Marine Pollution Bulletin 173 (2021) 113027

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Microplastics on plankton samples: Multiple digestion techniques assessment based on weight, size, and FTIR spectroscopy analyses

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ARTICLE INFO

Keywords:
Digestion
FTIR
Microplastics
Plankton
Plastic
Pollution

ABSTRACT

Digestion protocols are needed to determine microplastics abundance and features. This study assessed the organic matter (OM) digestion efficiency on plankton samples and the MPs' weight, size, and polymer changes under different digestion techniques. For this, 2-step (KOH and H_2O_2 + Fe^{2+}) and 3-step (2-step and enzymes) digestion techniques were assessed under different duration and temperature conditions. The results obtained for OM digestion with 2-step and 3-step techniques were satisfactory. Weight changes were registered for polyethylene terephthalate (PET), polystyrene foam, polyvinyl chloride, and polycarbonate with 2-step digestion, but with inconsistent values. Significant size changes were registered only for PET applying 2-step digestion techniques at 60 °C. Using 40 °C for 72 h prevailed all polymers from size changes. Polyethylene weathered MPs were also preserved, including an enzymatic step. Polymer fingerprints were not affected by any digestion technique. Based on these results, any method applying high temperatures will damage MPs.

1. Introduction

In microplastics (MPs) environmental research, digestion protocols are needed for a broad type of matrixes as biota tissue, water, and sediments (Yonkos et al., 2014; Li et al., 2018; Arias et al., 2019). Samples for MPs analyses in aquatic systems are usually obtained with a neuston or plankton net (50–300 μm), presenting organic matter (OM) at different proportions depending on the environmental conditions (e.g., trophic state, temperature, season). OM in samples is usually represented by plankton (phytoplankton, zooplankton, ichthyoplankton), suspended particulate matter, and occasional seaweed fragments. Among the studied MPs features, size ranges are critical because they provide information about the potential to be ingested by different organisms (Cole et al., 2014) and for long- or short-range dispersion (Besseling et al., 2017). So, any possible change in MPs size during sample analyses will bias the results and our knowledge about this emergent pollutant in the environment.

Digestion techniques are varied depending on the selected reagent (acids, bases, oxidants, enzymes), concentrations, digestion steps (1, 2, or more), duration (hours, days) and, temperatures conditions (Frias et al., 2014; Karami et al., 2017; Lusher et al., 2020). Also, previous sample processing could include oven drying, manual sorting, and density or mesh filter separation to optimize sample extraction (Li et al., 2018; Lusher et al., 2020). Depending on the reagents used, digestion could be classified as: basic (NaOH, KOH) (Karami et al., 2017; Hermabessiere et al., 2019), acidic (HNO_3, HCl, $HClO_4$) (Avio et al., 2015; Sun et al., 2018), enzymatic (e.g., lipase, amylase, chitinase, cellulose) (Cole et al., 2014; Li et al., 2018; Lusher et al., 2020) and one of the most widely used, oxidative (H_2O_2) (Karami et al., 2017; Li et al., 2018).

The result was a wide diversity of digestion techniques for MPs analyses, with only a few studies evaluating OM digestion efficiencies and possible MPs damage (Cole et al., 2014; Nuelle et al., 2014; Enders et al., 2017; Lusher et al., 2020). Changes in color, weight, and polymers condition were found for several digestion protocols (Claessens et al., 2013; Cole et al., 2014; Karami et al., 2017; Munno et al., 2018; López-Rosales et al., 2021). Most of the previous studies applying acidic digestions reported problems with many polymers (e.g., polystyrene, polyamide, polyurethane, polyvinyl chloride, Nylon) being the most aggressive and less recommended (Claessens et al., 2013; Collard et al., 2015; Enders et al., 2017), but still on use (Sun et al., 2018; Payton et al., 2020). According to previous research, KOH and Fenton's reagent (H_2O_2 + Fe (II)) were recommended for vegetal and animal OM digestions, respectively (Lusher et al., 2020). Nevertheless, when applied (individually or in 2-step digestions) at high temperatures,

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https://doi.org/10.1016/j.marpolbul.2021.113027
Received 7 June 2021; Received in revised form 29 September 2021; Accepted 30 September 2021
Available online 7 October 2021

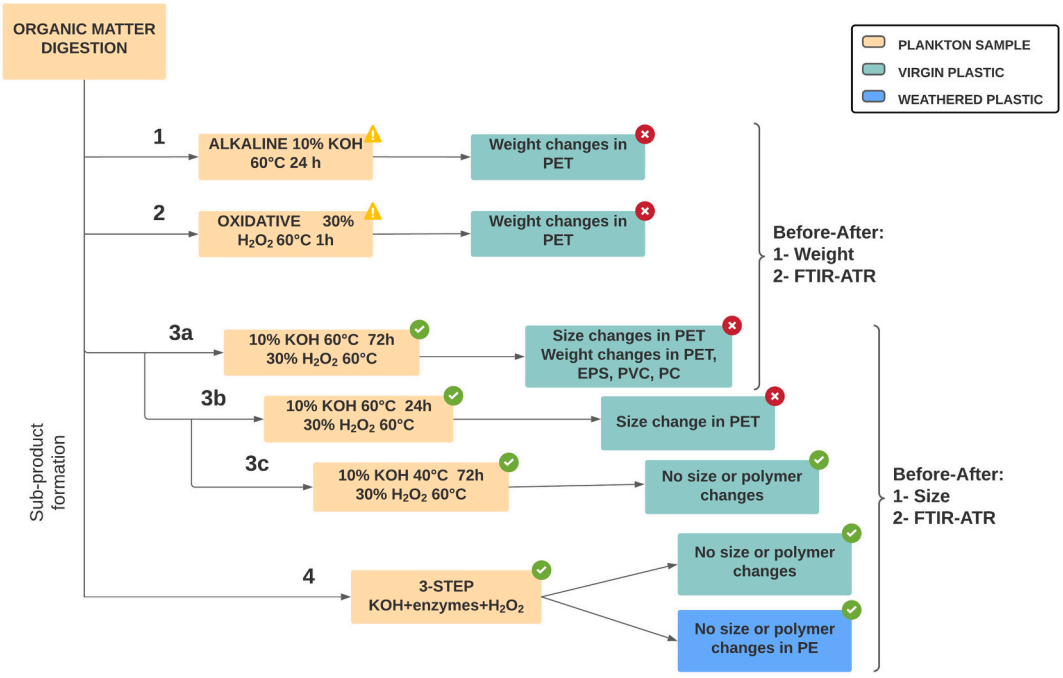

Fig. 1. Working flow diagram for chemical digestion trials on organic matter and microplastic samples, including weight, size, and polymer fingerprint assessments.

concentrations, or during long periods, MPs damage was also registered (Nuelle et al., 2014; Munno et al., 2018).

