Microplastics and Nanoplastics

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A novel proof of concept approach towards generating reference microplastic particles

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Abstract

For almost two decades now, scientists have increasingly focused on the occurrence of microplastic particles (MPs) in the environment and their impact on environmental and human health. Currently, the variety of analytical methods used in microplastic research result in data of different quality. This largely hampers comparability between data sets and consequently prevents a reliable risk assessment. In this context, the lack of suitable reference microplastic particles (RMPs) that can be added as an internal standard in an exactly known number further prevents quality assessment of, and harmonization in terms of comparability between different analytical methods. Although this challenge has been widely recognized, the availability of RMPs is currently limited to commercially available particles in the form of micro-beads or -fragments (powders). Manual addition of such RMPs to samples in a precisely defined number as an internal standard is inefficient and the alternative use of MP suspensions does not allow for the addition of an exactly defined particle number. The optimum solution to solve this issue would be RMPs embedded in an easy-to-use soluble matrix in exact numbers. This would allow for evaluating analytical quality during microplastic analysis as well as establishing harmonization in terms of comparability between different methods. In the present study we focused on the development of such RMPs. We used computerized numerical controlled (CNC) milling to produce small diameter plastic columns followed by gelatine embedment and subsequent cryosectioning. This results in gelatin slices containing an exactly defined number of RMPs with well-defined size, shape and polymer type / chemical composition that can be added to a sample easily with the dissolution of the gelatine. We successfully produced square shaped RMPs in a size range of 125–1000 µm of five different polymers. The overall size-deviation of the RMPs never exceeded ± 11.2% from the mean value of a set of particles. The highest percentage mass-deviation was 25.5% from the mean value of a set of 125 × 125 × 20 µm polystyrene (PS) RMPs. Our approach allows for the production of RMPs tailored to specific needs of all different analytical methods used in current microplastic research. Beyond analytical method validation, these RMPs furthermore open possibilities for experiments on MPs in different fields.

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Keywords Microplastic, Reference microplastics, Soluble matrix, Method validation, Method harmonization, Standard addition, Internal standard

Introduction

Currently the demand for plastics continues to rise, with 390 million tons produced worldwide in 2021, and the forecasts point to a continuing upward trend [1]. Because of the increases in manufacture and use of plastic across the globe, it is anticipated that the amount of plastic waste will also increase [1]. There are a variety of mechanisms that can cause plastic to be released into the environment, either intentionally or unintentionally, with plastic waste representing an important issue that is currently being addressed at the international level through discussions aimed at establishing an international treaty on plastic waste [1–3]. An important goal of actions aimed at reducing the release of plastic waste into the environment is to prevent its accumulation, such as in soils, oceanic gyres and sediment [4–6].

Once in the environment plastic waste can fragment and degrade into microplastic particles (MPs), commonly defined as plastic particles < 5 mm [1, 2, 7–9]. Next to such secondary MP originating from disintegration of lager fragments or from abrasion during daily usage of plastic products, so-called primary MP produced in MP size additionally enters the environment [2]. Interest and research regarding the possible environmental fate and effects of MPs, which have been reported in a variety of environmental compartments from the poles to the equator as well as in organisms, has significantly increased over the last several years [10].

Although research aimed at better understanding exposure and effects of small plastic particles for more than 50 years [11] and ever-increasing research efforts have been made since the term "microplastics" has been coined in 2004 [12–16], many open questions remain regarding their sources, fate, and the availability of reliable data on environmental concentrations [2]. A key challenge towards generating reliable and relevant information relates to a lack of standard methods used for sampling, extraction, purification and analysis, which yield results of different quality and resolution and therefore hamper data comparability [17, 18]. Since MPs represent a complex heterogeneous mixture of plastic particles with different physical and chemical properties such as, e.g., polymer type, mass, shape, size and aging stage it is broadly accepted that MPs must be analyzed by chemical methods to obtain reliable results. Currently there are two major analytical approaches that are commonly used – particle-based spectroscopic techniques like Fourier-transform-infrared (FTIR) or Raman spectroscopy and mass-based spectrometric techniques like pyrolysis gas-chromatography-mass spectrometry (GC/MS) or thermal extraction – desorption (TED) GC/MS [17]. One of the key challenges for a comparison of these analytical approaches is the lack of a reliable method for converting particle-to-mass based concentrations, and vice versa. Additionally, the use of different protocols for sample treatment can significantly influence results, since during sample processing, such as through the adoption of density separation methods and/or sample digestion techniques, some MPs can be lost or destroyed, depending on the polymer type, size or shape [17–19]. Consequently, it is widely understood that the challenges associated with not being able to directly compare data generated by different research groups using different methods represents a significant impediment to generating a reliable and robust risk assessment of MPs for both environmental and human health. Consequently, to support both international treaty discussions and regional actions, such as the EU's Green Deal, there is an urgent need for reliable data to be generated from monitoring programmes applying harmonized methods. Thus, a key goal of the EU's Plastics Strategy or the Zero Pollution Action Plan, is such a reliable monitoring of MPs in the environment [20, 21].

As a first step towards strengthening data comparability, there is a need to improve quality assessment and harmonization of the different methods currently used for the detection of MPs in the environment. Given that MPs represent a diverse suite of contaminants, there are thus numerous challenges associated with evaluating the analytical performance of a specific method, such as in terms of recovery rates, detection limits etc., that may result from the interplay of all steps used across the entire analytical process, and which may be further influenced by the complexity of the sample matrix itself. Consequently, evaluation of an analytical workflow must, for instance, consider a number of important factors that might influence the reliability and relevance of data generated. These include, the influence of the sample method, homogenization of the sample, the reliability of taking subsamples to support an accurate quantification of the concentration in the total sample, a quantitative evaluation of the influence of extraction and purification methods (where necessary), and finally the relative reliability of the measurement and analysis method adopted. A fundamentally important element that would enable harmonization of methods is thus an appropriate suite of reference microplastic particles (RMPs). In principle, RMPs should ideally reflect the main polymer types that have been reported in the environment and should represent different particle size classes and shapes. Furthermore,

the ideal RMPs should be capable of being dosed into a sample in an accurately predefined number of MPs and/or total MP mass, consistent with the use of any internal standard used in chemical analysis, to enable the transparent quantification of recovery rates, detection limits, and analytical quality of both particle-based and mass-based methods.

Recognition of the important and urgent need for RMPs towards supporting harmonization of methods is not necessarily novel [19]. Indeed, there has been considerable effort that has already been initiated towards producing RMPs [21–27]. The most common methods that have been adopted to generate RMPs include cryo-milling, which can generate fragments of MPs [22, 25, 26] or emulsion processes, which result in the generation of microbeads [29, 30]. Consequently, the majority of RMPs that are currently commercially available take the form of microbeads, fragments or powders [31]. Generally, manual counting and addition of such RMPs to samples in a precisely defined number as an internal standard is time-consuming and inefficient. We also suggest that the alternative use of MPs in suspensions or the adoption of a mass-based introduction of particles, does not allow for the addition of an exactly defined number of particles, since there are small inhomogeneities in the suspension or in the particle number per mass that can occur.

