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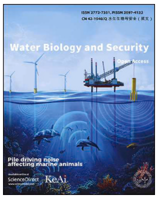

Research Article

Optimizing microplastic extraction from organic-rich and bristle polychaetes (*Hermodice carunculata*)

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ABSTRACT

Microplastics are ubiquitous and persistent contaminants in marine ecosystems worldwide. Benthic organisms, such as polychaetes, are particularly vulnerable to direct or indirect ingestion of microplastic and have recently been identified as potential microplastic concentrators. Understanding the impacts of microplastics pollution on polychaetes has garnered significant attention given their abundance, species richness, and key ecological role in marine food webs and benthos community structures. However, current extraction protocols are largely adapted from methodologies designed for seafood and fail to account for the morphological diversity of polychaeta species. Thus, harmonization approaches that are broadly applicable across taxa, enabling comparisons between studies, are needed. Herein we proposed an optimized and validated analytical method for the extraction and quantification of microplastics content in the bearded fireworm, *Hermodice carunculata*, a lipid-rich polychaete characterized by calcareous-chitinaceous structures. Following a comparative assessment in filterability of existing approaches, our optimized method was evaluated for digestion efficiency, microplastic recoveries and alteration on microplastic physio-chemical properties. The protocol included a pre-enzymatic digestion with trypsin, followed by thermo-oxidative treatment using hydrogen peroxide to remove organic matter and a mild acid digestion with acetic acid to dissolve chaetae. To further isolate microplastics, samples are pre-filtered with a stainless-steel mesh, subjected to a flotation step using canola oil, and finally concentrated onto a 1.2 μm filter. This method achieved high efficiency in removing organic and inorganic materials (98.4 ± 1.2% of the initial dry weight and 99.7 ± 0.2% of the initial wet weight of the *H. carunculata* specimens) while maximizing the recovery of common plastic polymers (ranging from 86.7 ± 5.8% to 94.4 ± 5.0% depending on the polymer type), preserving microplastic integrity. According to our results, the proposed method is a reproducible, sustainable and time-effective approach for microplastics detection in fireworms. Its applicability to other large benthic invertebrates can contribute to standardization of data collection and a better understanding of microplastic ingestion and ecological implications for Mediterranean benthic communities.

1. Introduction

Microplastic (MP) pollution ubiquitously affects the marine environment from coastal areas to the open ocean, representing one of the most pressing emergencies of our time (Leal Filho et al., 2025; Muñiz and Rahman, 2025; Napper and Thompson, 2020). MP can enter the seas as particles intentionally manufactured at such size (<5 mm, often added to cosmetics, cleaning products, or as industrial pellets) or through the physico-chemical breakdown of large plastic debris, shedding of microfibers from garments, agricultural textiles or clothes washing, and tire wear (Arias et al., 2022; Bajt, 2021; Mishra et al., 2019).

Once at sea, MP exhibit diverse distributional patterns depending on both polymer density and biological processes. Marine sediments are widely considered to be the final sink for MP (Knutsen et al., 2020; Van Cauwenberghe et al., 2015b), as particles which are denser than

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seawater tend to settle and accumulate on the seafloor. However, even low-density MP can eventually sink due to biofouling (Li et al., 2023). This process contributes to the widespread and long-term presence of MP on the seafloor, where they can affect benthic communities. Due to their ubiquity, persistence, and small size, MP can be ingested directly or indirectly by many benthic organisms, ranging from mollusks to echinoderms and cnidarians (Barboza et al., 2024; Courtene-Jones et al., 2017b; Rapp et al., 2021; Wootton et al., 2022), leading to reduced feeding activity, growth and survival rates, as well as behavioral changes, development and reproduction alterations (Haegerbaeumer et al., 2019; Mason et al., 2022).

Marine polychaetes (Annelida) represent one of the most species-rich, abundant, and biomass-dominant invertebrate groups (Díaz-Castañeda and Reish, 2009). They are characterized by significant morphological body plan variations, which enable them to exhibit diverse feeding strategies and lifestyle, being adapted to a broad range of ecological niches (Jumars et al., 2015; Kicklighter and Hay, 2006). Beyond their important ecological role as key organisms in marine food webs (Jumars et al., 2015), polychaetes are widely employed in biomonitoring studies due to their sensitivity to pollutants (Díaz-Castañeda and Reish, 2009; Giangrande et al., 2005; Pires et al., 2022).

Recently, a growing number of studies have assessed MP ingestion in this group (Keerthika et al., 2024; Schuab et al., 2023; Van Cauwenberghe et al., 2015a), suggesting that polychaetes can serve as potential bioindicators of MP in the benthic compartment (Calmão et al., 2023).

In literature several methods have been proposed to isolate MP from marine animals (Lusher et al., 2017). Current protocols are primarily designed for fish and bivalves (Thiele et al., 2019) because MP in seafood are a potential concern for human health; however, such protocol may be inadequate for organisms with different body structure, tissue composition and size. Therefore, methodological adaptations are needed to identify the most suitable extraction protocol based on animal specific characteristics (Karlsson et al., 2017; von Friesen et al., 2019). In the case of polychaetes, tissue digestion is commonly performed with acids (De Benedetto et al., 2024; Vecchi et al., 2021) and alkalis (Hamzah et al., 2021; James et al., 2023; Keerthika et al., 2024). The adoption of long incubation times and oxidative treatment is not uncommon (Knutsen et al., 2020). However, these reagents are reported to damage plastic polymers (Schrank et al., 2022). Still, the lack of standardization in methodology does not allow the comparison of outcomes in MP content from different studies (Silva et al., 2018; Tsangaris et al., 2021; Wesch et al., 2016).

The bearded fireworm *Hermodice carunculata* (Pallas, 1766) is a large amphinomid polychaete (up to 50–70 cm in length), widespread in coral reef, rocky habitats and shallow waters throughout the in the Western Atlantic Ocean, Caribbean, and Gulf of Mexico, and in the Red and Mediterranean Seas (Righi et al., 2020; Schulze et al., 2017). Over the last decade, *H. carunculata* has emerged as a native invader in the Central Mediterranean due to progressive sea warming, the flexible dietary and the absence of natural predators (Righi et al., 2020; Schulze et al., 2017). The bearded fireworm often reaches high population densities in coastal zones heavily influenced by human activity, including tourists and fishermen locations (Schulze et al., 2017; Simonini et al., 2021b; Righi et al., 2021). This species is notorious for opportunistic scavenging on carrion, injured animals and fish trapped in nets (Righi et al., 2020; Tiralongo et al., 2023). It is also a corallivorous and voracious generalist predator capable of consuming a wide variety of living benthic invertebrates (Righi et al., 2020; Simonini et al., 2018). This powerful predatory capacity results from its dorsal calcareous and chitinous bristles (notochaetae), capable of penetrating tissue and delivering bioactive compounds (Righi et al., 2021, 2022; Simonini et al., 2021a).