Previous studies reported their results differently when assessing MPs damage, depending on the experiment design, with MPs recovery rates as those most commonly used (Lusher et al., 2020). This parameter provided information about possible errors on MPs abundances by digestion techniques, but changes in MPs size will not be registered. Considering the exponential increase in MPs studies, with the smaller fractions (<100 μm) becoming more relevant (Besseling et al., 2017; Song et al., 2018; Fu et al., 2020; Zhang et al., 2020), avoiding MPs size damage results essential for their analysis. Therefore, this study aimed to assess the effect of different duration and temperature conditions for 2-step KOH and Fenton's H_2O_2 digestions on MPs weight, size, and polymer identification by FTIR. In addition, 3-step digestion, including enzymes, was also assessed with several virgin polymers and weathered PE MPs. The obtained results will improve our efficacy on digestion methods for MPs research, moving forward to standardize analysis methodology.

2. Materials and methods

2.1. Proposed digestions

Different digestion techniques were applied to determine the most efficient protocol for MPs analysis in water samples containing plankton. The work was organized in four stages (Fig. 1): First, the efficiency of one-step (1) alkaline (KOH) and (2) oxidative (H_2O_2 + Fe (II)) digestions were evaluated on sea plankton samples. In a second stage, the efficiency of 2-step digestion (3) combining KOH and H_2O_2 + Fe (II) at three different duration and temperature conditions was assessed (Fig. 1). Then, in a third stage, virgin MPs were subjected to 1- and 2-step digestions to evaluate the effect of digestions on plastic polymers (Fig. 1). Finally, in a fourth stage, virgin and weathered MPs from seawater samples were subjected to 3-step digestion, including (4) KOH, H_2O_2 + Fe (II) and, enzymes as reagents (Fig. 1). The details about the digestion's techniques are following:

2.1.1. One-step oxidative and alkaline digestions

For the alkaline digestion (1), the sample was soaked in 10% KOH solution at 60 °C for 24 h. For the oxidative digestion (2), 40 mL of 30% H_2O_2 were added three times every 20 min at 60 °C, including 40 mL Fe (II) 0.05 M solution only the first time. Selected reagents concentrations were based on previous studies (Masura et al., 2015; Prata et al., 2019; Lusher et al., 2020). Reagent KOH 85% (Kishida Chemical Co. Ltd., Japan) and purified water to prepare 10% KOH. In the case of 30% H_2O_2 (Kishida Chemical Co. Ltd., Japan), it was used directly. The use of iron catalysts in oxidative digestions led to increased digestion efficiency because of free radicals' formation (Masura et al., 2015; Prata et al., 2019). The Fe (II) 0.05 M was prepared according to Masura et al. (2015) using Iron (II) sulfate,7-hydrate, and H_2SO_4 (Kishida Chemical Co. Ltd., Japan). Stirring was performed very gently for 10 s only when reagents were added. The reaction temperature was always kept below 60 °C using a cold bath and adding purified water in small amounts when needed. Heating was performed when reagents were added until it reaches 50 °C. In both analyses, the sample was filtered through 0.2 mm mesh to collect any residual material.

2.1.2. Two-step digestions

In this case, the sample was first soaked in 10% KOH solution at three different temperature and duration combinations: (3a) 10% KOH at 60 °C, 72 h, (3b) 10% KOH at 60 °C 24 h, and (3c) 10% KOH at 40 °C 72 h (Fig. 1). After alkaline digestion, the sample was filtered with a 0.2 mm mesh to retain the remaining material, rinsing the surface with purified water. The pore size net used depends on the MPs size research target (≥ 300 μm). Then, the second step consists of oxidative digestion under the same conditions as those detailed for digestion (2) (Section 2.1.1).

2.1.3. Three-step digestions

For the (4) 3-step digestion, an enzyme complex treatment was applied following the (3c) alkaline digestion detailed above (Section 2.1.2). In previous trials, a remaining OM with a composition similar to cellulose was observed sometimes in environmental samples after 2-step digestions (Fig. 2). Therefore, a group of enzymes was chosen according to their potential to avoid its presence. A modified protocol from Löder et al. (2017) was applied with available domestic enzymes without the

