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Facilitating microplastic quantification through the introduction of a cellulose dissolution step prior to oxidation: Proof-of-concept and demonstration using diverse samples from the Inner Oslofjord, Norway

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ABSTRACT

Identifying and quantifying microplastic in marine samples can be facilitated by removing natural organic matter (NOM). Cellulosic material, like chitin, however, are a type of NOM that is resistant to chemical digestion, and difficult to eliminate from samples. To address this, a two-step digestion method was developed to remove or reduce cellulosic materials in diverse marine media. This method was applied to reference microplastics, reference cellulosic materials, and diverse marine samples from the Inner Oslofjord Norway. This included plankton, seabed sediments near a water treatment plant and driftline sand. The method was developed and tested for plastic particles >45 μm. The first-step was to pre-dissolve cellulosic materials using a mixture of urea: thiourea:NaOH. This was followed by an oxidative digestion step, here using H_2O_2 and NaOH. Most reference plastics were unaffected, except minor effects for PET and nylon. After sufficient repetitions, cellulosic materials in both reference and marine samples were largely removed. This method was compared to other digestion methods used for microplastic quantification, including single-step oxidation, alkaline treatment, acid treatment and enzymatic treatment. The results indicate that the pre-dissolution step greatly facilitates NOM and cellulosic material digestion for the purpose of microplastic quantification.

1. Introduction

Microplastic debris is spread throughout the world's oceans. There is currently a global effort to better survey its distribution, accumulation rate, transportation pathways, weathering behavior and impacts (Akdogan and Guven, 2019; Jahnke et al., 2017). To understand these diverse processes, more quantification is needed in diverse marine media, including coastal sand, surface water, water columns, benthic sediments and all the marine fauna within. Quantifying microplastic in these diverse media remains challenging. Many studies have attempted to quantify microplastic directly with a minimum amount of work-up, such as by sieving and counting suspected microplastics (Hidalgo-Ruz et al., 2012; Silva et al., 2018). However, any quantification method would be greatly assisted by introducing a purification step, in which all that is not microplastic is removed from the sample, or the microplastic is selectively extracted. How such purification can be done is dependent upon the sampled media. For coastal and sea sediments, it is important to remove mineral clay and sand particles. Methods to remove these include density separation using salt solutions (e.g. Yu et al., 2016), elutriation systems (Claessens et al., 2013), oil based separation (Crichton et al., 2017) and more recently chemical extraction of the specific polymers for the quantification of monomers (Müller et al., 2020). Arguably a more formidable challenge is the removal of other organic material from the sample, as organic material has a similar density range to plastics. These organic materials include algae, plankton, shell fish, oil residues, sea grass, organic detritus, and diverse marine organisms (Löder et al., 2017). Removing these materials would greatly facilitate quantification. For instance, consider the rapid method of microplastic identification based on fluorescent tagging with Nile Red staining (Maes et al., 2017), as Nile Red also stains organic material its

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quantitative removal is a pre-requisite.

The ideal isolation method would be to separate microplastic from organic and biological material, without causing any changes to microplastic. Many studies have tested and reviewed the stability of different types of microplastics to chemical digestion methods, such those using acids, bases, oxidation agents, enzymes and solvents (e.g. Claessens et al., 2013; Cole et al., 2015; Prata et al., 2019; Zarfl, 2019). From our experience and assessment of this literature, the most recalcitrant molecules to any chemical workup are cellulosic materials such as chitin.

The abundance of cellulosic materials gives them the potential to be "false positives" for microplastic. A study by Kanhai et al. (2018) showed that cellulosic materials, both natural and synthetic, can be abundant in samples as remote as the Arctic. In a study of benthic eelpout, only cellulose fibers were found in the stomachs, which the authors argue could have been mistaken for microplastic without spectroscopic techniques (Wesch et al., 2016). This echoes earlier conclusions for seagrass macrofauna (Remy et al., 2015) and North Sea fish (Hermsen et al., 2017). Cellulose fibers were one of the most dominating "microplastic"-like particles in the Bohai Sea (Yu et al., 2016). In waste water samples, without the possibility of spectroscopic techniques, it is not possible to discern cellulose fibers from microplastic fibers, even after commonly used digestion procedures (Dyachenko et al., 2017; Sutton et al., 2016). Complicating this issue, however, is the argument that semi-synthetic cellulose such as rayon or viscose, used in the textile industry, should be classified as a microplastic. Some scientists argue against (Hermsen et al., 2017) and others for (Hartmann et al., 2019; Yu et al., 2016). Such a debate is complex and is dependent on methods to distinguish between natural and semi-synthetic cellulose, as well as production volumes, occurrence and emissions of natural and semi-synthetic cellulose. Nevertheless, seeing that both natural and semi-synthetic cellulose structures can dominate environmental samples, methods to remove them would nevertheless assist in isolating different plastic particles.

Methods involving acids, bases or oxidizing agents alone are not capable of removing cellulosic interferents in marine samples for microplastic quantification (e.g. Biginagwa et al., 2016; Cole et al., 2015). To date the most effective method is exposure to enzymes like cellulase and chitinase followed by H_2O_2 and density separation (Löder et al., 2017; Mintenig et al., 2017), as will be presented further below. Though such enzyme-based methods are promising, a non-enzyme-based method to dissolve cellulose would be complimentary or a possible alternative to such methods; hence, the motivation for the development of a cellulose-dissolution step in this study.

The aim of this work is to see if a novel enzyme-free, two step-digestion method, that first uses a dissolution step for cellulose and chitin, followed by a second step of oxidation, can facilitate the purification of marine samples for microplastic analysis. The purpose of the dissolution step is to swell and break up cellulosic materials, to decrease their particle size and increase their surface area. Here we applied a cold mixture of 8% NaOH, 8% urea and 6.5% thiourea in deionized water (e.g. Hu et al., 2007; Jin et al., 2007; Yan et al., 2007). The second step involves digesting the remaining organic matter through oxidation. The oxidation step used here involved adding 30% H_2O_2 and 1% NaOH solution to the rinsed sample after dissolution. To test this method, reference microplastics, reference cellulosic materials and diverse marine samples from the Inner Oslofjord, Norway, were utilized. The results are compared to the performance of other methods used for removal of organic matter in environmental samples.

2. Material and methods

2.1. Chemicals

The chemicals used for the chemical digestion were Urea (Sigma Aldrich, Germany, $\geq$ 98%), Thiourea (Merck, Germany, $\geq$ 98%), NaOH pellets (Merck, Germany, 99–100%) and 50% H_2O_2 (VWR International, Germany, analytical grade). Sodium dodecyl sulfate (VWR International, Germany, $\geq$ 99%) was used as cleaning agent. $ZnCl_2$ (VWR International, 97%) and $CaCl_2$ (VWR International, 90–98% purity) were used to make a high density $ZnCl_2$:$CaCl_2$-solution ($\rho \geq 1.55$ g/cm^3), based on a weight ratio of 4.4: 3.6: 2 kg ($ZnCl_2$:$CaCl_2$:MilliQH_2O) (Hudgins, 1964).

