Quantitative image analysis of microplastics in bottled water following Nile Red staining and fluorescence microscopy

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Abstract

The ubiquitous occurrence of microplastics (MPs) in the environment and the use of plastics in packaging materials result in the presence of MPs in the food chain and exposure of consumers. Yet, no fully validated analytical method is available for MP quantification, which prevents the reliable estimation of the level of exposure and, ultimately, the assessment of the food safety risk associated with MP contamination. In this study, an integrated method for the analysis of MPs in bottled water based on Nile Red staining, fluorescent microscopy, and automated image analysis was developed and validated, featuring a partial interrogation of the filter, thereby boosting the analysis time. The image analysis provided the number of particles in the region of interest of the analyzed filter and size of their major and minor axis. From these data, a rough estimation of the mass of the individual MPs, and consequently of the mass concentration in the sample, could be obtained as well. The applicability of the method for accurate MP size measurements and mass quantification was critically evaluated: the method showed to be highly sensitive in sizing MPs down to 10 µm with a limit of detection and quantification of 1.1 ppb and 3.4 ppb, respectively. Linearity and linearity range were studied between 10 ppb and 1.5 ppm resulting in a regression coefficient of (R^2) of 0.99. Method precision was demonstrated by a repeatability of 11% - 12% RSD (n=7) and within-laboratory reproducibility of 16 - 29% RSD (n=21). Accuracy based on recovery was 92.6 ± 23.3 % - 97.3 ± 14.5 %. Finally, the method was successfully applied to the analysis of 15 commercial samples of bottled water with and without gas and additives.

Introduction

The extensive use of plastics and the wide spread occurrence of plastic debris in the environment have resulted in the presence of microplastics (MPs), defined as plastic particles whose longest distance measures from 1 µm to 5000 µm [1], in most ecosystems including oceans [2], freshwaters [3], and

agricultural soil [4]. As a consequence, the presence of MPs along the food chain has been reported [5], drawing attention on the possible exposure of consumers[6–8].

As it has not been clarified yet whether MPs may have the potential to represent a threat to human health, the understanding of the abundance and properties of this emerging contaminant has become a key societal and scientific challenge. However, no fully validated analytical method is available for the quantification of MPs [9] and the data available in the literature are insufficient for a reliable estimation of MP exposure, hence for the assessment of the food safety risk related to MP contamination [5]. The method fit-for-purposeness is usually assessed by comparing performance characteristics with regulatory requirements or defined target values. The unavailability of validated methods for the analysis of MPs is partially due to the lack of standard reference materials and official guidelines for MP analysis. As a matter of fact, it has even not been determined yet whether the quality parameters conventionally used for analytical method validation are suitable for the evaluation of imaging-based MP quantification methods.

The analysis of bottled water represents a convenient starting point for analytical development and method validation. Several analytical techniques have been applied to the analysis of MPs in bottled water, including optical [10–13] and fluorescence [14] microscopy, (micro-)Fourier-transform infrared spectroscopy [11,12,14,15], (micro-)Raman spectroscopy [11,13,14,16,17], and scanning electron microscopy [13,15] with Energy Dispersive X-Ray Analysis [18,19]. Among these, Nile Red staining coupled with fluorescence microscopy [12,14] stands out as a promising option for the development of a high-throughput, easy, and affordable protocol for quantitative analysis of MPs. Nile Red is a solvatochromic dye with intense fluorescence when sequestrated in an hydrophobic pocket, therefore it has successfully been applied to the selective staining of plastic particles in combination with quantification of the stained particles by semi-automated image processing [20–27]. Despite the potential of this strategy, a complete, time efficient and reliable method integrating sample preparation, image acquisition, and fully automated image and data processing has not been described yet.

In this paper, we present an integrated method for the quantitative analysis of MPs in bottled water based on Nile Red staining, fluorescence microscopy, and automated image processing (**Fig. 1**). The performance of the method was successfully validated against common criteria such as limit of detection and limit of quantification, linearity and linearity range, repeatability, in-laboratory reproducibility and recovery, and its applicability was tested by the analysis of 15 samples of commercial bottled water containing a range of different additives. The developed protocol resulted in a reliable, high throughput, and easy to benchmark method.

