 ml of 40% NaOH (10 M) were added to sub-samples, they were stirred for 1 min, and finally placed in an oven for 24 h, at 60 °C.

Protocol 3

Protocol 3 was adapted from Dehaut et al. (2016), and consisted of alkaline sample digestion. The sample was previously oven-dried at 60 °C, then 40 ml of 10% KOH (1.78 M) were added to the sub-samples, they were stirred for 1 min and maintained at 60 °C for 24 h in a drying oven.

Protocol 4

Protocol 4 is based on the work of Budimir et al. (2017), presented at MICRO 2016 International Congress. In this protocol, less concentrated NaOH was added to samples together with the detergent, SDS. Budimir describes an alkaline digestion procedure in which 10 ml of 4% NaOH (1 M) and 5 ml of SDS are added to the sub-samples, and in which only 2 h at 50 °C were enough to digest the biological material in the samples. The original protocol was modified in order to standardize all the procedures followed here. This was done by oven-drying the sample at 60 °C, adding 40 ml of NaOH and 20 ml of SDS, and maintaining it for 2 h in an oven at 50 °C. If no visual changes were observed in the sub-samples, they were maintained for 24 h at 60 °C.

Protocol 5

Protocol 5 was based on the Wet Peroxide Oxidation (WPO) method described by Masura et al. (2015). Here, only the WPO step was carried out despite Masura et al. (2015) describing several other steps for the analysis of microplastics on beach sediment samples. The sample was

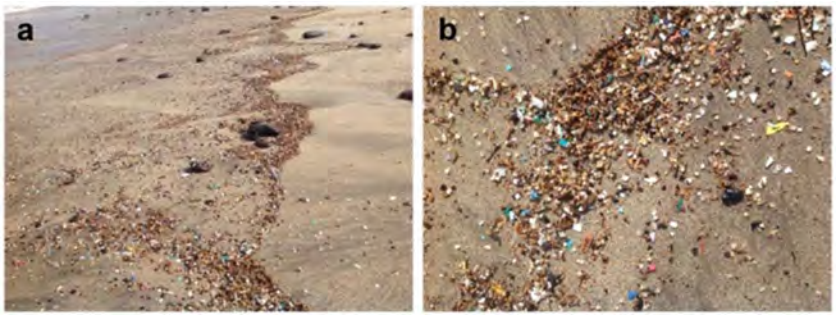

Fig. 1. (a) Microplastic pollution from the high tide line in Famara, Lanzarote. (b) Detail of microplastics and organic debris.

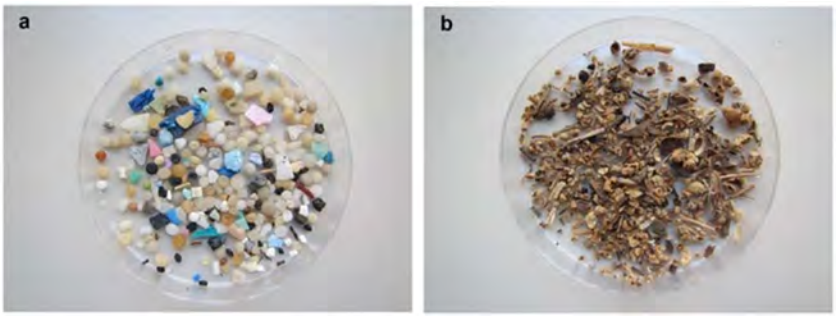

Fig. 2. Samples were composed of (a) 5 g of microplastics and; (b) 1 g of biological material.

previously oven-dried at 60 °C, 40 ml of aqueous 0.05 M Fe(II) were added to a large beaker (~800 ml) containing the sample, followed by 40 ml of 30% H_2O_2 (9.79 M). After incubating 5 min at room temperature, the mixture was heated to 75 °C on a hotplate for 30 min. **CAUTION**: this solution can boil violently if heated > 75 °C. Avoid this condition. If biological material remained in the mixture after that time, another 40 ml of hydrogen peroxide should be added. In this work, on three occasions, more hydrogen peroxide was added to the sub-samples.

