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RESEARCH PAPER

Optimising sample preparation for FTIR-based microplastic analysis in wastewater and sludge samples: multiple digestions

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Abstract

The lack of standardised methodologies in microplastic research has been addressed in recent years as it hampers the comparison of results across studies. The quantification of microplastics in the environment is key to the assessment of the potential ecotoxicological impacts that this new category of emerging pollutants could have on terrestrial and aquatic species. Therefore, the need for protocols that are robust, simple and reliable together with their standardisation are of crucial importance. This study has focused on removal of organic matter with Fenton reagent from wastewater and sludge samples. This step of analysis was optimised by implementing a multi-digestion treatment on these samples that have high concentration of complex mixtures of organic matter, which interfere with microplastic enumeration. Moreover, this study targeted the detection of microplastics in the sub-hundred-micron size range due to the potential higher risks associated with smaller-sized particles and the limited data available from previous wastewater research. To show the validity of the method, triplicate samples of raw sewage, final effluent and sludge were independently spiked with two different sizes and types of microplastic polymers. Due to the various analytical stages required for the isolation of microplastics, time is a limiting factor in sample processing. The sequential digestion with Fenton reagent represents an inexpensive and time-efficient procedure for wastewater research providing effective degradation of organic material. These advantages over other currently available methods mean the method is suitable for analysis of large numbers of samples allowing robust monitoring data sets to be generated.

Keywords Wet peroxide oxidation · Organic matter · Microplastics · Wastewater · Recovery · Extraction

Introduction

Various studies have identified that microplastic (MP) research suffers from the lack of standardised methodologies, from sampling to the characterisation of MP particles in environmental samples [1–7]. The analytical procedures currently used to isolate microplastics (MPs) are dependent on the type of matrix being studied. However, methodologies for extraction and quantification of MPs within the same environmental compartment can vary significantly between studies, hampering the comparison of spatial and temporal patterns of MP abundance. Both sampling strategies and equipment can affect the quality and quantity of MPs reported [2, 8–13]. A wealth of studies on MPs has focused on marine habitats and highlighted the need for future research to investigate further their environmental implications and potential impacts on aquatic organisms. In comparison, the body of knowledge in terrestrial and freshwater ecosystems is limited, although several studies addressed this issue in recent years [6, 14–17]. Among several other land-based sources of MP pollution, wastewater treatment plants (WWTPs) have received attention as they have been identified as terrestrial pathways of MP emissions into aquatic ecosystems [4, 5, 18–27]. Therefore, assessment of the presence and quantification of MPs in effluent discharges and sewage sludge applied to land is a high priority, to enable the risks to be assessed.

It is important that MP detection methodologies are developed that are simple, accurate and efficient, in terms of both time and cost, so that these dynamic and highly variable

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wastewater system flows can be effectively monitored [28–30]. With regard to the size definition of MPs, no official consensus has yet been reached. However, 1 mm or 5 mm are the most commonly used upper size limits [31–33]. The lower size bound has less consensus, and the definition of nanoplastics has to be taken into account. It has recently been suggested that a cut-off of 1 μm is considered [31, 34, 35]. Some studies have shown that around 60% of MPs found in the final effluent of WWTPs were smaller than 100 μm [4, 21, 23, 25, 36–38]. However, the sub-hundred-micron size range has not been frequently fully investigated because of the large mesh size often used for sample collection as well as technical challenges associated with examining smaller size fractions [6, 12, 29, 39]. The large surface area-to-volume ratio makes the assessment and quantification of smaller-size-range MPs important in understanding their potential eco-toxicological impacts [30, 40–44]. Difficulties in reaching consensus towards a methodological standardisation are hindered by wastewater and sludge being complex and organic-rich matrices. Degradation of organic matter is a major limiting step in the extraction process in terms of the time and costs associated with removal.

The following section reviews the methods currently applied to decompose the organic component for the detection of MPs in wastewater. It then discusses optimised digestion treatment, followed by density separation with zinc chloride ($ZnCl_2$) and analysis of the samples through Fourier transform infrared (FT-IR) spectroscopy. The performance of the chemical characterisation of MPs is imperative to correctly identify and quantify these pollutants in the environment [45–47]. Several techniques can be applied to identify polymer types, with the most widely used being spectroscopy methods such as FT-IR and Raman spectroscopy [47–49]. These techniques are non-destructive and allow for a relatively fast identification of MPs in environmental samples, when used in combination with automated chemical imaging processing to obtain information on abundance, size and polymer type. Moreover, when Raman and FT-IR spectroscopy are coupled with microscopy, particles down to a size of 1 μm and 10–20 μm can be detected, respectively [48–51]. These two analytical tools present their own advantages and disadvantages related to size resolution, measurement time and spectra acquisition modes. They therefore are considered complementary techniques, and the choice mainly resides on the scope of the research [48, 52–54]. In this study, micro FT-IR (μFT-IR) was chosen for MP characterisation as the targeted size range was between 38 and 100 μm.

