Semi-automated analysis of microplastics in complex wastewater samples

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Abstract

In order to assess risks to the natural environment from microplastics, it is necessary to have reliable information on all potential inputs and discharges. This relies on stringent quality control measures to ensure accurate reporting. Here we focus on wastewater treatment works (WwTWs) and the complex sample matrices these provide. Composite samples of both influent and effluent were collected over a 24 h period on two separate occasions from eight different WwTWs across the UK. Sludge samples were taken on five occasions from five WwTWs. The WwTW treatments included activated sludge, trickling filter and biological aerated flooded filter with or without tertiary treatment. Using micro-FTIR analysis, microplastics ≥ 25 µm were identified and quantified. Procedural blanks were used to derive limits of detection (LOD) and limits of quantification (LOQ). Where values were above the LOQ, microplastics in the influent ranged from 955 to 17,214 microplastic particles /L and in the effluent from 2 - 54 microplastic particles /L, giving an average removal rate of 99.8%. Microplastics could be quantified in sludge at concentrations of 301 – 10,380 microplastics /g dry weight, this analytical method therefore revealing higher concentrations than reported in previous studies. The most common polymers present overall were polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET). We also report on critical considerations for blank corrections and quality control measures to ensure reliable microplastic analysis across different sample types.

Capsule

This study describes robust, time efficient methods, used here to analyse microplastics in complex matrices including wastewater influent, effluent and sludge.

1. Introduction

The sources and pathways of microplastics into and within the environment are diverse, including road runoff (e.g. paints and tyre particles), in situ degradation of litter (e.g. food packaging, plastic bags), industrial and construction activities (e.g. nurdles, microbeads, construction dust) and domestic activities (e.g. textile fibres) (Browne et al., 2011; Horton et al., 2017b). Proportional inputs from different sources to the environment have been suggested (Boucher and Friot, 2017), but are difficult to accurately apportion. In order to assess environmental exposures, potential risks and practicable interventions, reliable data on microplastic emissions are required. This requires accurate, repeatable and efficient methods for sample processing and analysis.

Given their role in processing the majority of down-the-drain waste in developed countries, wastewater treatment works (WwTWs) play an important role in the conveyance and potential dispersal of microplastics. Whilst there is a growing body of literature on the presence of microplastics in wastewater and the products of treatment, the results are difficult to compare (Blair et al., 2017; Prata, 2018; Ziajahromi et al., 2016). For example, there is enormous variability between reported influent concentrations, ranging from 3 to 18,285 microplastics /L (Blair et al., 2019; Simon et al., 2018). This large range may indicate the high variability between different WwTWs based on influent loads, treatment technologies or day-to-day WwTW management. However, this discrepancy may also partly be an artefact of differences in sample processing, analytical techniques and/or reporting between studies. Visual identification is commonly used, however can lead to bias, for example through subconsciously selecting particles based on size, specific colours or shapes (Lusher et al., 2020; Tagg et al., 2015). Imaging micro-spectroscopy such as micro-FTIR (μFTIR) and Raman are capable of picking up particles that cannot be seen by the naked eye and therefore where analysis has been

undertaken only by visual quantification, this is likely to lead to a significant underestimate of particle numbers (Frère et al., 2016; Primpke et al., 2017).

Further, the microplastic sizes analysed across different studies are highly variable, for example minimum sizes reported include 10 µm (Mintenig et al., 2017), 45 µm (Carr et al., 2016), 250 µm (Lares et al., 2018) or in some cases are not clearly specified (Bayo et al., 2016; Leslie et al., 2017). Previous research has shown a greater number of microplastic particles present in the small size ranges (Enders et al., 2015; Erni-Cassola et al., 2017). Therefore, excluding small particles from sampling or analysis can lead to a significant underestimate of the number of particles. The wide range in the reported sizes of microplastics analysed complicates our understanding of the topic and our ability to compare studies and assess exposures. While analytical chemistry techniques such as GC-MS and LC-MS have been tried and tested for microplastics, and can quantify particles of any size providing sufficient mass within the sample (Fischer and Scholz-Bottcher, 2017; Wang et al., 2017; Zhang et al., 2019), these are not currently widely used and therefore provide yet fewer opportunities for inter-study comparison.

A number of studies have reported decreasing microplastic concentrations as wastewater passes through WwTWs. For example, Murphy et al. (2016) found 15.7 microplastics /L in influent, 8.7 microplastics /L after grit and grease removal, 3.4 microplastics /L in primary effluent and 0.25 microplastics /L in final effluent. A similar trend was reported by Gies et al. (2018), with 31.1 microplastics /L in influent, 2.6 microplastics /L in primary effluent and 0.5 microplastics /L in final effluent. Dris et al. (2015) also saw a decrease in particle concentration throughout the treatment process, although with concentrations 1-2 orders of magnitude higher than in the other two studies (maximum 260 microplastics /L in influent to maximum 50 microplastics /L in final effluent). Overall, studies of microplastics in wastewater suggest a high removal rate (> 95%) when comparing counts in effluent to those in influent (Magnusson and Norén, 2014;

Murphy et al., 2016; Talvitie et al., 2017b). However, due to the high persistence of many plastics, where these particles are 'removed' from the wastewater flow, they will usually be transferred to the sludge (Bayo et al., 2016; Gies et al., 2018; Li et al., 2018). Reported concentrations of microplastics in sludge range from 2-240 microplastics /g dry weight (DW) (Liu et al., 2019b; Zubris and Richards, 2005). In many countries, sludge is applied to land as a soil conditioner and fertiliser. Thus, even if present in low concentrations in treated effluent released to rivers, these particles will still enter the environment, albeit via a different route.

