

Skittles: A Multicolor Flow Cytometric Workflow for High-Throughput Characterization of Micro- and Nano-plastics

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Method Article

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Abstract

Micro- and nano-plastics (MNPs), polymer particles less than 5 mm in dimension, are abundant and globally pervasive anthropogenic pollutants. However, their true abundance in natural systems remains poorly constrained by the lack of high-throughput, standardized analytical methods for their characterization. Here, we present Skittles, a multicolor flow cytometric workflow for the high-throughput characterization of MNPs in liquid suspension that simultaneously provides fluorescence-based discrimination, particle enumeration, and SSC-calibrated PS-equivalent size distributions. The workflow was optimized and analytically validated using laboratory reference standards and environmental samples, demonstrating high precision (CVs typically <4%), quantitative recovery (mean 103%), and strong agreement with epifluorescence microscopy ($R^2 = 0.99$), while polymer classifications were independently corroborated using Raman spectroscopy. Application of Skittles to heterogeneous laboratory and environmental samples demonstrated its ability to characterize particle populations spanning multiple polymer types, morphologies, and sizes, including fragments, clumps, and fibers. Together, these results establish Skittles as a high-throughput analytical workflow for micro- and nano-plastic characterization in aquatic environments.

Short Synopsis Statement

Skittles enables single-instrument high-throughput characterization of micro- and nano-plastics, improving environmental monitoring and assessment of ecological plastic pollution risks.

Introduction

Plastics have become ubiquitous in modern society due to their low cost, lightweight, and versatile characteristics¹. However, the same durability and chemical resilience that underpins their utility have also led to the accumulation of mismanaged plastic waste in the environment². Consequently, plastic pollution is now considered one of the greatest threats to marine ecosystems^{3–6}. Once plastic enters the environment, it is subjected to mechanical (abrasion, erosion), chemical (photodegradation, oxidation), and biological degradation processes that drive its continuous fragmentation into smaller size classes^{7–11}. While the threat of entanglement, suffocation, and ingestion of larger plastic fragments by birds, turtles, fish, and marine mammals is well-documented^{4,12–17}, the extent to which smaller plastic particles, particularly micro- and nano-plastic, compromise environmental sustainability remains unclear. Studies on relatively large marine plastics (i.e., macroplastics) demonstrate that their abundance increases rapidly with decreasing size^{18–29}. Extrapolating this inverse abundance-size distribution to smaller size classes suggests that a substantial proportion of environmental plastics occurs within the micro- (MP; 1–5000 μm) and nano- (NP; <1 μm) size ranges^{27,30,31}. However, little is known about the abundance, size distribution, and composition of micro- and nano-plastics (MNPs) in aquatic environments. This gap persists largely due to the absence of rapid, high-throughput methodologies capable of effectively detecting, identifying, and quantifying MNPs in environmental samples^{10,32}.

Fluorescence staining, in combination with microscopy and spectroscopic techniques, is widely used for the detection and quantification of MPs^{33–37}. However, accurately quantifying and determining the polymeric composition of microplastics cannot typically be achieved with a single analytical technique. Consequently, microscopic and spectroscopic analyses are typically combined within the same workflow when assessing MNPs. These techniques are time-consuming, laborious, and limited to the detection of microplastic particles ($\geq 1\ \mu\text{m}$)^{38–41}. An alternative approach is to combine fluorescent staining with flow cytometry (FCM). FCM enables the high-precision detection and characterization of particles suspended in a liquid medium based on their light-scattering and/or fluorescence properties through the utilization of hydrodynamic focusing to pass suspended particles individually through a laser beam (or for a multicolor FCM, a series of laser beams). The scattering and fluorescence properties of particles are then collected and amplified through a series of photomultiplier tubes. This method enables the simultaneous measurement of multiple parameters for individual particles down to a size range of 30 nm at a rate of up to 50,000 particles per second⁴². Traditional FCM characterizes particles according to their size (forward scatter), surface and internal granularity (side scatter), and fluorescence properties (either autofluorescence or using a molecular fluorescence marker). The advent of multicolor or multi-laser flow cytometry (MFCM), along with a dramatic increase in commercially available fluorochromes, has expanded the number of accessible parameters, enabling more precise and detailed characterization of particles^{43–45}.

One promising fluorophore for microplastic detection using MFCM is 4-dimethylamino-4'-nitrostilbene (DANS)³⁹. DANS possesses several properties that make it especially well-suited for plastic detection. It exhibits solvatochromic fluorescence in response to different plastics, with its emission spectrum shifting across a broad wavelength range, from blue to red, according to the polarity of the bound plastic³⁹. Consequently, DANS produces distinct fluorescence emission patterns across different polymer types, enabling discrimination among common MNPs over a broad spectral range. DANS also provides strong polymer sensitivity due to the wide spectral shift⁴⁶, and its broad spectral response makes it particularly well suited for multicolor flow cytometric analysis, allowing simultaneous characterization of heterogeneous particle populations. Moreover, it provides reliable staining across various plastic types, including materials like polyethylene terephthalate (PET) and weathered particles³⁹.

Here, we introduce Skittles, a multicolor flow cytometric workflow for the high-throughput characterization of micro- and nano-plastics. We optimize the analytical workflow, evaluate its analytical performance, and demonstrate its application to environmental samples through cross-validation with fluorescence microscopy and Raman spectroscopy.

Materials and Methods

Precautions to reduce cross-contamination

To minimize plastic contamination, all experiments and samples were conducted or handled, respectively, under a clean and sanitized fume hood⁴⁷. To reduce contamination from clothing, 100% cotton shirts were worn with 100% cotton lab coats when handling samples or conducting experiments, and stored in wool garment bags when not in use. All tools and equipment were serially cleaned with detergent, rinsed with DI water, 70% ethanol, and autoclaved before use. Samples were contained in combusted borosilicate glass test tubes (450 °C, 24 h) and covered in combusted aluminum foil. Glass or metal tools were used for transfers whenever possible; plastic was restricted to necessary items (e.g., flow cytometry tubes and transfer pipettes), and the duration of plastic use was minimized. DANS stock solution and MNP stock solutions were stored in amber glass vials, with caps covered in combusted aluminum foil to prevent contamination from the polyvinyl chloride (PVC) cap.

MNP preparation

Method development and optimization experiments utilized commercially available and isolated nano- and micro-plastics (Table 1). Four polymer types were selected based on their commercial availability in the nano- and micro-size range and their documented prevalence in natural aquatic environments^{48–50}. For commercial particles, concentration was estimated based on material density and mean particle diameter. Although polyethylene terephthalate (PET) and polypropylene (PP) were not commercially available in nano- or micro-particle form, they were prepared due to their status as abundant and pervasive environmental pollutants^{4,16,49,51,52}. Furthermore, PET was included to evaluate method performance on irregular particle morphologies.

Polyethylene terephthalate particles were prepared from PET bottles following Rodriguez-Hernandez⁵³ and Hernandez et al⁵⁴. Polypropylene particles were prepared by dissolving isotactic pellets (average $M_n \sim 5,000 \text{ g mol}^{-1}$) (Sigma-Aldrich, St. Louis, MO) in 50 mL of ACS-grade xylene (Fisher Chemical, Waltham, MA) at 130°C for two hours under constant stirring. The polymer solution was then precipitated by slowly adding it to 200 mL of cold methanol (Fisher Chemical, Waltham, MA). Any excess methanol was decanted, and the resulting polymer powder was dried under a stream of N_2 for 8 h, followed by vacuum drying at 40°C for an additional 24 h.

Table 1
Types and sources of polymers used in this study.

Polymer	Shape	Size (µm)	Source
Polystyrene (PS)	Sphere	0.1–10.0	Cospheric & Sigma-Aldrich
Polyethylene (PE)	Sphere	0.2–9.9	Cospheric
Polyethylene Terephthalate (PET)	Fragment	< 0.2	PET Bottles
Polypropylene (PP)	Fragment	< 20	Sigma-Aldrich

Plastic particles were pre-treated to modify their surface wettability (i.e., reduce hydrophobicity) by suspending them overnight in 0.1% (w/v) Tween-20 solution (Sigma-Aldrich, Darmstadt, Germany).

