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Development of a fast and efficient method to analyze microplastics in planktonic samples

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ABSTRACT

Microplastics (MPs) affect plankton (a basis of the trophic chain) and planktivorous fish can ingest them through food confusion or by trophic transmission. Consensus to determine MPs in plankton is lacking and, so, three digestion treatments were evaluated: Alkaline (potassium hydroxide) and enzymatic (protease plus lipase) digestions, both combined with a hydrogen peroxide stage; and an oxidative method using a surfactant (sodium dodecyl sulfate) plus hydrogen peroxide. The alkaline method using potassium hydroxide was found to damage polystyrene. MPs were identified with a stereomicroscope and characterized by reflectance infrared microscopy in semi-automatic mode (using dedicated multi-well aluminium plates). Analytical recoveries for polypropylene, polystyrene, polyethylene, polyamide, polyvinyl chloride and polyethylene terephthalate were higher than 75%, 82% and 83% for the alkaline, enzymatic and oxidative treatments, respectively. The enzymatic method was successfully validated in a European interlaboratory exercise and the oxidative method was demonstrated to be a reliable, fast and cheaper alternative.

1. Introduction

It is estimated that anything between 4.8 and 12.7 million tons (Mt) of plastic enter the ocean each year (Miller et al., 2017), of which 80% proceed from the continents (Andrady, 2011). It is expected that by 2050 they will increase to 30 Mt/year (Jambeck et al., 2015) and microplastics (MPs) are a part of this problem. The situation is alarming and demands urgent attention and severe measures. In brief, MPs are polymer particles smaller than 5 mm, most of which come from the degradation of large plastic waste (Crawford and Quinn, 2017a), and it was found that their most reduced sizes can affect plankton (Desforges et al., 2015; Setälä et al., 2014; Sun et al., 2017) so that this critical basis of the food chain gets impacted.

Microalgae are indispensable for maintaining the balance of the aquatic ecosystems (Harris, 2012). They are the first step in the food chain, amounting up to 50% of the net primary production (Prata et al., 2019b), and any breakdown or contamination there would lead to subsequent effects, primary consumers being affected next. Therefore, their good preservation and healthy conditions are important for the equilibrium of the aquatic ecosystem and its flow of energy (Bergami et al., 2016). A recent review from Prata et al. (2019b), concluded that MPs can inhibit growth (Zhang et al., 2017; Sjollema et al., 2016), reduce chlorophyll (and hence photosynthesis; Bhattacharya et al., 2010; Besseling et al., 2014; Mao et al., 2018) and induce oxidative stress in microalgae. As a matter of example, it was seen in laboratory studies that phytoplankton can precipitate after forming aggregates with MPs due to adsorption forces (Chae et al., 2018; Lagarde et al., 2016; Long et al., 2017). Furthermore, biological functions are affected by the toxic effects of some compounds, like plastic additives (Zhang et al., 2017). Should this occur to a large extent, the phytoplankton population will be affected, reducing the available nutrients and, so, primary consumers.

Zooplankton represents the second stage in the marine trophic scale and is also an indicator of the level of bioavailable energy in the ecosystem. It is an important source of food for commercially important fish and cetacean and it was seen that it can confuse MPs with food (Cole et al., 2013). Under laboratory conditions, different species of zooplankton have been found to ingest MPs (Cole et al., 2013; Wilson, 1973; Wright et al., 2013), and even transform them into smaller plastics after digestion (Dawson et al., 2018). In addition, two reports revealed trophic transfers of MPs from mesoplanktonic species to macroplanktonic ones (Setälä et al., 2014), and coprophagous feeding (Cole

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et al., 2016). The influence of biofilm formation on the ingestion of MPs is also important, the older the MPs become the more likely they are to be ingested (Vroom et al., 2017). Further, the presence of MPs at the same locations as microalgae proliferation also increased the probability of MPs ingestion by zooplankton (Chae et al., 2018).

These findings are not restricted to laboratory experiments as they were observed also in Nature (Botterell et al., 2019). Studies in different regions worldwide showed that zooplankton ingests MPs; either in the Pacific (Boerger et al., 2010; Desforges et al., 2015), the Atlantic (Cole et al., 2014; Pazos et al., 2018; Payton et al., 2020), and Asia (Payton et al., 2020; Md Amin et al., 2020; Sun et al., 2017; Sun et al., 2018a; Sun et al., 2018b; Kang et al., 2015). This problem seems to be exacerbated both close to urbanized coasts (Desforges et al., 2014; Browne et al., 2010; Frias et al., 2014) and plastic accumulation areas where the abundance of MPs is high (Cózar et al., 2014).

With regard to the size of the items ingested by zooplankton, Sun et al. (2018b, 2017) and Md Amin et al. (2020) found that copepods accumulate many MPs, whose length can be around 300 μm or, even, 550 μm (Desforges et al., 2015). However Payton et al. (2020) reported smaller sizes, 43–104 μm. Likely, the size of MPs ingested is highly dependent on the different copepod species (whose size can range between 0.2 and 11 mm).

Whenever MPs accompany plankton, filter feeders and planktivorous organisms can incorporate them into their organisms, either by direct ingestion of zooplankton or because they can confuse zooplankton preys with MPs of similar size and color (Moore, 2008; Pazos et al., 2018; Alfonso et al., 2020; Cedervall et al., 2012; Germanov et al., 2018; Van Colen et al., 2020). Several sites where this occurs have been reported: *Decapterus muroadsi* in South Pacific confused blue microplastics (around 1 mm) with copepods (Ory et al., 2017); in the coastal planktonic community of Río de la Plata estuary (South America), up to 139 MPs/m^3 (500–1000 μm) were found, whose size coincides with the most common plankton sizes (Pazos et al., 2018). A saline lake of Argentina was found to contain 180 MPs/m^3 (50–950 μm) that affect *Odontesthes argentinensis* (Alfonso et al., 2020). It was verified that there is a 61% chance of finding MPs (500–150 μm) in planktonic samples along the Portuguese coasts (Frias et al., 2014). In the Mediterranean Sea, the average ratio between the weight of MPs and mesozooplankton has been found to be 0.5 (Collignon et al., 2012). At Northwestern Sardinia, near the Asinara National Park, MPs were found in 81% (size 1–2.5 mm) of the samples (Panti et al., 2015). In Las Canteras Coast (Gran Canaria, Spain) as much MPs (0,2–1 mm) as zooplankton was found (Herrera et al., 2020).

Therefore, MPs are expected throughout higher trophic levels. For example, Boerger et al. (2010) identified MPs in 35% of planktivorous fish (n = 670 fish); Neves et al. (2015) found MPs in 20% of 236 fish in the coast of Portugal. Giani et al. (2019) found MPs in 23% of Mediterranean fish. In Chile, 6 commercial species have been found to ingest MPs frequently (Pozo et al., 2019). Avio et al. (2019) observed a frequency of ingestion of MPs between 13% and 35% in the Adriatic sea.

