Enzymatic digestion method development for long-term stored chitinaceous planktonic samples

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Abstract

Different extraction methods have been proposed to study the ingestion of microplastics by marine organisms, including enzymatic digestion. While mussels have been the focus of research, crustaceans' enzymatic digestion has received little attention. An overlooked source of information for microplastic research is analysis of long-term time-series biotic samples. These collections are invaluable for the detection and monitoring of changes in ecosystems, especially those caused by anthropogenic factors. Here, crustacean larvae collected in two periods, 1985 and 2020, in the central North Sea were used to develop and optimise an effective and gentle enzymatic digestion method suitable for microplastic research. Sequential breakdown of these chitinaceous samples *via* a mechanical and surfactant (Sodium Dodecyl Sulphate 1% v/v) pre-treatment, followed by proteinase K (100 mU/mL) and chitinase (50 mU/mL) digestion, efficiently removed >96% of biomass of 1985 and 2020 samples. The optimised method was effective without interfering with the identification of naturally weathered microplastics *via* FTIR Spectroscopy.

Keywords

Microplastics, Chitin, Zooplankton, Plastic pollution, Crab larvae

1. Introduction

Microplastics (MPs) are a ubiquitous pollutant in the marine environment (Geyer, Jambeck, and Law 2017). Of particular concern is their prevalence in marine biota, as MPs have been found in every aquatic organism studied for microplastic research (Weston et al. 2020; Rezania et al. 2018). Information on the prevalence and fate of microplastics in the marine environment is key to assess their bioavailability and movement through the marine food web. This knowledge is also crucial to determine environmental quality standards that could lead to the implementation of appropriate control measures. Investigations into microplastics contamination have used several strategies of extraction including numerous physical (Therien et al. 2019), chemical (Thiele, Hudson, and Russell 2019), and enzymatic methods (Löder et al. 2017) . Diluted potassium hydroxide (10% v/v or 1M) has been proposed as the quickest and most cost-effective method despite its potential impact on certain synthetic polymers (i.e. polyethylene terephthalate, PET) (Thiele, Hudson, and Russell 2019). In contrast, enzymatic digestion methods have been deemed as laborious, slow, and expensive, albeit being gentle and effective at removing biomass for which the enzymes used are specialists (von Friesen et al. 2019).

Research on enzymatic digestion of marine biotic samples has focussed on the breakdown of soft-proteinaceous tissues (Piarulli et al. 2019; Karlsson et al. 2017), using molluscs as model organisms (von

Friesen et al. 2019; Catarino et al. 2017). However, little attention has been paid to the enzymatic breakdown of the polysaccharide chitin, the second most abundant natural polymer on Earth, and an essential component of the Arthropoda, the largest and most diverse group of organisms in the animal kingdom (Gupta 2011). One of the principal reasons for this Phylum's successful adaptation to live in every habitat on the planet is their exoskeleton, a hierarchically structured biomineralised chitin-calcium carbonate composite material that provides them with structural and mechanical support, as well as protection against predators and pathogens (Gupta 2011; Boßelmann et al. 2007). Additionally, understanding the uptake of microplastics by marine crustaceans is of particular concern, as these organisms are prevalent in the human diet (FAO 2020).

An overlooked source of information for MPs research are long-term-time-series biotic samples. These collections are extremely valuable for the detection and monitoring of changes in ecosystems (Mieszkowska et al. 2014), especially those caused by anthropogenic factors such as pollution (Clark and Frid 2002). However, many of these samples are not collected for the purpose of MPs analysis, and the collection of samples in the older, potentially most informative, time series predated our knowledge of marine plastics in the environment. The use of long-term time-series for this purpose therefore faces two main limitations: i) These samples are often preserved in formaldehyde and alcohol solutions (e.g. 70% ethanol (EtOH), 70% industrial methylated spirit), chemicals which could have deteriorated synthetic polymers after long exposure times (Table S1); ii) the lack of mitigation and control measures at the moment of collection and handling to prevent environmental contamination. As such, biotic samples of this nature must be considered differently to samples collected for the purpose of MPs analysis. These samples provide an opportunity for investigation using an enzymatic digestion treatment, a gentler approach to biomass breakdown that would prevent further potential damage to the plastics particles they might contain.

The robust nature of chitin requires a strong digestion method to break down the material and achieve an effective biomass removal. Indeed, few chemical and enzymatic digestion methods have been employed for chitin breakdown in MPs studies (Cole et al. 2014; Rodrigues et al. 2018; Kallenbach et al. 2021). However, previously successful enzymatic methods were supported by numerous manual handling steps (e.g. drawing and expelling sample through needles, multiple sieving) that could cause sample loss and contamination, and/or by the use of hazardous substances such as strong oxidizing and corrosive chemicals (e.g. sodium perchlorate, zinc chloride, hydrogen peroxide) (Cole et al. 2014; Rodrigues et al. 2018; Kallenbach et al. 2021), making such methods unsuitable for processing samples stored from long-term time-series.

Here, the enzymatic chitin removal of preserved crustacean (decapods) larvae from a zooplankton long-term collection was assessed. The aim of this work was to develop and optimise an enzymatic digestion method to allow for successful microplastic analysis of long-term time-series samples. The objectives of this study were to a) propose and deconstruct a chitinous digestion method assessing the essential steps for optimal chitin removal, b) evaluate the efficacy of different concentrations of the chemicals and enzymes used, and c) determine an easy, and effective way to measure successful chitin removal. The method developed was then tested on recently collected decapod samples (2020) and samples from a long-term time-series (1985) for comparison of efficacy.

2. Materials & Methods

2.1 Literature review

Piarulli and collaborators (Piarulli et al. 2019) comprehensive literature review comprising four distinctive enzymatic digestion approaches covering from 1985 up to 2018 was updated to include the methods

developed up to May 2021, following similar inclusion selection criteria (Piarulli et al. 2019). Only peer reviewed articles that accounted for the effectiveness of the methods and the relative effects on MP morphology and/or chemistry were selected. The search was performed on Web of Science Core Collection, using the following search terms: “Microplastics”, “Digestion”, “Biota” combined using the Boolean operator ‘AND’. The literature research resulted in twenty eight original peer-reviewed research articles from 2019 to 2021. The initial search was further refined by including the term “Enzymatic”, resulting in a total of five distinctive publications. The enzymatic methods were analysed for: different steps employed for biota digestion and classified according to the type of organism targeted; the enzyme(s) employed; any additional treatment conducted, and the digestion efficiency reported by the authors (Table 2).