Current digestion methods in wastewater research

The quality of an analytical methodology resides in the reliability of each step: from sampling, to organic and inorganic matter removal, and quantitative and qualitative MP identification techniques. Establishing the optimal digestion method for an application requires four factors to be considered: (1) the removal efficiency of the technique and digestion time; (2) the potential damage to MP particles including spectral changes before and after the treatment (if spectroscopic analyses are performed); (3) cost; and finally, (4) the recovery rate in spiking experiments. The most common methodologies in wastewater research are hydrogen peroxide (H_2O_2) and Fenton reagent where iron (II) sulphate ($FeSO_4$) acts as a catalyst to oxidise the organic component in the presence of H_2O_2 [7, 29, 55–58]. This is also referred to as the wet peroxide oxidation (WPO) method that was developed by the National Oceanic and Atmospheric Administration Marine Debris Program [59]. Other widely used methods to extract MPs from biota, sediments and seawater include alkaline (e.g. sodium hydroxide, NaOH; potassium hydroxide, KOH), acidic (e.g. hydrochloric acid, HCl; nitric acid, HNO_3; perchloric acid, $HClO_4$) and enzymatic treatments (e.g. protease-K) [11, 60, 61]. However, it has been shown that acids and alkalis can degrade some plastic polymers such as polyester fibres (PES), nylon (PA), polyethylene (PE), polyvinyl chloride (PVC), polyethylene terephthalate (PET), poly methyl methacrylate (PMMA) and polyurethane (PU) [58, 62–65].

In an important study, Hurley et al. (2018) investigated the impact of four reagents (H_2O_2 at 60 °C and 70 °C, Fenton, NaOH and KOH) on organic-rich samples such as soil and sludge matrices. They confirmed that NaOH treatment was inappropriate due to damaging effects on multiple polymer types. KOH caused less damage to MPs (except for polycarbonate), but both KOH and NaOH were found to be unsuitable due to their low efficiency at degrading cellulose and chitin, which are common components of soil and sludge. As for 30% H_2O_2, visual changes of the MP polymers were observed at both test temperatures (60 °C and 70 °C), whereas no impact was found when Fenton reagent was used (<40 °C) [55]. Although higher temperatures would promote degradation of organic matter [66], recent work has argued that temperatures above 60 °C cause losses of some plastic polymers. For this reason, a maximum temperature of 50 °C represents a safe cut-off to avoid any losses [22, 64, 67, 68]. Organic degradation rates using the WPO method were also greater than the H_2O_2 treatment alone, even at 70 °C. This might be explained by Fenton reagent requiring a low pH (range 2–4) to maximise degradation of organic material, allowing a more successful degradation than H_2O_2 treatment alone [55, 68, 69]. This has also been confirmed by visual comparison of different filtered sludge samples after separate treatment with H_2O_2 and Fenton reagent [58].

Other studies have also shown that PE, polypropylene (PP), PET and PES, PVC, polystyrene (PS), PU and PA are resistant to WPO, with no change observed to MP size and spectra before and after treatment. Together these polymers account for 92% of global plastic demand [22, 55, 56, 58, 63,

64, 68, 70, 71]. It is important to perform MP recovery experiments to validate the methodology applied, although it is challenging to cover the wide range of polymers present in the environment. Studies using Fenton reagent that have spiked their samples with a known concentration of MPs have obtained relatively high extraction efficiency. Moreover, when WPO is performed in combination with density separation using $ZnCl_2$ solution, the combination of both techniques and the order in which they are conducted do not impact recoveries [5, 7, 55, 72–74].

Finally, enzymatic treatments have been proved to be efficient at purifying organic material from wastewater samples. However, these methods have the disadvantage of being costly and time-consuming (taking up to 2 weeks or longer) and are not feasible for the processing of large numbers of samples [7, 38, 39, 75, 76]. Interestingly, this was further investigated by Rodrigues et al. (2018) who compared Fenton reagent alone and in combination with enzymes and concluded that both are equally effective, but WPO represented the ideal compromise due to simplicity and time and cost-effectiveness of the procedure [56].

Multiple digestions with Fenton reagent

A variation of the WPO technique has been suggested and presented by Dyachenko et al. (2017) [72]. They performed sequential digestions with Fenton reagent and obtained cleaner samples from their experiments, but highlighted the need for validation of this technique with samples spiked with MPs. In wastewater research, spiking experiments have sometimes been conducted with aqueous samples to avoid background interference or by testing manufactured MPs >100 μm [55, 72, 77]. Among those studies where the WPO method was applied and recovery experiments were performed with wastewater and sludge samples, the smallest sizes investigated ranged from 63 to 90 μm (Table 1) [5, 39, 78].

Another recent study reported using a multiple digestion method, but no details were given about the procedure and recoveries obtained [5]. The aim of this study was to optimise the digestion technique with Fenton reagent, by carrying out multiple digestions based on the work of Dyachenko et al. (2017) [72]. To verify the method's reliability, three different types of environmental matrices (raw sewage, final effluent and sludge) underwent one or more WPO treatments. Furthermore, recovery experiments with MP sizes down to 38–50 μm were conducted in triplicate on a separate set of samples using the same environmental matrices, which were also subjected to one or multiple digestion cycles. The paper presents a detailed description of the optimised technique that it is simple, cost-effective and more time-efficient compared to current alternative methods.