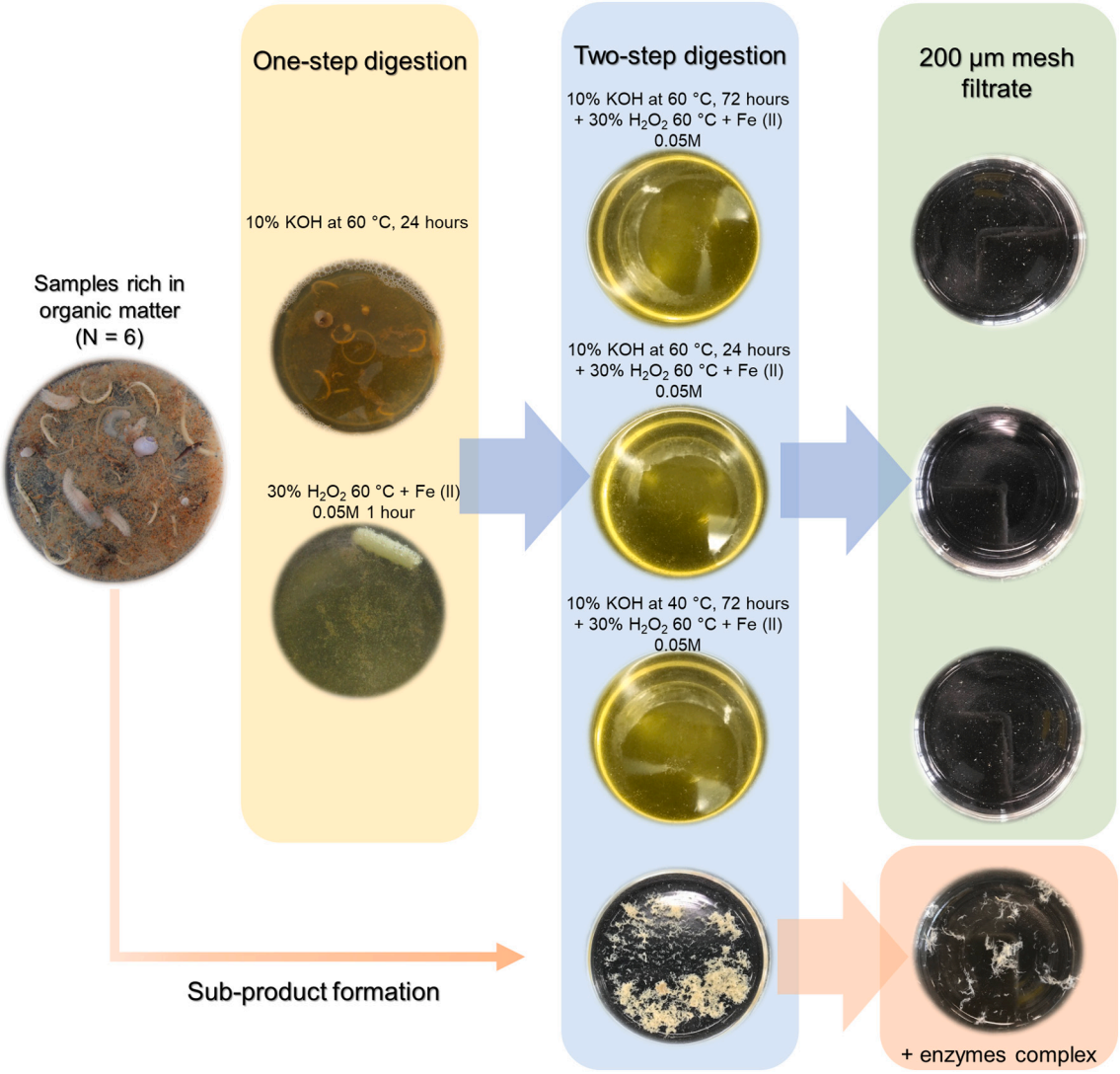

Fig. 2. Working flow diagram for the different organic matter digestion procedures with pictures of the results obtained.

detergent immersion step (sodium dodecyl sulfate) because no additional effect was found in previous trials. For this, 1000 U of cellulase (MP Biomedicals) were added three times (3000 U total) with 1000 U glucoamylase (Tokyo Chemical Industry), 40 mL amylase FL (ASA Spezialenzyme GmbH), 1 mL pectinase, and 500 mg of powder enzyme complex (cellulase A, cellulase T, hemicellulase, pectinase G, mannanase) (Amano Enzyme Co. Ltd.). The procedure was performed in an incubator system (Yamato Scientific Co., Ltd. IN604W) with a shaker (Yamato Scientific Co., Ltd. MK201D) at the enzyme's optimum pH and temperature conditions (40 °C, pH 5). Finally, the oxidative technique was applied as described above (Section 2.1.1).

2.2. Organic matter samples and analysis

Water samples obtained in previous sampling campaigns (July and August 2015) from the North Pacific Sea near Japan (142° 54′ E - 39° 55′ N) were used for OM digestions trials. A Neuston net (5552; RIGO Co., Ltd., Tokyo, Japan) provided with a flowmeter (5571A; RIGO Co., Ltd., Tokyo, Japan) was used for the sampling, with a mouth, length, and mesh size of 75 cm × 75 cm, 3 m, and 0.35 mm, respectively. The ship continuously towed the Neuston net around each station for 20–40 min at a constant 2–3 knots speed. These samples were selected based on their high proportion of plankton content. Plankton found in seawater samples were composed of individuals from the orders Calanoidea, and Cyclopoidea, the families Hyperiidae, Sagittidae, and Luciferidae, and in less proportion, some fish larvae and snail individuals. Between 4 and 5 g of plankton (wet weight) were concentrated using a 200 µm mesh (Fig. 2). OM digestion efficiency was qualitatively assessed six times for each digestion technique, documenting the results by taken photographs before and after treatments (Figs. 1 and 2). All tests were conducted in glass flasks previously cleaned and rinsed with purified water.

2.3. Microplastics weight, size, and polymer fingerprint assessment

To determine MPs' resistance to processes and possible polymer fingerprints changes, techniques (1), (2), and (3a) were applied with eight types of virgin plastic pellets (Japan Plastic Industry Federation): Low-density polyethylene LDPE, High-density polyethylene HDPE, Polypropylene PP, expanded Polystyrene EPS, Polystyrene PS, Polyvinyl chloride PVC, Polyethylene terephthalate PET, and Polycarbonate PC. Size details of plastic pellets are provided in Supplementary Table 1. The weight of ten pellets per polymer type was registered previous and after digestions (1) and (2) ($N = 80$) and five pellets per polymer for digestion (3a) ($N = 45$) with a precision digital scale (A&D GX-600, SD = 0.001 g). Pellets were previously dried at room temperature on a desiccator, and measurements were taken several times until stabilized weight values.

Table 1
Virgin MPs weight changes (g) before and after digestion trials and the corresponding recovery rates (RR, %) for LDPE, HDPE, PP, PS, EPS, PVC, PET, and PC. Grey values correspond to those different from 100%.

Polymer type	KOH			H_2O_2 + Fe			KOH + H_2O_2 + Fe		
	Before	After	RR%	Before	After	RR%	Before	After	RR%
LDPE	0.146	0.146	100	0.139	0.139	100	0.072	0.072	100
HDPE	0.161	0.161	100	0.164	0.164	100	0.077	0.077	100
PP	0.238	0.238	100	0.242	0.242	100	0.123	0.123	100
PS	0.167	0.167	100	0.159	0.159	100	0.086	0.086	100
EPS	0.013	0.013	100	0.028	0.028	100	0.006	0.007	116.7
PVC	0.367	0.367	100	0.436	0.436	100	0.197	0.195	99.0
PET	0.288	0.279	96.9	0.304	0.302	99.3	0.142	0.143	100.7
PC	0.148	0.148	100	0.145	0.145	100	0.073	0.072	98.6

Weight changes were presented as recovery rates (%). Then to evaluate possible polymer fingerprint changes, Fourier transform infrared spectroscopy with attenuated total reflection (FTIR-ATR) was applied to 3 pellets of each group to obtain the spectra previous and after digestions ($N = 24$). Bruker, ALPHA-P FTIR-ATR instrument at 4 cm^{-1} resolution, 4000–400 nm range, and 24 scans conditions were used to obtain the infrared spectrum of each polymer.

