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An assessment of methods used for the generation and characterization of cryomilled polystyrene microand nanoplastic particles



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Abstract

There is a critical need to generate environmentally relevant microplastics (MPs) and nanoplastics (NPs) to better investigate their behavior in laboratory settings. Environmental MPs are heterogenous in size and shape, unlike monodisperse and uniform microspheres commonly used as surrogates. Cryogenic grinding, or cryomilling, was successfully utilized to transform polystyrene (PS) bulk material into heterogenous micro and nano fragments. Fourier-Transform Infrared (FTIR) spectroscopy confirmed that this approach did not alter polymer surface chemistry. The number of milling cycles (time of milling) and frequency of grinding (intensity of milling) were varied to investigate the role cryomilling parameters had on generated MP characteristics. The resulting particle size distributions of cryomilled samples were measured and compared. Coulter Counter and Nanoparticle Tracking Analysis (NTA) were used to measure the particle size distributions at the micro and nanoparticle size ranges, respectively. Microspheres were used to determine what camera settings yielded more accurate sizing and to reduce bias in the NTA analysis. Increasing milling cycles generally increased the number of smaller particles. The evolution of the measured size distributions indicated that small nanosized fragments broke off from larger MPs during cryomilling, steadily eroding larger MP fragments. The number of milling cycles was observed to more consistently impact the size distributions of fragments compared to the frequency of milling. This study offers both analysis of the cryomilling process and recommendations for generating more realistic PS MP/NPs for examining environmental fate and effects.

Keywords Microplastics, Nanoplastics, Cryomilling, Particle sizing, Polystyrene

Introduction

Plastic pollution is of notable concern to marine and aquatic life with an estimated 1.15 to 2.41 million tons of these contaminants entering the ocean annually through

freshwater and terrestrial environments [1, 2]. Due to high plastic production and improper disposal, these plastics have entered the environment where they subsequently break down into secondary microplastics (MPs), plastics < 5 mm in diameter, and secondary nanoplastics (NPs), plastics < 1 μ m in diameter [3, 4]. These contaminants are broken down by processes such as mechanical abrasion, photooxidation, or biodegradation [3, 5]. MPs isolated from environmental matrices are heterogenous in shape, size, and surface chemistry [5, 6] and secondary NPs are assumed to have similar heterogeneity although data is quite limited and techniques to isolate and identify NPs from environmental matrices are still emerging



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[7]. However, current colloidal plastic fate, transport, and toxicity research is largely based on experiments using monodisperse plastic spheres (polystyrene, most commonly) as a surrogate for secondary plastics found in the environment [6, 8–12]. Monodisperse spheres of uniform chemical composition do not account for the chemical and physical heterogeneity exhibited in environmental samples where MPs are most commonly found as fragments and fibers [6, 10, 13, 14]. Particle shape has been shown to affect the behavior of microplastics in the environment and it is likely also important for understanding NP behavior. For example, Waldschläger et al. demonstrated that spherical particles infiltrate sediment deeper than fragments and fibers [15]. Particle size is also a key factor contributing to particulate contaminant mobility and bioavailability to organisms [16-18]. Additionally, surface area and polymer type have both been shown to affect the adsorption of organic pollutants onto MPs and NPs [11, 19]. Thus, commercially available spheres, while readily available and simple to use, may not be sufficient to accurately model the complexity of real MP and NP colloidal behavior in the environment.