To overcome inhomogeneity obstacles, attempts have recently been made to introduce a well-defined number of RMPs into a sample. For instance, Zobkov and Esiukova used 0.96 ± 0.39 mm x 0.96 ± 0.39 mm x 0.46 ± 0.02 mm square shaped PET particles for the validation of recovery rates of the density separator "MPSS" [32] in 2017 and recorded a recovery rate of 97.1% ± 2.6% for 100 particles per sample [22]. However, the RMPs had a relatively large variation in size and had to be counted manually to apply 100 RMPs to a sample [33]. Similarly, Möller et al. (2021) analyzed the impact of both enzymatic-oxidative digestion and the use of a $ZnCl_2$ density-separation technique on the structural integrity of MPs [19]. In this instance, Möller et al. (2021) used fibers, fragments and beads in a size range between 100 and 400 µm, for which there was a large variance in the size of the fiber- and fragment-shaped RMPs. Once again here, the adoption of manually counting particles represents a time-consuming procedure, which was used to introduce the RMPs into samples, and which does not reflect an efficient and reliable approach that could be adopted by the research community. The abovementioned examples, thus, demonstrate that recent attempts for evaluating data quality through the use of RMPs are inefficient and therefore the lack of efficiency represents a significant shortcoming that prevents a daily routine protocol that can be applied for data quality control.

Furthermore, RMPs should be well-characterized with respect to their properties (polymer type/chemical composition, size and shape), and there should be an efficient method to dose the RMPs into samples as an internal standard precisely and accurately. Access to a well-defined suite of RMPs that lend themselves to being efficiently added to samples would thus enable the evaluation of the influence of sample handling and processing on recovery rates, detection limits, etc., which would intuitively facilitate quality assurance of the analysis of MPs and harmonization between methods in terms of comparability. To our knowledge, there are currently no such RMPs.

To address the challenges summarized above, we propose a workflow that can be adopted for the production of RMPs, which can be designed with variable properties (polymer type, size, shape) and are fixed in a soluble matrix in a predefined number that can be introduced easily into samples as an internal standard. Here we present a proof of concept application, whereby RMPs were generated from plastic blocks, which are produced by injection molding as a base material for milling small diameter plastic columns on base plates using computerized numerical control (CNC) technology. The resulting column plates were embedded in gelatin and horizontal sections of the embedded columns were cut using cryo-microtomy. It is notable that the application of cryo-slicing has been previously used by Cole et al. (2016) for the preparation of short fibres, however, the application of CNC milling represents a novel component that allows more robust control over the shape of the particles, which has not been previously reported [34]. Following our procedure, a gelatin slice with a defined number of RMPs could be realized, which can be added to a sample and be dissolved by warming. As a proof of principle, our goal was to exemplarily produce square-shaped MPs from pure source material of five different relevant polymer types (low-density polyethylene (LD-PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET) and polylactic acid (PLA)), in defined quantities. After production, we characterized the properties of the RMPs using scanning electron microscopy (SEM), optical microscopy, mass-measurement as well as focal plane array (FPA) detector-based micro FTIR and Raman spectroscopy.

Materials and methods

Avoidance of contamination

To minimize airborne contamination with MPs during laboratory work, lab coats made of 100% cotton (Universal Labormantel # 1785048, Laborhandel Krumpholz, Selters, GER) were worn. Additionally, glass- and stainless-steel lids or aluminum foil were used to cover laboratory equipment and materials during all steps.

Embedding steps with warm gelatin were conducted under a Laminar FlowBox (Series „SuSi", Spetec Ltd., Erding, GER). The equipment was rinsed before and between every use with the sequence of milli Q water, 35% EtOH and again milli Q water. Ethanol and milli Q water used for rinsing etc. were filtered using filters with an average pore size of 0.2 µm (ME 24 Membrane Filters (Mixed cellulose ester) # WHA10401712, Merck KGaA, Darmstadt, GER). The respective solutions were applied with Polytetrafluoroethylene (PTFE) spray bottles and light hand pressure for rinsing. Whenever possible the use of plastic products was avoided.

Production of plastic polymer blocks

Plastic polymer blocks were manufactured by injection molding. We used a micro compounder (MC15 – Xplore Instruments BV, Sittard, NL) and an injection molder (IM 12 – Xplore Instruments BV, Sittard, NL) to melt, homogenize and injection mold different polymers. The polymers included in this study are LD-PE (Lupolen 1800P-1 – LyondellBasell AG, Rotterdam, NL), PP (Moplen HP526J – LyondellBasell AG, Rotterdam, NL), PET (CleanPET – Veolia AG, Paris, FR), PS (PS 158 N/L – Styrolution Ineos AG, Frankfurt, GER) and PLA (PLA IngeoTM 4043D – NatureWorks AG, Minnetonka, USA). Each of the polymers was used as raw material in the form of pellets to create blocks. LD-PE was processed at 230 °C, PP at 240 °C, PS at 220 °C and PET and PLA were both processed at 280 °C. All polymers were molded into 20 °C stainless steel molds at 8 bar for 2 × 8 s. The dimension of the blocks was 4 × 10 × 80 mm. After cooling, each block was cut into eight pieces of 4 × 10 × 10 mm (Fig. 1A) to prepare them for CNC milling.

CNC milling

Each piece of a plastic polymer block was processed using a CNC mill (CMX 600 V; DMG MORI Inc., Bielefeld, GER) equipped with a 1.5 × 4, 1.2 × 3 mm and 0.8 × 3 mm VHM micro milling cutter (Hoffmann SE, München, GER) (Fig. 1B). The milling process was performed using a maximum rotation speed of 12,000 rpm. During the milling process, an external water coolant feed was applied at 3.7 bar. Due to concerns that LD-PE would not withstand the thermal stress during the milling process, blocks of LD-PE were milled inside a bath of organic cooling lubricant (Biowas EP1; Wascut Industrieprodukte GmbH, Sierksdorf, GER). Milling was conducted according to a CAD model created using the open-source software FreeCAD v. 0.19. The final milling resulted in a base plate consisting of 25 columns on top (Fig. 1C). Each column had a height of 3 mm, and an intended x-y edge length of either 125, 250, 500 µm or 1000 µm, depending on the size of the intended RMPs to be generated. The columns were first cleaned from potential chippings using tweezers and then rinsed with milli Q water, followed by a second rinsing with 35% EtOH and milli Q water, aimed at ensuring complete removal of any loose chips that may have remained from the milling process. Ethanol and milli Q water used for rinsings were filtered according to the description in "Avoidance of contamination". The chips were discarded.

