# Validation of a method for extracting microplastics from complex, organic-rich, environmental matrices

*Rachel R. Hurley*[a], Amy L. Lusher[a], Marianne Olsen[a], Luca Nizzetto[a,b]*

[a] Norwegian Institute for Water Research (NIVA), Gaustadelléen 21, 0349 Oslo, Norway

[b] Research Centre for Toxic Compounds in the Environment (RECETOX), Faculty of Science, Masaryk University, Kamenice 753/5, Brno 62500, Czech Republic

ABSTRACT

Complex and organic-rich solid substrates such as sludge and soil have been shown to be contaminated by microplastics; however, methods for extracting plastic particles have not yet been systemically tested or standardised. This study investigated four main protocols for the removal of organic material during analysis of microplastics from complex solid matrices: oxidation using $H_2O_2$, Fenton's reagent, and alkaline digestion with NaOH and KOH. Eight common polymer types were used to assess the influence of reagent exposure on particle integrity. Organic matter removal efficiencies were established for test sludge and soil samples. Fenton's reagent was identified as the optimum protocol. All other methods showed signs of particle degradation or resulted in an insufficient reduction in organic matter content. A further validation procedure revealed high microplastic extraction efficiencies for particles with different morphologies. This confirmed the suitability of Fenton's reagent for use in conjunction with density separation for extracting microplastics. This approach affords greater comparability with existing studies that utilise a density-based technique. Recommendations for further method optimisation were also identified to improve the recovery of microplastic from complex, organic-rich environmental samples.

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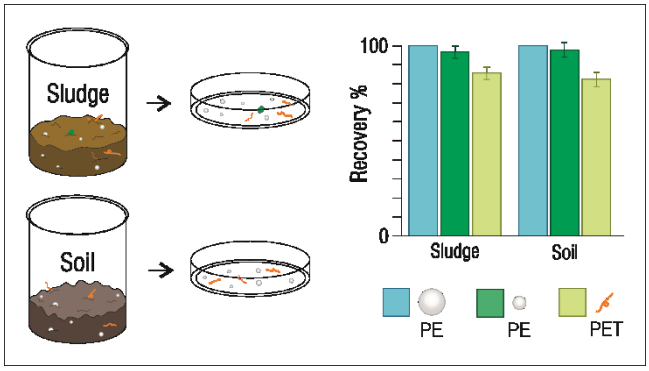


TEXT

## Introduction

Microplastic contamination has emerged as a major global environmental issue. Small plastic particles are now pervasive across marine and freshwater systems[1–5]. Recently, attention is beginning to focus on the occurrence of microplastics within other environmental compartments[6]. Wastewater treatment plants (WWTPs) have been shown to have a high trapping efficiency for microplastics[7,8]. However, particles are concentrated in the solid sludge phase[8,9], which is often applied to agricultural soils as fertiliser. Nizzetto et al.[10] estimate that between 63,000-430,000 and 44,000-300,000 tons of microplastic are added per year to farmlands in Europe and North America respectively. Hence, agricultural soils may represent a major environmental reservoir of microplastic. A small number of studies have examined microplastics in soil[11–14] and sludge samples[7–9,15–21], but no standardised method has emerged. The organic components, complexity of the solid matrix, and presence of additional contaminants complicates the extraction of small plastic particles[22]. Accurately assessing the magnitude of temporary stores, source inventories and emission rates of microplastics in terrestrial environments is crucial for the definition of management frameworks and the protection of both terrestrial and marine systems. There is an objective urgent need for validated analytical methods to effectively characterise microplastic dynamics in this specific area.

The majority of work extracting microplastics from solid matrices has been concerned with *aquatic sediments*. Most commonly, microplastics are extracted based upon their density[23–25]. This can be performed using density solutions or through elutriation-based methods[26,27]. However, this approach, when used alone, is not effective for the analysis of microplastics in sewage or soil samples based on the high organic matter content (up to 99%) and the presence of complex organic compounds and aggregates. For example, soil organic matter (SOM) typically exhibits a density of 1.0 – 1.4 g $cm^{-3}$ and therefore will not be effectively separated from microplastics during density extraction[22]. Hence, additional procedural steps are required.

Preliminary studies that have examined small quantities of *sewage sludge* have bleached, dried, or filtered samples prior to analysis[8,9,16,17]. This approach is not sufficient for analysing larger sample sizes, where the organic component will likely physically conceal microplastic particles during identification and quantification. More recently, studies have applied density-based separation[9,17–20]. Some studies have incorporated an organic matter removal step[19,20]; however, the efficacy of these techniques has not yet been systematically tested.

In contrast, analyses of microplastic in *soil* samples have, thus far, concentrated on direct extraction techniques, such as pressurised liquid extraction[13], thermal decomposition coupled with GC-MS[11,14], and rapid heat treatment[28]. These approaches negate the need for sample pre-treatment (i.e. the isolation of microplastic particles) and yield mass-based concentrations of common polymer types. However, they destroy particle information that is critical to current microplastic research directives e.g. particle numbers, shapes, and size. These details are presently more important for establishing potential sources or associated ecotoxicological implications than polymer concentration alone. As discussed by Fuller and Gautam[13], these approaches will likely complement existing methods.

The lack of a standardised approach to microplastic analysis has already been widely discussed[24,29]. An important additional note is the current lack of a sufficiently detailed, unique classification scheme for microplastics and related reference materials needed for the validation of methods. This is for example the case for microfibers, car tire debris and other types of microplastic.