A relevant issue about almost all the preceding papers is that they put in force different methods, including sample digestion. And so, it is difficult to directly compare their figures. Therefore, it is necessary to develop reliable methods to determine the amount of MPs and their constitutive polymers in planktonic samples, irrespectively of whether they have been ingested or not. It is worth noting that the size range of the particles considered in this study (70–400 μm) merits attention because the lowest can be ingested by copepods whereas the largest can be ingested by fish directly. So far, standardized operational guidelines for this purpose are not available nowadays and many different approaches were proposed.

There are not specific protocols reported for digesting planktonic matrices as the majority of studies focused mainly on fish and bivalves. However, a simpler, faster and cheaper digestion protocol can be envisioned for plankton due to its inherent less complex matrix (at least, when compared to fish stomachs, mussels, etc.). In some cases the amount of plastics were monitored directly from the water sample after filtration (Collignon et al., 2012; Frias et al., 2014), but if the plankton load is high the digestion of organic matter is necessary. This involves commonly a first decomposition of the biological material to isolate MPs. This can be done using acid (like hydrochloric acid, HCl, and nitric acid, HNO_3), alkaline (like sodium hydroxide, NaOH, and potassium hydroxide, KOH), oxidative (like hydrogen peroxide, H_2O_2) or enzymatic digestions or combinations among them. The main concern here is the impact these reagents can cause on the polymeric material. The key is to find a trade-off between matrix destruction and conservation of the MPs present in the sample. Each of these options has its own pros and cons and will be reviewed and evaluated in this paper.

Regarding polymer characterization, the most common analytical technique to identify polymers is Fourier transform IR spectrometry (FT-IR or, simply, IR) (Andrade et al., 2020; Crawford and Quinn, 2017b; Xu et al., 2019). IR is usually complemented with Raman spectrometry, as the latter provides better resolution, less water interference and can measure smaller particles (Joon Shim et al., 2017; Ribeiro et al., 2017). Both IR and Raman were recommended by international institutions (European Commission, 2013). However, there are still numerous analytical challenges and an important lack of information about the instrumental setups (Andrade et al., 2020) that hamper automation (Xu et al., 2019). In particular, if IR is to be used for high throughput studies, the way in which complex samples (e.g. with complex organic matrices) need to be prepared and the way in which the very many suspicious particles need to be characterized must be simplified and accelerated.

From the aforementioned discussions, it is concluded that there is an urgent need for standardizing digestion protocols to promote consistency in data collection and analysis (Lusher et al., 2017; SAPEA, 2019). Searching for effective digestion methods with a good compromise in terms of destruction of organic matter and high recovery of MPs, at the lowest possible cost, is one of the ongoing targets in this research field and, so, of this work. Therefore, the major objective of the present work is to evaluate two common working methodologies to get rid of the planktonic matrix: a digestion with an alkaline medium (using 10% KOH followed by H_2O_2) and a simplification of the BEPP (basic enzymatic purification protocol) proposed by Löder et al. (2017); and to propose a new purely oxidative method (based on 30% H_2O_2). The concentrations of the reagents and incubation times were selected among those reported in literature as the least aggressive for polymers. In all cases, the incubations will not exceed 60 °C (Munno et al., 2018). The analytical recoveries of MPs obtained with the 3 methods will be studied for polyethylene terephthalate (PET), polystyrene (PS), low-density polyethylene (LDPE), polypropylene (PP), polyamide (PA) and polyvinyl chloride (PVC). Some practical recommendations to speed up the IR analysis of MPs for the size range between 70 and 400 μm will be proposed.

2. Experimental

2.1. Reagents and materials

The reagents used for the alkaline treatment were KOH (85% purity, Panreac), and Triton X-100 (Sigma-Aldrich®). The enzymatic treatment required sodium dodecyl sulfate (SDS ≥ 98.5% purity), Tris (tris (hydroxymethyl)aminomethane), protease from *Streptomyces griseus* (Type XIV activity ≥3.5 units/mg), lipase (from *Thermomyces laguginosus* with activity ≥100.000 U/g), all them from Sigma-Aldrich®. From these, aqueous solutions of SDS (2% w/w) and Tris (1 M) were prepared. To adjust the pH of Tris, HCl (37% w/w, PA-ACS-ISO, Panreac) was used. H_2O_2 (≥30%) was from Sigma-Aldrich®.

AE100 Whatman cellulose nitrate filters with a pore diameter of 12 μm were used and stored in 60/15 mm glass Petri dishes from Pobel. The planktonic samples were prepared in 1 L Pyrex® bottles and 500 mL Pobel® Erlenmeyers were used for sample digestion and incubation.

2.2. Apparatus

The incubation system was a Rotabit P (Selecta, Spain), with temperature and agitation controls. A Pobel® vacuum filtration system was used in combination with a Millipore® vacuum pump. A 2001 micro-pH-meter from Crison® (Barcelona, Spain) calibrated in each working session was employed throughout.

The optical analysis was done using a Leitz Wetzlar stereomicroscope (10× ocular and manual adjustment of the objective zoom up to 5×, total magnification 50×). The IR microscopic analysis was performed with a Spectrum 400 FTIR Perkin Elmer spectrometer equipped with a Spotlight 200i Perkin Elmer microscope. The images obtained with the micro-FTIR were treated with the Spectrum® software. The FTIR experimental set up was: 4000–600 cm^{-1} working range; nominal resolution: 4 cm^{-1}; number of scans: 200; strong Beer-Norton apodization; reflectance mode; apertures: 70 × 70 and 100 × 100 μm; spectral processing: normalization 10% and Kubelka-Munk. All spectra were background corrected (a background per sample). This information complies with the proposed minimum information for publication of IR-related data on MPs characterization (MIPIR-MP) (Andrade et al., 2020). To identify the polymers, a library containing the reflectance spectra of different plastics, including their ageing, was developed in-house.

All suspicious particles found in the stereomicroscope were measured by IR microscopy after placing them on dedicated cavities of multi-well aluminium plates developed in our laboratory (80 mm × 40 mm × 2 mm –length × width × thickness-, 39 wells), see Fig. 1. It was verified that particles up to 70 μm can easily be handled in this way. This approach largely reduced the time required to locate the particles by the FTIR system.

A JEOL JSM-6400 Scanning Electron Microscope (SEM), coupled to an Energy-dispersive X-ray spectroscopic microanalysis device (EDXA, Oxford INCA Energy 200) was employed to evaluate the changes produced in the particles. All MPs particles were manually placed over an electrically conductive, nonporous carbon type (Agar Scientific, UK), covered with a gold layer using a cathodic spraying system (BAL-TEC SCD 004) prior to the SEM measurements. SEM and EDXA studies were done on PS particles, because they showed to be the more affected polymer by the digestion treatments in several preliminary experiments.

2.3. Samples

Representative planktonic samples of the coastline of A Coruña (Galicia, N.W. of the Iberian Peninsula) were prepared (Fig. 2) so as to simulate the maximum primary production period of the region (that occurs around May). They consisted of 500 mL of 1 μm-filtered-seawater that contains 40 individuals/L, formed mostly by copepods (Souto, 2017) and a phytoplanktonic mass of around 2.10^6 cells/mL (Valencia et al., 2003). Seawater was collected at the Northern coast of Galicia –including gross filtration, stored in a tank and delivered to the laboratory through a dedicated pipe. Microalgae *Tetraselvis suecica* and *Phaeodactylum tricornutum* were cultivated and provided by the UDC microbiology laboratory; and copepods (*Acartia* sp.) were from the Aquarium Finisterrae of A Coruña.