Materials and methods

Reagents and standards. Recent work [5] shows that polyethylene (PE) fragments are the most frequently found MPs in food samples. Therefore PE MPs (ultra-high molecular weight, surface-modified, powder, 40-48 µm, Sigma-Aldrich, Schnelldorf, Germany) were used in both method development and spiking inultrapure water. The accuracy of particle size measurements was assessed using polystyrene (PS) analytical standards from Sigma-Aldrich with certified particle sizes (10 µm, 30 µm, 100 µm, 150 µm, 200 µm). The staining protocol was also tested on low-density -polyethylene (LDPE) 300 µm, polyamide-6 (PA 6) 55 µm, poly(hydroxy butyrate) / poly(hydroxy valerate) 2% biopolymer 300 µm, poly(ethylene terephthalate) (PET) 300 µm, poly(tetrafluoro ethylene) (PTFE) 20 µm, poly(vinyl denechloride) (PVDC) <180 µm from Goodfellow (Hamburg, Germany); blue PE microsphere 125 – 150 µm was purchased from Cospheric (Santa Barbara, California); and, microparticles based on poly(methyl methacrylate) (PMMA) 100 µm were purchased from Sigma-Aldrich. Aqueous MPs suspensions were prepared in 0.02% Tween20 (Sigma Aldrich), 0.02% sodium dodecyl sulfate (SDS), (Sigma Aldrich), or 0.02% Triton X-100 (Merk, Darmstadt, Germany). A stock solution of Nile Red (Sigma-Aldrich) was prepared in HPLC-grade acetone (Actu-All Chemicals) and diluted to in HPLC-grade ethanol (Supelco, 64-17-5). The applicability of the method was tested on 15 bottled water samples purchased at a local store, for details see Table S1 in the supplementary information.

Filtration. Filtration was performed using a glass vacuum filtration device (Sartorius) connected to a mini diaphragm vacuum pump (VWR, model VP 86). Several filters were tested for their compatibility with the method: black polycarbonate membrane filters (pore size 0.2 µm, diameter 25 mm. Whatman, United Kingdom), alumina-based filter membranes (Anodisc, pore size 0.2 µm, diameter 25 mm. Whatman), cellulose nitrate filters (pore size 0.2 µm, diameter 25 mm, Whatman).

Contamination prevention. To prevent environmental contamination of samples and standards, the following good practices were incorporated into the method and considered mandatory preparatory steps prior to sample preparation: (I) lab work on samples may take place only under laminar bench flow; (II) 100% cotton lab coats must be worn during operations; (III) only 100% non-particle releasing nitrile gloves may be worn during the operations; (IV) any piece of glassware to be used during the operations shall be washed, additionally to the routine cleaning procedure, with ultrapure water and a suitable surfactant and shall be rinsed in analytical grade acetone prior the operation; (V) sample should always be covered with aluminum foil or clean glassware; (VI) metallic labware shall be rinsed with analytical grade acetone immediately before operations.

Development of Nile Red staining procedure. The staining procedure was adapted from the study by Konde *et al* [25]. Briefly, a 0.5 mg/mL stock solution of Nile Red in acetone was prepared and further diluted in ethanol to obtain a 20 µg/mL staining solution. An adequate volume of staining solution was added to each sample to a final level of 0.4 µg/mL sample. Samples were incubated for 15 minutes and filtered. Then, each filter was laid on a glass microscopy slide and protected with a coverslip tightly taped onto the microscope slide. The staining protocol was tested on a set of different polymers, namely PE, LDPE, PS, PA 6, PET, PTFE, PVDC and PMMA. Three different surfactants were tested to obtain a homogeneous dispersion of MPs in the water matrix: SDS, Triton X-100, and Tween20. 4 mg of PE were dispersed in 100 mL of a 0.2% solution of each surfactant in ultrapure water. After staining, three aliquots of 25 mL were sampled, filtered, and analyzed according to the protocol described below. Different filter

materials were tested for their suitability for the fluorescence detection of Nile Red stained MPs. All the filters had a 25 mm diameter and a pore size of 0.2 µm. 0.1 ppm and 1 ppm suspensions of PE in ultrapure water with 0.2% Tween20 were stained and filtrated using black polycarbonate, alumina or cellulose nitrate membrane filters.