This study aims to identify an additional processing step that can be added to existing methods for analysing microplastic in solid substrates (e.g. aquatic sediments). Namely, the removal of organic material from soil and sludge samples will be tested. Eerkes-Medrano et al.[30] highlighted several considerations for methodological development: techniques should be simple, affordable, precise, accurate, and have limited potential for contamination. This study will test four main protocols to establish the optimal method for extracting microplastics from organic-rich environmental substrates which satisfies these criteria.

## Methods

Review of existing organic matter removal techniques

A commonly applied technique for removing organic material from environmental matrices is oxidation using hydrogen peroxide ($H_2O_2$). Despite this, the efficacy of $H_2O_2$ has been called into question. Cole et al.[31] found that only 25% of *biogenic* material was removed following treatment with 35% $H_2O_2$ at ambient temperature for 7 days. This has been observed elsewhere, where hydrogen peroxide often has the effect of bleaching organic material rather than completely removing it [32]. Additionally, Nuelle et al.[32] noted the degradation of some polymer types as a result of $H_2O_2$ oxidation. These included polyethylene (PE) and polypropylene (PP), which are amongst the most commonly produced plastics globally. Despite this, further studies have observed no significant changes to microplastic particles following $H_2O_2$ digestion, including no evidence of microplastic bleaching[20,33]. To reduce the reaction time, some studies have utilised higher temperatures during peroxide oxidation. For example, Sujathan et al.[20] used 30% $H_2O_2$ at 70°C to decrease the reaction time to approximately 12 hours. Whilst 70°C is lower than the continuous operating temperatures (COTs) for most of the common polymer types, the authors noted that particles composed of PMMA may be affected[20]. A modified approach using lower temperatures may overcome this issue, although the effect on reaction time must be assessed.

A potential alternative to peroxide oxidation is the use of Fenton's reagent. This has previously been used to extract microplastics from organic-rich wastewater samples[34]. Fenton's reagent is an advanced

oxidation process using $H_2O_2$ in the presence of a catalyst ($Fe^{2+}$). This method is performed at ambient temperature, reducing the potential for exceeding COTs. Fenton's reagent is effective in destroying organic components such as highly chlorinated aromatic compounds or inorganic compounds, which are typically recalcitrant in $H_2O_2$[35,36]. This may prove more effective in removing all organic components from complex environmental substrates. Additionally, the reaction occurs more rapidly than traditional $H_2O_2$ oxidation, typically taking less than 1 hour to process wastewater samples[37]. Ferrous sulfate ($FeSO_4 \cdot 7H_2O$) is usually used as the iron catalyst component and is inexpensive and readily available. Although, the composition of sewage sludge may reduce the efficacy of organic matter removal; high concentrations of hydroxyl free radical scavengers, for example, will inhibit the degradation of organic material[38]. Furthermore, the pH of the reagent must be adjusted (to 3.0 – 5.0) to encourage the dissolution of the ferrous sulfate granules and optimise the degradation of organic material[39–41]. This acidity may begin to degrade some polymers, although this effect was not observed by Tagg et al.[34]. Therefore, the efficacy of this technique needs to be tested.

Other potential methods for the removal of organic matter arise from existing studies that extract microplastics from biota. Acid digests, such as hydrochloric acid (HCl) and nitric acid ($HNO_3$), have been shown to be highly effective in destroying organic matter but they also attack microplastic particles, leading to degradation and melting[31,33,42]. Hence, these have not been considered further. Alkaline digests have also been investigated, including potassium hydroxide (KOH) and sodium hydroxide (NaOH). Dehaut et al.[42] showed that use of 10 M NaOH led to the degradation of polycarbonate and polyethylene terephthalate; however, Mintenig et al.[19] used NaOH digestion to remove organic material from sewage sludge samples. 10% KOH at 60°C has been highlighted as the optimum procedure for the extraction of microplastics from *biota* [33,42–45]. However, the efficacy of KOH in extracting microplastics from *sludge* or *soil* must be tested. KOH breaks down humic acids; however, Bläsing and Amelung[22] point out that humins and alkali-insoluble compounds within soils will not be removed. Humins are likely to also be present in sewage sludge in the form of raw organic matter, bacteria, and fungi that may not been removed by the wastewater treatment process[46]. Therefore, testing

of this procedure on complex environmental samples is important to establish the degree of organic matter removal in this context.

Finally, a number of studies utilise enzymatic digestion to remove organic material prior to microplastic analysis. Cole et al.[31] first introduced the use of proteinase-K to extract microplastics from both seawater and biota. They report a removal of >97% of biogenic material present. However, this technique was applied on small sample volumes (0.2 g dry weight) and the enzyme used is expensive. Hence, it may not be feasible or cost-effective to process large samples with high organic content using this technique. Likely, a range of enzymes will be required to breakdown the different organic compounds found in these sample types. Mintenig et al.[19] apply an enzymatic-oxidative procedure to extract microplastics from wastewater samples. They used protease, lipase and cellulase, which are less expensive than proteinase-K. However, the procedure took over six days to complete and the same study goes on to utilise a different, non-enzymatic, approach to analyse sludge samples. This suggests that the technique may not be optimised for analysing solid environmental samples. For these reasons, enzymatic treatments were not tested in this study.