To validate the digestions, planktonic samples were prepared accordingly and spiked with 20 particles of each polymer; namely, PP, PS, PVC, PET, PA and PE. The pristine polymers used throughout this work were provided by the Universität of Bayreuth (Germany), within the framework of the JPI-Oceans-funded Baseman project. They were prepared in the form of powders, i.e., small particles in the 50–300 μm range.

The samples for the interlaboratory exercise were provided by AWI (Alfred Wegener Institute, Germany) within the framework of the Baseman project and consisted of replicates of two real plankton samples collected at Helgoland-roads, at the island of Helgoland in the German Bight, and close to Elbe, spiked with known amounts of PE, PS, PET and PA66 particles, whose size was 500–1000 μm in diameter.

2.4. Blanks and controls

In order to avoid contamination by MPs, all laboratory equipment and glassware were washed with alkaline soap solution (Extran® MA01) for 48 h and rinsed thoroughly with Milli-Q water (18 $M\Omega cm^{-1}$ resistivity) before and during all steps. All materials and glass recipients were covered with aluminium foil during storage and use. All the solutions were made with ultrapure Milli-Q water. Dedicated air flow cabinets were not used.

Negative controls as procedural blanks were made for each experiment using all reagents and filtration instruments, according to quality criteria proposed by Hermsen et al. (2018). The presence of fibers from the laboratory environment was detected in some blank filters although they do not constitute a target MP in this work, and only particles were analyzed. Fortunately, contaminant particles were absent from all blank filters evaluated (n = 12).

3. Methods

3.1. Digestion of plankton

Fig. 3 presents the general analysis workflow of the digestion protocols studied in this work, which are described in the following lines. The spiked plankton samples (ca. 500 mL) were vacuum filtered yielding a totally opaque filter holding all the organic matter and MPs; 2 or 3

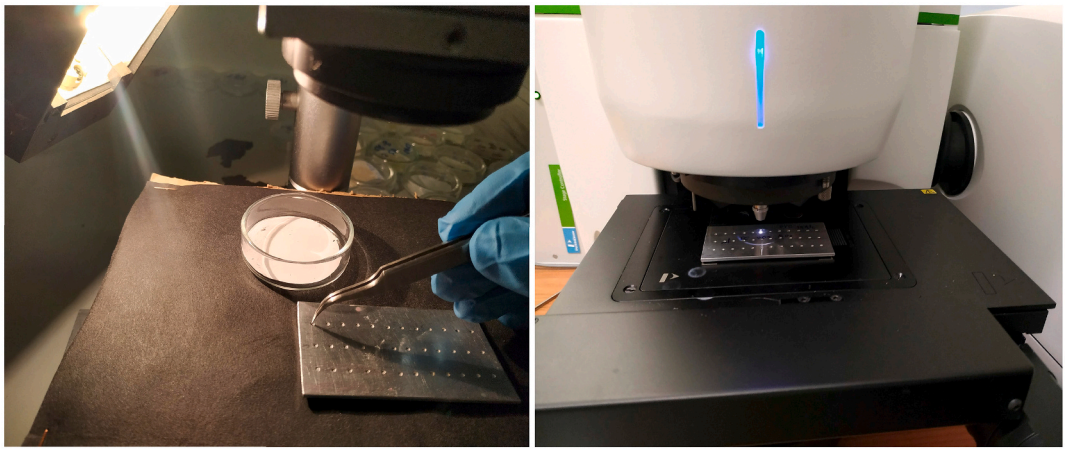

Fig. 1. Customized aluminium plates used to deploy the suspicious particles for IR microscopy analysis.

Fig. 2. Phytoplanktonic samples and copepod of Order Calanoida (*Acartia* sp.).

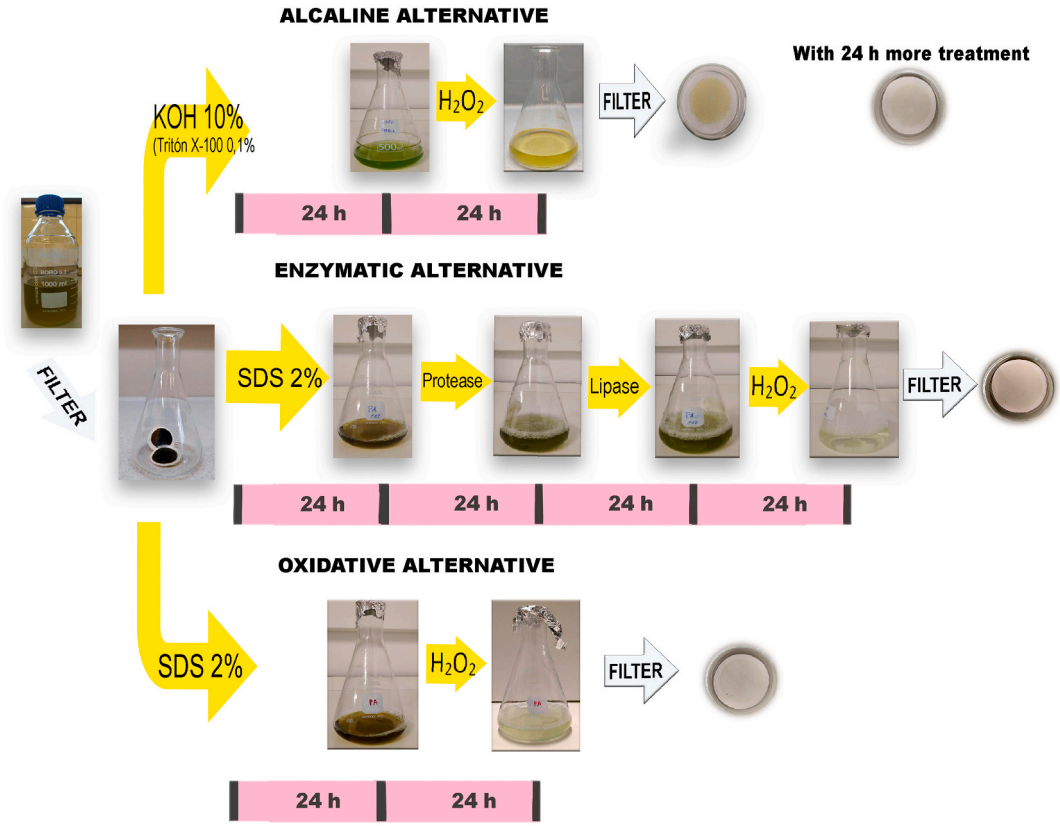

Fig. 3. Analysis workflow of the three digestion procedures considered in this study.

filters per sample were required due to their clogging by the organic matter and subsequent slow filtration process (Fig. 4). All filters were transferred to a 500 mL Erlenmeyer flask for further treatments. It is important to wash out the walls of the Pyrex bottle containing the sample with water jets, at least 1 L, adding 2% SDS to help all MPs slip to the filter.