Cryo-microtomy

After the cleaning process the cleaned columns were embedded in liquid gelatin (0.2 g/ml) at 60 °C (Gelatine Silber, extra pure, 140 Bloom; Carl Roth Ltd., Karlsruhe, GER) (Fig. 1D) as preparation for cryo-microtomy. Prior to this step, the warm liquid gelatin was ultrasonicated for 5 min to remove air bubbles. It is important to remove any air bubbles from the gelatin, as these can

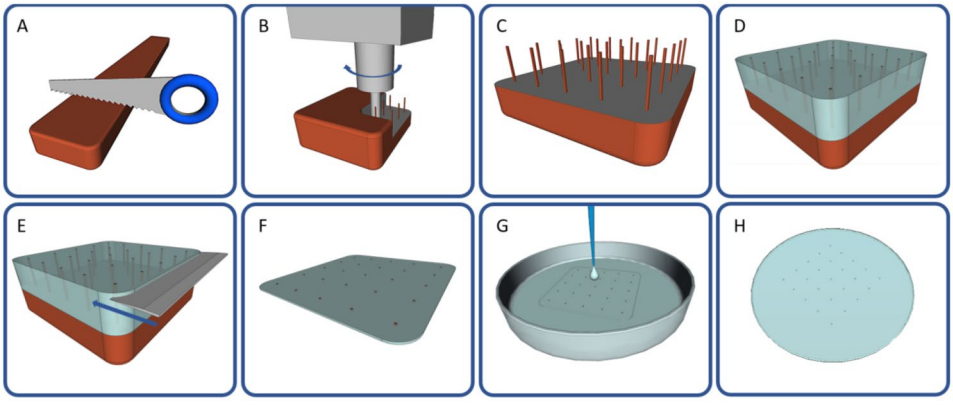

Fig. 1 Manufacture of RMPs using CNC milling and cryo-microtomy. (**A**) An injection molded polymer block is cut into square pieces. (**B**-**C**) The cut pieces are processed using a CNC mill and a polymer column plate is created. (**D**) The polymer column plate is embedded in gelatin. (**E**-**F**) the embedded column plate is cut into slices using a cryo-microtome. (**G**-**H**) The slices are further covered with a thin film of gelatin from both sides to facilitate handling and ensure no RMPs are lost during handling

interfere with the proper embedment of the columns and fixation of RMPs in the gelatin slices, respectively, after the columns are cut. The gelatin was kept at 60 °C in a 250 ml glass flask surrounded by a heat-insulating apron made of PS-foam on a hot plate for the entire time it was needed. We note that it was necessary to maintain the hot plate at a temperature of 100 °C in order to maintain a 60 °C temperature of the gelatin, which we attribute to dissipation losses of heat, despite the adoption of insulating materials.

For embedding, the column plates were placed in self-made stainless-steel casting molds, covered with warm gelatin using a glass pipette and subsequently frozen. Freezing was conducted inside the cutting chamber of a cryo-microtome (CM1950; Leica Camera Inc., Wetzlar, GER) at -19 °C for 10 min. Stainless-steel molds include a small hole on the bottom (⌀ ~ 2 mm), which is used to allow the embedded column plates to be manually pushed out by means of a pin after being frozen. The ejected column plates were next cut at -19 °C using the cryo microtome (Fig. 1E), which resulted in horizontal gelatin slices that each contained 25 RMPs (Fig. 1F). We note that the first 10 sections were cut to a thickness of 50 µm in order to trim the columns to an optimal cutting plane, with the trimmings being discarded. This procedure supported uniformity of RMPs and helped to remove any potential defects occuring at the tip of the columns. The step was monitored using a stereomicroscope (MS5; Leica Camera Inc., Wetzlar, GER) at 25 times magnification. The following 100 cuts were adjusted to 20 µm thickness each (resulting in 20 µm thick gelatin slices containing 20 µm thick RMP particles). These slices were placed in glass petri dishes (Ø 30 mm) filled with a thin layer of liquid gelatin, and were subsequently covered with an additional thin layer of liquid gelatin at room temperature (Fig. 1G). The addition of the thin gelatin layer was used to fix and seal the particles securely in the gelatin slice, which helps to prevent losses of the RMPs during handling. The slices were dried using an exicator under low pressure of 300 mbar for 48 h. After this step, the gelatin slices were observed to shrink, allowing for relatively easy removal of the slices from the glass petri dish, which were then ready for use or storage.

Analysis

Stereomicroscopy

To control the number of particles embedded in the gelatin slices we used a stereo microscope (MS5; Leica Camera Inc., Wetzlar, GER) at 25 times magnification. The RMPs were observed using top light resulting in good contrast to the gelatin (Fig. 2B). This microscope was not used for size measurements.

FTIR spectroscopy

To monitor for potential polymer changes introduced by the production procedure, we analyzed the chemical composition of the RMPs via FTIR spectroscopy. For preparation for FPA-µFTIR measurements we dissolved a gelatin slice in 200 ml of milli Q water with 60 °C filtered according to the description in "Avoidance of contamination" and filtered the RMPs directly onto aluminum oxide filters (pore size 0.2 µm, Whatman Anodisc inorganic

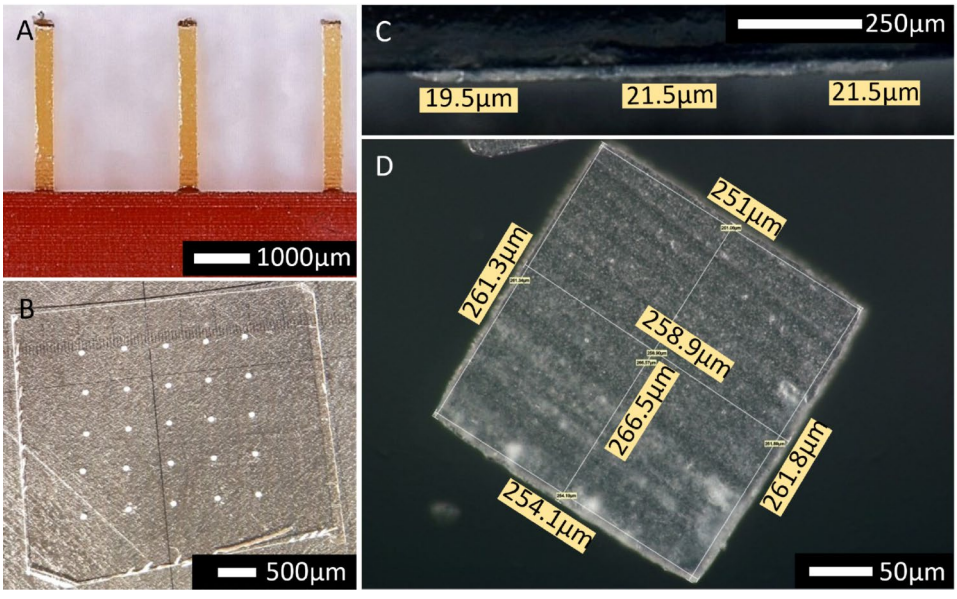

Fig. 2 Microscopic images of a polymer column plate manufactured using injection molding and CNC milling (**A**), a cut gelatin pad including a desired amount of RMPs (**B**), a measurement of the z-size of a 1000 µm x 1000 µm x 20 µm sized particle (**C**), a measurement of the x-y-dimensions of a 250 µm x 250 µm x 20 µm sized particle (**D**)

filter membrane # WHA68096022, Merck Ltd., Darmstadt, GER) using a custom-made glass funnel and a filter holder mounted on a suction bottle. During filtration the whole filtration setup was heated to 60 °C using a heating plate. We note that the heating step was necessary to prevent clogging of the filter from gelatin cooling due to prolonged filtration resulting from the small pore size of the filters (0.2 µm). After filtration, each aluminum oxide filter was dried for 24 h to minimize H_2O IR interferences during FPA-µFTIR analysis. We used a Hyperion 3000 µFTIR microscope with a 3.5 x IR objective and a 64 × 64 pixel FPA detector coupled to a Tensor 27 spectrometer (both Bruker Optics GmbH & Co. KG, Ettlingen, GER) for analyzing the filters containing the RMPs in the wave number range from 3600 –1250 cm^{-1} with a wavenumber resolution of 8 cm^{-1} as well as an accumulation of 32 scans and a resulting pixel resolution of 11 µm. The measurement was operated by the Bruker software Opus 7.5.