Based on the results obtained with weight pellets trials and OM digestion efficiencies (see following sections), techniques (3) and (4) were applied over six types of virgin plastic samples (Good Fellow Co.): LDPE, HDPE, PP, EPS, PVC, PET (Fig. 1). As technique (3) implies a combination of techniques (1) and (2) to shorten experiments, the assessment of MP resistance with (1) and (2) could be skipped if (3) showed no damage on plastic sizes. In this case, size changes were evaluated on a set of virgin plastics (Supplementary Table 1) carefully obtained from plastic sheets with a 3 mm diameter hole puncher tool (HDPE PP, PVC, PET, PET). Styrofoam granules (EPS) were obtained with tweezers from commercialized packaging material, and LDPE manufactured pellets (300–350 μm) were used (Cospheric Co.) (Supplementary Table 1). Twelve randomly selected virgin MPs of each polymer type were analyzed previous and after digestions to test technique (3a) ($N = 72$). In the case of digestions (3b) and (3c), only the polymers presenting problems with digestion (3a) were used for trials, using 20 virgin MPs per technique (Fig. 1, Supplementary Table 1). For technique (4), 20 virgin PET particles and ten of the rest of the polymers ($N = 70$) were used (Fig. 1, Supplementary Table 1).

Photographs of each particle were taken to register possible size changes. According to recent MPs research guidelines, size can be determined based on maximum Feret's diameter, defined as the distance between parallel tangents on opposite sides of particle's outline (GESAMP, 2019; Michida et al., 2019). Therefore, the maximum diameter was measured using free image processing software (ImageJ, National Institutes of Health). This definition and measuring method to determine MPs' size has been widely adopted in published literature (Serranti et al., 2018; Cowger et al., 2020; Rosal, 2021). Also, image analysis is widely used to measure the size in MPs studies (Lindeque et al., 2020; Cowger et al., 2020). Nevertheless, other techniques are also available for size measuring according to different definitions; therefore, we have to recognize the limitation of the present study to definitely conclude MPs size changes after digestions. Finally, FTIR-ATR was applied to obtain the spectra for three particles per group previous and after digestions to evaluate possible polymer fingerprint changes.

The last experiment applied technique (4) on naturally weathered MPs ($N = 9$) to determine their resistance to the selected digestion conditions (Fig. 1). Polymer's weathered fragments were collected from the same water samples as OM and identified as PE by FTIR-ATR. The obtained data has non-normal distribution for virgin MPs size changes, so the U-Mann Whitney test was applied to test the differences before and after digestions. Also, data were presented as median values and box plots. Whereas comparisons by pairs were applied for weathered MPs, as these were easily identified (color/shape). In this case, the obtained data followed a normal distribution (Supplementary Table 1), so a *t*-test for paired samples was applied. All statistical analyses were performed using XLSTAT software (Addinsoft, 2021).

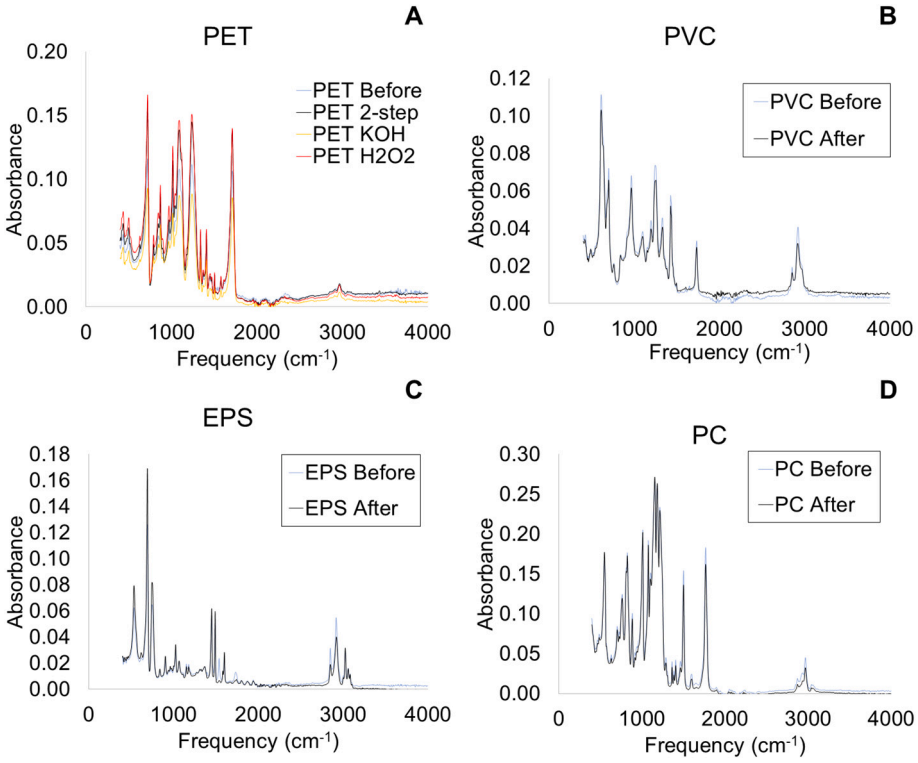

Fig. 3. FTIR spectra examples for virgin plastic pellets previous and after digestions for (a) PET pellets with 1-step and 2-step digestions and (b) PVC, (c) EPS, and (d) PC with 2-step digestion.