2.2. Reference materials

Reference microplastic samples. Low-density polyethylene granulates (LDPE (g), 5 mm) and fibers (LDPE (f), textile quality, fiber count 90 and tex value 110, diameter 380 μm, length 0.5-1.0 mm), polypropylene granulates (PP (g), 5 mm), polystyrene granulates (PS (g), 3.5 mm), polyethylene terephthalate granulates (PET (g), 3 mm), powders (PET (p), <300 μm) and fibers (PET (g), fiber count 24 and tex value 24) were obtained from Goodfellow Cambridge Ltd. (UK) (catalogue numbers LDPE-n ET316300, LDPE-f ET315710, PP-n PP306312, PS-n ST316310, PET-n ES30631, PET-p ES306030, PET-f ES305720, respectively). Nylon fishing wire (Trilene XL Smooth casting, 0.4648 mm thick) was purchased from Torshov Sportsfiske AS, Norway. The PET-p powder was sieved to a 75–300 μm fraction for use as a "recovery spike" for microplastic quantification in sand and sediment.

Reference cellulosic materials. Cotton pads (non-bleached, Creative Concept Nordic AB, Sweden) and printing paper (Multicopy original 80 g g/m2 by StoraEnso, Sweden, 100%) were used as reference sources of cellulose.

2.3. Field samples

Plankton trawl material. Plankton from two locations and three water column depths were sampled in June 2016 within Oslo harbor, near the outflow of the Akerselva river and near the outflow of the Bekkelaget water treatment plant (WTP). The different depths were chosen to get a first glimpse of variation in microplastic concentrations in the upper, middle and lower part of the water column at potential microplastic source areas. Coordinate data and depth are presented with the results. Sampling was performed onboard R/V Trygve Braarud (University of Oslo), using a custom-built sampler containing three Neuston nets made of nylon (dimensions 50 cm × 20 cm, mesh size 90 μm, Hydro-Bios Germany) called the "Multihaav" that was capable of being lowered to various depths. It is noted that nylon appearing in such a sample may occur from the net. The trawling speed was between 2.2 and 2.3 knots. Upon sample retrieval, the collected plankton material was filtered through the same nylon netting attached to the removable cod end using fresh water. The netting and material were then placed in a glass jar with an aluminum foil lined lid for storage and transport, and later dried at 60 °C for 14 h while still in the glass jar for dry mass quantification.

Harbor sediments. Harbor sediments from three nearby locations were collected from the area where the Bekkelaget WTP plankton trawl sample was collected on March 9, 2016 using a crane-mounted Van Veen sampler (depths and coordinates presented with the results) with a surface area of 0.15 m^2 following standardized methods (ISO 5667–19:2004). Either the top 5 or 10 cm of visually undisturbed sediment was carefully transferred using a regular stainless-steel spoon from the top of the van Veen sampler and into a pre-rinsed, 10 L polypropylene sample bucket (compromising potentially polypropylene in the sample). The three samples were processed separately and not mixed. The mineral fraction was removed in the laboratory using density separation. This was conducted using an inhouse built density separator (Knutsen et al., 2020; Mahat, 2017), which was inspired by an earlier design (Imhof et al., 2012). For this, 500 g subsamples were homogenized with a high-density $ZnCl_2$:$CaCl_2$-solution (density 1.55 kg/L) in an aluminum tray (note, this salt solution, pH = 5.17 ± 0.01. was found to not be corrosive to either the aluminum tray or the steel mesh used later,

unless water was allowed to evaporate to dryness). This slurry was then added into a glass column (10 cm in diameter and 65 cm in height) of the density separator that was ca 90% pre-filled with the same salt solution. Stirring was done via a propeller at the base of the column for 20 min at 40 to 60 rpm to break up any settling clumps, without causing a visible whirlpool. The salt solution in the column was then left stationary to ensure density separation (normally overnight). Separation was complete when the salt solution became transparent and free of visibly suspended particles. The floating fraction was then transferred into a separation chamber on the top of the column, by carefully increasing the amount of salt solution by an inlet at the base of the chamber, so as not to disturb settled sediments (Knutsen et al., 2020; Mahat, 2017). Particles were then filtered on a stainless-steel mesh (see below), carefully rinsing off particles that stuck to the chamber walls. If visual particles were remaining in the glass column, the process was repeated until the top of the glass column was visibly particle free.

Driftline sand. The driftline farthest from the seashore was visually identified within the Bygdøy Sjøbad beach area in Oslo in March 2017 (59.9109444 N 10.6663333 E) via the accumulation of vegetal debris. Washed up plastic litter was also evident in this area. Within the driftline, three sand samples covering 40 cm^2 in area and 2 cm sand depth were collected using a wooden frame and stainless-steel soil scoop. Larger debris was manually removed from the samples, e.g. macroplastics (bags), feathers and driftwood. The remaining samples underwent the same density separation procedure as the harbor sediments.

2.4. Two-step dissolution and oxidation

All samples were placed or filtered into a loosely packed pre-weighed stainless-steel mesh (#300 Mesh – 45 μm Aperture- 0.04 mm Wire Diameter - SS316 Grade - Woven Wire, purchased from the Mesh Company, Warrington UK), which was then folded into an envelope in a manner that allowed the material to move when the envelope was shaken, yet prevented the sample from escaping (Fig. 1). A pre-weighed nickel-copper wire (Alloy Wire, China) was then wrapped around the mesh envelope to prevent it from opening.

The first step of the digestion is based on existing methods to dissolve cellulosic materials (Hu et al., 2007; Jin et al., 2007; Yan et al., 2007). First, the steel-mesh envelope was placed in an Erlenmeyer flask, followed by a solution of 8% NaOH, 8% urea and 6.5% thiourea (by weight) in water solution in a ratio of 40 ml per 0.1 g dry weight sample and stored at −20 °C for 40 min to allow soaking and mini-crystal formation. Longer storage times resulted in complete crystallization of the NaOH:Urea:Thiourea solution. The Erlenmeyer flasks were then transferred to a fume hood at room temperature and immediately vigorously stirred until room temperature was reached, using a magnetic stirring plate. The solution was discarded, and flask and mesh were rinsed 15 times with 30 mL aliquots of deionized water, discarding the solution each time. Non-thorough rinsing led to overly-aggressive oxidation reactions in the subsequent oxidation step, likely due to residual urea/thiourea.

The oxidation step involved placing 30% H_2O_2 and 1% NaOH solution into the Erlenmeyer flask still containing the steel-mesh filter, by first adding H_2O_2 at ratios of 30 mL per 0.1 g dry weight sample aliquots of 0.75 mL 10 M NaOH per 0.1 g of sample. 30% H_2O_2 is a commonly used oxidizing agent for quantifying microplastic in biological material (Herrera et al., 2018; Hurley et al., 2018; Nuelle et al., 2014; Prata et al., 2019). The NaOH was added in order to increase the pH and favor the formation of nucleophilic HO_2 radicals, which has been found to be effective for digesting humic materials and lignin in soils (Mikutta et al., 2005). As violent reactions could occur during this step, the top of the flask was sealed with the same steel-mesh used for the samples and placed within a PP container to collect spillage from overpressure. The reaction assembly was placed on a magnetic stirrer to facilitate digestion for generally 5 h; though occasionally this was ended early in case of a violent reaction or allowed to continue overnight. Afterwards, rinsing with 15 times with 30 ml aliquots of distilled water was repeated as before.

The above two-step procedure was generally repeated three times, though sometimes more depending on the presence of organic matter after each round. Weight loss from digestion was determined by drying overnight at 60 °C and weighing the steel-mesh enclosed sample. In the case where a violent reaction occurred fast in the oxidation step, two to three repetitions could be repeated within the same day, though without weighing.