Experimental design

The majority of studies that analyse microplastics in solid samples (e.g. sediments) utilise a density separation procedure to isolate microplastic particles[23,24]. To increase potential for comparability, the aim of this study was to add an additional processing step to remove organic matter in conjunction with a density separation approach. Based on the review of existing literature, four main protocols were tested for removal of organic material from complex, organic-rich, environmental samples. Temperature and concentration variants were also tested for some of the selected reagents. As a result, this study tested a total of six protocols:

1. **30% (v/v) $H_2O_2$.** Sujathan et al.[20] used this reagent at 70°C; however, the authors noted that this may be above the COTs of some polymers. Microplastics have been shown to be preserved

147 by other reagents during continuous heating at 60°C[42]. Hence, this protocol was tested at two
148 temperatures:
149 a. 30% hydrogen peroxide at **70°C**
150 b. 30% hydrogen peroxide at **60°C**
151 2. **Fenton's reagent.** This reagent has two components: 30% (v/v) $H_2O_2$ with an iron catalyst.
152 The catalyst solution was composed of 20 g of iron (II) sulphate heptahydrate in 1 l of filtered
153 RO water. Tagg et al.[34] tested this reagent within the context of extracting microplastics from
154 wastewater. The authors identified this as the optimum concentration. The catalyst solution was
155 adjusted to pH 3.0 using concentrated sulfuric acid.
156 3. **NaOH** solution. A 10 M solution has been applied to sludge samples by Mintenig et al.[19],
157 although studies have identified some particle degradation with this concentration[42]. A lower
158 concentration solution may present a reduced potential for particle degradation. This technique
159 has previously been used at different concentrations to extract microplastics from biota[31,47,48].
160 Hence, this protocol was tested at two concentrations to observe differences in microplastic
161 preservation and organic matter removal:
162 a. **1 M** NaOH at 60°C
163 b. **10 M** NaOH at 60°C
164 4. **10% KOH** solution at 60°C. This protocol has been rigorously tested within the context of
165 biota microplastic studies[42]. The optimal operating conditions (10%, 60°C) were applied here
166 to test the efficacy of this technique in removing organic material from soils and sludge.

167 Protocol assessment was split into two main phases: 1) testing the effect of the selected protocols on
168 plastic particles; and 2) establishing the efficacy of the protocols in removing or reducing organic matter
169 content. Method validation was performed by assessing the extraction efficiency of the optimum
170 protocol. The optimum protocol was established by the outcomes of Phase 1 and 2 testing. A schematic
171 diagram showing the experimental design is provided in Figure S1.

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Phase 1: Effect of reagents on polymeric particles

The initial testing phase aimed to establish the preservation of microplastics following exposure to the reagents. Eight common polymer types were tested for indicators of degradation following treatment: PP, LDPE, HDPE, PS, PET, PA-66, PC, and PMMA (Table S1). These represent >70% of plastic demand in Europe[49]. Details of the particles used are provided in Table S2 and images are presented in Figure S2. The test particles were acquired through the JPI-Oceans BASEMAN project. The tested particles represent large microplastics. Particles of this size were tested to improve the quality of weight and mass measurements and to afford greater visibility of degradative changes to the particle surface.

Three replicates were analysed for each of the six protocols, in addition to three control samples. Three particles from each polymer type were tested in each replicate (total of 504 particles tested). The particles were placed into clean, pre-washed glass jars and 30 ml of each reagent was added. Filtered RO water was used in the case of the control samples. Protocols 1a, 1b, 3, 4 were placed into an incubator (60 or 70°C, as detailed above; 120 rpm). The samples for Protocol 2 and the control samples were performed at room temperature. The particles were exposed to the reagents for 24 hours. They were then removed from the jars, rinsed thoroughly in filtered RO water, and left to air dry in petri dishes.

Microplastic particles were characterised physically prior to and following exposure. Each particle was measured along the a- and b-axis using a Nikon SMZ 745T stereomicroscope at 10x magnification and the Infinity Analyse software package. Particle mass was also recorded before and after treatment. Each particle was photographed to assess for any visual evidence of degradation. Some particles exhibited surface degradation following treatment (see Results and discussion). In this case, the particles were first photographed and then gently brushed to remove loose fragments prior to taking mass and size measurements.

Following treatment, three particles of each polymer type from each treatment were analysed using FT-IR (n = 168). Particles were tested using an Agilent Cary 630 FT-IR spectrometer with a diamond ATR accessory. Spectral changes were noted, in addition to deviations in the library search hit quality index.

200 The library search was performed using the Agilent Polymers ATR library. Matches were calculated by
201 the MicroLab PC software which uses a scalar product algorithm to assign a hit quality index. For
202 particles exhibiting surface degradation, the fragments from the outer layer were analysed separately to
203 test for differences in the FT-IR spectra.

204

205 Phase 2: Efficacy of reagents in reducing organic matter content

206 The second phase of testing aimed to establish the proportion of organic material that is removed by
207 each of the selected protocols. For this experiment, test soil and sludge samples were collected from the
208 Oslo area. Details of sample characteristics including sampling, soil texture analysis and sludge
209 treatment are provided in the Supporting Information. Moisture content was established through the
210 percentage loss following drying at 105°C. The organic matter content of the samples was assessed
211 through loss-on-ignition (LOI): the samples were placed into a muffle furnace and heated to 550°C for
212 4 hours. The results are provided in Table S3.

213 10 g of soil and sludge were weighed into clean, pre-washed glass jars. Three replicates were performed
214 for both sample types, for each protocol (n = 36). The samples were first dried at 105°C to establish the
215 dry weight. For Protocols 1 and 2, 30 ml of $H_2O_2$ was added initially, followed by further additions in
216 5 ml increments until no further reaction (e.g. fixing, frothing) was observed. In the case of Protocol 2,
217 the reagent was added as a ratio 1:1 $H_2O_2$ and catalyst solution. The catalyst solution was added first
218 and $H_2O_2$ was then added slowly. Further additions of the reagents were added until no reaction was
219 observed. The samples were processed at room temperature, but an ice bath was used to modulate the
220 temperature when it exceeded 40°C (Protocol 2 only). For Protocols 3 and 4, 50 ml of reagent was
221 added, with no further additions during the reaction period.