The aim of this study was to use a combination of robust, unbiased and time-efficient methods to analyse microplastics in a range of complex matrices, providing a reliable representation of microplastic concentrations in the influent and effluent of eight different WwTWs, and in the sludge of five different WwTWs. Samples represented a range of common treatment types, including secondary and tertiary treatments, and diurnal variability was accounted for by collecting samples over a 24 hour period.

2. Materials and Methods

2.1. Study sites and sampling

The eight WwTWs which were examined for microplastics in influent and effluent were chosen to represent a range of treatment processes, including six activated sludge treatment plants with differing tertiary treatments, two trickling filters and one biological aerated flooded filter (BAFF). The wastewater 'strength' of the different WwTWs was broadly similar at 213-321 L /PEq/d based on dry weather flow (DWF) permit values (Table 1). Five WwTWs were selected for sludge sampling. Only two of the eight WwTW selected for influent/effluent sampling process sludge on-site, therefore these two corresponding sites were chosen for sludge collection, in addition to three new sites (Table 2). Not all WwTW have sludge processing

facilities and therefore sludge at any WwTW with sludge-processing capability is therefore likely a composite of sludges from multiple treatment works accumulated over a longer time period. For this reason, it was not possible collect sludge samples that exactly match the influent and effluent samples. Influent and effluent samples were collected on two separate occasions between November 2018 - February 2019 and sludge samples on five separate occasions between July 2018 - February 2019. This produced a total of 16 influent samples, 18 effluent samples and 25 sludge samples.

Table 1. Influent and effluent WwTW sampling sites, coded by processing type. PEq = population equivalent, DWF = dry weather flow. Simultaneous 24 h influent and effluent samples were taken on two occasions at each site.

Site code	Secondary treatment	Tertiary treatment	PEq	DWF (m^3/d)	L/PEq/d
ASTC1	activated sludge (AS)	cloth filter	264,000	56,160	213
ASTC2	activated sludge (AS)	cloth filter	157,000	40,300	257
ASTS1	activated sludge (AS)	sand filter	90,000	20,394	227
ASTS2	activated sludge (AS)	sand filter	38,000	11,476	302
AS1a*	activated sludge (AS)	none	320,000	70,000	219
AS2b* TFSb*	activated sludge (AS) or stone trickling filter (TF)**	sampled before tertiary	103,348	27,500	266
TFP	plastic trickling filter	none	40,000	12,860	321
BAFF	biological aerated flooded filter (BAFF)	none	42,350	9,484	217

*Plants with the suffix ‘a’ or ‘b’ are linked to plants with the same suffix in Table 2, where influent, effluent and sludge were collected at the same works.

** Two different treatment streams exist within the same plant, therefore the influent and both secondary effluents were sampled for comparison

Table 2. Sludge sampling sites, coded by processing type. Sludge cake samples were taken on five occasions from each site.

Site code	Description of process type(s)
AAD1a*	Advanced anaerobic digestion^
AAD2	Advanced anaerobic digestion^
AAD3	Advanced anaerobic digestion^
ADb*	Conventional anaerobic digestion
LS	Limed sludge

* Plants with the suffix 'a' or 'b' are linked to plants with the same suffix in Table 1, where influent, effluent and sludge were collected at the same works.

^ Advanced anaerobic digestion refers to a thermophilic digestion process with the inclusion of a heat treatment step to achieve a better pathogen reduction and/or improve gas yield.

Composite samplers (ISCO Avalanche refrigerated autosampler) were used to collect liquid (influent and effluent) samples over a 24 h period to account for within-day variability. Both influent and effluent samples were taken simultaneously at each WwTW. Sampling usually started around midday and was completed at midday on the following day. Due to residence time within the WwTW it is recognised that the effluent did not correspond exactly to the influent, however all samples were collected on weekdays and significant variability between adjacent days would not normally be expected. Influent samples were collected directly behind the coarse screen (≥ 6 mm) to prevent clogging of the sampling equipment with large items, and programmed to collect 100 mL every 30 min over the 24 period, obtaining a total volume of 4.8 L into a glass bottle. For effluent, the autosampler was set to sample 7.5 L every 30 min over the 24 period (nominally 48 * 7.5 L = 360 L). In this case, the sample was pumped through a filter system using a design modified from that used by Mintenig et al. (2017). In brief, this consisted of a large stainless steel filter holder (Spectrum Inox economic filter housing, EFH-SBR) containing a removable woven 10 µm stainless steel cylindrical filter cartridge inside (9 ¾" length, ca 500 cm^2 filter area, Wolftechnik Germany). The exact volume

passed through the filter cartridge was measured using a water meter (Fig. S1). All sludge was collected in 1 L glass Kilner jars using a metal trowel. Jars were sealed with aluminium foil between the jar and the lid, to avoid contact with the rubber coating on the inside of the lids.

2.2. Contamination control

Strict contamination control measures were undertaken throughout, during sampling, processing and analysis. All handling of sampling equipment, filters and samples in the laboratory was carried out within a Class II Microflow Biological Safety Cabinet, which filters air through a 99.999% high-efficiency particulate air (HEPA) filter (MDH Contamination Control, Hitchings Clinical Services, UK). All equipment was thoroughly washed before use: three times under a reverse osmosis (RO) water tap and three times using RO water within the safety cabinet. Cotton lab coats were worn at all times to cover clothing when handling samples and equipment. To avoid contamination from the reagents themselves, all reagents were filtered through a 1.2 µm glass-fibre filter (Whatman GF-C) before use.

