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Improvement of microplastic extraction method in organic material rich samples

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ABSTRACT

Because of their small size, microplastics are available for many marine organisms. Their consumption can be hazardous and have chemical, physical, and biological effects. Nowadays, the scientific community is focused on determining the microplastics abundance and distribution, to gain a better understanding of the magnitude of this global problem. Prior to that, it is essential to harmonize sampling, extraction, and quantification methodologies in order to get reliable and reproducible data of microplastic contamination. Several methodologies have been described for the digestion of animal tissue and zooplankton within microplastic samples, but methodologies for digesting algal and vegetal material are not well known, although this material is abundant in sediment samples (e.g. beach sediment samples). The aim of this study was to determine which digestion protocol was more efficacious at digesting vegetal and algal material, defining then a standard digestion procedure for the extraction of microplastics from algae and vegetal material rich samples. Not only digestion efficacy, but the potential support of density separation steps, and the safety of the plastic particles during the digestion procedure, were examined. Among five described digestion methodologies, using HCl, NaOH, KOH and H_2O_2, the protocol based on Masura et al (2015) methodology, was selected as the most promising procedure for the extraction of microplastics from algae and vegetal rich samples.

Key words: Microplastic, algae, vegetal debris, extraction, digestion, efficacy.

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Abbreviations and acronyms

CA	Cellulose acetate
%De	Digestion efficacy
DW	Dry weight
DW_f	Dry weight filters
DW_{fad}	Dry weight filter after filtration
Fe(II)	Iron (II)
FT-IR	Fourier Transform Infrared
HCl	Hydrochloric acid
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
KOH	Potassium hydroxide
NaCl	Sodium chloride
NaI	Sodium iodide
NaOH	Sodium hydroxide
PA	Polyamide
PC	Polycarbonate
PE	Polyethylene
PET	Polyethylene terephthalate
PCB	Polychlorinated biphenyl
POP	Persistent organic pollutant
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinyl chloride
PUR	Polyurethane
SDS	Sodium dodecyl sulfate
WPO	Wet Peroxide Oxidation
$ZnCl_2$	Zinc chloride

1. INTRODUCTION

1.1. Marine plastic debris. Sources and impacts of microplastics

Plastics are synthetic organic polymers, very resistant, lightweight, with great versatility, and with low production costs, what nowadays make them the perfect packaging material. They are produced in higher rates every year, as the plastic industry keeps growing. In 2014, for instance, the global production of plastics reached 311 million tons (PlasticEurope (PEMRG), 2015). As they degrade very slowly and persist for centuries, they are becoming a huge problem contaminating not only the land, but many freshwater ecosystems (Eerkes-Medrano et al. 2015; Fischer et al. 2016) and the oceans too. Jambeck et al. (2015) estimated that, just in 2010, from the 275 million tons of plastics produced in 192 coastal countries, between 4.8 and 12.7 million tons of them entered the ocean. There is so much plastic in it that, in some areas, the recorded mass of neustonic plastic debris was six times higher than the mass of zooplankton, in the samples collected (Moore et al. 2001).

Plastic litter in the oceans come from two main sources (Coe & Rogers 1997): it has been estimated that land-based sources are responsible for about an 80% of the plastic debris in the oceans, while the fishing industry accounts for about another 18% (Andrady 2011). Main sources for microplastic debris have been also described, categorized as waste management, ocean dumping, effluents, and litter from land (Arthur & Baker 2011). They usually float but, depending on their composition, plastics can be denser than water and be neutrally-buoyant, or sink to the sediments (Andrady 2011).

Microplastics are plastic particles with sizes up to 5 mm (Arthur et al. 2009; Andrady 2011; Fendall & Sewell 2009; Moore 2008; Thompson et al. 2004), and above 333 μm. Although the lower limit size for microplastics has not been defined, 333 μm is commonly used, as it is the usual neuston net mesh (Arthur et al. 2009).

Primary microplastics are produced specifically with these sizes, and they are, among others, exfoliants and scrubbers, microbeads, synthetic clothing fibres, plastic pellets, and airblast cleaning media. Secondary microplastics originate from the degradation of larger plastic items (Cole et al. 2011; Arthur et al. 2009). Plastics break down in the oceans much more slowly than they do on land, and related to the marine environment, the most probable site for weathering of plastic litter and generation of microplastics is the beach (Gregory & Andrady 2003). Microplastics are widespread worldwide, but they tend to accumulate along coastlines and within mid-ocean gyres (Moore et al. 2001).

Because of their small size, microplastics are available for many marine organisms, that can ingest them directly from the water or by feeding on contaminated prey (Setälä et al. 2014; Barnes et al. 2009). How they impact on the wildlife is still not well understood,

but their consumption seems to be hazardous and has chemical, physical, and biological effects (Wright et al. 2013; Setälä et al. 2014).

Microplastics, besides its inherent additives and toxic plasticisers, adsorb and accumulate persistent organic pollutants (POPs), which are present in sea water at very low concentrations, but have high affinity for plastics. The POPs, hydrophobic and with long half-life, concentrate on microplastics surface and, when consumed by organisms, may start disassociating from the microplastics. Due to its lipophilic nature, they can accumulate in the fatty tissue of organisms, being biomagnified as they move up the food-chain. These chemicals can penetrate into the cells and disrupt the endocrine system too (Teuten et al. 2009). Example of man-made POPs are polychlorinated biphenyls (PCBs), which have been reported to have a wide range of biological effects (Neal 1985).

Physical effects include damage of the feeding appendages and digestive tract, physical blockage, and even translocation of microplastics into the circulatory system. Although some of these effects do not show any lethal impact in the short term, they certainly have negative impacts in the organisms (Von Moos et al. 2012; Köhler 2010; Browne et al. 2008). Additionally, there is a biological risk related to microplastics, not involved with ingestion, that lies on their function as vectors for potential pathogens or harmful microorganisms able to live on the plastics' surface (Osborn & Stojkovic 2014; Zettler et al. 2013).

Even though education is the best way to battle the increasing marine pollution, and more strict laws should be implemented to protect the oceans, nowadays, the scientific community is focused on determining the microplastics abundance and distribution, to gain a better understanding of the magnitude of this global problem. Prior to that, it is essential to harmonize sampling, extraction and quantification methodologies in order to get reliable and reproducible data of microplastic contamination in marine biota.

1.2. Importance and current status of sampling, extraction and quantification methodologies

There are various sampling techniques that allow us to obtain microplastic samples from different environments (Hidalgo-Ruz et al. 2012). Among them:

- Sediment sampling provides benthic samples (e.g. from beaches), from which the plastic particles can be separated via flotation (Thompson et al. 2004).
- Several marine trawls and nets can be used to collect floating microplastics from sea surface and mid-water transects. Bulk sea water samples can be collected in bottles too. In all cases, microplastics must be concentrated, and this can be done by evaporation (Yamashita & Tanimura 2007), sieving, or filtration (Song et al. 2014; Masura et al. 2015; Hidalgo-Ruz et al. 2012).