Synthesized MNPs (PET and PP) were vacuum filtered through a 20.0 μm pore-sized polycarbonate (PC) filter (47mm PCTE, STERLITECH, Auburn, WA, USA) to remove larger particles that may obstruct the MFCM, and then transferred into an autoclaved flask, and stored under the fume hood at RT. For recovery rate experiments, plastic stock solutions were prepared at a starting concentration of 1.0×10^7 particles mL^{-1} . For all other treatments, 25 mg mL^{-1} suspensions of the different MNPs in UP water were used.

DANS staining Optimization

A 500 μM stock staining solution was prepared by dissolving DANS (Sigma-Aldrich, Darmstadt, Germany) in ethanol. For evaluating the optimal DANS stain concentration, stain concentrations ranging from 1.4 to 25 mM were used. For all other treatments, 50 μL of DANS solution was added to the samples.

To assess DANS staining conditions for micro- and nano-plastics (MNPs) analysis, samples were subjected to a series of controlled treatments and analyzed by multicolor flow cytometry (MFCM). The effects of DANS concentration, filtration, heating, stock stability, and post-staining storage on fluorescence intensity, particle recovery, and polymer discrimination were evaluated using MFCM. All treatments were compared to a reference condition consisting of MNPs stained with 50 μL of unfiltered DANS, heated for 60 min at room temperature in the dark, and analyzed within 12 h. Staining performance was assessed based on fluorescence intensity, discrimination among polymer types, variability in fluorescence emission maxima, and accuracy of particle concentration estimates.

Undissolved DANS particles were observed microscopically in stock solutions. Therefore, filtered and unfiltered DANS stock solutions were compared to evaluate the effect of dye filtration on MFCM performance. Specifically, DANS solutions were filtered through 0.2 μm polytetrafluoroethylene syringe filters (Finetech) before application to MNP samples. To test the shelf life of the staining solution, filtered and unfiltered DANS stock solutions were used to stain MNP fresh and after 10, 20, and 30 days of storage at RT. To test the effect of applying heat during MNP staining on fluorescent intensity measured by MFCM, filtered and unfiltered DANS stock solutions were used to stain MNP samples. Stained samples were either heated at 80°C for 60 min before analysis or measured immediately. To evaluate the shelf life of the stained samples, particle counts and fluorescence measurements were obtained immediately after staining and then re-measured after 12, 24, and 36 h of storage in the dark at RT. For both the sample-storage and DANS shelf-life treatments, only PS and PE were used.

Analysis of variance (ANOVA) was used to assess the effects of various staining treatments on MNPs measured by MFCM. Statistical analysis was performed using R statistical software (Posit PBC, Boston, MA) within the RStudio interface (version 4.4.1). Model assumptions were verified using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. Post-hoc pairwise comparison of means was based on Tukey's honest significance difference tests. When ANOVA assumptions were violated, non-parametric analysis was conducted using the Kruskal-Wallis H test, followed by the Mann-Whitney U test. The alpha level for all significance tests was set at 0.05.

Flow Cytometry configuration

Suspensions of DANS-labeled plastic particles were measured using a BD FACS-Celesta flow cytometer equipped with violet (405 nm), blue (488 nm), and red (610 nm) excitation lasers, along with corresponding emission filters configured to detect DANS fluorescence from each MNP type (Table 2). The BD FACS-Celesta supports analysis of particles approximately 30 nm to 20 μm in size. Before each analysis, the MFCM was cleaned, and the flow rate was measured manually ($\mu\text{L min}^{-1}$)⁵⁵. Detection was triggered on V525/50LP and V450/25LP channels, and the noise threshold was calibrated to < 20 events sec^{-1} using a blank consisting of UP water. Trigger channels were selected to maximize detection of DANS-labeled particles while minimizing background events. Sample concentration and flow rate were adjusted to achieve an event rate of 200–800 events sec^{-1} , providing statistically robust particle counts. Laser and emission configurations for MNP analysis are listed in Table 2. Obtained .fcs files were analyzed using FCS Express software version 7.20 (De Novo Software, Pasadena, CA). Bivariate plots used to distinguish individual MPNs and mixed MPN samples are also listed in Table 2.

Table 2
Optimal laser and emission configurations for MNP analysis and distinction.

Polymers	Laser	Threshold	Bivariant MNP discrimination
PET	B488	LP575/25	SSC vs B575/25
PS	V405	LP525/50	SSC vs V525/50
PE	V405	LP450/50	SSC vs V450/25
PP	V405	LP450/50	V450/50 vs V525/50
Three Mixed MNPs	B488; V405	LP575/25; LP525/50	V780/60 vs B575/25

Method validation

Method performance was evaluated following standard analytical validation criteria, including analytical blanks, recovery, repeatability, cross-method comparison, and environmental application. Analytical blanks (ultrapure (UP) water and artificial seawater (ASW) amended with DANS) were used to quantify instrumental and reagent background noise and to determine limits of detection (LOD) and quantification (LOQ). Filtered seawater and sterile (autoclaved) seawater blanks amended with DANS were included to assess the influence of matrix treatment on background particle detection. Matched seawater processed without DANS served as a negative staining control.

To assess method performance in complex natural matrices and evaluate potential interference from organic matter and biological constituents, spike-recovery experiments were conducted using commercially available polystyrene (PS) and polyethylene (PE) standard particles. Briefly, stock particle concentrations were estimated on manufacturer-reported material densities and mean particle

diameters. PS and PE particles were spiked into UP water, ASW, untreated natural seawater, and seawater amended with axenic and non-axenic algal cultures across a concentration range of 5.8×10^4 to 6.4×10^6 particles mL^{-1} . Axenic monocultures of *Gephyrocapsa huxleyi* coccolithophores (CCMP 371) and non-axenic cultures of *Tisochrysis lutea* (also known as *Isochrysis* sp. (T-ISO); obtained from NOAA Milford Laboratory, CT, USA) were grown and sampled as outlined in Supplementary Methods section.

For cross-validation of particle enumeration, MNP samples, including laboratory standards and environmental samples, were analyzed using the optimized Skittles workflow (Fig. 1) and subsampled for parallel enumeration by epifluorescence microscopy. For epifluorescence microscopy, 1–5 mL of the DANS-stained MNP sample was vacuum filtered onto a 0.2 μm black PC filter (Cytiva Marlborough, MA, 25mm), rinsed with UP water, transferred to a sterilized glass slide, overlaid with a glass cover slide, and examined immediately by fluorescence microscopy. Imaging of DANS-labeled samples was performed using an Olympus BX51 upright compound microscope, powered by the X-Cite 120Q fluorescent lamp (EXCELITAS, Waltham, MA), and equipped with an OLYMPUS DP71 digital camera. A minimum of 75 fields and 200 MNPs were recorded using a wide UV filter with ex 330–385, Dm 400, and Em 420 for PE, PP, and PET MNPs and a wide blue filter with ex 450–480, Dm 500, and Em 520 for PS MNPs. Enumeration derived from MFCM and epifluorescence microscopy was compared using Type II geometric mean regression in RStudio, which minimizes orthogonal deviations from the fitted line.

Raman spectroscopy was used to cross-validate the chemical composition of particles detected in environmental samples. Fifty-milliliter subsamples were chemically digested to remove organic material by combining samples with 150 mL of 10% KOH and incubating for 24 h at 40°C in a shaking incubator. Following digestion, samples were neutralized with 1 M citric acid and vacuum filtered onto glass fiber filters (47 mm; 0.7 μm pore size; Whatman Glass/Avantor, Radnor, PA). Filters were wrapped in aluminum foil for transport and analyzed within 3–5 days. Raman measurements were performed using a Thermo Fisher Scientific DXR 3 Raman microscope equipped with a 532 nm laser operating at 1–5 mW. Spectra were collected over the range of 500 to 3000 cm^{-1} with a spectral resolution of 1.7 nm^{-1} and an integration time of 0.5 s per acquisition (32 accumulations). Individual particles were selected manually from the filter surface for point analysis. Spectral processing, including baseline correction, was conducted using OMNIC software (version 9.16). Polymer identification was confirmed by comparison to reference spectra and validated using MNP standards.

Scatter-based particle size determination

Particle size calibration within the Skittles workflow was performed using forward and side scatter signals measured by flow cytometry. Four PS bead standards with certified mean diameters (0.1, 1, 5, and 10 μm ; Sigma-Aldrich, Darmstadt, Germany; Cospheric LLC, Somis, CA) were used to evaluate scatter-based particle sizing within the Skittles method. For each size class, approximately 10 μL of stock suspension was diluted to 2% (v/v) in UP water in autoclaved glass test tubes and stained following the optimized Skittles protocol (Fig. 1).