222 Following organic matter removal, the overlying liquid was decanted and vacuum-filtered onto pre-
223 weighed Whatman GF-D filter papers. The filter paper was dried and the retentate mass was established
224 gravimetrically. The total mass loss ($\Delta m$) was assumed to directly reflect the loss of organic material
225 and this was used to estimate organic matter removal (%).

Validation: Extraction efficiency of selected protocol

The final phase of testing included establishing the extraction efficiency of the optimum protocol, which was identified following Phase 1 and 2 testing. This aimed to assess whether the additional processing step affected the recovery of particles during the full microplastic extraction procedure.

The test sludge and soil used in this study represent environmental samples. Three control samples of sludge and soil were first tested for existing microplastic concentrations using the selected protocol. Microplastic abundance in both samples was low and no particles with similar physical characteristics (size, colour) were observed. The results and description of measurements are provided in the Supporting Information.

Different microplastic shapes were used to test the influence of particle shape on extraction efficiency. Thirty large PE microbeads (850-1000 µm), 30 small PE microbeads (425-500 µm), and 30 PET fibres (322-395 µm) were added to each replicate. Details on the particles are provided in Table S4 and images are shown in Figure S3. Orange fibres (Certified reference material CRM-FOPET-1-18, NIVA, Norway) were used to spike the solid samples. No orange clothing or textiles were permitted near the samples during testing to prevent artificially enriching samples through airborne contamination. No orange fibres were observed in ongoing laboratory contamination tests. All sample processing was performed in a sterile cabinet and samples were kept covered to prevent laboratory contamination. Only fibres within the predefined size range were considered, although no smaller or larger orange fibres were identified.

For each replicate, 10 g (d.w.) of sample (sludge/soil) was added to clean, pre-washed glass jars. The samples were then spiked with the microplastic particles. The particles were thoroughly mixed into the solid matrix. Samples were then partially wetted using a fine spray of filtered RO water and allowed to air dry. This was repeated three times to encourage the incorporation of microplastic particles into aggregates. This aimed to mimic environmental samples and establish environmentally-relevant extraction efficiencies.

Organic matter removal followed the same method as outlined in Phase 1 & 2. Only the optimal protocol underwent validation. Density separation was achieved using a) filtered RO water, to extract microplastics at freshwater density (1 gm $cm^{-3}$); and b) NaI solution (1.8 g $cm^{-3}$), to extract higher density microplastics. Sequential density extractions have been applied elsewhere to infer the potential environmental behaviour of particles[5]. Containers were filled to the top with each density solution, sealed, and agitated for 1 minute. The supernatant was decanted after the sample had been allowed to settle for 24 hours, and vacuum filtered through Whatman GF-D filter papers. Once air-dried, the filter papers were traversed at 20x magnification to count the extracted microplastics.

Several analytical parameters associated with density separation were tested. Firstly, the importance of the ordering of the analytical procedure was investigated. Extraction efficiencies were established for a) organic matter removal followed by density separation (OMR → Density); and b) density separation followed by organic matter removal (Density → OMR). Three replicates were tested for both approaches. For the 'Density → OMR' samples, the filter papers were placed into a jar after density separation and subjected to organic matter removal. The samples were then filtered again, and the original filter paper was carefully rinsed to ensure all particles were passed through the second filter.

Secondly, the optimum number of density extracts was examined. Three density extracts were performed for each density solution and the number of particles isolated in each was recorded. Finally, the labware used for density separation was tested. Three replicates were tested in 250 ml glass jars that were used in the previous phases and three additional replicates were tested using 50 ml tubes. For the latter, the 'OMR → Density' samples were transferred to the tubes prior to density separation (organic matter removal was always performed in glass labware).

## Results and discussion

### Phase 1: Effect of reagents on polymeric particles

#### Physical changes

Different protocols to remove organic material had different effects on the physical integrity of the polymers. In one replicate of Protocol 1b (30% $H_2O_2$ 70°C), all three PA-66 particles were destroyed. Small residual fragments were observed during filtering (Figure S4). This outlier had considerable influence on the average and variance of mass and size changes observed for this treatment (Table 1). The particles in the other two replicates for that treatment showed no signs of degradation. The reason for such a different outcome is unexplained. PA-66 is not resistant to hydrogen peroxide at concentrations ≥30%[50], which causes oxidative damage and degradation of the polymer structure. However, the exposure time of the three peroxide-based treatments (Protocols 1a, 1b, and 2) appears to be below the time required to have an observed effect on particle mass, size, or visual appearance. However, the temperature setting (70°C) used in Protocol 1b may just exceed the threshold tolerance of PA-66 particles.

In all three replicates performed for Protocol 3b (10 M NaOH), PET and PC particles were severely degraded. Surface degradation was observed for both polymer types (Figure 1bc). These visual changes were also recorded as significant decreases in particle mass and size (Table 1). This effect was observed to a lesser extent for Protocol 3a (1 M NaOH), with signs of 'peeling' (PET) and the development of a matte texture (PC) (Figure S5). However, no significant change in mass or size was measured. Notably, a decrease in weight of 16.1% was observed for PC following treatment with 10% KOH (Protocol 4), despite no associated visual or size-related changes. Polycarbonate is significantly affected by hydrolytic degradation, and alkali salt solutions such as NaOH (Protocol 3a,b) and KOH (Protocol 4) accelerate this process[51]. Alkaline solutions also degrade PET by saponification of ester linkages at the particle surface[52], although this was only observed for NaOH-based treatments in this study.