Wherever possible, all parts within the sampling and processing equipment were made of metal or glass. The only places where this was not possible were O rings to ensure sealing of the equipment, a combination of silicon and PVC hosing for sample collection through the peristaltic pump, and a distinctly coloured yellow polypropylene wash bottle for rinsing filters and equipment in the lab. Any possible contamination from these would be accounted for by blank correction (section 2.6.).

Filter units were assembled and sealed in the lab within the biological safety cabinet to prevent airborne contamination. These pre-assembled units were taken to the field where the filter unit was attached to the sampler without exposing the filter to the air at any point. When sampling, a flushing step was always carried out by using isolation taps on the filter unit, allowing all

sampling equipment (except the filter itself) to be rinsed with the wastewater being sampled, before sampling commenced (Fig. S1).

2.3. Sample processing in the laboratory

Recovered effluent solids were rinsed off the stainless steel filters into a glass beaker with reverse osmosis (RO) water from a wash bottle using a natural hair paintbrush to dislodge particles until the filter and the brush were visibly clean. Influent and effluent were then processed in the same way: A 200 mL sub-sample was placed in an ice bath and Fenton's reagent (70 mL of 30% H_2O_2 (Fisher Scientific, USA) followed by 30 mL Fe (II) solution (0.05 M $FeSO_4°7H_20$, Fisher Scientific, USA, > 98% purity) was added. This dilution allowed the reaction intensity, speed and temperature to be more easily controlled than if the Fenton's reagent was added to a dewatered sample, thus reducing the risk of damage to polymers (Liu et al., 2019a). The temperature was regularly monitored during the first hour using an infrared thermometer (Electronic Temperature Instruments Ltd, Worthing, UK), to ensure it did not exceed 50°C. The sample was covered loosely with aluminium foil and left overnight in the biological safety cabinet for the reaction to complete. If any iron precipitates formed during the digestion, these were removed by pipetting 1% H_2SO_4 drop-wise until they dissolved. Between each digestion step, the sample was concentrated onto a 10 µm stainless steel filter by vacuum filtration. The concentrated sample was then placed into a beaker with 20 mL cellulase (MP Biomedicals, > 60 000 U, solution made to 200 mg /L) in a shaking incubator for 48 h at 50°C, to aid with the removal of cellulosic fibres (Löder et al., 2017). Next, the re-concentrated sample was placed in a beaker with 16 mL RO water and 4 mL Trypsin (Sigma Aldrich, 25 g /L in 0.9% NaCl solution) to remove proteinaceous material (Courtene-Jones et al., 2017).

Sludge samples were dried in an oven at 50°C for approximately 1 week. When dry, a subsample of sludge was gently disaggregated using a pestle and mortar and sieved using a 1 mm stainless steel sieve, with 1 g (< 1 mm) taken for further processing. This was diluted to 200 mL with RO water and digested using Fenton's reagent as above. Due to the high levels of inorganic matter in sludge, a flotation step was necessary. The digested sample was sonicated with $ZnCl_2$ at a density of 1.7 g/mL (BonnyMans, UK, > 98% purity) for 5 minutes to disaggregate particles and then washed with more $ZnCl_2$ into a conical separation funnel. This was left to separate for 20 h as recommended by Wang et al. (2018), before discarding the high density (mostly inorganic) settled material. The sludge sample was then filtered and enzymatically digested as per influent and effluent. Finally, to reduce the amount of large particulate matter which could hinder the µFTIR analysis, the sample was vacuum filtered through 178 µm mesh size stainless steel mesh before analysis (this mesh size based on availability of suitable filters, Bridgewater Filters Ltd., UK). Given the complexity of the sludge processing procedure, to ensure the consistency and reproducibility of the sludge processing across samples, from one sample (AAD3) four replicate subsamples were taken and processed (Table S5). Following processing and digestion steps, all samples were concentrated and diluted in 5-10 mL 50 % ethanol for storage prior to analysis, as recommended by Liu et al. (2019a).

2.3. Sample analysis

For spectroscopic µFTIR analysis, samples were thoroughly mixed by vortexing for 10 seconds, then a subsample immediately deposited onto a 25 mm diameter 5 µm pore size silver membrane filter (Sterlitech, Washington USA) using a glass pipette. The volume of subsample was determined by weighing the whole sample before and after subsampling to 0.1 mg accuracy (Sartorius MC1 Balance). All microplastics within a selected filter area were

identified and quantified with an imaging µFTIR spectrometer (PerkinElmer Spotlight 400) set to collect spectra in the range between 4000 and 700 cm^{-1} wave numbers. A background spectrum of the silver filter was collected and removed from resulting data. The pixel size selected was 25 µm to give a reasonable compromise between resolution, processing time and resulting file size, this therefore being the minimum particle size that could be quantified. Mapping was carried out at a resolution of 8 cm^{-1}, with a total of 4 scans per pixel, with an interferometer speed of 2.2 cm/s. Due to software limitations regarding file size limits (Perkin Elmer SpectrumIMAGE), the infrared mapping area was selected to be 11.6 mm x 11.6 mm, which resulted in 92% of each filtration area being mapped. A calculation was subsequently applied to the data to account for the 8% of the filter that was not possible to scan.

