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A novel high-throughput analytical method to quantify microplastics in water by flow cytometry

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ABSTRACT

Microplastics (MPs) are pervasive contaminants with unclear toxicological impacts. Current research on MP pollution relies on low-throughput methodologies, which are time-consuming and cannot directly measure MP concentration in suspensions. This study presents a qualitative and quantitative flow cytometry-based method for analysing MPs in water, offering a faster and more sustainable alternative. The method involves density separation to remove interfering particles, UV irradiation to eliminate microorganisms, and filtration to remove particles above 100 μm. The sensitivity of the method for different types of MPs, such as polystyrene (PS), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polyamide (PA) microbeads, ranges from 2 μg/L to 1 mg/L. For these MPs, good linearity was found in matrix-matched calibration where the most concentrated standard was 5 mg/L (R^2 0.9820–0.9989) although the linear range can be larger (e.g. 42 mg MP/L for PS microbeads). The repeatability and reproducibility of the method for the model PS MP were <17.0% and 8.5%, respectively. The sample treatment method consisting of density separation and UV pretreatment, when carried out independently, led to 95.0% and 93.4% recoveries. The overall trueness of the optimized method for various sizes and compositions of microbeads is about 97%, according to validation supported by microscopy analysis. This method can substitute the traditional quantitative analytical approach based on counting microbeads with microscopy.

1. Introduction

Microplastic (MP) pollution is abundant and rising in all environmental compartments [1–6]. As such, quantification of MP pollutants will increasingly be needed by a wide range of laboratories. However, the analysis of MP pollutants is still at the exploratory level with the scientific community still studying pollution levels and toxic effects to know more about its fate and impact. Monitoring MP pollution is time-consuming as the existing methodologies involve extensive sample digestion (e.g. for soils, dust, sludge, tissues, faeces, plants, and biota) that can last several days [7–9]. The analysis of MPs in less complex matrices such as salt [4] and water [5] also requires MP separation from the media. In this regard, MPs are floated and separated from the digested samples by centrifugation and filtration steps, where filtration restricts the size of the MPs that will be detected. The quantification of MPs in filters using microscopy is sometimes aided by semi-automatic software [9,10]. Particles with no distinctive colour or shape near the minimum size that optical microscopy can detect may be equivocally identified as MPs. μFTIR and μRaman offer more selectivity in the identification although they still depend on visual detection and are sensitive to the presence of impurities sorbed onto the particles [11]. Pyro-GC–MS overcomes visual difficulties in the identification, however the presence of natural organic compounds coextracted can affect the speed and accuracy of the identification [11]. The identification of MPs by Pyro-GC-MS, μFTIR and μRaman is not automatic and as a result only a fraction of all MPs recovered in environmental studies are characterised by these methods, which can bring inaccuracy in overall amounts of MPs reported.

Overall, the existing analytical methodologies for MP quantification require long analysis time and are labour-intensive. These analytical limitations increase the cost of studies addressing MP pollution. This subsequently limits the number of samples; impacting the representativity and accuracy of the quantification and knowledge gained. Current microscopy and spectroscopy approaches used, offer non-automated low throughput analysis and cannot be used to directly measure the concentration of MPs (in mass/volume) from suspensions. They only provide the number of MPs estimated in the portion of the sample that have been filtered and dried. The visual field and magnification of the microscope limit even more the part of the sample that is directly measured. Therefore, automated and green MP detection and quantification meth-

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ods that can support the investigation of MP pollution in multiple areas are very much needed.

Flow cytometry is a well-established technique for the rapid and automatic analysis of cells and particulate biomolecules suspended in aqueous samples [12]. It can sort subpopulations of cells based on preselected specific fluorescent signals and non-fluorescent scattering signals [13]. The use of flow cytometry to detect MPs was pioneered in 2016 [14]. A main advantage of using flow cytometry is that it can distinguish MPs from false positives (e.g. bacteria), and it can detect particles ranging from 0.2 μm to 100 μm through fluorescent staining [15], hence it covers the low range of MPs (up to 1 μm) [16] rarely covered with commonly used filtration and detection with optical microscopy. The cost of a flow cytometer needed to perform the analysis is comparable to a mid to high end trinocular motorized metallurgical microscope. Therefore, the cost of flow cytometers is not unattainable. Apart from the relatively small price difference, flow cytometers offer additional advantages that contribute to their overall cost-effectiveness.

However, the need to dye MPs for detection with flow cytometry [14] makes its use difficult in the monitoring of MPs in natural water because it is impractical to stain these particles in samples, and dyed MPs are not spiked in these types of studies either. Also, unresolved fluorescent signals from the staining (dots in the output plot) have been observed at ≤10 μm elsewhere [14], which narrowed the working range for the analysis of MPs in environmental samples. Therefore, our research has developed and validated new methodology that includes sample pretreatment and flow cytometry for the analysis of MP microbeads without the need for staining. This will pave the way for the use of flow cytometry in environmental monitoring of MPs and enable high-throughput, automated analysis of MPs that is in alignment with green principles.

2. Materials and methods

2.1. Chemicals and materials

Microbeads made of polystyrene (PS) (10–100 μm), polypropylene (PP) (10–100 μm), polyvinyl chloride (PVC) (10–100 μm), polyethylene terephthalate (PET) (10–100 μm) and polyamide (PA) (10–100 μm) were purchased from Dongguan Xingwang Plastics Co., Ltd. K-12. Escherichia coli (E. coli), YB525 Yeast, 30% H_2O_2, 14% NaClO, 99.5% NaBr, HPLC grade methanol, 1 M NaOH and 1 M HCl were purchased from Sigma-Aldrich (UK). Bio-wash (BioWash Mold Extraction System) was purchased from Biocide Labs Ltd. (UK). Natural aquarium silica sand (100 μm) was purchased from TM Aquatix Ltd. (UK), and it was washed with ultrapure water before use.

"Model" water was prepared by mixing 10–100 μm MP microbeads in ultrapure water with 2% methanol. Standards and stock solutions included 2% methanol. K-12 E. coli and YB525 yeast were prepared to 1×10^8 ind./mL, where "ind." refers to individuals, cells in this case. When indicated, 1 L samples were also added with 1 mL K-12 E. coli (1×10^8 ind./mL), 1.5 mL YB525 yeast (1×10^8 ind./mL) and 5 mg natural aquarium silica sand (100 μm).

