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Chitinase digestion for the analysis of microplastics in chitinaceous organisms using the terrestrial isopod *Oniscus asellus* L. as a model organism

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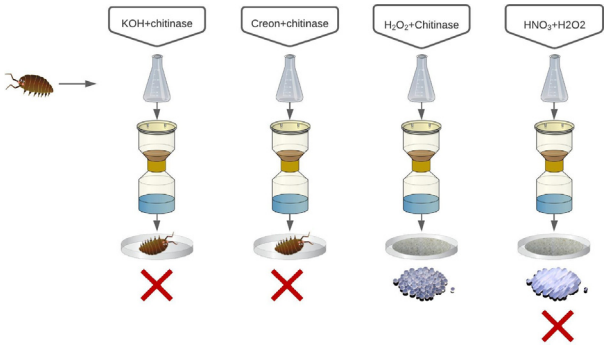
HIGHLIGHTS

- Chitinaceous organisms can be digested using H_2O_2 in combination with chitinase.
- This method does not affect microplastic.
- The method can be used for microplastic analysis in chitinaceous organisms.

GRAPHICAL ABSTRACT

Symbols in the graphical abstract derives from Adobe Stock (stock.adobe.com) and the Integration and Application Network, Univ. of Maryland Center for Environmental Science (ian.umces.edu/symbols/). The graphical abstract was made using lucid.app.



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ABSTRACT

Chitinaceous organisms have been found to ingest microplastic; however, a standardised, validated, and time- and cost-efficient method for dissolving these organisms without affecting microplastic particles is still required. This study tested four protocols for dissolving organisms with a chitin exoskeleton: 1) potassium hydroxide (KOH) + chitinase, 2) Creon® + chitinase, 3) hydrogen peroxide (H_2O_2) + chitinase, and, 4) Nitric Acid (HNO_3) + hydrogen peroxide (H_2O_2). The effects on microplastics composed of eight different polymers were also tested. The use of H_2O_2 followed by chitinase was found to be a highly efficient method. The three other protocols either did not digest the chitin sufficiently or negatively affected the tested polymers. A recovery test using microplastic fibres, beads and tyre particles revealed high recovery rates of 0.85, 0.89 and 1 respectively. This further supported the applicability of the H_2O_2 and chitinase (protocol 3) for dissolving chitinaceous organisms. Thus, we recommend that future investigations of microplastic (0.05 μm–5000 μm) in chitinaceous organisms (0.3 cm–5 cm) utilise the here presented methodology. This represents an important component of the ongoing validation and harmonization of methodological approaches that are urgently needed for the advancement of microplastic assessments globally.

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1. Introduction

Plastic contamination is now firmly established on the global environmental agenda (e.g. Rochman and Hoellein, 2020). Consequently, a large number of studies have been conducted in the past decade aiming at assessing the problem of plastic pollution, understanding ecological effects and deriving possible solutions from source to sink (Auta et al., 2017; Bucci et al., 2020; Gallitelli et al., 2020). In addition to the highly visible accumulations of plastic litter in the environment, microplastic has been described as an environmental pollutant, appearing ubiquitously in glacial ice, in the marine environment from the poles to equator, in soil, air, in streams and lakes and in biota (e.g. Ambrosini et al., 2019; Dris et al., 2015; Lacerda et al., 2019; Rochman et al., 2017).

One of the key focus areas of the microplastic research field is the potential ecological impact of microplastics in natural ecosystems. This goes beyond the aesthetical issues associated with visible plastic litter accumulations (Li et al., 2018; Wright et al., 2013). However, we don't have a complete understanding of the risk and effects that microplastic poses to the different environmental matrices. This has led to significant research effort aiming at elucidating the potential sources, occurrence, and effects of microplastics in biota (Beer et al., 2018; Bergami et al., 2020; Bour et al., 2018; Bråte et al., 2018; Catarino et al., 2018; De Witte et al., 2014; Digka et al., 2018; Foekema et al., 2013; Hurley and Nizzetto, 2018; Lusher et al., 2013; Lwanga et al., 2017).

Only a small number of studies have thus far focused on microplastic occurrence in organisms with an exoskeleton of chitin (Pastorino et al., 2020; Simmerman and Coleman Wasik, 2020; Wardlaw and Prosser, 2020). This is surprising given that this group of animals is abundant in both the terrestrial and aquatic environments. Previous environmental studies have shown that most of the investigated species (of the orders ephemeroptera, trichoptera, diptera, amphipodata, decapoda and odonata) contained plastic particles, demonstrating that these organisms interact with microplastics in the environment (Akindele et al., 2020; Leslie et al., 2017; Lourenco et al., 2017; Nan et al., 2020; Nel et al., 2018; Pastorino et al., 2020; Simmerman and Coleman Wasik, 2020; Wardlaw and Prosser, 2020; Windsor et al., 2019). Far fewer studies have been conducted on semiterrestrial/terrestrial species living in nature. For example, microplastics (polypropylene, PP, and polyethylene, PE) were found in the semiterrestrial sandhopper (*Talitrus saltator*) and in the Antarctic collembolan (*Cryptopygus antarcticus*) (Bergami et al., 2020; Iannilli et al., 2018). Further research is urgently required to better understand the uptake and potential risk of microplastics in these organisms.

The lack of a validated analytical method for isolating microplastics from chitinaceous organisms is a major research gap. Many different methods have been applied for extracting microplastics from organic material and biota (Lusher et al., 2020). Employed methods for dissolving organisms have primarily been based upon the use of chemicals e.g. KOH, HNO_3 or H_2O_2 (Zhu and Wang, 2020). However, these previously tested methods have been shown to be insufficiently effective in dissolving exoskeletons made of chitin. Chitin $(C_8H_{13}O_5N)_n$ is a recalcitrant natural polysaccharide with a highly ordered structure resembling cellulose. It is the second most abundant natural biopolymer (Shahidi et al., 1999). Some studies investigating chitinaceous organisms dealt with the presence of chitin by crushing or powdering the macroinvertebrates, but this is associated with a risk of fragmenting microplastics within the organism (Iannilli et al., 2019; Löder et al., 2017) Instead, the enzyme chitinase poses a viable method for removing chitin in microplastic samples. Chitinase cleaves the polymer into oligomers of chitobiose and *N*-acetylglucosamine by endohydrolysis. Thus, enzymes do not interact with polymers, and is thus "plastic-conserving" while removing organic material (Löder et al., 2017). Löder et al. (2017) developed a method for complex samples where chitinase was used to eliminate chitin exoskeletons. Uurasjärvi et al. (2020) dissolved marine samples containing chitinaceous organisms using a four-step and 8-day long purification process with filtration in between each step using H_2O_2, chitinase, and sodium dodecyl sulphate. Shortcomings of these methods include an elevated risk of contamination due to the many methodological steps, which includes several filtering steps. Furthermore, the methods described above were not specifically targeted for chitinaceous organisms. A simple, targeted, and efficient procedure is required to handle this complex sample type to facilitate important research on microplastics and chitinaceous organisms.

An important aspect which needs to be considered when developing methods for microplastic analysis, is whether the method is fit-for-purpose. Certainly, in the last few years many of the available methods for the analysis of microplastics have reached a baseline level of suitable sampling, extraction, and identification tools (e.g. Lusher et al., 2020; Primpke et al., 2020). Still, many of these techniques are targeted for method optimization and improvement in the time or cost efficiency of the analysis. Methods developed must be tested to ensure that they are reproducible, do not damage the target particles (at least before the chemical characterisation stage), and are validated for the relevant conditions of analysis. Well-validated methods are an important component of the ongoing method harmonization process; meaning that data generated by different researchers (and/or research groups) is comparable and can be used to build a global understanding of plastic pollution (Cowger et al., 2020; Provencher et al., 2020).