Given its broad distribution and opportunistic feeding behavior consuming a variety of prey species, *H. carunculata* is likely to be particularly susceptible to MP ingestion. Despite the bearded fireworm may represent a relevant model for studying MP accumulation in benthic predators, to date only a pilot study from Vecchi et al. (2021) investigated MP contamination in this species. Moreover, the previous workflow for the extraction of MP ingested by *H. carunculata* involved the use of strong acid and multiple filtration steps. Nevertheless, a thorough assessment of the potential effects of the treatments on the physical and chemical integrity of plastic polymers is lacking. Thus, a targeted and efficient method that maximizes tissue degradation, preserves the integrity of MP particles, and facilitates reliable extraction is required. Since the bearded fireworm presents several methodological challenges for MP isolation due to its complex tissue compositions and anatomical structures (i.e., high protein and lipid content, calcareous-chitinous chaetae, muscular pharynx and long intestine), this species represents an ideal benchmark for method development, applicable to other large benthic invertebrates with similar or simpler body complexity.

In the present study, we set up and validated an optimized method to extract MP from whole specimens of *H. carunculata.* The procedure was designed to maximize the removal of calcareous-chitinous and body tissues thereby yielding filters with minimal residual content suitable for analysis using chemical imaging techniques (e.g., μ-Raman spectroscopy). A sustainable design approach, which prioritizes the reduction of hazardous substances and minimizes risks to human health and the environment was adopted (e.g., Bellasi et al., 2021; Gohla et al., 2020; Mani et al., 2019). The efficiency of the proposed protocol was assessed in terms of MP recovery and removal of organic matter, while ensuring that polymer integrity was preserved to allow for accurate chemical identification following extraction. By providing a validated and effective workflow for MP detection in the bearded fireworm, our study contributes to the standardization of data collection and a better understanding of MP ingestion by large benthic invertebrates.

2. Materials and methods

To identify the best protocol to extract MP from *H. carunculata* whole specimens with negligible impact on the plastic particles, a three-step approach was followed: (i) selection of the best reagent for organic matter digestion between hydrogen peroxide (H_2O_2) and potassium hydroxide (KOH), based on available protocols for marine organisms; (ii) optimizing the process and the conditions for MP extraction; (iii) conducting validation tests to assess the method reliability and extraction efficiency (Fig. 1).

This polychaete species has a high moisture content, is rich in proteins, lipids, including polyunsaturated fatty acids, and minerals (Simonini personal communication). Its digestive tract, particularly the intestine, internally occupies most of the animal's body, and it is characterized by a muscular pharynx that is extruded through the mouth during feeding (Marsden, 1963). As amphinomid polychaete, *H. carunculata* is notorious for stinging bristles, which chemical and mineralogical composition has been recently clarified highlighting that chaetae are composed by chitin, crystalline $CaCO_3$ and clusters of apatite (Righi et al., 2021). However, most common chemicals (e.g., nitric acid, KOH, or H_2O_2) employed for MP isolation from biota have been proven to be ineffective in dissolving chitinaceous organisms (Kallenbach et al., 2021). Therefore, to establish the methodology for MP extraction from *H. carunculata*, various factors such as time and temperature of incubation, reagent quantities and concentrations were taken into account.

2.1. Specimen collection and conservation

Specimens of *H. carunculata* were collected by divers and fireworm traps following the methods developed by Simonini et al. (2021b) along the Ionian coast of Apulia (Santa Maria al Bagno, LE) in September 2022. Specimens were promptly rinsed with 0.2 μm filtered artificial seawater (Istant Ocean) to remove potential MP adhered to the animal's body and

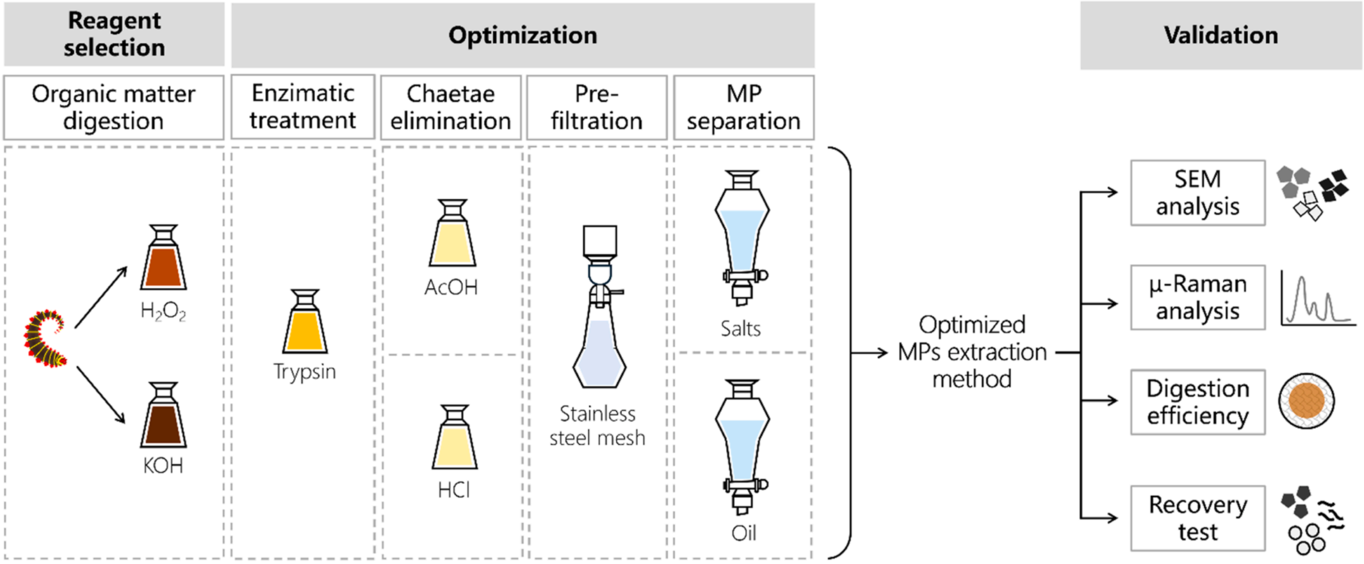

Fig. 1. Schematic workflow of the procedures tested, optimized and validated to extract microplastics (MP) from polychaetas as organic-rich organisms with chitinous and calcareous structures. Abbreviations: H_2O_2 = hydrogen peroxide; KOH = potassium hydroxide; AcOH = acetic acid; HCl = hydrochloric acid; SEM = scanning electron microscope.

preserved in 70% Ethanol (EtOH) in glass jars at 4 °C in darkness until laboratory processing.

2.2. *Reagents and solutions*

Two reagents for organic matter digestion were tested: 30% (v/v) H_2O_2 solution (PanReac AppliChem, Barcelona, Spain) and 10% (w/v) KOH (Carlo Erba, Cornaredo (MI), Italy), the latter prepared by dissolving tablets in ultrapure deionized (Milli-Q) water. For density separation, sodium chloride (NaCl, PanReac AppliChem), zinc chloride ($ZnCl_2$, Sigma-Aldrich, Darmstadt, Germany), sodium bromide (NaBr, Sigma-Aldrich) and commercial canola oil were tested. Saturated solutions of NaCl, $ZnCl_2$ and NaBr were prepared by dissolving the salts in 0.22 μm filtered ultrapure water to achieve densities in the range 1.2–1.5 g/cm^3. For the enzymatic digestion, 2.5% v/v Trypsin stock (Capricorn Scientific, Ebsdorfergrund, Germany) was diluted in Milli-Q water reaching the concentration of 0.3%. As acid reagents, 5% (v/v) hydrochloric acid (HCl, stock solution of 30%, Carlo Erba) and 10% (v/v) acetic acid (AcOH, stock solution of 99.8%, Sigma-Aldrich) were prepared by dilution in Milli-Q water. All reagents were filtered with cellulose esters filters of 0.2 μm (Millipore, Darmstadt, Germany) before use. Fireworm digestates were concentrated on glass fiber filters (GF filter; Cytiva Whatman (United Kingdom), Grade GF/C, pore size of 1.2 μm, diameter of 47 mm) and stainless-steel mesh with a diameter of 47 mm manually obtained from mesh pieces with porosity of 10 μm (Anping Teling Wire Mesh Products Co., Ltd, Anping County, Hebei, China).

