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Methodology optimization to quantify microplastic presence in planktonic copepods, chaetognaths and fish larvae

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ABSTRACT

Two of the groups most impacted by microplastics (MPs) are zooplankton and fish larvae, either through MPs ingestion or absorption. Although there has been an increase of studies focusing on MPs ingestion by these organisms, there is still no standardized methodology for the quantification of MPs present in plankton. For example, some reagents normally used to digest plankton and recover MPs appear adversely to affect some plastic characteristics. This can potentially lead to underestimating the amount and types of MPs present in the organisms analyzed. Hence, this work aimed to optimize a methodology to quantify MPs present in plankton, namely zooplankton and fish larvae, and ensuring MPs integrity. Hence, the planktonic organism tissues were digested using 30% (v/v) H_2O_2 solution at different temperatures and incubation periods while preserving the integrity and polymer characteristics of 13 types of MPs. MPs' characteristics were register before and after the tests, by visual inspection and Fourier Transform Infrared Spectroscopy (FTIR) analysis, to evaluate the integrity and features of MPs. With this methodology, MPs recovery was above 85% for all types of plastic tested. The proposed methodology is a rapid protocol, with a maximum of 7 h of incubation, that ensures simultaneously the full digestion of the organism tissues and the complete preservation of all the plastic characteristics, namely color, size and polymer type.

- A methodology was optimized to quantify microplastics present in zooplankton (copepods, chaetognaths and fish larvae).
- Thirteen types of microplastics (fibers and fragments of different polymers) were used to test the efficiency of the methodology ensuring the maintenance of the integrity of plastics.
- With this methodology, microplastic recovery was above 85% for all the types of microplastic tested and no changes in their characteristics were observed.

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Specifications table

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More specific subject area:	Microplastic pollution
Name of your method:	Quantification of microplastics present in planktonic organisms
Name and reference of original method:	Avio et al. [10]
Resource availability:	NA

Background

Plastic pollution, especially microplastic pollution (MPs – plastic particles < 5 mm), has received much attention in recent years since they are a potential threat to aquatic ecosystems and many marine organisms [1,2]. One of the most impacted groups is zooplankton since the size of MPs is the same as zooplankton prey. These organisms can either actively or passively ingest MP during filtration or as food [3]. Although an increasing number of laboratory experiments have been performed showing a variety of effects of MPs on zooplankton, the assessment of MPs ingestion by these organisms and their effects is still scarce. The small size of these organisms and the even smaller size of MPs can explain the scarcity of studies of MPs ingestion by zooplankton and fish larvae [2,4]. However, it is important to understand whether MPs are present in zooplankton, in what quantities, and with what effects of MP ingestion. Despite recent research, there is no standardized methodology for the quantification of ingested MP in zooplankton [5]. More importantly, from the small number of methodological studies, most did not assess whether that methodology affects the integrity of MPs [4].

To date, the quantification of MPs in biological samples has been made using several methods, including using acidic (HNO_3), enzymatic (lipase), and oxidative (H_2O_2) digestions of biological tissues [5]. For both water and sediment, H_2O_2 is the most common reagent used to digest organic matter [4]; however, to digest organisms such as zooplankton or bivalves, KOH is the most common digestion solution used [6]. HNO_3 has been also used to quantify MPs although previous studies showed that this solution may affect the integrity of some specific plastic polymers, namely nylon, polyethylene terephthalate, and biopolymers [7]. It is emphasized that there is a wide diversity of methodologies to eliminate (digest) biological tissues for MPs quantification, but only a few studies evaluated the organism tissue digestion efficiency and simultaneously the maintenance of MPs integrity (e.g., [8,10]). Changes in color, weight and polymer structure have been reported in several digestion methodologies that may affect the integrity of certain types of polymers, particularly when the reagent is used in combination with high temperatures [9]. This can potentially underestimate the abundance of MPs present in the organisms [1,5].

According to Hara et al., [5], the methodologies used to quantify MPs in biota have varied greatly between studies, hence a standardized methodology is especially important for quality control as well as comparability of results among studies. The methodology tested in this study was based on the method developed by Avio *et al.*, [10] that uses H_2O_2 to extract MPs from fish tissue. This is one of the most used methods to extract MP from organism tissues; however, this method was only tested with two types of plastic polymers (polyethylene and polystyrene fragments) and was developed to be applied to adult fish tissues. More recently, Aytan *et al.*, [13] applied this methodology to copepods using 30% (v/v) H_2O_2 solution with an incubation period of 48 h but there have been no tests with other plastic polymers. Hence, the present study advances the optimization of a methodology to assess MP contents in planktonic organisms. As such, the methodology optimized in this study was specifically designed to verify the most efficient and effective MPs extraction method from zooplanktonic organisms and to ensure both (i) the complete elimination of the planktonic organism tissues, as well as (ii) the preservation and integrity of 13 types of MPs in terms of size, color, visual aspect and polymer characteristics.