After sampling, extraction of microplastics must be done, since other materials, besides plastics, might have been collected (glass, wood, organisms, sand particles, detritus…), and all this non-plastic material could mask the presence of microplastics. The most used extraction technique, based on density separation via flotation, was developed by Thompson et al. (2004) and has been improved by other researchers (Claessens et al. 2013; Imhof et al. 2012). Density separation techniques need high density solutions, like NaCl (1.2 kg L^{-1}), NaI (1.6 kg L^{-1}) and $ZnCl_2$ (1.6-1.7 kg L^{-1}), since the specific density of plastics ranges from 0.8 kg L^{-1} to 1.56 kg L^{-1}. Other strategies for the separation of microplastic particles include filtration, sieving or visual sorting (Hidalgo-Ruz et al. 2012).

After extraction of microplastics, subsequent identification and quantification might be followed, using optical microscopy, electron microscopy, Raman spectroscopy or FT-IR (Fourier Transform Infrared) spectroscopy (Andrady 2011). Lipophilic dye (e.g. Nile Red) can be used to stain microplastics and visualize them using optical microscopy and even flow cytometry (Arthur & Baker 2011). Microplastics are described using several categories, including source, type, shape, erosion and colour (Hidalgo-Ruz et al. 2012).

1.2.1. Microplastics extraction in organic material rich samples

Biological material present in the samples needs to be treated differently in order to be eliminated, since it often has low density and floats along with the microplastics, and numerous digestion procedures have been described to achieve this goal. However, it is important to find a method that has maximum efficacy digesting the organic material but does not damage the microplastics, since they will be quantified and measured after the extraction. The types of plastics considered for validation in different microplastic extraction methodologies vary among studies. Some authors include just a small range of plastics, like polystyrene (PS) and nylon fibres (Claessens et al. 2013), or polyethylene (PE) and polystyrene (Avio et al. 2015); others expand it to five or seven families of plastics (Cole et al. 2014; Collard et al. 2015), and authors like Dehaut et al. (2016) evaluate up to 15 types, including polyethylene (PE), polyamide (PA), polycarbonate (PC), cellulose acetate (CA) and polypropylene (PP). These plastic families are chosen due to their high production worldwide, or because they are commonly encountered in the oceans.

Several methodologies have been described for the digestion of animal tissue and zooplankton within microplastic samples, but methodologies for digesting vegetal and algal material are not well known, although this material is abundant in sediment samples (e.g. beach sediment samples), and it has been proven that some seaweeds can retain suspended microplastics on their surfaces (Gutow et al. 2015).

Hidalgo-Ruz et al. (2013) reviewed some studies suggesting sieving or visual sorting and separation of dried algae and seagrasses among other residues present in the

microplastic samples, done by the naked eyed or with the aid of a microscope. Although these are good ways to get rid of the bigger fragments and pieces of algae and leaves, these procedures are time consuming and might cause a loss of microplastics.

Claessens et al. (2013) developed two methods for the extraction of microplastics from sediments and biota samples. The first method, for sediments like sand particles and heavy materials, used an upward water flow in an elutriation tube, and flotation, to separate the microplastics. The second methodology describes a digestion procedure for animal tissue. Applied to mussels, they learned that the most efficient digestion solution, among nitric acid (22.5 M HNO_3), hydrogen peroxide (32.6 M H_2O_2) and sodium hydroxide (52.5 M NaOH), was nitric acid (22.5 M HNO_3), in an overnight destruction of the organic matter at room temperature, followed by 2 h of boiling and warm filtration. This acidic digestion technique resulted in high extraction efficiencies, but when tested for polystyrene spheres and nylon fibres, the treatment damaged some of the microplastic, and nylon rope fibres could not be recovered. More authors (Dehaut et al. 2016; Avio et al. 2015) have obtained negative results for HNO_3 digestion too, as it always seems to damage the microplastic content of the samples. For this reason, although several nitric acid methods were recently used and recommended by other researchers (Van Cauwenberghe & Janssen 2014; Vandermeersch et al. 2015; De Witte et al. 2014), they were discarded and were not tested in this work.

An alternative to strong acids digestion is the use of non-oxidizing acids or alkaline hydrolysis. Cole et al. (2014) developed and optimized a protocol for digesting biological material with no microplastic destruction, in which they compared the use of acid (HCl), alkaline (NaOH) and enzymatic (Proteinase-K) digestion treatments on plankton-rich sea water samples and several types of microplastics (polyethylene, polyester, nylon, polystyrene and uPVC). Among different procedures and digestion solutions (1 M and 2 M HCl; 1 M, 2 M, 5 M and 10 M NaOH; and an adapted enzymatic digestion protocol from Lindeque & Smerdon (2003)), the best results were obtained for the optimized proteolytic enzyme treatment, having a digestion efficacy above 97% and showing no damage in any microplastic tested. Proteinase-K would not be effective in the digestion of algae or vegetal tissue, but the hydrochloric acid (1 M HCl) and sodium hydroxide (10 M NaOH) digestion methods were selected as potential procedures for the digestion of algae and vegetal material present in microplastic samples. It is fundamental to check on microplastics resistance to the digestion procedure, since not only acids but strong bases too, could damage and discolour several types of plastics (nylon, polyethylene, uPVC, cellulose acetate, polycarbonate) (Cole et al. 2014; Dehaut et al. 2016).

In addition to NaOH, the strong base potassium hydroxide (KOH) was selected for experimentation. Foekema et al. (2013) used 10% KOH to dissolve stomach, intestines and esophagus content at room temperature for 2 or 3 weeks. The modified protocol (Rochman et al. 2015; Dehaut et al. 2016) shortened the incubation time, using 10% KOH solution

with 24 h or overnight incubation at 60 ºC, and had a digestion efficacy in seafood products ranging from 99.6% to 99.8%. It also proved no detrimental effects on the tested plastic polymers except for cellulose acetate, which is actually a natural polymer, and which suffered modifications in shape and size after every protocol tested (Dehaut et al. 2016).

Oxidizing treatments using hydrogen peroxide are utilized by different authors, obtaining diverse results. Some referred to 30% and 35% H_2O_2 as the ideal solution to dissolve biogenic and organic matter without alteration of polymers chemistry, but others found it inefficient, as it resulted in an incomplete removal of the biological material and, sometimes, even significant losses of microplastics (Nuelle et al. 2014; Tagg et al. 2015; Liebezeit & Dubaish 2012; Mathalon & Hill 2014). According to Nuelle et al. (2014), diverse types of microplastics apparently suffer size reduction when subjected to 30% H_2O_2, but Masura et al. (2015) also used H_2O_2 in their methodology, described for a range of plastics including polyethylene, polypropylene, polyvinyl chloride, and polystyrene, without any deleterious effect on them. Masura et al. (2015) oxidizing method was selected for the present work, since it is the only protocol that could be found specifically described for beach sediment samples with algal and vegetal material on them. It is based on a wet peroxide oxidation (WPO) using hydrogen peroxide (30% H_2O_2) and a Fe(II) catalyst to digest labile organic material, followed by a density separation and a microscope exam. Besides the method for the analysis of microplastics in beach samples, they also described two other methodologies for the analysis of microplastics in water samples and bed samples.

Enzymatic methods were not included in the present work because of their high price. Although they apparently do not harm microplastics and yield high digestion efficacies (Cole et al. 2014), processing a large number of samples using an enzyme approach would not be cost-effective. Cellulase might be a suitable alternative for the digestion of algae and vegetal material in case none of the methods above is efficacious enough.