Protocol 6

Protocol 6 was a novel method based on density separation by ethanol (see graphical abstract). The sample was previously oven-dried at 60 °C. Forty milliliters of 96% (v/v) EtOH (16.44 M) were added to samples. They were then stirred at 600 rpm for 3 min and settled for 1 min. This allowed the separation of microplastics from imbedded organic material. Concentrated EtOH, at 96% has a density of 0.8 g/cm^3 (at 20 °C). This is lower than the most common plastics found in samples, except for some polystyrene polymers (PS), expanded foam (EPS), extruded foam (XPS) and polyurethane foam (PUR) (see Table 2). If the density of the biological material present in the samples is lower than 0.8 g/cm^3, the biological material will float with the polystyrene and polyurethane foams while the heavier plastics will sink (Fig. 3). PS and PUR foams should be identified by visual detection and removed with forceps. After density separation, the supernatant was removed and the remnant sample was filtered. **NOTE**: use glass containers because poly (methyl methacrylate) (PMMC) can be chipped in contact with 96% EtOH.

After applying each treatment, samples were filtered through a Whatman® filter paper grade 4 (20–25 μm), oven-dried at 60 °C and weighed on a high precision balance (0.1 mg). The efficiency of a digestion protocol depends on the relative removal of organic mass during the digestion procedure. If the method validation showed that

Table 2
Protocols applied. Original units (in bold) for the digestion solutions have been converted to their % (v/v or w/v) concentration equivalent.

Protocol	Methodology	Solutions	Concentration	Temp.	Exposure time	Adapted from
Protocol 1	Digestion	40 ml HCl (PANREAC 471020)	3% (v/v) HCl (**1 M**)	20 °C	24 h	Cole et al. (2014)
Protocol 2	Digestion	40 ml NaOH (Scharlau SO0420)	40% (w/v) NaOH (**10 M**)	60 °C	24 h	Cole et al. (2014)
Protocol 3	Digestion	40 ml KOH (Scharlau PO02660500)	10% (w/v) KOH (1.78 M)	60 °C	24 h	Dehaut et al. (2016)
Protocol 4	Digestion	40 ml NaOH (Scharlau SO0420) 20 ml SDS (Acros Organic 226,145,000)	4% (w/v) NaOH (**1 M**) SDS 0.5% (w/v) (17.34 mM)	60 °C	24 h	Budimir et al. (2017)
Protocol 5	Digestion	40 ml H_2O_2 (Panreac 121076) 40 ml Fe(II) $FeSO_4{\cdot}7H_2O$ (Panreac 131362) H_2SO_4 (VWR 20700.298)	30% H_2O_2 (w/v) (9.79 M) 0.05 M Fe(II) catalyst (7.5 g $FeSO_4{\cdot}7H_2O$; 500 ml H_2O; 3 ml H_2SO_4)	75 °C	30 min × 3	Masura et al. (2015)
Protocol 6	Density separation	100 ml C_2H_6O (Scharlau ET00031000)	96% v/v EtOH (16.44 M)	20 °C	3 min	Present work

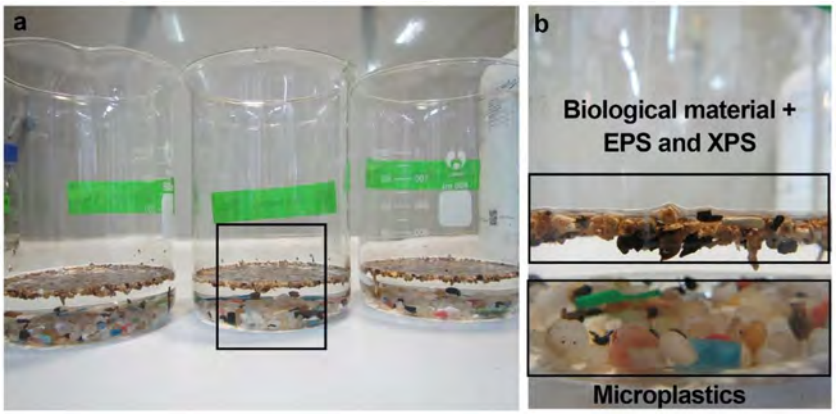

Fig. 3. Density separation by 96% EtOH. (a) Samples subject to protocol 6 (96% EtOH) (b) shows the organic matter floating together with the EPS and XPS foams, the rest of the microplastics are deposited in the bottom.