Method details

Preparation

Quality assurance/quality control (QA/QC)

As with any chemical analytical techniques, when working with MPs, it is of utmost importance to include QA/QC measures throughout the study to provide reliable and comparable results [11]. The present study included precautions to avoid any contamination, including: i) all the materials used were made of glass; only in a few exceptions polymers materials were used, e.g., sieving of water samples to collect fish larvae; ii) cleaning steps were applied before usage as also rising steps (including washing with deionized water and ethanol before use) to avoid any contamination; iii) cleaned equipment was covered when not in use with aluminum foil; iv) colored cotton laboratory coats were use at all steps thus minimizing potential synthetic fiber contamination; v) all the solutions were previously filtered and checked under a stereomicroscope (through 0.45 μm pore size nitrate cellulose membrane filter) before using; vi) all the materials were double-checked: glass material was checked under a stereomicroscope after the washing process, filters were checked under a stereomicroscope prior to use; and vii) all samples were processed in a fume hood at all times.

Procedural blanks are necessary to quantify and understand the sources of background contamination in the laboratory and so blank samples were included during the processing of samples. For example, during visual inspection in the stereomicroscope or filtration steps in the fume hood, a clean glass Petri dish with filtered deionized water was left exposed nearby to capture any possible airborne contamination, and immediately observed under a stereomicroscope (Leica EZ04). Also, blank samples (with only filtered deionized water or filtered H_2O_2) were subjected to the protocol together with the tests/field samples and inspected under a stereomicroscope at the end of the protocol. Both types of blanks were always inspected and none of them had any type of contamination.

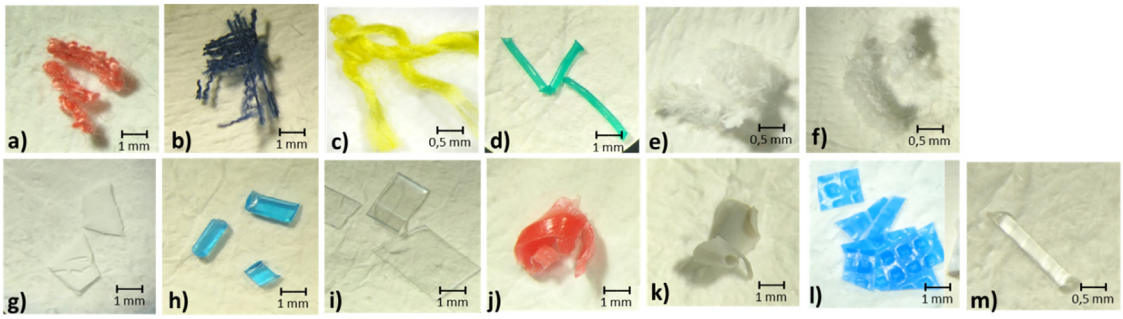

Fig. 1. Particles prepared for methodology testing: (a) Rayon fibers; (b) Polystyrene fibers; (c) Acrylic fibers; (d) Polyethylene fibers; (e) Polyamide fibers; (f) Lycra fibers; (g) Cellulose acetate fragments; (h) Polypropylene fragments; (i) Polyethylene Terephthalate fragments; (i) Polyethylene of high-density fragments; (k) Polyvinyl chloride fragments; (l) Polyethylene of low-density fragments and (m) Polyamide fragments.

Microplastics preparation

The MP types used in this study were selected based in the most common items and polymer types of marine litter (e.g., [16,23]) as plastic bottles (polyethylene terephthalate and polyethylene of high-density), fishing nets (polyamide and polyethylene), plastic bags (polyethylene of low-density) and cigarettes (cellulose acetate). Although fibers are the main type of MP collected (e.g., [16,23]), tests that use fibers are not usual. Hence, a variety of fibers from the most common types of polymers used in clothes (e.g., Rayon, Acrylic, Lycra) were also tested. The MPs were produced from larger objects acquired specifically for the tests as e.g., plastic bottle, PVC tube, fishing net and fabrics. Tests were performed in 6 types of fibers and 7 types of fragments. Fibers were made of rayon (Ra), polystyrene (PS), acrylic (Ac), polyethylene (PE), polyamide (PA) and lycra (Ly), and fragments were made of cellulose acetate (CA), polypropylene (PP), polyethylene terephthalate (PET), polyethylene of high-density (HDPE), polyvinyl chloride (PVC), polyethylene of low-density (LDPE) and polyamide (PA) (Fig. 1). Together, thirteen types of MPs were used in the tests (Fig. 1). All the particles were manually produced with scissors from larger products (fabrics, bottles, fishing nets, tubes, etc.) and sieved through 5 mm mesh sieves to discard particles >5 mm.

To ensure the maintenance of integrity and features of MPs, their characteristics were registered before and after the tests, both by visual inspection to observe their color, range of sizes and visual aspect (e.g., shape, relative appearance of the surface and edges, tightness of fibers), and by Fourier Transform Infrared Spectroscopy (FTIR) analysis to identify their polymer. Polymer spectra were registered in a PerkinElmer (Waltham, MA, USA) FTIR Spectrum 2 instrument coupled with attenuated total reflectance (ATR).