Particle detection was triggered on the V450 and V525 fluorescence channels to select DANS-stained particles. Flow cytometry event data were exported as CSV files and analyzed in MATLAB. Forward scatter area (FSC-A) and side scatter area (SSC-A) were extracted for all triggered events. For each bead size and replicate, scatter distributions were summarized using the geometric mean. Size calibration was performed using log–log linear regression relating geometric mean scatter intensity to nominal PS bead diameter, following the form $\log_{10}(D) = A + B \cdot \log_{10}(\text{scatter})$. Separate calibration models were fit for SSC-A and FSC-A, and equations were applied only within the bead-calibrated size range. Particle sizes are reported as polystyrene-equivalent diameters. Calibration precision was assessed by calculating coefficients of variation (CVs) of geometric mean scatter values across replicate measurements.

Environmental Samples

To evaluate the applicability of the optimized methodology to natural field samples, five independent seawater samples (800–1000 mL) were collected from the subsurface layer (0.3 m depth) at various locations within the Mississippi Sound (30.3140°N, 89.24319°W; 30.2649°N, 89.18379°W; 30.2052°N, 89.1067°W; 30.2236°N, 89.0607°W; 30.2483°N, 89.2494°W). Samples were collected in 1L autoclaved glass jars covered with aluminum foil and sealed with PP lids. For each sample, 5 mL subsamples were filtered through a 20 µm PCTE filter (47 mm; Sterlitech, Auburn, WA, USA). Four samples were processed and analyzed using the cross-validation framework described above, including enumeration by MFCM and epifluorescence microscopy. Due to the low abundance of MNPs in environmental samples, MFCM measurements were conducted at a medium flow rate with a 5-min acquisition time to maximize total particle detection, rather than targeting an optimal event rate. The remaining sample was processed to cross-validate polymer composition assignments obtained using the Skittles method with Raman spectroscopy.

Drinking water samples were also analyzed to evaluate method performance under lower particle abundance and lower background conditions. Samples were collected through a citizen science framework, in which volunteers across the Mississippi (MS) and Louisiana (LA) coastal region collected 500 mL water samples from household sources, including direct tap water, home-filtered tap water (e.g., pitcher-style activated carbon filtration), or commercially bottled water, using pre-cleaned glass containers. Samples were subsequently shipped to the laboratory for analysis, enabling evaluation of method performance under realistic, non-controlled collection conditions and low particle and low background matrices. Additionally, tap water from three independent locations was analyzed to evaluate PS particle size distributions.

Light scattering depends on particle refractive index and geometry; size calibration is inherently polymer and particle shape-dependent. In this study, size calibration was restricted to spherical PS particles due to the availability of certified bead standards. Accordingly, the calibration curve was only applied to environmental particles classified as putative PS based on their Skittles fluorescence signature. Event-level PS bead-equivalent diameters were calculated by applying the SSC-based calibration equation to fluorescence-triggered particles identified as PS. Events were flagged as within calibration range when

SSC-A values fell within the minimum and maximum SSC-A values defined by bead size standards, corresponding to spherical PS particles of diameters 100 – 10,000 nm. Size estimates for events within this range were considered quantitatively reliable. Events outside the calibration range were retained for qualitative assessment of size structure but interpreted as extrapolated estimates. All reported sizes are expressed as PS spherical-equivalent diameters. PS-equivalent size distributions are presented to evaluate the lower limits at which the Skittles method can resolve particle size structure under low-abundance conditions. All data processing and statistical analyses were performed in MATLAB (MathWorks, Natick, MA). For each sampling location and replicate, particle size distributions were evaluated as Log₁₀-transformed PS-equivalent diameter space and summarized using non-parametric statistics to account for skewed size distributions. Kernel density estimates (KDEs) were determined when event counts were ≥ 20 . To assess the internal consistency of staining and detection, fluorescence intensity was examined as a function of SSC-A for each location.

Use of Artificial Intelligence

During preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist with manuscript editing and scientific writing. Specifically, ChatGPT was used to improve grammar, readability, organization, and scientific clarity, and to assist with MATLAB code development for data processing and visualization. ChatGPT was not used to generate experimental data, perform data analyses, or independently interpret scientific results. All analyses, code, figures, interpretations, conclusions, and final editorial decisions were developed, critically reviewed, and approved by the authors, who assume full responsibility for the accuracy and integrity of the manuscript.

Results and Discussion

Development and Optimization of Skittles Workflow

To optimize DANS staining of MNPs, a range of DANS concentrations (1.38-50 mM) was tested. Quantitative MFCM analysis exhibited a typical saturation profile with increasing DANS concentration, consistent with binding site occupancy (Fig. 2a). Notably, the optimal DANS concentration was dependent on MNP surface area (Fig. 2b). Furthermore, when the stain concentration was optimized according to the power law equation, MNP concentration had no appreciable effect over the range tested.

Applying heat during staining had no significant effect on MNP counts obtained from MFCM (3-way ANOVA, p-value = 0.41). Polystyrene fluorescence intensity, however, was significantly reduced in unheated samples compared to heated ones (2-sample t-test, $p < 0.00$) (Fig. 3). Moreover, in heated PS samples, fluorescence intensity deteriorated significantly over time (36 h; ANOVA; p-value < 0.001); although signal intensity could be restored by reheating the samples within 1 h of analysis. Heat had no detectable effect on the fluorescence intensity of the other MNP types tested. Although heating

significantly increased fluorescence intensity, no measurable shift in SSC distributions or particle abundance was observed for either PS or PE particles (Fig. 3), indicating that the heating step did not alter the scatter-based particle characteristics used for enumeration and PS-equivalent size estimation.

Freshly prepared DANS maintained consistent staining performance over the 36-h evaluation period, with no significant change in particle counts (two-sample one-tailed t-test; p value 0.159). Conversely, the DANS stock solution showed substantial temporal degradation over the 30 days, evidenced by a significant alteration in particle counts applied to PS and PE samples (two-sample one-tailed t-test p-values of < 0.001 and 0.040, respectively). This degradation in staining efficiency suggests that prolonged storage of the DANS stock solution can compromise quantitative MFCM analysis by reducing signal strength and particle detectability. Accordingly, DANS stock solutions should be prepared fresh before use.

At high DANS concentrations, evidence of oversaturation (e.g., solid dye particles) was observed from epifluorescence microscopy. This may result in false positives and lead to overestimating MNP content^{42,56}. Filtering DANS stock solution improved epifluorescence visualization of MNP by removing unbound dye particles but did not affect fluorescence intensity or particle counts obtained by MFCM (One-way ANOVA, P-value = 0.520 and 0.880, respectively). This is likely due to the peak excitation and emission wavelengths (740–780 nm) of unbound DANS falling outside of the set detection range for our MFCM measurements of MNPs.

The final optimized Skittles workflow is outlined in Fig. 1. Briefly, samples were filtered through a 20 µm PCTE filter (47 mm, STERLITECH, Auburn, WA, USA), stained with 50 µL of 11.6 mM DANS solution, heated at 80°C for 1 h, shaken vigorously, transferred to a PS round-bottom flow cytometry tube (Corning, Phoenix, AZ), and analyzed by MFCM using the laser and emission configurations outlined in Table 2. Application of the optimized workflow enabled simultaneous fluorescence-based discrimination and enumeration of heterogeneous particle populations representing four polymer types across a particle size range of approximately 0.2–20 µm within a single analysis (Fig. 4). DANS fluorescence signatures generated by polarity-dependent shifts in emission spectra enabled discrimination among particle populations, while scatter measurements provided complementary information on particle size and morphology. Together, these results demonstrate the ability of the Skittles workflow to characterize heterogeneous MNP populations exhibiting a range of sizes and morphologies, including fragments, clumps, and fibers.