There is a need for reliable and reproducible methods for generating well-characterized micro and nanoplastics that better represent environmental plastic particles. Multiple MP generation techniques have been developed to mimic the aging and fragmentation of plastics in the environment. These include ultraviolet photooxidation, laser ablation, ultrasonic treatment, and mechanical milling [10, 14, 20]. Particles resulting from these methods vary in quantity, size distribution, and wettability; however, cryomilling, also known as cryogenic grinding, is currently the most common MP generation procedure [10, 21–23]. Here, bulk plastics are made brittle using liquid nitrogen and mechanically milled until reduced to the desired particle size. Particles derived from this method are heterogeneous in shape and span the micrometer and nanometer size range. This broad size distribution is difficult to characterize for polydisperse samples as no single instrument can simultaneously determine particle size distributions from nano-scale to micrometer sized particles without limiting the resolution of smaller particles [24, 25]. For example, Static Light Scattering (SLS) has been used regularly for micro- and nano-plastic research, however, the scattered light intensity scales with the cube of particle diameter. As a result, the resulting ensemble size measurements in polydisperse suspensions are biased towards larger particles in the sample and the presence of smaller particles, even if present at higher number concentrations, may be masked [25]. Although cryomilling is a commonly used method, there has not been a detailed investigation of how cryomilling parameters affect the particle size distribution of the resulting milled plastics because of the current challenges in characterizing non-spherical and nano-scale plastic particles.

Various techniques have been used to reliably measure the particle size distributions of monodisperse MPs [5, 26]. However, it is often necessary to combine multiple techniques to characterize the complete size distribution of polydisperse samples, akin to real plastic particle suspensions. Impedance spectroscopy, such as with a Coulter Counter apparatus, measures changes in electrical impedance as particles pass through an aperture. The impedance change is proportional to the volume of the particle [27]. Multiple size apertures, each capable of measuring particles 2 to 40% of its diameter, can be used together to capture broad size distributions. For example, particles between 0.6 µm and 80 µm can then theoretically be measured using a 30 µm aperture and a 200 µm aperture. Analytical tools such as Nanoparticle Tracking Analysis (NTA) have also been used to measure polydisperse samples. NTA is a light scattering technique that visualizes, records and tracks particle movement in solution due to Brownian motion by following the scattered light emitted by the laser beam passing through the sample. Because NTA tracks individual particles, it can provide good size distribution and concentration information for polydisperse and low-concentration samples. NTA has a working range of 10-1000 nm depending on the type of laser and particles measured. However, Lambert and Wagner reported a working range of 30-2000 nm for plastics, while Ekyall et al. reported a limit of detection around 90 nm [28, 29]. Despite this, NTA still provides a valuable opportunity to obtain detailed size distributions for the nano-fraction of polydisperse particle suspensions. Of note, NTA and Coulter Counter also measure particle size with a spherical assumption by default [5, 24, 30]. For Coulter Counter measurements, volume displaced is converted to an equivalent spherical diameter. NTA relates particle movement due to Brownian motion to an equivalent spherical hydrodynamic diameter through the Stokes-Einstein equation. With both instruments, the reported diameters will deviate from the actual dimensions of non-spherical particles. Further, because the basis of measurement is different for the two techniques, there are difficulties in overlapping/connecting the size distributions for nonspherical particles measured with the two instruments. Despite these challenges, Coulter Counter and NTA together provide high resolution information regarding relative particle size distributions in both the micrometer and nanometer size ranges, respectively. In doing so, they prove invaluable for quantifying changes in size distributions due to physical processes like milling or aggregation.

With the aim of developing a reproducible technique for generating heterodisperse populations of environmentally relevant microplastics, polystyrene (PS) bulk material was cryomilled for varying lengths of time and milling intensities. The physical and chemical properties of the resulting plastic particles were characterized and compared using a suite of common analytical techniques. The size and number distributions of the cryomilled MP suspensions were analyzed using NTA and Coulter Counter in an effort to characterize both the nano- and micro-sized fractions.