Methodology optimization

Preliminary tests (tests A and B)

Preliminary tests were divided into two types: tests A performed with all selected MPs at two different temperatures (room temperature (20–25 ˚C) and 65 ˚C) and three different periods of incubation (24, 48 and 72 h) at rest; tests B performed with fish larvae at the same two temperatures (room temperature between 20 and 25 ˚C) and 65 ˚C) and at the same periods of incubation (24, 48 and 72 h) at rest.

For tests A, the following methodology was applied (Fig. 2): an initial mass of 2 mg of each MP type was defined per sample. For each MP type and each condition, 3 replicates were prepared. MPs were weighed and transferred to 26 ml scintillation glass vials. For tests B the following methodology was applied (Fig. 2): fish larvae were selected from previous collected samples under a stereomicroscope, including specimens from different larval stages, sizes and families (Gobbidae, Labridae, Clupeidae, Gadidae, Soleidaea). Fish larvae were divided into three types: elongated and thin body (Clupeidae); average body length (Gobbidae; Gadiidae), and large and high body length (Labridae, Soleidae). The selected organisms were rinsed with deionized water several times and inspected under a stereomicroscope to ensure that no type of particle was attached to the organism. Groups of 30 organisms, in triplicate, were placed in 26 ml scintillation glass vials. After each sample being added to the respective scintillation vial, the methodology was similar to both tests (A and B). In summary, 2 ml of 30% (v/v) H_2O_2 solution was added to each scintillation vial (either with one type of MP or one type of fish larvae) ensuring the sample was completely submerged and then the vials were closed. The samples were placed at different temperatures (room temperature and 65 ˚C) and different periods of incubation (24, 48 and 72 h). After the period of incubation, samples were filtered in a glass filtration vacuum pump system through a 0.45 μm pore size nitrate cellulose membrane filter. The filters were then transferred into a clean glass petri dish and left to dry at room temperature. After that, filters were examined under a stereomicroscope to detect any change in color, size or visual aspect (e.g., shape, relative appearance of the surface and edges, tightness of fibers) in the case of the samples with MPs, and to ensure completeness of digestion of the tissues in the case of the samples with organisms. A representative sample of these MPs were also analyzed through FTIR before and after the tests to ensure that the polymers were still identifiable and that no significant change in FTIR spectra occurred. In addition, each sample with MPs was weighed before and after the protocol to calculate the recovery rate (Recovery rate = (Final weight/Initial weight) x 100%).

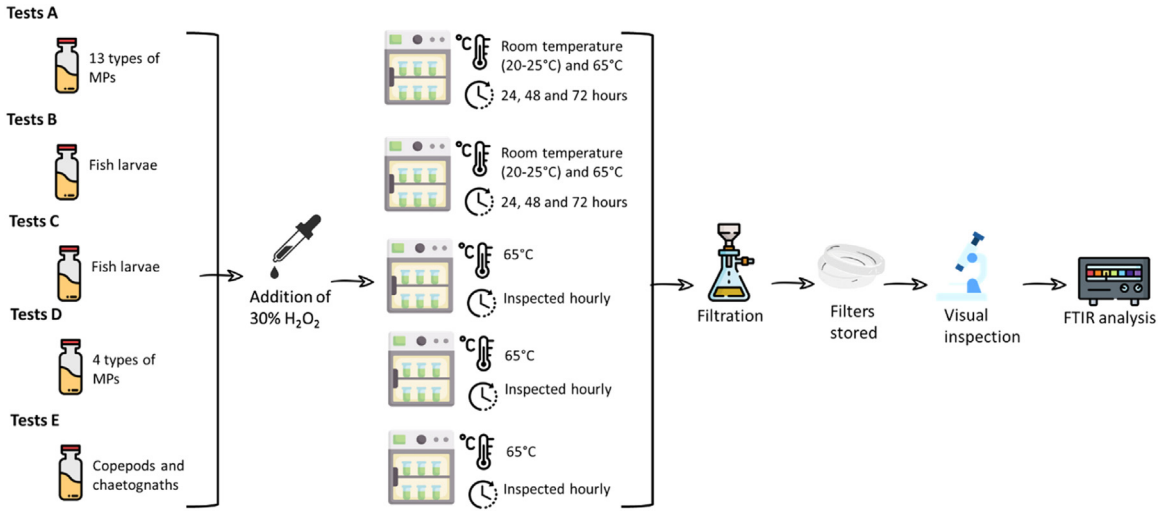

Fig. 2. Schematic representation of the different tests performed to optimize a protocol to quantify microplastics present in planktonic organisms: samples were prepared according to each test (A to E), 30% (V/V) H_2O_2 was added to all the samples, different temperatures and periods of incubation were applied; all the samples were then filtered, stored in petri glasses, visually inspected under a stereomicroscope and subjected to FTIR analysis.