All spectra were analysed using the MPhunter software with a linked reference database, developed by Aalborg University, Denmark. For a full description of the spectral matching and particle building approach used by MPhunter see Liu et al. (2019). We reported on the following nine polymers: polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), poly(methyl methacrylate) (PMMA), polyamide (PA), polystyrene (PS), polyurethane (PU), polyvinylchloride (PVC) and acrylonitrile butadiene styrene (ABS). These polymers are among the most commonly manufactured and are often reported in environmental samples (Liu et al. 2019, PlasticsEurope 2018).

2.5. Blank samples

Procedural blanks were run alongside samples, to account for any contamination that may occur during sample collection and processing. Eight effluent procedural blanks were run by setting up the filter rig to collect a sample of pre-filtered clean water (350-1300 L RO water or regular tap water, pre-filtered to 2 µm) on the 10 µm stainless steel filter within the cartridge, as would be used for collecting effluent samples in the field. Five sludge procedural blanks

were also run in empty vessels by carrying out the whole sludge extraction process including flotation and digestion, but without the addition of any dried sludge. Separately, three unused silver filters (used for the final presentation of the sample to the µFTIR) were also tested for contamination (no particles found).

2.6. Resolution, limit of detection (LOD) and limit of quantification (LOQ)

For a polymer to be detected, at least one particle of that polymer must be present on the final silver filter. The particle(s) detected on the filter represent a number of particles per litre or per gram in the original sample. This concentration that nominally results in exactly one particle on the final filter is the operational 'resolution' i.e. the minimum concentration required in order to observe one particle. Based on variable subsample volumes, this therefore varied between samples. LODs and LOQs are commonly used in analytical chemistry but have rarely been applied to microplastics research, with the exception of our parallel study on potable water (Johnson et al., 2020) and a recent study on blue crabs (Waddell et al., 2020). Procedural blank results were used to calculate LODs and LOQs for influent and effluent (effluent blanks were considered representative for both media as the extraction procedure was the same) and sludge (sludge blanks) separately for each studied polymer. The initial LOD was defined as 3.3 x the standard deviation of the blank, with initial LOQ being 10 x the standard deviation of the blank (AOAC, 2011), with each therefore giving a specific value based on blank contamination across the whole sampling and analysis process (Table S1). The final LOD/LOQ varied depending on sample volume and was therefore either the initial LOD/LOQ or the resolution, whichever was higher. A detailed explanation of the LOD, LOQ and resolution calculations is provided in the SI.

2.7. Spike recovery

Efforts to quantify the recovery efficiency of the extraction procedures were made for both the sludge processing and liquid media processing through assessment of spike recoveries (positive controls). In this case five replicates of a single sludge sample were separately spiked with a solution containing a known concentration of polyamide (PA) particles (size range between 63-90 µm) and PVC particles (size range between 106-150 µm) dispersed in RO water and Tween (0.025%) and processed as a normal sludge sample. Additionally, five spike recoveries were carried out for the effluent process by spiking the same stock solution of PA and PVC into the filter cartridges as used for sample collection, and carrying out the extraction and analysis process as if they were effluent samples. The recoveries were calculated as a percentage of the total particles added to the sample and the coefficient of variance calculated to determine consistency of recovery. No standard particles or mixtures currently exist for the validation of spike recovery efforts. These specific polymers were chosen due to their high density and known sensitivity to digestion using Fenton's reagent and/or H_2O_2, therefore representing the polymers that would be the most difficult to extract effectively (Cole et al., 2014; Hurley et al., 2018; Karami et al., 2017).

2.8. Statistical analysis

It was only possible to carry out statistical analysis on the sludge data, due to insufficient replication of the influent and effluent samples. The sludge data were log transformed for normality and a one-way ANOVA carried out to determine whether the sampling site (WwTW) significantly affected the concentrations reported. A post-hoc Tukey's test was used to assess differences between specific sites. Coefficient of variance was calculated for the five samples analysed from each site to assess the variability in concentration based on sampling occasion.

Site LS was excluded due to an inability to analyse some samples, and thus insufficient replication for this site. All analyses were carried out using R Studio (version 1.3.959).

3. Results and discussion

3.1. Microplastics in influent are dominated by PE, PP and PET

Due to the high quantity of obscuring material present (despite the processing steps), only a small fraction of the processed influent sample (0.6-2.3%, representing 1.1-4.2 ml) could be put onto the silver filter disc to allow microplastics to be quantified using µFTIR. Subsampling has been commonly used in previous comparable studies for the same reason (Liu et al., 2019a; Simon et al., 2018). It is recognised that repeated digestions may have helped in removing more organic matter and thus allowing for analysis of a greater proportion of the final sample. Regardless of the small sample size, microplastic particles were found above the LOQ in 13 of the 16 influent samples. Where above the LOQ, concentrations ranged from 955-17,214 microplastics /L (Table S2). If considering particles above the LOD, particles could be detected in 15/16 samples (Table S2). The polymers that were quantifiable were PE, PP and PET (Fig. 1A). These polymers are among the most commonly used and manufactured globally (Geyer et al., 2017) and have been found to be prevalent within environmental samples (Allen et al., 2019; Horton et al., 2017a; Rodrigues et al., 2018). This result implies that these plastics are routinely released down the drain from the average home.