Materials and methods

Microplastic extraction

Sample collection and storage

Wastewater and sludge samples were obtained from a municipal secondary wastewater treatment plant in the South of England, UK, that discharges into a nitrogen-sensitive watercourse. It serves a population equivalent of approximately 410,000 and treats almost 190,000 m^3 d^{-1} of wastewater under dry weather flow. After passing through a 6 mm screen, raw sewage undergoes primary sedimentation followed by secondary treatment in an activated sludge process configured for biological nitrogen removal, with aerobic and anaerobic zones to promote nitrification and subsequent denitrification. The activated sludge mixed liquor is then passed through a final sedimentation tank before the final effluent is discharged. The primary and secondary sludge are mixed before anaerobic

Table 1 Reported MP recovery experiments using Fenton reagent. Sample type and spiking materials are shown along with recoveries

Size and polymer type of manufactured MPs used	Type of sample used for recovery	Recovery (%)	Reference
200 μm PS beads	Blank aqueous sample	87	[72]
850–1000 μm PE beads	Sludge and soil	close to 100	[55]
425–500 μm PE beads	Sludge and soil	92–98	[55]
322–395 μm PET fibres	Sludge and soil	79–86	[55]
100 μm PS beads	Raw	77.7	[39]
80–150 μm high-density PE particles	Raw	57.6	[39]
90 μm PS beads	Not specified	89.34	[5]
1000 μm PS beads	Not specified	99.02	[5]
2–4 mm PVC, PP and low-density PE particles	Influent, waste activated sludge and effluent	100	[77]
< 63 μm low-density PE particles	Milli-Q water	93.6	[77]
63–90 μm PA particles	Sludge	52.4	[78]

digestion, pH amendment and disposal to land. Samples were collected on the same day in August 2019 at three stages of the treatment: raw sewage after 6 mm screens (500 ml), final effluent (2.5 L) and sewage sludge (10 g, wet weight: composed of 5 g of primary sludge and 5 g of secondary sludge).

Compared to other MP studies in wastewater research, smaller sample volumes were taken as it was intended to investigate MPs within the 38–100 μm size range [67, 79]. The sample volumes and weights were informed by preliminary tests (see Supplementary Information (ESM_1), Table S1), which had shown high concentrations of micro-particles. These volumes were appropriate to target the 38–100 μm MP size range and to avoid excessive numbers of MPs being retained on the filters, which would have hampered the identification and characterisation of the particles in the final step of the extraction process (see section "μFT-IR analysis"). On the day of collection, wastewater samples were transported to the laboratory in plastic storage containers (made of high-density PE) and sludge samples in glass bottles, where samples were filtered through 38 μm stainless steel sieves (Endecotts Ltd., London, UK). The material retained on the sieves was rinsed three times with ultrapure water (Milli-Q Direct 8 Water Purification System; Merck Millipore) and stored in the freezer at −18 °C until further analysis. The sludge sample was immediately weighed and stored in the freezer. All samples were stored in plastic jars previously cleaned and rinsed thoroughly three times with ultrapure water. Subsequently, the samples were defrosted at room temperature and poured into glass beakers, which were loosely covered with foil and transferred into an oven set at 50 °C to remove the excess water, until ca. 25–50 ml of sample was left in the beaker.

Organic matter removal

The first step of analysis was the removal of the organic matter with Fenton reagent, which was performed under a fume hood. As mentioned above, samples were not completely dried but kept damp as this facilitated the digestion reaction. Raw sewage, final effluent and sludge samples were all treated in the same way. All reagents were freshly prepared each time and filtered (except for H_2O_2) prior to analysis to reduce contamination; 100 ml of 0.05 M $FeSO_4$ solution (iron II sulphate heptahydrate, ACS reagent, >99%; Sigma-Aldrich) [75] was prepared and poured into the glass beaker, followed by the addition of 100 ml of 30% H_2O_2 (hydrogen peroxide 30% *w*/*v*, 100 volumes, Extra Pure SLR, Fisher Chemical; Fisher Scientific). $FeSO_4$ was pre-filtered using cellulose nitrate membrane filters (Sartorious™ cellulose nitrate membrane filters, 47 mm, and 0.45 μm pore size). Temperature, pH and H_2O_2/$FeSO_4$ ratio are important factors that play a key role in the catalytic oxidation. As this combination generates an exothermic reaction, temperature was kept below 50 °C using an ice bath to preserve the MP polymers. A 1 M sodium hydroxide solution (sodium hydroxide, Extra Pure, SLR, pellets, Fisher Chemical; Fisher Scientific), pre-filtered using a PTFE membrane filter (0.2 μm pore size), was used to maintain pH between 3 and 4, to prevent the reduction of soluble iron species reacting with H_2O_2 [39, 69]. To avoid overflow of the high volumes of H_2O_2 and $FeSO_4$, 600 ml glass beakers were used to treat raw and sludge sewage samples, while 400 ml glass beakers were used for final effluent samples. During the digestion, samples were loosely covered with foil. The total reaction time, measured by the presence of visible bubbles in the samples, ranged from half an hour up to 2 h, after which only small bubbles were present. The samples were then left to cool overnight covered with foil. In order to dissolve the excess of ferric precipitates present in the mixture, ca. 10 ml of sulphuric acid was slowly added with a glass pipette to each sample (ca. 250 ml). The solution was gently stirred with the same pipette for a few seconds prior to filtration through a 38 μm sieve to rinse off the reagents. The materials retained on the mesh of the sieve were transferred into a beaker after being rinsed three times with ultrapure water. Hexane treatment, as suggested by Dyachenko et al. (2017), was not used as it could have affected polystyrene and polycarbonate particles [72, 80]. The final effluent sample underwent one digestion cycle, sludge underwent two cycles and raw sample underwent three cycles, due to the visible organic matter present in solution after implementing the first digestion. In each cycle, 100 ml of $FeSO_4$ and 100 ml of H_2O_2 were added once again to the beakers, and the procedure was repeated.