Raman spectroscopy

Raman spectroscopy represents an alternative and reliable analytical approach that is frequently used for the chemical analysis of polymers and MPs. Thus, we additionally performed Raman measurements of the RMPs. We used an Alpha 300 RA + Raman microscope (Oxford Instruments plc, Abingdon, ENG) operated using a frequency doubled Nd-YAG laser with an excitation wavelength of 532 nm at 25 mW, a 50x magnification objective, an EMCCD detector with 1600 × 200 pixels, resulting in a resolution of around 2 µm, and the WITec Suite FIVE software. Each measured image was composed of 5000 spectra (100 points per line x 50 lines per image) in the wavenumber range between 3600 –500 cm^{-1}. We used a large area scanning mode for Raman imaging of the samples. Each sample comprised of 5 RMPs of each polymer type. The RMPs were placed on slides made of quartz. Prior to that, the RMPs were released from the gelatin slice using the filtration approach as described in "FTIR spectroscopy". We transferred the RMPs from the filters to the slides by using a hair glued to a pipette tip. A particle was touched with the tip of the hair, causing it to stick to it by electrostatic forces until it could be wiped off on the quartz slide.

SEM

To analyze the surface of the RMPs we used a SEM (JSM-IT500LA; JEOL Ltd., Präfektur Tokio, JPN). The SEM was operated using a 3 kV gun voltage, a contrast set of 1872 units and a brightness set of 1700 units. For SEM analysis, gelatin slices from all types of RMPs produced were dissolved using filtered milli Q water with 60 °C. The dissolution was performed inside of a funnel of a stainless-steel vacuum filtration system (threefold stainless steel filtration system # 16828, Sartorius AG, Göttingen, GER) equipped with stainless steel filters with an average pore size of 2 µm (diameter: 47 mm, Rolf Körner GmbH, Niederzier, GER). After dissolution the supernatant was removed with a pump (LABOPORT N820 # N 820 FT.18, KNF Neuberger GmbH, Freiburg, GER).

Prior to SEM measurement we performed three ethanol drying steps and mounted the RMPs on stabs for sputtering with platinum using an e-beam coater (EM ACE600; Leica Camera Inc., Wetzlar, GER). We transferred the RMPs from the filters to the stabs according to the procedure described in "Raman spectroscopy".

Size measurements

We measured the sizes of the RMPs using a digital microscope equipped with a PLANAPO FOV 43,75 mm objective (Leica DVM6M; Leica Microsystems, Wetzlar, GER) at 100 times magnification. For analyzing the images of the RMPs we used the software LAS X v. 3.0.8. We measured the edge lengths of a particle in the x-, y- and z-dimension (Fig. 2). For the measurements of the x- and y-dimension, we placed the particles on object slides according to the description in "Raman spectroscopy". For the measurements of the z-dimension, we placed two object slides on top of each other in a way that a step was created along the longer edges. We applied a thin layer of beeswax to this step and then lined up the RMPs so that their edges pointed upwards. This way the height of the RMPs could be measured using the same digital microscope. Each dimension was measured in the center and at both edges (Fig. 2). We recorded each of these three size measurements per dimension for five replicates per polymer type and size class of our RMPs.

Gravimetric measurements

To investigate the suitability in terms of mass constancy of the RMPs as internal standards for mass-based analyses we weighed the RMPs using an ultra-microbalance (Mettler-Toledo XPR6U, Bayerische Waagenbau Werkstätte - Althaus GmbH, GER). First, we dissolved a gelatin slice containing the RMPs using filtered milli Q water with 60 °C. For filtration of the RMPs we used the same stainless-steel filtration device as described in "SEM" with the same 2 µm stainless steel filters. 10–15 pieces of the released RMPs of one size class and polymer type were pooled for one mass measurement to exceed the lower sensitivity threshold of the ultrafine balance. We replicated each mass measurement five times for each size class and polymer type.

Data analysis

FTIR spectroscopy

After measurement, data were imported into the software ImageLab (Epina GmbH, Retz, AT) to analyze the resulting data in combination with the noncommercial,

custom-made software "Bayreuth Particle Finder v. 4.09" for automatic MP detection based on random forest decision classifiers [34, 35]. The automated polymer identification was checked manually for quality control by using reference spectra from our in-house reference spectral library for respective polymers.

Raman spectroscopy

For data analysis we performed a background subtraction for shape fitting as well as a true component analysis for identification of different spectral components using the software R v. 1.2.5033.

Size measurements

The mean and standard deviation of the size of a single particle for each dimension (x-, y- and z, three measurements per dimension - see "Size measurements") was determined based on the data obtained from five replicates, for each polymer type and size class.

Gravimetric measurements

The mean and standard deviation of the mass of a single particle for each size class and polymer type was determined based on the gravimetric measurement of five replicates, each of which included 10–15 particles per replicate.

Results

Physicochemical property characterization for generated RMPs

Particle size and shape characterization

Results for the characterization of the particle size of the RMPs of each plastic type generated for each of the four size classes 1000, 500, 250 µm and 125 µm are summarized in Fig. 3A and B. The x-y-dimension of all RMPs in total never exceeded ± 24.36 µm (9.67%) from the mean values for the corresponding particle size class (Fig. 3A). The mean z-size (height) of all RMPs measured ($n = 600$) was 21.53 ± 2.89 µm (Fig. 3B). With 20.49 ± 1.19 µm we observed the smallest z-size deviation of the mean value for the 125 × 125 µm x-y-edge length LD-PE RMPs and the largest z-size deviation of the mean value could be

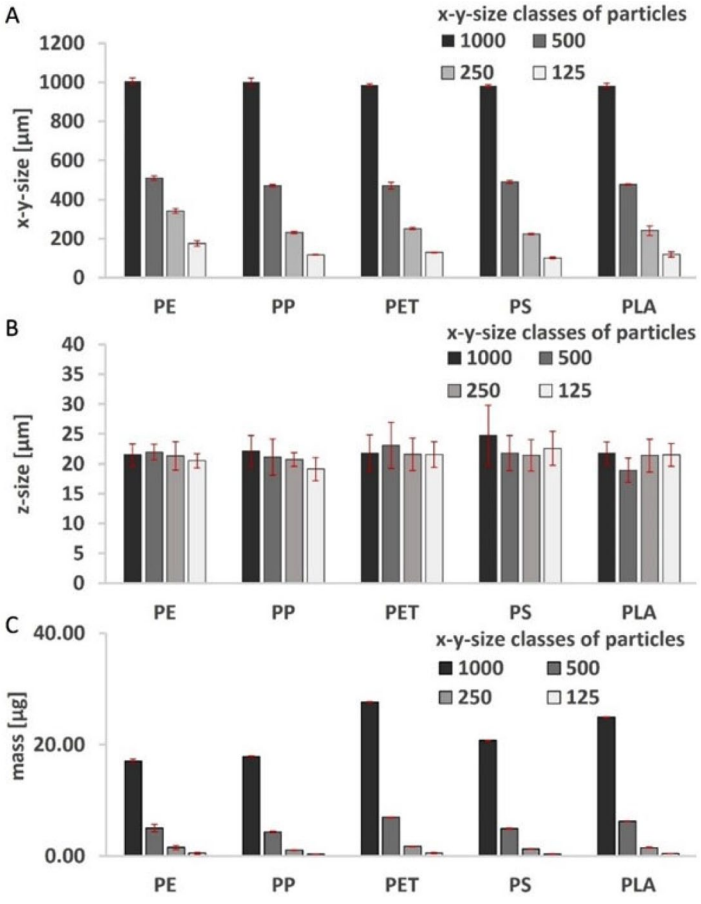

Fig. 3 Summary of the particle size and mass of RMPs, with (**A**) x-y-size, (**B**) z-size, and (**C**) mass of the RMPs. All RMPs were manufactured using CNC milling and cryo-microtomy. The mean results are shown for each of the respective measurements, the red bars show the standard deviations. The data are additionally given in tables in the supplementary information