2.2 Sample collection and preparation

Two time periods of samples spanning 35 years (August 1985 and September 2020), were selected from the Dove Time Series (DTS) for use in this study. The DTS is an ongoing monthly collection of zooplankton at a station in the central-west area of the North Sea (55° 07’ N 1° 20’ W), approximately 10 Km off the Northumberland coast, UK (Evans and Edwards 1993) running since 1969. The WP3 net employed to collect the samples used in this work has been designed as standard according to UNESCO (UNESCO 1968). The WP3 net has a mesh aperture of 1 mm, having a diameter of 1.13 m, equivalent to a mouth area of 1 m^2. This net is horizontally hauled for 10 minutes (about 700 m) at a net depth of 50 m (Evans and Edwards 1993). After collection, DTS samples have been preserved on formaldehyde 4%, and stored on 70% methylated spirit (95% v/v EtOH: 5% v/v methanol).

Subsamples from the DTS collection were taken using the Folsom splitter technique (Griffiths et al. 1984), and thoroughly washed with pre-filtered deionised water (DI water). Samples were then inspected under a stereomicroscope (Leica M205C, Leica Microsystems GmbH, Germany) for taxonomic identification and to ensure specimens were free from MP external contamination. Specimens of the later stages of Brachuryan larvae (zoea stages III-V and megalopa (Ingle 1981), size range 1274.2 μm – 4119.5 μm) were segregated and individually placed on pre-weighed glass tubes, after which the wet mass content of the tubes was recorded. Samples were then further processed according to the experimental design (Table 1). Unless otherwise stated, the pre-treatment, digestion, and filtration steps were conducted under a laminar flow cabinet (Purair, LS series, Air Science, USA LLC).

Specimens from samples collected between 2009 - 2017 treated and stored under the same DTS protocols, but not part of the DTS collection, were used for the pre-treatment tests, rather than experimenting on a finite volume of old samples. Due to availability, all enzymatic digestion tests (Table 1) were conducted in triplicate for 2020 samples, and only the full effective method was tested on 1985 samples.

The study of the microplastics content of these test samples was out of the scope of this report. Naturally weathered plastics tests (section 2.8) targeted distinctive particles, minimising the influence of background microplastics on the results.

2.3 Pre-treatment

To facilitate the enzymatic action, common stages for initial physicochemical breakdown of the samples were tested. The steps evaluated were initial dry vs wet biomass, sample grinding, and surfactant addition.

a) Dry vs wet samples. To reduce the processing time and exposure of the samples to high temperatures, initial digestion tests were conducted with both overnight oven-dried (60°C) and wet specimens.

b) Grinding. With the double purpose of preventing sample contamination and losing sample material it was assessed if grinding of samples was necessary. For this, glass rods (6 x 200 mm) together with or without glass microbeads (2mm) was assessed, as the use of microbeads for grinding has been proposed as an effective pre-treatment for chitin removal (Therien et al. 2019).
c) Surfactant addition. To reduce the total processing time and considering the size and macromolecular composition of crustacean larvae (~30.2% carbohydrates, 43.3% proteins, 20.8% lipids (Jamali, Ahmadifard, and Abdollahi 2015)), the need for the addition of surfactant, which would target the lipid components, the addition of Sodium Dodecyl Sulphate (SDS 20%, Sigma Aldrich) was assessed at different concentrations (1% v/v, 2% v/v). SDS was selected instead of milder surfactants available (Xia et al. 2020), as this commonly used, anionic compound is effective for solubilising most non-covalent bonds within and between bio macromolecules. This property was particularly relevant for the disruption of chitin's strong non-covalent hydrogen bonding interactions (Deringer, Englert, and Dronskowski 2016). Simultaneously, the low concentrations of SDS tested in this study would prevent damage to the chemical structure of MPs inside specimens.

2.4 Enzymatic digestion

Targeting the exoskeleton and proteinaceous content of the specimens, the addition of glycolytic (Chitinase) and proteolytic (Proteinase K) enzymes individually and in conjunction were tested at different concentrations. A commercial preparation of Chitinase from *Streptomyces griseus* (≥200 units/g solid, Sigma Aldrich) was dissolved on sodium phosphate buffer [50 mM, pH 6.0] (Sigma Aldrich) to a final concentration of 100 mU/mL. A further dilution of the chitinase solution [50 mU/mL] was prepared in Ultra-Pure water (Fisher Scientific, UK) and tested according to the relevant treatment conditions (Table 1). Unless otherwise stated, samples were then stirred continuously on a vortex fitted with a 9-tube insert (VariMix, SciQuip, UK) for 20 mins. The reaction was allowed to proceed for 48h at 40°C on a dry bath (Fisher Brand, UK).

A commercial solution of Proteinase K (20 mg/mL, equivalent to 600 mU/mL. Bioline, UK) was mixed on Tris-HCL buffer [20 mM, pH 8] (Trizma-HCl, Sigma Aldrich) to a final concentration of 100 mU/mL. A further dilution of the proteinase K solution was prepared [50 mU/mL] and used as described on Table 1. Calcium carbonate was added to the solution to a 1mM final concentration to activate the enzyme according to the manufacturer's protocol (Bioline, UK). Samples were stirred continuously on a vortex for 20 mins, following incubation for further 24h at 40°C on a dry bath.

After digestion, samples were left to cool inside a desiccator for ~30 mins. Samples were then stirred following vacuum filtration, ensuring the tubes were thoroughly rinsed with ultra-pure water during filtration.

Table 1. Experimental Design

2.5 Vacuum filtration

Although not part of the enzymatic digestion method *per se*, most microplastics studies rely on the visual detection of putative MPs under the microscope (Qiu et al. 2016). To further facilitate the method, an initial evaluation of three types of ϕ 47 mm filters was conducted: glass fibre (GF, GA-55, 0.6 μm pore size, ADVANTEC, MSF Inc., Japan. Pack of 100 = £ 45.60), aluminium oxide membrane (Al_2O_3, Anopore membrane disc, 0.2 μm pore size, Whatman GmbH, Germany. Pack of 50 = £ 452.36), and grided mixed cellulose esters membrane filters (MCE, 0.8 μm pore size, MF-Millipore, UK. Pack of 100 = £ 140.76).