2. OBJECTIVES

To eliminate the organic material present in microplastic samples, and correctly extract the plastic particles, a digestion procedure must be followed. The aims of the present work were to determine which method, among the existing digestion protocols, is more efficient at digesting vegetal and algal material, and to define a standard digestion procedure for the extraction of microplastics from algae and vegetal material rich samples.

In order to achieve these goals, two objectives were defined:

1. To evaluate the digestion efficacy of five existing protocols in the digestion of algae and vegetal material, including acid (HCl), alkaline (NaOH and KOH), and oxidizing (H_2O_2) treatments.
2. To test the resistance of six types of plastics to the chemicals, temperatures, and exposure times used in the previous digestion protocols.

3. MATERIALS AND METHODS

3.1. Biological material

Algae and vegetal material, like leaves and wood pieces, comprise the most common type of organic debris found at the beaches of the Canary Islands (Herrera, A., personal communication). Sediment samples collected in the tide line at Famara, Las Canteras, and Playa del Ámbar beaches, contain much of this type of material, which is very difficult to separate from microplastics, since they have similar low density and float together.

In this work, all algal and vegetal material used in the experiments was collected at Famara beach, in Lanzarote, Spain, between January and February of 2016. It was transported to EOMAR group facilities, at the University of Las Palmas de Gran Canaria, where it was washed with mili-Q water to remove salt and sand, and stored at -20 ºC. Prior to the experimentation, algae and vegetal material were oven-dried in glass Petri dishes at 60 ºC for at least 24 h, and the desiccated material was weighed and divided into 0.2 g dry weight (DW) sub-samples.

Identified species included the algae, *Caulerpa sp.* and *Sargassum sp*, and the seagrass, *Cymodocea nodosa*. Other vegetal material like leaves and wood pieces could not be identified.

3.2. Plastic material

The six major classes of plastic polymers that stand out in terms of their market share are called the “big six”. They are, ordered by European plastics demand, polypropylene (PP), polyethylene (PE), polyvinyl chloride (PVC), polyurethane (PUR), polyethylene terephthalate (PET), and polystyrene (PS) (PlasticsEurope 2015). The types of plastics that are most frequently recovered from the marine environment, among others, are PE, PP, PVC, PS, polyamide (PA) or nylon, and polyester (Hidalgo-Ruz et al. 2012; Andrady 2011).

Based on this, six types of plastics varying by polymer composition, colour, size, and shape, were selected for method validation (Table 1). These plastic polymers were polyethylene (PE), polypropylene (PP), polystyrene (PS), polyamide (PA), polyester and resin pellets. Resin pellets were included, although their composition was unknown, because they are very abundant in Canary Island waters and might have future importance in the study of marine debris (Herrera, A., personal communication).

Polymer type	Colour	Size (mm)	Original product	Source
PE	Blue	1.50-3.48	Lid of a water bottle	Supermarket
PP	Green, orange	1.51-4.52	Trays	Lab inventory
PS	White	1.8-4.55	Packaging	Lab inventory
PA	Green, orange, blue	6.84-16.50	Fishing nets	Beach sample
Polyester	Dark blue	0.5-8	Textile fibres	Blanket
Resin pellets	White, transparent	4.42-6.12	Resin pellets	Beach sample

Table 1. Polymer types, colours, sizes (mm), original product and sources used for method validation experiments.

Although some of the plastic particles used for methods validation were above the 5 mm limit that defines the microplastics, this simply facilitated the measurement and recovery of the particles and made it easy to observe changes in them.

3.3. Digestion solutions

Acid, alkaline, and oxidizing solutions were prepared in the laboratory facilities and stored at 8 ºC or at room temperature, until utilized in the digestion treatments. Since the authors of the tested protocols used different concentration units, and this might result in confusing and complicated prospective comparisons, original units have been annotated by their equivalent molar concentration in brackets.

Solutions prepared and used in this work were:

- 1 M HCl
- 10 M NaOH
- 10% KOH w/v (1.78 M KOH)
- 1 M NaOH
- SDS 5 g/l (17.34 mM SDS)
- 30% H_2O_2 w/w (9.79 M H_2O_2)
- 0.05 M Fe(II) (7.5 g of $FeSO_4 \cdot 7H_2O$; 500 ml of H_2O; 3 ml of H_2SO_4)
- NaCl 37 g/l (0.63 M)

3.4. Mitigating contamination

Usually, when researching microplastics and analysing field samples, control samples and steps to prevent external contamination of microplastics are needed, since there are many microplastic sources in any laboratory, specially textile fibres, that could obscure the real quantities of microplastics in the samples (Foekema et al. 2013; Rochman et al. 2015; Claessens et al. 2013; Hidalgo-Ruz et al. 2012). However, in this work, we did not follow mitigation steps, except for acid-washing all the containers and equipment, because microplastic contamination was not relevant in the digestion of biological material (first

objective), or in the validation of the techniques (second objective), as the number, size and type of microplastics in the samples were known.

3.5. Digestion procedures

To compare the efficacy of the selected methodologies in the digestion of algae and vegetal material, 0.2 g DW sub-samples were added to 40 ml of each digestion solution in glass flasks, and maintained at the protocol specific temperature for 24 h. Then, the resulting mixtures were diluted (1:5 v/v) with filtered deionised water, and vacuum-filtered through filter papers (FILTER-LAB, Filtros Anoia, S.A.) with a pore size of 30-50 μm. Filter papers were previously dried at 60 ºC for at least 24 h, and weighed before the experiments. After filtration, filter papers with the remaining biological material were folded, placed into glass Petri dishes, oven-dried at 60 ºC for at least 72 h, and weighed. Each experiment comprised six replicates. All experiments were photographed at t_1 and at t_2, that is, before and after the digestion procedure.

The procedure described above applies only to protocols 1 to 4, since protocol 5 required different proceedings.

Protocol 1 and protocol 2 corresponded to Cole et al. (2014)'s acid and alkaline digestion methods, respectively. 1 M HCl, in protocol 1, and 10 M NaOH solutions, in protocol 2, were added to sub-samples and maintained for 24 h, at 20 ºC and 60 ºC, in the order described.

Protocol 3 was adapted from Dehaut et al. (2016), and consisted of alkaline sample digestion at 60 ºC for 24 h, using 40 ml of 10% KOH per 0.2 g DW sub-sample.

Protocol 4 was based on the work of Budimir (2016), presented at MICRO 2016 International Congress. In this protocol, less concentrated NaOH was added to sub-samples together with detergent sodium dodecyl sulfate (SDS). Budimir described an alkaline digestion procedure in which 10 ml of 1 M NaOH and 5 ml of SDS were added to the sub-samples, and only 2 hours at 50 ºC were needed to digest the biological material in the samples. The original protocol was modified by adding 40 ml of NaOH and 20 ml of SDS to every 0.2 g DW sub-samples instead. Also, since no visual changes were observed in the samples after 2 h, they were maintained for 24 h at 60 ºC. This was done in order to standardize all the procedures followed in this work.