Subsequent tests (test C, D and E)

After the results of the preliminary tests, three other tests were carried out (Fig. 2): tests C) performed with fish larvae at 65 °C and hourly visual inspection until a maximum of 24 h of incubation (at rest) with 30% v/v H_2O_2 solution; tests D) performed with specific MP types (Acrylic fibers, Rayon fibers, PP fragments and LDPE fragments) at 65 °C and hourly visual inspection until a maximum of 24 h of incubation (at rest) with 30% v/v H_2O_2 solution; and tests E) performed with other groups of zooplankton, namely copepods and chaetognaths at 65 °C and hourly visual inspection until a maximum of 24 h of incubation (at rest) with 30% v/v H_2O_2 solution. In these three sets of tests (C, D and E), the samples were visually inspected to observe any changes in the MP or the stage of digestion of the organisms. For tests D and E, all the organisms selected were picked from previous collected samples and thoroughly rinsed with deionized water several times and inspected under a stereomicroscope to ensure that no type of particle was attached to the organism. Three replicates were prepared for each taxon, each with 30 organisms. For tests D, 2 mg of each type of plastic was created per sample in 3 replicates. In all the scintillation glass vials (from tests C, D and E) were added 2 mL of 30% v/v H_2O_2 solution, then the vials were closed, placed in the oven at 65 °C and inspected hourly for a maximum of 24 h. After that, the methodology followed was the same as that used in the preliminary tests: filtered using 0.45 μm pore size nitrate cellulose membrane filters, filters stored in glass petri dishes, dried, weighted, and visually inspected under a stereomicroscope. Again, the filters with MPs were inspected to detect any change in color, size or visual aspect; a representative sample of these MPs were also analyzed through FTIR to ensure that the polymers were still identifiable and that no significant change in FTIR spectra occurred. The samples were weighed before and after the protocol to calculate the recovery rate (Recovery rate = (Final weight/Initial weight) x 100%). In the case of samples with organisms, filters were inspected to ensure complete tissue digestion.

Statistical analysis

To assess the effect of temperature and type of MPs on the recovery rates of each type of MPs, a two-way ANOVA was used with "Temperature" and "Type of MP"' as fixed factors. To assess the effect of period of incubation on the recovery rates of each type of MPs, a one-way ANOVA was used with "Period of incubation" as fixed factor. ANOVA assumptions of homogeneous variance and normally distributed data were analyzed prior to the ANOVA [12]; homogeneity of variance was tested with the Cochran test and statistical analyses were performed with TIBCO Statistica^{TM} 14.0 software.

Field samples

After the methodology was optimized, planktonic samples previously collected from the field were used to validate the method. Fish larvae used were collected in November 2017 in the Douro River Estuary (NW Portugal) using a 500 µm mesh planktonic net. Samples were sorted and specimens of the flatfish *Solea senegalensis* were selected for further analysis. All selected fish larvae were rinsed several times and kept in 26 ml scintillation glass vials. Copepods and chaetognaths were also collected in the Douro River Estuary using a 150 µm mesh planktonic net. Zooplanktonic samples were sorted to select copepods and chaetognaths (the species were not differentiated); and groups of approx. 30 individuals in 1 replicate were placed into 26 ml scintillation glass vials for

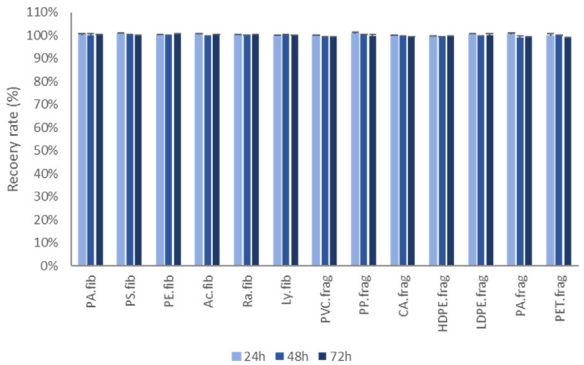

Fig. 3. Recovery rates (%) (mean ±standard deviation) for all the types of microplastics subjected to 30% v/v H_2O_2 solution for a period of incubation of 24, 48 or 72 h at room temperature (20–25 ˚C). Fibers (fib): PA (polyamide), PS (polystyrene), PE (polyethylene), Ac (acrylic), Ra (rayon) and Ly (Lycra); fragments (frag): PVC (polyvinyl chloride), PP (polypropylene), CA (cellulose acetate), HDPE (polyethylene of high-density), LDPE (polyethylene of low-density), PA (polyamide) and PET (polyethylene terephthalate).

digestion analyses. The zooplankton grouped together was always of the same group (i.e., copepods or chaetognaths). The organisms were also rinsed several times and kept in scintillation glass vials. In all the vials, 2 ml of 30% (v/v) H_2O_2 was added, being placed in an oven at 65 ˚C for 7 h (with the number of hours defined according to the results obtained after the preliminary and subsequent tests). Samples were subsequently filtered using 0.45 μm pore size nitrate cellulose membrane filter and stored in a glass petri dish. Following this treatment, each filter was analyzed under a stereomicroscope to examine and count MPs.