3.1.1. Alkaline digestion

The alkaline treatment requires digesting the filter with 80 mL of a solution of 10% (m/v) KOH (Zheng et al., 2020), and 0.1% Triton X-100 (v/v) as surfactant. This is then incubated at 50 °C at 130 U/m for 24 h. After this, 30% H_2O_2 is added to complete organic matter destruction and bleach the sample (see the Results and Discussion section for more details).

3.1.2. Enzymatic digestion

The enzymatic treatment is based on the first two steps of the BEPP approach proposed by Löder et al. (2017) although with slightly lower concentrations, with a subsequent step using lipase and, finally, H_2O_2 to completely destroy the organic matter (4th and 6th oxidative steps proposed by them). The objective of this simplified approach is to reduce the overall working time. Thus, 80 mL of a 2% SDS solution in a 500 mL Erlenmeyer flask (one per sample) were employed to initiate the degradation of the organic matter retained in the opaque filters as they initiate the lysis of the cellular membranes and increase the contact surface of the subsequent enzymatic treatments. This was incubated for 24 h at 50 °C. Next, 80 mL of 1 M Tris were added and, in order to get the optimal action of protease, the pH was adjusted to 9 with HCl. Afterwards, enough commercial protease to make its final concentration to 500 µg/mL at each flask was added (which meant adding 0.08 g in 160 mL) and the samples were incubated for 24 h, 50 °C. After this, 5 mL of

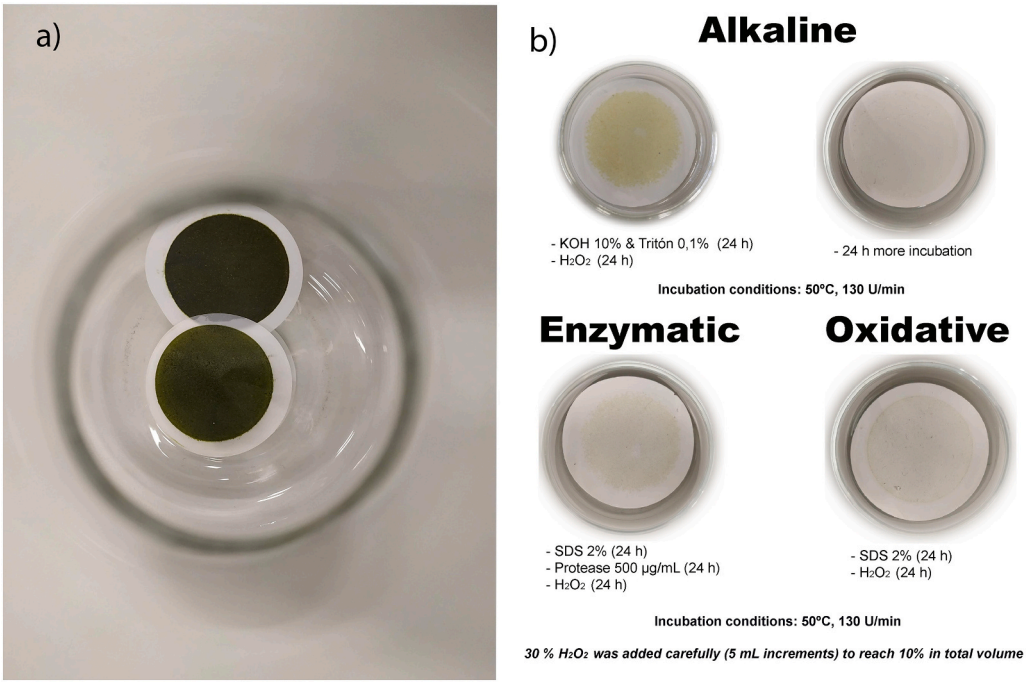

Fig. 4. a) Image of two filters obtained after 500 mL of sample filtration, before any digestion treatment. b) Photographs that exemplify the final appearance of the filters after each digestion treatment. They three offer an efficient destruction of the organic matter.

lipase (Mintenig et al., 2017) were added to each flask, followed by another 24 h incubation at 40 °C. The lipase used is from Thermomyces lanuginosus (TLL), whose maximum of activity occurs at pH around 9, according to Fernandez-Lafuente (2010) and Rodrigues et al. (2009), therefore pH is maintained in this range. Finally, H_2O_2 was poured carefully. These amounts were set after several optimization experiments.

3.1.3. Oxidative digestion

After studying the previous treatments, it was decided to try a new oxidative digestion to simplify the overall procedure. This consisted only of a first step using 80 mL of 2% SDS to macerate the planktonic organisms and increase the contact surface and a subsequent oxidative digestion with 30% H_2O_2. Note that in the three treatments considered in this report a final step using 30% H_2O_2 is required to destroy totally the remaining organic matter in the samples (and bleaching them).

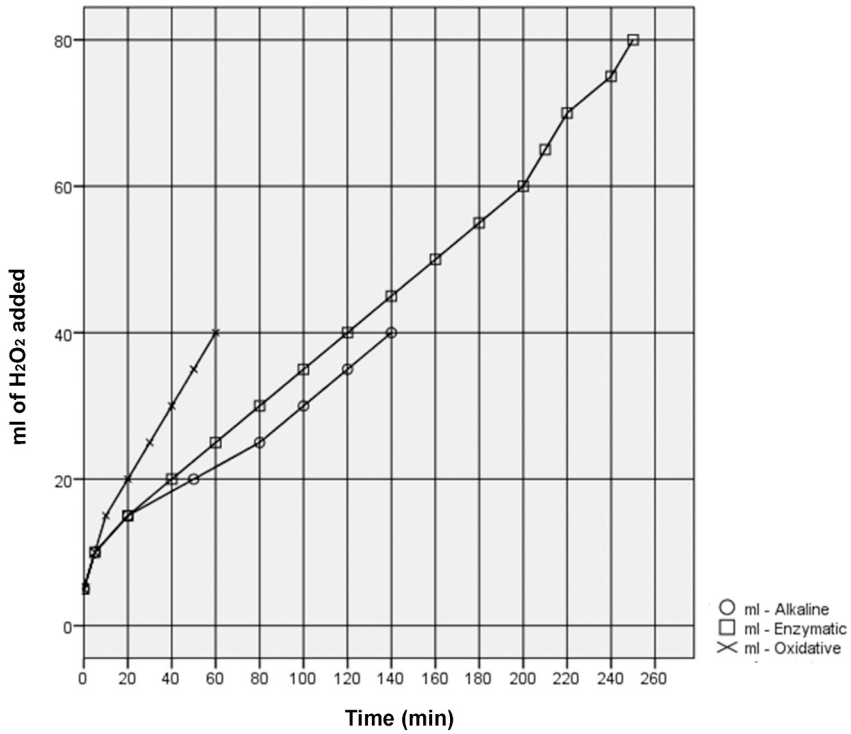

Fig. 5. Comparison of the sequences required to add H_2O_2 in each digestion treatment to avoid foam overflow.