The concentrations found in influent were higher than many of those reported elsewhere (Carr et al., 2016; Gies et al., 2018; Lares et al., 2018; Talvitie et al., 2017b), although similar to those reported by Simon et al. (2018), who used a similar semi-automated FTIR analysis approach, implying that this automated approach enables the detection of particles that may be missed through manual particle selection. Although differences in sludge concentrations

between studies would be expected based on (for example) WwTW location and sludge treatment processes, the large difference here, and correspondence with data from a study using similar methods (Simon et al., 2018), suggests that the lower concentrations reported in previous studies may in fact be primarily a result of the analytical methods used, leading to an underestimation of microplastic concentrations.

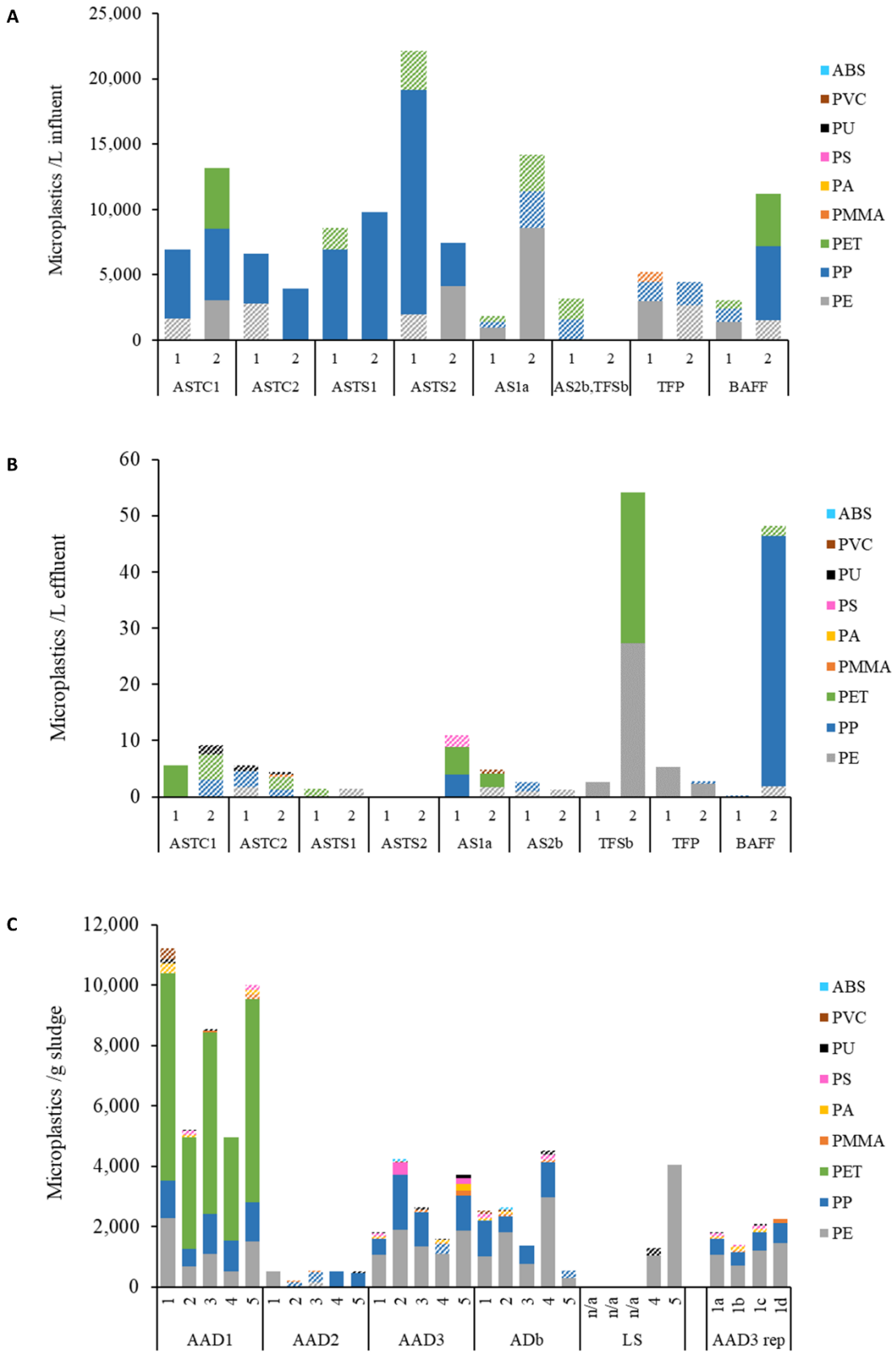

Figure 1. Concentrations of microplastics in influent (A) and effluent (B) at eight different WwTWs, sampled twice each (sampling occasions 1 and 2), shown by polymer type. At one influent site both activated sludge (AS)

and stone trickling filter (TFS) are used, therefore it has two influent codes. The resulting two streams of effluent were analysed separately and are reported in separate columns. Suffixes a and b indicate that sludge was also collected at that site. Concentrations of microplastics in sludge (C) at five different WwTWs, sampled five times each (sampling occasions 1-5). 'AAD3 rep' relates to the four repeat extractions taken from this sample to determine extraction efficiency. Samples labelled 'n/a' were not possible to analyse by μFTIR due to the high level of obscuring matter. Solid bars indicate quantifiable values (>LOQ). Shaded bars indicate values >LOD but <LOQ.