Image data acquisition. Images were acquired with an Olympus BX51 microscope, equipped with Olympus 4x, 10x, 20x and 40x objectives, an Olympus SC50 camera, an Olympus U-RFL-T UV lamp, and a band pass filter characterized by excitation and emission wavelengths of, respectively, 460-490 nm and >515 nm. The images, acquired by cellSens software (Olympus), were saved in Tagged Image File Format (.tif). 21 images (1.4 mm x 1.1 mm) were taken for each filter, accounting for 9.94% of the entire filter area. To keep into account the inhomogeneous distribution of the particles, the surface of the filter was divided into three concentric regions of equal areas, and the same number of pictures was taken for each region. A schematic diagram for the image data acquisition is given in the supplementary information (Figure S1).

Image data processing. A macro for image processing was developed in ImageJ (https://imagej.nih.gov/), and the script will be provided in a repository upon publication of the manuscript. Statical analysis was performed in RStudio (Windows Version 1.3.959) and Excel Office360 (Microsoft, California, USA). Image processing was performed on a PC HP Core (TM) i7-6600U CPU @ 2.60GHz (8.00 GB RAM) with a Windows 10 64-bit operating system. The 21 images from each filter were saved in a dedicated input folder to be processed in batch. For each image, a comma-separated values (CSV) file is generated. Briefly, the macro splits each image into three color channels and selects the green channel for further processing. An automated threshold ("Li" [28]) is then used to binarize the image, overlapping particles are distinguished by applying the watershed function, and the noise is reduced by applying default ImageJ functions. Once the pre-processing is completed, particle numbers are obtained by counting and particle sizes are measured, the major and minor axis of the ellipse that best fits on each particle are reported; size data

from combined particles provide the MPs range. In addition, the MPs mass can be calculated:first the particle volume is estimated by assuming that the individual particle shapes are ellipsoidic. Then, the volume of each particle is calculated assuming that the third dimension of the MP equals the minor axis of the best fitting ellipse, as previously reported [4,29]. Finally, using polymer density data, the MP mass can be estimated and reported in the csv file. The cumulative weight of all particles detected in each image is calculated and reported as well. Of course, such an MP mass estimation procedure based on 2D and 3D shape assumption requires a critical evaluation.

The Li threshold algorithm works by minimizing the cross-entropy between the foreground and the foreground mean and the background and the background mean [28], and is therefore dependent on the presence of at least one particle per image. In the case of pictures not containing fluorescent particles, the background noise interferes with the thresholds calculation and yields false positive results. To overcome this issue, our macro artificially adds a particle (Figure S2) in the top left corner of each picture. The particle is always reported in the first row of the csv file and ignored in the calculation of cumulative weight and in the data analysis. Finally, the total particle number or the total weight of MPs on the filter (either as absolute value or percentage recovery) was computed, and used to calculate the confidence interval of the analysis by bootstrapping.

Method performance assessment. The accuracy of particle sizing was estimated by measuring PS analytical standards with certified particle sizes ranging from 10 to 200 µm, linearity within this working range was studied.

The method limit of detection (mLOD) in terms of particle number and weight was estimated based on 7 blank samples (ultrapure water) as the mean number of particles plus 3 times the standard deviation. The limit of quantification (LOQ) was estimated as 3 times the mLOD. Linearity within the working range of 0.01 – 1.5 ppm was studied by the analysis of PE spiked ultrapure water. Similarly, the repeatability as

relative standard deviation (RSD, %) was determined from the analysis of 7 samples of ultrapure water spiked at a level of 0.10 ppm and 1.00 ppm. The reproducibility (here the within-laboratory reproducibility) was estimated from the analysis of 7 ultrapure water samples spiked at a level of 0.10 ppm and 1.00 ppm, the experiments were repeated on 3 different days. The computed recovery from the image data analysis was evaluated on ultrapure water samples spiked at a level 0.10 ppm and 1.00 ppm.

Analysis of microplastics in bottled water. An adequate volume of Tween20 was added to each sample to reach a 0.2% concentration (v/v). 500 mL of each water sample were filtrated on a black polycarbonate membrane filter. The filtration was stopped after the last 24.5 mL of sample were poured into the filtration unit, 500 µL of staining solution were added and the sample was incubated for 15 min. Afterwards, the filtration was completed and the filter stored as previously described.

Results and discussion

The objective of the development of this method for the analysis of MPs based on Nile Red staining, fluorescence microscopy, and automated image processing was to deliver a reliable, fast, and easy protocol for the quantification of MPs in drinking water. As for reliability, the performance of the method was assessed and will be discussed in the following paragraphs.