As the research community continues to call for harmonised and reproducible methods (Brander et al., 2020; Cowger et al., 2020; Provencher et al., 2020) it is imperative that all emerging or adapted methods undergo thorough validation. Therefore, the objective of the present study was to test a two-step process, that builds on formally established protocols and reduces analytical steps to minimize contamination risk (for example, with fewer filtration steps). Four protocols were tested based on the previous use of potassium hydroxide (e.g. Bråte et al., 2018; Dehaut et al., 2019), Creon® (von Friesen et al., 2019), hydrogen peroxide (e.g. Hurley et al., 2018) and nitric acid (Yu et al., 2019). These were used in combination with the enzyme chitinase, as the key digestive agent, in a simplified set-up, to separate microplastic from invertebrate tissue. The overall aim of this study was to develop a time- and cost-efficient method that is effective in dissolving organisms with a chitinaceous exoskeleton that does not affect plastic particles in the form of microplastic particles.

2. Materials and methods

2.1. Method testing

Fig. 1 depicts the experimental approach used in this study. To identify the optimum procedure for the removal of chitin that does not impact upon plastic particles, the initial methods testing phase was split into two components: Part A, testing of selected protocols on removal of chitin from woodlice; and Part B, testing of selected protocols on their impact on eight polymer types.

2.1.1. Selection of protocols

Four different protocols were tested for their capacity to efficiently dissolving chitinaceous organisms:

Protocol 1

Protocol 1 is based on dissolving the organisms using 10% potassium hydroxide (KOH). KOH is highly basic (pH 14) and has previously been used for dissolving soft tissue of biota for microplastic analysis through alkaline hydrolysis. This has been verified as one of the most appropriate methods for dissolving many forms of biota (Lusher et al., 2020; Lusher et al., 2017). Earlier studies found that 10% KOH was suitable at 60 °C for 24 h in dissolving soft tissue including fish guts and mussels, with limited impact on plastics (Dehaut et al., 2016; Dehaut et al., 2019). However, this method has been seen to impact polyacetate and rayon at 60 °C, and it has therefore been suggested to use lower temperatures (Thiele et al., 2019). Some researchers also apply variations to the

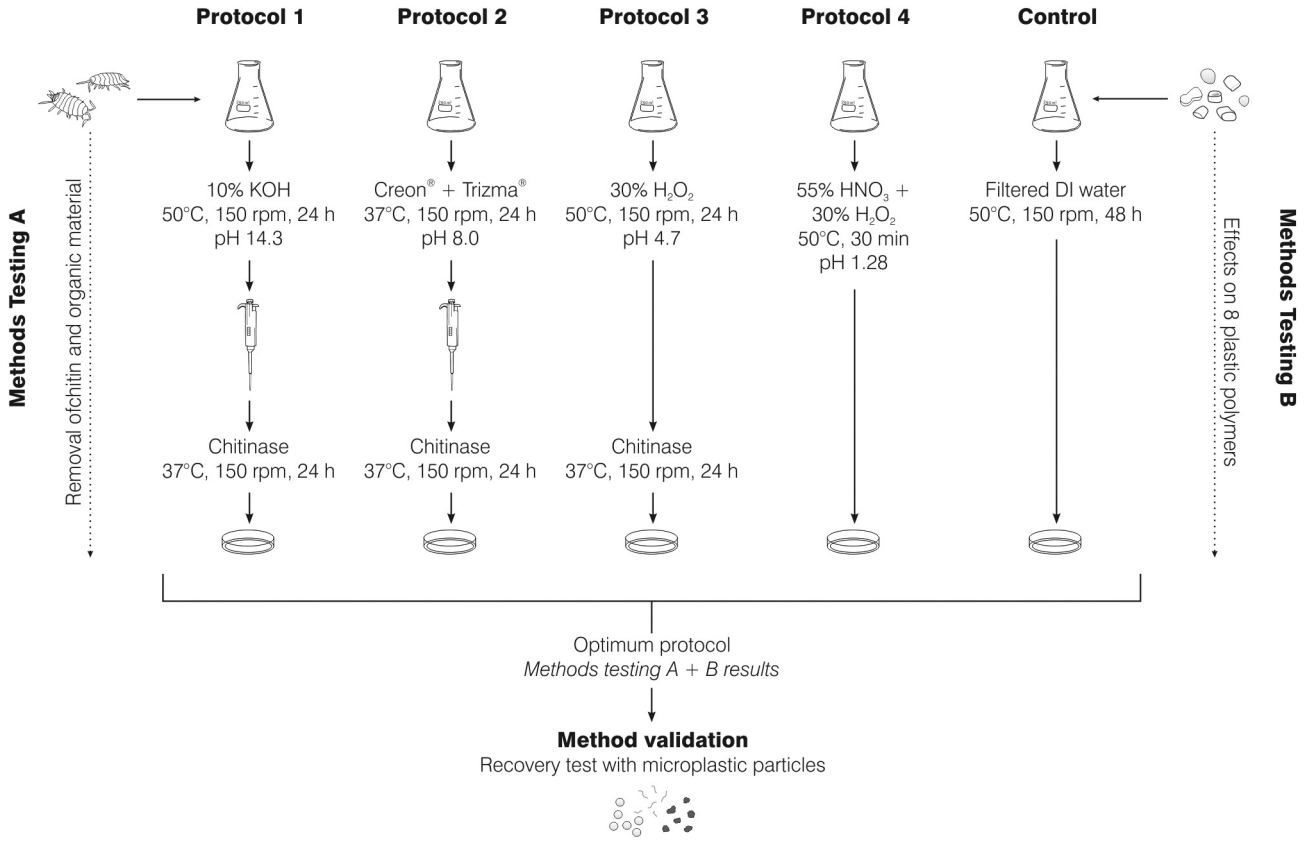

Fig. 1. Schematic summary of the four different protocols tested for suitability to dissolve organisms with a chitin skeleton.

methods, such as 20% solution or a longer digestion time (reviewed in Lusher et al., 2020). KOH alone cannot dissolve chitin, but it has not, to our knowledge, been tested in combination with chitinase (Zhu and Wang, 2020). KOH is used here to pre-treat the organisms prior to chitinase treatment.

Protocol 2

Creon® 40,000 (Abbott Laboratories GmbH, Germany, Mylan) is a pharmaceutical product that contains three different pancreatic enzymes: lipase (40,000 Ph.Eur), amylase (25,000 Ph.Eur) and protease (1600 Ph.Eur). It has been shown to be a highly efficient method for dissolving soft tissue of mussels but has not, to our knowledge, been tested on chitinaceous organisms (von Friesen et al., 2019). In addition, Creon® is a low-cost enzyme mix, that does not affect microplastic (von Friesen et al., 2019), and has the advantage of being non-hazardous. Here, we test Creon® in combination with chitinase.

Protocol 3

H_2O_2 together with the catalyst ferrous sulphate ($FeSO_4 \cdot 7H_2O$) has been successfully applied to the analysis of microplastics in complex matrices, such as sludge and soil (Hurley et al., 2018). Different studies have reported varying efficiencies of hydrogen peroxide (H_2O_2) in dissolving organic material (Avio et al., 2015; Mathalon and Hill, 2014; Nuelle et al., 2014; Tagg et al., 2015; Zhao et al., 2015). Only three studies have, to our knowledge, utilised H_2O_2 for dissolving chitinaceous organisms, although they did not report details on efficacy (Hu et al., 2018; Redondo-Hasselerharm et al., 2018; Windsor et al., 2019). Nuelle et al. (2014) found that most biogenic organic matter - including chitin - was either bleached, dissolved or fragmented after 7 days in H_2O_2 at room temperature. Moreover, effects were observed on virgin reference material polypropylene (PP) and polyethylene (PE). Method validation studies have shown that oxidation using H_2O_2 at temperatures below 60 °C for 24 h does not affect the polymers; hence, incubation time and temperature should be kept below this (Hurley et al., 2018; Tagg et al., 2017). Two studies have applied a combination of H_2O_2 and chitinase along with other chemicals to remove organic matter from complex environmental samples (Löder et al., 2017; Mintenig et al., 2017), showing promise for this approach. Yet, it was used as part of longer and more complex procedures. These multiple processing steps have the potential to introduce procedural contamination. It is desirable to minimize the number of procedural steps. Thus, testing of simplified procedure targeted chitin dissolution is required. H_2O_2 is used here as a pre-treatment step prior to the addition of chitinase.