Analytical Performance and Method Validation

Blank analyses were conducted to distinguish analytical background from matrix-derived particle signals and to establish method detection limits for micro- and nano-plastic quantification. Analytical blanks consisting of ultrapure (UP) water and artificial seawater (ASW) amended with DANS yielded no detectable events, indicating negligible instrumental and reagent background. In contrast, seawater-based blanks exhibited detectable DANS-positive particle signals. Filtered and sterile (autoclaved) seawater blanks yielded signal levels comparable to untreated seawater, indicating that these

treatments did not substantially reduce DANS-positive particles. Seawater samples processed without DANS exhibited markedly lower particle counts, demonstrating that detection under the selected trigger conditions was strongly dependent on DANS staining. Collectively, these results indicate that seawater blanks represent environmental particle baselines rather than analytical background. Accordingly, no blank subtraction was applied to reported environmental MNP concentrations. Detection limits were estimated from Poisson counting statistics, where the 95% upper confidence bound for zero observed events corresponds to approximately three events, yielding an estimated LOD of $\sim 3/V$ and LOQ of $\sim 10/V$, where V is the analyzed sample volume.

Spike recovery experiments using PS and PE demonstrated high within-run precision for MFCM particle concentrations, with coefficients of variation (CVs) typically $< 4\%$ and an overall mean recovery of 103%. In the UP water matrix, average CVs of 3.2% and 4.0%, with mean recoveries of 96% and 113% for PS and PE spikes, respectively. In untreated seawater, average CVs were 3.0% and 15%, with recoveries of 84% and 97% for PS and PE, respectively. All values fell within commonly accepted method validation criteria (recoveries of 80–120% and $CV \leq 15\%$), with particularly strong performance observed for the complex seawater matrix. No significant differences in polymer counts were observed between ASW and natural seawater (two-sample t-test, p value = 0.31 for PE and p -value = 0.13 for PS), indicating that dissolved organic matter and indigenous microorganisms alone had little effect on MNP detection. Conversely, ASW amended with *T. lutea* resulted in quantitative recovery of PS (100%) but substantially reduced recovery of PE (23%). Similarly, ASW amended with *G. hux* yielded recoveries of 71% for PS and 18% for PE. These results are consistent with previous studies demonstrating preferential interaction and removal of PE by algae cells, likely through surface adsorption or aggregation processes^{57,58}. Whether the reduced PE recovery reflects true biological removal, altered optical properties, or matrix-dependent detection remains unresolved.

Cross-method comparison of MNP laboratory standards in sterile UP water showed strong agreement between particle concentrations obtained by MFCM and epifluorescence microscopy across the full range of MNP types, sizes, and concentrations tested ($R^2 = 0.99$; Fig. 5). At the smallest size range evaluated, corresponding to PET nanoparticles, MFCM yielded higher particle counts than epifluorescence microscopy, consistent with increased detection of the smallest particles. Collectively, these observations indicate that MFCM provides precise, accurate, and sensitive quantification of MNP particles across a broad size range.

Scatter-Based Particle Size Characterization

In addition to particle identification and enumeration, the Skittles workflow was also evaluated for its ability to differentiate particle size from forward scattering and side scattering signals. In general, side scatter area (SSC-A) increased monotonically with PS bead size when particle detection was triggered on the V450 and V525 fluorescence channels (Fig. 6a). Deviations from linearity across the 1–5 μm size range likely reflect refractive index-dependent scattering and partial saturation of the SSC collection angle, which redistributes scattered light outside the detector's angular acceptance. Accordingly, particle sizes are reported as PS-equivalent diameters within the bead-calibrated range.

Replication precision for particle size determination was high, with CVs of the geometric mean SSC-A of 5.6%, 4.0%, 0.9%, and 0.4% for 100, 1000, 5000, and 10,000 nm beads, respectively (Fig. 7, blue line). As expected, SSC CVs were slightly higher at the smallest size class; however, CVs below 6% at 100 nm are considered very good for nanoscale flow cytometry. Precision improved markedly with increasing particle size, indicating that SSC-A provides a robust and reproducible size proxy for plastic particles measured using the Skittles method.

In contrast, forward scatter area (FSC-A) exhibited a compressed dynamic range and poor discrimination between 100 nm and 1000 nm PS beads, rendering FSC-A unsuitable as a primary size proxy for the Skittles method (Fig. 6b).

Application to Environmental Samples

Natural, unaltered seawater collected from multiple locations within the Mississippi Sound (MS Sound) contained DANS-stained particles at concentrations ranging from 231 to 1,178 particles mL^{-1} , with a mean of 762 ± 346 particles mL^{-1} . Consistent with results obtained using laboratory standards, cross-comparison of environmental samples revealed strong agreement between polymer concentrations measured by MFCM and epifluorescence microscopy ($R^2 = 0.99$), supporting the robustness of the method in complex environmental matrices. Particles exhibiting fluorescence signatures consistent with the targeted polymer types (PP, PS, PE, and PET) accounted for $83.9 \pm 8.3\%$ of total DANS-stained particles. Accordingly, the mean concentration of putative micro- and nano-plastics was 636 ± 296 particles mL^{-1} , equivalent to $\sim 6.4 \times 10^8$ particles m^{-3} . These concentrations are several orders of magnitude higher than previously reported estimates for microplastics in the Gulf of Mexico that were restricted to larger particle sizes (0.7–335 μm), which ranged from 6.0 to 15.7×10^4 particles m^{-3} . This discrepancy is anticipated given that the present study spans a substantially broader size range that includes nano- and submicron-scale particles. Direct field measurements of MNP abundance reported as particle number remain extremely limited. Recently, ten Hietbrink et al.⁵⁷ quantified oceanic nano-plastics by targeting particles $< 1 \mu\text{m}$ using TD-PTR-MS following removal of particles larger than $1 \mu\text{m}$, reporting polymer mass concentrations of approximately $1.5\text{--}32 \text{ mg m}^{-3}$ across the North Atlantic. Because these measurements report polymer mass rather than particle counts, direct comparison to number-based estimates is not straightforward. However, conversion of these mass-based concentrations to particle numbers using plausible assumptions for particle size and density (e.g., diameters of $\sim 100\text{--}1000 \text{ nm}$) yields inferred abundances on the order of $\sim 10^3\text{--}10^6$ particles L^{-1} . This suggests that the MNP concentrations reported here fall within a reasonable range, given the broader size range (30 nm to 20 μm) and coastal environment of our measurements. Particles exhibiting fluorescence signatures consistent with PP and PS were the dominant classified particle populations in MS Sound samples, while PE and PET contributed $9.1 \pm 2.9\%$ and $1.9 \pm 1.4\%$, respectively. Although the prevalence of PP is consistent with reported microplastic distributions across aquatic environments, PS is typically observed at lower relative abundance ($\sim 4\%$), while PE is often the dominant polymer ($\sim 23\%$) in microplastic surveys⁵⁸. Raman spectroscopy was used to independently validate MFCM-based

polymer identification using nearshore water collected at Pass Christian Harbor, showing spectral features indicative of PS, PE, and PET that were consistent with MFCM classifications (Fig. 8). Moreover, the relative peak intensity of PS in Raman spectra (Fig. 8b) suggested PS dominance at this site, in agreement with MFCM measurements. Cross-validation between MFCM, epifluorescence microscopy, and Raman spectroscopy underscores the reliability of this methodology for fluorescence-based polymer discrimination and enumeration. The successful application of Skittles to natural seawater demonstrates that the workflow is applicable to environmentally weathered particle assemblages. Future studies incorporating controlled weathering experiments will help determine how particle aging influences fluorescence signatures and analytical performance across polymer types.

Water samples collected across MS and LA through a citizen science framework exhibited a wide range of DANS-stained particle concentrations, with mean values ranging from 12.7 mL^{-1} (Glacéau® Smartwater) to 94.7 mL^{-1} (tap water from New Orleans, LA). These results demonstrate the sensitivity of the method for detecting low concentrations of MNPs in drinking water matrices. Particles classified as the targeted polymer types accounted for an average of $72.5 \pm 25.6\%$ of total DANS-stained particles. Among identified polymers, PP contributed the largest mean fraction ($35.3 \pm 25.3\%$), followed by PET ($9.3 \pm 11.3\%$), PE ($7.8 \pm 13.6\%$), and PS ($6.5 \pm 12.6\%$). Polymer distributions observed in our drinking water samples align with broad trends recently reported^{59,60}. Estimated particle concentrations, however, were two to three orders of magnitude higher than the pooled mean value of $56.98 \text{ particles L}^{-1}$ reported for micro-plastics in tap water based largely on measurements obtained from microscopy, Raman, and FTIR analyses. That pooled estimate largely reflects studies focused on particles $> 10 \text{ }\mu\text{m}$, with few extending measurements to $\leq 1 \text{ }\mu\text{m}$ (i.e., 2 out of 30 studies). As particle abundance is generally thought to increase with decreasing size in environmental systems, inclusion of smaller micro- and nano-plastic size fractions is expected to increase reported particle concentrations.