Filters were individually placed onto a glass Petri dish for later microscopic inspection. Once dried, filters were examined under a stereo microscope. The easiness to quantify and recover undigested material

using acupuncture needles (Acurea, Hansol Medical Co., South Korea) on the filters was assessed. The factors evaluated qualitatively (score 1-3, 1 being the simplest method (Fig. 1)) where: the easiness to visualise and quantify remaining particles; the integrity of the filter under needle pressure (i.e., fracture and perforation), and successful particle retrieval (e.g., microparticles embedded into filter material). The final 'user burden ' for each analysis was recorded by measuring the degree of difficulty of each case. All filter trials were performed in triplicate.

2.6 Effectiveness of enzymatic digestion analysis

The dry weight of biomass was determined before and after filtration. Wet specimens were placed inside pre-weighed glass tubes, their combined mass measured and left to oven-dry overnight at 60°C. After drying, tubes were allowed to cool down inside a desiccator for 30 mins and weighed. This measurement minus the weight of the corresponding empty tube provided the Biota Dry Weight (BDW). After enzymatic digestion treatment, samples were filtered into pre-weighed filters (FW), and their mass was measured (EDFW). The procedural control filters (PCF) were used to determine the weight that the solutions employed in each test contributed to the final weight of the digestate. The weight of the controls was subtracted from the corresponding sample filters. Thus, the digestion efficiency was calculated as follows: BDW - [(FW) – (EDFW – PCF)].

Additionally, as the printed grid on MCE filters divides the filtration area into squares, this was used to systematically inspect filters for undigested biota fragments after digestion treatments. Filters were examined under a stereo microscope (Leica M205C, Leica Microsystems GmbH, Germany) from top to bottom and from left to right using the grid as guide to count for the undigested material remaining. A ranking score was used as a multiplying factor to classify different undigested body parts according to their potential impediment on MPs analysis. For instance, a totally undigested specimen versus a long undigested limb both covering one square on the filter would receive 4 and 2 marks, respectively, e.g. a fully undigested individual might contain MPs inside that would be excluded from analysis, the body would obscure filter visualisation of the area, and/or it could be masking a MP attached to it, whereas an undigested limb could mostly interfere with the analysis due to the latter two reasons. Therefore, the scale used to assign multiplying factors was: fully undigested specimen – 4; fully undigested carapace and abdomen – 3; fully undigested limbs and telson – 2, and partially digested transparent fragments – 1. Transparent appendages (e.g., antenna) and eyes were not accounted for, since these where often too small, easily recognisable or fragmented to interfere with the filter visual inspection.

Negative controls (20 grinded specimens in phosphate and carbonate buffers, but without SDS and enzymes) were used to assess the maximum remaining material in the filter expected by undigested biomass, and this was used to compare the digestion efficiency of the different treatments.

2.7 Determination of the effect of the enzymatic method on weathered plastics chemical identification

Visually weathered plastic litter was collected from the Blyth beach, North East coast, UK (55° 06’ N, 1° 29’ W) in April 2020. The litter was thoroughly rinsed with deionised (DI) water and chemically characterised *via* Fourier-transform infrared spectroscopy (FTIR) analysis. Only fragments with chemical identity of at least 70% similar to that of the most commonly discarded plastics (Supplementary Table 1, S1) were used for further study. Four different types of distinct resins were found i) a blue stick, likely belonging to a cotton bud 91.1% similar to polypropylene (PP), ii) a transparent plastic bottle, 91.25% similar to polyethylene terephthalate (PET), iii) a piece of rope/net 70.71 % similar to polyethylene (PE), and iv) an orange sponge-like fragment, likely belonging to a shoe sole, 83.26% similar to polyurethane (PU). A scalpel and scissors were used to produce smaller fragments (<5 mm in size) from the plastic litter. Three of each of these particles were combined in a test tube (n= 12/tube) and subjected to the optimised

enzymatic digestion method. Polymers' FTIR spectra matches were compared before and after enzymatic digestion to evaluate the effect that the proposed enzymatic digestion method might have on the identification of said weathered plastics.

2.8 Microplastics chemical analysis

Plastic particles were placed onto gold platted slides (Thermo Fisher Scientific Inc., US) for FTIR analysis. A Nicolet iN10 FTIR micro spectroscope (Thermo Fisher Scientific Inc., UK) was employed to obtain the particle's infrared transmittance spectra, using the liquid nitrogen cooled Mercury Cadmium Telluride detector (spectral range 4000 to 675 cm^{-1}) and the Attenuated Total Reflectance (ATR) germanium crystal tip (NICOLET iN10 Ge Tip ATR, Thermo Fisher Scientific Inc., US). High resolution spectra were collected in reflection ATR mode for five seconds and sixteen scans at an aperture of 100 μm x 100 μm. Results were then visualised and matched against a series of inbuilt reference spectra libraries using the instrument's software (OMNIC Picta v1.7, Thermo Fisher Scientific Inc., US) to determine the chemical identity of the analysed particles. Preference to determine the polymer identity was given to matches against the ATR and corrected ATR libraries (HR Spectra Polymers and Plasticisers by ATR - corrected- Copyright 2008 Thermo Fisher Scientific Inc. for Nicolet FT-IR, Thermo Fisher Scientific Inc., US).

2.9 Microplastics contamination control and monitoring

Due to the ubiquity of microplastic fibres in the environment, preventive measures were taken to reduce and monitor for potential sources of contamination. Experiments were performed in a clean laboratory with restricted access, where only one researcher, always wearing a 100% cotton clean lab coat, was present conducting the experiment. At the start of any work session, benches were wiped with 70% EtOH using a 100% cotton cloth and allowed to dry fully. Only non-plastic equipment (glass and metal) was used to process the samples. Glass graduated piston pipettes, test tubes, and Petri dishes, were thoroughly washed with pre-filtered DI water, rinsed with acetone, covered with aluminium foil, and allowed to dry at 90 °C in a drying oven. The digestion and filtration steps were conducted under a laminar flow cabinet (Purair, LS series, Air Science, USA LLC). The equipment and samples were covered wherever possible to minimize environmental exposure. Additionally, procedural blanks were run in parallel with samples to monitor environmental contamination. For this, a glass petri dish with a damped grided membrane filter was left open next to the microscope during the specimens' segregation, while glass tubes with the enzymatic/chemical solutions but without specimens were processed as described above for each of the digestion treatments (Table 1). The resulting control filters were examined under a stereo microscope to correct for potential air-borne and/or procedural plastic contamination.