For PP treated with Protocol 1b ($H_2O_2$ 70°C), one particle in a single replicate was significantly reduced in size and coated with an opaque white layer (Figure 1a). This degradation may have been catalysed

by the destruction of PA-66, which occurred in the same single replicate. All other PP particles were unaffected by the Protocol 1b treatment.

Some limited surface degradation, noted as 'crazing', was observed for PS particles following treatment with hydrogen peroxide (Protocols 1a and 1b) (Figure 1de). Protocol 2 (Fenton's reagent) also uses hydrogen peroxide but no degradation was observed (Figure 1f). This may be linked to the influence of temperature, where more degradation was observed following Protocol 1b (70°C) than Protocol 1a (60°C). Oxidation of polystyrene occurs in air when temperatures are elevated[53]. Protocol 2 was performed at temperatures <40°C.

Interestingly, an increase in the weight of PS following treatment with 10% KOH (Protocol 4) was measured. This does not correspond to any size or visual changes. This effect was not observed during other methods testing studies[42], but could influence the density of the particle and effect subsequent microplastic extractions based upon density. The authors were not able to identify the cause of this change during testing.

Spectral changes

The majority of the post-treatment FT-IR results exhibited no major deviations from the control samples (Figure S6). The only significant alteration is observed for PC following treatment with Protocol 3b. The alkaline hydrolysis appears to have initiated depolymerisation, demonstrated by the introduction of breakdown products to the spectrum. The same spectrum is produced when analysing the degraded outer layer as well as the newly-exposed surface of the particle (Figure S6g and S7c). The degradation of *PET* caused by the Protocol 3b did not alter the FT-IR spectra of the particle. However, the loose fragments taken from the surface of the degraded particles had altered FT-IR spectra (Figure S7). Some reduction in intensity is observed for PA-66 following a range of treatments; however, this is likely associated with variations in the polymer structure of the virgin particles.

Library searches were performed for each analysed particle. With the exception of PC following Protocol 3b, all particles were successfully matched to the correct reference spectra with satisfactory

329 hit quality index (HQI) scores ≥ 0.88 (on a 0-1 scale). The loose fragments taken from the degraded
330 particles all recorded deviations from the control spectra. The spectra from the degraded PC and PET
331 fragments could not be reliably matched to any compound in the library, with HQIs <0.30. However,
332 fragments from the single PP particle that was affected by Protocol 1b, which developed a white outer
333 layer, matched with polyamide (HQI = 0.90). In the same replicate, PA-66 was destroyed. The
334 solubilised fragments apparently adhered to the outside of the degraded PP particle, which would have
335 led to the incorrect characterisation of the particle if the degraded layer had not been removed.

336

337 Phase 2: Efficacy of reagents in reducing organic matter content

338 Table 2 shows the total average mass loss (Δm) and organic matter removal of soil and sludge samples
339 following treatment with the selected protocols. For both sludge and soil, peroxide-based treatments
340 removed significantly more of the organic material than the alkali salt solutions (Table 2). Peroxide
341 oxidation is already used to reduce the organic content of solid environmental samples prior to other
342 analyses. For example, 30% hydrogen peroxide is commonly used to pre-treat samples before
343 measuring particle size distribution[54,55]. However, the completeness of peroxide digestion of organic
344 material varies based on the composition of the organic content[56]. In this study, peroxide-based
345 treatments (Protocols 1a, 1b, & 2) removed approximately 80-87% of the organic content of the sludge
346 samples and 96-108% of soil organic material (Table 2). The higher temperature used in Protocol 1b
347 appears to have improved the removal efficiency of the treatment. Fenton's reagent achieved
348 comparable removal rates to the 70°C hydrogen peroxide treatment. This removal may have been
349 enhanced by the low pH of the reagent, which introduces optimal conditions for the treatment of
350 organic-rich samples such as soil[57].

351 Treatment with alkaline salt solutions (Protocols 3a, 3b, & 4) removed between 57-67% of organic
352 material in sludge and 35-68% of soil organic matter. Alkaline hydrolysis is effective at destroying
353 proteins[31], which is why it is commonly utilised for the extraction of microplastics from biota. In
354 contrast, cellulosic and chitinous material is resistant to KOH and NaOH treatment[58], and may be

present in both sludge and soil. Additionally, alkali-insoluble humins are often the most abundant organic fraction found in soils[59]. This explains the lower removal efficiencies of NaOH and KOH. The higher percentage of organic matter removal by 10% KOH in sludge than in soil may reflect the composition of organic material within the test samples.

Critical selection of optimal clean-up method

Based on the results of Phase 1 testing, Protocols 1a, 2, and 4 could be considered to preserve microplastics satisfactorily, causing minimal to no damage. Only the use of Fenton's reagent (Protocol 2) did not cause any observed changes to the eight tested polymer types. Phase 2 testing showed that the use of alkaline salt solutions is not appropriate for the removal of organic material in complex, organic-rich, environmental matrices. In contrast, Protocols 1b and 2 were the most effective at reducing organic material. However, Protocol 1b caused degradation of several polymer types during Phase 1 testing. Based on these outcomes, Fenton's reagent was identified as the optimum protocol for preserving microplastic particles whilst also effectively reducing the organic components of soils and sludges.