Protocol 5 was based on the Wet Peroxide Oxidation (WPO) described by Masura et al. (2015). Despite their describing several steps for the analysis of microplastics on beach sediment samples, only their WPO step was carried out in this work. In brief, 20 ml of aqueous 0.05 M Fe(II) were added to a large beaker (~800 ml) containing 0.2 g DW sub-sample, followed by 20 ml of 30% H_2O_2. After five minutes of resting at room temperature, the mixture was heated to 75 ºC on a hotplate for at least 30 minutes. If heated >75 ºC the

solution could boil violently. Since this is a highly reactive mixture, laboratory safety practices and policies should always be carefully followed when handling this mixture. If biological material remained in the mixture after that time, another 20 ml of hydrogen peroxide were added, repeating until no organic material was visible. In this work, additional hydrogen peroxide was added to the samples up to three times, but to get rid of the thicker pieces seven times were needed. As in the previous protocols, samples were filtered through filter papers and oven-dried at 60 ºC.

3.5.1. Changes in sample density and buoyancy

A small experiment was developed to evaluate if changes in the density of the samples occurred during the digestion procedure, along with variations in their buoyancy. If that occurred and if the digestion of the organic material was not 100% effective, microplastics could still be separated from the remaining biological material via flotation in saline solution.

Five samples (S1-S5) were subjected respectively to the five digestion protocols mentioned above. All samples were placed in a sodium chloride solution before digestion (t_1) and after digestion (t_2), and photographs were taken at t_1 and t_2 to compare their flotation properties. Only those protocols that modified the density of the samples and made all the remaining material sink, were suitable for this density extraction approach. A sodium chloride solution (NaCl 37 g/l) was used at sea water concentration (Costa et al. 2010).

3.6. Efficacy of digestion

The efficacy of a digestion protocol depends on the relative removal of organic mass during the digestion procedure. Digestion efficacies were calculated using a modified version of Dehaut et al. (2016)'s equation (1), where *%De* was the digestion efficacy, DW_f and DW_{fad} were equivalent, respectively, to the dry weights of the filter papers before filtration and the filter papers covered by the remaining biological matter after filtration. Finally, T_w corresponded to the exact weight of biological material subjected to each digestion, i.e. the DW of the sub-samples.

$$\%De = 100 - \left(\frac{DW_{fad} - DW_f}{T_w} \times 100\right) \qquad (1)$$

Digestion efficacies were compared among protocols in order to find the protocol with the highest efficacy in removing algae and vegetal material. This measurement was always accompanied by images to verify the absence of organic debris and remaining tissue in the samples.

3.7. Methods validation

Every digestion protocol that was tested on biological material, was tested as well on plastic particles for methods validation, regardless of its digestion efficacy in the algal and vegetal samples. For every plastic family, three replicates of five units of plastic were subjected to the digestion protocols.

Microplastics were photographed before and after the experimentation (t_1 and t_2, respectively), and images of microplastics were compared among each other looking for changes in colour, number, and shape, to determine potential deleterious effects of digestion procedures (Dehaut et al. 2016; Cole et al. 2014; Nuelle et al. 2014). Microplastics were photographed using a stereomicroscope (Optika SZM-LED2; 0.7x magnification) and a digital camera (Canon Powershot D30). Using the software ImageJ 1.50b, microplastics colour histograms were generated. Since a colour image is a digital array of pixels containing colour information, each of these images can be decomposed into three different layers according to the three colour channels encoded: Red, Green and Blue (RGB) (Coste, 2012). A colour histogram is actually composed of three independent grayscale histograms, one each for the R, G and B channels, and they represent the pixels distribution over the grayscale intensity levels from 0 to 255 of each colour. Black colour is represented by (0, 0, 0) and white colour by (255, 255, 255).

Protocols were qualified as valid only when there was no microplastic type damaged during the digestion process.

3.8. Statistical analysis

Statistical analysis of digestion efficacies results were performed with R Statistics 3.3.0 software and the extension R studio.

Since digestion efficacy was a quantitative, continuous variable, the data had to be tested to confirm its normal distribution. Normality of the digestion data was examined using a Shapiro-Wilk test, since our sample size was less than 50. Q-Q plots and histograms were represented too, in order to support the previous test results.

Since the assumption of normality was rejected, non-parametrical Kruskal-Wallis test was utilized to look for significant differences among the protocols extraction efficacies, with statistical significance attributed where $P < 0.05$. Tuckey and Kramer (Nemenyi) post hoc analysis was used to examine differences between pairs of groups, and to identify which protocols were significantly different from the others. The obtained results were represented in box plots.

4. RESULTS

4.1. Digestion procedures

4.1.1. Digestion efficacy

The *%De* values of the treatments ranged from 45 to 100% (means and standard deviation are presented in Table 2). Protocol 1, protocol 3 and protocol 4 presented the lowest yields at digesting algae and vegetal debris, with mean *%De* ranging from 57.34 to 58.82%. Protocol 2 showed a mean *%De* of 61.88% and a high standard deviation. Conversely, protocol 5 revealed the highest values, with an average *%De* of 96.51 ± 4.64%.

Digestion procedure	Digestion solution	Mean digestion efficacy
Protocol 1	HCl	58.83 ± 4.81%
Protocol 2	NaOH	61.88 ± 15.07%
Protocol 3	KOH	57.34 ± 3.85%
Protocol 4	NaOH+SDS	57.88 ± 4.87%
Protocol 5	H_2O_2	96.51 ± 4.64%

Table 2. Digestion efficacies (*%De*) of the protocols in algae and vegetal samples. Digestion protocols; digestion solutions; means and standard deviation (mean ± SD) of the digestion efficacies.

Visual examination of all the samples at t_1 and t_2 (Figure 1) revealed qualitative differences especially among protocol 5 samples, at pre- and post-digestion times. Material digested following protocols 1 to 4 remained almost the same, while the material exposed to protocol 5 was completely digested at t_2, in a way that biological remnants could not be distinguished in the solution, and the debris collected in the filters was negligible.

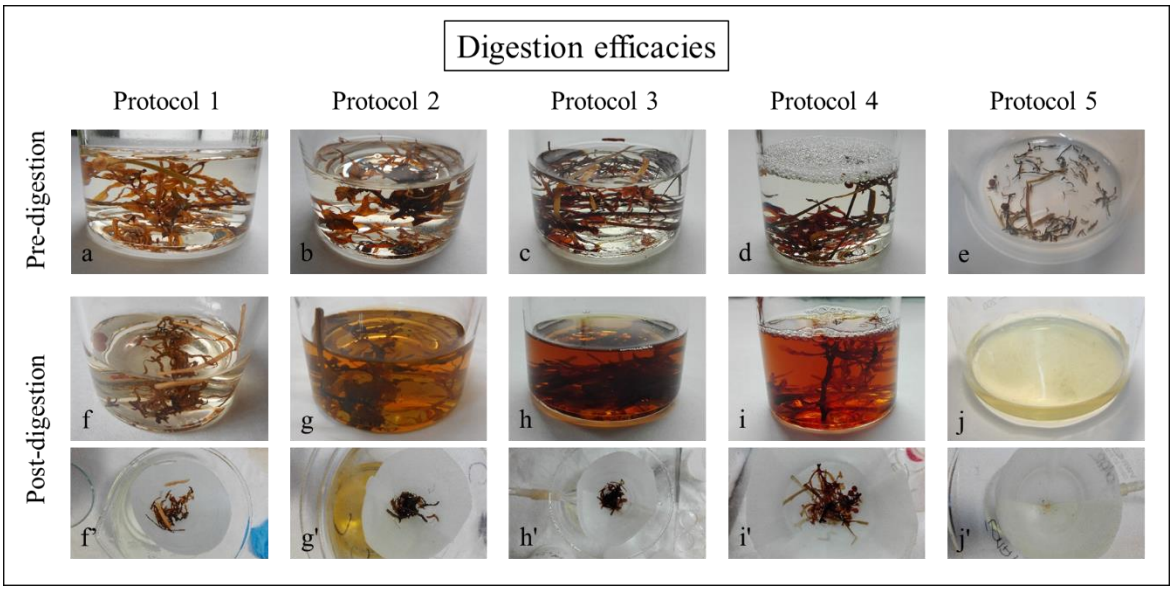

Figure 1. Results of the digestion of algae and vegetal 0.2 g DW samples. Samples are shown at t_1 and t_2, before and after the digestion: HCl protocol 1 (a, f, f'), NaOH protocol 2 (b, g, g'), KOH protocol 3 (c, h, h'), NaOH+SDS protocol 4 (d, i, i'), and H_2O_2 protocol 5 (e, j, j').