2.3. *Reagent selection for organic matter digestion*

An efficient treatment for organic matter removal is fundamental for the recovery of MP. In the present study, two approaches were compared, based on the alkaline-acid treatment reported for this species (Vecchi et al., 2021) and based on an oxidative treatment commonly used with marine animals (Bergami et al., 2023; Bråte et al., 2018; Lusher et al., 2017; Rosales et al., 2024). Six fireworms were first rehydrated and washed with Milli-Q water filtered at 0.2 μm, cut into pieces to facilitate tissue digestion and individually transferred to 250 mL glass bottles to which H_2O_2 (final concentration of 25%) or 10% KOH were added, with three independent replicates for each treatment. The volume of KOH was 3 × the tissue weight of *H. carunculata* specimen (Vecchi et al., 2021). Bottles, covered with a glass stopper, were then incubated in a thermostatic water bath (Memmert, Germany) with gentle shaking (65 rpm) under controlled conditions (H_2O_2: 50 °C; KOH: 40 °C) for 48 h. Following treatment, the samples were filtered on GF filters using a vacuum glass filtration apparatus (Advantec, Japan), rinsed with Milli-Q water and EtOH and stored in glass Petri dishes. The organic matter removal by H_2O_2 and KOH was then evaluated through inspection under a stereomicroscope and based on the digestion efficiency (DE), as described in paragraph 2.5.2.

2.4. *Optimization of MP extraction protocol*

2.4.1. *Enzymatic pre-digestion treatment*

As the reaction of both H_2O_2 a KOH with organic matter can lead to the formation of foam (López-Rosales et al., 2021; Tanoiri et al., 2023; Thiele et al., 2019; Vecchi et al., 2021), resulting in a potential underestimation of the MP content, the extraction method was implemented by adding a first enzymatic treatment (20 mL of Trypsin at 0.3%). Such enzyme concentration was previously reported for highest digestion efficiency in the mussel *Mytilus edulis* and no detrimental impacts to various plastic polymers (Courtene-Jones et al., 2017a). Based on our previous study, this enzyme is also effective in digesting animals with cuticle and high lipid-rich content (Wilkie Johnston et al., 2023). Samples (n = 3) treated with Trypsin were incubated at 37 °C for 1 h in a thermostatic water bath with gentle shaking.

2.4.2. *Chaetae elimination*

After the digestion of soft tissues, an acid attack to fully degrade the chitinous-calcareous chaetae was considered. For this purpose, HCl (Karami et al., 2017) and AcOH solutions (Reineccius et al., 2021) were tested, considering three replicates for each treatment. Following the organic matter digestion step, 10% AcOH was added to each sample and left at room temperature (RT) for 2 h, while 5% HCl was laid on the GF filter paper, on which each sample was concentrated, and kept for 10 min at RT. As for organic matter digestion, a quantitative assessment of chaetae removal was performed by measuring the residual mass on the filters, calculating the DE for both acid treatments (see paragraph 2.5.2).

2.4.3. Sample filtration and MP separation

Upon completion of the digestion of organic matter and chaetae, samples were filtered using a glass vacuum filtration system (Advantec) on 1.2 μm GF filter. Since organic matter residues and chaetae not fully digested could hinder the accurate detection of MP, a density separation or flotation step was included. Different high-density solutions of NaCl ($\rho = 1.2$ g/cm^3, Cutroneo et al., 2021), $ZnCl_2$ ($\rho = 1.3$–1.5 g/cm^3, Cutroneo et al., 2021), NaBr ($\rho = 1.5$ g/cm^3, Schütze et al., 2022), and a mixture of NaCl and sucrose ($\rho = 1.3$ g/cm^3, Bellasi et al., 2021) were tested as well as canola oil (Radford et al., 2021).

Plastic buoyancy test was carried out using different plastic polymers (High-Density Polyethylene (HDPE), Low-Density Polyethylene (LDPE), Polyvinyl Chloride (PVC), Polyamide (PA), Polyethylene Terephthalate (PET) and Polystyrene (PS)). Fragments ($n = 3$ for each polymer) were obtained from reference materials by manually cutting them with a scalpel. Fragments were individually placed under the surface of saturated solution of NaCl, $ZnCl_2$ or NaBr in 250 mL graduated cylinders, and their behavior (i.e., buoyancy or vertical distribution in solution) was observed for 1 min. For oil separation, canola oil and Milli-Q were added in the graduated cylinder at ratio 1:30 containing the reference fragments. After shaking to generate an emulsion, the separation of plastic fragments between the phases was assessed.

2.5. Validation of the extraction method

Based on the optimization outcomes, the MP extraction protocol was validated by scanning electron microscopy (SEM) and spectroscopic analyses to assess the potential effect of reagents on MP physical and chemical properties and their identification. Moreover, the digestion efficiency (DE, %) of the polychaetae tissues and the Recovery Rates (RRs, %) of reference polymers were determined.

2.5.1. Impact of chemical treatments on reference plastics

To ensure that the chemical treatments did not alter the characteristics of plastic synthetic polymeric materials such as color, weight and morphology, physico-chemical characterization of reference plastics subjected to treatments was carried out. Fragments of PVC (2.0 ± 0.4 mm), PA (1.9 ± 0.3 mm), PS (2.2 ± 0.8 mm) and PET (2.4 ± 0.2 mm) were obtained by mechanical shearing using a metal scalpel under a stereomicroscope (Fig. S1) and treated as follows:

Method 1: 0.3% Trypsin (37 °C, 1 h), 25% H_2O_2 (50 °C, 48 h), and 10% AcOH (RT, 2 h)

Method 2: 0.3% Trypsin (37 °C, 1 h), 25% H_2O_2 (50 °C, 48 h), and 5% HCl (RT, 5 min)

Six fragments were considered for each treatment and untreated (pristine) fragments were included as a control group.

Prior and after treatments, all fragments were washed with filtered Milli-Q water, dried at 30 °C for 24 h, weighed (Crystal 100, Gibertini, d = 0.1 mg) and photographed using an Olympus TG6 camera with a scale to determine changes in their weight and size, respectively. SEM (Fei Burker Nova NanoSEM 450) was used to determine potential morphological alterations in polymer surface following chemical treatments, focusing on changes in roughness, presence of cracking or deformation. Images were acquired at the following conditions: high vacuum, 20 kV, 4.5 spot, 11.1–12.3 mm working distance and 30 s live time acquisition.