Attention is required here because the oxidative process yields abundant foam that can overflow the flask, especially in the KOH treatment, therefore 30% H_2O_2 was added gradually to avoid this problem. The volume added in each solution was calculated to be 10% H_2O_2 in the total volume: 40 mL for the alkaline and oxidative treatments and 80 mL for the enzymatic one, in 5 mL steps. In some cases, interruptions of stirring were needed to allow the foam to diminish, so the process can take several hours. This was not so in the oxidative treatment (SDS + H_2O_2), because foaming was much less than in the other two treatments. The pace of the additions is depicted in Fig. 5: In the oxidative protocol the first 20 mL can be added during the first 20 min (5 mL aliquots). Then the foam formation was more violent, and the 5 mL additions were spaced every 10 min. In the enzymatic and oxidative protocols, 15 mL could be added during the first 20 min. From that moment on, in the enzymatic protocol, 5 mL aliquots were added every 20 min to avoid foam overflow. In the alkaline method there is a sudden surge of foam around 15 mL and it was necessary to wait 25 min for the next addition, and another 35 min for the next one. At both times, stirring had to be interrupted so that the foam did not overflow the flask. Afterwards, it was possible to return to a 5 mL/20 min rate.

Finally, the digest is filtrated, and the filter stored in Petri dishes. To standardize all treatments the Erlenmeyer flasks were washed with 1 L of milli-Q water, using water jets, and the Erlenmeyer flask as inclined as possible. Whenever smaller volumes were used, the recoveries for PS were poor. Finally, the filters were dried at 50 °C.

3.2. Analytical characterization

The main disadvantage of IR-based measurements is the need to locate each and every particle in the filter. This is hugely time-consuming even when focal plane array detection is used (Löder et al., 2015; Löder and Gerdts, 2015). The working approach followed in the present report consisted of a first collection step where suspicious solid particles observed through a stereomicroscope were collected using microtweezers (Fig. 1). The particles were placed on dedicated wells of aluminium plates developed in our laboratory (see Experimental section). Each was located with a marker and finally all of them measured automatically by the micro-FTIR device.

It is worth considering the spectra of weathered polymers in the searching database because the natural degradation of the polymers into the environment can affect the appearance of their spectra, as reported elsewhere (Costa et al., 2018), and because the surface of the polymers might be affected by the chemicals used during the digestions. In particular, the spectra of PS showed relevant differences among the treatments, which was not the case for the other polymers studied here (Fig. 6), and the alkaline treatment modified the shape and relative appearance of many bands, which was not so dramatic for the other treatments.

IR identification was successful in almost all particles but in a few cases where the spectra were very different from typical plastic polymers and these became unidentified. It was hypothesized that they might be some sort of residue of the reagents or salt that crystalized on the filter. It has to be noted that the irregularity of the surface plays an important role in the spectral noise and it can influence the intensity of some bands. So, it is recommended to perform the measurements in a flat part of the particle.

4. Results and discussion

In this section, the three methods will be studied, and their results

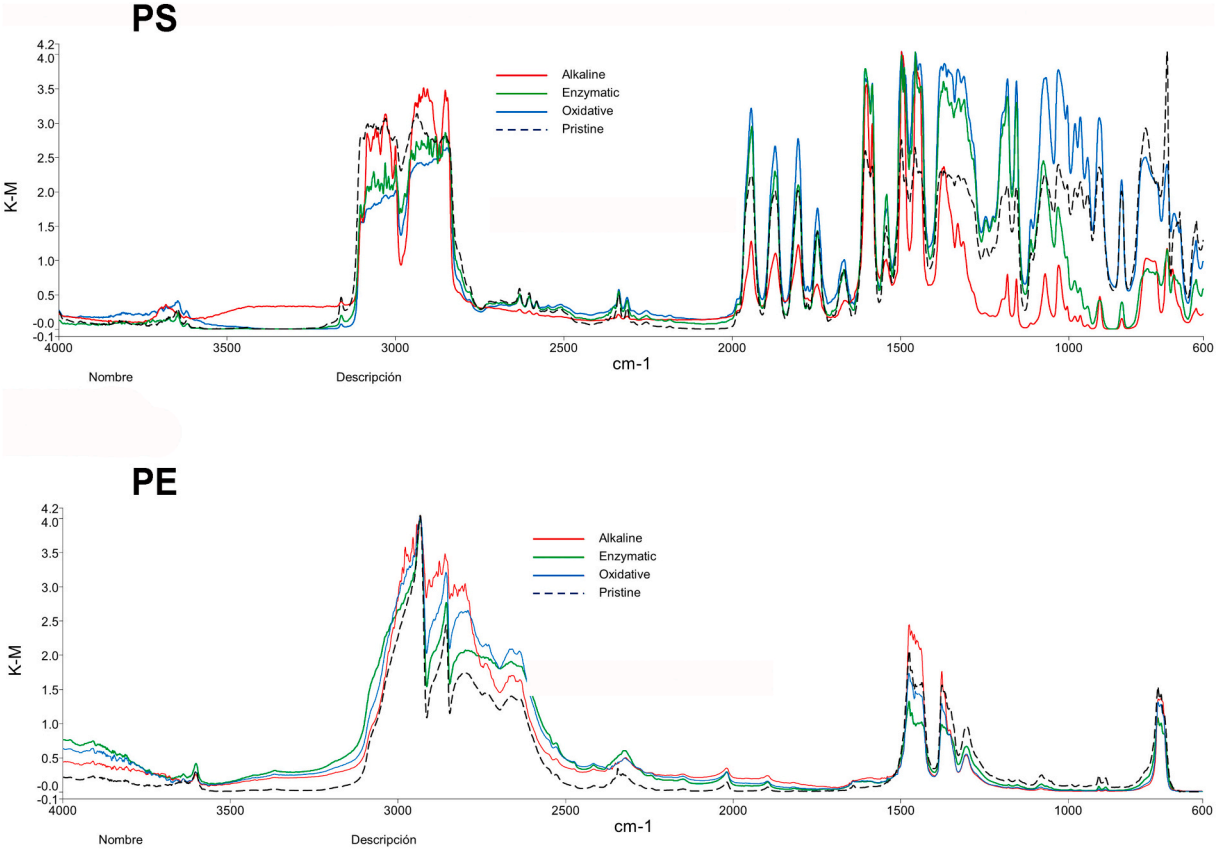

Fig. 6. Comparison of the IR spectra obtained after each digestion treatment. PS exemplifies the case when the polymer is affected by the treatment whereas PE represents a situation when the polymer is almost unaffected by the treatments (see text for more details). The spectra of the 'pristine' (*as received*) polymers are also displayed for comparison.

Table 1
Recoveries (%) for the studied plankton digestion methods (n = 3, ±SD). H_2O_2 was used as final treatment in all cases. The p-value corresponds to the ANOVA (analysis of variance) test; critical p-values are 0.05 or 0.01 (95% or 99% confidence levels, respectively).