Density separation and filtration

Density separation was performed using zinc chloride solution ($ZnCl_2$; 98 + %, extra pure, ACROS Organics™) with a density of 1.7 g cm^{-3} to remove inorganic debris and allow extraction of the heavier polymers [74]. $ZnCl_2$ solution was freshly prepared each time and filtered before use over 0.7 μm glass microfiber filters (Fisherbrand™ Microglass Fiber Filter Discs, 47 mm; Fisher Scientific). At the end of the last digestion cycle and after filtration through a 38 μm sieve, samples were rinsed three times with $ZnCl_2$ instead of ultrapure water, to prevent a change in density. Samples were first poured into small beakers and then into 100 ml glass separation funnels previously rinsed three times with $ZnCl_2$, kept closed with lids and left to settle for a minimum of 15 h, after which 2/3 of the solution was drained out through the valve [81]. The remaining solution was poured into a sieve stack with a 100 μm sieve on top and a 38 μm sieve below to discard the fraction larger than 100 μm. The separation funnels were rinsed three times with ultrapure water to ensure all particles were transferred from the funnel to the sieves. Finally, the samples collected on the 38 μm sieve were rinsed

with copious amounts of ultrapure water to wash the $ZnCl_2$ off and poured into small glass beakers. However, sometimes, residues of $ZnCl_2$ were still present in solution. To avoid any interference with the spectral acquisition, two drops of HCl acid were added with a glass pipette to the sample to dissolve the residual salts. Immediately after, the samples were vacuum-filtered using a 13 mm glass filter holder (Cole-Palmer Advantec 311100 All-Glass Microanalysis filter holder, 13 mm; item #WZ-06644-84) fitted with a 25 mm silver membrane filter (Sterlitech, 5 μm pore size) for the subsequent μFT-IR analysis. After filtration, the silver filters were dried overnight (>15 h) in an oven at 50 °C and then stored in small petri dishes in the dark.

μFT-IR analysis

Once microplastics had been extracted through digestion and density separation, the subsequent analysis steps were performed at the UK Centre for Ecology and Hydrology (UKCEH, Wallingford) and following the protocol reported by Horton et al. (2021) [78]. The spectroscopic analysis was carried out using a PerkinElmer Spotlight™ 400 μFT-IR imaging system, in reflectance mode using a liquid nitrogen-cooled linear array detector, covering the IR spectral range from 4000 to 700 cm^{-1}. The spectral resolution was 8 cm^{-1}, using four scans per pixel, and the pixel resolution was 25 μm, representing a good compromise between mapping time and signal-to-noise quality of the spectra. This study used 38 μm as lower size cut-off to give good detection at the 25 μm resolution. A background spectrum collection was also carried out on a clean area of the silver filter, and the settings used were the same as for the samples, except for the number of scans per pixel (90 scans/pixel). An optical image of an area of ca. 13 × 13 mm was first generated (Fig. 1a). Because of the size limit of the spectrum image file created by the μFT-IR imaging system, a filter surface area of 11.6 mm × 11.6 mm was mapped, which was equal to 92% of the whole filter area being scanned [82–84]. This was considered sufficient to reliably estimate MP concentrations and polymer types present in the samples [85]. The analysis of one sample on the μFT-IR took between 2.5 and 3 h. Once the spectra map images were acquired and the spectra of the particles generated, they were analysed through siMPle software to obtain information about MP numbers, polymer type and size [84]. An image with a map of the pixels representing the MPs that have been identified was also obtained (Fig. 1b), and the spectrum for each of the MPs was visualised and compared to a spectral reference database (Fig. 1c).