2.10 Statistical analysis

All data were tested for normality (Shapiro-Wilk normality test) to select further parametric or non-parametric statistical analysis. If data were normal and the homogeneity of variances was met (Levene's test), analysis of variance (ANOVA) were used to compare treatment groups, following post-hoc analysis (Tukey HSD). Non-normal data were examined *via* Kruskall-Wallis test, and when relevant, multiple pairwise-comparison between groups (Wilcoxon-Whitney-Wilcoxon Test, using Bonferroni correction) was performed. The statistical analyses were conducted in R v 4.1.0 (Core Team 2014), and results considered statistically significant at $p\text{-}value < 0.05$. The ± values represent the standard error of the mean (SE) n=3, unless otherwise stated.

3. Results and discussion

3.1 Enzymatic digestion methods review

From the five enzymatic methods reviewed, 40% used oven dried biomass, 60% included the use of surfactant, and 40% fundamentally relied on additional chemicals for the digestion strategy (Table 2). Among these studies, 80% targeted soft tissue biomass, with mussels being the organism of preference, while only two studies were directed towards chitinous biomass breakdown. The findings from this review informed the design of the methods detailed herein.

Table 2. Enzymatic methods

3.2 Vacuum filters performance

Visual selection of putative microplastics after filtration and before chemical analysis is open to bias. Therefore, the simplification for particle visualisation, counting and retrieval is key in the accurate study of microplastics. From the three types of filters compared in this study, grided membrane filters, traditionally used for microbiology applications (Wolochow 1958), were deemed as more appropriate for microplastics recovery from zooplankton samples as these facilitated the visibility and recovery of both microplastics and undigested material while retaining their physical integrity after manipulation with needles (Figure 1). Furthermore, the grid lines on the membrane surface eliminated eye fatigue and facilitated methodical filter examination. Although glass fibre filters are the most widely used type of filter in microplastics research (Qiu et al. 2016), this study found that smaller undigested particles could be lost within the fibres of the filter when pressing with the needle during particle manipulation. However, the lower cost of GC filters might add to the appeal to select this type of filter over gridded membrane discs. Based on our assessing criteria, aluminium oxide filters proved to be the least compatible for the proposed method due to their brittleness, since these invariably fractured when trying to remove the filters from the filtration system (Figure 1C). In addition, aluminium oxide filters are considerably more expensive than the other two alternatives tested.

Figure 1.

3.3 Determination of digestion efficacy

Numerous studies on MPs ingested by biota, including zooplankton (Cole et al. 2014; Löder et al. 2017) have used the differential weight loss of filters after biota digestion to determine the efficacy of the proposed methods (Kallenbach et al. 2021; Piarulli et al. 2019; von Friesen et al. 2019). However, due to the small mass of the organisms analysed here (3.4 mg ± 14.1 n = 1055), this gravimetric method to assess digestion efficacy produced variable, including negative, inconsistent values (Supplementary data table). This was not prevented despite measuring the filter's weight after drying overnight at 60 °C, cooling down in a desiccator until constant weight was reached, and calculating their individual mean weight after at least three independent filter measures. Digestion efficacy was therefore determined using the counts of remaining undigested body fragments as described above (section 2.6). The calculations for Chi50FullTr treatment, replicate A, are presented as an example:

$$Chi50FullTr_{Undigested} = [Complete\ (0*4) + Carapacea\ (0*3) + Abdomen(0*3) + Telson\&Limbs(0) + Transparent(2*1)] = 2;$$

$$Digestion\ Effectiveness\ (\%) = \left[\left(\frac{2}{60.67*}\right)*100\right] = 96.7\%\ removal\ compared\ to\ negative\ ctrl.$$

**Where 60.67 is the mean value for the negative control remaining fragments.*

3.4 Pre-treatment

Digestion with wet biomass was tested to reduce processing time and exposure to high temperatures that could impact the physical integrity of potential MPs inside the decapods. However, the physical breakdown of wet specimens was noticeably lower than for those that have been oven dried (one-way ANOVA, DF =1, F = 17.521, *p*-value = 0.001). Water acting as a plasticiser in the exoskeleton is known to reduce the stress of fracture (Jagadish C. et al. 2017), likely contributing to the minimal breakdown of this biological polymer observed in this experiment (Supplementary Data table). Therefore, subsequent tests were all conducted on oven dried biomass.

Equally, the need for a grinding step was assessed aiming to reduce direct contact with the sample. The filter coverage results indicate that grinding of biomass significantly reduced the filter coverage (Two-way ANOVA, DF =2, F = 12.988, *p*-value = 0.013) compared to un-treated specimens. However, the different grinding strategies tested did not produce significant different outcomes (TukeyHSD, *p*-value = 0.48423).

The use of a glass rod together with glass microbeads has been proposed as an effective method for the breakdown of dry chitinaceous biomass (Therien et al. 2019). Nevertheless, here the use of microbeads complicated the method, as the biological material got attached to the beads, which were difficult to fully wash and to recover after filtration. Thus, the use of microbeads to grind chitinaceous zooplankton could increase the risk of losing MP particles. In contrast, the use of a glass rod alone to grind the dry material visibly reduced the specimen's size and physical integrity of the chitinous material in a simple and quick manner. Upon removal from the test tube, rods were rinsed with 1mL of buffer (TRIS or phosphate, depending on the test (Table 1) easily recovering any biomass attached to the rod's surface, which was verified under a stereo microscope. Since the grinding of the biomass would increase the surface area available for the enzymes to act, and the use of a glass rod was straightforward, and it was decided to include this step as part of the method.