observed for the 1000 × 1000 µm x-y-edge length PS RMPs with 24.43 ± 5.12 µm. It was observed that the mean values differed on average by -3.59 µm from the targeted x-y-size. For RMPs generated for the particle size class 1000 × 1000 µm we observed the highest average x-y-deviations from the targeted x-y-edge lengths for LD-PE RMP particles, with an average x-y-edge length of 1006.59 ± 16.19 µm. Whereas the 500 × 500 µm (x - y) particle size PS RMPs were observed to have the highest deviation from the x-y-average with 489.02 ± 7.72 µm (Fig. 3A). For the target size class 250 × 250 µm x-y-edge length, and below, the deviations of the measured mean values from the target sizes were observed to occasionally be larger. Specifically, the RMPs with intended x-y-edge lengths of 250 × 250 µm for LD-PE had a measured x-y-average of 341.13 ± 12.11 µm, 231.30 ± 6.29 µm for PP, 251.04 ± 5.31 µm for PET, 224.33 ± 3.02 µm for PS and 240.556 ± 24.36 µm for PLA (Fig. 3A). Finally, for RMPs with a targeted x-y-edge length of 125 × 125 µm, we observed an x-y-average of 175 ± 13.92 µm for LD-PE, 116.41 ± 1.29 µm for PP, 128 ± 2.16 µm for PET, 101 ± 3.86 µm for PS and 118.52 ± 13.62 µm for PLA (Fig. 3A).

Using SEM, we observed that the targeted square shapes were successfully generated. For all RMPs of all polymer types, except LD-PE, we observed that the corners and edges were milled accurately (Fig. 4). For LD-PE RMPs, in particular for the size classes < 250 × 250 µm x-y-edge length, we observed the presence of protruding chips, and the shape of the particles did not consistently conform to the intended square shape (Fig. 4A3-5), whereby deformations in other polymers occasionally resulted in the formation of rhombus-like shape particles (Fig. 4C1, D1). On the surface of the majority of RMPs analysed, we observed the presence of variable prominent parallel grooves in the SEM images (Fig. 4).

Particle mass characterization

The results of the gravimetric mass measurements were generally observed to be similar to the results reported for the particle size measurements. Specifically, we observed that the standard deviation for each of the mean values of the particle mass of the RMPs in each particle size was typically small. The largest standard deviation was observed for LD-PE RMPs, at ± 0.31 µg for the 1000 × 1000 µm x-y-edge length, ± 0.66 µg for the 500 × 500 µm, ± 0.38 µg for the 250 × 250 µm as well

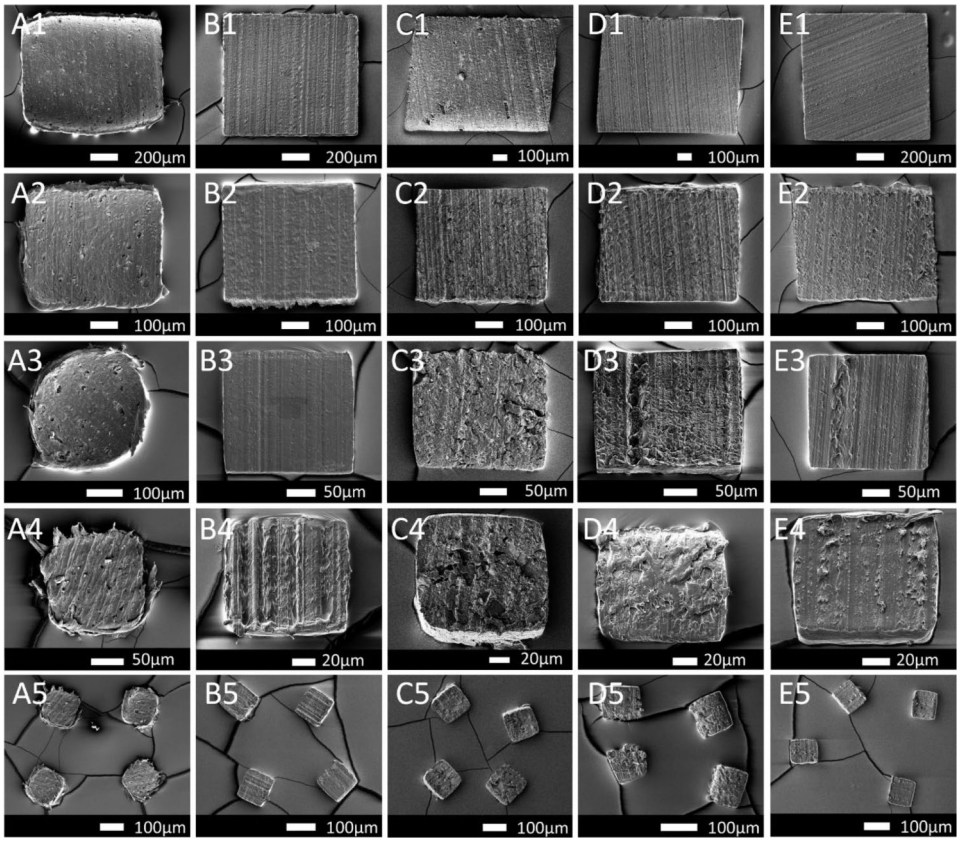

Fig. 4 SEM images of LD-PE- (**A**), PP- (**B**), PET- (**C**), PS- (**D**), PLA RMPs (**E**), with 1000 µm (1), 500 µm (2), 250 µm (3), 125 µm (4) and an overview of four 125 µm RMPs (5)

as ± 0.11 µg for the 125 × 125 µg particles (Fig. 3C). The standard deviations for all other RMPs were < ± 0.13 µg.

Particle chemical composition characterization

To account for potential chemical surface changes that may have resulted from the production and preparation process of the RMPs, we analyzed the chemical composition of the RMPs using FPA-µFTIR and Raman spectroscopy. We observed that the RMPs spectra were consistent with the pure polymer spectra of the respective polymer type after the whole production and preparation process (Fig. 5A-J). Results were consistent for both FPA-µFTIR- and Raman-measurements, which further confirmed an absence of gelatine, although we note that we cannot conclusively exclude minimal traces of gelatine with our approach. We did not observe any indication of polymer degradation (Fig. 5).