siMPle has been developed by Aalborg University (Denmark) in collaboration with Alfred Wegener Institute (Germany) by combining the software MPhunter [82] with the automated analysis of Primpke et al. 2017 [86]. siMPle is freely available for download together with a reference database (https://simple-plastics.eu/) [84]. In this study, the AAU pipeline has been used for image analysis, and it is based on a score threshold system [78, 81, 82, 84, 87]. This software is used to compare the infrared spectra collected from the μFT-IR against the spectra of the reference database for automated analysis. A score for each particle is obtained based on the quality of this match, ranging from 0.01 (misassignment) to 1 (certain assignment). This score is generated by an algorithm, through which the raw spectra, their first derivatives and their second derivatives are correlated by a Pearson correlation. This yields three Pearson's correlation coefficients, to which the user assigns global weights (k_0, k_1, k_2) [84]. The score is calculated by using the equation reported in Liu et al. (2019) [82]. In this study, the Pearson's correlation coefficient for the first probability threshold was set at 0.60, and the default settings of siMPle were used ($k_0 = 0$, $k_1 = 1$, $k_2 = 1$). The second and third probability thresholds, which help define the size of the particle, were left unmodified. At present, there are no guidelines in the literature as to the most appropriate thresholds [81]; therefore, we manually evaluated and compared the spectra across different polymers types by assigning different weights and score thresholds. A threshold setup of 0.60 using the first and the second derivatives of the raw spectra data yielded the best fit.

Method validation

Recovery experiments

In order to validate the multiple digestion steps, recovery experiments (positive controls) were carried out with two polymer types of manufactured MPs of different sizes. As the targeted MP size in this study is in the 38–100 μm range, two sizes of 38–50 μm and 100 μm were chosen for testing. PMMA particles of 38–50 μm in size (Merck Sigma-Aldrich, product no. 463183) and 100 μm PS beads (Merck Sigma-Aldrich, product no. 56969) were separately added to two sets of raw sewage (500 ml), final effluent (2.5 L) and sludge (5 g, wet weight) samples, processed in triplicate. The PS stock solution was prepared by dissolving 100 μl of PS in 100 ml of ultrapure water. For the PMMA stock solution, 0.01 g of PMMA was dissolved in ultrapure water and filtered through the 38 μm sieve, as the presence of PMMA particles smaller than 50 μm (down to 15 μm) was observed under the microscope in preliminary experiments. Particles were collected from the sieve and dissolved in 100 ml of ultrapure water. A 1:50 dilution was made to obtain the final PMMA working solution. The first set of raw, final effluent and sludge samples was spiked with 2 ml of PMMA final working solution, while the second set of samples was spiked with 1 ml of PS stock solution. The stock and final working solutions were stirred gently on a magnetic mixer prior to use. At the same time, separate controls for PMMA and PS particles were performed to limit the margin of variability, as a high variation in the numbers of MPs added to each triplicate