SSC-based PS spherical-equivalent particle size distributions spanned approximately two to three orders of magnitude across all samples (Fig. 9a). The locations varied in their geometric mean particle diameter (GMD), with Gulfport having the smallest particles (84 nm; GSD = 10) and Ocean Springs the largest (605 nm; GSD = 18) (Table S1). Quantitative size inference was restricted to the bead-calibrated SSC range (100 – 10,000 nm), which encompassed 25–67% of detected events depending on sampling location. Fluorescence intensity scaled linearly with side scatter across all samples (Fig. 9b), indicating coherent staining of particle populations across the entire size spectrum. Although only a subset of events fell within the bead-calibrated SSC range, the preserved fluorescence–scatter relationship suggests that out-of-range events represent real particles extending beyond the current calibration domain rather than background or noise. Thus, quantitative size inference was limited by the density of bead calibration coverage relative to the observed SSC range, rather than by particle detection or fluorescence classification. Improved quantitative size resolution will require bead calibration ladders that more densely bracket the SSC domain occupied by fluorescence-classified particles, particularly within the nanoscale size range. In addition, reliable characterization of particle size distributions requires sufficient event counts; based on this analysis, a minimum of ~ 50 events per polymer type is

recommended to support stable summary statistics and density-based analyses. Finally, extension of size characterization to additional polymers will require polymer-specific bead standards matched in both material and size range.

Conclusion

Various methodologies have been developed to identify and quantify plastic polymers in aquatic environments, including dye-based approaches^{36,37} and analytical instruments^{41,61–63}. However, none are capable of simultaneously detecting both micro- and nano-plastic-sized particles, and comprehensive MNP characterization typically requires the use of multiple instruments^{47,61}, increasing cost, analysis time, and the risk of cross-contamination^{32,64,65}. In this study, we developed and optimized a high-throughput workflow for the enumeration, fluorescence-based discrimination, and size characterization of micro- and nano-plastics in aquatic samples by combining DANS-based polymer labeling with multicolor flow cytometry. This single-instrument approach enables characterization of MNPs in complex aqueous matrices while reducing sample handling and analysis time and extending analytical capability across the nano- to micro-size range. By integrating particle enumeration, fluorescence-based discrimination, and PS-equivalent size characterization within a single workflow, Skittles provides a practical platform for high-throughput characterization of micro- and nano-plastics in aquatic environments. The current implementation targets four environmentally abundant polymer classes and reports PS-equivalent particle sizes based on scatter calibration. Future work will focus on expanding the polymer reference library, extending scatter calibration to additional polymer types, and evaluating performance across a broader range of environmental matrices. These developments will further broaden the applicability of Skittles as a flexible platform for high-throughput micro- and nano-plastic characterization.

Declarations

Competing interests

The authors declare that they have no competing interests.

Ethics declaration

Not applicable.

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Author Contribution

Conceptualization, K.D.A.M; C.P.D.B spearheaded sample collection, executed experiments, performed MFCM measurements and initial analysis with guidance of K.D.A.M. C.P.D.B drafted the original manuscript with guidance of K.D.A.M. K.D.A.M. and C.P.D.B contributed to final analysis and writing of the submitted manuscript. All authors have read, contributed to manuscript revision and agreed to the submitted version of the manuscript.

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References

1. Andrady AL, Neal MA. Applications and societal benefits of plastics. *Philosophical Trans Royal Soc B: Biol Sci.* 2009;364(1526):1977–84. 10.1098/rstb.2008.0304 . PubMed PMID: 19528050.
2. Andrady AL. *Pastics and the Environment*. Wiley; 2003.
3. Devriese LI, van der Meulen MD, Maes T, Bekaert K, Paul-Pont I, Frère L, et al. Microplastic contamination in brown shrimp (*Crangon crangon*, Linnaeus 1758) from coastal waters of the Southern North Sea and Channel area. *Mar Pollut Bull.* 2015;98(1–2):179–87. 10.1016/j.marpolbul.2015.06. .051 PubMed PMID: 26456303.
4. Li HX, Ma LS, Lin L, Ni ZX, Xu XR, Shi HH, et al. Microplastics in oysters *Saccostrea cucullata* along the Pearl River Estuary, China. *Environ Pollut.* 2018;236:619–25. 10.1016/j.envpol.2018.01.083 . PubMed PMID: 29433102.
5. Walkinshaw C, Lindeque PK, Thompson R, Tolhurst T, Cole M. Microplastics and seafood: lower trophic organisms at highest risk of contamination [Internet]. 2019. 10.1016/j.ecoenv.2019.110066
6. Diaz-Basantes MF, Nacimba-Aguirre D, Conesa JA, Fullana A. Presence of microplastics in commercial canned tuna. *Food Chem.* 2022;385. 10.1016/j.foodchem.2022.132721 . PubMed PMID: 35305437.
7. Hadri E, Gigault J, Maxit B, Grassl B, Reynaud S. Nanoplastic from mechanically degraded primary and 1 secondary microplastics for environmental assessments. 2020.

8. Zettler ER, Mincer TJ, Amaral-Zettler LA. Life in the plastisphere: Microbial communities on plastic marine debris. *Environ Sci Technol*. 2013;47(13):7137–46. 10.1021/es401288x . PubMed PMID: 23745679.
9. Costa MF, Ivar Do Sul JA, Silva-Cavalcanti JS, Araújo MCB, Spengler Â, Tourinho PS. On the importance of size of plastic fragments and pellets on the strandline: A snapshot of a Brazilian beach. *Environ Monit Assess*. 2010;168(1–4):299–304. 10.1007/s10661-009-1113-4 . PubMed PMID: 19680758.
10. Gigault J, Pedrono B, Maxit B, Ter Halle A. Marine plastic litter: the unanalyzed nano-fraction. *Environ Sci Nano*. 2016;3(2):346–50. 10.1039/C6EN00008H.
11. Gewert B, Plassmann M, Sandblom O, Macleod M. Identification of Chain Scission Products Released to Water by Plastic Exposed to Ultraviolet Light. *Environ Sci Technol Lett*. 2018;5(5):272–6. 10.1021/acs.estlett.8b00119.
12. Azzarello MY, Van Vleet ES. Marine birds and plastic pollution. Published May. 1987.
13. Napper IE, Thompson RC. Plastic Debris in the Marine Environment: History and Future Challenges. *Global Challenges*. 2020;4(6). 10.1002/gch2.201900081.
14. Scircle A, Cizdziel JV, Tisinger L, Anumol T, Robey D. Occurrence of microplastic pollution at oyster reefs and other coastal sites in the Mississippi sound, USA: Impacts of freshwater inflows from flooding. *Toxics*. 2020;8(2). 10.3390/TOXICS8020035.
15. Weitzel SL, Feura JM, Rush SA, Iglay RB, Woodrey MS. Availability and assessment of microplastic ingestion by marsh birds in Mississippi Gulf Coast tidal marshes. *Mar Pollut Bull*. 2021;166. 10.1016/j.marpolbul.2021.112187 . PubMed PMID: 33639379.
16. Craig CA, Fox DW, Zhai L, Walters LJ. In-situ microplastic egestion efficiency of the eastern oyster *Crassostrea virginica*. *Mar Pollut Bull*. 2022;178. 10.1016/j.marpolbul.2022.113653 . PubMed PMID: 35447440.
17. Gad AK, Toner K, Benfield MC, Midway SR. Microplastics in mainstem Mississippi River fishes. *Front Environ Sci*. 2023;10. 10.3389/fenvs.2022.1065583.
18. Collignon A, Hecq JH, Galgani F, Collard F, Goffart A. Annual variation in neustonic micro- and mesoplastic particles and zooplankton in the Bay of Calvi (Mediterranean-Corsica). *Mar Pollut Bull*. 2014;79(1–2):293–8. PubMed PMID: 24360334.
19. Collignon A, Hecq JH, Galgani F, Voisin P, Collard F, Goffart A. Neustonic microplastic and zooplankton in the North Western Mediterranean Sea. *Mar Pollut Bull*. 2012;64(4):861–4. 10.1016/j.marpolbul.2012.01.011 . PubMed PMID: 22325448.
20. Cózar A, Echevarría F, González-Gordillo JI, Irigoien X, Úbeda B, Hernández-León S et al. Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences*. 2014;111(28):10239–44. 10.1073/pnas.1314705111
21. Song YK, Hong SH, Jang M, Kang JH, Kwon OY, Han GM, et al. Large accumulation of micro-sized synthetic polymer particles in the sea surface microlayer. *Environ Sci Technol*. 2014;48(16):9014–21. 10.1021/es501757s . PubMed PMID: 25059595.