Critical reagents and materials used for filtration. Staining conditions as optimized by Konde *et al* [25] resulted successful in the staining of our heterogenous set of polymers in form of MPs. However, the addition of a surfactant was needed to prevent MPs from agglomerating or adhering to the walls of the glassware, and to yield satisfactory recovery values. No statistical significance was observed in the recovery of PE MPs dispersed in ultrapure water and SDS, Triton X-100, and Tween20 (one-way ANOVA test, $p = 0.75$). Tween20 was selected for further experiments in an effort to minimize contamination as it was available in a glass packaging. Then, we evaluated the effect of different filters on the ability to quantify MPs abundancy. Figure 2 illustrates Nile Red stained PE particles isolated on different filters (**Fig.**

2A-C). The filter material significantly affects particle quantification (one-way ANOVA test, $p = 2.3*10^{-7}$ at a concentration of 0.1 ppm and $p = 2.2*10^{-4}$ at a concentration of 1 ppm). Alumina-based and Cellulose nitrate filters (Fig 2C) showed a more intense background fluorescence which interfered with the image segmentation and resulted in computed recovery values higher than 200%. A Student t-test was performed to compare polycarbonate and alumina based filters, the different performance ($p = 2*10^{-3}$ and $p = 3.4*10^{-2}$ at 0.1 ppm and 1 ppm respectively) might be explained by the more tricky handling of alumina filters: MPs isolated on their surface are easily displaced. This results in particle agglomerates that interfere with the correct image segmentation, object classification, and ultimately quantification. The use of polycarbonate filters yielded the best computed recovery values at both 0.1 and 1 ppm levels (Fig 2D).

Automation potential of the protocol. Sample preparation - including contamination prevention, staining, incubation, filtration, and slide preparation for microscopic analysis – can be considered as the time limiting step in the proposed protocol. However, multiple samples can be processed in parallel. Thus, despite the extra care necessary for contamination prevention and in the handling of the filters to prevent particle loss or displacement, the protocol can be routinely performed by non-expert – but obviously trained – personnel. Automated image acquisition of a subsample of the whole membrane filter and fully automated image and data analysis allow the effortless completion of the analysis.

Performance characteristics. While the resolution of microscopy techniques depends on the underlying physics and is well established, the performance of algorithms for the automatized image segmentation can affect size measurement and the estimation of particle weight thereof. Therefore, the accuracy in particle size measurement was assessed by the analysis of analytical standards of PS microspheres with certified diameter. **Figure 3A** shows the average measured diameter as a function of the nominal diameter of 10, 30, 100, 150, and 200 µm microspheres. As expected, the measurements lie on a straight line with an unitary angular coefficient. 10 µm is considered as the lower detection limit of the method.

Accordingly, only fluorescent particles with a major dimension larger than 10 µm were included in the quantitative analysis.

In the full or partial validation of analytical methods, the performance characteristics must meet target values established by the applicable legislation and guidelines. As no guidelines nor dedicated legislation is available for MP analysis, general criteria derived from the Association of Official Analytical Chemists (AOAC) Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [30] were used to set target values for quantitative performance assessment of the developed method.

As illustrated in **Figure 3B** (insert), the method mLOD in terms of mass concentration is $1.1*10^{-3}$ ppm, calculated as the mean MP weight in 7 blank samples plus 3 times the standard deviation. The LOQ = $3.4*10^{-3}$ ppm, calculated as 3 times the mLOD. When calculated per number of particles in the 7 blank samples, mLOD and LOQ are respectively 102 and 305 items per filter. The working range in mass concentration spans from 1.1 ppb to 1.50 ppm (**Fig. 3B**). Linearity was studied in the concentration range of 0.01 – 1.5 ppm and resulted in a very good regression coefficient of $R^2 = 0.99$ (target value 0.98-0.999).

The repeatability of mass concentration, determined from the analysis of 7 spiked ultrapure water samples (**Fig. 3C**), is 12% RSD (relative standard deviation) at the concentration of 0.10 ppm and 11% RSD at 1.00 ppm (target values 5.5 to 22% 4 to 16%, respectively). The within-laboratory reproducibility, determined from the analysis of 7 spiked ultrapure water samples repeated on 3 different days (Fig 3C), is 29% RSD at 0.10 ppm and 16% RSD at 1.00 ppm (target values 11.5 – 46% and 8 – 32% for 0.1 ppm and 1.0 ppm, respectively).