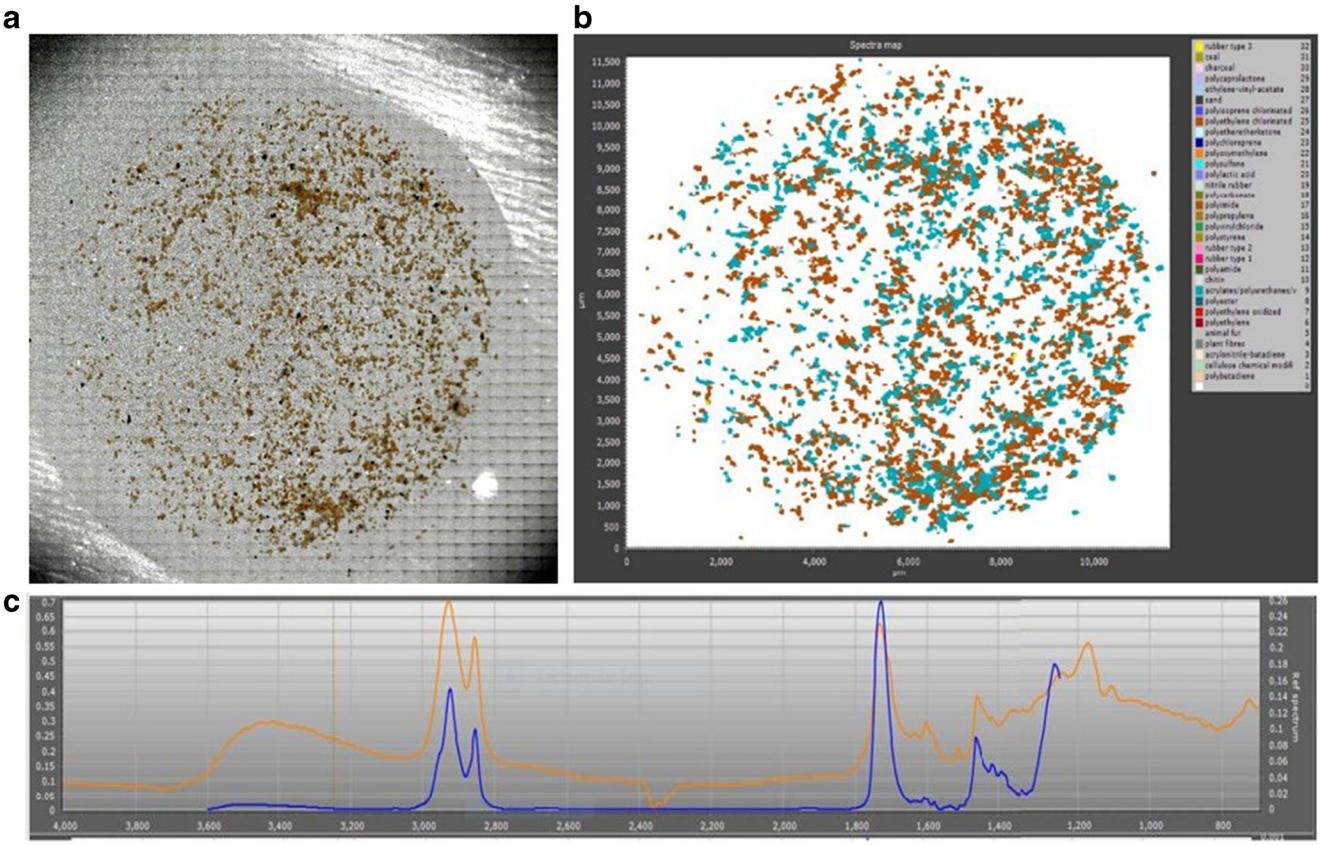

Fig. 1 **a** Optical image of the raw sample created by the μFT-IR imaging system; **b** spectra map of the MPs identified in the raw sample; **c** example of a particle spectrum identified as belonging to the acrylates-polyurethanes-varnish polymer group (orange line) and its reference spectrum (blue line)

sample had been observed in preliminary tests, due to agglomeration and fragmentation. Controls were carried out by vacuum-filtering 2 ml of final working solution and 1 ml of stock solution respectively over black polycarbonate filters (0.2 μm pore size, 25 mm, Whatman™, Nuclepore™) using a 25 mm Millipore glass filter holder to facilitate counting at the microscope.

Subsequently, raw, final effluent and sludge samples underwent the digestion and the density separation treatments described in sections "organic matter removal" and "density separation and filtration", followed by the recovery filtration step described above. The filtration through the 100 μm sieve was not performed after density separation to avoid the retention of the 100 μm beads on the sieve. Final effluent samples received one digestion cycle, sludge underwent two cycles and raw samples underwent three cycles, as per section"organic matter removal". Prior to filtration, 40 μl of sodium dodecyl sulphate (SDS; dodecyl sulphate sodium salt, 99%, Acros Organics™) solution (4 g/L) was added to the final extract solution (50 ml) to prevent agglomeration of virgin MP particles. Finally, for both controls and samples, the whole surface of each filter was screened, and particles were visually counted under the light microscope (Olympus BH2-RFCA; ×32 and ×100 magnification for the PS beads and PMMA particles, respectively) (Fig. 2).

Procedural blanks and contamination

Careful attention was paid to prevent airborne contamination in the field and the laboratory. Plastic containers and glass bottles for wastewater and sludge collection were covered with lids at all times on site, except for when collection was carried out. Field blanks were taken in triplicate in order to monitor the potential extent of contamination on site by using ultrapure water in glass bottles. In the lab, all surfaces were wiped down with a high-level disinfectant (Chemgene HLD_4L) prior to the experiments. All treatments were run under a fume hood, except for the initial filtration step through the sieves before sample storage. Lab coats made of 100% cotton were worn at all times. To mitigate airborne contamination, samples, glassware and equipment were always covered with aluminium foil both in and out of the fume hood, except for when the reagents were poured into the beakers. However, we acknowledge that although the use of materials made of plastic was minimised, it could not be completely avoided. All glassware and equipment (e.g. stainless steel sieves and filter rigs) were cleaned using a 2% Decon™ detergent solution (Decon 90; Fisher Scientific) before and after use to prevent cross-contamination among samples. Glassware and equipment were then rinsed thoroughly with reverse osmosis water and then rinsed three times with

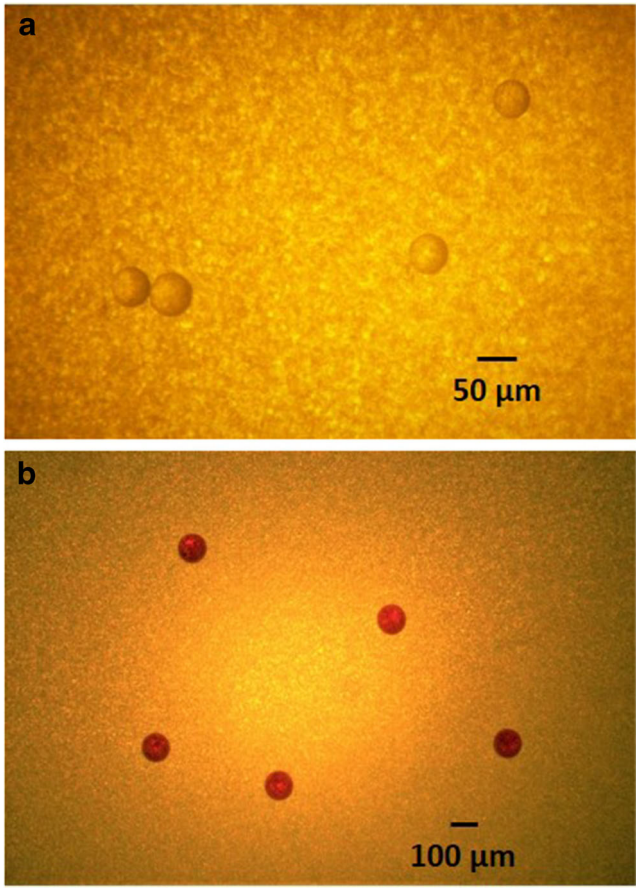

Fig. 2 Photos of manufactured MPs used in the recovery experiments taken under the light microscope (Olympus BH2-RFCA): **a** PMMA, ×100 magnification; **b** PS, ×32 magnification