3.2. High removal efficiency of microplastics between influent and effluent

Of the 18 effluent samples, two had no polymers above the LOD (both at site ASTS2), eight samples had detectable polymers above the LOD but below the LOQ, whilst only eight samples had quantifiable numbers of microplastics (Fig. 1B and Table S3). Where > LOQ (n = 8/18), the concentrations of microplastics in effluent across all sites ranged from 2-54 microplastics /L (Table S3). These values are in line with those reported by Michielssen et al. (2016) and Murphy et al. (2016), although higher than many other studies which found fewer than one microplastic particle per litre of effluent (Lee and Kim, 2018; Magni et al., 2019; Talvitie et al., 2017a; Ziajahromi et al., 2017). As in the influent, the most commonly quantified polymers were PE, PP and PET. There were two instances where effluent concentrations were disproportionately high (BAFF and TFSb WwTWs during the second sampling event only). A greater number of repeat samples would be needed to determine whether these results were anomalous, or representative of normal temporal variability.

Whilst there are insufficient data to statistically compare the microplastic removal performance of different WwTW types, compared to the concentrations in the influent, the WwTWs achieved an average removal of 99.8%. There was consistently high removal performance of the activated sludge plants with tertiary treatment with all achieving ~100% removal (Table S8). These values are comparable to the removal efficiency reported by previous studies

(Mintenig et al., 2017; Talvitie et al., 2017a). Combining all WwTW data, average removal efficiency values were also consistent across the top three polymers PE, PET and PP, at 99.7%, 99.8% and 99.9% respectively (Table S9). It was not possible to calculate removal for other polymers as they were only occasionally detected (Table S3).

Based on our data, it is possible to extrapolate the values for numbers of microplastic particles entering and leaving these eight WwTWs to suggest fluxes for the whole of England and Wales. The starting point for this calculation is the consented wastewater dry weather flow (DWF) of 14,322,627 m^3/d for the 6,047 WwTWs of England and Wales as reported in Johnson et al. (2007). Taking the median influent and effluent concentrations reported in this study (6940 and 5.45 microplastics /L respectively), this would suggest 9.9 x 10^{13} microplastics /d are entering these WwTWs from domestic and industrial premises and 7.8 x 10^{10} microplastics /d are discharged into receiving waters for England and Wales. It is worth bearing in mind that these calculations do not consider seasonal fluctuations such as high flows, nor combined sewer overflow (CSO) discharges, therefore these values are likely an underestimate.

3.3. High concentrations of microplastics in sludge

Of all the sample types, sludge was the most challenging from which to extract and identify microplastics. For µFTIR analysis it is essential that particles are not overcrowded on the filter. Due to the required subsampling to sufficiently reduce the amount of non-plastic matter on the filter before µFTIR analysis, the samples analysed represented a very small mass of sludge (3.2-32 mg, 0.32-3.2% of the original 1 g processed sample), while three samples could not be analysed (out of a total of 25) due to high particulate content. In addition to the requirement to subsample, based on the requirement to size fractionate the sludge, the analysed samples represented only particles between 25-178 µm in size. Although this means that larger particles

would not have been reported, the influence of this when concentrations are expressed on a number basis is likely to be small, as the vast majority of particles in these samples were in the lower particle size ranges < 100 µm (Fig. S2). Even considering the small mass of sludge processed for each sample, the majority of samples contained a high number of microplastics: 20 of 22 samples had microplastics >LOQ, with concentrations ranging from 301-10,380 microplastics /g DW (Table S4). PE and PP were the most common polymers found in sludge. Overall, WwTW sludge had a wider range of detectable polymers than reported in the influent and effluent, including PA and ABS which were never detected in influent and effluent (Fig. 1C, Table S4). This may simply be due to the higher numbers of particles being concentrated in sludge compared to the liquid phases, and thus a greater chance of them being observed here.

Concentrations varied significantly depending on the site from which the sludge was sampled ($p < 0.001$, one-way ANOVA, Fig. 1C). Despite the fact that many of the sites used the same sludge treatment processes there were also significant differences between individual sites: AAD1-AAD2 ($p < 0.001$), AAD2-AAD3 ($p < 0.05$), AAD1-ADb ($p < 0.01$) (Tukey's Test; all other site comparisons were not significantly different, site LS was excluded from the analysis due to lack of replicates.). This suggests that factors in addition to sludge treatment processes also influence sludge concentrations. For example, sludge samples from site AAD1 showed consistently higher microplastics concentrations than at other sites, and were dominated by PET (Fig. 1C), a polymer that was not detectable in sludge at any of the other sites. Site AAD1 corresponds with influent/effluent site AS1a where PET was always detected in influent (>LOD) and quantified in effluent (>LOQ), implying local sources of PET at this site (Tables S2 and S3). However, based on the available data it was not possible to investigate this in any further detail. Further, concentrations in sludge were variable within sites across different sampling occasions (coefficients of variance AAD1 = 33%, AAD3 = 50% and ADb = 68%, only considering the three sites where all five replicates had values > LOQ) (Fig. 1C and Table

S4). This suggests that temporal differences in sludge concentrations, for example based on seasonal or weather conditions, may be significant and should be further investigated.