Recoveries (%) in each polymer (n = 3). ANOVA test				
Polymer	Alkaline	Enzymatic	Oxidative	ANOVA (p-value)
PS	75.0 ± 5.0	81.7 ± 2.9	85.0 ± 5.0	0,079
PP	80.0 ± 5.0	83.3 ± 5.8	91.7 ± 2.9	0,055
PE	78.3 ± 5.7	81.7 ± 2.9	83.3 ± 2.9	0,373
PVC	90.0 ± 5.0	86.7 ± 2.9	88.3 ± 2.9	0,579
PET	90.0 ± 5.0	85.0 ± 5.0	86.7 ± 5.7	0,533
PA	86,7 ± 2,9	86.7 ± 2.9	88.3 ± 7.6	0,897

compared in terms of analytical recoveries and damage to the MPs. Table 1 shows the analytical recoveries for each digestion process considering samples spiked with 20 microparticles of each polymer. When they were compared (Anova test –Analysis of Variance-, 95% confidence level) it was observed that the means for PS and PP were very close to be significantly different (the other polymers are clearly not different). Taking into account that the number of replicates was 3 and, therefore, the so few degrees of freedom (that underestimates the variances) it was decided to accept a worst-case scenario and assume that the recovery means for PS and PP are not totally comparable.

4.1. Digestion with strong acids and bases

4.1.1. Acid digestion

The use of strong acids appears at first sight as a natural way to destroy organic matter. Desforges et al. (2015) digested zooplankton (mainly copepods and euphrasides) using HNO_3 at 80 °C for 3 h. The same approach was used by Sun et al. (2017, 2018a, 2018b). Md Amin et al. (2020) proposed the use of 60% HNO_3 for 30 min. Despite these studies did not include recovery or validation tests, Desforges et al. (2015) noted that the use of acids could underestimate the results due to the probable damage to the polymers. In fact, it was reported frequently that HNO_3 (and other acids like HCl) can destroy some nylon fibers and PET, PS, PP, PU (polyurethane), LDPE, HDPE (high density polyethylene, PVC, PE and PA (Avio et al., 2015; Catarino et al., 2017; Claessens et al., 2013; Dehaut et al., 2016; Enders et al., 2017; Karami et al., 2017; Naidoo et al., 2017; Prata et al., 2019a). Therefore, acids are not recommended (Dehaut et al., 2016; Enders et al., 2017; Karami et al., 2017; Prata et al., 2019a; Thiele et al., 2019) and they will not be considered here.

4.1.2. Alkaline digestion

Alkaline treatments, such as those with KOH, were suggested as a good alternative to acids because they respect more the integrity of the polymers (Enders et al., 2017). Recently Zheng et al. (2020) used 10% KOH at 60 °C for 48 h to digest plankton samples from the Bohai Sea (China). However, different authors reported that it could be harmful to PET (Dehaut et al., 2016; Hurley et al., 2018; Karami et al., 2017). Also, Hurley et al. (2018) observed a 16% reduction in weight on PC contents. Problems with PVC (reduced recovery rates) and Nylon 66 (changes in color) were found at 50 °C and 60 °C (Karami et al., 2017). The cellulose acetate (CA) is destroyed by KOH (Dehaut et al., 2016; Kühn et al., 2017) and LDPE was degraded in 1 M KOH during 2 days at room temperature (Kühn et al., 2017). Following, it seems that concentrated KOH is not the best option and caution is needed when using it (Prata et al., 2019a).

The studies performed in this work showed that after treating the filters with 10% KOH and 0.1% Triton X-100 the solutions presented a green color (Fig. 3). The next oxidative step based on bleaching with H_2O_2 yielded a transparent solution (Figs. 3 and 4), easy to filtrate and without operational problems to search for the microplastics in the stereomicroscope. An additional oxidative period for 24 h clarified the filter even more. It is worth noting that the alkaline treatment destroyed the filters without further residues (whereas the enzymatic alternative, shown in next section, preserved them). This is not a problem and indeed it is an advantage for it simplifies the subsequent steps and avoids manipulation to handle off the filter. Although H_2O_2 has traditionally been considered a safe digestion method (Nuelle et al., 2014; Prata et al., 2019a) there are nowadays some doubts about its ability to destroy organic matter, since sometimes it only manages to bleach it (Nuelle et al., 2014). Indeed its use is frequent to clarify the solutions obtained after acid or alkaline treatments (see next sections for more details). A remarkable problem when using H_2O_2 is that excessive foam formation can yield poor recoveries due to loses (Avio et al., 2015).

The episodes of foam formation when adding H_2O_2 were visually more violent in this treatment than in the others. This may be because H_2O_2 generates more effervescence in alkaline media than in neutral ones (Galindo and Kalt, 1999; Muangrat et al., 2010). So, it is mandatory to wait longer until the foam remits (30 min in some cases). The overall addition of H_2O_2 was optimized for each treatment and it required up to 140 min (Fig. 5) for the KOH treatment.

The recoveries for the polymers were good, >75%, although PS behaved worst. This was systematically so in different trials and, consequently, PS particles were studied in detail. Some preliminary results suggested that a reduction in their size may occur (observation through the stereomicroscope). Therefore, it was decided to carry out new experiments to check for the possible damage of this treatment to PS (the other polymers did not showed problems in this respect).

Three trials were undergone. Two essays with the same treatment (10% KOH for 24 h, and a next step adding 30% H_2O_2, until obtaining 10% in the total volume) although during 48 h and 72 h in total. The third essay differed in that the concentration of H_2O_2 in the final solution was 15% and 72 h were considered. Besides, the washing volume was increased to 2 L due to the difficulty in recovering PS. 20 microparticles of a similar size were selected (and their normal distribution tested by the Saphiro-Wilk's test) and the average areas were measured by IR microscopy before and after each treatment, see Table 2. Two

Table 2
Reduction of the area of PS particles after three different conditions for the alkaline digestion. The p-value* corresponds to the Shapiro-Wilk's normality test to check that 20 particles are in the same size range. The p-value** corresponds to the Student's *t*-test.

Treatment		Average (n = 20) (μm^2)	SD (μm^2)	p-Value*	Recovery	Area reduction
10% KOH for 24 h and H_2O_2 (10% in total volume)	Before	$1.9 \cdot 10^5$	$5.9 \cdot 10^5$	0.054	95%	27%
Total: 48 h	After	$1.4 \cdot 10^5$	$2.5 \cdot 10^4$	0.362		
	t-Student, before-after (p-Value**): 0,002					
10% KOH for 24 h and H_2O_2 (10% in total volume)	Before	$1.8 \cdot 10^5$	$5.7 \cdot 10^4$	0.113	90%	28%
Total: 72 h	After	$1.3 \cdot 10^5$	$3.0 \cdot 10^4$	0.052		
	t-Student, before-after (p-Value**): 0,003					
10% KOH in 24 h and H_2O_2 (15% in total volume)	Before	$1.5 \cdot 10^5$	$3.0 \cdot 10^4$	0.106	75%	32%
Total: 72 h	After	$1.0 \cdot 10^5$	$5.1 \cdot 10^4$	0.177		
	t-Student, before-after (p-Value**): 0,001					

conclusions can be derived from it: First, that the average area of the PS particles decreased significantly (by 27%) even with the mildest alkaline treatment. After that, another 24 h period to improve the oxidation of the organic matter lead only to some minor area reduction (on average ca. 1%). When the amount of H_2O_2 was increased to 15% of total volume, an additional reduction in the average areas (4%) occurred, reaching a significant 32% of size reduction. The sizes of the 20 particles considered at each trial followed normal distributions (Shapiro-Wilk's test, 95% confidence) and it was verified (Student's *t*-test, 95% confidence level) that the average areas of the particles before and after the digestions were different (Table 2).