The addition of the surfactant SDS also reduced the filter coverage area compared to non-chemically pre-treated specimens (Two-way ANOVA, DF =2, F = 13.0, *p*-value = 0.019), with no statistical difference between the two different concentrations tested (TukeyHSD, *p*-value = 1.000). along with the surfactant effect, the addition of up to 2% v/v SDS is known to activate protein K action (Hilz, Wiegers, and Adamietz 1975). Nevertheless, the higher surfactant concentration tested in this study tended to produce foam, causing digestate adhesion to the walls of the tube. The addition of 1% v/v SDS was selected for subsequent method stages, as this concentration equally improved material breakdown without producing a large amount of foam during agitation, thus not impeding digestate removal from tubes after rinsing with ultrapure water.

Based on statistical analysis and empirical evidence, the most effective combination of steps to reduce the size and physical integrity of chitinaceous zooplankton were oven-dried biomass, ground with a glass rod, following 1% v/v SDS addition. Despite of their contribution to fracture the samples, the pre-treatment steps were not sufficient to fully break down the biological material (Fig 2).

Figure 2.

3.5 Enzymatic Digestion

The proteolytic breakdown of pre-treated specimens reduced the filter coverage by the digestate when compared against non-enzymatically digested biota (Kruskal-Wallis, *p*-value = 0.029), with no significant difference between the two proteinase K concentrations tested (Pair-wise Wilcox, *p*-value = 0.048). Although proteinase K has been deemed as sufficient for efficient exoskeleton breakdown (> 97%) by a pioneering enzymatic digestion method study (Cole et al. 2014), and despite the improvements in biomass lysis observed here, the addition of proteinase K was not sufficient to achieve a material breakdown that would ensure full filter visibility, which could mask the presence of microplastics (Figure 2). The difference in the results between Cole *et al*. (2014) and this study could be attributed to the proteinase k

concentration used in the previous report (500 µg mL^{-1} of proteinase-K per 0.2 g DW), in combination with the several additional physicochemical steps before and after enzymatic activity said method involved.

As the focus of this study was on crustacean larvae, it was decided to include chitinase, an enzyme ubiquitous in the natural world to achieve chitin breakdown. The direct addition of chitinase to pre-treated and non-pre-treated samples lead to similar results to those achieved by proteinase K in pre-treated samples (Kruskal-Wallis, pair-wise Wilcox *p*-value = 0.4583) (Fig. 2). However, including this step before the addition of proteinase k in pre-treated samples sharply increased the digestion efficacy of the proposed method (1.8% filter coverage, or 98.2% digestion efficiency, Kruskal-Wallis, pair-wise Wilcox *p*-value = 0.0099). No distinguishable difference was observed when using 50 mU/mL or 100 mU/mL chitinase concentrations (Kruskal-Wallis, *p*-value = 0.058).

To determine whether the method developed could be use in larger sample sizes, the lower chitinase concentration (50 mU/mL) was tested using 40 and 60 specimens. Chitin at 50 mU/mL efficiently removed biomass corresponding to 40 specimens. However, when 60 specimens were included, using this enzyme concentration did not achieve a full biomass removal. Nonetheless, this was achieved by using Chitinase 100 mU/mL (Fig 2. B).

The optimal sequential steps to achieve up to 98% digestion efficiency of chitinaceous zooplankton samples such as crustaceans involve a gentle physicochemical pre-treatment and the combination of chitinase and proteinase k. This method is illustrated in Figure 3.

Figure 3.

3.6 Effect of enzymatic digestion methods in long-term crustacean time-series

The chitin – proteinase K method developed herein was equally effective in digesting up to 97.7 % of 2020 and 1985 crustacean samples when using 100 mU/mL of chitinase (Kruskal-Wallis, pair-wise Wilcox *p*-value = 0.23).

Although the 1985 samples were marginally harder to digest than fresher 2020 samples at low chitin concentration (98.2% vs 96.5%, Kruskal-Wallis, pair-wise Wilcox *p*-value = 0.0467), the results from our study sharply contrast with a recent investigation (Kallenbach et al. 2021) where the exoskeleton of 11-month EtOH preserved terrestrial arthropods (~3-5 specimens *per* replicate) were left intact after treatment with H_2O_2 plus chitinase (~96 mU/mL). Despite being stored for 35 years in alcohol solution, here decapod larvae (20 specimens *per* replicate) were at least 96 % digested.

EtOH is known to swiftly penetrate tissues, removing water from them, and consequentially slowing down enzymatic degradation (Marquina et al. 2021). This mechanism is thought to aid the uniform tissue fixation and the minimal loss of tissue integrity (Marquina et al. 2021; Srinivasan, Sedmak, and Jewell 2002). Specifically, EtOH has been shown to improve the mechanical stability of the exoskeleton cuticle (Wang et al. 2019). These mechanisms could have provided 1985 samples with a stronger/stiffer exoskeleton, mildly affecting the enzymatic digestion effectiveness. However, further studies would be required to test this hypothesis.

3.7 Effect of enzymatic digestion methods in naturally weathered microplastics

The chitinase + proteinase K digestion method had no noticeable effect on the chemical identification via FTIR of naturally weathered PE (71.74 % ± 2.14 % similarity), PP (92.1 % ± 2.88 % similarity), PET (91.25 % ± 1.56 % similarity), and PU (81.93 % ± 3.36 % similarity), four of the most discarded plastic polymers worldwide (Table S1). This is in agreement with the literature, as it has been recognised that enzymatic methods do not interfere with plastics physical or chemical structure, due to enzymes specific activity.

Furthermore, the method here proposed does not rely on high concentrations of additional chemicals to achieve almost complete chitinaceous material removal. Future work should investigate the application of this method in other sample matrices as well as its effect on biodegradable plastics.

Conclusions

The use of grided membrane filters facilitates the study of microplastics collected by vacuum filtration by facilitating micro particles visualisation, counting, and retrieval, reducing processing time and the user burden. The combination of chitinase and proteinase k together with the surfactant Sodium Dodecyl Sulphate (SDS), has been shown to be an effective method to digest chitinaceous zooplankton samples such as crustaceans for microplastics analysis. This method is a gentle and effective method for processing long-term time-series samples that have been stored in alcohol solutions over time as well as freshly collected samples. This method expands the possibility to use time-series samples to investigate MPs contamination in key chitinaceous marine species. The method developed allows samples that have been stored in alcoholic solutions with potential negative effects on microplastics integrity to be investigated, and therefore expands opportunities to explore the temporal change in MPs contamination under a range of different scenarios.

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Artwork and Tables with captions

Table 1. Experimental design to assess the need of different stages for enzymatic digestion of chitinaceous zooplankton.