As no reference or test material is available, a recovery study was performed to estimate the trueness of the method (**Fig. 3D**). The computed recovery of MPs spiked in ultra-pure water is 92.6 ± 23.3 % (mean ±

SD, n = 21) at a concentration of 0.1 ppm and 97.3 ± 14.5 % (mean ± SD, n = 21) at 1.0 ppm. The recovery complies with the target values of 70-120 % and 75-120 % at 0.1 and 1 ppm, respectively.

The results of the quantitative performance evaluation not only proof the suitability of this method for the analysis of MPs in bottled water, but also demonstrates that conventional performance parameters used in regulatory frameworks, can also be applied to the field of MP analysis.

Analysis of microplastics in bottled water. 15 samples of bottled water purchased at a local store were analyzed for MP contamination. Details on the sales denomination, water origin, bottle material and volume are available in SI (Table S1). The sample set was quite different and comprised still, sparkling, carbonated, mineral, spring, filtered, and flavoured waters and combinations thereof.

The number of Nile Red stained particles was found to be below the method detection limit in five samples and below the limit of quantification in two samples. **Figure 4A** illustrates the quantification of fluorescent particles in the samples showing a contamination level above the limit of detection. A boxplot chart was used to visualize the bootstrap estimate and the 95% confidence interval of each quantitative measurement, the image output? data used to build the chart are available in SI (Table S2). As the analysis is performed on a sub-section of the whole membrane filter, the bootstrap estimation is necessary to provide each quantitative data with an expanded measurement uncertainty, that is an interval for the result of a measurement expected to contain a large part of the distribution of values that can result from the measurement. The contamination level ranges from a minimum of 392 to a maximum of 6329 items in 500 mL of bottled water, a graphic representation of the expanded measurement uncertainty is given in **Figure 4A** and the related exact values are available in SI (Table S2).

For the study of particle size, the major dimension of the analyzed particles was evaluated. The 86% of the analyzed particles measured less than 50 µm, while only the 4% measures more than 100 µm. The size of the smallest detected particles overlaps with the detection limit of 10 µm, the largest detected particle

measures 506 µm. The size distributions of the fluorescent particles isolated from each sample are reported in figure 4B, the data used to build the chart are included in SI (Table S3). The medians range from 17 to 40 µm and all of the distributions are right-skewed, coherently with the considerable portion of the analyzed particles measuring less than 20 µm (38%).

Conclusions

In this study, a critical evaluation of MP quantification based on Nile Red staining, fluorescence microscopy, and automated image processing was performed. The accuracy of the method in sizing and quantifying MPs was assessed. Repeatability, within-laboratory reproducibility, and recovery complied with the AOAC general criteria for analytical methods. Conventional quality parameters, regularly used for the validation of analytical methods, showed to be applicable to the assessment of method performance. The method was applied to a wide range of commercial bottled water samples and their ingredients were found not to interfere with the developed method.

A reliable, high throughput, and effortless protocol for the quantitative analysis of MPs is now available for further development in the field of MPs analysis in food, following the future optimization and validation of specific sample preparation procedure for the isolation of MPs from complex matrices.