4.1.2. Statistical analysis

The non-parametrical Kruskal-Wallis test revealed significant differences among the protocols' extraction efficacies (H=14.6; 4 df; p-value=0.0069). The Tuckey and Kramer (Nemenyi) post hoc test made homogeneous groups to separate the significantly different protocols. Group "a" contained the protocol 5, while group "b" gathered protocols 1 to 4. Therefore, WPO protocol 5 yielded a significantly greater digestion efficacy than the other protocols efficacies, according to what can actually be observed in Figure 2.

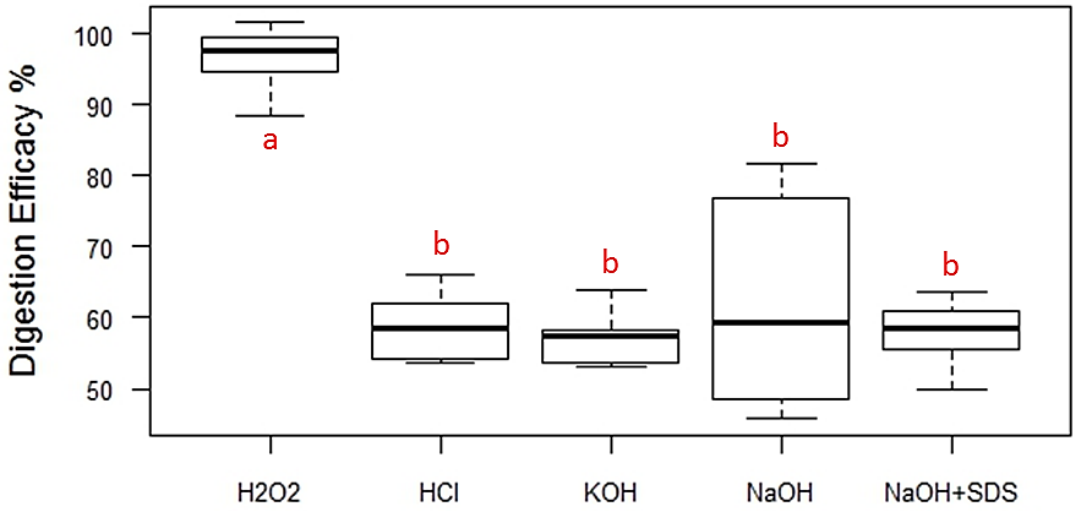

Figure 2. Digestion efficacy of the protocols in the vegetal and algae samples. Protocol 5 (H2O2) is clearly more efficient than protocol 1 (HCl), protocol 2 (NaOH), protocol 3 (KOH) and protocol 4 (NaOH+SDS). Different letters (a, b) separate homogeneous groups with significant differences between them. The central thick line of each box designate the median, the box height shows the interquartile range, and the whiskers indicate the lowest and the highest values.

4.1.3. Changes in density and buoyancy

How the density and buoyancy of the biological samples changed, depended on the digestion approaches.

Sample S1, subjected to HCl digestion, did not show any change in its density after the acid digestion, and continued to float when placed again in NaCl solution (Figure 3).

The density of all the samples that were digested following alkaline digestion protocols, that is, S2, S3 and S4, seemed to be modified during the digestion procedure, which resulted in changes in their buoyancy.

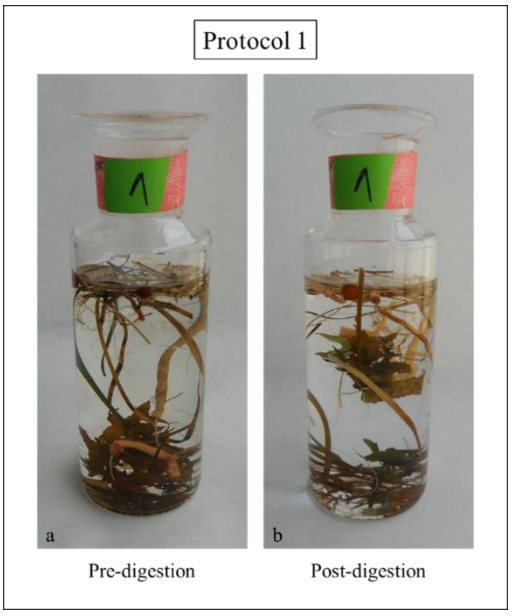

Figure 3. Buoyancy of S1 in saline solution, at t_1 (a) and t_2 (b), before and after being subjected to protocol 1 acid digestion.

Sample S2, corresponding to 10 M NaOH protocol 2, exhibited a reduction in its flotation (Figure 4). More material sank to the bottom after the digestion, comparing to t_1, but approximately half of it remained in the surface.

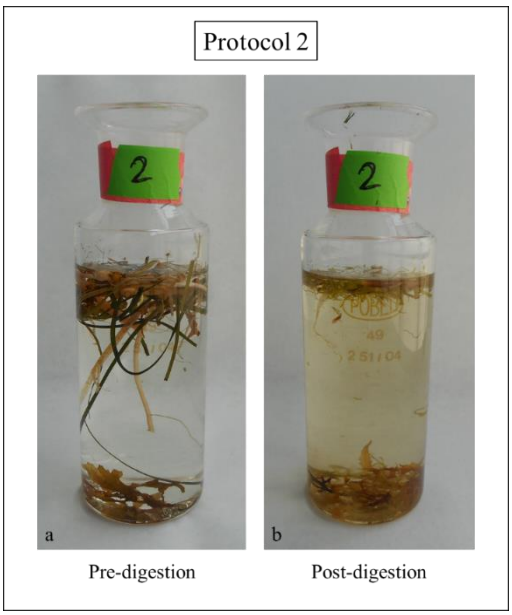

Figure 4. S2 buoyancy, at t_1 (a) and t_2 (b), before and after being subjected to protocol 2 alkaline digestion.

The digestion procedure applied to sample S3, corresponding to 1 M NaOH protocol 3, caused an increment in S3 density and a reduction in its buoyancy, so that all the remaining material in the mixture sank to the bottom when placed in the saline solution (Figure 5).