To understand why this happened all the recovered particles were examined visually, and it was found that some fragments were flat. Fig. 7 shows two PS particles, before and after the alkaline treatment, each. The particle before the treatment shows the typical spherical shape whereas that after the treatment is essentially flat. Four of them were picked up and sticked to a carbon adhesive tape (see Section 2.2) to perform a more detailed study using SEM. That suggested that PS particles may degrade through exfoliation and the layers exposed to chemical/mechanical attack (Fig. 8). Note how clear these layers are in the original particles (Fig. 8). This surface "crazing" degradation in PS after H_2O_2 was also suggested by Hurley et al. (2018).

The flat shape of these particles (likely due to degradation) may explain why more water was needed to recover the MPs after the digestion treatment as their flatness impeded the particles to roll down easily. This justifies the problems encountered to get good recoveries for the PS particles in the samples.

It was concluded that the combination of H_2O_2 with a KOH alkaline treatment could significantly damage PS particles. That may increase with larger incubation times and more H_2O_2 added. Therefore, this treatment is not recommended if the integrity of all types of polymers (mainly PS) is to be preserved. This is consistent with the problems shown by Karami et al. (2017) and Hurley et al. (2018) when using 10% KOH; and those reported by Nuelle et al. (2014), Budimir et al. (2018) and Hurley et al. (2018) with the H_2O_2; and the general review reported by Thiele et al. (2019) and Prata et al. (2019a).

4.2. Enzymatic treatment

Enzymes have been shown in literature to be the most conservative treatment to preserve the integrity of plastics (Löder et al., 2017; Prata et al., 2019a). Cole et al. (2014) used Proteinase-K (500 μg/mL) and ultra-sonication to decompose previously dried and ground zooplankton samples. They selected this option after comparing it with 1 M HCl and 1 M NaOH without destruction of plastics. But a significant drawback of this protocol is the high cost of the enzymes. Therefore, Löder et al. (2017) proposed a basic enzymatic purification protocol (BEPP) with low cost enzymes which was applied to samples with a high content of phytoplankton and zooplankton (diatoms and copepods). The steps consist of a first treatment with 5% SDS, followed by protease, cellulase, H_2O_2, chitinase, H_2O_2 again, and a final density separation. The results showed good analytical recovery (84.5 ± 3.3%). However, this treatment is long (up to 13 days) and complex, it also requires multiple filtration steps (6–7), which could lead to big losses of MPs (Löder et al., 2017; Nakajima et al., 2019; Prata et al., 2019a).

In our study, the enzymatic treatment yielded a steady clarification of the sample after incubation with protease (Fig. 3). However, the use of lipase did not show any visual change. Finally, H_2O_2 oxidized the digested organic matter and yielded transparent, clear solutions (Fig. 3). The filters obtained thereafter were clean and without visual organic remains (Fig. 4). The recoveries were good (Table 1); higher than 81% for all polymers, slightly lower for PS (although higher than those for the alkaline treatment). Since lipase seemed not to produce relevant changes, additional studies were made to test if that step could be avoided. The final solutions and associated filters were still clear (Figs. 3 and 4) and recoveries were good (and similar to those in Table 1, values not shown here). Therefore, it seems this step can be avoided for the kind of samples considered here, thus accelerating the sample treatment. Even with this simplification the last step involving H_2O_2 is lengthy until 10% v/v is completed, with an operating time around 4 h. This was so because the volume from 20 to 50 mL had to be added through 5 mL increments every 20 min to avoid overflows (Fig. 5).

The enzymatic method was considered by that time as more conservative and polymer-friendly than other alternatives and it was validated by participation in a European interlaboratory exercise organized within the JPI-Oceans Baseman project along 2018. Each partner developed and applied its own analytical protocol and they were compared there (some details cannot be disclosed due to confidentiality issues). Two target criteria to consider that a protocol was fit-for-purpose were stated before the interlaboratory exercise: i) the overall general recovery should be above 90%, and ii) individual polymer recoveries should be above 80%. Each participant received coded (blind) containers containing two blanks and three replicates of each bulk sample (Helgoland Roads and Close-to-Elbe). Their contents had to be analyzed without further subdivision and both the overall number of particles and their nature reported. Table 3 shows that excellent and

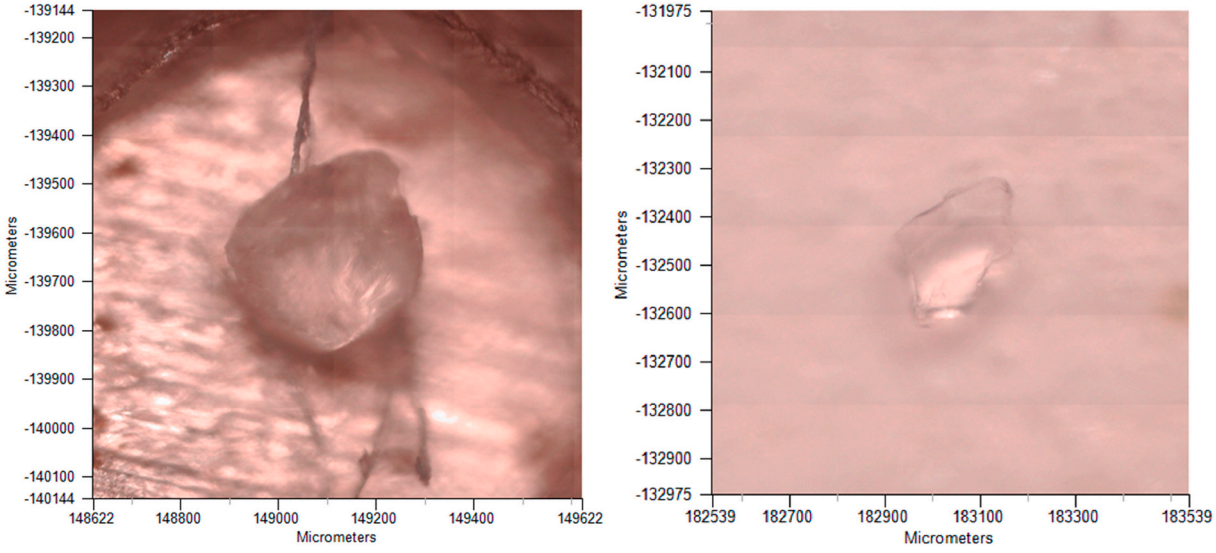

Fig. 7. Microphotographs of a PS particle before (left) and after (right) the alkaline digestion (IR microscopy camera).