To ensure that any variability observed across samples was not related to our analytical procedures, for one sludge sample (AAD3), four replicate samples were analysed. These AAD3 replicate results were reasonably consistent (Table S5, coefficient of variance = 26.7%, RStudio, package 'GoeVeg'), and showed almost identical coefficient of variance to the spike recovery values (CV = 26.9% for sludge, CV = 26.6% for effluent, section 3.5). This is likely a combination of natural variability in subsamples due to the heterogeneity of the solutions measured and some unavoidable variability in extraction efficiencies. As the coefficients of variance for both spiked samples and repeat sludge samples were similar, this indicates that this variability was consistent across samples and different matrices. This supports the reproducibility of the subsampling method even with the most complex samples. Any further variability observed (for example based on WwTW or collection date) can therefore be attributed to real variability between the samples.

It is recognised that repeat digestion(s), or a less dilute Fenton's reagent, might have led to more effective digestion of organic matter, and should be explored in future sludge studies (Enders et al., 2020; Hurley et al., 2018; Park et al., 2020). However, given the very high numbers of microplastic particles even in 1 g sludge (Table S4), subsampling would have been required regardless of the efficiency of the organic digestion, to ensure microplastics themselves were not touching or overlapping (which would have led to errors in distinguishing particles). Despite the presence of residual organic matter, the unbiased approach of spectroscopic mapping, combined with the thresholds used for polymer matching (as per Liu et al., 2019a) and the stringent blank correction, affords confidence that the particles reported were unlikely to be false positives.

The concentrations found in this study were high in comparison to the majority of other sludge studies, which have usually found only tens or hundreds of microplastics per gram (Edo et al., 2020; Lares et al., 2018; Liu et al., 2019b; Rolsky et al., 2020; Xu et al., 2020). A likely significant factor in the reporting of these high concentrations compared to other studies is the FTIR microscopy methods used, identifying particles of a size that could not be detected using more common visual identification techniques.

Based on reported sludge-to-land application of 1,118,159 tonnes in 2010 (Ofwat, 2015), using the median sludge concentration from our data (2406 microplastics /g), the contribution of microplastics to land via sludge application can therefore be calculated as roughly 2.7×10^{15} microplastics annually. Again, it is worth noting that high variability between samples from different WwTW and across years, with sludge application continuing to increase in the UK, may lead this to be an underestimate. It is also worth bearing in mind that many sources of microplastics to the environment will bypass wastewater treatment systems (Horton et al., 2017a).

Given the variability in concentrations in sludge from different treatment plants, despite often using the same sludge treatment processes (Fig. 1C), it is recommended that further research be undertaken to investigate how different factors such as inputs from different sources (for example industry or urban areas) may influence microplastic concentrations and polymer types in sludge. Such understanding is essential for predicting the implications of sludge application as a potential contributor of microplastics to land. To further investigate temporal differences, a mass-balance approach considering influent, effluent and corresponding sludge in more detail at the same WwTW(s) would enable a better understanding of how concentrations in these matrices can be linked, and the fate and possible interventions of microplastics throughout the treatment process.

3.4. The importance of blank corrections

In the procedural blanks, there was a high level of contamination across all replicates (n = 8 for influent and effluent, n = 5 for sludge), despite the implementation of stringent contamination control measures. This can be understood in the context of other studies which highlight the prevalence of microplastics throughout the laboratory, domestic and outdoor environments, and show that background contamination in samples is unavoidable (Mintenig et al., 2019; Nuelle et al., 2014; Simon et al., 2018; Talvitie et al., 2017b). This highlights the critical importance of accounting for blank contamination throughout the whole process of microplastic extraction to prevent the reporting of false positives, particularly in relatively clean matrices such as effluent.

Contamination by PE, PP and PET was detected in almost all blanks, although the level of contamination varied. No contaminating particles from the polymers PMMA or PU were found, and there was only limited contamination by the polymers PVC, PA, PS and ABS (Table S1). The method was therefore very sensitive to the presence of PMMA, PU PVC, PA, PS and ABS microplastics but less so (higher LODs/LOQs) for PE, PP and PET. Despite using LOD and LOQ, microplastics at levels above the LOQ (including PE, PP and PET) were detected in most samples and, in the case of sludge, at high concentrations. Where samples are highly contaminated with microplastics (such as sludge), the effect of blank correction will be proportionally less significant compared to very clean samples, where applying LOD and LOQ correction to an already small number of particles may lead to a reduced capacity to report any microplastics above the LOD or LOQ.

Our approach of using LOD and LOQ is novel for microplastics and is one of the most rigorous that has yet been applied, despite being a common (and recommended) approach in the analysis of chemical contaminants (AOAC, 2011; Vial and Jardy, 1999). This is a far more robust approach than is usually used for microplastics analysis. Indeed, of 50 reported values for

microplastics in freshwater or drinking water published in peer reviewed literature, 32 of these studies either did not run full procedural blanks (negative controls) or correct for this contamination (Koelmans et al., 2019). Our results demonstrate the importance of not only conducting full procedural blanks but also correcting the data to account for this using the LOD and LOQ approach, so as to avoid reporting false positive results and thus overestimating microplastic particle concentrations. As such, we recommend such an approach become routine in the monitoring and detection of microplastics in environmental samples.

3.5. Spiked samples as a means of assessing recovery efficiency

The mean recovery of PA particles spiked into the sludge was 52.4% (± 14.1% SD, Table S6) and recovery was relatively consistent across replicates (coefficient of variance = 26.9%, RStudio, package 'GoeVeg'). The mean recovery of PA particles from the effluent processing was 101% (± 26.8% SD, Table S7) and recovery was similarly consistent across replicates (coefficient of variance = 26.6%, RStudio, package 'GoeVeg'). Again, these coefficients of variance are comparable across all samples analysed (26.6-26.9%) highlighting the variability between samples, yet the consistent ability to account for this in our processing. The PVC particles could be observed but had aggregated, and therefore accurate quantification of these particles using µFTIR was not possible.