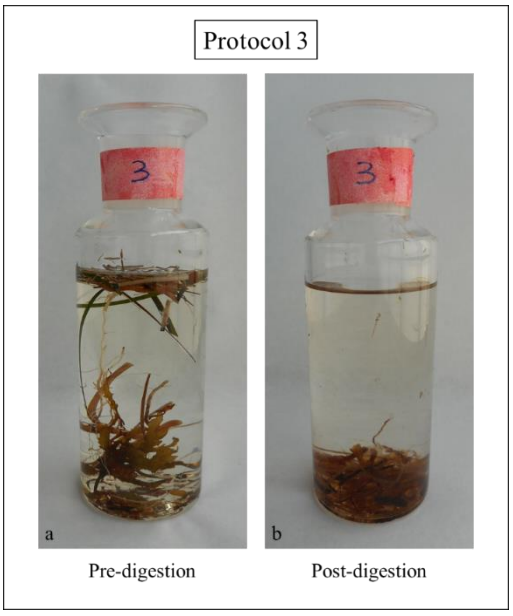

Figure 5. S3 buoyancy, at t_1 (a) and t_2 (b), before and after being subjected to protocol 3 alkaline digestion.

Sample S4, digested with KOH solution following protocol 4, displayed the same results as S3. Density was increased after the digestion protocol, and flotation decreased (Figure 6). No material remained in the solution's surface.

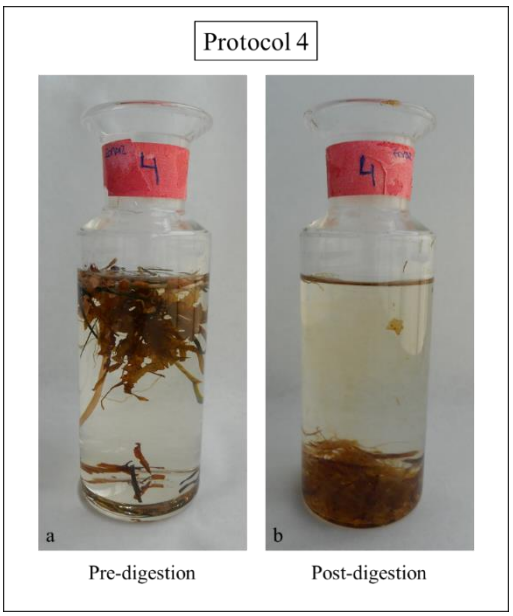

Figure 6. S4 flotation, at t_1 and t_2, before (a) and after (b) being subjected to protocol 4 alkaline digestion.

Sample S5, exposed to an oxidizing treatment following protocol 5, showed that the remaining material, although it was a small quantity, still floated in the high density solution, remaining on its surface and indicating that no changes in density or buoyancy occurred (Figure 7).

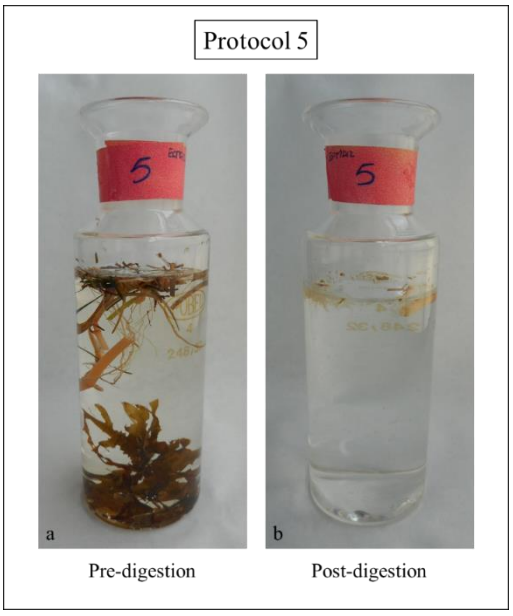

Figure 7. S5 buoyancy, at t_1 and t_2, before (a) and after (b) being subjected to protocol 5 alkaline digestion.

4.2. Impact of digestion protocols on microplastics

The results obtained after the method validation experiments are presented in the table below (Figure 8), were photographs and colour histograms of the plastic particles, at t_1 and t_2, are exposed. All plastic particles, except for polyester fibres subjected to protocol 2, were successfully recovered after the digestion procedures, at t_2, and their colours and shapes remained the same. Images presented in Figure 8 allowed to examine possible changes in microplastics shape at t_1 and t_2. Changes in microplastics colour were studied using colour histograms and their RGB mean values.

Polyester fibres subjected to protocol 2 (Figure 9) were damaged and degraded by the highly concentrated NaOH. As a result, the colour of the fibres changed from dark blue to pale red, and their shapes were altered from fibres to dust-like particles that could barely be recovered. Histogram RGB intensity values at t_2 were displaced to the right, toward lighter intensity values. RMean values rose from 73.66 to 178.19; GMean, from 92.13 to 168.37; and BMean, from 136.56 to 177.26.