Protocol 4

Protocol 4 is based on digestion with nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a fast and simple one-step process. For environmental samples, HNO_3 is commonly used for digesting organic material but at higher temperatures and for longer exposure times it has found to be corrosive for certain polymers including PA, PS, PE and PET (Pfeiffer and Fischer, 2020; Van Cauwenberghe and Janssen, 2014; Vandermeersch et al., 2015). The method has been applied to biota samples (fish and mussels) for 30 min at 50 °C; however, this methodology has not yet been tested on chitinaceous organisms nor has its potential effect on some polymers such as polypropylene (PP) and polycarbonate (PC) been specifically investigated (Yu et al., 2019). The method has been applied on a protein – keratin (human hair and nails) – and was found to successfully dissolve the sample but has not, to our knowledge, been tested for dissolving chitin (Liu et al., 2015).

Addition of chitinase

As depicted in Fig. 1, protocols 1–3 all include the addition of chitinase following chemical priming. Protocol 4 was not tested in combination with chitinase as preliminary studies have indicated that this approach might be efficient at removing chitin without the need for additional enzymatic digestion (Liu et al., 2015; Zhu and Wang, 2020). One of the main goals of this current study was to establish a method that employs fewer processing steps, in an effort to minimize the potential

for laboratory contamination. Therefore, chitinase was added after the removal of residual reagents by pipetting (Protocol 1 and 2) or following neutralization of the reagent pH (Protocol 3). This prevented the need for filtering the samples in between processing steps. In the case of Protocol 3, the reaction between organic material and H_2O_2 leads to the production of water, which does not interact with the chitinase. This is a modification from previous studies which removed H_2O_2 from the samples before adding chitinase (Löder et al., 2017; Mintenig et al., 2017).

2.1.2. Selection of test organism

Woodlice (*Oniscus aséllus* L.) are a common terrestrial isopod that live in humid and dark habitats and primarily feed on decaying organic matter, such as plant material. This organism was chosen as a test organism as they have a thick exoskeleton of chitin compared with most terrestrial arthropods and aquatic macroinvertebrates. Furthermore, woodlice are abundant and have a relatively large body size in the adult stages, making them well suited as test organisms. The woodlouse *Porcellio scaber* has been found to ingest microplastic particles under laboratory conditions (Wood and Zimmer, 2014). As inhabit environments where plastic is likely to occur (e.g. home composts, roadsides) they may also interact with microplastics in their natural environment. The underlying assumption in the choice of test organism was that if the protocol were able to dissolve woodlice, it would be applicable to most other terrestrial and aquatic invertebrates with smaller body sizes and thinner exoskeletons. The woodlice were sampled in a wild garden in Søborg, Denmark. No studies on their microplastic content were performed, as this was not expected to affect the digestibility of the woodlice. The spiked recovery tests targeted specific particles, thus there will not be any expected influence of background microplastic particles on the results.

2.1.3. Methods testing A: efficacy of chitin removal

For all protocols, woodlice were first euthanized by freezing them at −18 °C and were then dried at 40 °C for 2 days. Thereafter, 0.1 (±0.01) gram of dried woodlice (corresponding to 3–5 individuals) was added as whole individuals to each conical flask and the 4 different protocols were tested (Fig. 1). Each protocol was tested using 6 replicates. In addition, three controls were included.

Protocol 1–3: Chitinase (ASA Spezialenzyme GmbH, Germany) was stored at −18 in glycerol until use and was then mixed with a NaOAc buffer to reach an activity of ~96 U L^{-1}. The NaOAc buffer was made from 800 mL filtered deionized water mixed with 82.03 g of sodium acetate. Thereafter glacial acetic acid was added to the solution until it reached pH = 5. The chitinase was added to the samples and incubated at 37 °C for 24 h (Fig. 1).

Protocol 1: A solution of 10% KOH was made from 10 g KOH and 90 g of filtered deionized water (Dehaut et al., 2016) and 10 mL of the solution was added to a conical flask with woodlice 0.1 g (dw). The samples were incubated at 50 °C, 150 rpm for 24 h, pH = 14.3. Thereafter KOH was removed via pipetting and the organisms rinsed with filtered deionized water before 10 mL of chitinase was added (Fig. 1). The pipetted liquid is reserved for filtering to check for microplastic.

Protocol 2: The content of one capsule corresponding to 0.7 g of Creon® 40,000 was added to 15 mL Trizma® hydrochloride solution (1 M, pH 8.0, 0.2 μm filtered, Sigma-Aldrich, ID T3038, USA) and the solution was added to a conical flask with woodlice 0.1 g (dw). The organisms were incubated at 37 °C, 150 rpm for 24 h, pH = 8. Creon® was removed via pipetting and the organisms were rinsed with filtered deionized water before 10 mL of chitinase was added (Fig. 1). The pipetted liquid is reserved for filtering to check for microplastic.

Protocol 3: 5 mL H_2O_2 30% was added to a conical flask containing 0.1 g woodlice (dw). The organisms were incubated at 50 °C for 24 h. Thereafter 10 mL of chitinase was added directly into the conical flask without removing the H_2O_2, since the pH are the same for the two solutions. The sample was incubated at 37 °C for 24 h, pH = 4.7. The reaction between organic material and H_2O_2 leads to the production of water, which does not interact with the chitinase. This is a modification from previous studies which removed H_2O_2 from the samples before adding chitinase (Löder et al., 2017; Mintenig et al., 2017) (Fig. 1).

Protocol 4: HNO_3 (55%) was mixed with H_2O_2 (30%) 4:1 and added to a conical flask containing woodlice. It was heated to 50 °C and left to incubate for 30 min, pH = 1.28. 8 mL HNO_3 and 2 mL H_2O_2 was used for 0.1 g of woodlice (dw). Initial tests were made, to find the optimal volume and incubation time. Six replicates were made using Protocol 4.

Controls were performed by heating up woodlice in filtered deionized water. 3 replicates were made by adding 0.1 g of dried woodlice to 3 conical flasks followed by adding 10 mL of filtered deionized water. The flasks were incubated at 50 °C, at 150 rpm for 48 h (Fig. 1).

The final solution of each treatment was vacuum filtered onto Whatman GF/C-filters (47 mm, pore size 1.2 μm) that were weighed prior to filtering using a Dual Range XS105 scale (Mettler Toledo, USA). Filters transferred to petri dishes and placed into an oven at 40 °C until dry. When filters were completely dry, they were weighed again to establish the weight of the remaining woodlice/chitin material. This was used to assess the percentage removal of chitinaceous material following treatment with each protocol. The filters were also photographed using an Olympus SZX10 stereomicroscope (3.5× magnification) attached to an Olympus DP22 camera to visualise the reduction in chitin material. The center point of the image was lined up with the center point of the filters. Each picture had an area of 14 mm × 10.5 mm.

2.1.4. Methods testing B: effect on plastic polymers

To test if the selected protocols caused any impacts of plastic particles, eight different plastic polymers: polyethylene terephthalate (PET), polymethyl methacrylate (PMMA), polypropylene (PP), polystyrene (PS), high-density polyethylene (HDPE), low-density polyethylene (LDPE), polycarbonate (PC), and polyamide-6,6 (PA-6,6) were selected and exposed to the four chemical treatments. These eight polymers were chosen as they are relatively common and make up >65% of the European plastic production in 2019 (PlasticEurope, 2020). The tested particles were pre-production pellets in the size range of 3–5 mm and represented virgin polymers. This was used to correspond with previous methods testing exercises (Hurley et al., 2018) and to facilitate testing of multiple different effects. Full details of the reference polymers are provided in Supplementary material 7.