Water samples from the Thames River and Regent's Park Lake were collected daily between May 29 and June 3, 2020, in volumes of 10 L each. Specifically, the sampling point in Regent's Park is displayed in Fig. S1a (Decimal degrees (DD): 51.5243897, −0.1538166) and in Thames River, in Fig. S1b (Decimal degrees (DD): 51.5083535, −0.1204094). Water sampling was done using a metal bucket prewashed at least 3 times with natural water. The bucket was immersed 50 cm deep from the surface to collect water. The aqueous samples were stored in glass bottles in the dark at 3 °C until analysis (performed within 3 days of the sampling). In experiments where natural water was used as a matrix and spiked with microplastics, methanol was added (to a final concentration of 2%) to improve the suspension and dispersion of microbeads in the sample.

2.2. Equipment

MPs were sorted and detected with a Guava easyCyte™ 5 HPL flow cytometer (Merck, Germany) with 488 nm Fluorescence Detection Channel, Green-B 525/30 nm laser, Yellow-B 583/26 nm laser and Red-B 695/50 nm laser. MPs were measured with forward scatter (FC), and side scatter (SC) (Alexa Fluor 488, cyan green; excitation: 495 nm; emission: 519 nm). The threshold was set at side scatter at 100, and the optimum voltages for forward scatter (FSC-H), side scatter (SSC-H), and BL1 tested were 340, 340, and 260 respectively.

UV lamps operating at 254 nm and 5 W, 10 W, 15 W, and 20 W (one at a time) (QTX, UK) were used for irradiating the aqueous samples for the removal of microorganisms at the second stage of the sample treatment procedure. The first stage involves the separation of MPs from inorganic particles.

A HWIR200A drying oven (Thermo Fisher, UK) was used at 105 °C for 12 h for drying filter paper containing filtered MPs in experiments assessing the recovery of MPs and comparing the quantification of microbeads with the new method and with the traditional microscopy approach. MPs recovered from aqueous samples following sample treatment were examined with Scanning Electron Microscopy (SEM). A JEOL JSM-6700F SEM equipped with EDX (JEOL, Japan) was used for the analysis, with an accelerating voltage of 10 kV.

2.3. Design of the experiments

The density of the suspension was optimised for removing insoluble inorganic components of the aqueous sample that could cause interference and lead to false positives (e.g. sand). Afterwards, four different microorganisms removal treatments (H_2O_2, NaClO, Bio-wash and UV) were compared by varying lamp power and irradiation time. The optimised method for removing insoluble inorganic substances and microbial treatment was used to process the samples. A calibration curve of 10, 50, and 100 μm MPs (PS, PP, PVC, PET, PA) count was used for the quantification. Also, natural water samples were tested to verify the scope of the method. In addition, SEM-Energy Dispersive X-ray Spectroscopy (EDS) was used to confirm that the particles identified were organic and therefore they were not sand.

2.3.1. Separation of MPs from insoluble inorganic interferences

Flotation steps for separating MPs from aqueous samples were optimised with ultrapure water (including 2% methanol) spiked with PS microbeads (5 mg of 100 μm MP/ L). NaBr was used to adjust the density of the aqueous samples to float the MPs. The densities tested were 1, 1.1, 1.2, 1.3, 1.4, and 1.5 g/cm^3. In addition, parallel control samples were spiked with natural aquarium silica sand (100 μm) to a final concentration of 5 mg sand/L instead of 5 mg of 100 μm PS MP/L. A glass funnel (250 mL, Fisher Scientific UK) was used to separate MPs from sand with 3 consecutive extractions after shaking.

2.3.2. Separation of MPs from potential microorganism interferences

For synthetic samples, K-12 E. coli and YB525 yeast were prepared to 1×10^8 ind./mL. 1 L water samples were added with 1.5 mL K-12 E. coli, 1.5 mL YB525 Yeast from their corresponding 1×10^8 ind./mL cell cultures. Following this, the sample density was adjusted to 1.4 g/cm^3 by adding NaBr (99.5% purity). After adjusting the density, the pH of all the samples was adjusted to 7 with the addition of 1 M HCl or 1 M NaOH. In separate assays, either 30% H_2O_2 or 14% NaClO or bio-washing powder (Biocide Labs Ltd. UK) were added to samples to final concentrations of 3% H_2O_2, 500 mg/L NaClO, 500 mg/L bio-washing powder, respectively. Finally, these samples were irradiated with a 254 UV lamp with the optimised conditions (given in 2.3.4). The lamp was located 5 cm at one lateral of the water sample.

2.3.3. Experimental design for the removal of microorganisms and statistical data analysis

A Design Of the Experiment (DOE) model was used in Minitab ® version 21 with a four-factor four-level statistical design. Specifically, the four factors studied were reaction time (min): 5, 15, 30, 45; UV lamp power (W): 5, 10, 15, 20; pH: 3, 5, 7, 10; and reaction temperature (°C): 15, 25, 35, 45. An incubator was used to modulate the temperature. The levels used were 1, 2, 3, and 4.

A 4-way ANOVA, with 95% confidence, was used to assess the effect of the 4 studied factors on the removal of microorganisms from the samples. The recovery of the analysis was studied at 5 mg 100 μm MP PS /L spiking level ($n = 6$). The UV lamp set-up is detailed in Section 2.3.2

2.3.4. Optimised sample treatment and quantification method of MPs with flow cytometry

Different types and sizes of MP (10–100 μm PS, PP, PVC, PET, PA) beads were spiked in ultrapure water or in surface water, in every case adjusted to 2% methanol, to prepare standards of individual composition and size (10, 50, 100 μm). The calibration curve had a concentration of 1, 2, 3, 4, and 5 mg MPs/L, although lower and higher concentrations were tested to find the best work range. A standard calibration curve for individual types of microbeads (PA, PET, PP, PS, PVC) was obtained, relating the MPs' mass concentration with the flow cytometer counting results. The density of water samples with 2% methanol was adjusted to 1.4 g/cm^3 by adding 400 g NaBr/ L. Afterwards, samples were irradiated with UV (15 W, 30 min, 25 °C, 254 nm). Before the introduction of the sample in the flow cytometer, the samples were filtered with an aluminium sieve (100 μm, Jin Yuan Ltd., China), and the filtrate liquid was shaken for 10 min and measured with flow cytometry. MPs were assessed on forward scatter (FC), side scatter (SC) and fluorescence intensity. The 488 nm laser of the cytometer was used for excitation, and the emitted fluorescence signal was detected at 530/540 nm. From the density dot plot, the particle (size range 10–100 μm) count results were used as a response for the calibration curve.