In addition, both untreated and treated fragments were analyzed using two complementary spectroscopic techniques such as μ-Raman and Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) to determine the effect of the proposed extraction protocol (i.e., H_2O_2 and canola oil) on polymer chemical fingerprint. Measurements at μ-Raman (Horiba Scientific LabRAM HR Evolution coupled with an Olympus BXFM-ILHS microscope) were carried out at 600 gr/mm grating, 300× hole and 100× objective, using a 532 nm excitation laser with a nominal output power of 10%, at 4 s and 4 accumulations. Further characterization by ATR-FTIR (Bruker Vertex 70, equipped with a MCT detector) was performed according to Bergami et al. (2023) in transmittance at 4 cm^{-1} resolution in the range 3200–600 cm^{-1} by averaging 32 scans.

The identification of polymers was carried out using the optical spectroscopy software Spectragryph (version 1.2.16) by the best match rate based on Pearson's correlation coefficient between acquired spectra and reference ones from available Raman and FTIR spectral libraries (Munno et al., 2020; Miller et al., 2022; Nicodom Ltd). These included an extensive spectral dataset of particles aged in the environment, pigments and paints, anthropogenic and biological ocean polymers, as well as natural, artificial and synthetic textile fibers of various colors.

2.5.2. Digestion efficiency

Specimens ($n = 3$) of *H. carunculata* were individually oven-dried at 105 °C for 6 h and their dry weight recorded before starting the extraction procedure (Fig. S2). After obtaining the digestates, they were concentrated on GF filter papers (1.2 μm), previously dried in an oven at 50 °C for 24 h and weighed on a scale with 0.1 mg precision. Finally, samples deposited on the filters were dried again in an oven at 50 °C for 24 h to obtain the dry weight of the non-digested organic matter. The DE was calculated according to von Friesen et al. (2019) and Dawson et al. (2020), as reported in the Supplementary materials.

2.5.3. Recovery rate

To evaluate MP recovery efficiencies, the following plastic materials were considered: fragments of PVC (1.5 ± 0.3 mm; Goodfellow Cambridge Ltd., Huntingdon, United Kingdom), PS obtained from commercial plastic cups (1.5 ± 0.3 mm), PA derived from commercial fishing line (2.5 ± 0.7 mm), tire wear (485 ± 101 μm) and textile fibers (2.6 ± 0.7 mm) were cut using a metal scalpel under a stereomicroscope. Moreover, low-density polyethylene (LDPE) fragments (575 ± 170 μm) were selected from commercially-available powder (Thermo Fisher Scientific, Waltham, United States) and PS green-fluorescent microspheres (90 μm; Cospheric LLC, California, United States) were prepared from stock aliquots and manually counted to obtain the final spiking suspension (Fig. S3).

PVC and PA were selected as potentially most ingested plastic polymers by fireworms: PVC is a high-density polymer ($\rho = 1.38$ g/cm^3) frequently found on the seabed (Fernández-González et al., 2022; Liu et al., 2019; Phuong et al., 2018), while PA is one of the main polymers of fishing nets, in which fireworms often get trapped trying to consume dead fish (Simonini et al., 2021b). PS and PE were chosen as low-density polymers commonly found in marine waters (Avio et al., 2015; Dekiff et al., 2014; Karami et al., 2017). Tire wear particles were also included in recovery tests because they are now widely recognized as a major source of MP contamination in marine ecosystems and analytically challenging for MP extraction due to their high density, complex chemical composition and irregular surface morphology, strongly interacting with organic and mineral matrices (Sun et al., 2025; Rødland et al., 2023).

Specimens of *H. carunculata* ($n = 3$) were individually added to glass bottles and spiked with 30 particles of each reference material, to be mixed into each sample prior to digestion treatment. All samples were treated following the final protocol chosen to extract MP from polychaetes. The RR was calculated as the number of reference fragments retrieved after processing, compared to their initial number (Karami et al., 2017; von Friesen et al., 2019). Analysis of variance was performed using non-parametric Kruskal-Wallis test followed by Dunn's post-hoc test to compare the recoverability of PVC, PA and PS reference fragments. Differences were considered significant as the p-value was under 0.05. Statistical analysis was performed using the software Past (version 4.03; Hammer et al., 2001).

3. Results

3.1. Reagent for organic matter digestion

From the comparison of alkaline or oxidative treatment, a high amount of undigested residues and bristles were found in the digestate treated with KOH with respect to H_2O_2, which appeared more clear suspension (Fig. 2a). Notably, H_2O_2 allowed for filtration onto a single GF filter with porosity of 1.2 μm, while KOH led to rapid filter clogging. Filtration of the latter sample was achieved only by using multiples filters, additionally including two of larger porosity (20 μm). Weights of the residual matter on the filters confirmed a low amount of undigested debris after H_2O_2 treatment (28.9 ± 12.3 mg; Fig. 2b), while, after treatment with KOH, organic and chitinous residues covered the entire surface of the filter (217.3 ± 70.0 mg; Fig. 2c), potentially hampering MP detection. The DE further supported such difference in the tissue digestion degree, reaching 99.5 ± 0.3% for H_2O_2 and 96.4 ± 0.8% for KOH, considering the initial wet weight of the samples. As a consequence, H_2O_2 was selected for the digestion of *H. carunculata* soft body tissues.

3.2. Method optimization

The selected thermo-oxidative method was optimized to maximize the removal of calcareous-chitinous and lipidic residues as well as preventing MP degradation and loss. The additional step with 0.3% Trypsin proved to be effective in softening tissues prior to chemical treatment, particularly when combined with H_2O_2, and a reduction in foam formation in the sample was observed. Regarding the acidic digestion, both 5% HCl and 10% AcOH exhibited high efficiency in dissolving the bearded fireworm bristles, with DE values of 96.9 ± 0.9% and 96.1 ± 2.6% based on dry weight of samples, and 99.7 ± 0.1% and 99.6 ± 0.2% considering wet weight, respectively.

During filtration onto 1.2 μm-GF filters, the amount of partially undigested matter in the samples reduced the flow rates. Therefore, a pre-filtration step using a 10 μm stainless steel mesh was added, according to Wilkie Johnston et al. (2023) to accelerate the subsequent concentration of the samples on 1.2 μm-GF filters. The use of a stainless-steel mesh also simplified the subsequent flotation step from a procedural perspective.

Results of the buoyancy tests showed that canola oil retained all reference polymers tested, unlike the high-density salt solutions (Table 1). Furthermore, canola oil exploited a good performance in elimination of organic residues and bristles from filters. Indeed, observation of the coverage level by undigested material on filters after canola oil step was lower than without this treatment (Fig. 3), enhancing the reliability of microscopy examination.