Three pellets were used in each replicate, and three replicates were performed for each of the four protocols. Prior to treatment, individual particles were each photographed using an Olympus DP22 camera attached to an Olympus SZX10 stereomicroscope, and weighed using a Dual Range XS105 scale from Mettler Toledo. An FTIR spectrum was also produced for each particle. This allowed for an assessment of any effect of the protocols on the chemical spectra, the surface characteristics (colour, appearance) and the weight of the plastic material. Plastic spectra were obtained using an Agilent Cary 360 FT-IR with a diamond ATR crystal accessory (sample scans: 8, resolution: 4 cm^{-1}, range: 4000–650 cm^{-1}). Treatment with the protocols followed the procedure outlined in Section 2.1.3, with the absence of woodlice organisms.

Following treatment, the particles were removed from flasks, rinsed with filtered deionized water and dried, and then weighed, photographed and analysed with FTIR again. The spectra of reference material after treatment with the protocols were compared manually to reference material that had not been treated with any protocols, to observe any deviations. In addition, a hit quality index was generated by the Agilent MicroLab PC software. This was based on a scalar product algorithm to assess the similarity between the measured spectrum and reference spectra stored in the library, resulting in a score between 0 and 1. The reference library used was the Agilent Polymers ATR library. The same analytical method file was used on the FTIR, so all measurements were taken with the same data treatment. The hit quality index is a measure of similarity to the library spectra (Agilent Polymers ATR library). The hit quality index generated prior to and following treatment

was recorded to check for any changes resulting from the selected protocol in the efficacy of library matching. For particles exhibiting surface degradation, fragments from the outer layer were analysed separately to test for differences in the FT-IR spectra at the particle surface and in the deeper layers. Changes in surface characteristics were noted from the photograph images and changes in the particle mass were observed through discrepancies in pre- and post-treatment weights.

2.2. Method validation

Based on the results of the methods testing phase, the optimum protocol was then subjected to a validation procedure. Namely, recovery tests were performed using a range of microplastic particle types to assess the capacity of the protocol to effectively reduce chitin and isolate microplastics for further analysis.

To test the recovery efficiency, 0.1 g of woodlice was first added to a conical flask. Six replicates were included. Several microplastic particles were then added to each replicate: 20 car tyre particles (250–500 μm), 20 PET fibres (<2 mm), and 20 PE beads (420–500 μm). The samples were then treated following the identical chemical treatment procedure as defined above for the optimum protocol. The samples were swirled to mix the reference material with the woodlice. Following treatment, the final solution was filtered on Whatman GF/C-filters and analysed visually under an Olympus SZX10 stereomicroscope. Photographs were taken using an Olympus DP22 camera accessory and these were used to quantify the microplastic particles. The images were processed using the open-source image processing software ImageJ/Fiji (version 2.35). The pictures were processed by subtracting background, which even out unequally illuminated areas at the filter. Thereafter the pictures were transformed to 8-bit, and then processed using the build in Fast Fourier Transformation (FFT) with a bandpass filter, that filters out noise and uneven background. These processing steps were followed by thresholding using the default method. The threshold was adjusted manually based on inspection of the unprocessed pictures. The recovery was assessed based on the number of microplastic particles that were isolated from each replicate following treatment.

2.3. Additional test on the effect of ethanol storage

Preliminary testing for this study observed a potential influence from ethanol storage on the efficacy of the method. Hence, an additional test was included to assess this effect. Environmental samples of chitinaceous organisms are often stored in ethanol following collection in the field. The optimum protocol was also applied to organisms that had been stored in ethanol for 11 months, to test if this affected the digestion. The ethanol was first removed by pipetting and the organisms were rinsed with filtered deionized water before treatment. 0.1 g of woodlice were placed into conical flasks and treated to an identical procedure as outline above for the optimum protocol. A total of six replicates were included. The effect of ethanol was assessed by comparing photographs with the results from Part A of the Methods testing (Section 2.1.3).

2.4. Statistical analysis

Statistical analyses were carried out in RStudio. A Kruskal-Wallis test followed by a Dunn post-hoc analysis was performed to test for statistical differences between mean percentage biomass remaining of the woodlice before and after treatment with the four protocols. In addition, a Welch two-sample *t*-test was carried out to compare the area of undegraded chitin calculated from the photographs. Differences in weight change of the reference polymers before and after treatment was also tested for the four different protocols. A linear regression analysis in RStudio was carried out using the lm-function. Model check was carried out via residuals vs. fitted values, normal Q-Q, Cook's distance and Scale location.

3. Results and discussion

3.1. Efficiency of digestion protocols for organisms with a chitinaceous exoskeleton

Protocol 1 and 2 were inefficient for dissolving chitin. This was immediately visible following a qualitative inspection of the filters that revealed intact exoskeletons. The method based on degradation by KOH (Protocol 1) did not break down the chitinaceous exoskeleton of the woodlice. The woodlice exoskeletons were bleached but remained intact (Fig. 2a). If this protocol were to be applied to environmental samples, microplastic ingested by the organisms may be trapped inside which will hinder subsequent analysis. A similar outcome was observed for Protocol 2, where intact, unbleached organisms with no sign of degradation of the exoskeletons were observed. Thin chitin structures such as antennas and uropods also remained intact (Fig. 2b). Woodlice in Protocol 1 and 2 had an average weight reduction following the treatment of 48% and 70% respectively (Fig. 3). This weight reduction was a result of digestion of less recalcitrant organic matter within the organism, while the chitin exoskeleton was left intact. The inefficacy of protocol 1 and 2 is suggested to be due to the alkaline nature of the treatment, which is ineffective at digesting calcium compounds within the exoskeleton.

Protocol 3 (H_2O_2 + chitinase) was found to be a highly efficient method for dissolving the macroinvertebrates. The first step using H_2O_2 at 50 °C removed all organic material with the exception of chitin. It also caused the organism to fragment into pieces of chitinaceous exoskeleton. These fragments were then dissolved following the addition of chitinase. Some smaller chitin fragments were left after the treatment, but nothing that would preclude undertaking chemical characterisation of microplastics (Fig. 2c). The mean percentage biomass remaining was 9% after applying Protocol 3 (Fig. 3). Similarly, Protocol 4 (HNO_3: H_2O_2) was also effective at digesting chitin, as well as the rest of the organism. This represents an efficient method as it utilises a one-step process, with just 30 min incubation time. Small pieces of chitin remained at the end of the treatment but in sufficiently small pieces that would not drastically interfere with chemical characterisation of microplastics (Fig. 2d). The mean percentage biomass remaining was 10% when applying Protocol 4 (Fig. 3). Protocol 3 and 4 are both acidic which will degrade the calcium compounds and improve the bioavailability of chitin for the subsequent chitinase step.

A Kruskal-Wallis test followed by a Dunn post-hoc analysis revealed no differences between mean percentage biomass remaining between the Protocol 1 and 2 ($p = 0.117$) and between Protocol 3 and 4 ($p = 0.999$). However, Protocol 1 and 2 differed significantly from Protocol 3 and 4 (Fig. 3). As both Protocol 3 and Protocol 4 were able to digestion chitin, a Welch two-sample *t*-test was applied to look for differences between the results. There was a significant difference between the mean area of remaining chitin when applying the Protocol 3 and 4 ($p = 0.0016$, $df = 8.63$, $t = -4.53$). The six replicates from Protocol 3 had a mean area of 1.24 mm^2 (±0.53 SD) chitin left on the filter, whereas Protocol 4 had a mean area of 5.60 mm^2 (±0.80 SD) of chitin remaining. No statistical test was carried out on the area of remaining chitin when applying Protocol 1 and 2 as the extent of degradation when using these protocols was deemed to be insufficient.