This study highlights the unsuitability of NaOH as a reagent for removing organic matter in microplastics studies. Based on the degradation of multiple polymer types, it is recommended that NaOH is no longer used for microplastic analysis. Dehaut et al.[42] reported similar effects on PET and PC following treatment with 10 M NaOH, however, this study demonstrates that lower concentrations of this reagent (1 M NaOH; Protocol 3a) still exhibit surface degradation in these polymer types. Thus far, NaOH has only been used in a single study of microplastic contamination in sludge samples by Mintenig et al.[19]. However, in this case, the authors highlight that the method was as yet untested and microplastic results were subsequently presented as estimates.

Validation: Extraction efficiency of selected protocol

The validation phase focused on assessing the recovery of microplastics following treatment with the selected optimal protocol: Fenton's reagent. Figure 2 shows extraction efficiencies for the spiked microplastic particles. The ordering of the analytical procedure (organic matter removal followed by density separation, and vice versa) had no significant effect on the recovery of the different microplastic particles. Hence, the organic matter removal step can be added within existing protocols for microplastic isolation through density separation based on preference or convenience. The overall extraction efficiencies were very high. Large PE beads had close to 100% recovery for both the sludge and soil test. Small PE beads were also mostly recovered, with extraction efficiencies between 92-98%. The spiked PET fibres presented the lowest recovery (79-86%) but this was still considered to be satisfactory. These results are higher than or comparable to the extraction efficiencies observed following density separation alone by Claessens et al.[26]. Hence, the inclusion of an organic matter removal step using Fenton's reagent does not negatively affect the recovery of microplastic particles from complex, organic-rich, environmental matrices. Only low density microplastics (small and large PE beads) were observed in the freshwater density extracts, whilst only PET fibres were extracted during the subsequent NaI steps. Crucially, no evidence of degradation was observed for the spiked microplastic particles following treatment, confirming observations during phase 1 testing.

There is no difference between the recovery of small or large PE beads using either 250 ml glass jars or 50 ml tubes (Table S5). However, the extraction of fibres is slightly increased by using the tubes. Extraction efficiencies when using jars were 76-78%, compared to 79-86% for the tubes. The lower recovery of irregularly-shaped particles, such as fibres, during density separation is often speculated as the effect of particles adhering to the walls of the apparatus[32,60]. These results indicate that this is likely to be a contributing factor, whereby the container with the smallest internal surface area led to higher recovery of fibres. Furthermore, there was no significant difference between the ordering of the analytical procedure for either container. Hence, methods which have been shown to have high

406 extraction efficiencies for a range of particles types[26,27,61], may also be used for soil and sludge samples
407 in conjunction with an organic matter removal step.

408 During the density separation procedure, three extracts were processed for both density solutions (low,
409 freshwater density: 1 g $cm^{-3}$ & high density 1.8 g $cm^{-3}$). The recovery data for the different particle types
410 associated with each extract are provided in Table S6. For low density microplastics (PE beads), the
411 majority were recovered in the first extraction. The extraction efficiencies for large PE beads was close
412 to 100%, whilst the mean recovery of small PE beads after one extraction was 87.2%. For higher density
413 PET fibres, only 50.8% of particles, on average, where extracted in the first step. A further 28.6% were
414 recovered in the second extract. This may relate to the adhesion of particles to the inside of the tubes
415 during decanting, which are then successfully recovered in a second extract. Alternatively, the settling
416 of the solid matrix may trap higher density particles with complex shapes and prevent them from
417 floating to the surface of the density solution. Very few particles of any type were recovered in the third
418 extract (<4.4%). Based upon this testing, it is recommended that two extracts are taken for each density
419 solution used to ensure optimal recovery of microplastic particles, particularly for higher density
420 extractions (e.g. NaI or $ZnCl_2$). Performing a third extract may slightly increase recovery of plastics
421 from environmental samples; however, the use of two extractions for each density solution represents
422 a more time-effective approach that is capable of recovering the majority of plastic particles.

423

424 Method optimisation

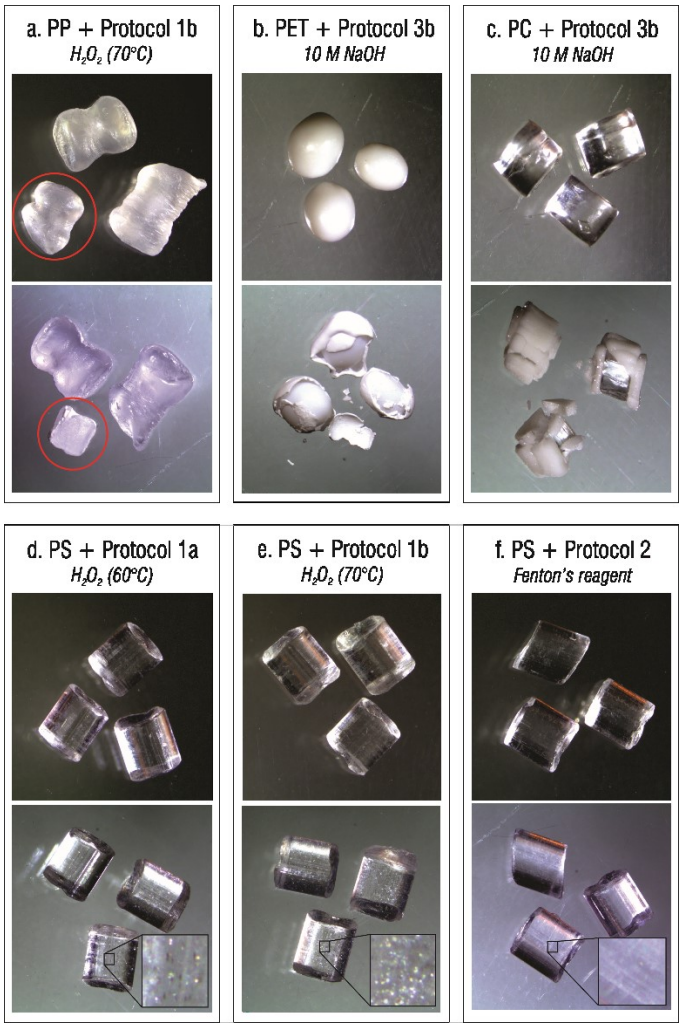
425 Organic matter removal using Fenton's reagent is an exothermic reaction. Reaction temperatures in the
426 context of organic matrices can reach as high as 89°C[62]. This may negate the benefit of using Fenton's
427 reagent, where degradation of polymers was observed for peroxide-based treatment performed at 70°C
428 in Phase 1 testing (Protocol 1b). However, an ice bath can be used to lower the reaction temperature.
429 This can also limit the occurrence of violent reactions improving safety conditions in the laboratory. It
430 is recommended to keep the temperature below 40°C to decrease the decomposition of hydrogen
431 peroxide[40]. This will also better preserve microplastic particles. During testing, reactions using Fenton's