Method validation

Preliminary tests (tests A and B)

Regarding temperature, the MPs subjected to the protocol at room temperature showed no difference in size, color, or visual aspect (e.g., shape, relative appearance of the surface and edges, tightness of fibers). FTIR analysis also showed no differences in the spectra of MPs polymers before and after the protocol (Supplementary material – Fig. S1). Moreover, all the types of MPs tested had recovery rates > 99% (Fig. 3). However, four types of MPs showed a decrease in weight with the increase in time of incubation (PVC fragments, CA fragments, HDPE fragments and PA fragments). However, according to the 1- way ANOVA results these decreases of weight were not statistically significant (1-way ANOVA DF= 3, $F = 0.45$ and $p = 0.72$) (Supplementary material – Table S1). Moreover, the recovery rates were always > 99% and no other type of changes/degradation was observed (e.g., changes in color, visual aspect or size) or even difference in FTIR spectra.

At 65 ˚C, the Ra and Ac fibers showed some loss of color after 24 h, and LDPE fragments completely lost their color after 24 h. After 72 h, also Ac fibers completely lost their color and PP fragments showed some loss of color (Fig. 4 and Table 1). The remaining MP types (PS fibers, PE fibers, PA fibers, Ly fibers, CA fragments, PET fragments, HDPE fragments, PVC fragments, PA fragments) showed no changes in color, size or visual aspect after any of the three times of incubation tested. Recovery rates of MPs were high, with values above 71%. At 65 ˚C for 24 h, the lower recovery rate was around 85% for Ac fibers; for 48 h it was 71% for PS fibers, and for 72 h it was 81% for PA fibers (Fig. 5). It is of note that these slightly lower values were not always observed for the same polymer, perhaps indicating a slight loss of fibers during the filtration process (lost in the filtration or passing the filters from one place to another). PVC fragments, CA fragments and PA fragments showed some increasing loss of weight with the increasing of time of incubation; however, these decreases of weight were not statistically significant (1-way ANOVA: DF=3, F=0.45 and p=0.72) (Supplementary material – Table S1) and the recovery rates for these three types of MPs were always >83 % (Fig. 5). There were no significant differences in the recovery rates of the different types of MPs (2-way ANOVA: DF=12, F=1.12, p=0.35), nor significant differences in the recovery rates between MPs exposed to 30% v/v H_2O_2 solution at room temperature and 65 ˚C (2-way ANOVA: DF=1, F=0.92, p=0.34), and there was no significant interaction between the two factors (2-way ANOVA: DF=12, F=1.13, p=0.34) (Supplementary material – Table S1). The three types of MPs that showed a decrease of weight at 65 ˚C (PVC fragments, CA fragments and PA fragments) also showed a decrease of weight at room temperature, hence, these MPs may be affected by the 30% v/v H_2O_2 solution regardless of the temperature of incubation. It is possible that this loss of weight could be due to the degradation of the additives on the surface of some weathered polymers during the digestion; however, according to the FTIR analyses, no changes in spectra were observed for any of the MPs used in tests (Supplementary material – Fig. S1). So, the use of this methodology should not compromise the polymer identification by FTIR. In summary, it is important to denote that although some types of plastic showed loss of color (Ra fibers, Ac fibers, LDPE fragments and PP fragments) and others a slight decrease of weight at 65 ˚C (PVC fragments, CA fragments and PA fragments), none of the MPs tested showed more than one type of change (e.g., change in color and change in size or change in color and change of format).

In terms of fish larvae subjected to the protocol at room temperature, even after 72 h of incubation, only the loss of body pigmentation was observed, but the bodies were still intact (Table 1). Although at room temperature MPs showed slightly better

Table 1
Resume of the results obtained in the tests performed with all microplastics (MPs) selected and planktonic organisms at different temperatures (room temperature and 65 °C) and different periods of incubation (7, 24, 48 and 72 h). ✓ means that there was no changes in color, size, visual aspect (e.g., shape, relative appearance of the surface and edges, tightness of fibers), weight or FTIR spectra, in the case of MPs, or that the organisms were completely digested, in the case of plankton. ✗ means that some type of change (e.g., color, weight) was observed, in the case of MPs, or that the organisms were not digested, in the case of plankton. Microplastics: PA (polyamide), PS (polystyrene), PE (polyethylene), Ac (acrylic), Ra (rayon), Ly (Lycra), PVC (polyvinyl chloride), PP (polypropylene), CA (cellulose acetate), HDPE (polyethylene of high-density), LDPE (polyethylene of low-density) and PET (polyethylene terephthalate).