22. Peeken I, Primpke S, Beyer B, Gütermann J, Katlein C, Krumpen T, et al. Arctic sea ice is an important temporal sink and means of transport for microplastic. *Nat Commun.* 2018;9(1). 10.1038/s41467-018-03825-5 . PubMed PMID: 29692405.
23. Shim WJ, Hong SH, Eo S. Marine microplastics: Abundance, distribution, and composition. *Microplastic Contamination in Aquatic Environments: An Emerging Matter of Environmental Urgency.* Elsevier; 2018. pp. 1–26. 10.1016/B978-0-12-813747-5.00001-1.
24. Cordova MR, Purwiyanto AIS, Suteja Y. Abundance and characteristics of microplastics in the northern coastal waters of Surabaya, Indonesia. *Mar Pollut Bull.* 2019;142:183–8. 10.1016/j.marpolbul.2019. 03.040 PubMed PMID: 31232293.
25. Han M, Niu X, Tang M, Zhang BT, Wang G, Yue W, et al. Distribution of microplastics in surface water of the lower Yellow River near estuary. *Sci Total Environ.* 2020;707. 10.1016/j.scitotenv.2019.135601 . PubMed PMID: 31767320.
26. Jiang Y, Yang F, Zhao Y, Wang J. Greenland Sea Gyre increases microplastic pollution in the surface waters of the Nordic Seas. *Sci Total Environ.* 2020;712. 10.1016/j.scitotenv.2019.136484 . PubMed PMID: 31931187.
27. Lindeque PK, Cole M, Coppock RL, Lewis CN, Miller RZ, Watts AJR, et al. Are we underestimating microplastic abundance in the marine environment? A comparison of microplastic capture with nets of different mesh-size. *Environ Pollut.* 2020;265:114721. 10.1016/j.envpol.2020.114721.
28. Mhired Gela S, Aragaw TA. Abundance and Characterization of Microplastics in Main Urban Ditches Across the Bahir Dar City, Ethiopia. *Front Environ Sci.* 2022;10. 10.3389/fenvs.2022.831417.
29. Qian N, Gao X, Lang X, Deng H, Bratu TM, Chen Q, et al. Rapid single-particle chemical imaging of nanoplastics by SRS microscopy. *Proc Natl Acad Sci U S A.* 2024;121(3):e2300582121. 10.1073/pnas.2300582121 . PubMed PMID: 38190543.
30. Ferreira I, Venâncio C, Lopes I, Oliveira M. Nanoplastics and marine organisms: What has been studied? [Internet]. 2019. 10.1016/j.etap.2019.01.006
31. Yin K, Wang Y, Zhao H, Wang D, Guo M, Mu M, et al. A comparative review of microplastics and nanoplastics: Toxicity hazards on digestive, reproductive and nervous system. *Science of the Total Environment.* Elsevier B.V.; 2021. 10.1016/j.scitotenv.2021.145758.
32. Provencher JF, Covernton GA, Moore RC, Horn DA, Conkle JL, Lusher AL. Proceed with caution: The need to raise the publication bar for microplastics research. *Sci Total Environ Elsevier B V.* 2020. 10.1016/j.scitotenv.2020.141426 . PubMed PMID: 32814297.
33. Liu S, Shang E, Liu J, Wang Y, Bolan N, Kirkham MB et al. What have we known so far for fluorescence staining and quantification of microplastics: A tutorial review **H I G H L I G H T S A R T I C L E I N F O** [Internet]. 10.1007/s11783-021-1442-2
34. Erni-Cassola G, Gibson MI, Thompson RC, Christie-Oleza JA. Lost, but Found with Nile Red: A Novel Method for Detecting and Quantifying Small Microplastics (1 mm to 20 µm) in Environmental Samples [Internet]. 2017. 10.1021/acs.est.7b04512

35. Maes T, Jessop R, Wellner N, Haupt K, Mayes AG. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. *Sci Rep*. 2017;7. 10.1038/srep44501 . PubMed PMID: 28300146.
36. Karakolis EG, Nguyen B, You JB, Rochman CM, Sinton D. Fluorescent Dyes for Visualizing Microplastic Particles and Fibers in Laboratory-Based Studies. *Environ Sci Technol Lett*. 2019;6(6):334–40. 10.1021/acs.estlett.9b00241.
37. Gao Z, Wontor K, Cizdziel JV. Labeling Microplastics with Fluorescent Dyes for Detection, Recovery, and Degradation Experiments. *Molecules*. 2022;27(21):7415. 10.3390/molecules27217415.
38. Maes T, Jessop R, Wellner N, Haupt K, Mayes AG. A rapid-screening approach to detect and quantify microplastics based on fluorescent tagging with Nile Red. Nat Publishing Group. 2017. 10.1038/srep44501.
39. Sancataldo G, Ferrara V, Bonomo FP, Chillura Martino DF, Licciardi M, Pignataro BG, et al. Identification of microplastics using 4-dimethylamino-4'-nitrostilbene solvatochromic fluorescence. *Microsc Res Tech*. 2021;84(12):2820–31. 10.1002/jemt.23841 . PubMed PMID: 34047435.
40. Wiggin KJ, Holland EB. Validation and application of cost and time effective methods for the detection of 3–500 µm sized microplastics in the urban marine and estuarine environments surrounding Long Beach, California. *Mar Pollut Bull*. 2019;143:152–62. .2019.03.060 PubMed PMID: 31789151.
41. Song YK, Hong SH, Jang M, Han GM, Rani M, Lee J, et al. A comparison of microscopic and spectroscopic identification methods for analysis of microplastics in environmental samples. *Mar Pollut Bull*. 2015;93(1–2):202–9. 01.015 PubMed PMID: 25682567.
42. Kaile N, Lindivat M, Elio J, Thuestad G, Crowley QG, Hoell IA. Preliminary Results From Detection of Microplastics in Liquid Samples Using Flow Cytometry. *Front Mar Sci*. 2020;7. 10.3389/fmars.2020.552688.
43. O'Donnell EA, Ernst DN, Hingorani R. Multiparameter Flow Cytometry: Advances in High Resolution Analysis. *Immune Netw*. 2013;13(2):43. 10.4110/IN.2013.13. .2.43 PubMed PMID: 23700394.
44. Sewell WA, Smith SABC. Polychromatic flow cytometry in the clinical laboratory. *Pathology*. 2011;43(6):580–91. 10. 1097/PAT.0B013E32834A69AE PubMed PMID: 21881537.
45. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods*. 2000;243(1–2):77–97. 10.1016/S0022-1759(. 00)00229-5 PubMed PMID: 10986408.
46. Lakowicz JR. In: Lakowicz JR, Boston MA, editors. *Principles of Fluorescence Spectroscopy*. Springer US; 2006. 10.1007/978-0-387-46312-4.
47. Kutralam-Muniasamy G, Shruti VC, Pérez-Guevara F, Roy PD, Elizalde-Martínez I. Common laboratory reagents: Are they a double-edged sword in microplastics research? *Sci Total Environ*. 2023;875. 10.1016/j.scitotenv.2023.162610 . PubMed PMID: 36894090.
48. Guo X, Wang J. The chemical behaviors of microplastics in marine environment: A review. *Marine Pollution Bulletin*. Elsevier Ltd; 2019. pp. 1–14. 10.1016/j.marpolbul.2019.03. .019 PubMed PMID:

31232281.