plastic particles were not degraded or damaged, then any difference between the samples weight, before and after being exposed to the protocols, was attributed to a loss of biological material. The percentage separation efficacy (%*Se*) was calculated as:

$$\%Se = \frac{T_0 - T}{B_0} \times 100$$

where B_0 is the initial dry mass of biological material, T_0 is total dry mass before exposure, and T is total dry mass after exposure.

2.3. Statistical analysis

Statistical analyses and graphics of digestion efficacies were performed with R statistical software (R Core Team, 2017) and its extension, RStudio. Data normality was confirmed by the Kolmogorov-Smirnov test and data homoscedasticity was assessed graphically. ANOVA and Tukey post-hoc tests were applied to determine significant differences among protocols. The results were represented in box plots.

2.4. Method validation

Each protocol was tested on plastic particles selected according to European plastics demand: polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET; polyester fibers), and polystyrene (PS) (PlasticsEurope, 2015). Pre-production pellets were also included because they are very abundant in Canary Islands beach samples and have importance in the study of marine debris (Herrera et al., 2017). A subsample of 7 pre-production pellets collected from Famara beach were analyzed by Fourier transform infrared spectroscopy (FTIR) and calorimetry to determine their composition using an infrared spectrometer (Perkin-Elmer Spectrum BX from Perkin-Elmer Spain S.L., Madrid, Spain). 20 scans between 4000 and 600 cm^{-1} were performed with a resolution of 32 cm^{-1} in the reflection mode. Differential scanning calorimetry (DSC) was conducted in a Mettler-Toledo 821 calorimeter (Schwerzenbach, Switzerland) in air atmosphere, the heating program was from 30 to 300 °C at a heating rate of 10 °C min^{-1} (details in Appendix A).

Five pellets, and five small pieces < 5 mm of each type of plastic polymer (PP, PE, PV, PUR, PS and PET), were subjected to protocols 1 to 6 (Table 3). Each experiment was conducted in triplicate. Microplastics were visually inspected under a stereomicroscope, counted, measured and photographed before and after experimentation (t1 and t2, respectively). Recovery rates were calculated for pellets and fragments of polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR) and polystyrene (PS). Polyester (PET) fibers were not counted, only visually inspected for changes.

Table 3
Polymer types, colours, sizes (mm), original item and sources used for method validation experiments.

Polymer type	Colour	Size (mm)	Original product	Source
PE	Blue	1.50–3.48	Water bottle cap	Supermarket
PP	Green, orange	1.51–4.52	Plastic container	Supermarket
PS	White	1.8–4.55	Packaging	Supermarket
PA	Green, orange, blue	6.84–16.50	Fishing nets	Beach sample
Polyester	Dark blue	0.5–8	Textile fibers	Blanket
Resin pellets	White, transparent	4.42–6.12	Resin pellets	Beach sample

Microplastics' images were compared among t1 and t2 in order to detect changes in colour, size, number, and shape, to determine "destructive" effects of digestion procedures (Cole et al., 2014; Dehaut et al., 2016; Nuelle et al., 2014). Using the software ImageJ 1.50b, microplastics length and area were digitally measured, and colour histograms were plotted (Appendix A, Fig. 4).

3. Results

3.1. Sampling collection and preparation

The biological material was mainly composed of vegetal debris composed of leaves, seeds, wood, seaweed, and seagrasses (Fig. 2b). Seagrass *Cymodocea nodosa* and algae *Sargassum vulgare* were identified in the sample. Other material like leaves, seeds and wood pieces could not be identified.