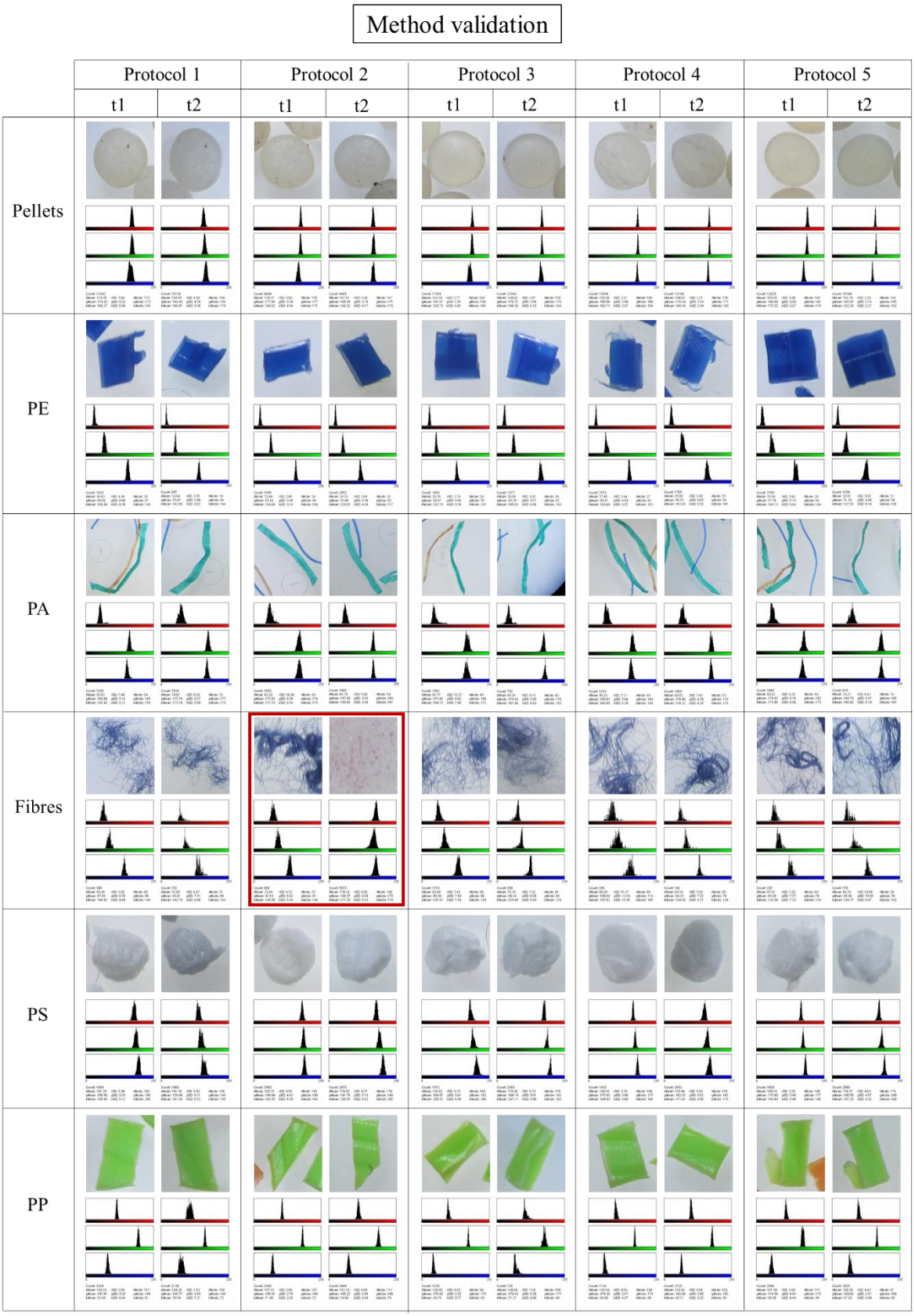

Figure 8. Method validation results. Plastics photographs of each type and tested for each protocol are presented at t_1 and t_2, accompanied by a colour histogram.

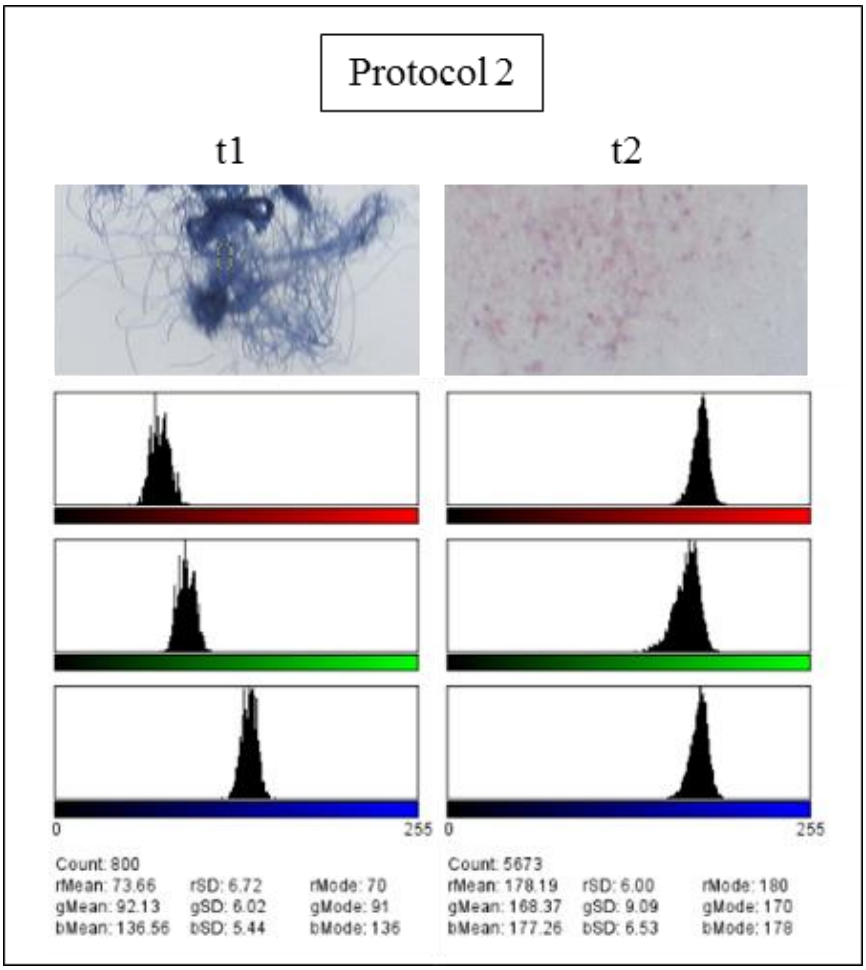

Figure 9. Photographs and colour histograms of polyester fibres at t_1 and t_2, before and after being subjected to protocol 2. At t_1, RGB mean values were: rMean = 73.66, gMean = 92.13, bMean = 136.56. At t_2, RGB values were rMean = 178.19, gMean = 168.37, bMean = 177.26.

5. DISCUSSION

After studying the protocols efficacy in the digestion of algae and vegetal material, after testing the possibility of combining a digestion method with a density separation step, and after examining the impact the digestion procedures had on the plastic particles, the best options to standardize a protocol for the digestion of algae and vegetal material, present in microplastic samples, are here discussed.

Protocols 1 to 4 displayed digestion efficacies of such low values that they should not be considered for the digestion of algae and vegetal material. Methodologies using HCl had not been efficient before, at digesting zooplankton (Cole et al. 2014), or at digesting biogenic organic matter like chitin carapaces or leaves, even at higher concentrations than the concentration used here (Nuelle et al. 2014). Methodologies using NaOH, however, showed efficacies ranging from less than 50% (Nuelle et al. 2014) to 91.3% (Cole et al. 2014) and even >99% (Claessens et al. 2013), depending on the concentration used and the procedure followed. Here, the digestion efficacy of NaOH in our samples was inconsistent and varied from 45 to 81%. The high efficacies of Cole et al. (2014) and Claessens et al. (2013) were not found. SDS detergent did not seem to contribute to the improvement of the NaOH digestion efficacy. Protocol 3 appeared promising before testing, since digestion procedures using KOH have previously displayed digestion efficacies ranging from 99.6 to 99.8% in the destruction of mussel tissues (Dehaut et al. 2016), but here, its digestion efficacy in algae and vegetal material was as low as the other protocols'.

It was considered, at the beginning of the present work, to use enzymes like cellulase for the removal of algae and vegetal material, since enzymes have been proven to be highly efficient at the digestion of biological material (Cole et al. 2014). Cole et al. (2014) certainly obtained a high digestion rate using enzymes (97%), but this might be too expensive, considering the large number of samples that usually need to be processed, and the great amount of organic debris present in the samples. Therefore, enzymatic approaches were not taken into consideration.

Among the five tested protocols, only WPO protocol 5 yielded digestion efficacy values over 95%, with an average efficacy of 96.51 ± 4.64%, and some of the replicates having values of 100%. From all the protocols using H_2O_2 as oxidizing reagent, the Masura et al. (2015) method seemed to be the most efficient one. Values of 50 and 56% had been obtained by Nuelle et al. (2014) in the digestion of <1 mm and >1 mm biogenic organic particles, respectively, but it required seven days to reach that percentage of degradation. Other authors found better results employing Nuelle et al. (2014)'s method, but they only achieved a biological material removal efficacy of 82% after seven days of treatment (Tagg et al. 2015). It must be mentioned that these procedures did not include any heat treatment, nor Fe(II) as a digestion catalyst. The main differences between the protocols of Nuelle et

al. (2014) and Masura et al. (2015), were the digestion temperature and the exposure time. While Masura et al. (2015) heated the samples at 75 ºC for 30 minutes, Nuelle et al. (2014) exposed the sample for seven days at room temperature (≈20 ºC). The differences between both protocols in the resulting digestion efficacies might be due to the reaction temperature.