reagent were completed in less than 2 hours for both sludge and soil samples when using an ice bath intermittently to adjust reaction temperatures[63].

As stated previously, the optimal pH for Fenton's reagent is close to 3.0. However, it is important to monitor the pH of the reaction, as if it exceeds pH 5-6, an iron hydroxide precipitate will form. This precipitate floats out during density separation and hinders visual analysis and chemical characterisation through physical obscuration. Although, during the testing of sludge and soil samples, this effect was not observed.

Fenton's reagent represents an effective, low-cost, and rapid treatment for removing organic material from complex, organic-rich environmental matrices. Coupled with density separation, the majority of microplastics are recovered, where the organic matter removal step does not significantly affect extraction efficiencies compared to other solid matrices.

444 FIGURES:




445

446 **Figure 1.** Micrograph (10x) images of selected plastic particles before (top) and after (bottom)
447 treatment. Small pitting in the surface of PS granule was observed for Protocols 1a (d) and 1b (e), but
448 not following treatment with Protocol 2, which also utilises hydrogen peroxide as an oxidising agent
449 (shown in the magnified images).

**Table 1.** Changes in mass (a) and size (b) of the tested plastic particles following treatment. Results are presented as the mean ± SD of the three replicates per treatment (3 particles per replicate). Significant changes, defined as a change greater than analytical error (± 10%), are highlighted in bold.

**a. Mass**

| | **Protocol 1a** $H_2O_2$ (60°C) | **Protocol 1b** $H_2O_2$ (70°C) | **Protocol 2** Fenton's reagent | **Protocol 3a** 1 M NaOH | **Protocol 3b** 10 M NaOH | **Protocol 4** 10% KOH | **Control** |
|---|---|---|---|---|---|---|---|
| **PP** | -0.11% ± 0.16% | -5.96% ± 8.52% | 0.14% ± 0.11% | -0.16% ± 0.14% | 0.18% ± 0.26% | -1.30% ± 1.31% | 0.27% ± 0.10% |
| **LDPE** | -0.05% ± 0.28% | 0.00% ± 0.00% | -0.05% ± 0.20% | 0.00% ± 0.14% | 0.01% ± 0.14% | -2.39% ± 2.78% | 0.17% ± 0.01% |
| **HDPE** | 0.07% ± 0.05% | -0.01% ± 0.17% | 0.07% ± 0.05% | 0.03% ± 0.05% | -0.10% ± 0.01% | 0.07% ± 0.05% | 0.07% ± 0.05% |
| **PS** | 0.06% ± 0.09% | -0.01% ± 0.24% | 0.00% ± 0.14% | -1.81% ± 2.44% | 0.16% ± 0.13% | **12.1% ± 2.08%** | -0.89% ± 1.13% |
| **PET** | 0.25% ± 0.24% | 0.59% 1.09% | 0.19% ± 0.16% | -6.98% ± 7.52% | **-29.2% ± 1.52%** | -0.86% ± 0.05% | 0.19% ± 0.16% |
| **PA66** | 7.42% ± 0.74% | **-26.7% ± 51.8%** | 5.49% ± 0.55% | 1.55% ± 1.14% | 2.54% ± 1.31% | 4.00% ± 0.21% | 4.45% ± 1.98% |
| **PC** | 0.15% ± 0.21% | 0.39% ± 0.25% | -1.58% ± 2.65% | -8.24% ± 11.0% | **-59.9% ± 3.97%** | **-16.1% ± 3.67%** | 0.00% ± 0.12% |
| **PMMA** | 1.35% ± 0.33% | 3.28% ± 2.73% | 1.15% ± 0.10% | 0.57% ± 0.42% | 0.54% ± 0.10% | 0.03% ± 0.76% | 0.57% ± 0.08% |