3.2. Separation efficacy

Visual examination of all the samples after the extraction procedures (Fig. 4) revealed qualitative differences, especially among samples subjected to protocol 6. Biological material remained almost the same after applying protocols 1, 2 and 3, while the material exposed to protocols 4 and 5 was partially digested. However, the density separation by the 96% EtOH (protocol 6) showed that microplastics were separated almost completely from biological material (Fig. 4f). The ANOVA test revealed significant differences among the separation efficacies of protocols ($F = 140.6$; 5 df; p-value < 0.001). A Tukey post-hoc test showed no significant differences between efficacies of protocols 1, 2 and 3 (p-value > 0.01) (Fig. 5). The separation efficacy (%*Se*) showed significant differences, ranging from 9 to 97% (means and

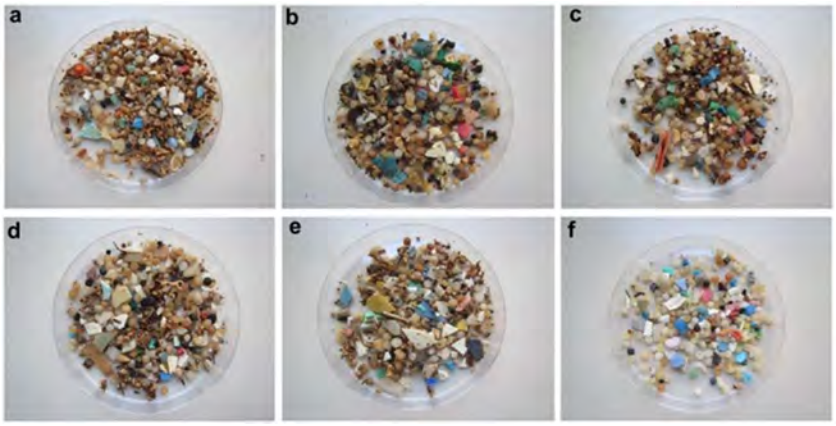

Fig. 4. Microplastic samples that contained algae and plant debris after being subjected to: (a) HCl protocol 1, (b) NaOH protocol 2, (c) KOH protocol 3, (d) NaOH + SDS protocol 4, (e) H_2O_2 protocol 5, and (f) EtOH protocol 6. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

standard deviation are presented in Table 4). Protocols 1, 2, 3 and 4, were not efficient at digesting algae and plant debris, with the mean % *Se* ranging from 9 to 40.9%. Protocol 5 was the one that obtained a greater digestion efficiency, with an average %*Se* of 64.6 ± 7.1%, but the highest %*Se* and the most efficient separating microplastics from algae and plant material was protocol 6. This simple procedure incorporated density separation using 96% EtOH. After the ethanol addition, an average of 97% of biological material floated and separated from the microplastics that had sunk to the bottom. Polystyrene extruded foam and expanded foam floated along with the biological debris, but were easily detected and removed from the sample with forceps.

3.3. Method validation

From the analyzed pellet sub-sample, 6 pellets were identified as polyethylene (PE) and 1 pellet was identified as polypropylene (PP) (detailed results can be found in Appendix A).

The recovery rates were 100% in all treatments for pellets and fragments of polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR) and polystyrene (PS). Polyester fibers could not be recovered after subjected to 40% NaOH treatment (protocol 2). Microplastics were successfully recovered after the experiments, and their colours, shapes and sizes remained intact, except for fibers subjected to protocol 2. Changes in shape, size and colour were observed in polyester fibers subjected to this protocol (40% NaOH) by visual examination and by comparing colour histograms before and after treatments (Fig. 6).

4. Discussion

Claessens et al. (2013) developed a nitric acid digestion-based method for animal tissue using 22.5 M HNO_3 to digest mussels (The HNO_3 concentration reported by Claessens et al. (2013) was 22.5 M (~95%). This is probably an error in the manuscript). It employed overnight organic matter oxidation at room temperature, followed by 1 h heating at 60 °C and by 1 h boiling at 100 °C, and finally a warm filtration (approx. 80 °C). This acidic digestion technique resulted in high digestion efficacies of tissues, but when tested for polystyrene spheres and nylon fibers, nylon rope fibers could not be recovered. Other authors have also reported damage to plastic particles using HNO_3 digestion (Avio et al., 2015; Catarino et al., 2017; Dehaut et al., 2016). For this reason, although several nitric acid methods have been recently used (De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014; Vandermeersch et al., 2015), here, because they cause damage, nitric acid methods were not included.