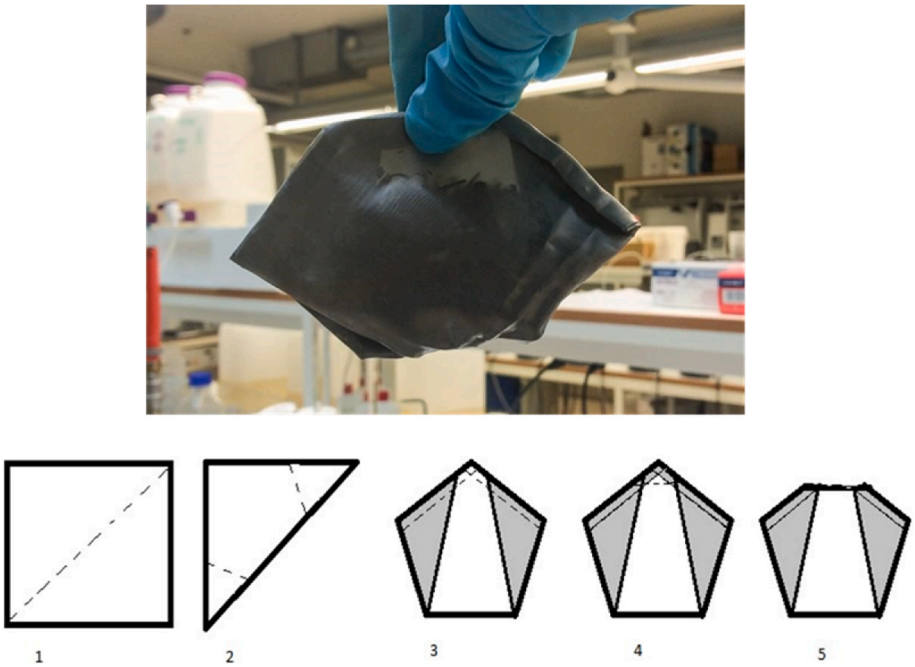

Fig. 1. Illustration of sample placed in a steel-mesh "tea-bag" and the folding technique used.

2.5. Alkaline digestion

As a basis for comparison in the lab, the two-step method above was compared with the "optimized alkaline digestion protocol" by Cole et al. (2015), which was reported to have a digestion efficiency of 91.3 ± 0.4% for zooplankton trawls. Here pre-weighed dry samples are placed in a steel mesh as before, though exposed to 10M NaOH (20 ml per 0.1 g of dry weight of material) at 60 °C for 24 h. The samples were rinsed thoroughly with distilled water before being dried at 60 °C for 14 h before being weighed.

2.6. Microplastic quantification

The samples were weighed using a METTLER AE 240 Analytical Balance (Marshall Scientific) with an accuracy of ±0.1 mg after chemical digestion to detect potential weight loss. In addition, visual analyses were performed using polarization microscopy (Nikon Eclipse E400) to detect potential degradation of the reference plastics and to inspect the remaining material in environmental samples. Quantified subsamples were taken for counting of suspected microplastic particles. Further identification of microplastics was performed using a micro Fourier-transform infrared spectroscopy (FT-IR) imaging system (Perkin Elmer Spotlight 200i FT-IR microscope, wave number range: 4000-600 cm^{-1}, 8-16 cm^{-1} resolution, 4 accumulations). Particles large enough to be picked up by tweezers (0.6 mm–5 mm and visible fibers) were analyzed by the Frontier (Attenuated total reflection) ATR assembly. Particles from the driftline sand, which were dominated by particles >0.6 mm, were exclusively identified with this assembly. For smaller particles, subsamples of plastic were transferred to a pre-cut 13 mm steel mesh (same material as the digestion filter) for analysis with the FT-IR microscope in transmittance mode. The FT-IR libraries "Polymer", "ATR-Spectra", "Transmission-Spectra" and "Fluka" provided by PerkinElmer were used to identify obtained spectra, and only spectra matches above 0.7 were considered.

2.7. Quality control

An important aspect of doing the work-up in a steel mesh filter (Fig. 1) is that it locks the sample in, avoiding contamination throughout the entire digestion procedure. However, contamination can occur during field sampling, density separation, and after opening the steel mesh filter for analysis. Method blanks (n = 6) of the two-step chemical digestion resulted in no observed weight change or particle contamination of the steel mesh filter. Method blanks (n = 9) of the density separation and chemical digestion combined resulted in a weight gain of 1.0 ± 1.0 mg, corresponding to 0.4 ± 0.4% of the steel mesh, indicating that the density separation step introduced particulate contamination. These particulates were identified as mostly polypetrafluoroethylene (PTFE, identified to originate from a valve in the density seperator), organic particles (including suspected cotton lab coat fibers), minerals (suspected salt crystals) and occasionally other microplastic (PS, PET and polyvinylfluoride particles were observed as a single particle in 1 out of 9 blanks, and polymethymethacrylate in 2 out of 9 blanks). Dry mass yields and the particle counts for specific microplastics in the blanks were corrected for. Recovory blanks for sand and sediment (ca 500 g) were prepared by spiking weighed amounts reference PET powder (75–300 μm, ca 100 mg), PE fibres (5–10 mm length, ca 50 mg) and LDPE granulates (3–5 mm, 5 granulates) into pre-cleaned driftline sand (n = 3 per type of plastic spike) and sediments collected from the Norwegian Continental Shelf (n = 8) using density separation to remove microplastic and other low density material prior to spiking (Knutsen et al., 2020). This resulted in recovery rates of 93 ± 2%, 82 ± 7% and 100 ± 0% respectively for sand, and 63 ± 18%, 91 ± 6% and 100 ± 0%, respectively for sediment. This indicated good recoveries for PE-fibres and LDPE granulates, but that recoveries were less quantitative for PET powder in fine-grained sediments. No recovery correction was performed in this study. Field blanks were not conducted for the environmental samples, which were mainly sampled for method validation not microplastic quantification. All results presented for nylon (plankton) and PP (sediment, debris sand) should be taken with caution due to the sampling equipment used. In future studies it is recommended to use field blanks that are deep sediments/sand dated before the advent of plastic to account for sampling contamination using plastic free equipment. It is also recommended to use weathered microplasics as the spiking medium for recovery blanks.

3. Results and discussion

An overview of the results from the testing of the digestion method are presented in Fig. 2, with a detailed comparison with literature studies in Tables 1 and 2.

3.1. Digestion of reference microplastics

In initial experiments, a subset of the reference microplastic materials (LDPE (f), LDPE (g), PS (g), PET (g)) did not exhibit any statistically significant weight loss (n = 3 per reference microplastic) when exposed exclusively to the NaOH/urea/thiourea dissolution step; it was therefore concluded that the dissolution step was benign to these microplastics. The complete set of reference microplastics (granulates: LDPE (g), PS (g) and PP (g); fibers: LDPE (f), PET (f) and nylon (f); mesh: nylon (m)) were then exposed to the two-step dissolution and oxidation treatment. The recovery rates after up to three rounds of the dissolution and digestion protocol is presented in Table 1 and Fig. 2. LDPE (g) (three rounds, n = 3), PS (g) (three rounds, n = 3), PP (g) (one round, n = 3) did not undergo any weight change from the procedure (100 ± 0% change in mass); PET (g, three rounds, n = 3) and nylon mesh (m, three rounds, n = 3) exhibited a weight loss that was not statistically significant (99 ± 1%). However, the nylon mesh did become brittle and easily broken after treatment, indicating structural change. Fibers of nylon (f) (three rounds, n = 3) and PET (f, three rounds, n = 3) showed some slight signs of weight loss, at 94 ± 1% and 96 ± 1% recoveries, respectively.