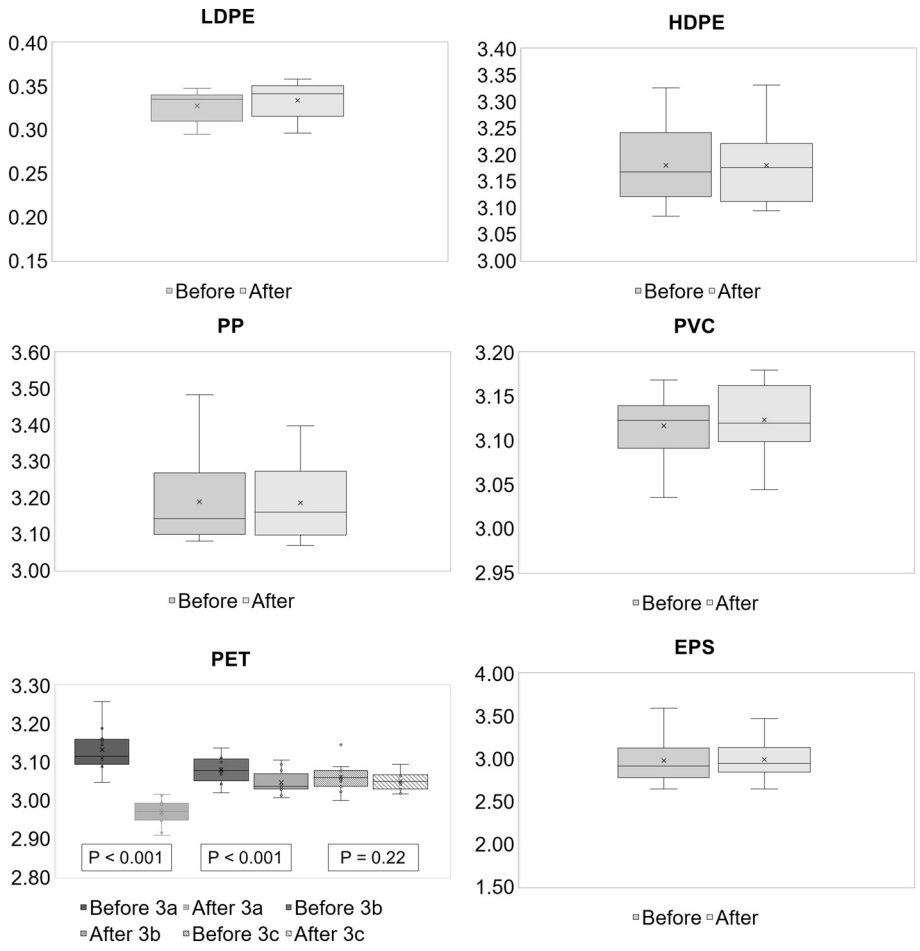

Fig. 4. Box plots for microplastics size changes before and after applying 2-step digestions (3a) for all polymers and (3b) and (3c) for PET.

3. Results and discussion

3.1. Organic matter digestions

From the qualitative assessment of OM digestions, it was found that 1-step alkaline and oxidative digestions at 60 °C presented a good performance, but with some material remaining (Fig. 2). After the alkaline digestion (1), a snail shell and a few shrimps remained after 24 h of digestion (Fig. 2). Other studies also reported good digestion efficiencies with alkaline and oxidative digestions (Cole et al., 2014; Karami et al., 2017; Prata et al., 2019). Nevertheless, besides the differences in duration and temperature in the procedures, the samples used as OM sources (e.g., fish tissue, mesozooplankton, algae, driftwood, feathers) and the used quantities (Prata et al., 2019) are varied, making it difficult to compare among studies. Prata et al. (2019) obtained good results with one oxidative digestion but using smaller OM quantities for digestion trials compared with this study. For example, Cole et al. (2014) use plankton samples but previously removed and rinse any large macrozooplankton. In this study, the remaining snail shell could be easily removed with tweezers, but the large crustacean presented more resistance to the tested chemical digestion. So, before sample digestion, large zooplankton removal could be included with these techniques, but this is highly time-consuming for regular monitoring.

Digestion efficiency assessment by gravitational methods was discarded in this study because of initial samples' water content bias or problems with efficiency values over 100% reported in previous studies (Prata et al., 2019). Other studies as Cole et al. (2014), avoid this issue by drying samples previously and after digestions. The recommended temperatures to eliminate water content with gravitational methods are around 60 °C, but some polymers have heat deflection temperatures at 55 °C (PP), 35 °C (LDPE) 50 °C (HDPE) (Qiu et al., 2016). Therefore, we recommend to do not use this procedure unless necessary to avoid MPs damage or deformation. The 2-step digestions presented better results, considerably reducing the remaining OM in samples (Fig. 2), with similar results at the three different conditions (Fig. 2). After filtrating the samples with a 0.2 mm mesh, the sample was almost completely clean, with little OM retained in the net filter. Another study applying 2-step digestion in similar conditions also registered good digestion efficiencies in freshwater samples with high OM content (de Carvalho et al., 2021).

In some cases, the presence of a dense sub-product after oxidative digestion was registered in 2-step digestions, mainly in plankton samples rich in organic matter, with algae presence (not identified) (Fig. 2). When analyzed under FTIR, this sub-product presented a composition similar to the cellulose compound. Other studies also registered a similar situation with OM-rich samples (Avio et al., 2015; Zhao et al., 2017) where MPs get entangled in "dense subproducts," hampering the following analyses. Nevertheless, no photographs of the sub-products were taken to compare results. Therefore 3-step digestion was tried, adding an enzyme complex to eliminate any exceeding material that possibly triggers the presence of sub-products after oxidative digestion. As a result, the presence of this material was significantly but not completely reduced (Fig. 2). However, as reported in previous studies, the results will depend on each sample's OM matter content (Avio et al., 2015; Zhao et al., 2017), and the sub-product was not always present. So, it is not easy to set a priori if the enzyme digestion step will be necessary considering it is time and cost consuming (Löder et al., 2017). In conclusion, 2-step digestion was a good selection in terms of the