49. Hidalgo-Ruz V, Gutow L, Thompson RC, Thiel M. Microplastics in the marine environment: A review of the methods used for identification and quantification. *Environ Sci Technol*. 2012;46(6):3060–75. 10.1021/es2031505 . PubMed PMID: 22321064.
50. Andrady AL. The plastic in microplastics: A review. *Marine Pollution Bulletin*. Elsevier Ltd; 2017. pp. 12–22. 10.1016/j.marpolbul.2017. 01.082 PubMed PMID: 28449819.
51. Alam FC, Sembiring E, Muntalif BS, Suendo V. Microplastic distribution in surface water and sediment river around slum and industrial area (case study: Ciwalengke River, Majalaya district, Indonesia). *Chemosphere*. 2019;224:637–45. 10.1016/j.chemosphere. 2019.02.188 PubMed PMID: 30849624.
52. Quintana R, Manzano-Medina S, Pérez-López L, Oyón-Sanz A, González-Fernández D, González-Gordillo JI, et al. Vertical Distribution and Composition of Plastics in Coastal Areas of the Gulf of Cádiz: Insights into Transport Dynamics. *Environ Sci Technol*. 2025;59(33):17760–72. 10.1021/acs.est.5c03513 . PubMed PMID: 40762161.
53. Hernandez LM, Xu EG, Larsson HCE, Tahara R, Maisuria VB, Tufenkji N. Plastic Teabags Release Billions of Microparticles and Nanoparticles into Tea. *Environ Sci Technol*. 2019;53(21):12300–10. 10.1021/acs.est.9b02540 . PubMed PMID: 31552738.
54. Rodríguez-Hernández AG, Muñoz-Tabares JA, Aguilar-Guzmán JC, Vazquez-Duhalt R. A novel and simple method for polyethylene terephthalate (PET) nanoparticle production. *Environ Sci Nano*. 2019;6(7):2031–6. 10.1039/C9EN00365G.
55. Marie D, Partensky F, Vaulot D, Brussaard C. Enumeration of Phytoplankton, Bacteria, and Viruses in Marine Samples. *Curr Protoc Cytom*. 1999;10(1):11. 10.1002/0471142956.CY1111S10 . PubMed PMID: 18770685.
56. Bianco A, Carena L, Peitsaro N, Sordello F, Vione D, Passananti M. Rapid detection of nanoplastics and small microplastics by Nile-Red staining and flow cytometry. *Environ Chem Lett*. 2023;21(2):647–53. 10.1007/s10311-022-01545-3.
57. ten Hietbrink S, Materić D, Holzinger R, Groeskamp S, Niemann H. Nanoplastic concentrations across the North Atlantic. *Nature*. 2025;643(8071):412–6. 10.1038/s41586-025-09218-1 . PubMed PMID: 40634739.
58. Erni-Cassola G, Zadjelovic V, Gibson MI, Christie-Oleza JA. Distribution of plastic polymer types in the marine environment; A meta-analysis. *J Hazard Mater*. 2019;369:691–8. 10.1016/j.jhazmat.2019. 02.067 PubMed PMID: 30826562.
59. Malekzadeh M, Dehghanzadeh R, Ebrahimi SM, Sarbakhsh P, Fathifar Z, Aslani H. Occurrence, sampling, identification and characterization of microplastics in tap water: A systematic review and meta-analysis. *Ecotoxicology and Environmental Safety*. Academic; 2025. 10.1016/j.ecoenv.2025.118347 . PubMed PMID: 40393317.
60. Gambino I, Bagordo F, Grassi T, Panico A, De Donno A. Occurrence of Microplastics in Tap and Bottled Water: Current Knowledge. *Int J Environ Res Public Health* MDPI. 2022.

10.3390/ijerph19095283 . PubMed PMID: 35564678.

61. Prata JC, da Costa JP, Duarte AC, Rocha-Santos T. Methods for sampling and detection of microplastics in water and sediment: A critical review. *TrAC - Trends in Analytical Chemistry*. Elsevier B.V.; 2019. pp. 150–9. 10.1016/j.trac.2018.10.029.
62. Stock F, Kochleus C, Bänsch-Baltruschat B, Brennholt N, Reifferscheid G. Sampling techniques and preparation methods for microplastic analyses in the aquatic environment – A review. *TrAC - Trends in Analytical Chemistry*. Elsevier B.V.; 2019. pp. 84–92. 10.1016/j.trac.2019.01.014.
63. Yang L, Zhang Y, Kang S, Wang Z, Wu C. Microplastics in freshwater sediment: A review on methods, occurrence, and sources. *Science of the Total Environment*. Elsevier B.V.; 2021. 10.1016/j.scitotenv.2020.141948 . PubMed PMID: 32916488.
64. Prata JC, Castro JL, da Costa JP, Duarte AC, Rocha-Santos T, Cerqueira M. The importance of contamination control in airborne fibers and microplastic sampling: Experiences from indoor and outdoor air sampling in Aveiro, Portugal. *Mar Pollut Bull*. 2020;159. 10.1016/j.marpolbul.2020.111522 . PubMed PMID: 32771665.
65. Lenz R, Labrenz M. Small microplastic sampling in water: Development of an encapsulated filtration device. *Water (Switzerland)*. 2018;10(8). 10.3390/w10081055.

Figures

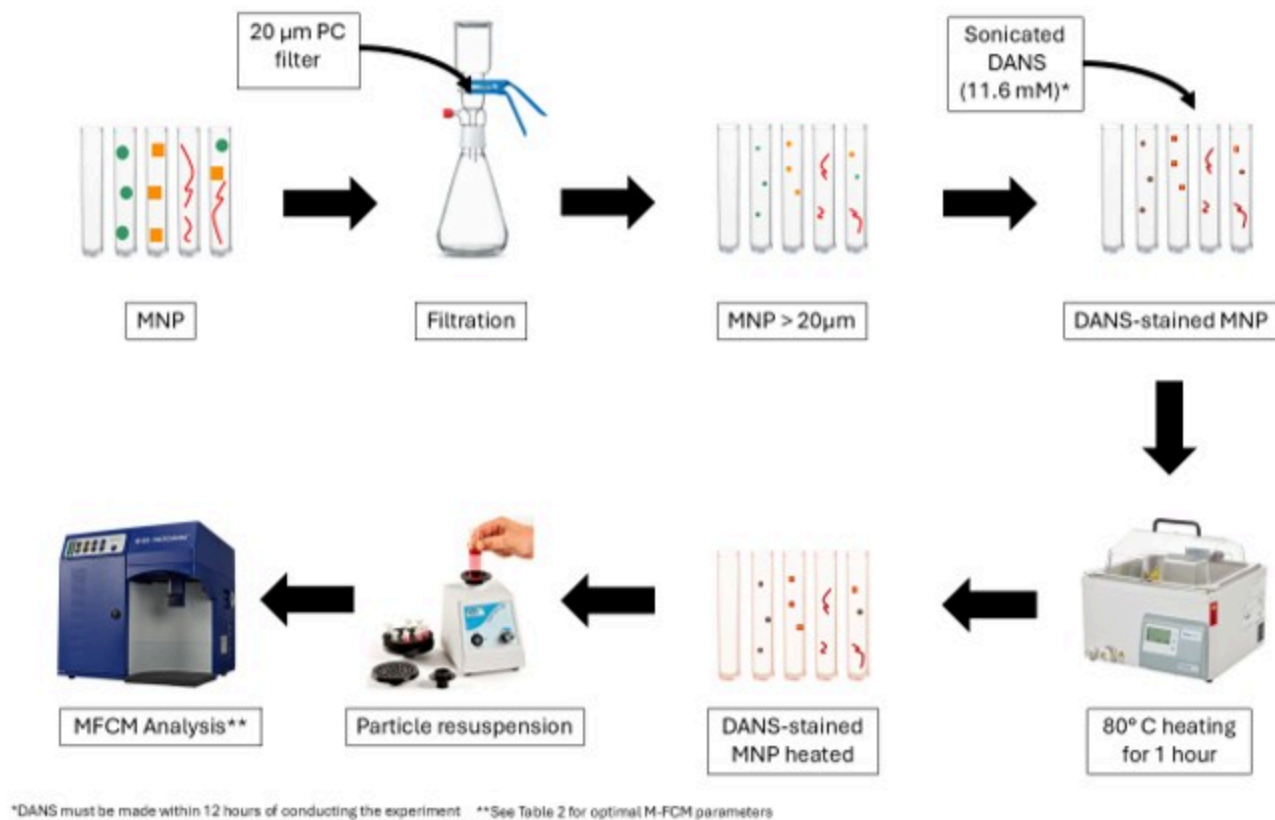


Figure 1

Basic workflow of MNP preparation and analysis in a liquid medium using the Skittles method.