The high PA recovery in the effluent samples implies that it is the more involved sludge processing, including flotation, rather than the digestion or FTIR analysis, which leads to the loss of particles. While the use of LOD and LOQ reduce the likelihood of reporting false positives, the underestimation of concentrations as a result of particle loss during processing of the sludge samples therefore likely leads to the reporting of lower concentrations than are actually present.

Due to different particle densities and characteristics, to accurately correct for recovery, a range of microplastic sizes and polymers would be necessary. While it was therefore not appropriate to do a recovery-correction based on the results for only one polymer, based on these data it must be borne in mind that an underestimation of microplastic particles in the most complex matrix of sludge is possible. PA is denser than water at 1.05-1.1 g/cm^{-3} (Herrera et al., 2018) and is notoriously susceptible to damage by chemical digestion (Karami et al., 2017; Lusher et al., 2017). Therefore, the recovery of PA here likely represents a 'worst case' recovery; other polymers, which are more buoyant and/or more resistant to damage, may have been more effectively extracted following flotation and digestion procedures.

3.6. Semi-automated characterisation of small microplastics (> 25 µm) to prevent bias

Following µFTIR analysis, the MPhunter software was used to provide an output of the size distribution of plastic particles found. When reviewing this output, it was clear that whilst a few larger particles existed, the smaller the particles, the greater the number appeared to be present (Fig. S2), as is common in wastewater and wider environmental studies on microplastics (Enders et al., 2015; Park et al., 2020; Simon et al., 2018). It is essential that studies report the operational boundaries for what is defined as a 'microplastic' within all studies, not only by polymer type, but also in terms of the size distribution investigated. Particles < 100 µm (or even < 500 µm, depending on the visual acuity of the operator) are incredibly difficult to identify and handle using common manual microscopic analysis, and therefore such analysis can be biased (Käppler et al., 2016; Löder and Gerdts, 2015; Shim et al., 2017). This highlights the value of semi-automated mapping methods, such as those used here, to quantify the smaller and more abundant particles. Had we been reporting on a different (larger) size range e.g. only particles > 100 µm or > 200 µm, the observed concentration of microplastics in our samples would be very different (and far lower) than those reported here.

Based on the automated analysis using MPhunter software, we did not characterise particle shapes. However, it should be noted that fibres are notably difficult to observe and identify using µFTIR. Given that the lower limit of the µFTIR resolution was set at 25 µm, it is possible that thin fibres were under-represented in the final dataset if they had a width below 25 µm (Primpke et al., 2019).

3.7. Methodological limitations

As with all microplastic studies to date, there are recognised methodological limitations, and further efforts need to be made to improve digestion efficiencies, to reduce blank contamination and to better account for specific recoveries of different polymers. The processing method of Fenton's reagent and enzymatic digestion did not eliminate all the obscuring matrix in any of the sample types. This could probably be improved by increasing the incubation time used, although that also increases the risk of damage to the polymers.

It should be noted that due to the residual organic and inorganic matter, it was not possible to analyse entire samples, and thus subsampling was required. Other studies employing digestion techniques on complex environmental samples have used similar methods and have found similar challenges with removing the obscuring (non-plastic but IR absorbing) matrix (Bläsing and Amelung, 2018; Liu et al., 2019a; Scheurer and Bigalke, 2018). More successful attempts at removal of organic matter have usually been carried out over prolonged periods, with multiple steps over several days or weeks (Löder et al., 2017; Simon et al., 2018). Due to time constraints, it was not possible to employ long digestion periods in this study, but longer or repeated digestions would be advisable for future work. It should be noted that even if all organic matter could be removed, due to the high concentrations of microplastics found in sludge, subsampling would still have been required to ensure an even spread of particles across the final filter.

3.8. Conclusions

The methods used in this study allowed for accurate, semi-automated, unbiased analysis of microplastics in a range of sample types derived from wastewater. The µFTIR method enabled identification of particles > 25 µm and therefore allowed the analysis of microplastics that it would not be possible to extract and manipulate by eye. Further, the methods used were time-efficient, allowing both quantification and polymer analysis of all particles within a filtered sample to be undertaken simultaneously. Running procedural blanks and correcting for blank contamination was shown to be essential to prevent the reporting of false positives.

This study confirmed the effectiveness of all tested wastewater treatment works at removing the vast majority of microplastics from the influent, regardless of treatment types (average 99.8% reduction). However, the corollary of this success was the retention of much of this load in the sewage sludge. Given the differences seen between sampling occasions, a more in-depth survey would be needed to assess specific differences based on flow conditions and daily or seasonal differences in wastewater production. Composite sampling is essential to account for within-day variability, while filtering on-site enables the collection of larger and more representative samples than is possible with grab sampling.

While microplastic presence and removal in wastewater systems has been often reported, the robust and repeatable approach taken here should provide greater confidence in the data. The combination of digestion, flotation and µFTIR analysis allowed high concentrations of microplastics to be discovered in sludge, using a semi-automated method that was capable of giving consistent results.

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Supporting Information

SI contains data for blank contamination and equations for blank correction, full final data tables of concentration values for microplastics in influent, effluent and sludge, repeat sludge and spike recovery data, and particle size distribution graphs.

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