Based on the results of the first phase of methods testing, both Protocol 3 and Protocol 4 were shown to be effective at significantly reducing chitin exoskeletons and other organic biomass. However, there was a significant difference between the amount of chitin that remained following treatment with the protocols. Protocol 3 was slightly more effective than Protocol 4, resulting in fewer small chitin fragments on the filter. Yet, the amount of chitin that was left of the filter following both protocols was considered to be sufficiently low as to not impede subsequent analysis of microplastic particles using visual or chemical analysis. With respect to processing time and efficiency, Protocol 4 represents the treatment with the shortest processing time and fewest

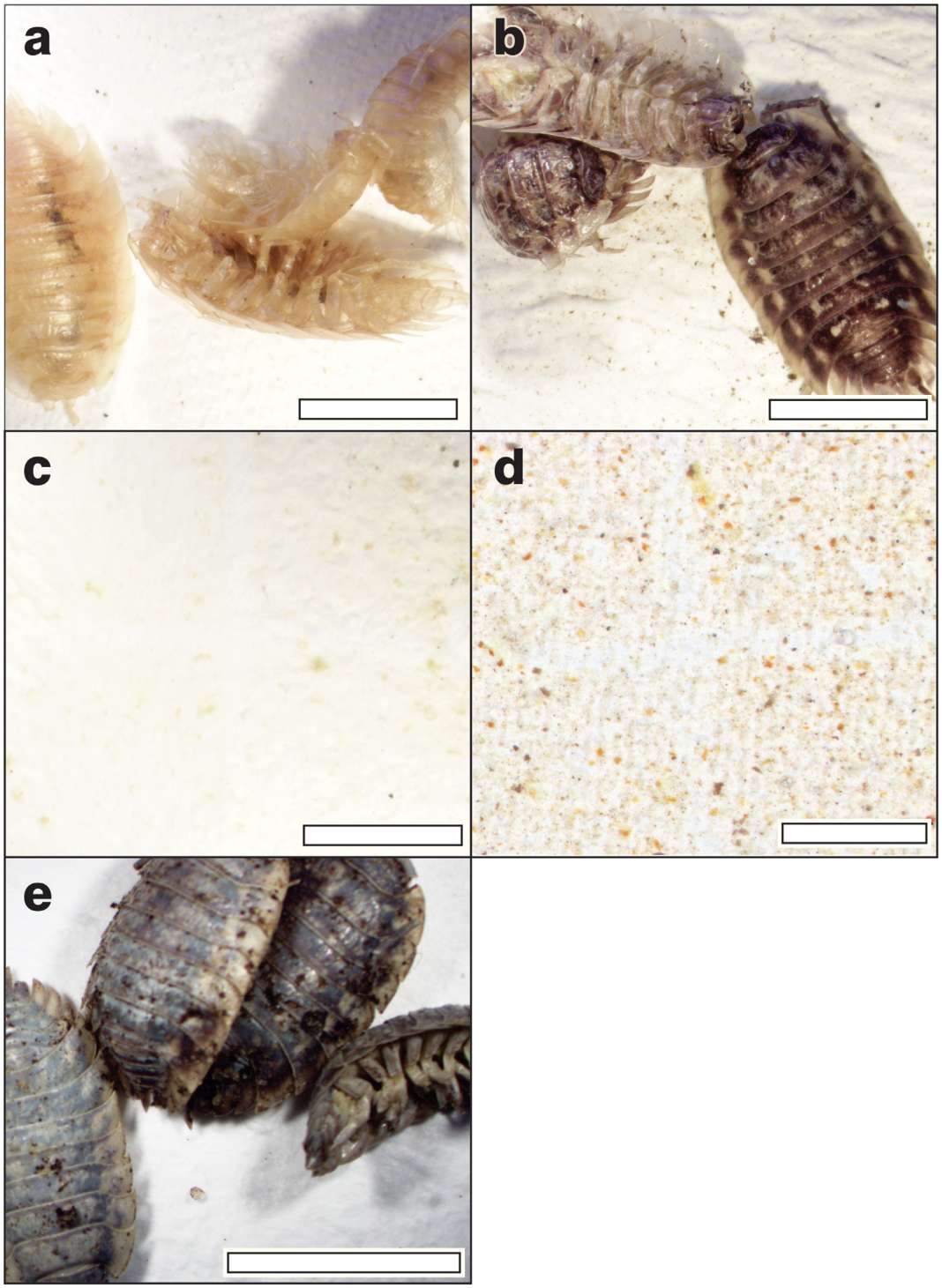

Fig. 2. Pictures visually showing the remains of dissolved woodlice on GF/C-filters when applying a) Protocol 1 (KOH + chitinase), b) Protocol 2 (Creon® + chitinase), c) Protocol 3 (H_2O_2 + chitinase), d) Protocol 4 (HNO_3 + H_2O_2) and e) control with water. All bars represent 5 mm. Pictures of all replicates can be seen in Supplementary material 1.

processing steps. This is advantageous given the reduced risk for potential laboratory contamination. However, for Protocol 3 the chitinase was added directly to the H_2O_2 solution following neutralization, so this protocol also had reduced processing steps compared to Protocols 1 and 2 (which included a pipetting step). Even though the samples need to be in incubated for 48 h in total, the actual person-hours associated with Protocol 3 are in fact similar to Protocol 4. Thus, both protocols represent effective and efficient methods for reducing chitin exoskeletons and other organismal biomass on the tested organism. The chemical composition of the exoskeleton of different chitinaceous organisms vary, depending on habitat and life style (Neues et al., 2007), and future studies should test the protocol on, for example, aquatic organisms.

3.2. Effect of four protocols on 8 plastic polymers

Fig. 4 summarises the effect of the different protocols on the eight selected polymer types, following treatment. Effects were defined based upon the visual images. Particles were classified as "No effect", when

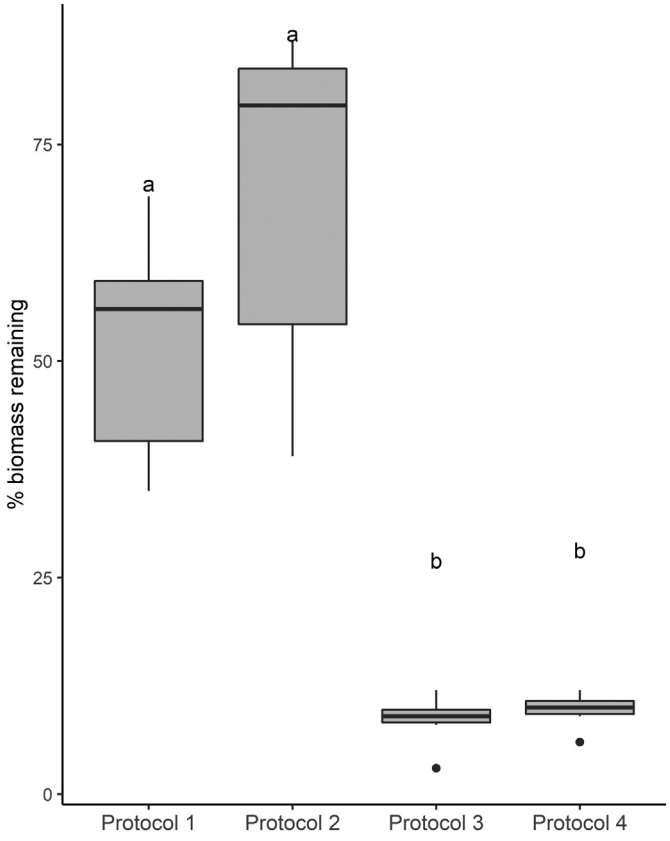

Fig. 3. A boxplot illustrating the percentage biomass remaining change of woodlice using the four protocols 1) Protocol 1 (KOH + chitinase), 2) Protocol 2 (Creon + chitinase), 3) Protocol 3 (H_2O_2 + chitinase), 4) Protocol 4 HNO_3 + H_2O_2. Where letters are different, they indicate significant differences between means (Kruskal-Wallis test followed by a Dunn post-hoc analysis, $p < 0.005$).

no visible effect was observed, "Little effect" was assigned when colour changes were observed, while particles with changes in surface characteristics or shape as categorised as "Major effect".

Protocols 1 and 4 both caused effects to several different polymer types. Treatment with KOH followed by chitinase (Protocol 1) induced visible changes to the integrity and structure of PET and PC (Fig. 5). Surface degradation occurred for PET, while PC fragmented completely into crystalline structures. The weight change measurements supported these findings, recorded as a significant reduction in the weight of PC and PET following treatment (Fig. 6). The chemical spectra for PC, PA-66 and HDPE were also slightly affected by Protocol 1. The PC spectra were missing the absorption peak at 1762 cm^{-1}, and the area between 1863 cm^{-1} to 630 cm^{-1} contains many dissimilarities. The spectra for PA-66 had an additional adsorption peak at 1732 cm^{-1}, while HDPE had an additional adsorption peak at 1575 cm^{-1} (Fig. S6). Despite these changes, the hit quality index scores remained about 0.65 for all polymers treated with Protocol 1 (Fig. 6).