Materials and methods

Cryogenic milling

Polystyrene culture test tubes (Fisherbrand, 1119382) were first cut into 2-4 mm pieces. Approximately 5 g total mass of cut pieces were then milled using a stainless-steel ball mill (Retsch CryoMill, Haan, Germany) in a 50 ml chamber with a single 25 mm diameter stainless steel ball such that the total milling volume (including the ball) was approximately 70-80% of the total chamber volume. The milling procedure followed a sequence of 4 min of pre-cooling at 5 Hz then alternating cycles of 5 min of grinding followed by 1 min of intermittent cooling at 5 Hz. The precooling time was sufficient for the chamber to reach -196 C, well below the glass transition temperature of most plastics. It should also be noted that the cryomill in this study surrounds the sample with a continuous supply of liquid nitrogen during the milling cycle, which is different than cryomilling where plastics are precooled then allowed to generate frictional heat during milling. The number of grinding cycles (1, 2, 3, 6) and frequency of grinding (15 Hz and 30 Hz) were varied. Resulting MPs were stored in amber glass vials and kept away from direct sunlight to minimize the effects of UV photooxidation. The bulk cryomilled material was suspended in 18.2 M Ω cm distilled, deionized (DDI) water. The MP suspension was then ultrasonicated for 30 min (VWR B1500A-DTH Ultrasonic Cleaner, 35 kHz for Coulter Counter suspensions and Sonics Vibra Cell 750 Sonics & Materials, Inc, 20 kHz for NTA suspensions) to fully suspend MPs. Aliquots of this suspension were then fractionated by vacuum filtration prior to characterization by various techniques following Fig. 1.

Figure 2 illustrates the analytical techniques used for particle characterization along with the relevant size range for each technique. FTIR and SEM measurements were performed on bulk powders containing particles spanning the micro- and nano-size range.

Surface analysis

FTIR

Fourier-transform infrared (FTIR) spectroscopy was used to verify polymer chemistry before and after the cryomilling process using a Bruker VERTEX 70v FTIR Spectrometer with a platinum diamond attenuated total reflectance (ATR) crystal in the wavenumber range from 4000 cm⁻¹ to 400 cm⁻¹. Data was collected at a spectral resolution of 4 cm⁻¹.

Scanning electron microscopy

The morphology of the resulting MPs was observed using a Quanta 500 FEG SEM. All samples were coated with an Au/PD layer using a Cressington sputtering instrument before analysis.

Fractionating/Characterization



Cryomilling





Fig. 2 Overview of characterization techniques and relevant size ranges

Particle sizing

Sieve analysis

Mass fractions of cryomilled fragments in different size ranges were measured by filtering through mesh filters. Briefly, ~ 0.2 g of each cryomilled fragment type were added to 14 mL ultrapure water and sonicated for 5 min. Suspensions were then fractionated through either a 100 μ m or 20 μ m Nylon Net filter. The filtrate was then dried and the mass of each fraction was determined.

Coulter counter

Particle size distributions were measured using a Beckman Coulter, Multisizer 3, Coulter Counter. 500 mg/L suspensions were analyzed with 200 µm and 30 µm apertures to obtain the size distribution. For the 30 µm aperture, the MP solution was fractionated using a 20 μ m Nylon Net filter (Millipore Sigma, R1EB01319) to prevent blocking the aperture. MP samples were diluted in a weak electrolyte solution (ISOTON II) for measurement. Time Resolved Dynamic Light Scattering (TRDLS) was used to verify that MPs remained stable in the electrolyte solution (Fig. S1). Data was collected as number of particles counted into set bin widths $(\Delta \log(d_n) = 0.1, d_n)$ in µm). Data from multiple apertures were combined as described in the SI (Fig. S2). The particle size distribution function (PSDF), $n(d_p)$, was calculated using the Coulter Counter raw data [31]. The PSDF is defined in Eq. 1:

$$n(d_p) = \frac{\Delta N}{\Delta d_p} \tag{1}$$

where *N* is the number concentration (number/mL) and d_p is the particle diameter in μ m. The number distribution (ND) was calculated by dividing Δ N by $\Delta \log(d_p)$. Using a spherical particle assumption, the volume distribution (VD) can be derived from the number distribution. Triplicates for all samples were analyzed to verify

reproducibility and statistical significance. Resulting data was averaged for size distribution figures.