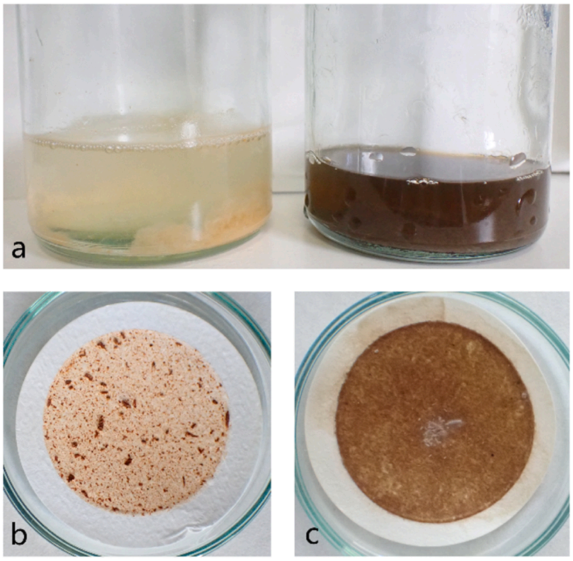

Fig. 2. Processing of *H. carunculata* whole body with (**a**) hydrogen peroxide (H_2O_2) (on the left) and potassium hydroxide (KOH) (on the right) after 48 h of incubation; membrane filters obtained after filtration of samples treated with H_2O_2 (**b**) and KOH (**c**).

3.3. Proposed workflow for MP extraction

Following the reagent selection and the optimization steps, the final method proposed to digest whole specimens of *H. carunculata* is illustrated in Fig. 4 (additional images of the main protocol steps are shown in the Supplementary materials). Briefly, specimens (initial wet weight and dry weight of 9.1 ± 3.8 g and 1.6 ± 0.7 g, respectively; $n = 3$) are first rinsed with Milli-Q water and cut into pieces to facilitate digestion in 250 mL glass bottle (Fig. S4). Then, the method consists of a pre-enzymatic step followed by a thermo-oxidative treatment: samples are incubated with 20 mL of 0.3% Trypsin (1 h at 37 °C) and subsequently with 25% of H_2O_2 (48 h at 50 °C) (Fig. S5a and b). After soft tissue digestion, fireworm chaetae are removed by an acidic digestion step by adding AcOH at 10% (Fig. S5c). Following 2 h incubation at RT, the resulting digestate undergoes initial vacuum filtration through a stainless-steel mesh with a 10 μm pore size (Fig. S6). MP are further separated using canola oil. The organic and inorganic residues previously concentrated on the stainless-steel mesh are carefully recovered from the mesh using a stainless-steel spatula in a glass beaker. To ensure complete sample recovery, stainless steel meshes are thoroughly rinsed with Milli-Q water. The collected material is then transferred to a glass separatory funnel, where 10 mL of canola oil and 300 mL of Milli-Q water are added. The separatory funnels are shaken vigorously and left to settle for 2 h to allow complete separation of the oil and water phases (Fig. S7). Afterwards, the aqueous phase is replaced twice, and the oil phase is vacuum concentrated on 1.2 μm GF filters, by dissolving oil micelles with filtered EtOH. To maximize the recovery of MP adhered to glass walls, separatory funnels and filtration apparatus are also rinsed with both filtered EtOH and Milli-Q water. After filtration, filters are transferred into glass Petri dishes for analysis (e.g., microscopy examination and chemical spectroscopic characterization).

The amount of biological matter concentrated on GF filters was 30.3 ± 29.6 mg ($n = 3$). Compared to the previous method by Vecchi et al. (2021) for fireworms, requiring at least 2–3 weeks processing, our approach is less time-consuming, reducing sample processing to a maximum of 5 days, from the preparation of the specimen to MP collection onto a single 1.2-μm GF filter. This enhances the practical applicability and scalability of the proposed protocol for monitoring studies requiring the processing of multiple specimens.

3.4. Method validation

3.4.1. Digestion efficiency and MP recovery

The proposed method efficiently removed 98.4 ± 1.2% of the initial dry weight (1.6 ± 0.7 g) and 99.7 ± 0.2% of the initial wet weight (9.1 ± 3.8 g) of *H. carunculata* specimens.

As shown in Table 2, high RR values were obtained for different types of MP added to the samples: RRs ranged from 77.0 ± 8.8% for PS microspheres to 97.8 ± 1.9% for tire particles. RR values of the tested materials were not significantly different, suggesting no effect of polymer type and MP category on RRs.

3.4.2. Reagents effect on MP physico-chemical properties

Regarding the acid treatments, no sign of degradation was observed in the plastic polymers tested (i.e., PET, PVC, PA, PS; average size of 2.29 ± 0.40 mm) following visual inspection, with no change in weight, size or color compared to pristine materials (see Table S1). Differently, compared to virgin untreated materials such as PS (Fig. 5a–c), SEM analysis showed alterations in the surface morphology following HCl-

Table 1
Behavior (i.e., buoyancy or vertical distribution in solution) of different plastic polymers placed in both high-density salt solutions and oil observed during preliminary buoyancy tests. For Zinc chloride ($ZnCl_2$) 1.5 g/cm^3 only polymers that sink at lower density were tested.

Plastic polymer	FLOATATION SOLUTION					
	NaCl (1.2 g/cm^3)	$ZnCl_2$ (1.3 g/cm^3)	$ZnCl_2$ (1.5 g/cm^3)	NaBr (1.5 g/cm^3)	NaCl (1.3 g/cm^3) + sucrose	Milli-Q water + canola oil
HDPE	✓	✓	Not tested	✓	✓	✓
LDPE	✓	✓	Not tested	✓	✓	Not tested
PVC	×	×	✓	✓	✓	✓
PA	✓	✓	Not tested	✓	✓	Not tested
PET	×	×	✓	✓	—	✓
PS	✓	✓	Not tested	✓	Not tested	✓
PP	✓	✓	Not tested	✓	✓	✓

✓ = float; × = sink; — = neutral. Abbreviations: HDPE, high-density polyethylene; LDPE, low-density polyethylene; PVC, polyvinylchloride; PA, polyamide; PET, polyethylene terephthalate; PS, polystyrene; PP, polypropylene.

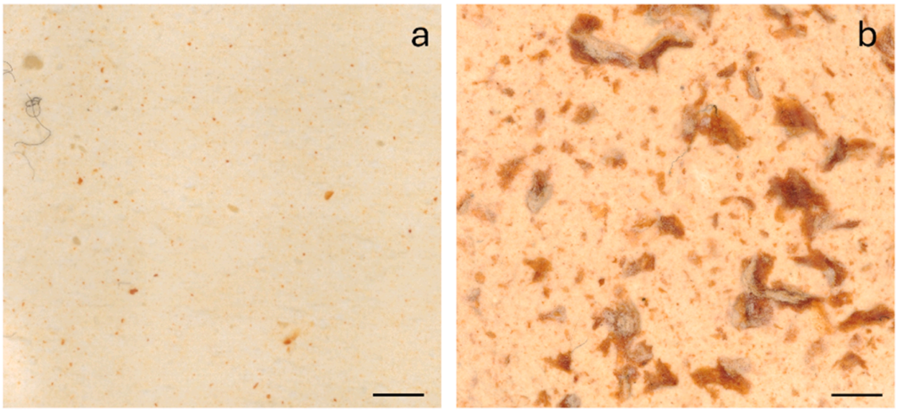

Fig. 3. Glass fiber (GF) filter portion of sample treated with canola oil floatation step (**a**) and omitting it (**b**). Scale bar: 2 mm.