		Room temp			*65°C*			
		24 hours	**48 hours**	**72 hours**	**7 hours**	**24 hours**	**48 hours**	**72 hours**
MPs	**PA fibers**	✓	✓	✓	-	✓	✓	✓
	PS fibers	✓	✓	✓	-	✓	✓	✓
	PE fibers	✓	✓	✓	-	✓	✓	✓
	Ac fibers	✓	✓	✓	✓	✗ Loss of color	✗ Loss of color	✗ Complete loss of color
	Ra fibers	✓	✓	✓	✓	✗ Loss of color	✗ Loss of color	✗ Loss of color
	Ly fibers	✓	✓	✓	-	✓	✓	✓
	PVC fragments	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight	-	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight
	PP fragments	✓	✓	✓	✓	✓	✓	✗ Loss of color
	CA fragments	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight	-	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight
	HDPE fragments	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight	-	✓	✓	✓
	LDPE fragments	✓	✓	✓	✓	✗ Complete loss of color	✗ Complete loss of color	✗ Complete loss of color
	PA fragments	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight	-	✗ Loss of weight	✗ Loss of weight	✗ Loss of weight
	PET fragments	✓	✓	✓	-	✓	✓	✓
Plankton	**Fish larvae**	✗ Not digested	✗ Only loss of pigmentation	✗ Only loss of pigmentation	✓ Completely digested	✓ Completely digested	✓ Completely digested	✓ Completely digested
	Copepods	-	-	-	✓ Completely digested	-	-	-
	Chaetognaths	-	-	-	✓ Completely digested	-	-	-

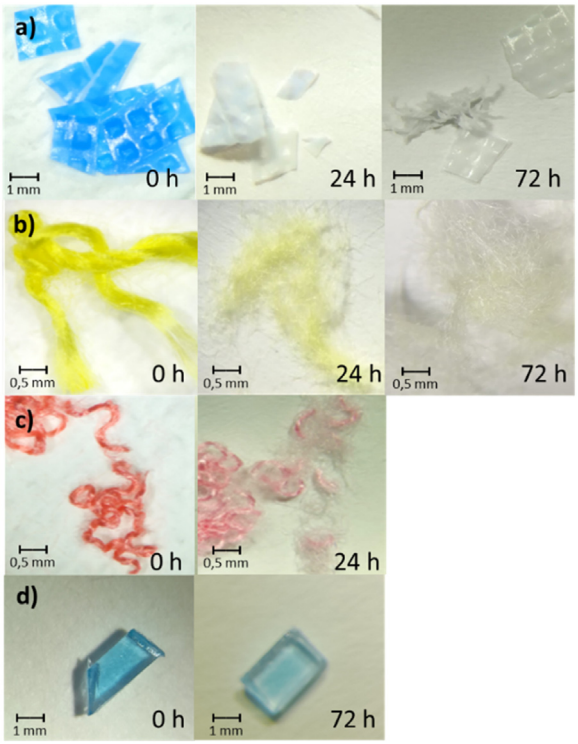

Fig. 4. Example of plastic polymers that showed loss of color: (a) polyethylene of low-density fragments before the methodology, after 24 h and 72 h; (b) acrylic fibers before the methodology, after 24 h and 72 h; (c) rayon fibers before the methodology and after 72 h and (d) polypropylene fragments before the methodology; after 24 h and 72 h.

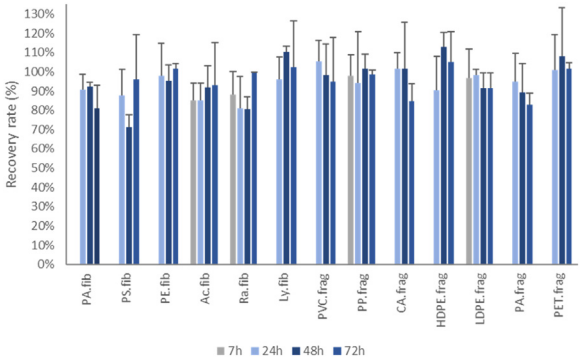

Fig. 5. Recovery rates (%) (mean ±standard deviation) for all the types of microplastics subjected to the 30% (v/v) H_2O_2 solution for a period of incubation of 24, 48 or 72 h at 65 °C. For some types of polymers (Ac and Ra fibers and PP and LDPE fragments) recovery rates after 7 h of incubation are also presented (subsequent tests D). Fibers (fib): PA (polyamide), PS (polystyrene), PE (polyethylene), Ac (acrylic), Ra (rayon) and Ly (Lycra); fragments (frag): PVC (polyvinyl chloride), PP (polypropylene), CA (cellulose acetate), HDPE (polyethylene of high-density), LDPE (polyethylene of low-density), PA (polyamide) and PET (polyethylene terephthalate).

recovery rates and no loss of color, a protocol of > 72 h to fully digest small organisms is unnecessarily long. In contrast, fish larvae subjected to 65 °C showed full digestion in the first 24 h of incubation (Table 1), independently of their size. All categories of fish larvae were completely digested after only 24 h.

Subsequent tests (tests C, D and E)

The preliminary results (tests B) showed that fish larvae were easily digested after 24 h at 65 °C. However, given that some types of MPs (Ac and Ra fibers and PP and LDPE fragments) lost some color at this temperature (Table 1), additional tests were made with fish larvae (tests C) and these specific types of MPs (tests D). In these tests, samples were analyzed hourly to evaluate their progress. In the case of fish larvae (tests C) after only 1 hour, pigmentation loss was observed, and the elongated and thin body fish larvae