2.4. SEM-EDS analysis

Filters with deposited MPs were oven-dried at 100 °C for 12 h. The dry samples were coated with gold and examined with SEM-EDS (magnification 1.00 KX, 10 μm, EHT: 10 KV, WD: 5.4 mm, tilt angle: 45°).

2.5. Quality parameters

Quality parameters of the proposed analytical method, including assessment of sensitivity, precision, trueness and robustness of the sample treatment and instrumental analysis were measured with ultrapure and freshwater samples spiked with 100 μm PS microbeads following the procedure detailed in Section 2.3.4. The instrumental Limit of Detection (LOD) and Limit of Quantification (LOQ) were estimated from the calibration curve prepared with MPs in ultrapure and surface water (matrix-matched). The response of the LOD and LOQ corresponded to the intercept of the regression line plus 3 and 10 times the standard deviation of the residuals (S y/x) [17]. The repeatability of the method (intra-day precision) was determined from 6 measurements of 5 mg/L 100 μm PS microbeads in ultrapure water with 2% methanol on the same day. With the same concentration and type of microbeads, the reproducibility (inter-day precision) of the method was assessed by carrying out 2 independent analyses over 3 non-consecutive days. An independent analyst from this study was given the detailed experimental protocol and quantified 5 blind freshwater samples (with 2% methanol added) which MP concentration was 0–5 mg PS microbeads/L spiked in natural water (from Reagent's Park Lake). The independent analyst did a triplicate sample treatment for every sample following the procedure detailed in Section 2.3.4 and the trueness of the analysis was assessed by comparing the mass of MPs quantified with the method based on flow cytometry with the mass of MPs weighed when preparing the samples. The robustness of the method was also assessed with tap and freshwater water samples spiked with MPs of different sizes (i.e. 10, 50, 100 μm) and microbead polymer types (i.e. PA, PET, PP, PS, PVC). Prior to quantification of MPs by flow cytometry, samples were first purified (see Section 2.3.4), and then during analysis, the signal (measured in counts/mL or ind./mL) was converted to mg of MP/L using the established calibration curve. The MPs counted using flow cytometry were also compared with the MPs counted from the same samples using microscopy. For the latter procedure, MPs were extracted from a controlled water sample volume. The comparison of both orthogonal approaches was carried out with confidence limits at p 0.05. The optical microscope used was microscope (model Euromex Oxion Material Science, Netherlands) using Countess™ cell counting chamber slides (C10228, Thermo Fisher Scientific, UK) [18]. Briefly, 5 mg of 100 μm PS microbeads were spiked in 1 L ultrapure water with 2% methanol. The water sample was treated (as described in Section 2.3.4), filtered with a syringe filter (2.5 cm wide, cut off 100 μm nylon from Sigma Adrich, UK) and at this point, the filtrate was either measured using the flow cytometer or filtered under vacuum using a filter paper (PVDF 0.22 μm, from Sigma Aldrich) and dried (105 °C, 12 h) before counting using the optical microscope. The recovery rate was obtained by comparing the number of MPs counted onto the dried filters (for both spiked freshwater and ultrapure water samples) and the mass used for the preparation of the spiked solution. Control blanks of the analysis using both microscope and flow cytometer were carried out ($n = 3$ per approach) in parallel.

3. Results and discussion

This work involved optimizing a sample treatment and detection method of MP microbeads using flow cytometry without the need for staining the MPs. Although microfibers are generally the most abundant MP found in the environment, microbeads can also make a significant contribution to overall MP pollution. For instance, in a wetland, spherules accounted for 36% and 46% of the overall MP pollution found in surface sediments [19]. Furthermore, microbeads are commercially available, making them a convenient material to use for investigating the effectiveness of water treatment methods in removing MP pollution. The analytical procedure has been optimised with 100 μm PS microbeads. That size of MPs was selected because it raised toxicity concerns [20] and these MPs have an intermediate density of 1.05 g/cm^3 compared to other plastics (e.g., PP: 0.92 g/cm^3, PET: 1.38 g/cm^3, PVC: 1.38 g/cm^3, PA: 1.44 g/cm^3); therefore, PS was considered a good model to represent MPs. Furthermore, PS was among the 4 most abundant types of plastics found in freshwater worldwide according to our review [20]. The method has been validated with MPs of different sizes (10, 50, 100 μm) and compositions (PA, PET, PP, PS, PVC) in different water qualities to assess whether compositional changes can affect the calibration curve.

3.1. Method development for the analysis of MP microbeads (10–100 μm)

3.1.1. Overview

The optimization of the sample treatment started with finding a suitable sample density that could make 100 μm PS microbeads float and at the same time remove insoluble inorganic particulates that could interfere with the MP signal and identification thereof. The sample treatment method was designed to differentiate microbeads from other potential interferences, such as microorganisms, that may overlap in size range (≤100 μm) with the MPs studied in this work. The study compared 4 different treatments for removing microorganisms (H_2O_2, NaClO, enzymatic degradation (with Bio-wash) and UV) (see Section 2.3.2) and the most effective approach was selected for further studies. Once the selectivity of the method and recovery of PS microbeads were suitable, external standard series with the PS microbeads were prepared and used to calibrate the quantification. Natural water samples (from Reagent's