Treatment with Protocol 4 also caused several visual, weight, and chemical changes to some of the polymer types. PA-66 and PMMA were both significantly degraded after 30 min in the solution: The PMMA pellets were melted and stuck together, while PA-66 was completely destroyed (Fig. 6). In addition, Protocol 4 caused discolouration of PP (Fig. 6). This was also observed in the mass measurement: the weight of PA-66 was significantly reduced, as no particle was left after treatment, while the weight of PMMA actually increased significantly (Fig. 7). In the FTIR analysis, several small deviations from the reference spectra were observed for some of the polymer types. PMMA varied from the reference spectrum by with a large shoulder around the absorption peak at 1724 cm^{-1} and a reduction in the size of the peaks at 703 cm^{-1}, 749 cm^{-1} and 1434 cm^{-1}. PS was changed at two of the characteristic absorption peaks (1600 cm^{-1} and 1497 cm^{-1}) (Supplementary material 6). A summary of the hit quality index scores is given in Fig. 6. Based on these results, Protocol 4 was found to have a severe effect on two of the eight tested polymers, i.e. PA-66 and PMMA, which both melted during the 30 min incubation. Additional smaller changes were also observed for other polymer types.

Catarino et al. (2017) previously reported that PET and HDPE melted after an hour-long exposure of 35% HNO_3. In addition, Dehaut et al. (2016) observed similar melting of PA-12 as observed for PA-66 in this study after exposure to 65% HNO_3 at 60 °C for two hours. The previous study investigating HNO_3:H_2O_2 for digesting organic material for plastic analysis did not report any alterations of the tested polymers (Yu et al., 2019). However, upon studying the chemical resistance sheets from different plastic producers, it is clear that HNO_3 at concentrations >40% and temperatures ≥50 °C has a moderate to severe effect on tested polymers (Bürkle GmbH, 2018; Ted Pella, 2019). For example, Bürkle GmbH (2018) tested 50% HNO_3 at 50 °C and found that HDPE, LDPE, PA, PC, PP, PS were all significantly affected. The tested polymers in this study as well as the commercial studies are all pristine plastic polymers of a relatively large size. An even more severe effect could be expected, had the methods been tested on environmentally deteriorated plastic particles and smaller sized particles. Desforges et al. (2015) used HNO_3 at 80 °C for 30 min to dissolve marine zooplankton without testing the recovery of plastic polymers. Similar high temperatures of HNO_3 were applied by Claessens et al. (2013) who reported 0% recovery of nylon fibres. This has also been observed in other method evaluation studies (Avio et al., 2015; Catarino et al., 2017; Dehaut et al., 2016). Thus, this study supports the consensus that in applying HNO_3 there is a risk of destroying some polymer types and thereby underestimating the number of particles. Consequently, Protocol 4 was deemed to be unsuitable for microplastic analysis in chitinaceous organisms.

The enzymatic treatment with Creon® followed by chitinase (Protocol 2) did not show any visible effect on the tested plastic polymers when inspected under a stereoscope (Supplementary material 3). However, a significant increase in weight was observed for PA-66 (Fig. 7). The only chemical spectra from polymers that varied from the reference spectra were for PC, where an additional absorption peak was observed at 2962 cm^{-1} (Supplementary material 6). Yet, all of the hit quality index scores were ≥0.70 for all of the polymers (Fig. 6).

The eight different plastic polymers showed no sign of degradation from the H_2O_2 + chitinase treatment (Protocol 3) following visual inspection (Supplementary material 4). However, the weight of PA-66 and PMMA did significantly increase in response to the treatment (Fig. 7) and there were minimal changes in the chemical spectra generated by FT-IR analysis (Supplementary material 6). A summary of the spectral analysis is given in Fig. 6. Thus, Protocol 3 did not show any signs of degradation of the eight different polymers exposed to the treatment – neither when inspected visually or when chemical FT-IR spectra were analysed. Also, no reduction in weight was observed for any of the polymers in response to the treatment.

3.3. Method validation

The test of chitin removal (Methods testing Part A) revealed that Protocols 3 and 4 were both effective treatments, while the testing of effects resulting from exposure of plastic particles (Methods testing Part B) identified that Protocols 2 and 3 did not cause any negative impacts. Therefore, Protocol 3 was identified as the optimum procedure by this study.

The validation test for Protocol 3 revealed high recoveries of the different microplastic particle types (Fig. 1 Supplementary material 8). 100% of tyre particles were recovered, while 89% and 85% of beads and fibres were observed after treatment, respectively. A mean recovery rate equal to or higher than 85%, is considered satisfactory for analysis (Hurley et al., 2018). This is especially the case for fibrous particles, which have been observed to have a lower recovery rate from a range

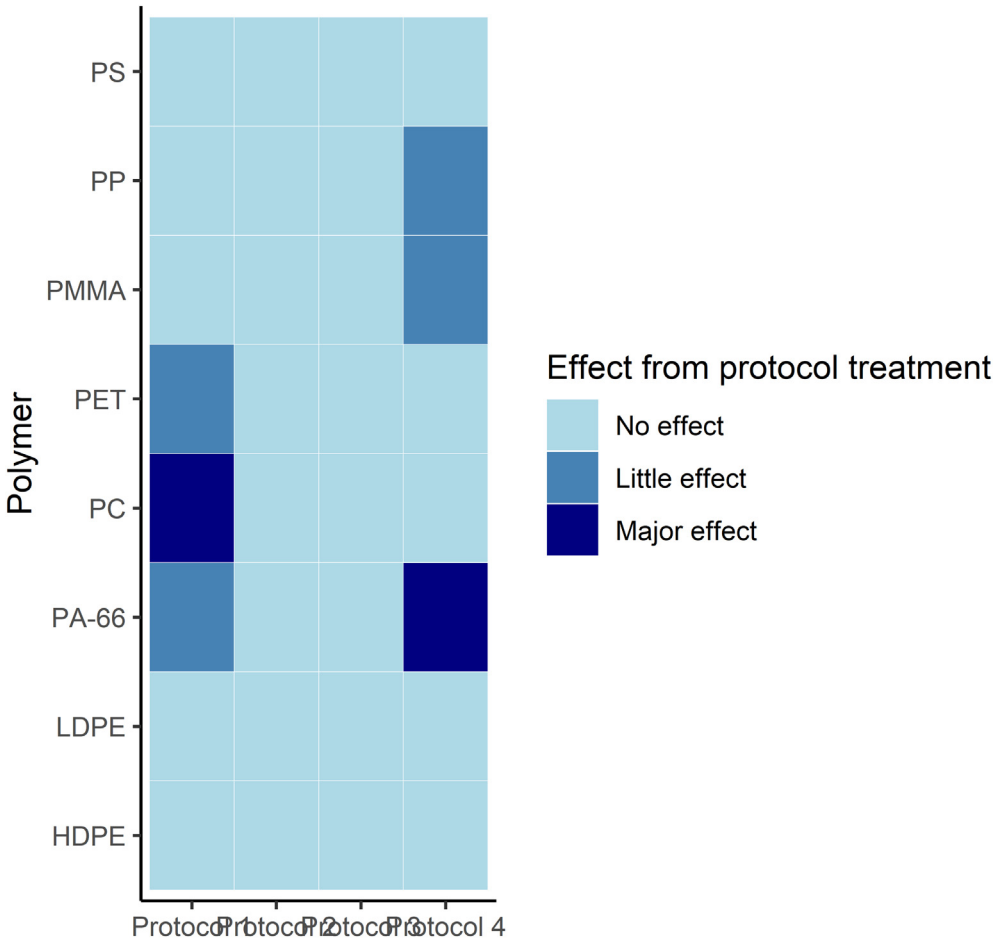

Fig. 4. Schematic overview of the effect of treatment with the four protocols on 8 difference plastic polymers. "No effect" = no visible effect, "Little effect" = changes in colour, "Major effect" = Changes to surface structure or polymer shape.

of different sample types (Hurley et al., 2018; Karlsson et al., 2017; Nuelle et al., 2014). Relatively high recovery rates might be a result of the few steps in this protocol, as the solution is only filtered once, which reduces the risk of losing plastic particles.