	Digestion stage	Test	Water		Grinding (RT)			SDS [% v/v] (RT)			Proteinase K [µU/mL] (40°C)		Chitinase [µU/mL] (40°C)		n
			wet	dry (60°C)	none	rod+b	rod	0.5	1	2	50	100	50	100	
Pre-Treatment	Physical	A	x		x										15
		B	x			x									15
		C	x				x								15
		D		x	x										15
		E		x		x									15
		F		x			x								15
	Chemical	A		x			x	x							15
		B		x			x		x						15
		C		x			x			x					15
Enzymatic digestion	Proteolytic														
	PK only	A		x							x				20
		B		x								x			20
	+preTr	C		x			x		x		x				20
		D		x			x		x			x			20
	Glycolytic														20
	Chi only	A		x									x		20
		B		x										x	20
	+preTr	C		x			x		x				x		20
		D		x			x		x					x	20
	FullTr	E		x			x		x		x		x		20
		F		x			x		x		x			x	20
Scale up		G		x			x		x		x		x		40
		H		x			x		x				x		60
		I		x			x		x					x	60

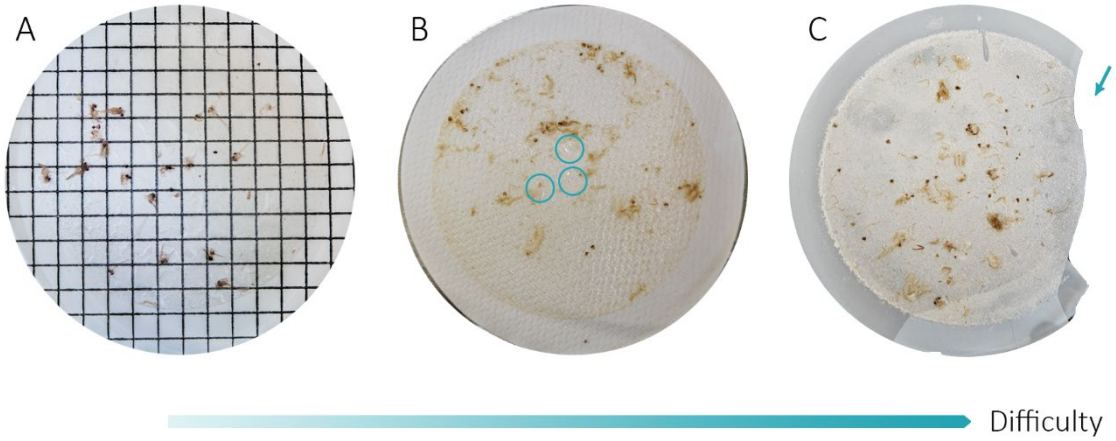
Water: water content, either wet or dry specimens. RT: stages conducted at Room Temperature. Rod: using a glass rod only. rod+b: glass rod plus glass microbeads. SDS: Sodium dodecyl sulphate. n: number of specimens digested *per* replicate for a given test. +preTr: a given step plus pre-treatment. PK: Proteinase K. Chi only: chitinase only. FullTr: Full treatment up to that stage.

Table 2. Enzymatic digestion methods for microplastics extraction from biotic samples.

Enzyme(s)	Organism/tissue	Method steps	Test MP particles	Digestion Efficacy	Particle changes	Reference
Commercially available multienzymatic detergent composed by proteases, lipases, amylases and cellulases	Water fleas (cladocerans) in an artificial water mixture comprised of duckweed, chestnut leaves, and sand. / Chitinaceous organism in a complex mixture	1. Samples passed through two sieves (55 µm and 0.2 mm). 2. Samples oven-dried (90°C). 3. SDS 25% (w/v), stirring for 10 min. Overnight incubation (70 °C). 4. Enzymatic detergent [100 g/L] addition. Incubation 3 days RT, constant oxygenation (>0.05 mgO_2 L^{-1}). 5. Sieving (0.2mm) 6. Density separation using zinc chloride. 7. Filtration onto 0.45 µm membrane filter.	Fragments of HDPE, LDPE, PP, PS, PVC, PET	Organic matter degradation ≤80%	None, although caused loss of MPs	(Rodrigues et al. 2018)
Commercially available pancreatic enzyme (PEz: lipase, amylase, and protease)	Greenland smoothcockle (*Serripes groenlandicus*) / soft tissue	1. PEz [~75g/L : 15 g WW] in TRIS-HCL addition. Overnight incubation (37.5 °C) on a shaking table (126 rpm). 2. Sequential filtration through 300 µm & 20 µm nylon filters. 3. Rinsing with warm water (~40°C).	Pre-weathered fragments of LDPE, ePS, & PP. Microfibres of PLA, PET, Nomex, PP, modacrylic, PS.	97.7 % bivalve tissue removal	None	(von Friesen et al. 2019)
Commercially available Biozym F (lipase) and Biozym SE (protease and amylase)	Wild crabs (*Carcinus aestuarii*)/ soft gastrointestinal tract tissue	1. 25% SDS. Incubation at 50°C for 24h. 2. Enzymes' addition [total 10 mL]. Incubation RT 48h. 3. Rinsing & filtration onto 20 µm nylon filters.	Microfibres of PES & PP	Incomplete digestion of biological tissues	None	(Piarulli et al. 2019; Löder et al. 2017)
Enzymatic-alkaline Pepsin and KOH (optimised method)	Fish fillets (9 different species, see (Süssmann et al.	1. Pepsin 0.5% (w/v) in 0.063 M HCl added to the 10 g sample.	Particles of PP, PS.	the enzymatic-alkaline approach (protocol No. 10b)	No significant changes as	(Süssmann et al. 2021)