ultrapure water prior to use. Procedural blanks (in triplicate) were taken by using ultrapure water and underwent two digestion cycles and the same treatment to which the samples were subjected, covering all the steps from sample preparation to filtration in order to assess contamination from plastic containers and air deposition. The blanks were vacuum-filtered using a 13 mm glass filter holder unit (Cole-Palmer Advantec 311100 All-Glass Microanalysis filter holder, 13 mm; item #WZ-06644-84) over 25 mm silver membrane filters (Sterlitech, 5 µm pore size). Both field and procedural blanks were analysed via µFT-IR, and the acquired infrared spectra were run through siMPle software using the same settings reported in section "µFT-IR analysis".

Results and discussion

Method validation

Recovery experiments were carried out to assess the efficiency of a multi-digestion-steps procedure with Fenton reagent. Raw, final effluent and sludge samples were separately spiked with PS beads (100 µm) and PMMA particles (38–50 µm), and experiments were performed in triplicates per sample type. A mean count of the MPs added in the controls was calculated within each set of triplicates. This value was then used to obtain the recovery (%) for each replicate based on the count of MPs recovered in the correspondent spiked sample. The mean recovery by sample type for PMMA and PS beads are shown in Fig. 3. In particular, with regard to PMMA particles, the mean recovery for raw, final effluent and sludge was 78.8 ± 23.2%, 60.9 ± 16.3% and 82.2 ± 9.9% (see ESM_1, Table S2), respectively. As for the PS beads, it was 106.1 ± 5.5% for raw wastewater, 84.3 ± 19.4% for final effluent and 67.1 ± 10.3% for sludge (see ESM_1, Table S3). As the spiked concentration was unknown, a control was carried out for each sample to estimate the number of manufactured MPs that were being added. In particular, the recovery for the PS beads in the raw samples was over 100%. This is possibly an artefact of the inherent variability of the spiking process when adding the manufactured MPs. Table S3 (see ESM_1) shows that the number of MPs recovered in the raw samples slightly exceeded the average number counted in the controls. The results of the spiking experiments show that multiple digestions do not cause loss of MPs. The majority of studies that have conducted recovery experiments have tested manufactured particles that were larger than the size range of the MPs being investigated. In this experiment, the MP sizes selected included the lower and upper limit cut-off of the

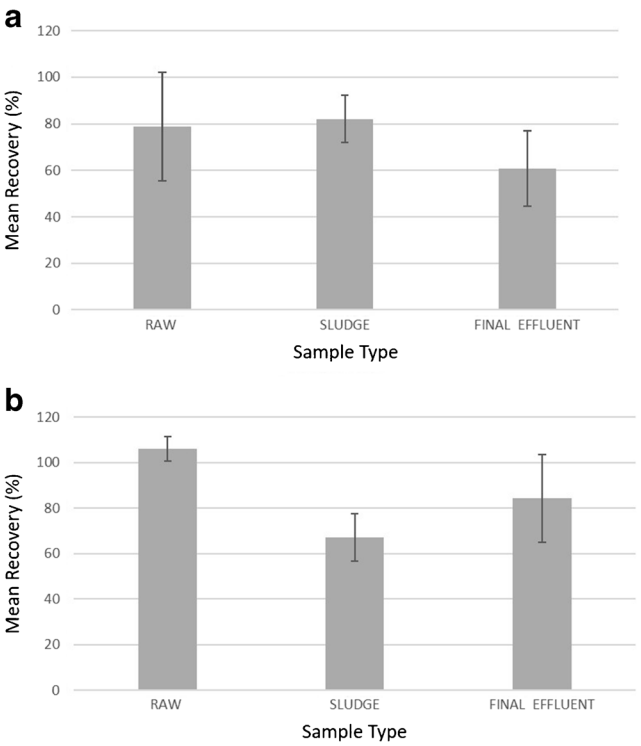

Fig. 3 Mean recovery by sample type for **a** PMMA particles and **b** PS beads

targeted size range (38–100 μm) and underwent all the steps of the analysis.