Nanoparticle tracking analysis

The nanosized range of MPs was characterized using a Malvern NanoSight NS500 Nanoparticle Tracking Analysis (NTA) instrument (405 nm violet laser). Although NTA has a reported working size range of under 1000 nm, latex PS spheres of various sizes were measured to assess NTA detection limits. A lower limit of 100 nm was determined as 100 nm spheres were able to measured but 75 nm spheres were not detected. Additionally, it is difficult to reliably measure close to the 1000 nm threshold [24, 30]. To remove larger MPs, 135 g/L particle suspensions were filtered using a 20 µm Nylon Net filter (Millipore Sigma, R1EB01319) followed by a 1 μ m cellulose ester filter (Advantec, A100A047A). Samples were loaded into the NanoSight Instrument using a peristaltic pump. Multiple 60 to 180 s videos were recorded, and sample fluid was advanced between each recording. As users may introduce bias when choosing camera settings and detection threshold, 0.1 mg/L 750 nm PS microsphere suspensions (Polybead, 0730915) were measured while varying these parameters (Figs. S3-6) [24, 30]. A camera setting of 10 displayed the smallest particle size deviation for the 750 nm PS microspheres used (Figs. S4 and S6). At this camera setting, a detection threshold between 20 and 25 displayed the smallest concentration deviation from 0.1 mg/L for the 750 nm spheres (Fig. S6). However, smaller particles are less visible at this detection threshold so a detection threshold of 10 was used for all samples as this allowed for detection of 100 nm spheres as well as 750 nm spheres. As the cryomilled plastics are heterogeneous in size, multiple measurements were needed to get a more representative size distribution. Samples were analyzed until a desired 100,000 valid tracks were achieved as described in the Supporting Information (Fig. S7). Number concentration

was collected for each cryomilled sample with bin widths of 1 nm and converted to PSDF, ND, and VD. A one-way ANOVA test was used to determine if number of milling cycles had a significant effect of the generated particle concentration of samples. Tukey's HSD test was then used to analyze significance between these paired samples. Welch's T Test was used to determine significance of frequency of milling between paired samples as variance and sample size were unequal between these groups. For all statistical tests, a *p*-value < 0.05 was determined to be significant.

Results and discussion

Surface properties

FTIR analysis was used to examine if the cryomilling process changed the surface chemistry of the polystyrene test tubes. Figure 3 shows the FTIR spectra for both the unmilled polystyrene and MPs generated by cryomilling for 3 cycles @ 30 Hz. Images of the bulk and cryomilled plastics are included as insets. Functional groups associated with polystyrene (= C-H stretch and 3025 cm⁻¹; C-H stretch at 2919 cm⁻¹; C–C in aromatic ring at 1604, 1492, 1418 cm⁻¹; out of plane bending -C-H at 757, 694 cm⁻¹; C–C bend out of spectral window at 541 cm⁻¹) were seen before and after the milling process showing Page 5 of 15

that cryomilling did not alter the surface chemistry of the materials. Surface chemistry of the unmilled PS tube was verified independently by a second lab using Thermo Electron iN5 μ FTIR (Thermo Fisher Scientific) (Fig S8).



Fig. 4 SEM image of PS MPs generated by cryomilling for 3 cycles @ 30 Hz with scale bar representing 30 μm



Fig. 3 Fourier-transform infrared spectroscopy (FTIR) spectra of PS test tube before and after cryomilling

The SEM image for cryomilled PS in Fig. 4 shows the heterogeneity of particle size and morphology of PS fragments generated by cryomilling. Micro- and nanosized fragments akin to these generated fragments have consistently been found to be the dominant type of MP shape found in various aquatic environments such as wastewater, seawater, and rivers [6, 13, 32]. The microspheres that are frequently used in fate, transport and toxicity experiments do not exhibit the heterogeneity in size and morphology of MPs found in the environment [10].

Particle sizing

Sieving was used to get a coarse measure of the size distribution of the bulk cryomilled sample. Figure 5 shows the percent of mass of the original plastic in the <100 μ m and <20 μ m size fractions. Fragments larger than 100 μ m in diameter make up the majority of mass for each cryomilled sample. However, increasing the time and intensity of milling increased the mass fraction present in both the <100 μ m and <20 μ m ranges. Further analysis of the size distributions was performed on the fraction of mass in the micron and sub-micron range.