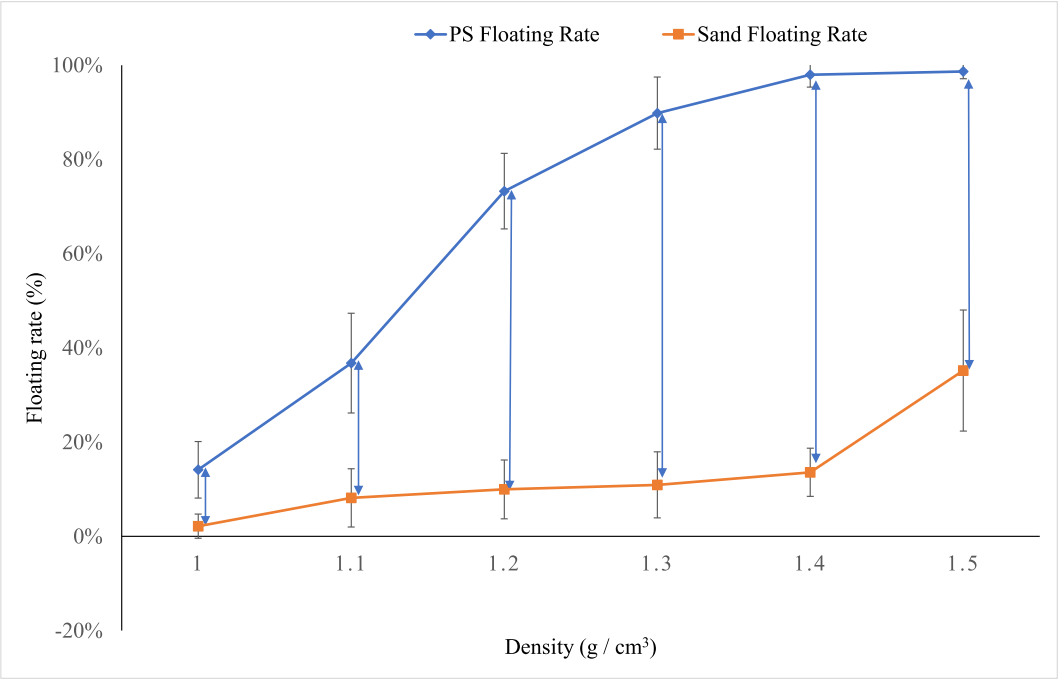

Fig. 1. Curves showing PS (100 μm) floating rate (recovery) (top curve) and sand (100 μm) floating rate curve (bottom curve) at different solution densities adjusted with NaBr.

Park Lake) and microbeads of different polymer compositions and sizes spiked in freshwater and tap water were tested to verify the practicability of the method. The quantification was carried out using flow cytometry, and qualitative data on composition was carried out with SEM-EDS to demonstrate that inorganic particulate matter was not counted as MP. Quality parameters of the method were established, and its validation included the analysis of broad types of microbeads spiked in tap water, urban lake water, and river water. The results from flow cytometry assisted quantification were then compared to the currently used traditional method on counting MPs by optical microscopy.

3.1.2. Separation of MP microbeads from insoluble inorganic particles

A way to separate MPs from grit is to change the density of the suspension [21]; by doing this, the inorganic solid particles will sediment. EDX supported this work by helping to differentiate organic from inorganic particles with the compositional spectrum.

The recovery of MPs from samples adjusted to different densities with NaBr is shown in Fig. 1. At 1.4 g/cm^3, a large portion of the plastic particles floated and was recovered (94.5%), while ~11% of the sand floated. The sand selected for this study had a similar size as the studied MPs, hence the separation of MPs from the sand was assessed under the most challenging conditions. Low densities (up to 1.38 g/cm^3) led to 95% of the sand being settled and removed through this step, however, MPs did not float efficiently at densities <1.4 g/cm^3 (Fig. 1). Therefore, the density of 1.4 g/cm^3 was chosen for the removal of inorganic solids, while being effective at floating MPs from samples before the flow cytometer analysis. Other studies carrying out environmental monitoring of a wide range of plastics in soil (samples rich in inorganic particles) adopted NaI at a density of 1.68 g/cm^3 [22].

Studies characterizing MPs in the environment usually carry out MP counting with microscopes with a limit of identification of ~50 µm due to the limitations of the microscope [22]. The scope of the new analytical method developed in this study includes microbead MPs ≤100 µm, hence the working range of both microscopy and flow cytometry is complimentary, and just overlaps ~50–100 µm. Flow cytometry's largest application is in the characterization of populations of cells and bacteria, these are generally 0.2–150 µm [23], and microbeads, in a way have large similarity with cells; therefore, the scope of flow cytometry may be broadened to the analysis of MPs.

3.1.2. Separation of MP microbeads from microorganisms

The addition of H_2O_2, NaClO, bio-washing powder in the samples, and irradiation with UV (see Section 2.3.4) were selected as approaches that could remove microorganisms and be easily applied in the lab with the consumption of minimum energy. This work adopted E. coli and yeast as model microorganisms. The effectivity of the treatments is shown in Fig. 2. The removal of microorganisms by UV (91%) was about 2 times greater than the effect of H_2O_2 (46% removal, p 0.000050), NaClO (51% removal, p 0.00013) and Bio-washing powder (45% removal, p 0.00012).

The superior removal of E. coli and yeast through UV justifies its selection as a method for microorganism removal, although the power and time of the treatment were yet to be optimised for a more sustainable treatment. UV irradiation is a commonly used sterilization method in laboratories for bacteria and fungi. Furthermore, it may cause less weathering of MPs after short exposure than the addition of oxidant chemicals. However, PS' aromatic rings can absorb 254 nm radiation and be affected, whereas MPs with no π system may be less degraded.

3.1.3. Sample treatment by UV irradiation

The effectivity of UV treatment could be boosted by conditions such as the pH of the sample, power of the UV lamp, treatment duration and water temperature. A DOE statistical model was used to optimize the best conditions to reduce the number of trials. The 4-way ANOVA results of DOE statistical analysis that result from the combination of the 4 conditions are shown in Table 1. Table Supplmentary material 1 (S1) was generated from Table 1 by the Minitab DOE analysis tool. The values calculated by a 4-factor DOE analysis in Table S1 show the influence of the studied factors on the results. Greater quotients in Table S1 imply a greater effect of the treatment on the studied microorganisms. From Table (S1), the impact levels of each factor are power, which had greater impact than time, then pH and finally temperature. Considering economic and environmental factors, 15 W, pH=7, 30 min, 25 ˚C, and 254 nm were chosen as the best conditions. The presence of PS MPs in