An alternative to strong-acid digestion is the use of non-oxidizing acids or alkaline hydrolysis. Cole et al. (2014) compared the use of HCl at concentrations of 1 M and 2 M, NaOH at different concentrations (1 M, 2 M, 5 M, 10 M) and the enzyme, Proteinase-K, in digesting marine plankton in samples containing polyethylene, polyester, nylon, polystyrene and unplasticized polyvinyl chloride (uPVC) based microplastics. Methodologies using 1 M (3%) HCl and 2 M (6%) HCl to digest zooplankton had the lowest efficacies of 82.6% and 72.1% respectively

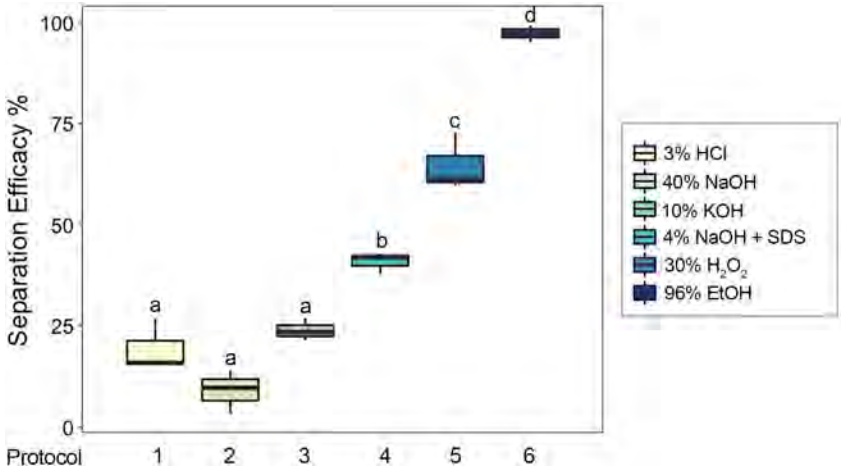

Fig. 5. Separation efficacy (%) of the protocols. The central thick line of each box designates the median, the box height shows the interquartile range, and the whiskers indicate the lowest and the highest values. Different letters indicate significant differences (Tukey post-hoc test p-value < 0.01).

Table 4
Results of separation efficacy and recovery rates. Separation efficacy values (%*Se*) of the protocols in algae and plant samples, displayed by means and standard deviation (mean ± SD). n/o = no observed changes.

Procedure	Solution	Separation efficacy (%*Se*)	Impact on microplastics	Recovery rates (%)
Protocol 1	3% HCl	19.5 ± 6.4%	n/o	100% PE, PP, PS, PA, pellets
Protocol 2	40% NaOH	9.0 ± 5.4%	Damage to polyester fibers	100% PE, PP, PS, PA, pellets. Not recovery of polyester fibers
Protocol 3	10% KOH	24.0 ± 2.8%	n/o	100% PE, PP, PS, PA, pellets
Protocol 4	4% NaOH + SDS	40.9 ± 2.7%	n/o	100% PE, PP, PS, PA, pellets.
Protocol 5	30% H_2O_2	64.6 ± 7.1%	n/o	100% PE, PP, PS, PA, pellets.
Protocol 6	96% EtOH	97.3 ± 2.1%	n/o	100% PE, PP, PS, PA, pellets.

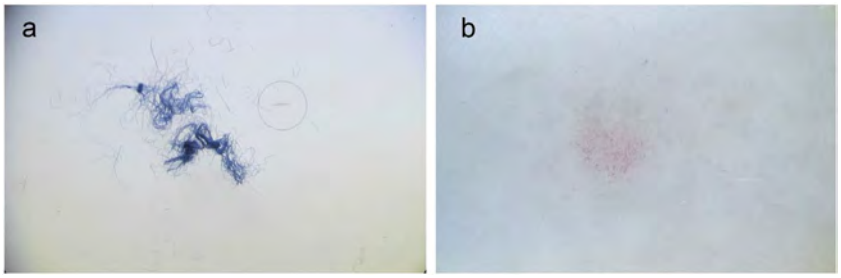

Fig. 6. Photographs of polyester fibers, (a) before and (b) after being subjected to 40% NaOH (protocol 2). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

(Cole et al., 2014). In the study of Nuelle et al. (2014), none of the biogenic organic particles like chitin carapaces or leaves had dissolved or were discoloured, even at a higher concentration (20% HCl) than the one used here.