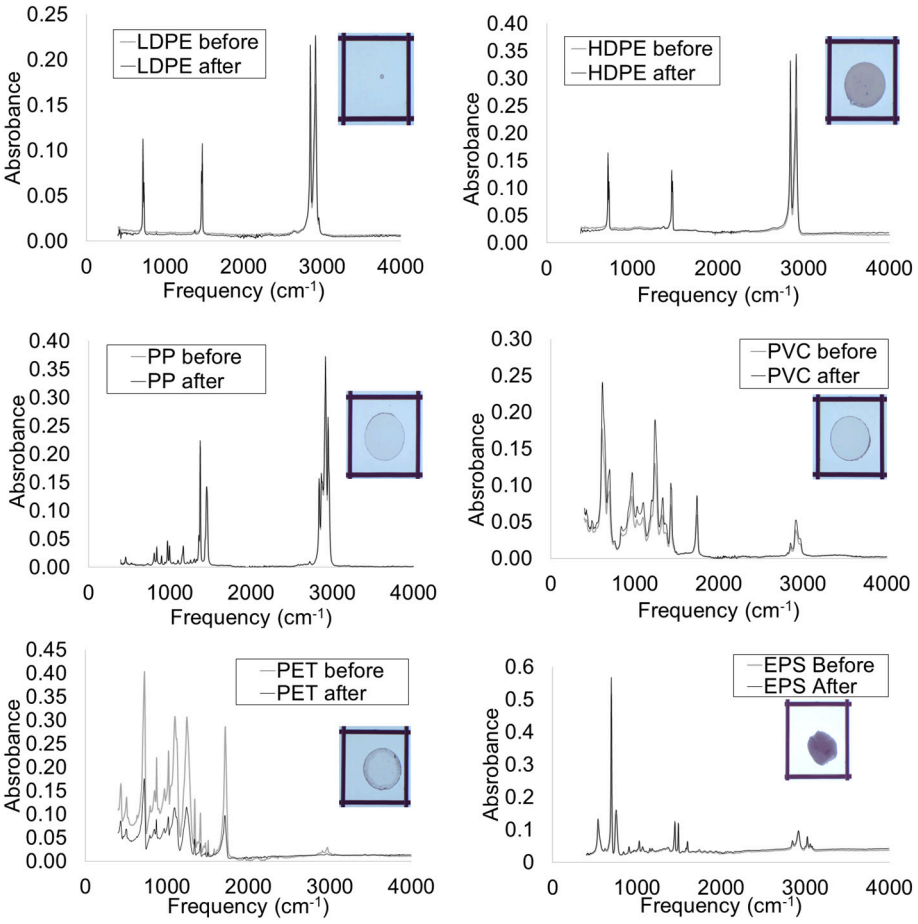

Fig. 5. FTIR microplastics spectra obtained previous and after digestions for virgin microplastics samples.

obtained results, time-consuming, and costs, but 3-steps digestion could be considered if a problem with sub-products is registered.

3.2. Microplastics resistance assessment

Based on OM digestion results, we evaluate possible MPs weight and size changes under different temperature and duration conditions, including polymer fingerprint changes for virgin and weathered plastics. Following the main results are presented:

3.2.1. Microplastics weight and polymer fingerprint

The weight changes and recovery rates for virgin MPs after digestions (1), (2), and (3a) are presented in Table 1. PET pellets presented weight reduction after alkaline digestion. However, when 2-step digestion was tried, PET and EPS pellets presented an unexpected increase in recovery rates (Table 1). In contrast, PVC and PC pellets decreased weight recovery rates with 2-step digestion (Table 1). Another study registered PET damage and weight loss (17%) with 2-step digestion applying KOH at 60 °C (de Carvalho et al., 2021). Notably, the authors registered significant weight changes for PET pellets from Sigma Aldrich Company, but no significant weight changes from PET samples from Good Fellow Co. They attributed this difference to the plastic formulation and chemical stability. Also, Treilles et al. (2020) registered PET damage on fibers using KOH at 60 °C. Previous studies also registered unexplained weight increases, with Kühn et al. (2017) attributing these changes to moisture presence, especially for foams. Prata et al. (2019) also recorded PP and LDPE weight increases discarding moisture, salts, and iron presence on particles. Dehaut et al. (2016) also registered unexplained weight variations in HDPE and PP. This study discarded moisture presence as samples were dried on a desiccator, and weight measures were made up to stabilized values. Therefore, based on this study and previous research, explaining MPs damage based on weight recovery rates presents some difficulties and unexplained values, and conclusions should be taken cautiously. Concerning polymer fingerprints, these did not present significant differences before and after digestions (Fig. 3), making it possible to identify polymers independently of the digestion method.

3.2.2. Microplastic size and polymer fingerprint assessment with 2-step digestion

When comparing 2-step digestion effects on virgin plastics under different conditions (Fig. 1), significant changes in MPs size were found only for PET with techniques (3a) ($p < 0.001$) and (3b) ($p < 0.001$) (Fig. 4, Supplementary Table 1). PET MPs were reduced in size after (3a) and (3b) digestions (Fig. 4). These results partially differ from weight changes where a decrease with technique (1) and an increase with (3a) were observed (Table 1). In contrast, the rest of the polymers did not present significant differences (Supplementary Table 1, Fig. 4). Assuming that weight loss should imply a size reduction, the results registered by de Carvalho et al. (2021) differs for PET polymers from Good Fellow Co. (same as this study), as the authors stated no significant changes with this brand but significant ones with those from Sigma Aldrich Co. With technique (3c), where temperature conditions were lower in the KOH step (40 °C), PET MPs did not present significant size differences ($p = 0.22$) (Supplementary Table 1, Fig. 4). Treilles et al. (2020) also registered PET preservation applying these digestion conditions on microfibers. Although the 2-step digestion (3a) was certainly efficient in reducing the remaining OM in samples (Fig. 2), adopting moderate digestion conditions as shown here is recommended for the safety of the accurate quantification of MPs that may include PET. All