Discussion

Here we present a proof of concept method that we suggest represents an efficient and reliable approach towards generating RMPs. The method is based on the use of injection molded plastic polymer blocks of five different polymers, which are used to manufacture columns of a pre-defined size using CNC milling. The application of embedding the columns in gelatin followed by cryosectioning, results in the generation of gelatin slices with a defined number of RMPs. The success of the proof of concept method is evaluated based on careful and thorough monitoring of the production process through each of the steps. We observed that, during both CNC milling and further processing, the columns generated rarely broke. In instances when broken columns were observed, however, the respective column plates could still be used to produce RMPs. The efficiency, in terms of the number of particles obtained, with respect to broken columns is notably lower relative to each broken column. We strongly suggest that the number of RMPs per finished gelatin slice must be recorded accurately using a stereo microscope. This represents an important element of the quality assurance and quality control of generated RMPs, whereby the number of RMPs contained in each embedded gelatin slice is quantified relatively quickly by microscopy, with subsequent photo documentation

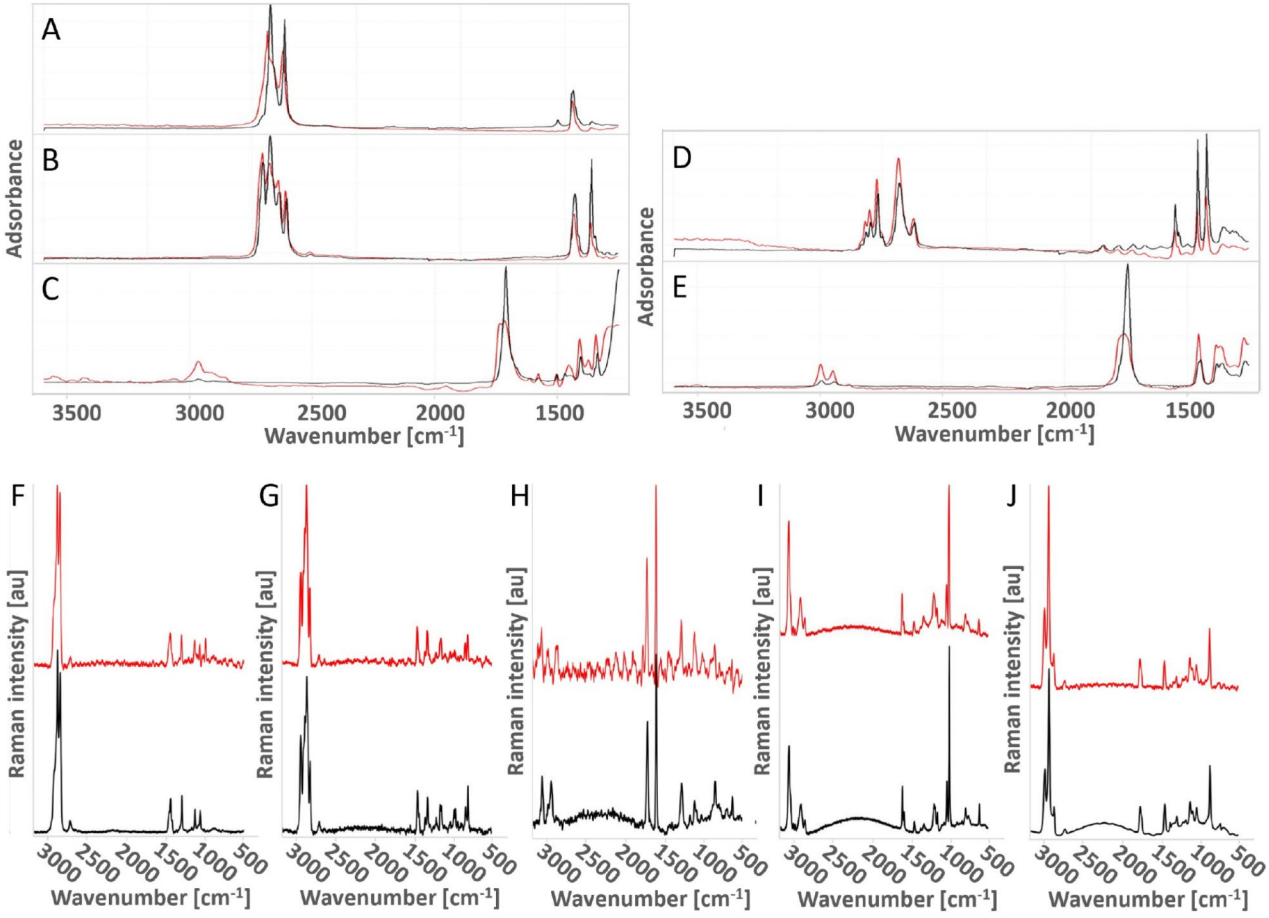

Fig. 5 FPA-µFTIR spectra of our RMPs are shown for LD-PE (**A**), PP (**B**), PET (**C**), PS (**D**) and PLA (**E**) in red as well as the corresponding reference spectra in black. Raman spectra of our RMPs are given for LD-PE (**F**), PP (**G**), PET (**H**), PS (**I**) and PLA (**J**) in red as well as the corresponding reference spectra in black

Table 1 Average tensile strengths and glass transition temperatures for the 5 polymer types included in this study (LD-PE, PP, PET, PS, PLA) as well as PMMA and tire rubber. The specified data are from matweb [38] and Greene (2021) [40]

Polymer	Average tensile strength	Average glass transition temperature
LD-PE	10.8 MPa	-115 °C
Tire rubber	14.0 MPa	20 °C
PP	33.1 MPa	-5 °C
PS	41.0 MPa	100 °C
PMMA	48.0 MPa	105 °C
PET	50.0 MPa	75 °C
PLA	59.5 MPa	60 °C

representing a transparent and robust protocol that ensures both traceability and reliability.

We further demonstrate the efficacy of our proof of concept method of cutting polymer column plates using cryo-microtomy, for the column sizes 1000 × 1000, 500 × 500, 250 × 250 µm and 125 × 125 µm x-y-edge length. Our results show that using our approach, RMPs down to an x-y-size of 125 × 125 µm can be produced with relatively low variance in particle size. We note, however, that the results obtained for RMPs generated at particle sizes down to 50 µm in additional tests resulted in a number of defects to the columns during production, which increased with decreasing size classes < 125 µm. Consequently, we suggest that the use of our proof of concept approach to generate RMPs < 125 µm will require additional research. We are optimistic, nevertheless, that lower sizes of RMPs can be generated using the method presented here, since we are aware that there exists specialized CNC milling equipment capable of obtaining accuracies of up to 1 µm [36], which we suggest represents an exploitable opportunity that can support the generation of even smaller RMPs with high precision. Currently, therefore, it is important to emphasize that the results reported here are limited to the generation of square RMPs > 125 µm for the five types of polymers included in this study.