The method validation test included here showed that Protocol 3 is effective for the analysis of a range of microplastic particles types in samples containing chitin exoskeletons. It is important that studies utilizing this method continue to include recovery tests to examine the

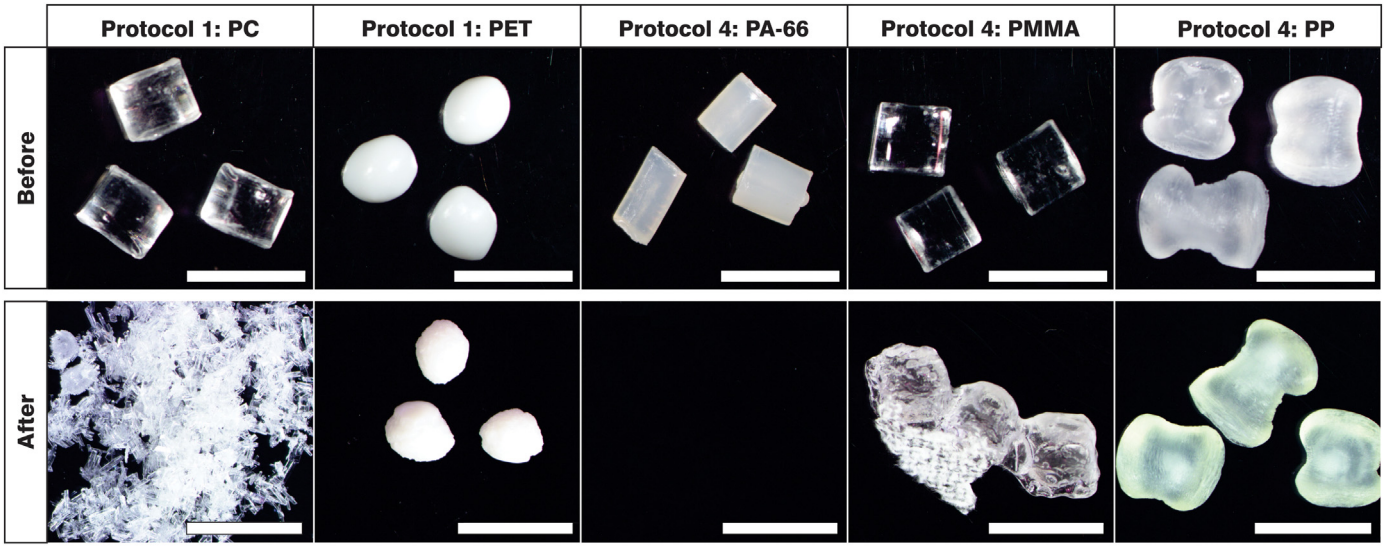

Fig. 5. Pictures of reference plastic before and after treatment. Pictures are only shown for those polymers that have changed due to the treatment. Pictures of all polymers before and after treatment using the 4 different protocols can be found in Supplementary material 2–4.

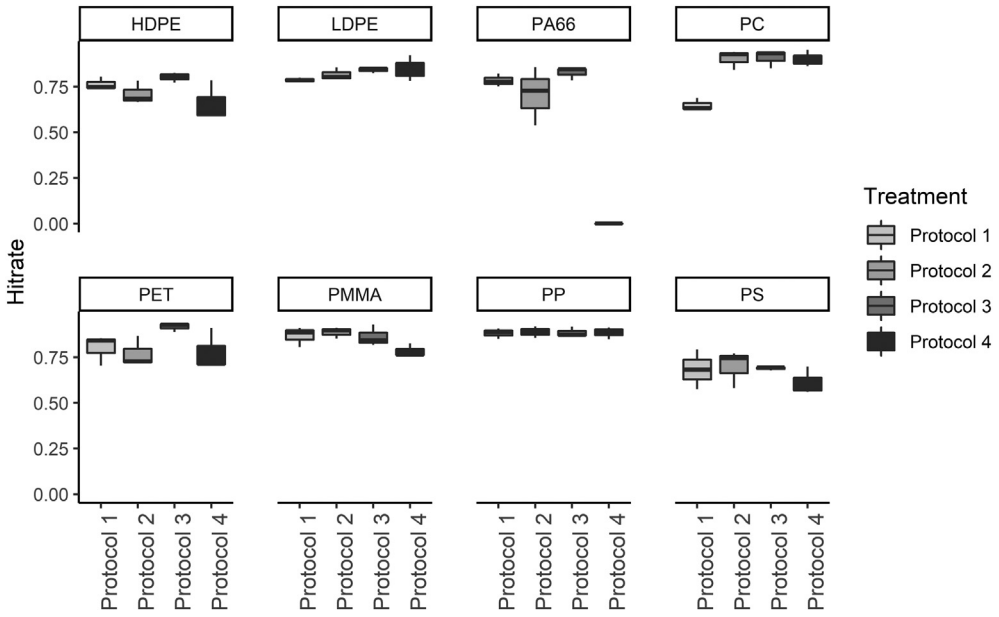

Fig. 6. Hit quality index value (SD) of the 8 tested reference plastic polymers after treatment with the four protocols.

study-specific recovery of particles from different chitinaceous sample types (Cowger et al., 2020).

3.4. Effect of ethanol storage

Protocol 3 was also tested to examine the effect of ethanol storage on method efficiency. The results from organisms stored in ethanol for 11 months were compared to the data from Part A of the methods testing, which was conducted on organisms that had instead been frozen. This clearly showed that ethanol prevents the digestion of the organism with H_2O_2 + chitinase, as an intact exoskeleton was observed after treatment. The frozen macroinvertebrates, in contrast, were completely dissolved (Fig. 2). The inability to digest chitin from ethanol preserved samples limits the possibility of retrospectively extracting plastics from previously collected material, e.g., from earlier research projects and monitoring programmes. As the method did not dissolve macroinvertebrates stored for a longer time in ethanol, storage of environmental samples by freezing is recommended as a preservation method. For field collection, it is suggested to euthanize the organism using dry ice, for example. This will also prevent the macroinvertebrates from emptying their gut, which may occur when they are placed into ethanol, as observed in other studies. Current protocols for sampling of macroinvertebrates, such as those in the context of the Water Framework Directive (WFD) include preservation in ethanol. The results of this study therefore suggest that sampling protocols will need to be modified for future assessments of chitinaceous biota for the presence microplastic, especially within the context of environmental impact assessment where multiple species and individuals are often collected.