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Figures

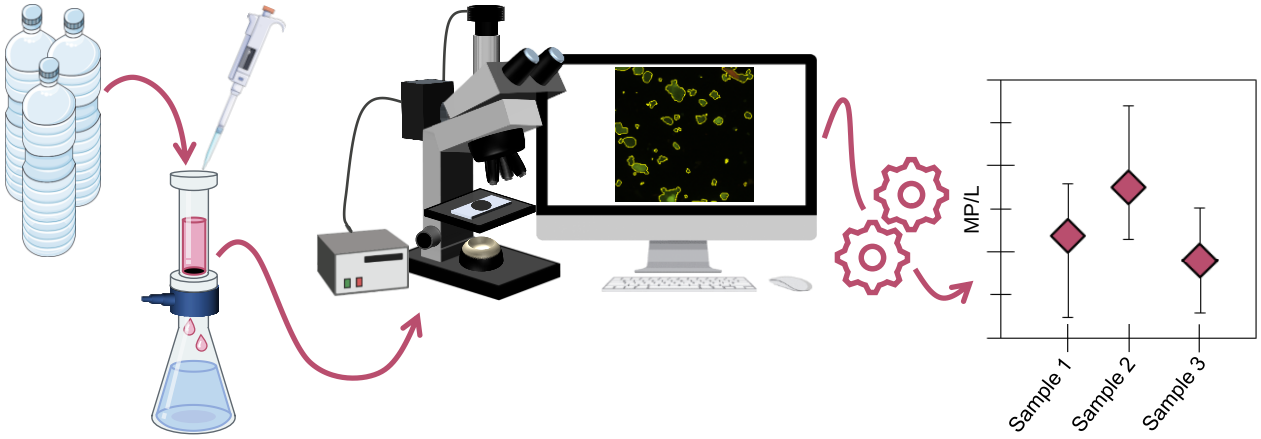

Figure 1. *Workflow for the analysis of microplastics in bottled water by Nile Red staining and fluorescence microscopy. The sample is preconcentrated and stained in the filtration unit. After an incubation of 15 min, the filtration is completed. Images are acquired under the fluorescence microscope. The automated image processing yields both qualitative as well as quantitative analysis data of the stained particles.*

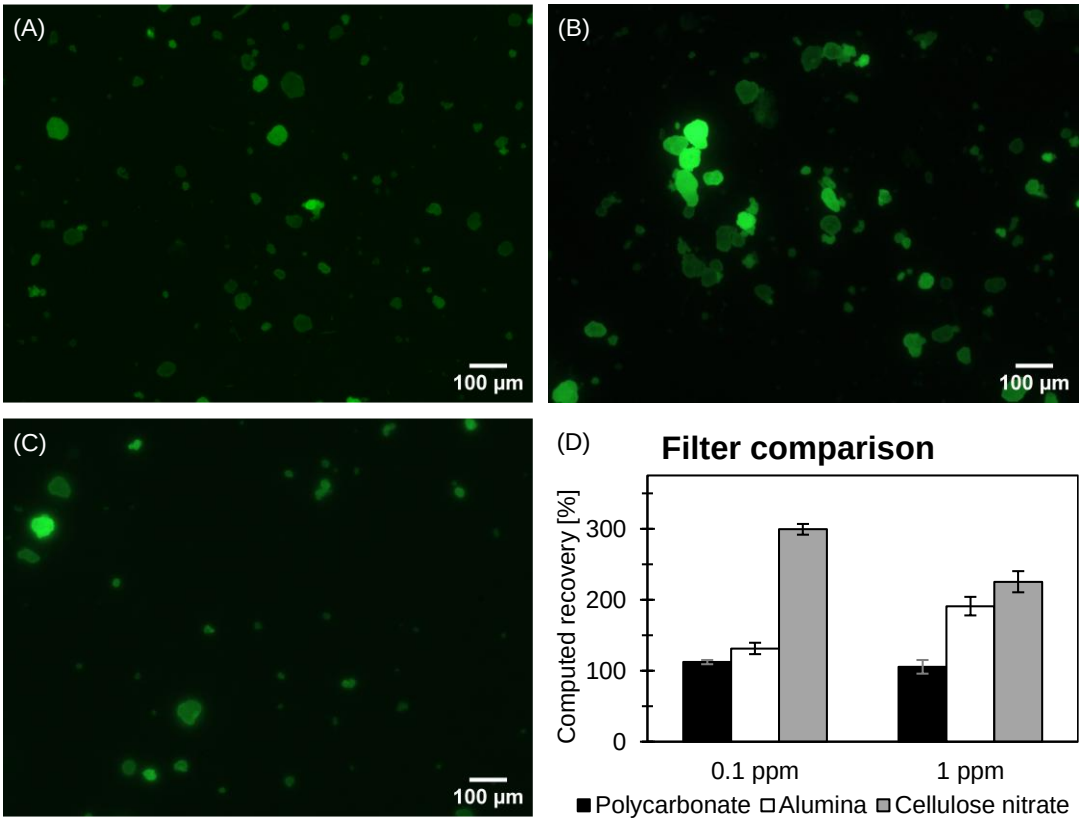

Figure 2. *Comparison of polycarbonate, alumina and cellulose nitrate filters for their suitability for the analysis of MPs by Nile Red staining and fluorescence microscopy. Nile Red stained polyethylene (PE) microplastics (MPs) isolated on (A) black polycarbonate membrane filter, (B) alumina filter membrane, and (C) cellulose nitrate filter. (D) computed recovery from image data analysis of PE MPs dispersed in ultrapure water, isolated and analyzed on filters of the three different materials; average ± SD (n = 3).*