3.1.1. Comparison with single-step oxidation methods

A survey of the literature indicates a wide variation in reported weight loss for reference polymers with different single-step oxidation methods (Table 1). The greatest mass loss of microplastic quantified through oxidation we could find in the literature was for a mixture of LDPE and PS exposed to 30% H_2O_2 for 55 °C for seven days, with a recovery of 70 ± 3% (Avio et al., 2015) where in another study LDPE was observed to decrease in size to 84.1% with a similar treatment using 35% H_2O_2 for 7 days (Nuelle et al., 2014). A change of shape and size for PP after oxidative treatment has also been reported when exposed to H_2O_2 (30 or 35%) at room temperature for 7 days (Nuelle et al., 2014) or with 30% H_2O_2 at 70 °C for 24 h (Hurley et al., 2018). Therefore, digestion in 30% H_2O_2 at high temperatures or several days appears to degrade microplastics. Exposure times of 1 day or less to 30% H_2O_2 at temperatures of 60 °C or lower, with or without exposure to Fe (II)/Fenton's reagent as a catalyst under ambient conditions, appear so far to have negligible effects on most polymers, with even very little effects on surface FT-IR spectra (Hurley et al., 2018; Tagg et al., 2016). One exception is a difference in mass recovery was seen when PS foam was exposed to 4M KOH at 60 °C for 1 h, followed by addition of 35% H_2O_2 after cooling (solutions were stirred for 15 min and allowed to stand covered for 2 h), resulting in a mass recovery 77.5 ± 8.4%; or, when using Fenton's reagent followed by 35% H_2O_2 once the reaction settled (no boiling) and the solution cooled somewhat, resulting in a mass recovery 81.7 ± 8.3% (Munno et al., 2018). Though in general PET granules seem inert to oxidative treatments (Table 1), PET fibers seem less so. Few studies in the literature have looked at how oxidation affects PET (f), but a negligible loss was reported by Herrera et al. (2018) after exposure to 30% H_2O_2 (7 days). Mesh fragments and fibers of nylon also

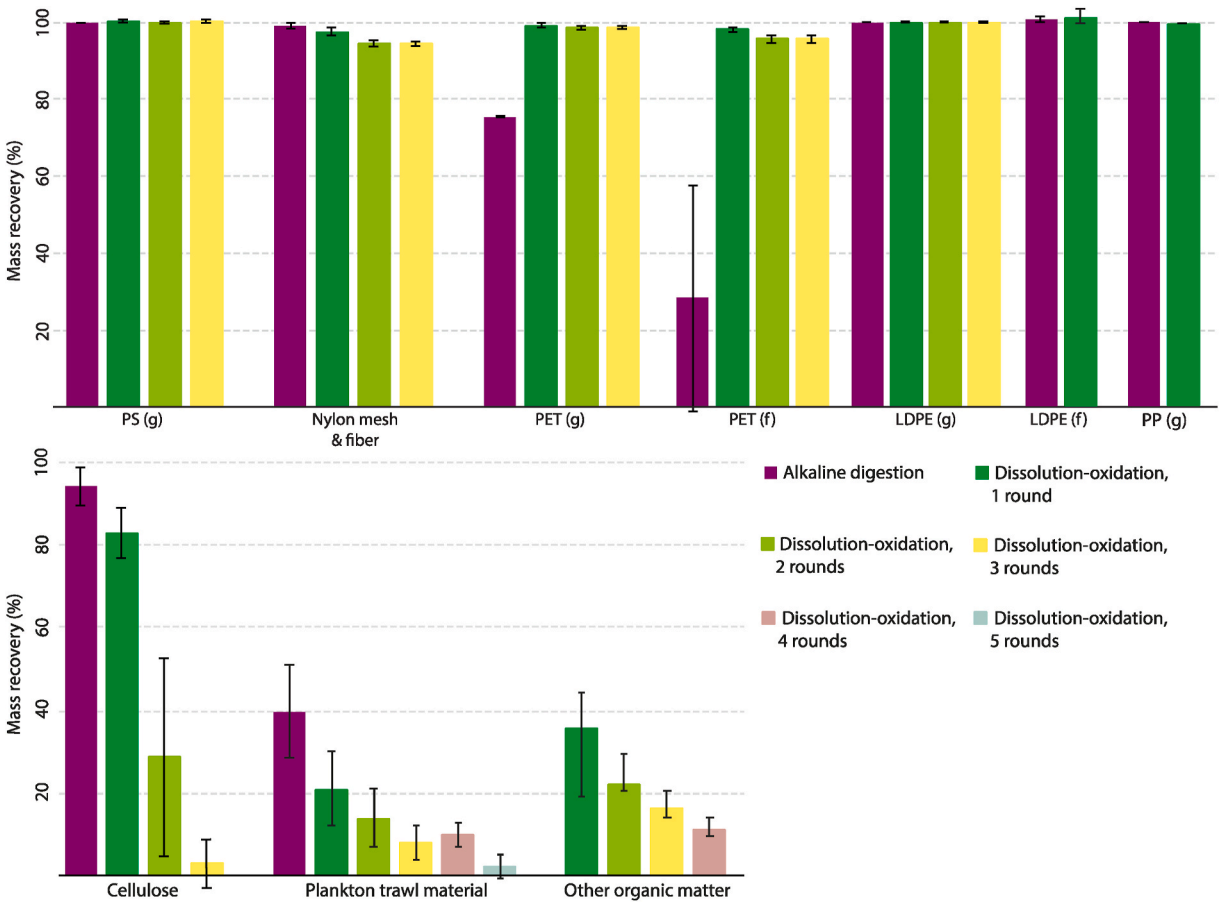

Fig. 2. Overview of mass recoveries of various reference microplastic, cellulose, plankton trawl material and low-density driftline material after the alkaline digestion or multiple-exposures to the dissolution-oxidation protocol presented in this study.

exhibit minor to negligible weight loss during exposure to various oxidative treatments, ranging from 88.0 ± 2.5% to 99.4 ± 1% mass recovery (Munno et al., 2018; Prata et al., 2019; Tagg et al., 2016).