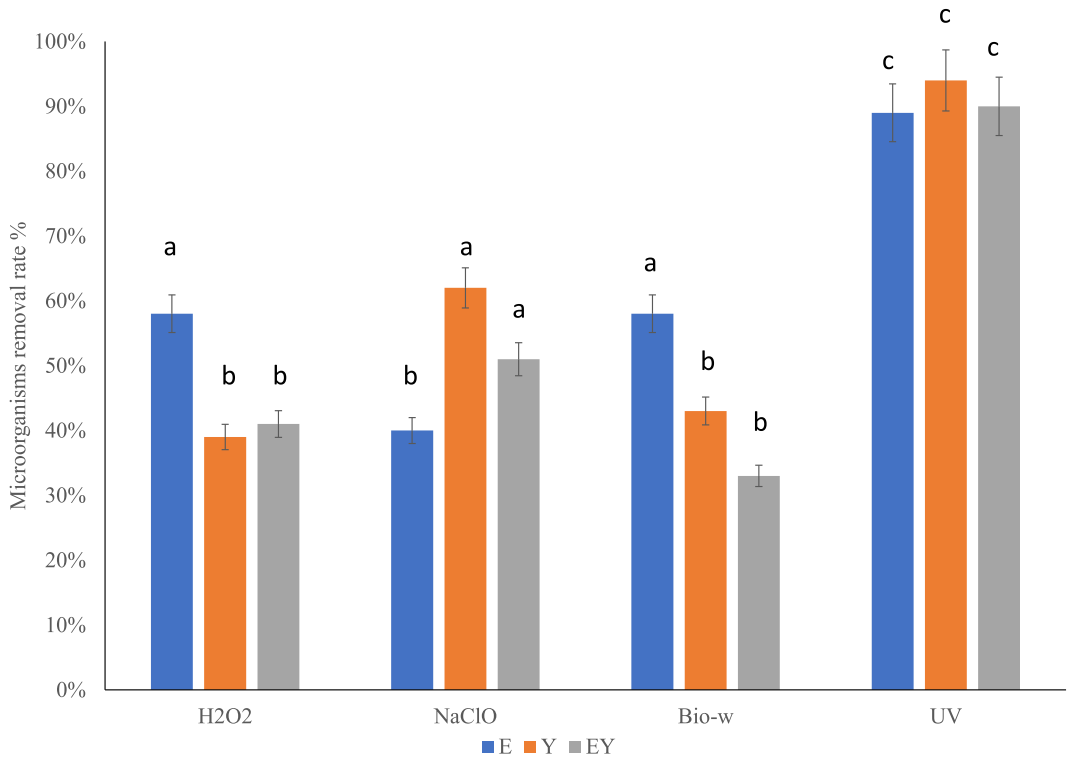

Fig. 2. Removal (%) of E. coli (E) and Yeast (Y) with four different methods (1.5 mL, 1×10^8 ind./mL K-12 E. coli and 1.5 mL, 1×10^8 ind./mL YB525 yeast, sample density adjusted to 1.4 g/cm^3 with NaBr (99.5% purity), pH adjusted to 7). In separate systems, 30% H_2O_2 or 14% NaClO or bio-washing powder were added to samples to final concentration of 3% H_2O_2 or 500 mg/L NaClO or 500 mg/L bio-washing powder. In all systems a 254 UV lamp located 5 cm from the water sampler was used at 15 W, 25 °C (temperature of the aqueous sample) and 30 min treatment in a closed box. Error bars correspond the standard deviation of $n = 3$. Means with the same letter are not significantly different from each other at the significance level of $p < 0.05$ as determined using t-test.

Table 1
E. coli and yeast removal rate in absence and presence of 100 μm PS microbeads in ultrapure water (5 mg/l) with 2% methanol.

Sample n	pH	Watt (W)	Time (min)	Temp (°C)	Mean removal rate in absence of MPs (%) ± SD ($n = 3$)	Mean removal rate with presence of MPs (%) ± SD ($n = 3$)
1	3	5	5	15	57.7 ± 3.06	59.6 ± 1.17
2	3	10	15	25	90.0 ± 2.65	89.5 ± 3.52
3	3	15	30	35	94.0 ± 1.00	99.4 ± 1.05
4	3	20	45	45	93.7 ± 1.53	97.3 ± 2.00
5	5	5	15	35	67.7 ± 4.16	71.4 ± 1.17
6	5	10	5	45	79.3 ± 4.16	82.9 ± 2.85
7	5	15	45	15	96.0 ± 1.73	97.0 ± 2.63
8	5	20	30	25	91.7 ± 2.31	99.3 ± 3.00
9	7	5	30	45	83.3 ± 2.08	83.3 ± 1.62
10	7	10	45	35	93.7 ± 2.08	98.7 ± 2.29
11	7	15	5	25	93.3 ± 1.53	96.5 ± 1.41
12	7	20	15	15	97.7 ± 1.15	97.5 ± 4.45
13	10	5	45	25	71.0 ± 2.65	74.7 ± 3.52
14	10	10	30	15	91.7 ± 6.81	89.9 ± 3.08
15	10	15	15	45	97.3 ± 1.15	99.2 ± 1.21
16	10	20	5	35	87.3 ± 5.13	96.2 ± 2.93

water caused increased removal of E. coli and yeast (see Table 1). This may be because the small microbeads may reflect or scatter UV light and by doing so increase the efficiency of the treatment. Through this sample treatment step, around 93.4% of the microorganisms were removed. Fig. 3 shows that before UV treatment, the microbes accounted for 38.2% (microorganisms/ total particles), and after UV treatment, the dead microbes accounted for 35.7% of total particles, hence the UV step was effective.

3.2. Quantification of microbeads by flow cytometry

3.2.1. Assessment of calibration curves and analysis of MPs in surface water

A significant correlation (following a t-correlation test at p 0.05 where R^2 >0.982, Table 2) was found between the response in the flow cytometer and concentrations of MPs of different sizes (10, 50, 100 μm) spiked in natural water (adjusted to 2% methanol) where MPs were