Methodologies using NaOH, however, have shown a wide range of efficacies (Claessens et al., 2013; Cole et al., 2014; Nuelle et al., 2014) depending on the concentration and the procedure followed. The optimized alcaline digestion protocol proposed by Cole et al. (2014) (NaOH 40% during 24 h at 60 °C) showed a digestion efficacy of 91.3%. However, after the treatment, several polyester fibers were lost, nylon fibers were partially destroyed, and physical changes were observed in polyethylene fragments and uPVC granules. In addition, Dehaut et al. (2016) reported degradation in cellulose acetate (CA), polycarbonate (PC) and PET. The digestion efficacy of the same protocol used by Cole et al. (2014) (protocol 2), in our samples was lower and varied from 3.4 to 14%. Furthermore, damage in the polyethylene fibers was also detected. The higher efficacies, previously found by Cole et al. (2014) were not found here when digesting vegetal material, probably due to the different composition of the organic material. Biogenic matter of plant origin is composed mainly of cellulose, hemicellulose and lignin, compounds that are very difficult to digest.

In protocol 4, we used less concentrated NaOH solution (4%) to avoid damage in fibers. As previously shown by Budimir et al. (2017), the SDS detergent improved the 4% NaOH digestion efficacy. We obtained a digestion efficacy of 40%, however, it was significantly lower than in protocols 5 and 6. Finally, because 40% NaOH caused drastic changes in the colour and shape of polyethylene fibers (Fig. 6) and because of its low digestion efficacy, even with SDS, NaOH is not recommended for digesting vegetal material.

The strong base, KOH, was also investigated. Foekema et al. (2013) used 10% KOH to dissolve the contents of fish stomachs, intestines and esophagus. To completely dissolve the organic material, 2 or 3 weeks of incubation time was necessary (Foekema et al., 2013; Lusher et al., 2016; Tanaka and Takada, 2016). This protocol was then modified and adapted by Dehaut et al. (2016), who shortened the incubation time to 24 h and applied higher temperatures (60 °C). They obtained high digestion efficacies, ranging from 99.6% to 99.8%, when applying this protocol to mussels (*Mytilus edulis*), velvet crabs (*Necora puber*) and black seabreams (*Spondyliosoma cantharus*) tissues. They also proved that KOH had no detrimental effects on plastic polymers except cellulose acetate, which was altered in shape and size after every digestion protocol tested (Dehaut et al., 2016). Similar results were found by Kühn et al. (2017). They confirmed the resistance of most plastic polymers to KOH, with the exception of cellulose acetate from cigarette filters, some biodegradable plastics, and a polyethylene sheet. The 10% KOH method described by Dehaut et al. (2016) has been used to digest organic matter in fish gastrointestinal tracts and in shellfish tissues (Rochman et al., 2015; Štindlová et al., 2017). Here we use the protocol modified by Dehaut due to the shortened digestion time and high digestion efficiencies obtained. Long exposure of 2 or 3 weeks to 10% KOH could improve the digestion of vegetal organic matter, but it is not suitable for large-scale beach monitoring programs due to the time needed to process the samples. Protocol 3 (10% KOH) appeared promising before testing (Lusher et al., 2017), but when applied to algae and plant material, its digestion efficacy was found to be lower (24 ± 2.8%) than reported previously for animal tissues and for the gastrointestinal tract of fish. As has been mentioned previously, this could be due to the different composition of plant and algae material, since they contain cellulose, hemicellulose, and lignin that are more resistant to 10% KOH.

In summary, protocols 1 to 4 were inefficient in digesting vegetal material and were not considered further.