Coulter Counter was used to measure the size distribution of the cryomilled MPs in the micrometer size range (0.68 μ m to 86 μ m). The resulting number distributions are compared in Fig. 6. The PSDF and the volume distribution for these samples can be found in the Supplementary Information (Figs. S9 and S10). Figure 6a demonstrates the impacts of an increasing number of cryomilling cycles, showing that the sample generated

by cryomilling for 3 cycle @ 30 Hz has more smaller particles than samples cryomilled for 1 and 2 cycles at the same frequency. Additionally, the particle concentration of the sample generated by cryomilling for 6 cycle @ 30 Hz is significantly greater than the other samples between 1 and 10 μ m. Beyond 10 μ m, differences in the size distributions generally follow the same trends and are more easily seen in Figs. S9 and S10. The frequency of milling was also varied for samples cryomilled for 1 and 3 cycles (Fig. 6b). Below 10 μ m, there were generally more particles generated in samples milled at 30 Hz. At particles sizes greater than 10 μ m these trends were more significant for the samples milled for one cycle and can more easily be seen in Figs. S9 and S10.

To further illustrate these trends, the total number concentration of particles between 0.68 and 86 μ m are compared in Fig. 7. It was observed that increasing the number of milling cycles at 30 Hz led to a greater number of particles in this size range (Fig. 7a). For example, milling for 6 cycles generated around 6.5 times more MPs than milling for 1 cycle. In regards to the effect of milling frequency on MP generation, there was not a statistically significant difference in mean number concentration between 1 and 3 cycle samples milled at different frequencies (Fig. 7b), indicating little impact of milling frequency on the generation of micro sized fragments (Welch's T Test, p-value = 0.378 and 0.358, respectively). Milling time did not have a significant effect on the mean number concentration between samples generated by cryomilling for 1 cycle @ 30 Hz and



Fig. 5 Percent of original plastic mass remaining after 100 µm and 20 µm sieving



Fig. 6 Average number distributions measured by Coulter Counter for MPs generated by cryomilling for varied milling cycles (a) and varied milling frequencies (b)

2 cycles @ 30 Hz samples and 2 cycles @ 30 Hz and 3 cycles @ 30 Hz samples (*One-way ANOVA with Tukey's HSD* post hoc *test*, *p*-value = 0.800 and 0.065, respectively). However, all other pairwise comparisons were statistically significant, indicating a stronger impact of milling time as time progresses (1 and 3 cycles, *p*-value = 0.018; 1 and 6 cycles, *p*-value = 0.000; 2 and 6 cycles, *p*-value = 0.012).

NTA was used to investigate the role of cryomilling parameters on particle sizes in the nanometer size range (Fig. 8). As noted in the methods, particles < 100 nm could not be quantified on NTA. The PSDF and the volume distribution for these samples can be found in the Supplementary Information (Figs. S11 and S12). Generally, increasing the number of milling cycles increased the number of submicron particles (Figs. 8a and 9a). Similar



Fig. 7 Total number concentration of microplastics from 0.67 – 86 µm measured by Coulter Counter obtained from a) varied milling cycles and b) varied frequency of milling. * signifies a *p*-value < 0.05 for **a**) One-way ANOVA with Tukey's HSD post hoc test, and **b**) Welch's T Test. Error bars indicate standard deviation of samples

to the Coulter Counter results, increasing the grinding frequency at 1 cycle, increased the number of submicron particles. After 3 cycles, the number of NPs generated was similar at frequencies of 15 and 30 Hz (Fig. 8b). Nanoplastics generated were mainly distributed around 250 nm in the 100-1000 nm size range (Fig. 8).

The total number concentration of particles generated in the sub-micron range are compared in Fig. 9. Following the number distribution in Fig. 8, more milling generally led to a greater number of particles. For example, milling for 6 cycles at 30 Hz generated 2.5 times the number of NPs than milling for a single cycle (Fig. 9a).