3.1.2. Comparison with other digestion methods

Alkaline treatments. Only minor loss of LDPE and PS (except foam) has been reported after exposure to various alkaline treatments (Cole et al., 2015; Dehaut et al., 2016; Herrera et al., 2018; Hurley et al., 2018; Munno et al., 2018; Prata et al., 2019; Tagg et al., 2016) similar to this study (Table 1). The lowest recovery of PS was for PS foam after exposure to 4 M KOH for 14 days at ambient temperature, with mass recovery of 71.3 ± 9.0% (Munno et al., 2018, Table 1). PET (g and f) was shown to be very sensitive to alkaline digestions in the literature (Table 1). In this study, using 10 M NaOH at 60 °C for 24 h we encountered a recovery of 28 ± 29% (n = 9) and 75 ± 0% (n = 6) for PET (f) and (g), respectively. Granulates have been shown to be sensitive to this treatment (Cole et al., 2015; Dehaut et al., 2016; Hurley et al., 2018; this study), and the PET granules in this study displayed surface changes, visible by the development of surface pits and crumbling. A complete destruction of PET fibers after exposure to 10 M NaOH (60 °C, 24 h) has been reported in other studies (Cole et al., 2015; Herrera et al., 2018). Fibers have a higher surface to volume ratio and will be more prone to degradation during the various chemical treatments. In the consideration that fibers have been reported as the most common microplastic material in marine water samples (Kanhai et al., 2018), alkaline digestion methods should be avoided to accurately quantify PET fibers. Previous reviews have reported that PET was "not resistant" during treatment with either KOH (1, 10, 30 and 50%) nor NaOH (30 and 50%) (Lusher et al., 2017), but the use of KOH seems to be less aggressive to PET, resulting in a higher recovery compared to the use of NaOH (Dehaut et al., 2016; Hurley et al., 2018; Prata et al., 2019). Particles of nylon (mesh, fibers and fragments) exhibited minor weight change during alkaline treatment, ranging from 87.6 ± 13.6% (1M KOH at ambient temperature for 14 days, Munno et al., 2018) to 99.4 ± 0.4% (10% KOH at 50 °C for 1 h, Prata et al., 2019). For PP, only minor or no weight loss has been reported for various alkaline treatments presented in Table 1 (Dehaut et al., 2016; Herrera et al., 2018; Hurley et al., 2018; Prata et al., 2019), and the same was found in this study.

Acid Treatments. For treatment with acid, the use of 1M HCl (room temperature for 24 h) appears benign to LDPE (g), PS, PET (f) and PP (Herrera et al., 2018). However, boiling microplastics with concentrated HNO_3 (22.5 M) has been shown to result in major loss of LDPE and PS, with mass recovery of only 4 ± 3% (Avio et al., 2015).

Enzymatic Treatments. According to Cole et al. (2015) enzymatic digestion with Proteinase-K (37 °C for 30 min; and optimized protocol at 50 °C for 2 h) resulted in no or negligible changes in reference materials LDPE (g), PS, PET (f), and PP.

In summary, the two-step digestion method presented here appears to perform amongst the gentlest methods for the microplastic; in the next section this is compared to its ability to remove cellulosic and other natural organic matter.

3.2. Digestion of cellulose and organic media

In initial experiments with reference cellulose materials, negligible or non-significant mass loss was observed when exposed to just the dissolution step (n = 3) (Table 2, Fig. 2), despite the formation of some precipitate outside of the filter and an observable color change of the solution from colorless to brown. It was therefore concluded that some decomposition of cellulose occurred, the resulting particle size of the

Table 1
Mass recovery (%) of the two-step dissolution-digestion process for reference polymers, as well as similar data for single-step oxidation processes from the literature. In the dissolution and oxidation method from this study, n = 6 for PE (f), PP (g) and n = 3 for the remaining materials. During alkaline digestion, n = 9 for PET (f), n = 3 for nylon mesh and n = 6 for the remaining materials. Neg. = negligible. RT = room temperature.

Protocol	PE (f)	PE (g)	PS (g)	PET (f)	PET (g)	PP (g)	Nylon (mesh/ fragment)	Nylon (f)	Ref
Dissolution, then oxidation with 30% H_2O_2/1%NaOH									
1 round	102 ± 2%	100 ± 0%	100 ± 0%	98 ± 1%	99 ± 1%	100 ± 0%	99 ± 1%	96 ± 1%	This study
2 rounds		100 ± 0%	100 ± 0%	96 ± 1%	99 ± 1%			94 ± 1%	This study
3 rounds		100 ± 0%	100 ± 0%	96 ± 1%	99 ± 1%			94 ± 1%	This study
Oxidation digestion									
15% H_2O_2 (50 °C, overnight)		95 ± 2% (mixed with PS)	95 ± 2% (mixed with PE)						Avio et al. (2015)
30% H_2O_2 + 0.05 M Fe(II)SO_4 (75 °C, 30 min x 3)		neg.	neg.	neg.		neg.			Herrera et al. (2018)
30% H_2O_2 (RT, 7 days)		optical change			optical change	shape change			Nuelle et al. (2014)
35% H_2O_2 (RT, 7 days)		84.1% (size)				82.8% (size)			Nuelle et al. (2014)
30% H_2O_2 (60 °C, 24 h)		100 ± 0%	100 ± 0%		100 ± 0%	100 ± 0%			Hurley et al. (2018)
30% H_2O_2 (70 °C, 24 h)		100 ± 0%	100 ± 0%		99 ± 1%	94 ± 9%			Hurley et al. (2018)
30% H_2O_2 (55 °C, 7 days)		70 ± 3% (mixed with PS)	70 ± 3% (mixed with PE)	–					Avio et al. (2015)
KOH (4M, 60 °C, 1 h) + 35% H_2O_2 (2 h)		neg.	77.5 ± 8.4% (foam)	–				88.0 ± 2.5%	Munno et al. (2018)
Fenton (Fe(II)SO_4 + 30% H_2O_2) (1 day)		100 ± 0%	100 ± 0%		100 ± 0%	100 ± 0%			Hurley et al. (2018)
Fenton (Fe(II)SO_4 + 35% H_2O_2) (RT until cool)		neg., except one sample	81.7 ± 8.3 (foam)	–				91.3 ± 1.2%	Munno et al. (2018)
Fenton (Fe(II)SO_4 + 30% H_2O_2) (RT, 10 min)		neg.	neg.			neg.		neg. (g)	Tagg et al. (2016)
0.27 M $K_2S_2O_8$/0.24M NaOH (65 °C, 24 h)		neg.	neg.	–	neg.	neg.			Dehaut et al. (2016)
30% H_2O_2 + Fe(II), 0.05M (50 °C, 1 h)	96.2 ± 5.0% (fiber, weathered)	101.1 ± 1.1%, 99.2 ± 0.9% (frag.)	101.4 ± 4.5%	–	99.6 ± 2.9%	100.6 ± 1.3%	99.4 ± 1.0%		Prata et al. (2019)
Alkaline digestion									
10M NaOH (RT, 64 h)	100 ± 1%	100 ± 0%	100 ± 0%	28 ± 29%	75 ± 0%	100 ± 0%	99 ± 1%	n.a.	This study
1M NaOH + SDS (50 °C, 2 h, or if no visible change 50 °C, 24 h)		neg.	neg.	neg.		neg.			Herrera et al. (2018)
1M NaOH (60 °C, 24 h)		100 ± 0%	98 ± 2%		93 ± 8%	100 ± 0%			Hurley et al. (2018)
10M NaOH (60 °C, 24 h)		neg.	neg.	–	weight change	neg.			Dehaut et al. (2016)
10M NaOH (60 °C 24 h)		melting	neg.	destroyed			visible change		Cole et al., 2015
10M NaOH (60 °C, 24 h)		100 ± 0%	100 ± 0%		71 ± 2%	100 ± 0%			Hurley et al. (2018)
10M NaOH (60 °C, 24 h)		neg.	neg.	destroyed		neg.			Herrera et al. (2018)
1M KOH (RT, 14 days)		neg.	94.1 ± 2.6% (foam)	–				87.6 ± 13.6%	Munno et al. (2018)
4M KOH (RT, 14 days)		neg.	71.3 ± 9.0% (foam)	–				96.8 ± 0.9%	Munno et al. (2018)
10% KOH (60 °C, 24 h)		neg.	neg.	neg.		neg.			Herrera et al. (2018)
10% KOH (60 °C, 24 h)		neg.	neg.	–	neg.	neg.			Dehaut et al. (2016)
10M KOH (60 °C, 24 h)		98 ± 3%	112 ± 1%		99 ± 0%	99 ± 1%			Hurley et al. (2018)
10% KOH (50 °C, 1 h)	96.7 ± 7.7% (fiber, weathered)	99.7 ± 0.4%,	99.9 ± 0.4%	–	99.3 ± 2.3%	99.9 ± 1.1%	99.4 ± 0.4% (mesh)		Prata et al. (2019)
Acid digestion									
3% HCl (1 M, RT, 24 h)		neg.	neg.	neg.		neg.			Herrera et al. (2018)
				–					