	2021)) and soft tissue of crustaceans (*Macrobrachium rosenbergii*, *Penaeus monodon*) and molluscs (*Mytilus edulis*).	Incubation under constant stirring for 2 h at 37 ◦C. 2. KOH 50% (w/ v) addition resulting in a concentration of approximately 10% KOH in the sample solution. Incubation at 37 ◦C for 4 h. 3. Filtration with large GF filters or PC filters depending on targeted MP size. 5. Filters rinsed using sonication and SDS 0.5%.	Microbeads of PA6, PC, PET, PSu, PU Commercial powder of PAN, PTFE, LDPE. PS nanoparticles.	resulted in a digestion efficiency of 99.6%	assessed by ATR-FTIR, μ-Raman or py-GC/MS except for PAN. Degradation due to alkaline hydrolysis at 60 ◦C for polyesters (PET, PC)	
Chitinase and H_2O_2	Wood lice (*Oniscus aséllus L.*) / Chitinaceous organisms	1. Specimens dried at 40 °C for 2 days. 2. H_2O_2 30% added to 3-5 specimens (0.5 g dw). Incubation at 50 °C for 24 h. 3. Chitinase in NaOAc buffer (96U/L, pH 5) added. Incubation at 37 ◦C for 24 h. 4. Filtration onto GC/C filters (pore size 1.2 μm).	pre-production pellets of virgin polymers: PET, PMMA, PP, PS, HDPE, LDPE, PC, PA-6,6	91% biomass removal.	No sign of degradation. However, the weight of PA-66 and PMMA increased significantly.	(Kallenbach et al. 2021)
Chitinase and Proteinase	Crustacean larvae from 2020 and 1985	1. Specimens dried at 60 °C overnight. 2. Biomass grinded with glass rod. Rod washed with 0.5mL 1% v/v SDS. Continuous stirring for 20 mins at RT. 3. Chitinase (100 mU/mL) addition. Continuous stirring for 20 mins at RT. Incubation at 40 °C for 48h. 4. Proteinase K (50 mU/mL) addition. Continuous stirring for 20 mins at RT. Incubation at 40 °C for 24h. 5. Filtration onto grided MCE filters (pore size 0.8 μm).	Naturally weathered fragments of PE, PET, PP, PU.	97.7 % digestion efficiency on specimens (n = 60) from 2020 and 1985.	No effect on chemical ID as assessed by ATR-FTIR	(Carrillo-Barragán et al. 2022)

RT: Room Temperature. Particles changes: Include physical (i.e., colour and shape) and chemical structure determined by FTIR/Raman. Digestion efficacy as reported by the authors. In all methods, filtration refers to vacuum filtration. All methods allowed filters to dry before visual inspection for mps. Abbreviations: PAN – Polyacrylonitrile. PC – Polycarbonate. PET - Polyethylene Terephthalate. PS – Polystyrene. Psu – Polysulfone. PTFE – Polytetrafluoroethylene. PU – Polyurethane. PMMA - polymethyl methacrylate. PP- Polypropylene. HDPE - High-density polyethylene. LDPE - low-density polyethylene. PA-6,6- polyamide-6,6. NaOAC buffer - sodium acetate in filtered deionized water.



	Grided MCE	GF	Al_2O_3
Particle			
Visualisation	1	2	1
Quantification	1	2	2
Retrieval	2	2	3
Filter			
Int. after filtration	1	1	3
Int. under pressure	1	2	2
User burden	6	9	11

Figure 1. Membrane filters after vacuum filtration of pre-treated chitinaceous zooplankton samples. A) MCE: Mixed Cellulose Esters. B) GF: Glass Fibre, circles highlight areas pierced by needles when trying to recover particles from the filter. C) Aluminium oxide filter, arrow highlights missing area due to fracture. Int.: Integrity. Images' brightness was increased by 60-70%.