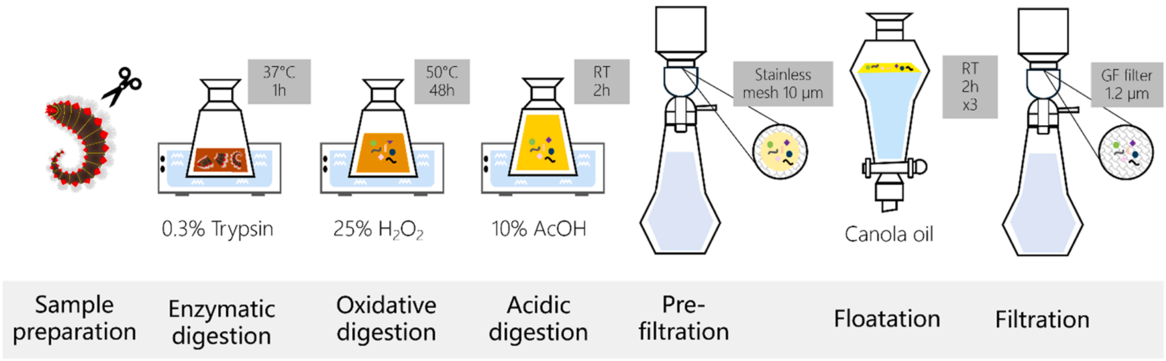

Fig. 4. Schematic overview of the proposed method to isolate microplastics (MP) from fireworms. Abbreviations: H_2O_2 = hydrogen peroxide; AcOH = acetic acid; RT = room temperature; GF filter = glass fiber filter.

treatment, while no changes were observed with AcOH. With the exception of PET fragments obtained from plastic bottles, which displayed a smooth surface regardless the extraction method (Fig. S8), all reference fragments were characterized by an increase in surface roughness when the extraction method included the HCl-treatment (Method 2) with widespread cracking, etching and more evident sharp grooves (Fig. 5g–i, see also Fig. S9–10 for PVC and PA) compared to AcOH (Method 1) (Fig. 5d–f and Fig. S9–10).

After floatation with canola oil, μ-Raman showed no relevant change in plastic chemical fingerprint with respect to untreated fragments, with comparable primary characteristic peak shapes intensities, as shown in Fig. 6. Although the polymer match rating slightly decreased after the floatation step, for instance from 99.1% for untreated PET to 91.8% after oil treatment and from 99.8% to 88.7% for PVC, the overall polymer identification was not impaired. Indeed, μ-Raman spectra showed characteristic bands at 632 cm^{-1} (symmetric bending C=O), 860-1288-1611 cm^{-1} (stretching C=C), 1104-1288 cm^{-1} (stretching C-C), 1734 cm^{-1} (stretching C=O) for PET and 360 cm^{-1} (C-Cl in the trans configuration of polymer), 638-690 cm^{-1} (C-Cl stretching), 1102-1179 cm^{-1} (C-C stretching), 1334–1403 cm^{-1} (C-H symmetrical stretching in CH_2 group), 2916 cm^{-1} (C-H asymmetrical stretching in CH_2 group) for PVC (Baibarac et al., 2021; Luo et al., 2022). These results were further supported by ATR-FTIR analysis, which confirmed that canola oil did not hinder polymer identification. The position and relative intensity

Table 2
Percentage of recovery rate (RR, average ± SD) for different plastic polymers (Polyvinyl chloride, PVC; Low-Density Polyethylene, LDPE; Tire particles; Polyamide, PA and PA66; Polystyrene, PS), types, colors, and sizes, obtained with the proposed method.

Polymer	Type	Color	Size (mm)	RR (%)
PS	Beads	Fluorescent yellow-green	0.09 ± 0.0	77.0 ± 8.8
	Fragment	White	1.5 ± 0.3	88.3 ± 2.5
Tire	Fragment	Black	0.5 ± 0.1	97.8 ± 1.9
LDPE	Fragment	White	0.6 ± 0.2	88.9 ± 2.6
PVC	Fragment	Grey	1.5 ± 0.3	94.4 ± 5.0
PA	Fiber	Transparent	2.5 ± 0.7	86.7 ± 5.8
PA66	Fiber	Black	2.6 ± 0.7	95.5 ± 5.1

of the characteristic absorption bands were retained in the FTIR spectra of the treated reference polymers (Fig. S11). Overall, high matching values based on Pearson's correlation coefficients were obtained for most plastic polymers following canola oil treatment (e.g., 97.9% for PET and 98.4% for PS). The lowest value was observed for PVC (88.5%), potentially reflecting a spectral interference associated with the flotation step.

4. Discussion

The isolation of MP from marine polychaetes presents specific challenges due to their complex body composition, characterized by high fat content and calcareous-chitinaceous structures. In this study, we developed a suitable multi-step method to extract MP from the whole body of the fireworm *H. carunculata* by implementing protocols available in the literature, with the goal to obtain negligible damages to plastic polymers and acceptable MP recovery.

4.1. Optimization

4.1.1. Chemicals for organic matter degradation

For the bearded fireworm *H. carunculata*, Vecchi et al. (2021) proposed a first digestion method based on 10% KOH. Despite alkaline digestion being generally effective in removing organic matter without significant degradation of most plastic polymers (Dehaut et al., 2016; Kühn et al., 2017; Lopes et al., 2022), in rich lipid-content samples this reagent tends to trigger a saponification reaction. Such reaction hinders the concentration of samples on single filters and thus the isolation of potential MP, hampering detection and slowing quantification. Instead, H_2O_2 tested in the present study overcomes this limitation, and it has been successfully employed to extract MP from several marine organisms, from mussels to fish (Karlsson et al., 2017; Song et al., 2022). Although a universal method for MP analysis is not feasible due to the complexity and diversity of biological samples, existing techniques need to be compared, finding the most suitable for biota digestion based on animal's characteristics. In this context, the efficiency of this oxidative treatment supports its use as a valid option for lipid-rich invertebrates and contributes to ongoing efforts toward harmonization and standardization of MP extraction methodologies.

4.1.2. Enzymatic pre-treatment

Enzymes are known to attain higher DE and plastic RRs, being gentler and with negligible or limited impact on plastic polymers (Karlsson et al., 2017; Thiele et al., 2019). Nevertheless, they are often considered expensive and time consuming (López-Rosales et al., 2021; Tuuri et al., 2024; von Friesen et al., 2019). Considering marine invertebrates, proteinase-K resulted in reliable for zooplankton processing (Avio et al., 2015; Cole et al., 2014), whereas commercially isolated pancreatic