(*continued on next page*)

Table 1 (*continued*)

Protocol	PE (f)	PE (g)	PS (g)	PET (f)	PET (g)	PP (g)	Nylon (mesh/ fragment)	Nylon (f)	Ref
22.5M HNO_3 (RT, 12 h + 100 °C, 30 min)		4 ± 3% (mixed with PS)	4 ± 3% (mixed with PE)						Avio et al. (2015)
Enzyme digestion									
Proteinase-K (37 °C, 30 min) Proteinase-K (50 °C, 2 h)		neg.	neg.	neg.			neg.		Cole et al., 2015

dissolved/dispersed cellulose could not be removed via filtration at the pore-size of the mesh filter used (45 μm) by simple rinsing to result in significant mass change. However, when this pre-treated reference cellulose was exposed to oxidation with 30% H_2O_2 for approximately 5 h, it resulted in substantial weight loss. The mass-recovery of cellulose after three rounds of dissolution and oxidation was 3 ± 6% (n = 5) (Table 2). For the plankton trawl material, after three rounds, the mass recovery was 8 ± 4% (n = 21) (Table 2); greyish particles in these samples were identified by FT-IR to be chitin residues (Fig. 3). Further repetition of the procedure for six rounds (n = 5) with a remaining amount of material being 2 ± 3%. The mass recovery of low-density seperated driftline sand debris was 35 ± 16% (n = 12), isolated using $ZnCl_2$:$CaCl_2$ solution, after only one round of digestion and oxidation (Table 2). FT-IR analysis subsequently revealed a large portion of the remainder to be microplastic and charcoal materials (Section 3.3.3).

3.2.1. Comparison with single step oxidation

In comparison to microplastic, much fewer studies have investigated the removal of cellulose after oxidation. Substantial weight loss of cellulose acetate was reported after exposure to 0.27 M $K_2S_2O_8$ and 0.24 M NaOH (65 °C, 24 h) (Dehaut et al., 2016), though less so with 30% H_2O_2 with Fe(II) (50 °C, 1 h, 87.4 ± 0.7% mass recovery) (Prata et al., 2019). Another study also found cellulosic materials like cotton clothing fibers and toilet paper were resistant to a similar procedure (Fe(II)SO_4 + 30% H_2O_2) (70 °C, 30 min), requiring several digestions to remove them (Dyachenko et al., 2017). Similarly, studies with driftline vegetal matter found that after oxidation with 30% H_2O_2 and Fe(II) (75 °C, 30 min x 3) the mass remaining was 35.4 ± 7.1% (Herrera et al., 2018), after 30% H_2O_2 (ambient temperature, one week) the mass remaining was 50% (Nuelle et al., 2014), or for driftwood, after 30% H_2O_2 with Fe(II) (50 °C, 1 h) the mass remaining was 74.8 ± 2.6% (Prata et al., 2019). After oxidation of sewage sludge with 30% H_2O_2 at 60 or 70 °C for 24 h, or with 30% H_2O_2 with Fe(II) (ambient, 1 day) the organic matter mass recovery was 19.8, 13.4 or 13.1%, respectively (Hurley et al., 2018). In comparison, the results in this study for cellulose-rich driftline debris show a mass recovery of 16 ± 2% (n = 3) after 3 rounds of dissolution and oxidation, with the remainder dominated by plastic and chars (Table 2, Figs. 2, Figure 4). Therefore this method is considered one of the best performing, and unlike many of the above methods that involve heat or long exposure times, it is considered gentler to microplastic as presented previously.

3.2.2. Comparison with other digestion methods

Alkaline treatment. In this study, only a minor removal of cellulose (94 ± 5% mass recovery) (Table 2) was found after treatment with 10 M NaOH at 60 °C for 24 h, similar to a previous observation in the literature (Biginagwa et al., 2016). Slightly better losses were reported by Prata et al. (2019), with a mass recovery of 78.3 ± 0.8% (cellulose acetate) and 87.6 ± 3.0% (driftwood) after treatment with 10% KOH at 50 °C for 1 h. For the plankton samples in this study, the mass recovery was relatively high after alkaline treatment (39 ± 11%), which is considerably lower than those reported for the plankton in e.g. Cole et al. (2015) which reported organic matter recoveries < 10% (1 M NaOH at 20 °C for 24 h, and treatment with 10 M NaOH at 60 °C for 24 h). Removal rates of sewage sludge OM using various alkaline treatments, such as 10 M KOH or NaOH (60 °C for 24 h) were lower than corresponding treatments with 30% H_2O_2 (Table 2) (Hurley et al., 2018). In general, alkaline digestive methods appear less efficient than oxidative methods for complex samples.

Acid Treatment. Acidification has also been tested in some studies. Cole et al. (2015) used 1 M HCl at 20 °C for 24 h, which proved inconsistent and inefficient in digesting plankton (digestion efficiency of 54%); whereas, alkaline hydrolysis with 1 M NaOH at room-temperature digested 90% of the plankton material (which could be even further enhanced by increasing molarity and experimental temperature).

Enzymatic Treatment. An alternative, and reportedly effective way of removing organic matter is through a multi-step enzymatic/H_2O_2, protocol, which uses, amongst other enzymes, cellulase and chitinase, in combination with a purification protocol (Löder et al., 2017). For plankton trawl this resulted in mass recoveries as low as 1.7 ± 0.1%, which is similar to our approach after 6 rounds with our two-step procedure (Table 2). It is noted, however, that 6 rounds of the procedure would require up to six days to complete (or less if more than one repetition was done per day), which is shorter than the 10–13 day enzymatic procedure by Löder et al. (2017) which resulted in a similar performance for plankton.

3.3. Microplastics in the marine samples

After several rounds of digestion of the plankton material, FT-IR identification of the remaining material showed that approximately >90% of the residual weights were chitin, but this resulted in plastics being more visible and easier to identify (Fig. 3). The particles in the plankton trawl samples were identified as PE, PP, epoxy resin, polyvinyl chloride (PVC), polyoxymethylene (POM) and nylon (Table 3). A high concentration of microplastic was detected near the seabed by the WTP outlet flow of Bekkelaget (from 0.23 to 0.157 MP items/L). By the outflow of the Akerselva river, the concentration microplastic of was linear over the water depth (from 0.001 to 0.006 MP items/L). In the sediment samples by the WTP outflow of Bekkelaget (Table 3), the identified microplastics were PE, PP, epoxy resins and PVC and trace amounts of others (rubber, PU and phenoxy resin). The presence of low-density microplastic below the sea surface is an indication of how biotic processes (e.g., ingestion by plankton, sinking of microplastic containing fecal matter or detritus) can bring floating particles below the sea surface (Gorokhova, 2015). For the driftline sand, after density separation and digestion, the remaining sample was dominated by char residues (presumably from barbeques and bonfires) and microplastic (Fig. 4, Table 4). Due to the abundance of large microplastics in this sample (>0.6 mm), which were considered to dominate the weight fraction, these were identified with FT-IR, and found to mostly be comprised of PE, PP and PS (Table 4). It is of relevance to note that PET was not frequently detected in these samples, and PET fibers appeared the most sensitive to the work-up provided. It could be that PET fibers were rare in these samples, or that they were not detected to them being partly destroyed by this method; however, PET fibers were identified using this method in a parallel study (Knutsen et al., 2020).