**b. Size**

| | **Protocol 1a** $H_2O_2$ (60°C) | **Protocol 1b** $H_2O_2$ (70°C) | **Protocol 2** Fenton's reagent | **Protocol 3a** 1 M NaOH | **Protocol 3b** 10 M NaOH | **Protocol 4** 10% KOH | **Control** |
|---|---|---|---|---|---|---|---|
| **PP** | -2.35% ± 1.88% | -4.99% ± 9.12% | 1.66% ± 4.27% | -3.57% ± 2.52% | -1.52% ± 4.32% | -3.61% ± 4.15% | -0.47% ± 3.88% |
| **LDPE** | 1.64% ± 4.13% | -0.61% ± 3.64% | 0.50% ± 3.20% | -3.38% ± 1.20% | -1.02% ± 3.53% | -2.26% ± 3.59% | -0.24% ± 4.61% |
| **HDPE** | -0.79% ± 2.38% | -1.13% ± 2.27% | 1.26% ± 2.23% | -2.57% ± 0.23% | -0.95% ± 3.06% | -3.53% ± 2.82% | 1.58% ± 1.46% |
| **PS** | -2.41% ± 4.22% | 3.34% ± 5.77% | -0.27% ± 3.23% | -2.40% ± 0.26% | -0.95% ± 2.60% | -4.42% ± 4.37% | -0.80% ± 4.76% |
| **PET** | -0.68% ± 5.32% | 0.18% ± 4.13% | 1.79% ± 2.38% | -0.88% ± 1.52% | **-10.4% ± 6.37%** | -3.13% ± 5.53% | -0.31% ± 3.09% |
| **PA66** | -0.78% ± 3.35% | **-33.4% ± 47.2%** | 2.10% ± 3.98% | -0.30% ± 4.11% | 0.20% ± 4.26% | 2.36% ± 4.23% | 0.89% ± 4.59% |
| **PC** | 0.10% ± 0.06% | -1.33% ± 4.64% | 2.93% ± 6.33% | -3.14% ± 1.64% | **-27.8% ± 7.13%** | -4.70% ± 5.36% | 0.17% ± 3.95% |
| **PMMA** | -0.82% ± 3.60% | -1.08% ± 3.90% | 1.54% ± 2.46% | -2.21% ± 0.03% | -3.28% ± 4.43% | -3.87% ± 2.80% | -3.60% ± 4.74% |

**Table 2.** Total mass loss following treatment (Phase 2 testing) and the corresponding proportion of organic material removed for each of the tested protocols for sludge (a) and soil (b). Results are presented as the mean of the three replicates ± SD.

**a. Sludge**

| | Mass loss | Organic matter removal |
|---|---|---|
| **Protocol 1a** $H_2O_2$ (60°C) | 41.3% ± 2.16% | 80.2% ± 4.20% |
| **Protocol 1b** $H_2O_2$ (70°C) | 44.6% ± 6.76% | 86.6% ± 13.1% |
| **Protocol 2** Fenton's | 43.8% ± 6.61% | 86.9% ± 9.87% |
| **Protocol 3a** 1 M NaOH | 31.4% ± 2.88% | 60.9% ± 5.60% |
| **Protocol 3b** 10 M NaOH | 34.6 ± 3.01% | 67.2% ± 5.84% |
| **Protocol 4** 10% KOH | 29.2 ± 8.56% | 56.8% ± 16.6% |

**b. Soil**

| | Mass loss | Organic matter removal |
|---|---|---|
| **Protocol 1a** $H_2O_2$ (60°C) | 6.54% ± 1.01% | 96.3% ± 14.9% |
| **Protocol 1b** $H_2O_2$ (70°C) | 7.36% ± 0.74% | 108% ± 10.9% |
| **Protocol 2** Fenton's | 6.81% ± 1.56% | 106% ± 13.8% |
| **Protocol 3a** 1 M NaOH | 4.59% ± 1.39% | 67.6% ± 20.5% |
| **Protocol 3b** 10 M NaOH | 4.38% ± 2.90% | 64.4% ± 42.7% |
| **Protocol 4** 10% KOH | 2.34% ± 1.53% | 34.5% ± 22.5% |

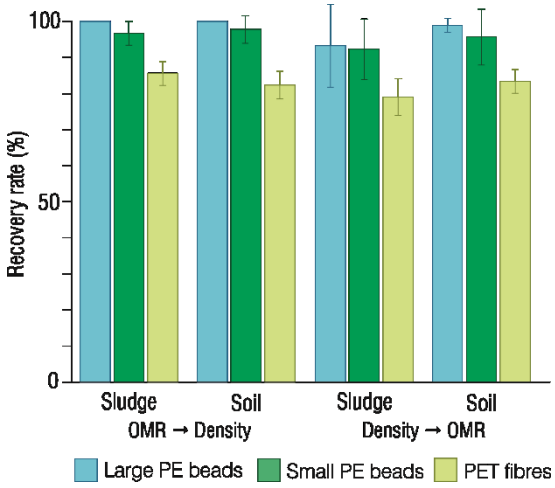



**Figure 2**. Extraction efficiencies for three microplastic types following treatment (Protocol 2: Fenton's reagent) and density separation. The extraction method was tested as 1) organic matter removal (OMR) followed by density separation, and 2) Density separation followed by organic matter removal. Results are reported as the mean of the three replicates ± SD.

## ASSOCIATED CONTENT

**Supporting Information**. Details of the experimental design; details of the polymeric material used in the study including the fibre production method; information regarding the test sludge and soil samples including the control assessment for microplastic content; FT-IR spectra of the analysed particles; results of the extraction efficiency studies.

## AUTHOR INFORMATION

### Corresponding Author

* rachel.hurley@niva.no; NIVA, Gaustadelléen 21, 0349 Oslo, Norway.

### Notes

The authors declare no competing financial interest.

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