Oxidizing treatments using hydrogen peroxide (H_2O_2) have been widely used in microplastics studies (Avio et al., 2015; Dubaish and Liebezeit, 2013; Güven et al., 2017; Liebezeit and Dubaish, 2012; Majewsky et al., 2016; Mathalon and Hill, 2014; Mintenig et al., 2017; Nuelle et al., 2014; Tagg et al., 2015; Zhao et al., 2017). Some of these studies (Liebezeit and Dubaish, 2012; Mathalon and Hill, 2014; Tagg et al., 2015) obtained high efficacies using H_2O_2 to digest biogenic and organic matter without altering the microplastic polymer chemistry. Nuelle et al. (2014) compared different solvents (H_2O_2, HCl and NaOH) to digest biogenic matter of animal and plant origin. In their studies, samples of organic matter and microplastics, were subjected to 4 ml of different solvents (30% H_2O_2, 35% H_2O_2, 20% HCl and NaOH (20, 30, 40 and 50%)) for 7 days. Results showed that with NaOH and HCl solutions none of organic particles had dissolved completely or became transparent. However, both 30% H_2O_2 and 35% H_2O_2 solutions engendered visible changes in organic particles, mostly of animal origin. According to Nuelle et al. (2014), after 7 days 35% H_2O_2 treatment, 92% of the biogenic material had been dissolved completely or had lost

its colour. As a result, this digestion procedure was considered safe for plastic polymers. This method may be promising for digesting organic matter of plant origin. However, we aim to improve it by finding a method that, in addition, reduces sample processing time.

Avio et al. (2015) tested two methodologies using H_2O_2 (Avio's protocols 4 and 6) for extracting microplastics from the gastrointestinal tract of the fish mullet (*Mugil cephalus*). Avio's protocol 4 was based on 7 days digestion of dried samples in 30% H_2O_2, and Avio's protocol 6 was a new method based in a density separation with NaCl 1.2 g/cm^2 followed by digestion of organic matter with 15% H_2O_2. They obtained extraction efficiencies of 70% for the 30% H_2O_2 and 95% for the new method. To validate the new protocol polyethylene and polystyrene particles were analyzed by FT-IR before and after the extraction procedure. Their results have confirmed that microplastics were efficiently extracted without any damage to the polymers (Avio et al., 2015). These findings cannot be compared with our observations, because they did not report the digestion efficiency data. Furthermore, we used the (WPO) method that, in addition to the 30% H_2O_2-based digestion, employs 0.05 M Fe (II) as a catalyst. Masura et al. (2015) recommend this method as suitable for determination of polyethylene, polypropylene, polyvinyl chloride, and polystyrene in organic-matter rich samples from water, beach sediments and bed sediments. Recovery rates or digestion efficiencies were not reported. Here, we tested this protocol because it reduced processing time and had been used successfully, previously, to digest vegetal organic matter from beach samples (Masura et al., 2015), organic debris from water samples (Free et al., 2014; Masura et al., 2015; McCormick et al., 2014) and wastewater (Sutton et al., 2016). Masura et al. (2015) described a complete procedure to follow, from which we only selected and tested the part corresponding to the WPO, since our objective was to test the digestion of the organic material. The complete protocol might be more efficient than found here.

Dyachenko et al. (2017) test the effectiveness of the catalytic WPO procedure on non-plastic contaminants, such as human hair, cotton clothing fibers, cigarette filters, and toilet paper fragments, that are commonly found in wastewater. None of the contaminants analyzed, because they were composed by cellulose fibers, were digested by the catalytic WPO method. Here, among the five digestion protocols tested, protocol 5 (WPO), yielded the highest digestion efficacy, with values of 64.6 ± 7.1%. However, a high remnant of biogenic material was observed (Fig. 4b), probably due to the fact that cellulose and other compounds were not digested, as has been demonstrated by Dyachenko et al. (2017). These investigators proposed an optimized WPO method by performing a sequence of catalytic WPO. After each digestion cycle the solution was filtered through a 0.125 mm sieve and then rinsed with hexane (HPLC grade) three times followed by a rinse with deionized H_2O. The optimized WPO method could probably improve digestion of plant organic matter, but it would also require more processing time, which is a potential difficulty for the analysis of a large number of samples.