Table 2
Mass recovery (%) of the two-step dissolution-digestion process for reference cellulose, plankton and other natural organic matter. RT = room temperature.

Protocols	Cellulose	Plankton	Other natural organic matter	Source of natural organic matter	Ref
Dissolution, then oxidation with 30% H_2O_2/1% NaOH					
1 round	83 ± 6% (n = 6)	21 ± 9% (n = 21)	35 ± 16% (n = 12)	Density separated organic matter using $ZnCl_2$–$CaCl_2$ sol'n.	This study
2 rounds	29 ± 24% (n = 6)	14 ± 7% (n = 21)	22 ± 2% (n = 3)		
3 rounds	3 ± 6% (n = 5)	8 ± 4% (n = 21)	16 ± 2% (n = 3)		
4 rounds		10 ± 3% (n = 5)	11 ± 2% (n = 3)		
6 rounds		2 ± 3% (n = 5)			
Oxidation digestion					
0.27M $K_2S_2O_8$/0.24M NaOH (65 °C, 24 h)	substantial weight loss (cellulose acetate)				Dehaut et al. (2016)
30% H_2O_2 + 0.05M Fe(II)SO_4 (75 °C for 30 min x 3)			35.4 ± 7.1%	Driftline OM	Herrera et al. (2018)
30% H_2O_2 (RT, 7 days)			50% dissolved/ discoloured	Driftline, > 1 mm	Nuelle et al. (2014)
30% H_2O_2 (60 °C, 24 h)			19.8 ± 4.2%	Sewage Sludge OM	Hurley et al. (2018)
30% H_2O_2 (60 °C, 24 h)			58.7 ± 2.2%	Sewage Sludge total	Hurley et al. (2018)
30% H_2O_2 (70 °C, 24 h)			13.4 ± 13.1%	Sewage Sludge OM	Hurley et al. (2018)
30% H_2O_2 (70 °C, 24 h)			55.4 ± 6.8%	Sewage Sludge total	Hurley et al. (2018)
35% H_2O_2 (RT, 7 days)			8% dissolved/ discoloured	Driftline, > 1 mm	Nuelle et al. (2014)
30% H_2O_2 + Fe(II), 0.05M (50 °C, 1 h)	87.4 ± 0.7% (cellulose acetate)				Prata et al. (2019)
30% H_2O_2 + Fe(II), 0.05M (50 °C, 1 h)			74.8 ± 2.6%	Driftwood	Prata et al. (2019)
30% H_2O_2 + Fe(II), 0.05M (50 °C, 1 h)			−8.3 ± 16.5%	Algae	Prata et al. (2019)
Fenton (Fe(II)SO_4 + 30% H_2O_2) (1 day)			13.1 ± 9.9%	Sewage Sludge OM	Hurley et al. (2018)
Fenton (Fe(II)SO_4 + 30% H_2O_2) (1 day)			56.2 ± 6.6%	Sewage Sludge total	Hurley et al. (2018)
Fenton (Fe(II)SO_4 + 30% H_2O_2) (70 °C, 30 min)	neg.				Dyachenko et al. (2017)
Alkaline digestion					
10 M NaOH (RT, 64 h)	94 ± 5% (n = 3)	39 ± 11% (n = 18)			This study
1M NaOH (20 °C, 24 h)		10.0 ± 2.9%			Cole et al. (2015)
1M NaOH + SDS (50 °C, 2 h, or if no visible change 50 °C, 24 h)			59.1 ± 2.7%	Driftline OM	Herrera et al. (2018)
1M NaOH (60 °C, 24 h)			39.1 ± 5.6%	Sewage Sludge OM	Hurley et al. (2018)
1M NaOH (60 °C, 24 h)			68.6 ± 2.9%	Sewage Sludge total	Hurley et al. (2018)
10M NaOH (60 °C, 24 h)			91 ± 5.4%	Driftline OM	Herrera et al. (2018)
10M NaOH (60 °C, 24 h)	weight loss (cellulose acetate)				Dehaut et al. (2016)
10M NaOH (60 °C, 24 h)		8.7 ± 0.4%			Cole et al. (2015)
10M NaOH (60 °C, 24 h)			32.8 ± 5.8%	Sewage Sludge OM	Hurley et al. (2018)
10M NaOH (60 °C, 24 h)			65.4 ± 3.0%	Sewage Sludge total	Hurley et al. (2018)
10% KOH (60 °C, 24 h)	weight loss (cellulose acetate)				Dehaut et al. (2016)
10% KOH (60 °C, 24 h)			76 ± 2.8%	Driftline OM	Herrera et al. (2018)
10M KOH (60 °C, 24 h)			43.2 ± 16.6%	Sewage Sludge OM	Hurley et al. (2018)
10M KOH (60 °C, 24 h)			70.8 ± 8.6%	Sewage Sludge total	Hurley et al. (2018)
10% KOH (50 °C, 1 h)	87.6 ± 3.0%			Driftwood	Prata et al. (2019)
10% KOH (50 °C, 1 h)	78.3 ± 0.8% (cellulose acetate)				Prata et al. (2019)
10% KOH (50 °C, 1 h)			57.8 ± 5.6%	Algae	Prata et al. (2019)
Acid digestion					
1M HCl (20 °C, 24 h)		17.4 ± 3.7%			Cole et al. (2015)
3% HCl (1 M, RT, 24 h)			80.5 ± 6.4%	Driftline OM	

(*continued on next page*)

Table 2 (*continued*)

Protocols	Cellulose	Plankton	Other natural organic matter	Source of natural organic matter	Ref
					Herrera et al. (2018)
Enzyme digestion					
Multi-step enzymatic/H_2O_2 purification protocol		1.7 ± 0.1%			Löder et al. (2017)
Proteinase-K (37 °C, 0.5 h)		11.1 ± 1.5%			Cole et al. (2015)
Proteinase-K (50 °C, 2 h)		<3%			Cole et al. (2015)

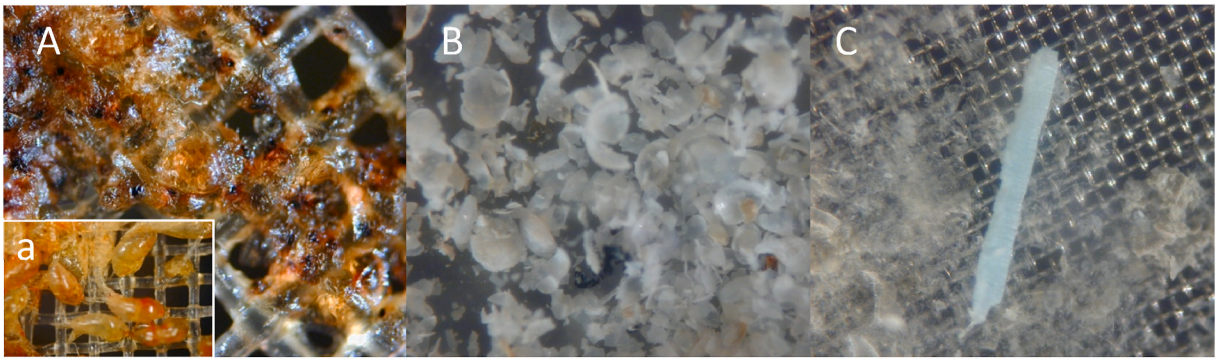

Fig. 3. Plankton trawl material A) after drying (porosity of mesh 90 μm); B) After the two-step digestion). The spherical shapes in B was later determined to consist of chitin, but a particle of polypropylene (C) was still easily detected within this residue after digestion (porosity of mesh 45 μm).