For all RMPs > 125 µm, our results show little relative standard deviation across all three particle size dimensions (x, y, z). A notable exception to this observation, however, are results obtained for LD-PE, for which we observed protruding chippings and round edges. We suggest that it is likely that this is due to wear of the material during milling and cutting [36, 37], which is likely caused by the lower tensile strength of LD-PE as compared to PP, PET, PS and PLA (Table 1) [38]. Tensile strength is understood to strongly influence the properties of particles generated from a milling process [36, 37]. Increasing tensile strength is associated with increases to the shear strength and thus also the cutting force. While this combination of properties results in good RMP milling results, it also enhances the stress to the milling tools, which potentially results in surface changes of the workpiece. Given that we observed defects in shape and the presence of chippings only for the lowest tensile strength polymer LD-PE, with an average of 10.8 MPa [38], we assume that polymers with even lower tensile strengths will not lend themselves to the generation of reliably produced RMPs via CNC-milling, as compared to polymers with higher tensile strengths. Consequently, we would anticipate that a polymer, such as polymethyl methacrylate (PMMA), which has a tensile strength between 30 and 50 MPa and an average of 48 MPa [38] (Table 1), would lend itself to be easily processable using the method described here, since the high tensile strength is consistent with that of PET, with an average of 50 MPa for instance, and which was processed successfully. Tire rubber, on the other hand, with a tensile strength < 18 and an average of 14 MPa (Table 1), would likely be harder to process by CNC milling under ambient temperature conditions [38]. We suggest that a possible solution to the difficulties encountered for lower tensile strength polymers would be milling the polymer at temperatures below the glass transition temperature of the polymers (Table 1). Indeed, cryogenic CNC-milling has been shown to represent a viable approach and, therefore, could potentially be used in such cases [39]. Cryogenic CNC-milling typically requires the temperature to be cooled to -196 °C, using liquid nitrogen that is applied through a nozzle directly to the milling tool and the work piece [39]. It is likely that application of cryogenic CNC-milling would thus also improve the milling results shown here for LD-PE, which has a glass transition temperature of approximately − 115 °C [40] (Table 1).

The majority of polymer column plates embedded in gelatin could easily be cut during cryo-microtomy. However, problems occasionally occurred when cutting 1000 × 1000 µm x-y-edge length PLA columns. This is most likely influenced by the material characteristics of PLA (Table 1). Specifically, we encountered difficulties cutting through the entire embedded column block for PLA, without experiencing technical issues, such as skipping of the blade. Through trial-and-error we discovered that the issue could be resolved by using a fresh blade but note that success was limited to a certain number of sections. Since we did not encounter similar issues for smaller RMP particle sizes, we suggest that this is most likely due to the larger PLA surface area associated with 1000 × 1000 µm x-y-edge length columns, which when coupled with the tensile strength of the comparably hard polymer PLA resulted in deterioration of the blades, and therefore the associated difficulties encountered. Thus, for production of larger RMP particles made of harder polymer types, we suggest processing smaller blocks of embedded column plates (x-y-wise) with a smaller

number of columns so that less force is needed to cut through the block. Furthermore, we observed that it was important to ensure the use of fresh blades for each column plate, particularly when processing polymers with higher tensile strength. Lastly, we frequently observed the formation of grooves in the cut RMPs. The formation of grooves occurred regardless of whether a fresh blade was used for the first time or had been subject to frequent use processing other blocks. Therefore, we conclude that the formation of the grooves is not something that can be prevented by simply using fresh blades.

The number of particles that can be produced per column plate, as described here, depends on the thickness of the cuts made. In the proof of concept approach presented in this study, we are able to demonstrate the ability to produce at least 100 cuts per column plate, resulting in the generation of 2500 particles. If a new blade would be used each time, we estimate that the cost (at the time of conducting the study) for generating 2500 particles would be < 10 €. The cost per particle, however, is not only influenced by the cost of the blades used for cutting, but also on other parts subject to wear, such as the micro-milling cutter used for CNC-milling, which has a cost (at the time of conducting the study) of about 10 € [41]. When considering the consumable costs of the produced polymer blocks and the gelatine, we observed these to be negligible on a per particle basis. Generally, however, the cost for personnel, the milling machine as well as the cryo-microtome, represent much higher costs, which we have not included in this estimate. Finally, when generating RMPs using the approach presented here, we note that a weakness of the method is the relative amount of milling waste that is generated, which from a sustainability perspective does not represent the most efficient approach towards generating RMPs. Consequently, we suggest a need to consider how the amount of waste might be minimized in future, which for example, could be accomplished by designing base plates where the space between the columns is reduced to a minimum.

Producing RMPs was always performed using cleaned equipment, as well as filtered solutions, with the sample preparation steps conducted under laminar flow boxes to prevent contamination. However, we note that for the procedure reported here we did not include a filtration step of the gelatine. We decided not to filter the gelatine, since we used only extra-pure grade gelatine. Furthermore, given that the gelatine was removed following the production of the RMPs prior to their characterisation and analysis - where we did not see any residual evidence of background contamination - we suggest that the potential for contamination was negligible. For future applications, however, we would strongly recommend that the gelatine be filtered to minimize any potential contamination of MPs, which might be present in the gelatine powder used, even if it was of extra-pure grade.

Towards quality assessment, quality control and harmonization in terms of comparability of methods

It is generally understood that a major shortcoming of data reporting MPs relates to the challenges associated with comparability of the data generated by different studies and research groups. In an effort to address this challenge, standardized methods that adhere to strict QA/QC procedures and standard operating protocols (SOPs) are needed to support the MP research community [42–50]. For example, research projects, such as 'BASEMAN' or 'WEPAL-QUASIMEME/NORMAN' have reported results from some of the first global inter-laboratory comparison studies on MPs, and 'EURO-qCHARM' have worked towards harmonizing sampling, sample processing and methods of identification, all of which have aimed towards the ambitious goal of enabling comparability of MP data between different researchers [42, 43]. In these projects, all relevant forms of MPs have been specifically produced for the specific studies conducted (e.g. pellets, fragments, fibers, films, spheres) [42, 43]. However, in general, a suite of commercially available RMPs, except for microbeads or powders, is currently not readily available [31].

Given the heterogeneous properties of environmentally relevant MPs of varying shape and size, a key challenge relates to the production of RMPs that might be consistent with the complex mixture of properties found in the environment. Currently, commercially available particles are limited to a suite of largely homogenously shaped particles. For instance spherical-shaped MPs, such as PMMA and PS, can be generated at commercial scale (e.g. tons), using emulsion polymerization methods [29, 30], and there have been some recent efforts to produce spherical MPs from other polymer types, such as PE, for which melt emulsification can be used [27]. In terms of generating fibers, the method presented by Cole (2016) applied a microtome to produce fibers in a soluble matrix. Using the microtome it was possible to produce polymeric fibers with a defined size (10–28 µm in diameter and 40–100 µm in length) [45]. Finally, the generation of fragments has been successfully demonstrated using cryomilling, which is currently the most commonly used technique [18, 19, 46–48]. The disadvantage of cryomilling, however, is that the particles produced tend to have large variances with respect to the particle size distribution. Thus, fragments of MPs produced by cryomilling require a sieving step to produce RMPs of a certain size fraction, as applied in the project 'BASEMAN' [42].