Fig. 8 Averaged NTA number distribution of cryomilled MPs obtained from **a**) varied milling cycles and **b**) varied frequency of milling. Measurements were averaged over multiple runs until the number of valid tracks reported were at least 100,000

A larger impact of milling frequency was observed in the nano size range when compared to the Coulter Counter measurements (Fig. 9b). For 1 milling cycle, the sample milled at a higher frequency resulted in a significantly greater quantity of nano-sized fragments (*Welch's T Test, p*-value = 0.000). However, the 3 cycle @ 15 Hz sample had a significantly greater quantity of nano-sized fragments to 3 cycles @ 30 Hz (*Welch's T Test, p*-value = 0.000).

In regards to the effect of milling cycles on NP generation, there was not a statistically significant difference in number concentration between 1 cycle @ 30 Hz and 2 cycles @ 30 Hz samples (*One-way ANOVA with Tukey's HSD* post hoc *test, p*-value=0.318). However, milling time had a significant effect on the mean number concentration among all other NTA samples (1 and 3 cycles, *p*-value=0.001; 1 and 6 cycles, *p*-value=0.000; 2 and 3 cycles, *p*-value=0.000; 2 and 6 cycles, *p*-value=0.000; 3



Fig. 9 Total number concentration of NTA measured nanoplastics from 100-1000nm obtained from **a**) varied milling cycles and **b**) varied frequency of milling. * signifies a *p*-value < 0.05 for **a**) One-way ANOVA with Tukey's HSD post hoc test, and **b**) Welch's T Test. Error bars indicate standard deviation of samples

and 6 cycles, p-value = 0.000). These results are consistent with the trends observed for the Coulter Counter number concentration and sieve analysis.

Although the results for each instrument demonstrate how milling parameters affect size distributions and number concentrations in their respective size ranges, a greater understanding of how cryomilling impacts PS particle size distributions can be obtained when looking across the entire size range. In order to combine Coulter Counter and NTA distributions, 750 nm PS microspheres were measured on each instrument at the same concentration and compared in Fig. S13. For these uniform, monodisperse spheres, the measured size of the microspheres aligned between the instruments. However, the number concentration of the distributions slightly varied despite the suspensions being the same concentration. Coulter Counter measurements yielded a total number concentration 1.08 times more than NTA measurements. This may be due to the difference in particle sizing techniques. As briefly mentioned, NTA camera and detection threshold settings can be susceptible to user bias and affect resulting measurements. Decreasing the camera level yielded a more accurate size measurement for 0.1 mg/L 750 nm PS microspheres (Fig. S4). We also observed that at this optimal camera level, increasing detection threshold led to a decrease in concentration error up to a threshold setting of 20 (Fig. S6). A more thorough investigation into NTA settings is needed to address these inconsistencies in number concentrations across the two instruments. However, a preliminary analysis of the combined size distribution was performed by normalizing number concentrations using the starting concentration of MP suspensions used for each analysis (500 mg/L for Coulter Counter and 135 g/L for NTA) (Fig. 10). Because the number concentrations are dominated by particles in the nano-range, volume distributions are used here to better illustrate the evolution of the particle size distributions during cryomilling. Although Fig. S13 demonstrates that particle sizes match between the two instruments, the number concentration still does not align. NTA has a fixed field of view, tracking around 100 particles, thus there are not many large particles that can be tracked while also resolving smaller particles in the same frame. Detection threshold can be adjusted to better see different sized particles; however, the preferential counting of smaller particles may account for the mismatch of distributions in the overlapping size range. This may be attributed to the camera and detection settings of NTA, as well as the non-spherical nature of the cryomilled fragments and basis for measurement for each instrument. Because Coulter Counter converts volume displaced to a spherical diameter and NTA calculates a spherical diameter through the Stokes–Einstein equation, differences in calculated diameter can shift the distributions to the left or right of one another, depending on the shape factor, and thus lead to distributions that don't overlap.