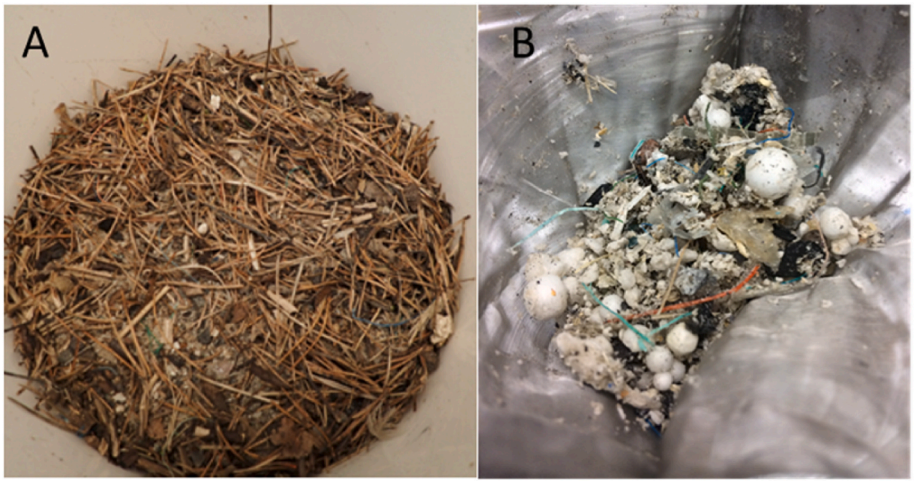

Fig. 4. The low-density matter in the driftline sand sample (A) after density separation ($\rho \leq 1.52$ g/cm^3) and (B) after the two-step digestion protocol (B).

4. Conclusions and further method development

Introducing the cellulose dissolution step can be a cost-effective and efficient way of removing most of the organic and cellulosic materials for microplastic quantification. It seems, however, challenging to achieve complete removal of all organic material with this method, but one could attempt to approach this if digestions are repeated several times. Such repetitions are, however, potentially harmful to more sensitive microplastics (e.g. PET fibers and oxidized PET fibers, which were not observed in the plankton and sediment samples in this study, and were rare in the driftline sand), so this would have to be controlled for. For many quantification methods, however, complete removal is not necessarily needed, and simple enrichment is sufficient.

For future method testing and development, it would be of interest to test the sensitivity of the two-step method for particles below 45 μm, to see if optimization is needed for smaller size fractions or more sensitive microplastics (e.g. PET, nylon), such as using gentler oxidation procedures. Further, for future method validation, weathered reference microplastics are recommended to use as recovery standards, rather than or in addition to pristine reference plastics as used here. Application of a cellulose dissolution step can facilitate studies of the distribution, dynamics, exposure and risk of microplastic in various marine ecosystems.

CRediT authorship contribution statement

Linn Merethe Brekke Olsen: Writing - original draft, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing - review & editing. **Heidi Knutsen:** Writing - review & editing, Investigation. **Sabnam Mahat:** Methodology, Investigation. **Emma Jane Wade:** Investigation. **Hans Peter H. Arp:** Writing - original draft, Conceptualization, Methodology,

Table 3
Microplastic particles identified in plankton trawl samples and sediment near Bekkelaget WTP and the outflow of the Aker river after the two-step digestion method. Particle size cut-off was 45 μm "Unknown" = The FT-IR analysis was not able to identify the particle above a library match score of 0.7. PE = polyethylene, PP=polypropylene; PVC = polyvinyl chloride; POM = polyoxymethylene. Nylon in these samples may originate from the plankton sampling net.

Media	Location	Water Depth (m)	N (WGS84)	E (WGS84)	Plankton mass ($g_{d.w.}/m^3$)	MP Items (max-min items/m^3)	FT-IR identification (percent of MP particles identified)
Plankton trawl material	Bekkelaget WTP	8	59.5296	10.4542	0.067	3–4	unknown (100%)
		20	59.5282	10.4552	0.053	3	PE (100%)
		46	59.8824	10.7507	0.052	23–157	PP (50%) PVC (20%) POM (20%) nylon (10%)
Plankton trawl material	Akerselva outflow	1	59.5408	10.4486	0.021	2–3	epoxy resin (100%)
		8	59.5394	10.4482	0.053	1–4	unknown (100%)
		14.5	59.5398	10.4487	0.088	1–6	PE (100%)
					MP weight (mg/kg)	**MP particles (items/kg)**	
Sediment 0–10 cm	Bekkelaget WTP	50.5	59.53096	10.45225	3.6	270	PP (57%), rubber (29%) PE (14%) PVC (7%) PU (7%) Epoxy resin (7%) unresolved (7%)
Sediment 0–5 cm		52.5	59.52926	10.4541	3.3	240	PVC (33%) PE (17%) PE-oxidized (17%) Epoxy resin (17%) Phenoxy resin (17%) rubber (17%)
Sediment 0–5 cm		51	59.52985	10.45373	20	1500	PE (44%) PP (22%) PE-oxidized (11%) Phenoxy resin (11%) PU (11%)
Average					9.0 ± 9.6	670 ± 719	

Table 4
Microplastic particles identified in the driftline sand samples (0–2 cm) from Bygdøy Sjøbad beach area), after density separation and the two-step digestion method. na = not applicable. LOD = limit of detection.

FT-IR identification	Shape	MP weight (mg/kg_{sand})	MP weight (%)	MP Items 0.6–5.0 mm (items/kg_{sand})	MP Items 0.6–5.0 mm (%)
Total charcoal		2524 ± 2238	na	na	na
PE	Granule	1346 ± 855	42.6%	124 ± 79	16.5%
	Film	146 ± 93	4.6%	78 ± 50	10.4%
	Fiber	178 ± 113	5.6%	117 ± 75	15.7%
PP	Granule	321 ± 204	10.2%	65 ± 41	8.7%
	Film	44 ± 28	1.4%	39 ± 25	5.2%
	Fiber	358 ± 228	11.3%	130 ± 83	17.4%
PS	Granule	745 ± 473	23.6%	170 ± 108	22.6%
PMMA	Granule	7 ± 4	0.2%	7 ± 4	0.9%
Nylon	Granule	15 ± 10	0.5%	7 ± 4	0.9%
PVC	Granule	< LOD	0.0%	7 ± 4	0.9%
PET	Fiber	< LOD	0.0%	7 ± 4	0.9%
Total MP	average	3160 ± 2010	100%	750 ± 477	100%
	range	1420–5360		338–1270	

Validation, Formal analysis, Data curation, Supervision, Project administration, Funding acquisition, Writing - review & editing.

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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