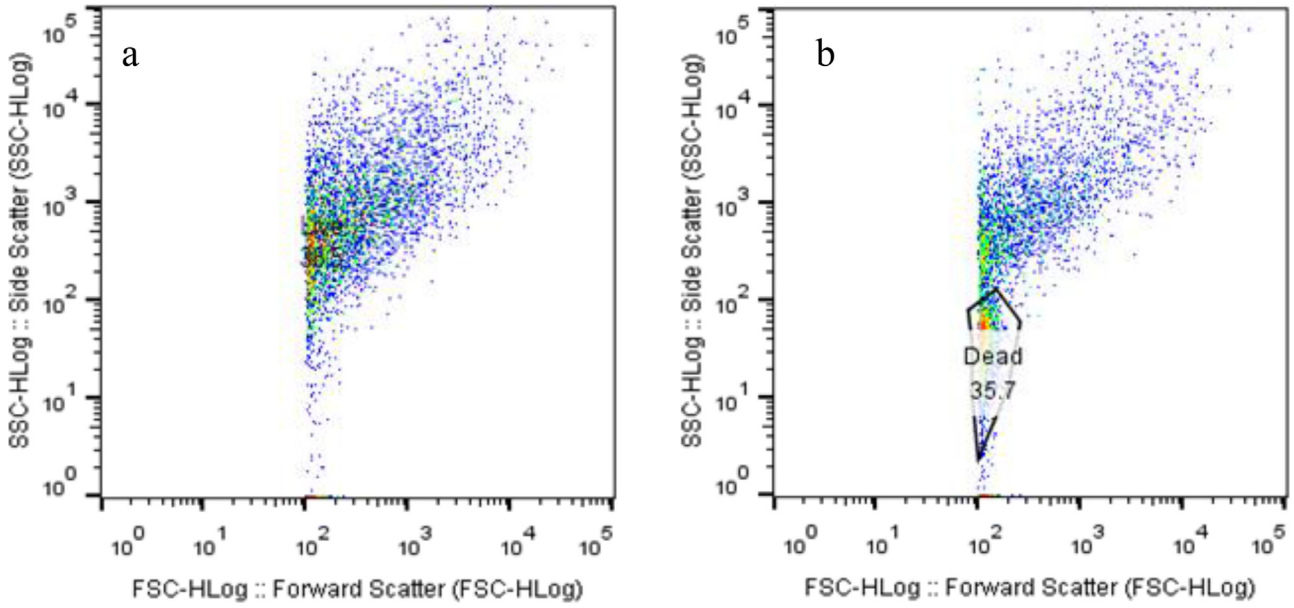

Fig. 3. Flow cytometry output from a) PS (100 μm, 5 mg/L, 485 ind./ μL) with E.coli (150 ind./ μL), yeast (150 ind./ μL) and 400 g NaBr in ultrapure water; b) same than a) having had irradiation with UV (254 nm UV lamp located 5 cm from the water sampler working at 15 W, 25 °C (suspension temperature), pH=7 and 30 min treatment in a closed box).

Table 2
Sensitivity of the quantification estimated from the calibration curve prepared in surface water from Reagent's Park urban lake (matrix matched).

Quantification parameters		Instrumental sensitivity with sample matrix (mg/L)			
Polymer	Mean size (μm)	Calibration curve	R^2	LOD	LOQ
PP	10	$y = 160.80x - 8.00$	0.9989	0.0018	0.010
	50	$y = 140.74x - 0.86$	0.9905	0.61	2.04
	100	$y = 155.69x - 38.05$	0.9867	0.63	2.43
PS	10	$y = 150.85x - 4.78$	0.9864	0.96	2.98
	50	$y = 159.26x - 3.81$	0.9953	0.43	1.43
	100	$y = 147.11x + 14.30$	0.9883	0.36	1.94
PET	10	$y = 147.00x - 10.00$	0.9962	0.39	2.01
	50	$y = 146.49x + 5.95$	0.9909	0.60	2.04
	100	$y = 151.40x + 1.00$	0.9941	0.48	1.60
PVC	10	$y = 157.97x - 16.76$	0.9923	0.34	1.84
	50	$y = 135.46x + 27.19$	0.9926	0.54	1.81
	100	$y = 153.29x - 10.05$	0.9930	0.53	1.26
PA	10	$y = 159.20x - 22.67$	0.9913	0.59	1.96
	50	$y = 168.80x - 32.00$	0.9973	0.70	2.34
	100	$y = 156.37x - 20.76$	0.9820	0.85	2.83

PA, PET, PP, PS, PVC, and the concentration was expressed in their mass/volume (see Fig. 4). The method uses the density of signals in the dot plot output and the concentration of MPs in standards prepared using an analytical balance. The calibration curve for the model PS 100 μm microbeads in ultrapure water ($y = 148.09x - 22.94$, $R^2 = 0.9848$) maintained sensitive when applied to pond water ($y = 147.11x + 14.30$, R^2 0.9883) although the intercept, that may denote the presence of interferences, increased in natural water. There was no evident relationship between the calibration results and the size or characteristics of the MPs from the calibration curves shown in Table 2. However, the calibrations in Fig. 4 were not different among them ($p > 0.05$), hence a universal calibration curve for quantifying any type of spherical MP may be feasible. The intercepts appeared to vary more across analytes than the slope, the latter is linked with the sensitivity of the analysis. In the case of adopting a common calibration curve for quantifying different types of microbeads, the participation of the intercept in the quantification could introduce error. Further work could address minimising the intercept values. In addition, it will be useful to test quality parameters for the quantification of other MP shapes in addition to microbeads. Ideally, a single calibration for any type of sample would be beneficial, in a similar way to quantifications of suspended particles using turbidimetry.

A total of 112 water samples were purified following the steps in Section 2.3.4. The MP counting with flow cytometry and separately using microscopy is compiled in Table S2. A *t*-test carried out at $p < 0.05$ comparing the quantification of MPs with independent approaches (Table S2) indicated that the counting through the flow cytometry method is significantly correlated with the counting MPs recovered onto a filter paper by optical microscopy ($R = 0.9928$). Confidence levels around the mean quantified values indicated that both methods led to a statistically similar number of microbeads ($p > 0.05$), and this constitutes an important result of the validation of our new method. The agreement of MP counts for PA, PET, PS and PVC microbeads between both approaches is illustrated in Fig. 5. There was a somewhat lower agreement for PP microbeads although the differences were not significant for some of the other types of microbeads. There is not a clear effect of the size or composition of MPs on the recovery observed when using the method developed in this work. The agreement between the method developed and microscopy counting of filtered MPs across the studied MP polymers in spiked surface water with 2% methanol was overall 90.1% (Table S2). Natural water poses the greatest challenge to the method developed. The sample treatment optimised, consisting of density separation, UV irradiation and syringe filtration of remaining particulate matter >100 μm, has been sufficient for reducing false positive signals due to the sample matrix according to the agreement between the quantification using flow cytometry and microscopy counting (Table S2). Compared with the study by Summers (2018) [24], our proposed method introduces a step of removing the insoluble inorganic matter by the density method and removing the microorganism by the UV method. The accuracy of measuring the concentration of MPs by flow cytometry is therefore improved and allows quantifying non-dyed MPs.