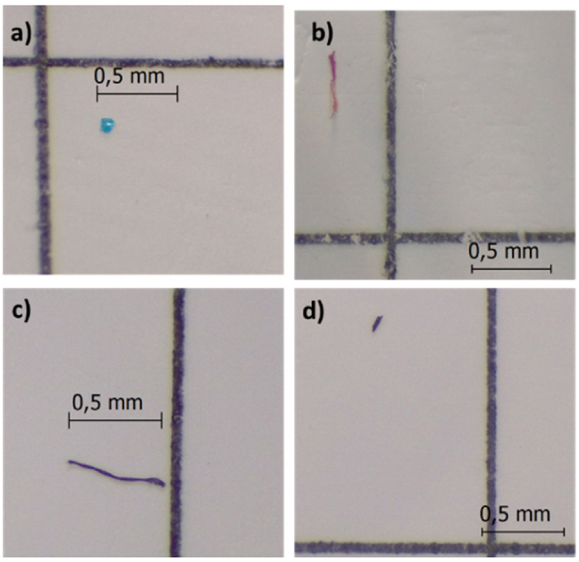

Fig. 6. Examples of microplastics collected from plankton samples after applying the optimized method of quantification: (a) fragment present in a fish larva (b) fiber present in a fish larva, (c) fiber present in a chaetognath and (d) fragment present in a copepod.

were already partially digested. After 3 h also the large and high body length fish larvae had begun to be digested. After 5 h almost every fish larva (either elongated and thin body, average body length or large and high body length fish larvae) were digested. After 6 h, only some parts of the fish larvae, as eyes and rays, remained. After 7 h fish larvae were completely digested, regardless the type of body and length, and the incubation period was ended. Since one of the main goals of the method was to test the effectiveness of digestion of fish larvae to quantify MPs present in them, the other two additional tests (D and E) were made afterwards with visual inspection hourly but with also an incubation period of 7 h. With regard to tests D, with selected MPs, after 7 h of incubation at 65 ˚C, neither Ac fibers, Ra fibers, PP fragments nor LDPE fragments showed any change in color, size, or visual aspect. Also, recovery rates were all > 85% (85% for Ac fibers, 88% for Ra fibers, 96% for LDPE fragments and 97% for PP fragments) after 7 h of incubation (Fig. 5). Regarding tests E, both copepods and chaetognaths were completely digested between 4 and 5 h at 65 ˚C (Table 1).

Field samples

In the case of the field samples, all the samples analyzed were contaminated with MPs. The MPs retrieved were fibers and fragments, of several colors (e.g., blue, red, pink) and measured between 0.01 and 3 mm (Fig. 6). After applying the optimized methodology, a total of 8 fish larvae of the flatfish *Solea senegalensis* were checked and a total of 28 MPs were found (approx. 3.5 MP per fish larvae), including 12 fibers and 16 fragments of blue, grey, transparent, orange, red, pink and purple colors and with size between 0.01 and 1 mm. A total of 30 copepods were digested and 29 MPs were retrieved (approx. 1.0 MP per copepod), including: 2 fibers and 27 fragments of blue, orange, red, and green colors and all of them measuring less than 0.5 mm. Lastly, for the 84 chaetognaths, a total of 57 MPs (approx. 0.7 MP per individual), were retrieved, including 6 fibers and 51 fragments, of blue, orange, and red colors, with size between 0.5 and 3 mm.

Final considerations

After all the tests, the optimized methodology ensures no changes in color, size or visual aspect (e.g., shape, relative appearance of the surface and edges, tightness of fibers) of all MPs studied, of different format (fibers and particles) and of different polymer type (Polyamide, Polystyrene, Polyethylene of low and high density, Acrylic, Rayon, Lycra, Polyvinyl chloride, Polypropylene, Cellulose Acetate and Polyethylene Terephthalate). Moreover, FTIR analysis showed that the polymers were still identifiable, and their spectra remain identical after the methodology and the MPs recovery rates were above 85%. Also, this method is efficient for different groups of zooplankton, including fish larvae, copepods and chaetognaths. The final protocol is presented below:

1. All glass material should be previously cleaned with deionized water and 96% ethanol and dried in a closed container (at room or higher temperature).
2. The organisms are placed in a clean glass petri dish and examined under a stereomicroscope.
3. The organisms should be isolated, transferred to another clean glass petri dish, examined individually with the help of metal tweezers, and rinsed with deionized water two or three times to ensure that no particle is attached to their body.
4. The organisms are separated by species and transferred to 26 ml scintillation glass vials (each vial can have only one organism or a group of organisms from the same taxon).

5. It is advised to add one or two blanks as a procedural control to check for any possible contamination during the methodology.
6. Two mL of 30% v/v H_2O_2 should be added to each scintillation vial ensuring the organisms are completely submerged (if not, a higher volume of 30% v/v H_2O_2 should be added) and then the vial is closed.
7. The samples are placed in the oven at 65 ˚C for a maximum of 7 h. If the organism tissues digest completely before 7 h, samples can be removed immediately.
8. After the digestion, the sample is collected by filtration through a glass filtration system using a 0.45 μm pore size cellulose membrane; the samples are poured into the glass filtration system and the vial should be rinsed with deionized water several times to ensure that all the possible particles in the vials are transferred to the filter.
9. The filters are stored individually in a clean glass petri dish and left to dry at room temperature (20–25 ˚C). The filters can also be dried at a low temperature for 24 h in the oven to accelerate the process.
10. At the end, the filters should be visually inspected for the presence of MPs. The MPs found should be characterized in terms of color, size, and type of particle (using for instance a stereomicroscope) and their polymer should be identified (using for example FTIR or Raman analysis or other as Py-GC/MS).