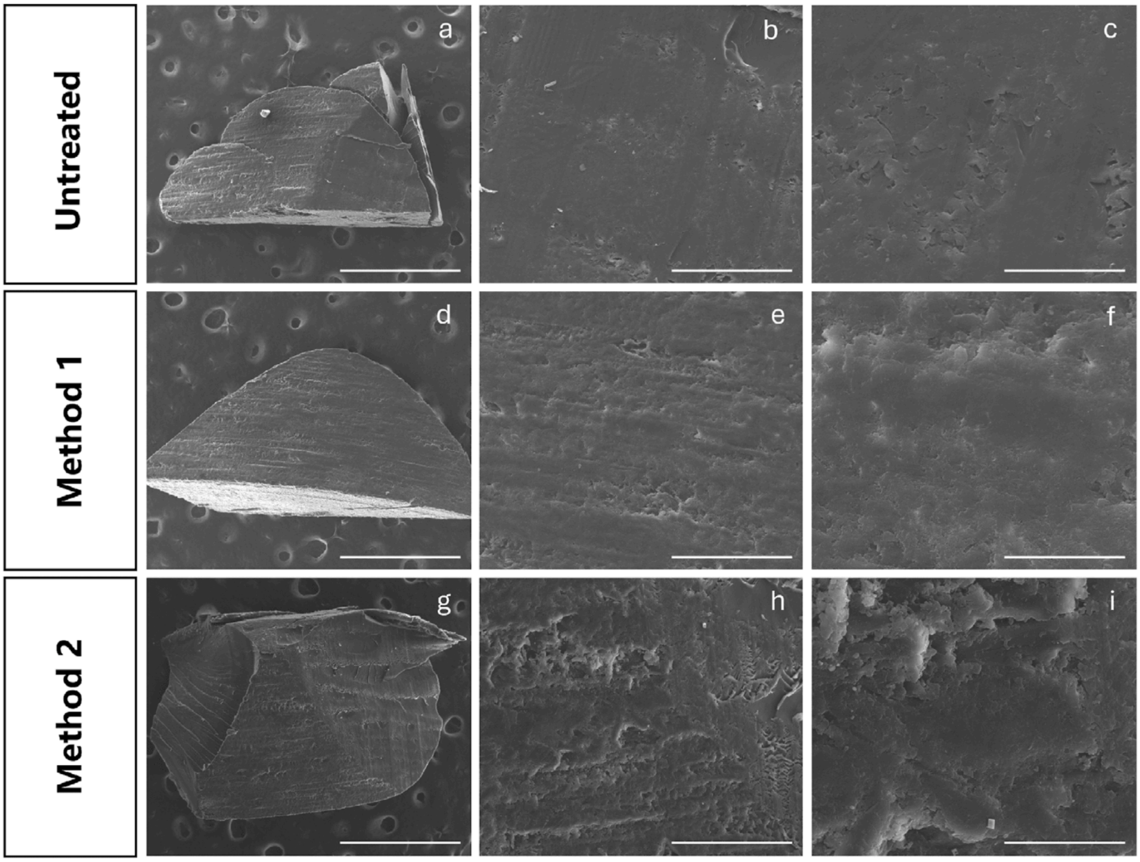

Fig. 5. Scanning electron micrographs of polystyrene (PS) fragments: untreated (**a**–**c**); treated with Method 1 (**d**–**f**; 0.3% Trypsin, 25% hydrogen peroxide, and 10% acetic acid); treated with Method 2 (**g**–**i**; 0.3% Trypsin, 25% hydrogen peroxide, and 5% hydrochloric acid). Magnification and scale bar: 100 × , 1 mm (**a**, **d**, **g**); 500 × , 200 μm (**b**, **e**, **h**); 6000 × , 50 μm (**c**, **f**, **i**).

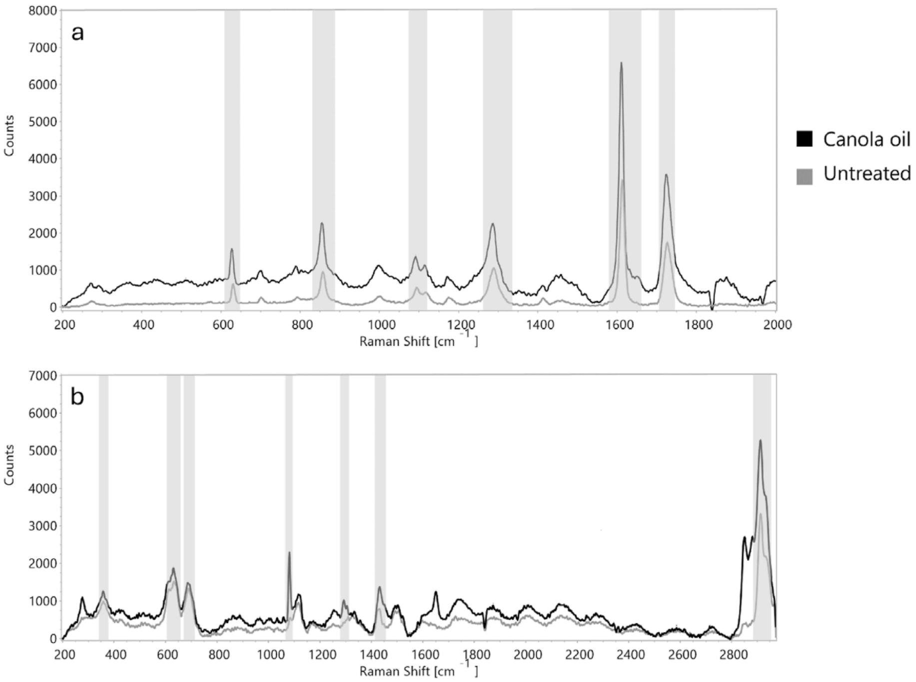

Fig. 6. μ-Raman spectra of high-density microplastics: (**a**) polyethylene terephthalate and (**b**) polyvinyl chloride after floatation with canola oil (black line), compared to the untreated one (light-grey line). The spectra were baseline-corrected, smoothed and normalized. Characteristic peaks are shown with grey rectangles.

enzymes were applied to bivalves (von Friesen et al., 2019). Several enzymatic protocols have been applied exclusively to gastrointestinal contents (e.g., Karlsson et al., 2017) or to whole organisms of low biomass (e.g., Railo et al., 2018), without assessing their effectiveness for larger organisms. Furthermore, some of these methods require prolonged digestion times, which may be impractical for use in routine monitoring programs (e.g., Löder et al., 2017). Here, the inclusion of a pre-treatment using Trypsin mitigated foaming during oxidative digestion, previously documented as an important limitation of H_2O_2-based methods (López-Rosales et al., 2021; Tanoiri et al., 2023; Thiele et al., 2019). Trypsin thereby represents a suitable alternative to other commonly used enzymes by preventing the adhering of MP to the flask walls and the potential underestimation of the particle count. This finding highlights the advantages of enzymatic digestion as a preparatory step in protocols targeting organisms with protein- and lipid-rich tissues, as it is also safer to handle than many other purification agents and should therefore be preferred (Schrank et al., 2022; Zhao et al., 2018).

4.1.3. *Acidic digestion*

Previous studies focused on polychaetes characterized by calcareous-chitinous structures employed acidic treatment to dissolve them, such as 5% nitric acid (HNO_3; Vecchi et al., 2021) and 25% formic acid (De Benedetto et al., 2024). Despite their digestion effectiveness, these strong acids have been reported to chemically and physically damage MP (Lusher et al., 2017; Schrank et al., 2022; Tuuri et al., 2024). Therefore, we compared the efficacy of HCl and AcOH in removing *H. carunculata* chaetae: HCl is frequently mentioned in literature for environmental sample purification for MP analysis but is noted to be less destructive to plastic polymers compared to other strong acids (Lusher et al., 2017), while AcOH is a weaker acid, and it has been verified to be efficient in dissolving large amounts of calcareous material (e.g., shell; Reineccius et al., 2021).