3.2.2. SEM-EDS analysis

The optimised flow cytometry method can be affected by counting inorganic particles as MPs, although the density was adjusted to prevent this. The optimisation of the developed method, especially the analysis of natural water samples, was supported by the examination of particles using SEM-EDS to confirm that the particles recovered were organic

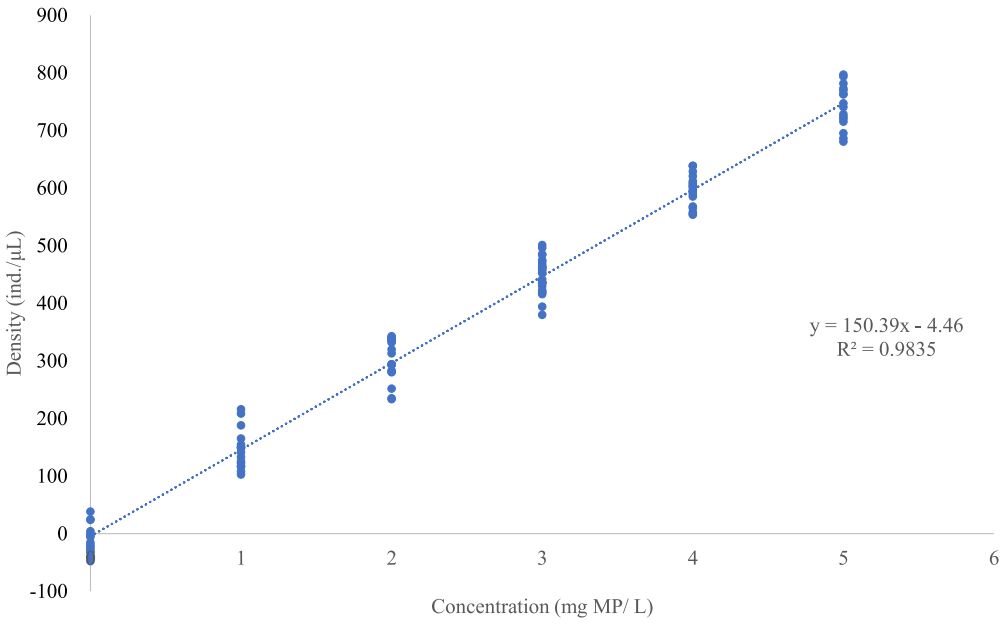

Fig. 4. External calibration curve with presence of matrix showing the relation between signal density and concentration of all study MPs (10, 50, 100 µm), and compositions (PA, PET, PP, PS, PVC). The sample matrix was Reagent's Park water and included 2% methanol.

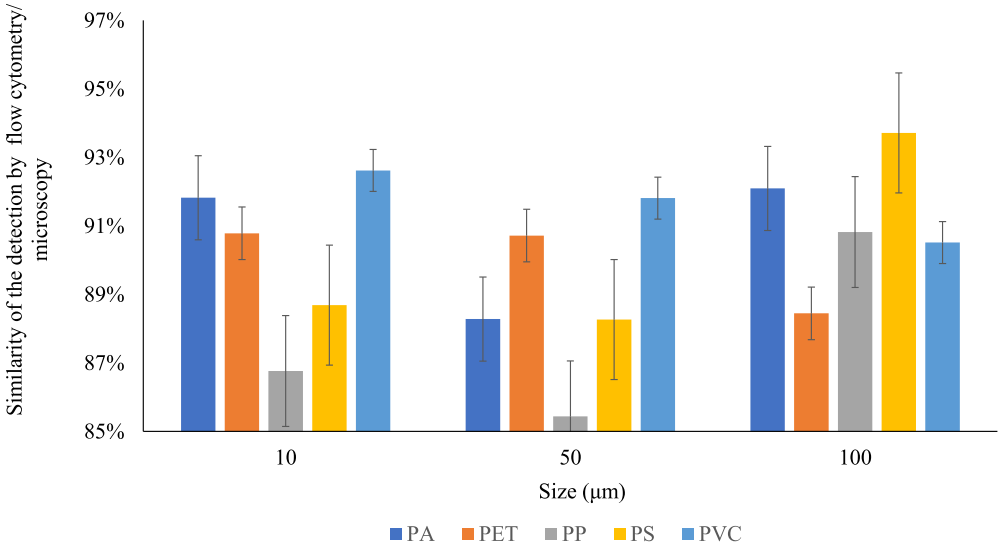

Fig. 5. Similarity between number of MPs counted with the proposed flow cytometry method and microscopy. Error bars correspond the standard deviation of $n = 3$. All means were found to be statistically similar ($p > 0.05$).

(with a high proportion of carbon and oxygen) in the EDS spectrum. Examples of the analysis and average element distribution results are shown in Fig. S2. In Fig. S2a, the two larger plastic particles can be differentiated. According to the EDS spectrum results in Fig. S2b, carbon in that potential MP exceeds 66.47%. Moreover, the contents of oxygen, nitrogen and chlorine are also high and their EDS spectra supports that these particles are not inorganic.

3.2.3. Quality parameters

Raw data related to the experimental assessment of the sensitivity of the method in ultrapure, tap water and natural water samples, repeatability, reproducibility, recovery of the beads in different water qualities, accuracy and robustness are available in Supplementary Materials (Tables S2-S7). LOD and LOQ were estimated from the calibration curve (see Section 2.1) [17]. The instrumental LOD and LOQ were 2 µg/L-0.96 mg/L and 10 µg/L –2.96 mg/L respectively for the studied microbeads (10, 50, 100 µm PS, PP, PVC, PET, PA), as presented in Table 2. LODs estimated from the calibration curve that included the freshwater matrix have been adopted because they consider the standard deviation of residuals of different levels of concentration and can be more robust than the evaluation with a single data point. The sensitivity in detection can be increased by preconcentrating the sample. However, in these cases, the concentration of potential interferences can increase too, and subsequently affecting the sensitivity. In this study, the maximum quantifiable concentration was ~40 mg/L (Tables S3 and S4), beyond which the signal becomes saturated, and agglomeration of beads may occur despite the 2% of methanol added to samples and standards to favour the stability and dispersion of the microbeads.