A bimodal volume (mass) distribution was observed in Fig. 10 between 100 nm and 86 µm. One peak exists in the nano-range and a second peak exists in the 10-100 µm range. As samples are milled for longer times (increasing number of milling cycles) two trends are observed. First, the volume concentration of plastics in the nano-range increases. Second, the peak in the 10-100 µm range shifts steadily to smaller particle sizes. Together, these trends suggest that the milling process is characterized by small, nano-sized fragments breaking off of larger particles during the fragmentation process. Herein, the number of smaller particles increase while the larger particles become gradually smaller over time. This is in contrast to a process where fragments are sequentially broken into roughly equally sized pieces and the entire size distribution shifts towards smaller sizes with continued



Fig. 10 Volume distribution of cryomilled PS fragments obtained from NTA and Coulter Counter

milling and the smallest fragments don't appear for some time. It is important to recall that only a small fraction of the bulk cryomilled sample exists in the range accessible by the NTA and Coulter Counter (Fig. 5). As such, we don't have resolution regarding how the particle size distribution is changing for particles > ~ 100 μ m.

Changing the number of cycles and milling frequency both had an effect on the number concentration of MP/NPs generated. To better understand which parameter is predicted to have greater influence on MP generation, the cryomilling "dose" (D_m) , or a measure of how much the plastics were milled, was estimated for each sample and compared (see Supplementary Information for details) [33, 34]. Theoretical cryomilling dose was found to be proportional to both frequency and time of milling for these experiments (Eq. 2).

$$D_m \propto f^3 \cdot t \tag{2}$$

where f is the frequency of milling and t is the time of milling. Relative cryomilling dose for each sample is compared in Fig. S14, where the frequency of milling is expected to have a much greater effect on the dose, and presumably, the resulting size distribution. The expectation was that a larger number of smaller particles would be produced with increasing dose. However, the data presented in Figs. 7 and 9 indicate that the frequency of milling only had a significant effect on the resultant size distribution for the nano sized fraction. Of all the parameters used to characterize the resulting MPs, only the number concentration of particles in the nano-range was somewhat correlated with the cryomilling dose (Fig. 11a).

The mass fraction of particles < 20 µm was also correlated with cryomilling dose (Fig. S15a). However, in both cases, cryomilling time was a better predictor than the relationship predicted in Eq. 2 (Figs. 11b-S15b). Cryomilling time was also a better predictor of the other parameters (mass fraction < 100 µm, and Coulter Counter number concentration), but the correlations were not as strong ($R^2 < 0.85$). Additional work is necessary to reconcile the differences between the predicted and observed impacts of cryomilling parameters on the resulting size distribution. However, it is clear that cryomilling time increases the number concentration of both nano- and micron-sized PS fragments, with a significant increase in the number concentration of fragments in the $1-10 \,\mu\text{m}$ range being observed only after 6 milling cycles. This relationship was found to be linear within the bounds of the milling times studied. Further research is needed to explore the boundaries of that relationship across multiple materials and cryomilling techniques.

Conclusions

The cryomilling approach presented here serves as a valuable method to generate both micron and submicron PS plastic particles. These fragments exhibited characteristics of secondary plastics found in the environment with respect to their size and shape when compared to simplified, monodisperse spheres. Cryomilling did not alter the surface chemistry of polystyrene and produced particles spanning both the micro- and nanometer size range. However, fragmentation is not the only transformative process plastics undergo in the environment. Therefore future work would involve aging of cryomilled fragments utilizing ultraviolet light to simulate photooxidation effects due to sunlight [20].

Coulter Counter and NTA were utilized to obtain high resolution size distributions of the resulting PS plastic fragments in both the micro- and nano- size ranges, respectively. This data shows the utility of commonly used particle sizing instrumentation to measure polydisperse suspensions more akin to environmental MPs, especially in the nano range. One limitation of this technique is that plastics collected from the environment could be contaminated with sediment or microbes if not well cleaned that would result in erroneous particle size distributions with either instrument; thus, generation from pristine plastics is preferred. It has been reported that NTA has the potential to introduce user bias via camera and detection settings [24]. In this work, monodisperse microspheres were used to investigate what camera settings and detection thresholds together counted particles most efficiently. Further, NTA measurements can be reprocessed if a more accurate detection threshold is determined after data collection. Increasing the length and number of NTA videos increased the likelihood of detecting larger particles. By insuring that the number of valid tracks were greater than approximately 100,000 for each sample, more continuous size distributions over the entire measured size range were obtained. In addition, we have shown that PS particles < 100 nm will not be included in the size distributions from the NTA. It is unclear how colored plastics or other laser light sources may influence the resolution of NTA and should be studied further.