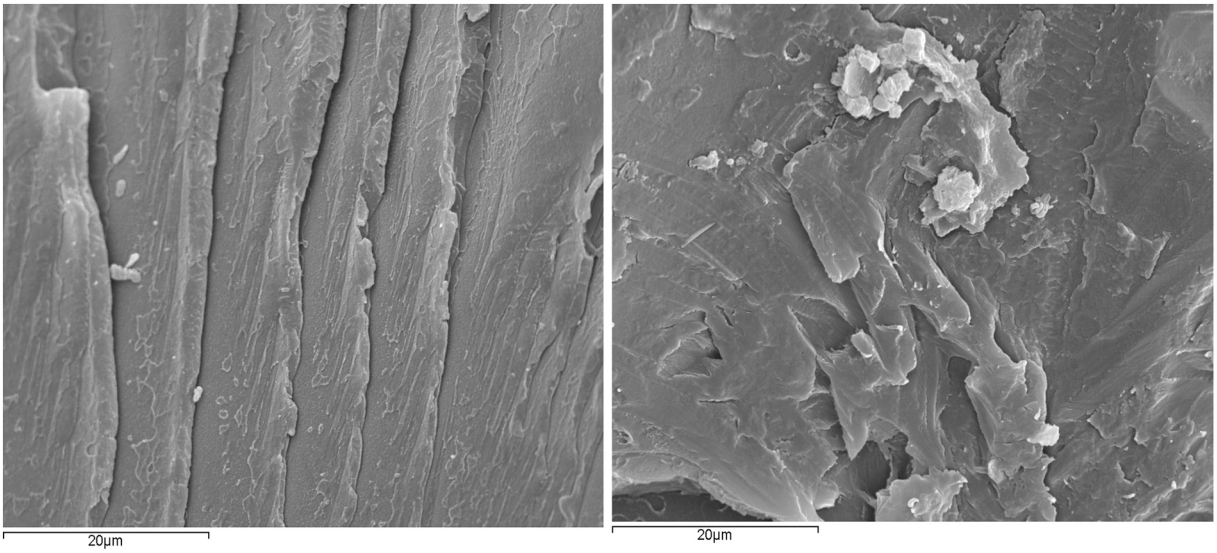

Fig. 8. SEM microphotographs (2000×) of PS particles before (left) and after (right) alkaline digestion.

consistent results were achieved with the enzymatic method used in the present work. The overall recoveries ranked second for both samples and individual polymer recoveries were good: 87, 87, 103 and 103 (PS, PE, PA and PET) for Helgoland Roads and 107, 93, 100 and 90 (PS, PE, PA and PET) for Close-to-Elbe. It is worth noting that the sample from Helgoland Roads contained pretty much plankton than the Close-to-Elbe one (visual observation) and that the interlaboratory exercise dealt with particles in the 500–1000 μm range (slightly larger than those used here to validate our studies, which was 70–400 μm).

4.3. Oxidative treatment

The filters after the simple oxidative method presented a clear appearance, similar to the enzymatic method (Figs. 3 and 4), and leaded to slightly higher recoveries (Table 1), >83%. The foam episodes were not as violent as in the other approaches, which allowed to quickly add 40 mL of H_2O_2 in 60 min (5 mL/10 min) (Fig. 5). This approach reduced notably the long delays required by the enzymatic method, to only ca 48 h, and its overall costs. No specific degradations in the particles (not even in PS) were observed in the stereomicroscope, their shapes and sizes seemed as they were originally and, therefore, SEM studies were not done.

Our results seem advantageous when compared to others reported in literature, likely because a moderate H_2O_2 concentration (10%) for only 48 h preserve the polymers considered here.

Oxidizing agents, mostly H_2O_2, were also used to digest plankton. Pazos et al. (2018) applied 30% H_2O_2 and a 0.05 M Fe(II) solution, in a 1:1 ratio (Fenton's reaction), at 75 °C. Alfonso et al. (2020) exposed samples to 30% H_2O_2 and considered only 8 h. Kang et al. (2015) used 20% H_2O_2 to digest samples from the South Korean Sea for 2 weeks and Payton et al. (2020) combined 30% H_2O_2 with 1 M HCl to treat estuarine plankton samples.

Despite H_2O_2 has been considered safe for MPs this is not obvious, as concentrated H_2O_2 may damage some polymers. For instance, ca. 16% reduction in volume of PP and PE particles <1 mm was seen after digestion with 35% H_20_2; and visible changes in PA, PC and PP <1 mm were detected when 30% H_2O_2 and 7 digestion days were tried (Nuelle et al., 2014). Total PA-66 degradation, size reduction of PP and superficial surface degradation ("crazing") in PS were found after their treatment with 30% H_2O_2 at 70 °C and 24 h (Hurley et al., 2018). Karami et al. (2017) found color changes on PET and bad recoveries on PA-66 after using 50 °C for 96 h. As a conclusion, it seems clear that H_2O_2 should be used with caution. Further, the Fenton's reaction can yield high temperatures that can harm polymers (Munno et al., 2018) and we experienced that it can boil off foam so that it overflows the container.

Table 3
Overall microplastics recoveries (%) obtained in the JPI-Ocean Baseman's. European interlaboratory exercise (QANAP-UDC-Treatment: Enzymatic + 30% H_2O_2).

	Sample Helgoland Roads		Sample Close-to-Elbe
Laboratory 1	96	Laboratory 1	94
QANAP-UDC	95	QANAP-UDC	98
Laboratory 3	95	Laboratory 3	93
Laboratory 4	92	Laboratory 4	82
Laboratory 5	90	Laboratory 5	80
Laboratory 6	88	Laboratory 6	99
Laboratory 7	86	Laboratory 7	93
Laboratory 8	84	Laboratory 8	96

5. Conclusions

Three methods were studied in this work to get rid of the planktonic organic matrix in order to recover the microplastics presented therein. It was found that the oxidative treatment consisting on the only use of SDS (2%) and H_2O_2 (10% in the final solution) was enough to destroy the organic matrix, yielding clear filters and without affecting the shape of the six types of plastic particles considered here (PS, PP, PE, PVC, PET and PA). The enzymatic treatment lead also to unaltered plastics and good recoveries for all tested plastics (>81%) although a bit lower than the oxidative approach (whose recoveries were >83%). In addition, it was observed that for the kind of plankton samples considered in this work the lipase step suggested within the enzymatic treatment for other purposes is not needed. So, the enzymatic approach was reduced by 1 day and only protease was enough.

Anyway, the oxidative treatment appears to be the best option because of economic and time/workload considerations. Roughly, the enzymatic, alkaline and oxidative digestions expenses were ca. 23, 10 and 10 €/sample, respectively, and required 96, 48 and 48 h, each.

Although the alkaline treatment seemed appealing at first sight, the combination of H_2O_2 and KOH was found to affect PS at least, so it should be avoided. It must be acknowledged that the plastics used in this study do not contained additives, further than those required for their fabrication, and so their resistance to chemical agents may differ from other commercial plastics.

From an operative viewpoint, the use of a stereomicroscope to locate suspicious particles and their subsequent analysis by IR microspectrometry using multi-well aluminium plates to locate them easily reduced the overall measurement time.

CRediT authorship contribution statement

ALR performed the experiments and FTIR determinations, discussed the data and wrote the first draft of the manuscript. GGN conducted enzymatic digestion. VFG collaborated in sample treatment experiments and microplastic identification by FTIR microscopy, discussed and interpreted the data. SML, JMA, PLM designed the study, discussed the data, review & editing manuscript and acquisition of funding. All authors were involved in writing and revision of the manuscript.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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