Since more than optimizing a method to a specific group of organisms (fish larvae, copepods and chaetognaths), we also aimed to standardize a method to extract MPs from these and other planktonic groups of organisms, it is important to give some further clarifying comments for others to be able to adapt this same methodology to their work:

Hydrogen peroxide (H_2O_2)

Other methodologies use KOH or HNO_3 solutions [14]; however, previous studies show that this type of reagent tends to degrade plastic polymers. In fact, previous studies reported that MPs partially melted and deformed or even attained defects in their structure when exposed to KOH or HNO_3 solutions [5,8,15]. The use of H_2O_2 solution in this methodology and previous methodologies developed by our team [16–18] was showed to be very successful, with high MPs recovery rates and only for a small number of polymers a loss of color was reported. However, that can be avoided with a period of incubation lower than 7 h. Although Bessa and Frias [19] reported partial degradation of plastic particles (e.g., changes in shape or size) using 30% v/v H_2O_2 solution, in the current study no degradation was observed. Other methodologies use a lower H_2O_2 solution concentration (e.g., [13,22]) but with an incubation period of 48 h. However, our results showed that incubation with 30% v/v H_2O_2 solution at 65 ˚C the organisms to be digested more easily, with no need to have a protocol with more than one day of duration.

Temperature

The results here show that room temperature does not have any effect on the digestion of the planktonic organisms, even in smaller organism such as recently hatched fish larvae. Other studies reported that a slightly higher temperature, 25 ˚C, was sufficient to digest macroinvertebrates [22]. However, the latter protocol has a period of incubation of 48 h, and it is important to denote that even at room temperature our results showed a decrease of the weight of some MPs after 24 h. From our experience ([16,17]; Silva et al. [18]), the increase of temperature is necessary to decrease the period of incubation to ensure the preservation of the integrity of plastic characteristics. Moreover, it is of note that in the plastic polymers that showed some losses of weight, this was both at 65 ˚C and room temperature, which may indicate that loss is not associated with the temperature of incubation. Nevertheless, the use of 30% v/v H_2O_2 solution seems to be less harmful to the plastic characteristics since only loss of color and a slight loss of weight is reported (in this and other studies) compared to other reagents where MPs melted and became deformed (hampering, for example the identification of the type or size of the MPs).

Period of incubation

Up to 24 h of incubation, MPs did not show changes in any of their characteristics, except for loss of color in Ac and Ra fibers and LDPE fragments; after 48 h, PP fragments also started to lose some color. However, if the incubation has a maximum duration of 7 h, none of these MP types showed loss of color or any other change in their characteristics. Previous studies showed some changes in plastic characteristics when exposed to 30% v/v H_2O_2 solution at high temperatures, namely size changes in PP particles after 96 h [20] or visual changes in the aspect on PA and PP particles after 7 days [21]. However, in those cases, MPs were exposed to this solution for several days. Despite this, if the identification of MPs colors collected is not an aim for other studies, according to our results, higher periods of incubation can be used with only loss of color of some types of MPs (after 48 or 72 h) (e.g., [18]).

Filters

The methodology here used 0.45 μm pore size cellulose membrane filters; however, different size pores and types of filters can be used according to the aims of each study. If the weight of the MPs collected is an aim in other studies, filters should be dried in the oven after filtration (drying of filters closed in Petri dishes at room temperature can last for weeks until completely dried).

Recovery rates

Our results showed that some types showed a decrease in recovery rates of MPs with the increase of the period of incubation, more specifically, four types of particles at room temperature and three types of particles at 65 ˚C. However, all of them were fragments. Fragments are rugged and can move a lot; hence, these findings can be due to some loss of particles when handling. Alfonso et al. [9] reported a loss of weight in PET (from water samples) when subjected to 30% v/v H_2O_2 and Fe (II) 0.05 M solution; however, the current study PET did not show any type of change. Lopez-Rosales et al. [8] reported that from the alkaline, enzymatic and oxidative methodologies used to analyze MPs in planktonic water samples, the oxidative (H_2O_2 solution) methodology gave the higher recovery rates (> 83%) and was also the methodology with lower costs. Overall, in our study, all selected MPs showed a recovery rate between 85% and 100% and no other changes in MPs characteristics were observed, being a quick, easy, efficient, and effective methodology to extract MPs from zooplanktonic organisms.

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.

CRediT authorship contribution statement

S.M. Rodrigues: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **F. Espincho:** Methodology, Formal analysis, Investigation, Writing – review & editing. **M. Elliott:** Supervision, Writing – review & editing. **C. Marisa R. Almeida:** Validation, Supervision, Project administration, Funding acquisition, Writing – review & editing. **S. Ramos:** Validation, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2023.102466.

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