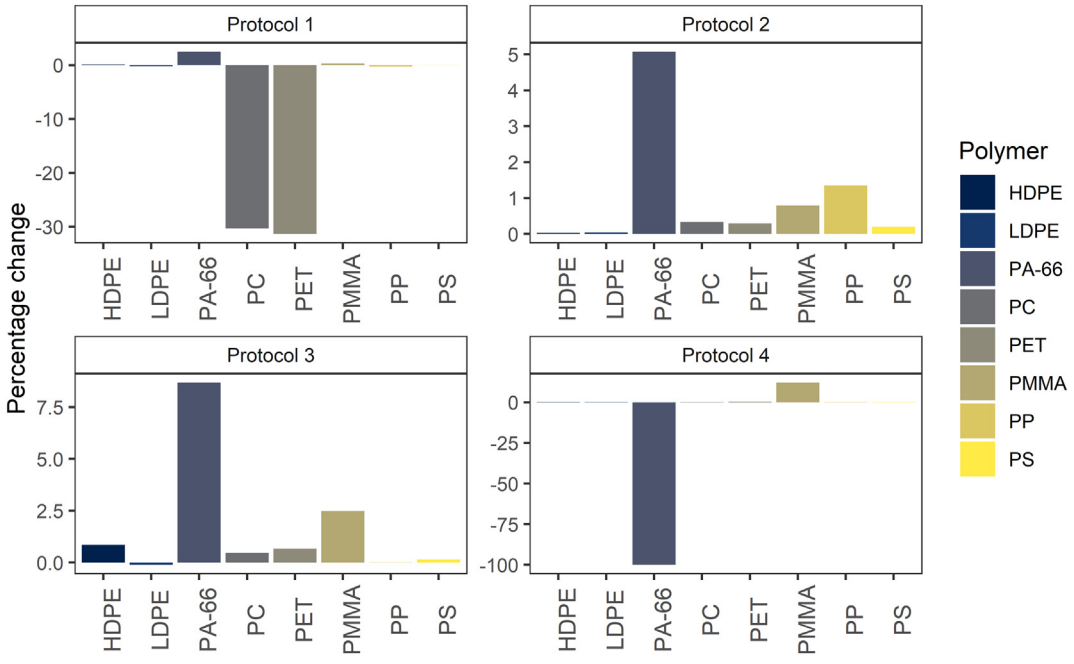

Fig. 7. Percentage change in mass of the tested plastic polymers after treatment. Five particles of each polymer were exposed to the treatments.

3.5. Analytical cost and safety

Several studies have reported that enzymatic digestion of environmental samples is expensive (Miller et al., 2017; Wagner and Lambert, 2017). This may have led to the lack of studies investigating the effect of chitinase on chitinaceous organisms. Yet, the method tested here is not only time efficient, it has also low costs. The price associated with enzymatic digestion varies significantly based on the enzyme that is used. In the case of chitinase, the cost of procuring the enzyme is relatively inexpensive. For example, dissolving 10 g of dried woodlice (corresponding to approximately 400 individuals >5 mm) using chitinase would cost less than 5 euros. The preparation of the NaOAc-buffer and the pre-treatment with H_2O_2 would cost around 3 and 9 euros, respectively, resulting in a total cost of 17 euros for the analysis at the time of writing. Compared to other costs associated with analysis of microplastics in environmental samples, and the costs associated with filters, machinery, salary etc., this must be considered a marginal cost.

Processing samples should always aim to minimize the risk to personnel, for example related to exposure to hazardous chemicals. Therefore, this aspect should be taken into consideration when assessing the applicability of a new method. H_2O_2, which is used in both Protocol 3, is hazardous when it comes into contact with the skin or eyes or is inhaled or ingested. Chitinase is an enzyme and is not hazardous. However, glacial acetic acid which is used to adjust the pH of the buffer can cause irritation if inhaled or if in contact with skin. The NaOAc buffer itself can cause mild skin irritation, eye irritation and be harmful if ingested or inhaled. Thus, for Protocol 3 safety precautions should be taken, and it is recommended to carry out the work in a fume cabinet, and to wear safety glasses and safety gloves. If these safety precautions are made, the method should be safe to apply.

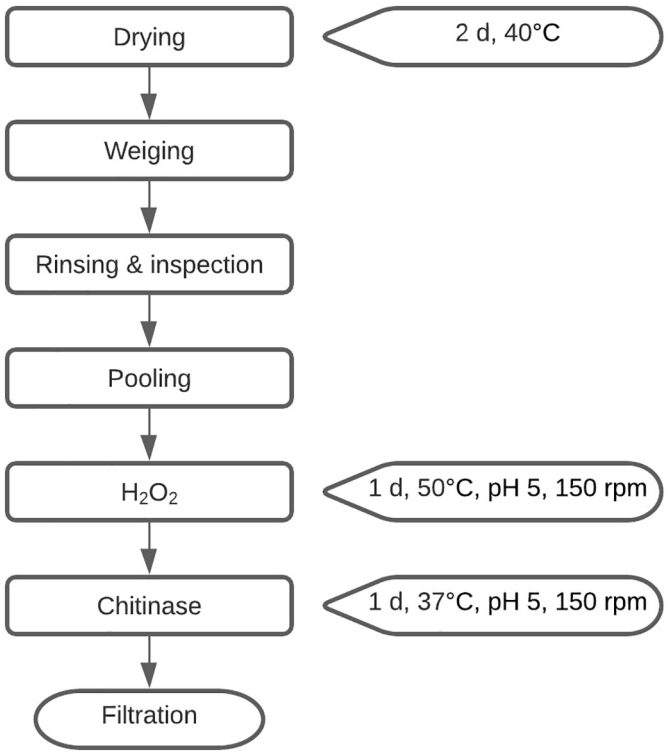

Fig. 8. Flow chart illustrating recommended processing of organisms with a chitin exoskeleton collected for microplastic analysis.

4. Future methodological recommendations

Outcomes of the study presented here suggest that future studies of microplastic content in organisms with a chitin exoskeleton could follow a structure as illustrated in Fig. 8, to make the analysis standardised and the results comparable between studies. The method is tested on woodlice, but is suitable for both terrestrial and aquatic organisms with a chitin exoskeleton of approximate 0.3 cm–5 cm e.g. isopods or macroinvertebrates. The organisms should be dried, and the dry weight should be measured. If organisms will be pooled for analysis, a known number of organisms should be included, and all organisms should be rinsed with filtered deionized water three times an inspected using a stereomicroscope to ensure that no microplastic particles are adhering to the outer surface of the organisms. By measuring, weighing and counting, results can be reported with in different units, which can ease comparability between studies and the applicability of results.

Currently, there are several initiatives developing recommendations for protocols to access the presence of microplastics in biota (including GESAMP, OSPAR, ICES, and AMAP). The available recommendations do not yet include chitinaceous organisms, which implicates the study of some of the most abundant species in the terrestrial and aquatic environments. This is a possibly related to the earlier reported challenges in efficiently processing chitinaceous materials. We have shown here that it is possible to process samples following an effective and cost-efficient procedure utilizing woodlice as a model/test organism. As isopods have been identified as appropriate bioindicators for biodiversity, habitat quality and environmental contaminants (e.g. Longo et al., 2013; Paoletti and Hassall, 1999), adopting them for the assessment of microplastics shows promise. Moving forward, the method presented here should be applied to environmental samples including organisms collected from marine, terrestrial, and freshwater ecosystems to verify the efficacy for a wider range of sample types. Chitinaceous organisms may prove a group of organisms suitable to derive differences in microplastics abundance between terrestrial and marine ecosystems. For example, isopods are wide-spread, easily identifiable and form dominant components of the soil macrodecomposter communities (Paoletti and Hassall, 1999) and coastal intertidal and shoreline communities (Longo et al., 2013), thus their application for environmental monitoring of microplastics must not be overlooked. Further investigations should build on early research which identified microplastics in marine isopods and compare similar species from different environmental compartments for their potential adoption as bioindicators of microplastic contamination of the global environment.

5. Conclusion

This work presents an efficient, cost-effective a validated analytical method that is capable of dissolving organisms with a chitin exoskeleton using a combination of H_2O_2 and the enzyme chitinase. The method was highly effective in dissolving the chitin exoskeleton of our test organism, making it easy to separate ingested plastic particles from the negligible animal remains left after treatment. Moreover, recovered microplastic particles were intact with no obvious signs of deterioration relating to the treatment. Given that the method yielded very good overall results and is cost-efficient in terms of reagents and processing time, it appears to be superior to methods currently used. Considering the global importance of arthropods, and other chitin containing biological organisms in terms of species diversity and biomass, we expect that our method will have a high acceptance among scientists working with plastic contamination in the environment. This method will facilitate the assessment of microplastic occurrence in chitinaceous organisms globally, significantly increasing our understanding of uptake and interaction of microplastic by biota in both aquatic and terrestrial ecosystems.

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CRediT authorship contribution statement

Emilie M.F. Kallenbach: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Rachel R. Hurley:** Conceptualization, Methodology, Resources, Writing – original draft, Visualization, Supervision. **Amy Lusher:** Conceptualization, Writing – original draft, Supervision. **Nikolai Friberg:** Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition.

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.scitotenv.2021.147455.

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