Despite the comparable effectiveness of HCl and AcOH in dissolving the bearded fireworm chaetae, SEM analysis carried out on reference polymers (PVC, PA, PS and PET) commonly found in the benthic compartment (Coyle et al., 2020; Schrank et al., 2022) revealed that AcOH better preserved the integrity of plastic polymer surfaces. Considering lower risks for both operators and the environment due to its reactivity and toxicity, AcOH represents a safer alternative to stronger acids, facilitating sample filtration and improving MP identification. Taken together, these results support the selection of AcOH as a practical and efficient reagent for MP extraction protocols targeting organisms with calcareous–chitinous structures and highlight its suitability for inclusion in standardized methods.

4.1.4. *Oil flotation*

Chemical digestion alone does not ensure optimal isolation of MP from marine biota, as residual materials such as shells, chitin, bones, or sand often remain on the filter and hinder the accurate identification of MP (Dehaut et al., 2016; Jaafar et al., 2020). Among post-digestion techniques, oil-based separation is increasingly employed for sediments and water samples (Crichton et al., 2017; Lechthaler et al., 2020), while most validated protocols for marine animals rely on density separation using hypersaline solutions after chemical digestion (Dehaut et al., 2016; Li et al., 2015; Rosales et al., 2024). Only a few studies previously applied oil extraction following digestion of soft tissues to separate MP from marine invertebrates, primarily concerned on mussels (e.g., Song et al., 2022; Yu et al., 2022), and to our knowledge no assessment has been conducted on polychaetes. This is the first study to evaluate the effectiveness of oil-based methods in removing non-plastic residues from digested residues of these animals. We selected canola oil as Kononov et al. (2022) found through an experimental comparative analysis that it was one of the most efficient in MP recovery.

Oils might interfere with MP characterization, as shown by MP analysis using FTIR spectrometry (Crichton et al., 2017; Lechthaler et al., 2020). Here, no effect of the canola oil on PVC and PET identification by μ-Raman and ATR-FTIR was found, as the correlative matching rates were far above 65%, indicating that polymer chemistry was well preserved (Rosso et al., 2023). These findings indicate that canola oil is a suitable option to isolate MP from marine invertebrates, representing a simple, easy, inexpensive, valuable solution for flotation step in substitution of density separation without compromising its efficiency to detect and identify target MP. Being also non-hazardous for both the environment and humans, canola oil meets the recent imperative of adopting a Green Analytical Chemistry, which aims to minimize toxic reagents, waste and energy consumption in analytical method design (Gałuszka et al., 2013; Sajid and Płotka-Wasylka, 2022).

Washing steps with an organic solvent is crucial to reduce interferences from oil that may occur during MP identification analysis. Some authors suggest removing the oil with normal detergent (Karlsson et al., 2017), however we observed excessive foam formation, requiring thorough washing to ensure complete removal. EtOH was thus selected as a washing solvent because it does not compromise Raman identification (Garzón-Vidueira et al., 2024).

In the context of determining MP content, the multi-step nature of the proposed protocol can potentially bias the results because of background contamination (Foekema et al., 2013; Karami et al., 2017). Standardized approaches on quality assurance, quality control and collection of samples must be included (Brander et al., 2020) in a perspective of quantification and identification of MP ingested by fireworms, such as procedural blanks and use of pre-filtered reagents (Brander et al., 2020). In addition, working in appropriate conditions (e.g., dedicated hood, cleaned with filtered EtOH prior to and after use) is essential to minimize potential airborne particle deposition.

4.2. *Suitability of the protocol*

As a widely accepted criterion, an MP extraction method is considered suitable when the DE is equal to or greater than 95% (Cole et al., 2014; Karami et al., 2017; Thiele et al., 2019). In terms of performance, our protocol demonstrated highly effective elimination of organic and mineralized material, with higher DE values than those obtained by Vecchi et al. (2021) for *H. carunculata* (DE of 96% obtained after long KOH incubation and multiple filtration).

Recovery tests further demonstrated that a wide range of MP types and sizes were retained and collected on a single filter support. With RRs ranging from 77.0% to 97.8%, our protocol can thus be considered efficient and reliable (Hurley et al., 2018; Kallenbach et al., 2021). Indeed, McIlwraith et al. (2025) suggested 70% as a suitable cut-off for MP isolation.

Although recovery tests are commonly conducted to validate MP extraction protocols in marine invertebrates such as bivalves, data for polychaetes remain scarce. Vecchi et al. (2021) did not report RR for *H. carunculata,* thus our study constitutes the first methodological reference for this species. Nevertheless, the RRs for small MP are below those reported in other polychaete species, such as on *Sabella spallanzanii* (RR of 96.8 ± 0.5% for 6 μm PS beads; De Benedetto et al., 2024). This variability could be related to differences in the spiking method. While in the present study approximately 60 microspheres were added per sample, De Benedetto et al. (2024) spiked higher numbers (ca. 2.3×10^4 particle/mL). Our results align with recovery rates reported in studies conducted on different marine organisms (e.g., von Friesen et al., 2019; Jaafar et al., 2020; Song et al., 2022). The recovery of additional small MP (<100 μm) should be tested in future applications.

While our protocol was validated using a relatively large benthic invertebrate characterized by a complex body structure, which poses substantial analytical challenges for MP extraction, its design allows straightforward adaptation to other taxa by adjusting reagent volumes, incubation times and handling steps. Future studies testing the method efficacy in MP isolation across a broader range of invertebrates of different sizes will support its wider applicability. More detailed characterization of reference polymers (e.g., through Nuclear Magnetic Resonance) could be also valuable in future investigations focusing on chemical modifications or degradation of a broad range of reference plastic polymers associated with digestion treatments.

5. Conclusions

Existing analytical methods for extracting MP from polychaetes often lack validation steps, hindering data consistency and comparability across studies. This posed a significant limitation to the development of harmonized and standardized methodologies for MP analysis. In this study, we focused on optimizing the protocol for isolating MP from the polychaete *H. carunculata*, with the goal of developing a more time-efficient, environmentally sustainable approach. Our proposed method enables effective isolation and clear identification of MP from *in toto* fireworm specimens. A key feature of the protocol is the efficient digestion of both soft tissue and chaetae, which allows the concentration of the sample onto a single 1.2 μm GF filter while preserving polymer integrity and ensuring high MP recovery. The procedure is simple from an operational point of view and minimizes toxicity risks to both human health and the environment. Given its promising performance, this approach represents a strong candidate for the development of a standardized method for MP analysis in organic-rich marine organisms characterized by calcareous and/or chitinous matrices.

CRediT authorship contribution statement

Valentina Ferrari: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marco Scaramelli:** Methodology, Investigation, Formal analysis. **Roberto Simonini:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Daniela Prevedelli:** Writing – review & editing, Resources. **Elisa Bergami:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Ethical statement

As this study involved the use of invertebrate animals (Annelida, Polychaeta), no ethical approval was required under Italian national law and European Union's Directive 2010/63/EU on the protection of animals used for scientific purposes.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watbs.2026.100621.

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