Proteinase-K treatment was 97% effective in digesting the plankton and did not damage the microplastics (Cole et al., 2014). A recent study found digestion efficacies of 88% in animal tissues using trypsin (Courtene-Jones et al., 2017). Enzymatic methods were not included in the present work because of their high price. However, they apparently do not harm microplastics and yield high digestion efficacies (Cole et al., 2014; Courtene-Jones et al., 2017; Lusher et al., 2017), but processing many samples using an enzyme approach would not be cost-effective. Nevertheless, cellulase might be a suitable alternative for the digestion of algae and vegetal material in case none of the other methods proved sufficiently effective.

Finally, the novel methodology based on density separation, tested as protocol 6, succeeded in isolating the microplastics except for the polystyrene and polyurethane foams (EPS, XPS and PUR). They were recovered by visual detection and physical removal. This protocol did not damage any type of plastic and, in addition, was inexpensive and required less time than the other protocols. In addition, according to chemical resistance chart (Thermo Scientific Nalgene, 2018), 96% EtOH at 20 °C does not cause damage to most plastic polymers after 30 days of exposure. Damage was only reported in polyethylene terephthalate copolymer (PETG) and the Flexible PVC after 7 days of exposure. Therefore, these polymers should not be affected by a brief exposure of < 10 min. Immediate damage occurs only in polymethyl methacrylate (acrylic) (PMMA) and styrene acrylonitrile (SAN), but these polymers are rarely found in marine debris. Other chemical resistance charts (Bürkle GmbH, 2018; Curbell Plastics, 2013) showed a partial resistance of PE and PC to 96% EtOH exposure at 20 °C, but not one of them report the exposure time.

Furthermore, the 96% EtOH protocol was safe and did not require any specific equipment, protocol 6 was therefore, considered the best option for the extraction of microplastics from vegetal-rich samples. The density separation methods currently used are based on solutions with higher density than most plastics polymers (NaCl (1.2 g/cm^3), NaI (1.6 g/cm^3), $ZnCl_2$ (1.6 · 1.7 g/cm^3)), these solutions are not suitable for the separation of organic matter, because they float together with plastics. This is why this method is proposed. It consists of using a 96% EtOH solution that is less dense than the density of most plastics, but denser than biogenic material of plant origin. This difference allows plastics to sink and organic matter to float, making their separation easy. This method is not effective in separating the sediment from the plastic, because both have a higher density than EtOH. This means that the 96% EtOH method should be used after separating the sediments by density using NaCl, NaI or $ZnCl_2$. The review of analytical techniques for quantifying microplastics in sediments, published by Hanvey et al. (2017), shows the importance of performing a matrix removal step. The authors indicate that organic matter is ubiquitous in sediment samples, however matrix removal was carried out in only 5 of 43 microplastic studies that they listed. According to the results obtained here, we recommend including a density separation step using 96% ethanol to remove vegetal matter. This step is suitable to be included in the protocols for extracting microplastics from beach samples, in order to harmonize the methodologies to meet the monitoring requirements of the European Marine Strategy Framework Directive (MSFD, 2008/56/EC).

Abbreviations

B	biological material
C_2H_6O	ethanol
EtOH	ethanol
EPS	expanded foam
XPS	extruded foam
HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
Fe(II)	iron (II)
HNO_3	nitric acid
CA	cellulose acetate
PE	polyethylene
PET	polyethylene terephthalate
PETG	polyethylene terephthalate copolymer
PMMA	polymethyl methacrylate (acrylic)
PP	polypropylene
PS	polystyrene
PUR	polyurethane
PVC	polyvinyl chloride
SAN	styrene acrylonitrile
KOH	potassium hydroxide
SE	separation efficacy
NaCl	sodium chloride
NaI	sodium iodide
NaOH	sodium hydroxide
SDS	sodium dodecyl sulfate

T	total dry weight
uPVC	unplasticized polyvinyl chloride
WPO	Wet Peroxide Oxidation
$ZnCl_2$	zinc chloride

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.marpolbul.2018.02.015. These data include the Google map of the most important areas described in this article.

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