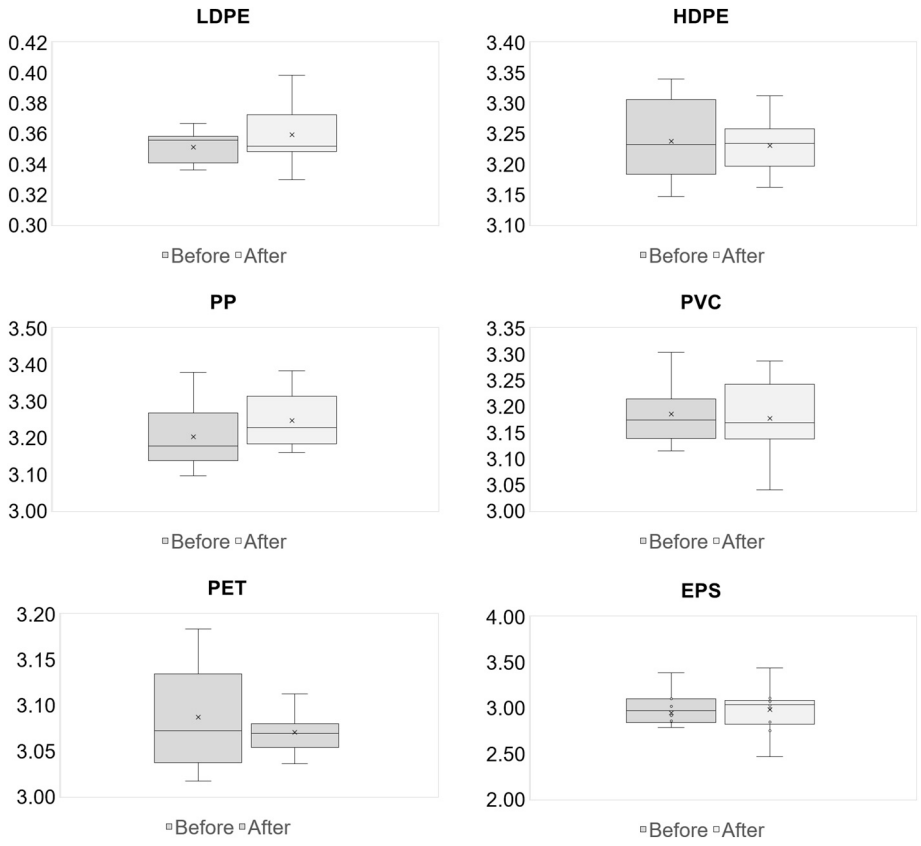

Fig. 6. Box plots for microplastics size changes before and after applying 3-step digestion 10% KOH at 40 °C, 72 h, enzymatic complex and, 30% H_2O_2 60 °C with Fe (II) 0.05 M.

photographs taken before and after digestion looks similar in shape and size, which indicated that this kind of change could not be perceived by only visual inspection (Supplementary Data 1). Concerning polymers conditions, FTIR spectra before and after digestion did not present any particular difference (Fig. 5), with the same fingerprints for each polymer tested. These results coincided with those for plastic pellets (Fig. 3) and other studies where polymers characterization was preserved after digestion trials (Munno et al., 2018).

Previous studies also registered MPs damage with alkaline digestions at temperatures above 40 °C (Munno et al., 2018; Thiele et al., 2019). A recent study in plankton samples registered PS damage with 10% KOH digestion using 50 °C temperatures (López-Rosales et al., 2021). Also, Thiele et al. (2019) registered Rayon damage with KOH at 60 °C. Karami et al. (2017) registered color loss with KOH at temperatures ≥50 °C. In addition, Nuelle et al. (2014) registered size damage in polyethylene (PE) and polypropylene (PP) MPs < 1 mm applying 30 and 35% H_2O_2, seven days. Studies applying 2-step digestions also registered MPs damage at higher temperatures or for extended digestion periods. Munno et al. (2018) applied KOH (224 g L^{-1}) and 35% H_2O_2 up to 68 °C, and Fenton's 35% H_2O_2 up to 93 °C with significant differences in MPs recovery rates. Additionally, Cole et al. (2014) found damage on Nylon (Ny), PVC, and PE MPs applying 10 M NaOH at 60 °C, 24 h. Furthermore, problems as color loss were registered in PE, PA, and PET at high digestion temperatures (Munno et al., 2018). Therefore, according to the results obtained in this and previous studies, MPs could present significant size, number, weight, and color changes applying high temperatures, prolonged digestions, or high reagents concentrations. In contrast, polymer fingerprints are less likely to present identification problems.

Other studies also found damage in different polymers to those analyzed here as cellulose acetate (CA) (Dehaut et al., 2016). A recent study stated that CA in cigarette butts is a significant source of microfibers for the environment but received less attention probably because current digestion protocols destroy these particles (Belzagui et al., 2021). They proposed a modified Fenton's digestion (75 °C, 20 min total) to avoid CA damage; however, those time/duration conditions will probably work for samples poor in OM content. Similarly, another study found AC with up to 20% of sample composition using 1 M sulfuric acid to clean water samples from treatment plants (Pivokonský et al., 2020). So, it is clear that CA is probably widely distributed in the environment; however, new digestion techniques are still needed to study these particles in water samples rich in OM.

3.2.3. *Microplastic size and polymer fingerprint assessment with 3-step digestion*

With 3-step digestions, polymers did not present significant size changes after digestion trials (Fig. 6, Supplementary Table 1). These results confirm that lower temperatures prevent PET from size change ($p = 0.24$) as determined with technique (3c), even when enzyme digestion is included (Fig. 6). The obtained FTIR spectra did not present any significant change in polymer's fingerprints, confirming that the selected conditions are suitable for MPs analyses (Fig. 7). When weathered PE MPs were tested under digestion technique (4), no significant change in size was registered ($p = 0.34$) (Fig. 8). Polymer fingerprint was also preserved after digestion (Fig. 8), presenting a "cleaner" spectrum, probably explained by the loss of any biofouling or OM in MPs surface. The photographs also registered this difference, where particles looked cleaner after digestions (Fig. 8, Supplementary Data 1). These results confirm that using the proposed method also prevents weathered PE from size change as determined with other polymers in virgin plastic samples with technique (3c).