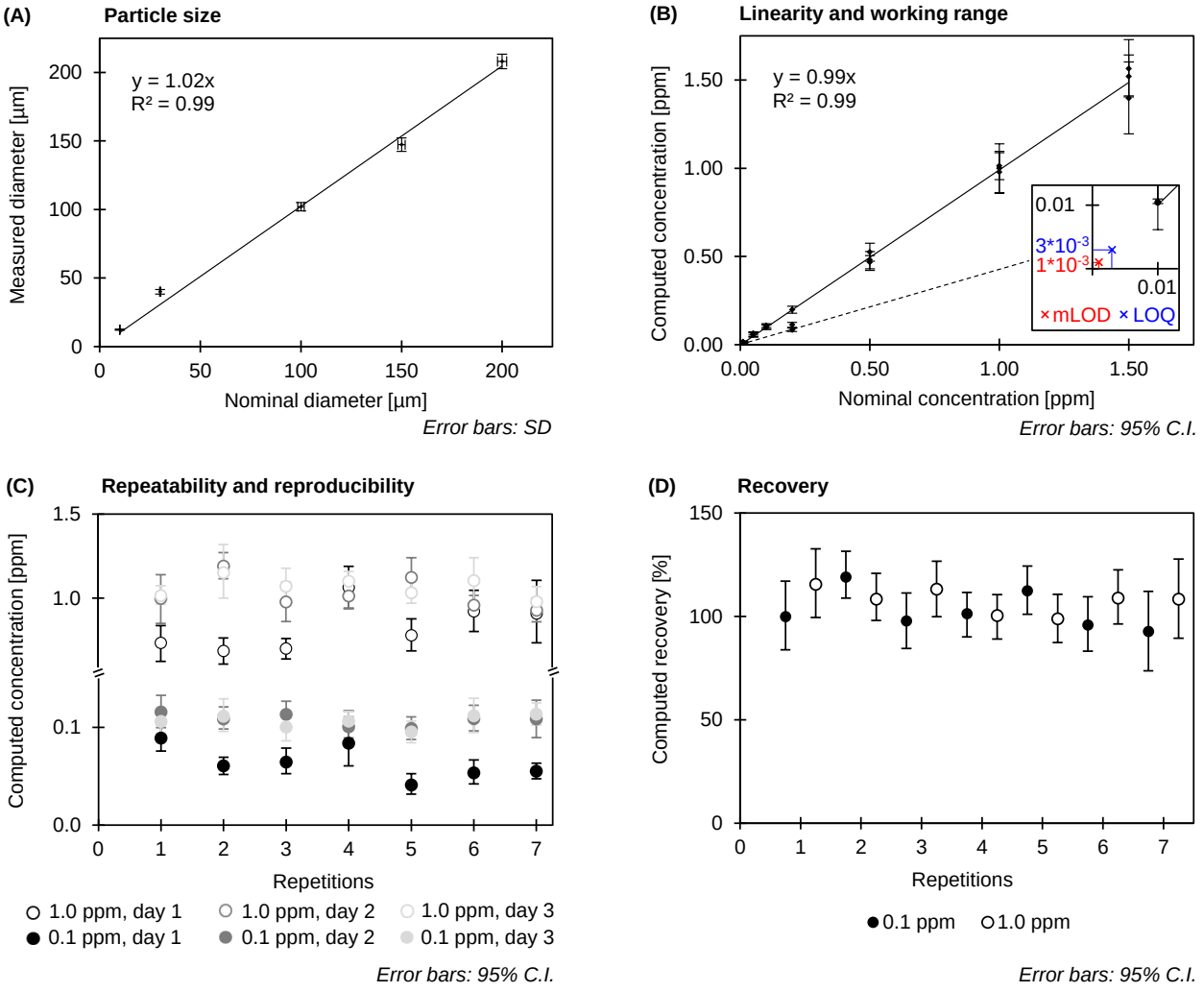

Figure 3. *Method performance characteristics. (A) Evaluation of the accuracy and precision in particle size measurements. Vertical error bars refer to standard deviation (SD) for n = 6 individual particles, horizontal error bars refer to SD of particle size as reported in the size certificate of the analytical standard. (B) Study of linearity and working range in microplastics quantification. Error bars represent 95% confidence interval (C.I.). The insert illustrates the method limit of detection (LOD) and limit of quantification (mLOQ). (C) Repeatability and within-lab reproducibility for microplastic quantification. (D) Recovery study.*