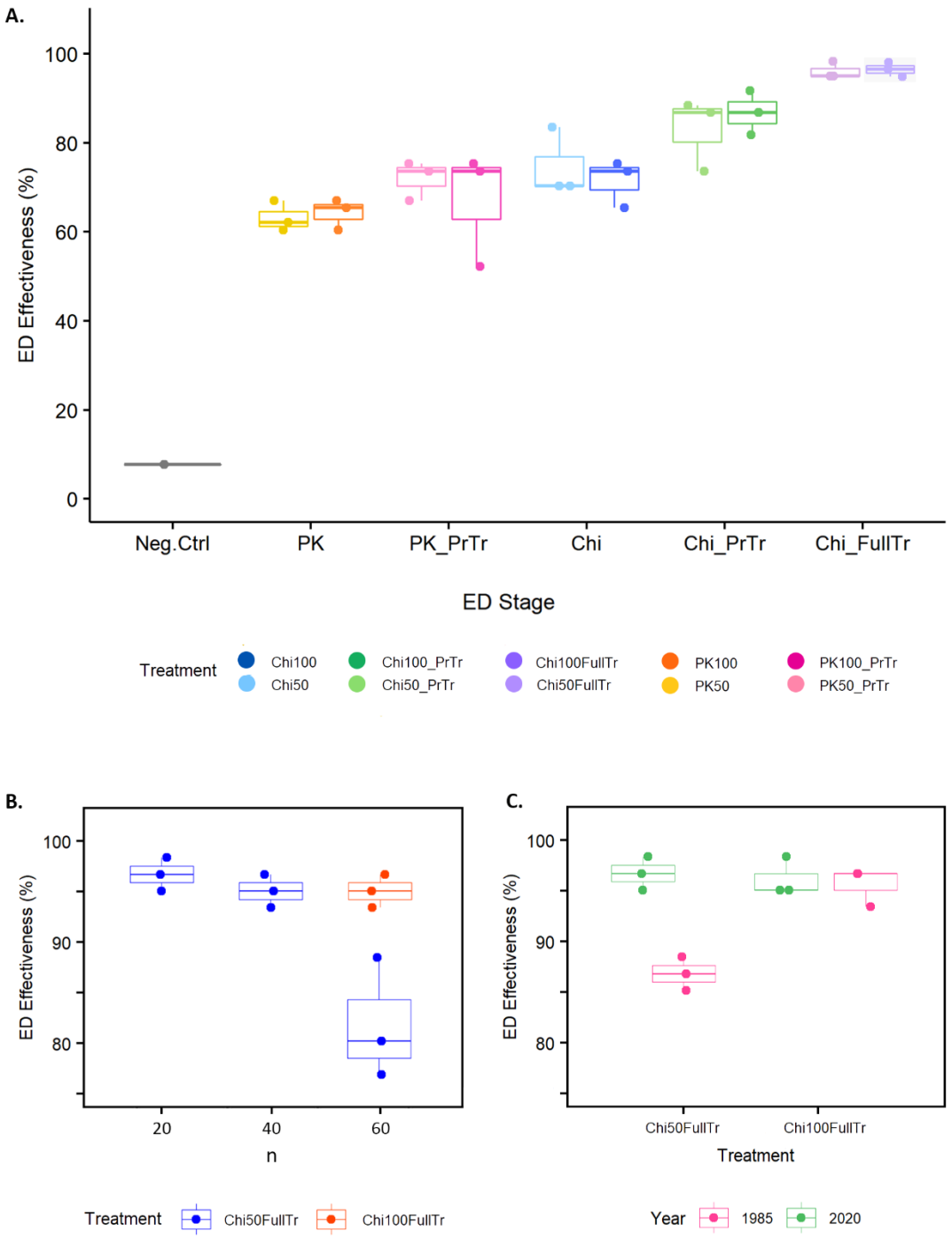


Figure 2. A. Effectiveness (%) of the different enzymatic digestion treatments. B. Effectiveness of enzymatic digestion at different sample sizes under two different concentrations of chitin. C. Effectiveness of enzymatic digestion of 1985 and 2020 samples under two different concentrations of chitin. Abbreviations: Chi100 – dried specimens + chitin [100mU/mL]. Chi50 – dried specimens + chitin [50mU/mL]. Chi100_PrTr – pre-treated samples + chitin [100mU/mL]. Chi50_PrTr – pre-treated samples + chitin [50mU/mL]. Chi100_FullTr – pre-treated samples + chitin [100mU/mL] + proteinase K [50 mU/mL]. Chi50_FullTr – pre-treated samples + chitin [50mU/mL] + proteinase K [50 mU/mL]. PK100 – dried specimens + proteinase K [100mU/mL]. PK50 – dried specimens + proteinase K [50mU/mL]. PK100_PrTr – pre-treated samples + proteinase K [100mU/mL]. PK50_PrTr – pre-treated samples + proteinase K [50mU/mL].

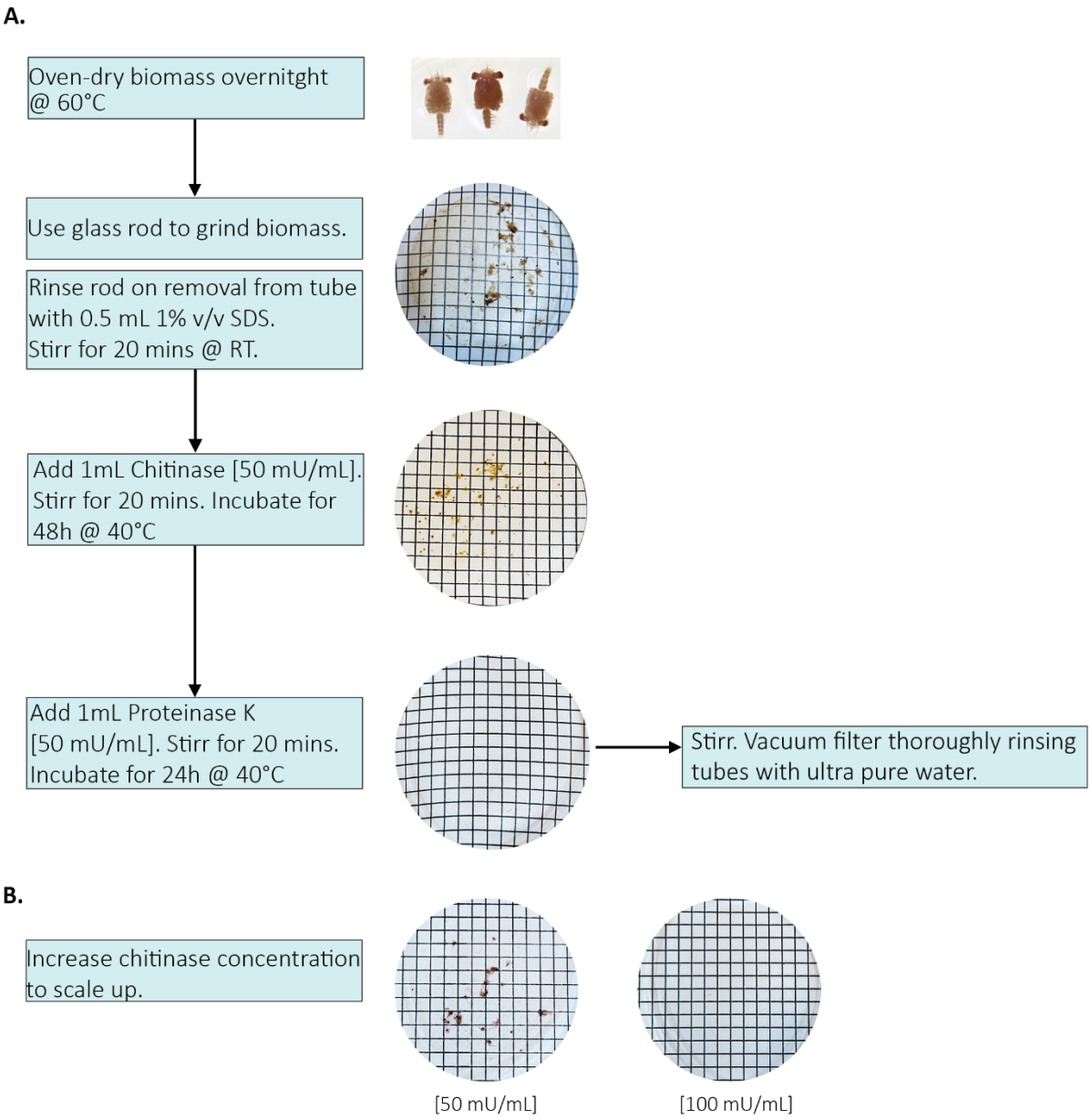


Figure 3. A. Chitinase – proteinase K digestion method for microplastics research on crustacean larvae (n = 40). B. Illustration of increment in sample size (n = 60). Filters illustrate the state of filter coverage by chitinaceous biomass after each treatment stage.

Highlights

- Effective enzymatic digestion of long term stored crustacean larvae with chitinase & proteinase K.
- Decapod samples from 1985 and 2020 were gently & effectively digested (>96%).

- This method did not influence weathered microplastics identification via FTIR.
- This method can be reliably used to extract and study MPs in crustacean larvae.

Graphical abstract