Warnings of significant losses of microplastics due to boiling and gas bubbles have been notified when using H_2O_2 (Nuelle et al. 2014; Mathalon & Hill 2014), but such problems did not occur during protocol 5 development. It should be mentioned, however, that in the present study the remaining material became discoloured after H_2O_2 treatment, something Nuelle et al. (2014) actually noted. Since microplastics in marine samples are often coloured white or transparent (Hidalgo-Ruz et al. 2012), H_2O_2 could actually make the visual analysis more complex, instead of facilitating it. The possibility of combining H_2O_2 digestion protocols with density separation steps, in order to increase their extraction efficacy and dodge bleaching problems, has been considered.

Biological material present in microplastic samples, like algae or vegetal debris, normally has a low density and floats. However, its density might suffer alterations when subjected to a digestion process. The density of the non-digested, remaining biological material might change and become denser, so when placed in a high density solution, instead of floating, it could sink to the bottom of the container. Indeed, Avio et al. (2015) suggested a density separation step should always be present in every extraction methodology to also remove sediment particles, potentially present in the samples.

Protocol 1, since the density of the material did not increase during the digestion process, would not be suitable for further density separation, so its effectivity in the extraction of microplastics from the samples lies in its digestion efficacy only. Protocol 2, due to a large amount of the biological remnants staying at the surface of the saline solution, would not be convenient for further density separation of microplastics either, even though the buoyancy of the material partially decreased.

Protocol 3 and protocol 4, regardless of their digestion efficacies, would be appropriate for further extraction of microplastics via flotation, after a previous digestion step, since microplastics would float in the saline solution but the remnants of the algae and vegetal debris would completely sink.

Oxidizing protocol 5, despite its high digestion efficacy, would not be appropriate for additional density separation steps, owing to the lack of changes in density and buoyancy of the samples. The negative results for protocol 5 were unexpected, considering that Masura et al. (2015) themselves followed a density separation step after the WPO, using even a more concentrated NaCl solution. The possibility of repeating the experiment with a longer decantation time was considered, but not done yet. In any case, since protocol 5 yielded the highest digestion efficacy, further density separation of remaining biological

material would not be necessary, but considering the bleaching of the remaining tissues and the possibility of interference in the microplastic quantification, density separation support would have been convenient.

Some researchers (Avio et al. 2015; Claessens et al. 2011) have tried to extract microplastics from biological material rich samples using modifications of Thompson et al. (2004)'s flotation method, but the recoveries of the plastic particles have not been ideal, with values of 73%, or ranging from 68.9 to 97.5%. But when a density gradient separation and an oxidant treatment were combined, the extraction efficiency increased to 95% (Avio et al. 2015). This, together with the results of the buoyancy experiment of the present work, suggests that a density separation step, before and/or after the digestion procedure (Masura et al. 2015; Dehaut et al. 2016), would help increase the extraction efficacy of plastic particles.

The experiments for evaluating the methods, which revealed that all protocols except protocol 2 were safe for the plastic particles, must be considered valid only for the tested parameters. The number, colour, and shape of most plastic particles remained the same after these digestion procedures.

Plastic particles remained resistant to the acid protocol 1. Probably due to the low concentration and low temperature at which HCl was used, this method did not damage any tested microplastic type, but it did not digest the algae and vegetal debris either, and did not increase the density of the biological material, so this method was discarded and it is not recommended for the extraction of microplastics from algae and vegetal-rich samples.

The results for protocol 2 were similar to Cole et al. (2014)'s results regarding the polyester fibres, that could not be recovered. However, the results differed in the destruction of other plastics. While Cole et al. obtained partially destroyed Nylon fibres and melded PE after the 10 M NaOH treatment, here all PA fibres and PE fragments were recovered in perfect conditions. Also, Dehaut et al. (2016) found this method was safe for PA and PE, but led to the degradation of two other plastics types, not included in this study (polycarbonate, PC, and polyethylene terephthalate, PET). Protocol 2, due to its insufficient digestion efficacy and the detrimental effect it had on some plastic types, is not advisable for the extraction of microplastics from any marine environment samples.

The other tested alkaline methods, that is protocols 3 and 4, did not cause any damage to the plastic particles. The strong base KOH method, from protocol 3, showed no detrimental effects on any of the six plastic types examined, neither in the fifteen types tested by Dehaut et al. (2016). The less concentrated NaOH protocol 4, unlike protocol 2, did not degrade any plastic and allowed the recovery of all fibres. Although these two alkaline protocols seemed to be harmless for the polymers, due to its poor digestion efficacy on algae and vegetal material, they are not recommended for the extraction of microplastics

from beach sediment samples. These two digestion methods should only be considered when combining the digestion treatment with density separation steps.

The most successful method in the digestion of algae and vegetal matter, protocol 5, also did not damage any plastic particles. Here, with protocol 5, all microplastics were recovered and changes in colour or shape of the plastics were not observed. Other researchers, however, obtained very different results when using hydrogen peroxide. These varied from reductions in size and thickness of PP, PE, PA and PC particles, to changes in PET colours, and LLDPE fragmentations (Nuelle et al. 2014), to no bleaching of plastic particles (Avio et al. 2015), or to no significant impacts on the FT-IR spectra of polymers like PP, PE, PVC, PA and PET after seven days of treatment (Tagg et al. 2015). The fact that results are so varied might be due to the diverse exposure times utilized (less than an hour vs. several days). In any case, the H_2O_2 oxidizing method developed by Masura et al. (2015) has been confirmed to be a respectful, harmless reagent for many polymer types.

More and more researchers are combining different methodologies to make the most of the extraction procedures and to get the highest extraction efficacies possible. Avio et al. (2015) described a methodology for the extraction of plastic particles, combining a density gradient separation followed by an oxidizing digestion (30 % H_2O_2) of the organic material, which yielded particles recoveries of approximately 95 %. Bergmann et al. (2015) combined these two steps as well for the extraction of microplastics, and so did Masura et al. (2015). Their methodology actually included two density separation steps and the WPO of the biological material, but their method also lasts a few hours instead of an entire day or a week, which is indeed more time-efficient.

The Masura et al. (2015) method, tested as protocol 5, succeeded in digesting algae and vegetal samples, yielded the highest digestion efficacy, needed less time than the other protocols, was not expensive compared to enzymatic methodologies, and did not damage any type of plastic. This is why protocol 5 is considered to be the best option for the extraction of microplastics from algae and vegetal-rich samples, and is suggested as the standard method for the extraction of microplastics from marine environment samples.

6. CONCLUSIONS

1. Among the five tested protocols, it was protocol 5, based on the methodology of Masura et al. (2015), that was the most efficacious at digesting algae and vegetal material.

2. Protocols 3 and 4, adapted from the methodologies of Dehaut et al. (2016) and Budimir (2016), would be appropriate for the extraction of microplastics from algae and vegetal rich samples, only if the digestion treatments were combined with density separation steps.

3. The five protocols, except for protocol 2, were safe for the tested plastic particles regarding their number, colour, and shape. These characteristics remained the same after the digestion procedures.

4. Due to its high efficacy at digesting algae and vegetal material, its time- and cost-efficient procedure, and the fact that it does not damage any tested plastic particle, protocol 5, based on Masura et al (2015) methodology, was selected as the most promising procedure for the extraction of microplastics from algae and vegetal rich samples.

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