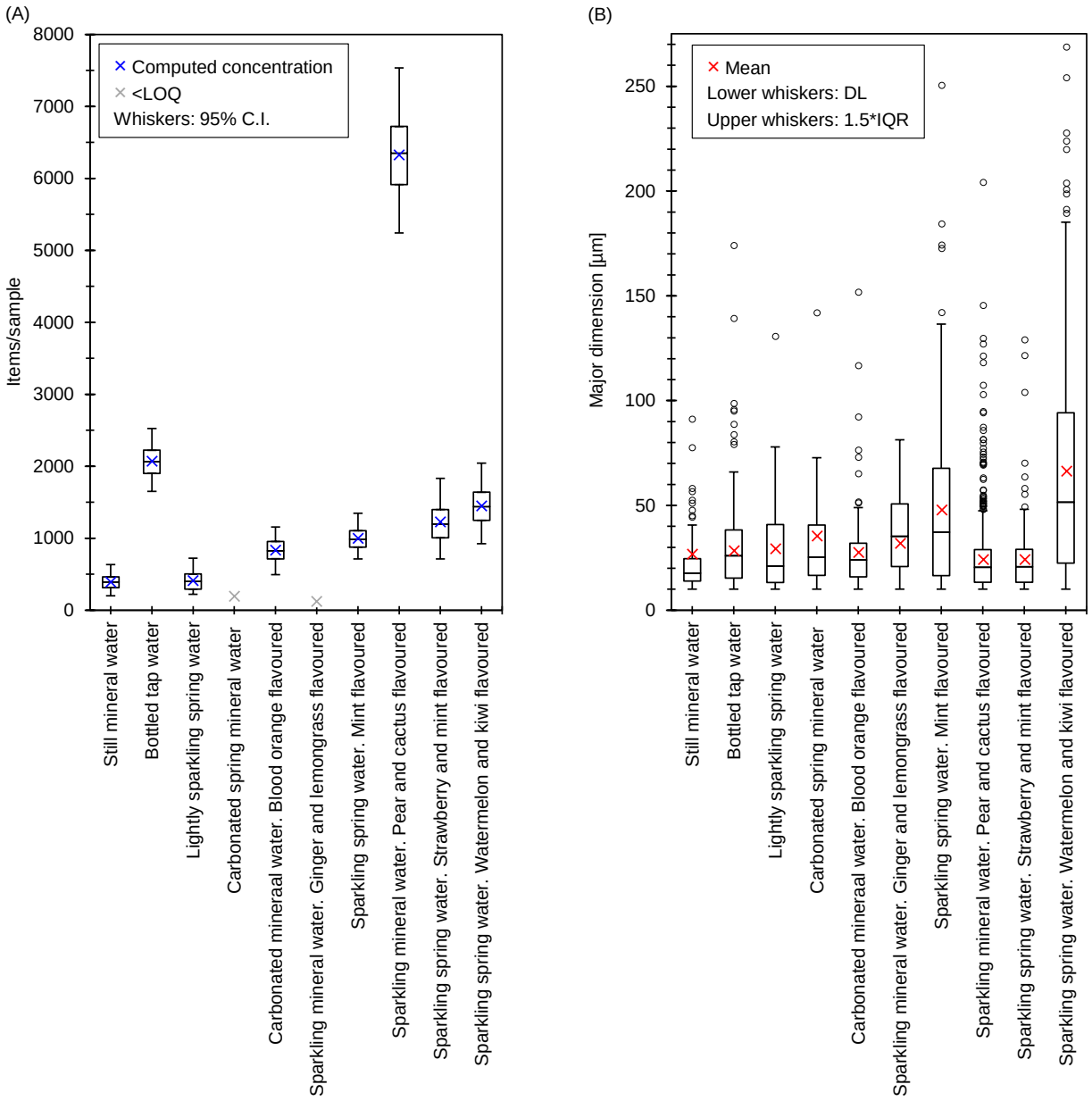

Figure 4. *Analysis of real samples. (A) Number of Nile Red stained microparticle quantification in 500 mL of bottled water. Boxplots of bootstrap estimates, see text. Each box illustrates first quartile, second quartile, and median of the estimated distributions, whiskers represent 95% confidence intervals (C.I.). (B) Size distribution of the analyzed Nile Red stained microparticles. DL, detection limit as per particle size; IQR, interquartile range.*