Generally, the addition of an exactly predefined particle number or mass of either bead-, fiber-shaped or cryomilled RMPs into a sample matrix represents a significant

challenge. Manual counting and addition does not represent a practical or efficient solution towards dosing large numbers of samples with RMPs. An alternative is to add a defined mass of RMPs to a sample [42, 43]. In these instances, however, there exists an uncertainty regarding the exact number of particles associated with the mass of particles, which may be important particularly where it may be necessary to understand a direct comparison between number-based and mass-based concentrations. Another method to adding RMPs to a sample is the use of suspensions, which again are based on a defined mass of of RMPs in the suspension [46]. In the case of suspensions, there exists concerns regarding the homogeneity of the distribution of RMPs in the suspension. The issues associated with variability resulting in differences between the nominal amount added to a sample versus the actual concentration in the sample are further confounded by various losses of RMPs that can occur during the handling of suspensions, such as particles adhering to the equipment, e.g., pipette tips, due to static charge. Consequently, there are various opportunities where the actual number of particles added to a sample likely differs from the nominal or intended amount, which can also result in significant differences between replicates of the same experimental treatment. In some studies, such as 'WEPAL-QUASIMEME/NORMAN', the use of soda tablets into which MPs are embedded has been adopted, whereby a mixture of soda salt is combined with a mass-based addition of MPs [43]. We suggest, however, that as a result of issues noted above and related to the preparation of the tablets, discrepancies are likely to exist between the intended nominal amount and the actual concentration in the soda tablet. Probably due to discrepancies, the evaluation of both number-based and mass-based analytical methods during the interlaboratory comparison study reported in the 'BASEMAN' project observed significant difficulties, which resulted in problems with the harmonization of results in terms of comparability for both number and mass-based analysis techniques [42].

To produce a defined number of RMPs, Bamshad and Cho (2021) proposed a procedure where they used a laser jet printer for printing particles [24]. These RMPs only showed a 5.2% deviation in the x-y-sizes which is lower than the average x-y-size deviation of 9.67% we measured across all size classes and polymer types of our RMP particles. However, the particles that were produced by laser jet printing were black, made of PS and contained multiple additives to make the polymer printable [24]. Thus, the RMP polymer properties are inconsistent with the original PS material. Consequently, the application of RMPs generated using the laser jet printer method appears to be limited to a narrowly defined group of particles, which will limit their application in MP research.

While we note that there have been various ambitious attempts to generate RMPs, we suggest that there are various limitations to the approaches that have been developed and applied to date. These include difficulties associated with the handling of the RMPs, the unknown, variable and inconsistent distribution of particle sizes generated and/or limitations and challenges associated with particle characterization. When considering all of the associated challenges that have been encountered, it is thus, not difficult to appreciate that there is still a need for RMPs of a defined chemical composition/polymer type, size and shape that can be handled easily and added to a sample reliably and accurately to support analytical method development, standardization and harmonization of data generated.

We suggest that the proof of concept method presented in this study represents a novel opportunity to generate RMPs, which we think can contribute to minimizing many of the current issues associated with other methods. The results presented here, consequently, suggest strengths associated with the method towards enabling the addition of an exact number of RMPs to a sample as internal standard for the calculation of recovery rates after sample preparation without manual counting or the use of suspensions. This in turn, for the first time, allows for a number-based validation of sample transfer processes, extraction, purification, subsampling etc. of all analytical methods for all relevant polymer types. Here the suitability of RMPs includes analysis of environmental samples of virtually all matrices since the defined shaping of our RMP particles ensures differentiability between MPs occurring in environmental samples. The use of our RMPs as internal standard for evaluation and quality control in mass-based analysis techniques is also possible, and thus comparability between number-based techniques like FPA-μFTIR or Raman and mass-based analysis techniques like TED- or pyrolysis-GC/MS can be realized. For pyrolysis- and TD-GC-MS/MS our RMP particles already have been used as an internal standard by Bartnick et al., in 2023 [50].

Although as part of our proof of concept approach we focused on generating square-shaped RMPs, which are generally consistent with films of MPs, as opposed to fragments or fibers, we suggest that generating other shapes of RMPs can be accomplished by simply using different CAD-models for CNC-milling, and different cutting heights during cryo-microtomy as part of future studies. Consequently, we suggest that the approach has the potential for broad application towards generating a large variety of RMPs.

Conclusion

Here we present a proof of concept approach for generating a suite of RMPs of varying polymer composition and size, which includes the ability to add the particles reliably and accurately to a sample matrix using both particle-number and/or mass-based concentrations. Consequently, we suggest that our proof of concept approach represents an important opportunity to address many of the MP research issues related to analytical quality assessment and comparability that the MP research community currently faces. While the results of our proof of concept approach are limited to the generation of RMPs down to a size of 125 µm x-y-edge length with low standard deviations in size and mass, we are optimistic that the approach could be applied to reliably generate even smaller MPs. Given the availability of specialized CNC milling instruments, which have the capability to reach accuracies of up to 1 µm [36], this suggests being realistic.

We are aware that our proof of concept approach represents a first step into a new direction. With our approach, it is now possible to use RMPs of different well-defined polymer types, shapes, sizes, etc. in exact numbers in laboratory experiments. With the availability of such RMPs, and their application in future MP studies, we perceive that a high standard of QA/QC can be achieved by improved evaluation of laboratory handling processes and analytical tools via the use of internal standards during the analysis of environmental samples. Thus, having access to a reliable source of RMPs can help in the development of standardized methods and the harmonization of data, which is important towards supporting comparability of different analytical methodologies. The ease of use of our RMPs by directly adding a defined number of MPs with the gelatine discs represents an opportunity to more reliably and accurately add MPs to samples, which prevent losses of the reference particles that might occur using other techniques, such as the use of particle suspensions. Ultimately, it is only through the generation of a broad, reliable and comparable data basis regarding the environmental concentrations of MPs that a more realistic design of ecotoxicological experiments and/or studies on the environmental transport of MPs can be achieved which is fundamental towards enabling a realistic risk assessment of MPs for both human health and the environment.

Abbreviations

FPA-µFTIR	Focal plane array detector-based micro-Fourier-transform-infrared
LD-PE	Low-density polyethylene
MP	Microplastic
MPs	Microplastic particles
PET	Polyethylene terephthalate
PLA	Polylactic acid
PMMA	Polymethyl methacrylate
PP	Polypropylene
PS	Polystyrene
RMPs	Reference microplastic particles
SEM	Scanning electron microscopy
TED GC/MS	Thermal extraction – desorption gas-chromatography-mass spectrometry

Supplementary Information

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Supplementary Material 1

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Author contributions

S.D.J.O.: Research idea, development of the production method for RMP particles, sample processing, acquisition of data, data analysis, writing of the manuscript. P.E.B.: QA/QC of the production of the RMP particles, sample processing, acquisition of data. D.W.: Sample processing, interpretation of data. M.R.: Sample processing, acquisition of data, interpretation of data. M.F.: Sample processing, interpretation of data. V.K.B.N.: acquisition of data, interpretation of data. E.H.: Sample processing, interpretation of data. H.M.: Sample processing, interpretation of data. E.C.V.: Revision of the manuscript, interpretation of data. M.S.: Acquisition of data, interpretation of data. C.L.: Substantive supervision and revision of the manuscript. M.G.J.L.: Substantive supervision for the development of the production method for RMP particles, revision of the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request. Further data is given in the supplementary information file.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree to the publication.

Competing interests

The authors declare no competing interests.

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