In order to reduce the inherent variability of preparing controls during the spiking process, a mean value of MPs counted in the controls was used. The recoveries obtained fall within the 60–100% range of recovery efficiency that has been reported in the literature (Table 1). In our study, smaller-sized manufactured particles (38–50 μm) were tested in real environment samples. It is important to note that when performing recovery experiments, the sizes of the spiked MPs should be chosen based on the targeted size range investigated, and that environmental samples should be used instead of clean aqueous solutions (Table 1). The recovery experiments have shown the validity of this method with efficient recovery of the added manufactured MPs. The size of the manufactured particles used in the spiking experiments could affect their recovery, as smaller particles are more likely to adhere to the vacuum filtration unit funnel due to a greater surface charge resulting from their higher surface-to-volume ratio. However, the surface characteristics of MPs present for a long time in the environment might differ from those of virgin plastic. Furthermore, the surface particle properties can also vary based on the polymer type [88]. Therefore, future work is required to explore the influence of polymer type and size on the recovery of manufactured MPs, as well as other factors such as other surfactant solutions and their interactions with both pristine and environmentally weathered MPs. Previous research has used SDS solution, and this has often been coupled with sonication. This may facilitate the recovery of particles [38, 39], but the brittle nature of MPs means that they could potentially break down in an ultrasonic bath. Therefore, the use of more invasive techniques has been avoided in this study [1, 10].

Analysis of MPs

Here we report the results on the MP concentrations of the non-spiked samples after analysis with siMPle software. Three types of environmental samples were collected and treated with single digestion (final effluent) or multiple digestions (raw and sewage sludge). As mentioned in section "μFT-IR analysis", 92% of the surface area of the filter was analysed via μFT-IR. The total concentration of MPs present in each sample was estimated by extrapolating the number of MPs found to the whole surface area of the filter. Estimates of MP counts ranged from 2102.16 MPs l^{-1} in raw wastewater, to 129.13 MPs l^{-1} in final effluent and 1979.74 MPs g^{-1} of dry weight in sewage sludge (Table 2). Procedural blanks were performed in triplicate, and the mean of MP concentration was 7.25 MPs l^{-1} (Table 2). Field blanks were also analysed to monitor the contamination, and the results show an average of 2.37 MPs l^{-1} (Table 2).

The raw data of both samples and procedural blanks obtained from siMPle software on polymer type, shortest and longest dimensions of the particles and their estimated mass and volume are reported in the Supplementary Information (ESM_2). MP concentrations have not been corrected for recovery as only two polymer classes (PS and PMMA) were tested [39]. It is acknowledged that these results do not reflect the behaviour of all the different classes of polymers. Sequential digestions with Fenton reagent could also be applied for batch processing and monitoring purposes. For instance, up to 10 samples (with similar volumes to those used in this study) could be processed by one person in 1 day. If three digestions are performed, it would take a total of 3 days to digest 10 samples. Compared to recent studies where enzyme purification has been performed, the digestion time alone ranged from 4 days up to 13 days depending on the amount and type of enzymes used [38, 39, 76]. In the method presented in this paper, the density separation and filtration of 10 samples over silver filters could be performed in 2 days and the μFT-IR analysis in 2–3 days. Therefore, the total duration of the analysis process for 10 samples would be 7–8 days. Other chemical analysis methodologies for polymer characterisation could be performed as an alternative to μFT-IR or to complement this technique, such as μRaman, pyrolysis-gas chromatography-mass spectrometry and thermos-extraction and desorption gas chromatography-mass spectrometry [89]. However, in the present study, the suitability of silver filters has not been tested with other techniques, as this was beyond the scope of this work. We acknowledge that the processing time for this last step of analysis could vary depending on what method is applied and the research question, subsequently affecting the whole duration of the analysis. For instance, μRaman, compared to μFT-IR, is more time-consuming (up to several days for one sample). This is mainly due to its lower detection thresholds (down to 1 μm), leading to a smaller area of the filter being scanned, and to Raman imaging systems and particle-finding algorithms that are in early stages of development [89]. With regard to thermal degradation methods, they have the disadvantage of not measuring particle size, but the advantage of providing information about chemicals and additives present on MPs. According to Primpke et al. 2020, these methods are less time-efficient

Table 2 Estimates of MP counts (per litre or grams of dry weight) obtained in the raw sewage, final effluent and sludge samples examined and average of MPs l^{-1} present in procedural and field blanks

Sample type	Estimate of MPs l^{-1} (or MPs g^{-1} of dry weight*)	Average of MPs l^{-1} in procedural blanks	Average of MPs l^{-1} in field blanks
Raw	2102.16	7.25	2.37
Final effluent	129.13		
Sludge	*1979.74		

compared to Raman and FT-IR [89]. It should be further highlighted that all these methods for the analytical characterisation of MPs are complementary as they differ in the benefits that they offer. Given the scope of this study, μFT-IR was chosen because it provides a reliable and fast polymer characterisation for the analysis of MPs >10 μm, and unlike thermal degradation methods, it is non-destructive.

Conclusions

This study has demonstrated the optimisation of the digestion treatment with Fenton reagent by performing multiple digestion cycles to target MPs in the sub-hundred-micron size range in wastewater and sludge samples. In order to validate the method, recovery experiments were conducted in triplicate testing two polymer types of different sizes. It has been shown that the WPO treatment performed in multiple cycles represents a valid alternative to current methods and a good compromise as a low-cost and time-efficient procedure, which also preserves the microplastic particles. The development of methodologies that are reliable, simple and relatively fast to perform is important for the accurate detection and quantification of MPs in the environment. Future research could investigate other polymer types and the recovery of smaller manufactured MPs.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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