4. Recommendations and conclusions

This study assessed the OM digestion efficiency on seawater plankton

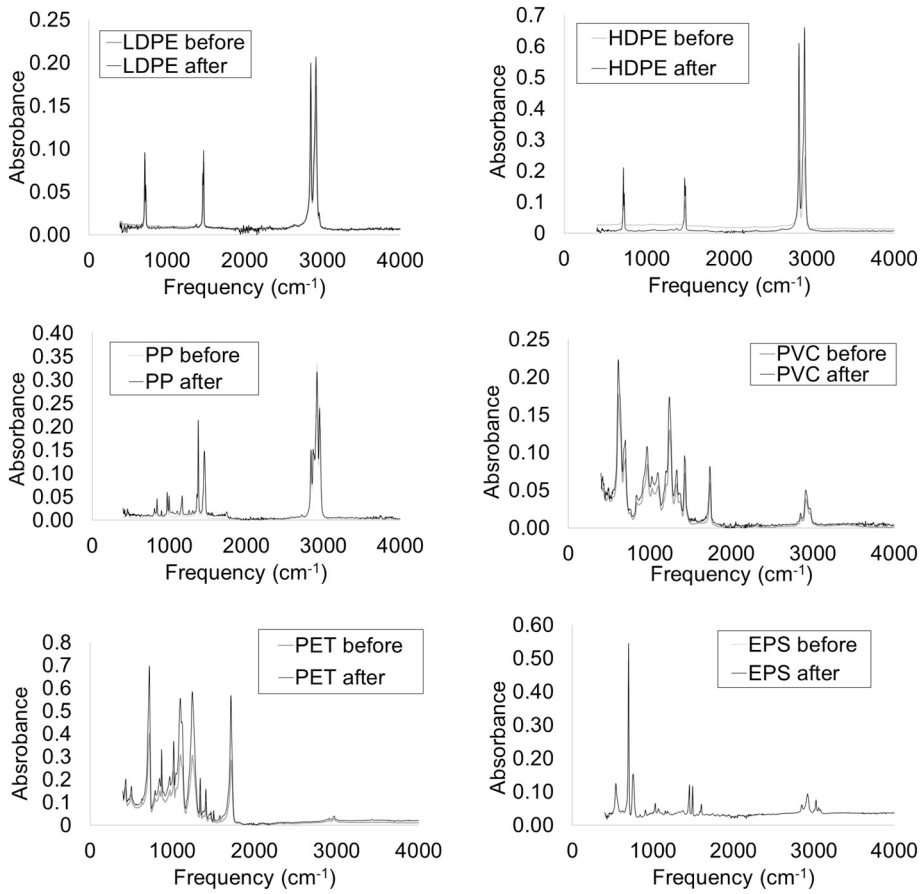

Fig. 7. FTIR microplastics spectra obtained previous and after 3-step digestion for virgin microplastics samples.

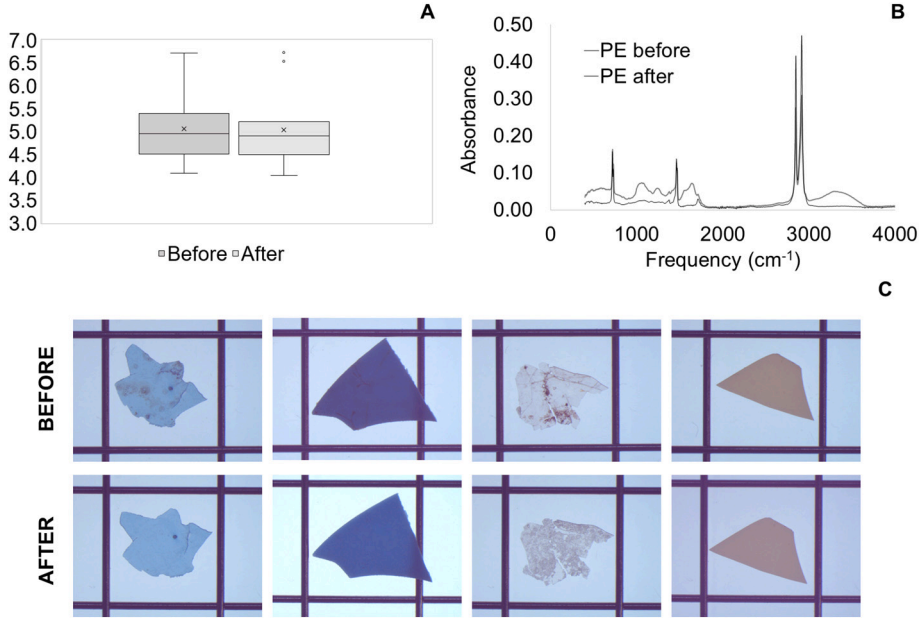

Fig. 8. Representation of the results obtained for weathered PE microplastics: (a) Box plot for size changes, (b) FTIR spectra example and, (c) photographs obtained before and after digestion for different items.

samples and the MPs weight, size, and polymer changes under different steps, duration, and temperature conditions techniques. According to our results, plankton samples rich in OM obtained the best results with 2-step and 3-step digestions using KOH and H_2O_2 + Fe (II) as reagents. Nevertheless, including enzymes in digestion protocols is recommended only for particular situations because they are costly and time-consuming.

Based on the results obtained in the present research, any method

applying aggressive reagents as acids, high temperatures, and prolonged digestions will damage MPs. Weight changes were found for PET pellets with 1- and 2-step techniques and EPS, PVC, and PC with a 2-step technique at high temperatures (60 °C). However, weight damage assessment presents some unexplained increased values, and further analyses are needed. Concerning size changes, PET particles presented size decrease applying 2-step digestion. The use of 60 °C temperature for 72 and 24 h in the first step with KOH lead to PET damage. Whereas KOH digestion at 40 °C for 72 h prevailed all the polymers tested from size changes. However, the arguments concerning size changes should be further examined based on different definitions of MPs sizes and measuring protocols that may be adopted in other studies. Concerning polymer fingerprints, they were not affected by any digestion techniques, assuring polymer identification. It was also confirmed that enzymatic digestions, when necessary, did not present a risk under the proposed conditions, even for weathered PE particles.

In conclusion, these results suggest that previous studies applying aggressive digestion techniques could be reliable about polymer identification but could present biased results on size values. Future studies should not base their digestion protocol on OM digestion efficiency and ignoring possible MPs' damage. Considering the exponential increase in the number of MPs studies, with the MPs <100 μm and nanoplastic fraction becoming more relevant, avoiding MPs size damage results essential for their analysis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.113027.

CRediT authorship contribution statement

María B. Alfonso: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Kayoko Takashima:** Conceptualization, Methodology, Investigation, Data curation, Writing – review & editing. **Sayaka Yamaguchi:** Investigation, Data curation. **Mie Tanaka:** Investigation, Data curation. **Atsuhiko Isobe:** Investigation, Writing – review & editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This study was carried out when MBA stayed at the Research Institute of Applied Mechanics (RIAM), Kyushu University, with an invitation program for overseas researchers. AI was supported by the Environmental Research and Technology Development Fund (JPMEERF18S20201) of the Ministry of the Environment, Japan, KAKENHI (Grant number 21H05058) of Japan Society for the Promotion of Science and SATREPS of Japan International Cooperation Agency and Japan Science and Technology Agency.

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