Neither Coulter Counter or NTA measurements were sufficient to capture the entire size distribution. Further, while trends in the measurements from the two instruments agree, additional work is necessary to combine the data from the two instruments into a single particle size distribution. Experiments with monodisperse polystyrene latex spheres indicated that particle size measurements via the two instruments agree with respect to particle size. However, there appear to be discrepancies in the measured number concentrations using the two



Fig. 11 Correlation of the number concentration of submicron fragments with a) theoretical cryomilling dose and b) milling time. Triangle markers indicate samples milled at a frequency of 15 Hz. Circles were samples milled at 30 Hz. Error bars indicate standard deviation of samples

instruments. These discrepancies were observed in the fragment data as well (Fig. 11 inset). Discrepancies in NTA derived number concentrations have been reported previously and are the subject of ongoing investigation [24].

The impacts of milling frequency on the resulting size distribution were less than theoretically predicted, with milling frequency having a greater impact in the initial stages of milling and the time of milling being a stronger predictor of the extent of milling. All cryomilling conditions resulted in broad and heterogeneous size distributions with particles spanning the nano- to micrometer range. Further, Coulter Counter and NTA measurements indicate that longer milling times result in larger numbers of smaller plastic fragments. Although all cryomilling conditions resulted in nanoplastic fragments approximately 250 nm in diameter, the concentration of fragments in this size range increased with increasing milling time. Therefore, if large quantities of PS nanoplastics are desired, longer cryomilling times are recommended. If it is desired to produce PS fragments in the 1–10 µm range, long cryomilling times are also required. Trends in measured volume distributions suggest that the nano-sized fragments are cleaved from the starting materials, gradually eroding the parent fragments to smaller and smaller sizes. Significant concentrations of PS particles in the 1–10 µm range did not appear until 6 cycles of milling @ 30 Hz. This work yielded a procedure for generating PS MPs that will enable more realistic fate, transport and toxicological studies. Future investigations are needed to assess 1) if these methods can be adapted to generate realistic microplastics of other common polymer types found in the environment and 2) if similar trends are observed between cryomilling parameters and particle size distributions for other polymer types.

Abbreviations

MPs	Microplastics
NPs	Nanoplastics
NTA	Nanoparticle tracking analysis
PS	Polystyrene
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy

Supplementary Information

The online version contains supplementary material available at https://doi. org/10.1186/s43591-023-00069-z.

Additional file 1: Fig. S1. Validation of MP stability in ISOTON II Coulter Counter diluent. Fig. S2. Combining data from multiple Coulter Counter apertures. Fig. S3. NTA evaluation of camera settings on PS ND with a set detection threshold. Fig. S4. NTA particle size and mass concentration error using different camera settings and a set detection threshold. Fig. S5. NTA evaluation of detection thresholds on PS ND with a set camera setting Fig. S6 NTA particle size and mass concentration error using different detection thresholds and a set camera setting. Fig. S7. NTA PSD data averaging with increasing valid tracks. Fig. S8. µFTIR confirmation of PS test tube before and after cryomilling. Fig. S9. Coulter Counter measured mean PSD for cryomilled polystyrene samples. Fig. S10. Coulter Counter mean Volume Distribution obtained from cryomilled polystyrene samples. Fig. S11. Averaged NTA PSD obtained from cryomilled polystyrene samples. Fig. S12. Averaged NTA Volume Distribution obtained from cryomilled polystyrene samples. Fig. S13. Cross-instrumental distribution of PS microspheres. Fig. S14. Theoretical cryomilling dose for each cryomilled sample. Fig. S15. Correlation of the percent of mass of original plastic < 20 µm with theoretical cryomilling dose and milling time.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation and data collection were performed by CJM and BJH. Data analysis was performed by CJM with oversight from JAN. The first draft of the manuscript was written by CJM with editing and input from JAN, BJH and SLH. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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