Relative standard deviation (RSD) is used to indicate the precision of the analytical test. Under repeatability conditions and based on the signals in data C from Table S5, RSD was 16.8%. Reproducibility was 8.5% (RSD, data C provided in Table S6). The trueness of the method was assessed with the model PS microbeads spiked at different concentrations in natural water through triplicate analyses. This study was done by an independent analyst not knowing information about the samples and following a standard operating procedure. These results are shown in Table S7, the experimental value from samples 0–5 mg/L quantified is highly similar to the concentrations of MP known from the preparation of concentrations independently with analytical scale: the error found was 0.62–9.59%. The average trueness achieved for these 5 levels

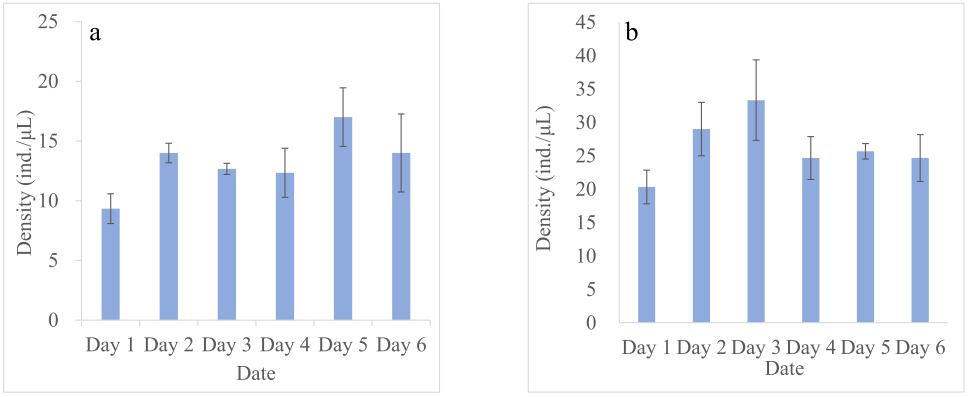

Fig. 6. MP signal density in natural water sample (a) in Regent's Park Lake water; (b) is Thames River. Error bars correspond the standard deviation of $n = 3$.

of concentration was 97.1%. The quality parameters obtained with the optimised sample treatment and detection method, and the comparable calibration across microbeads (Fig. 4), indicates that the developed methodology can be universal and be used for the calibration of possibly any MP within the established working range.

With the optimised sample treatment and flow cytometry method (Section 2.3.4), samples taken from Regent's Park Lake and River Thames on 6 independent days were treated. The treatment included 3 sequential flotation steps, which caused the collection of MPs from 3 volumes of sample into 1, and UV irradiation. Finally, the purified suspension of MPs was analysed by flow cytometry. The counts of MPs across most days were found to be statistically similar ($p > 0.05$) although, in this case, the composition of the water column on the sampling date was a factor to consider in the dispersion of the results (Fig. 6). The concentration of the MPs on Day 1 (Fig. 6) was the lowest compared to those from other days, maybe because it was raining on that day which increased the river flow. The RSD (%) of the quantification of MPs sampled on the same sites (Reagent's Park and Thames River) on 6 different days was 15.0% and 14.8% and this indicates the level of reproducibility of this approach for environmental monitoring. Nevertheless, the results displayed in Fig. 6 demonstrate the suitability of the method for monitoring MPs in surface water. When using the calibration curve relating the density (here it refers to a signal in the flow cytometer) with the concentration obtained with the adopted model microbeads (100 µm PS), the average total concentration of MPs, not all microbeads, sampled from the Regent's Park Lake (0.12 ± 0.018 mg/L), was significantly different to the concentration in the river Thames River sampling site (0.21 ± 0.031 mg/L) ($p < 0.05$). The MPs in the Regent's Park Lake and Thames River may originate from the discharge of industrial and domestic sewage (treated or untreated) [25], as well as from MPs settling in the lake from the surrounding urban environment [26]. Regent's Park is a tourist attraction, and the potential improper disposal of plastic waste, weathering of that plastic and runoff from roads [27] may contribute to its MP pollution. In any case, this study does not have sufficient sampling data points to compare levels of MP pollution.

Compared with the current flow cytometry methods [15,28,29], this method can be applied to the detection of natural water. Current flow cytometry detection methods require the staining of MPs or the direct use of fluorescent MPs to distinguish MPs from other interfering substances [14,15], and this is not possible in the environmental monitoring of MPs. The fact our method does not require MP staining, it falls under the principles of green chemistry. This work has analysed the effect of different factors affecting the selectivity of the method. The suitability of the quality parameters proposed (i.e. high sensitivity and precision), including analysis of MPs with varied sizes and densities, in different types of water, gives this method a broad scope beyond the analysis of microbeads in waters. Finally, our proposed method for detecting and quantifying microbeads is fully automated and can provide measurement in counts or mass/volume. In contrast, the traditional microscopy-based methods used for quantifying MPs are limited to counting MPs and have lower throughput. However, it was important to use counting through microscopy for validation. Future work should address whether fragments MPs can be measured with the proposed method and establish whether there are polymers or particle sizes that can lead to very different recoveries in the sample treatment or significantly different calibration curves than other MPs to find limits of the method proposed in this work.

4. Conclusions

Currently, there is no automated qualitative and quantitative method for the detection of MPs suitable for real-time monitoring in wastewater treatment plants, rivers, and drinking water. The use of microscopy methods for counting MPs is time-consuming. This research proposes a simple, environmentally friendly and low-cost method for the automated quantification of small MPs (10 - 100 µm) in waters using flow cytometry following flotation, UV irradiation and filtration. The analysis offered is sensitive (LOD ~0.3 mg MP/L), without considering sample preconcentration factors), precise (<17%), with high trueness (97%) and has low consumption of reagents. This method has been validated in parallel with flotation-filtration and counting following visual inspection with optical microscopy (standard method) leading to non-significant differences in the quantification of microbeads ($p > 0.05$). This work has included the analysis of MPs in ultrapure water, tap water and surface water, MPs with different compositions, densities and MP sizes (10, 50, 100 µm). The quantification has been tested with the analysis of a total of 112 spiked natural water samples. The understanding of the effect of the different parameters on MP analysis and results from the validation of the method demonstrates that the developed analytical procedure is advantageous as it allows for the automation of the quantification of MP pollution in mass/volume or in counts in waters. Our method is intended for use in optimising water treatment with microbeads and providing a broad estimate of MP pollution without distinguishing between different particle shapes. Further assessment is needed to test if other MP shapes (e.g. fragments <100 µm) can be analysed with this method.

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Declaration of Competing Interest

The authors of this work declare not conflict of interest with the content of this manuscript

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2023.100057.

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