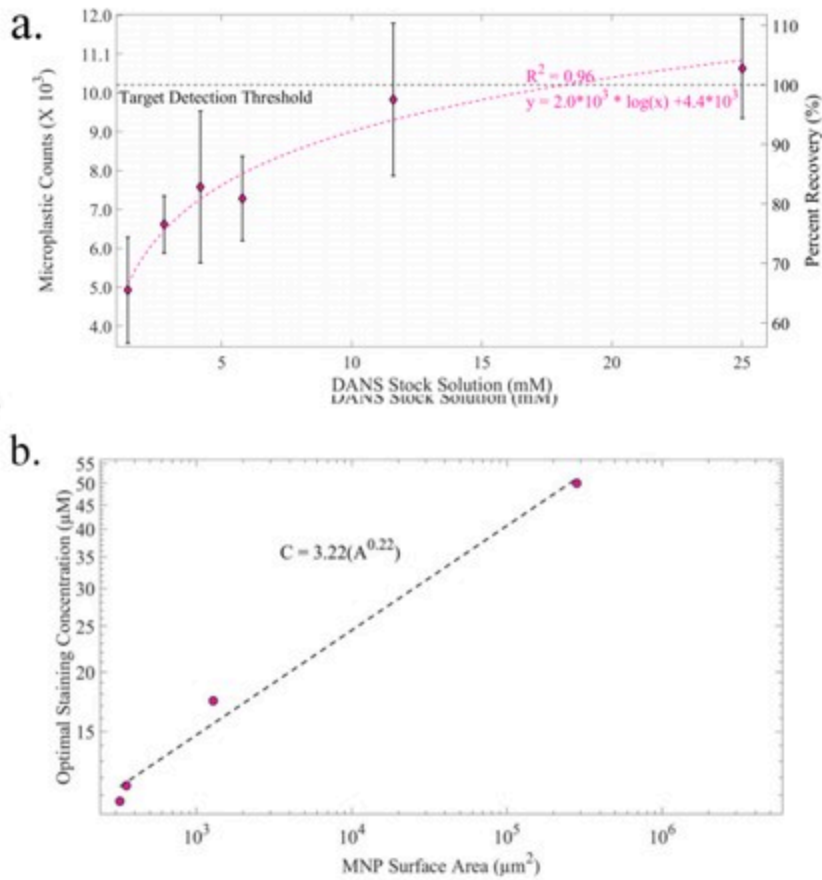


Figure 2

Optimization of DANS labeling for MNP detection by MFCM. (a) Representative plot (PS shown) illustrating a typical saturation profile of MNPs detected as a function of DANS concentration. The dashed line indicates the target detection threshold, with percent recovery estimated from material density and manufacturer-provided mean particle diameter. (b) Optimal DANS concentration required to achieve target detection, shown as a function of MNP surface area. (c) MNP particle concentrations detected by FCM as a function of the volume of MNP stock solution added, using optimal DANS concentration.

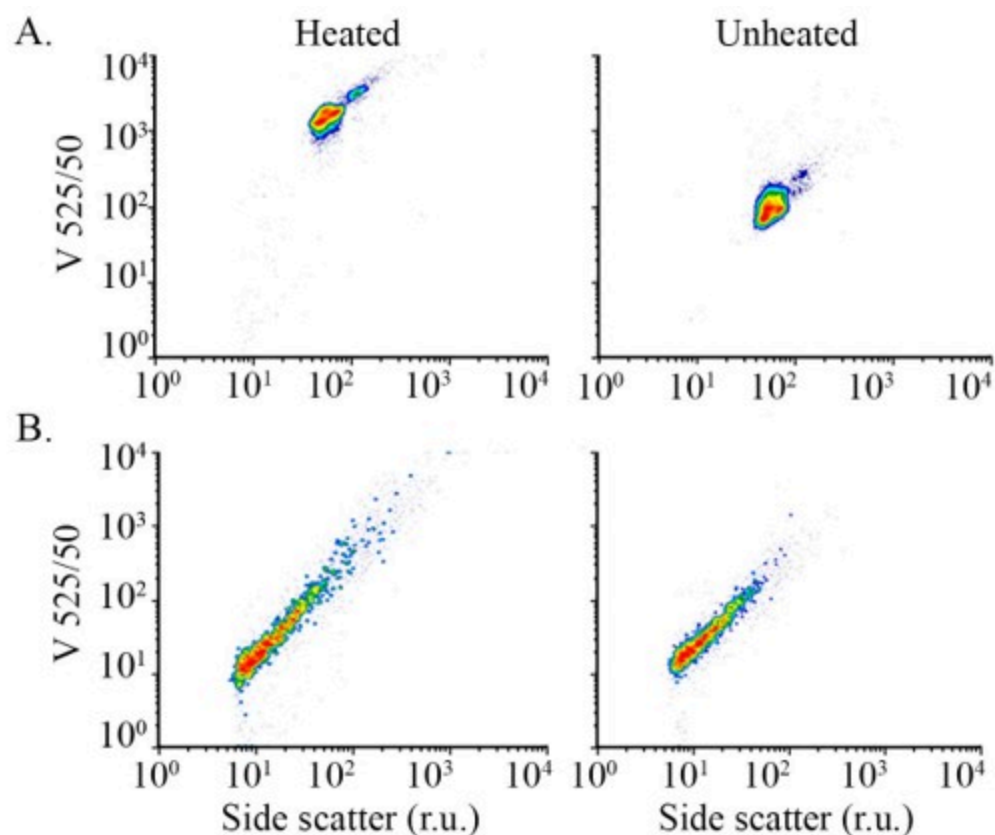


Figure 3

Effect of heating on the optical properties of DANS-stained micro- and nano-plastics. Representative multicolor flow cytometry density plots of (A) polystyrene (PS) and (B) polyethylene (PE) particles following staining with DANS under heated (80 °C, 1 h) and unheated conditions. Fluorescence intensity (V525/50) is plotted against side scatter (SSC). Heating substantially increased PS fluorescence intensity while producing no detectable shift in SSC distributions for either polymer, indicating that the heating step enhanced staining efficiency without measurably altering the scatter-based particle characteristics used for enumeration and PS-equivalent size estimation.

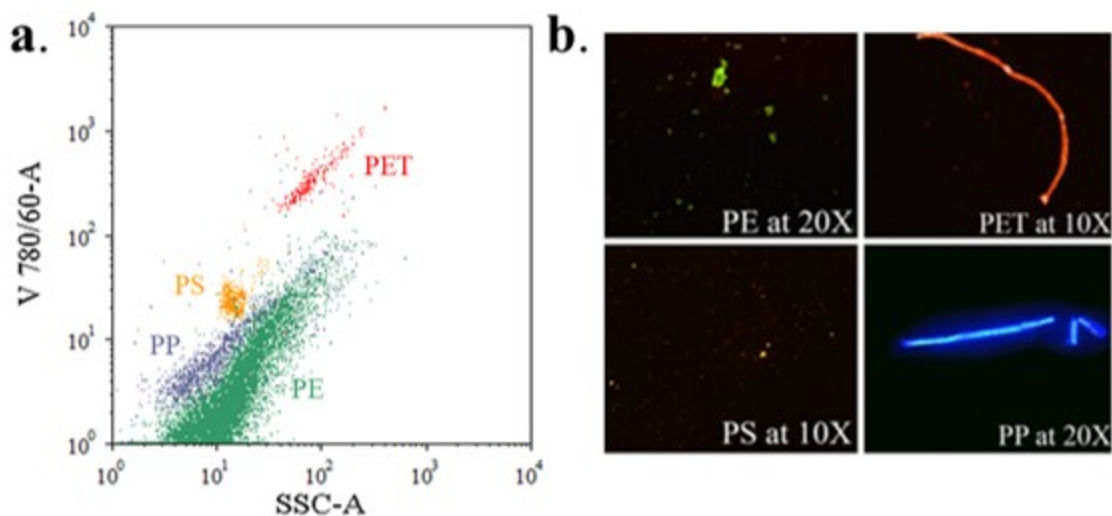


Figure 4

Visualization and enumeration of micro- and nano-plastics (MNPs) using (a) multi-color flow cytometry (MFCM) and (b) epifluorescence microscopy. (a) Representative MFCM dot plot of a mixed MNP sample suspended in UP water, showing separation of different plastic types based on fluorescence emission at 575 nm (488 nm excitation, B) and 525 nm (405 nm excitation, V). (b) Epifluorescence microscopy images demonstrating the functionality of DANS staining in distinguishing MNP composition via emission spectrum shifts under 405 nm excitation. Differences in MNP morphology (e.g., shape and structure) are also evident.

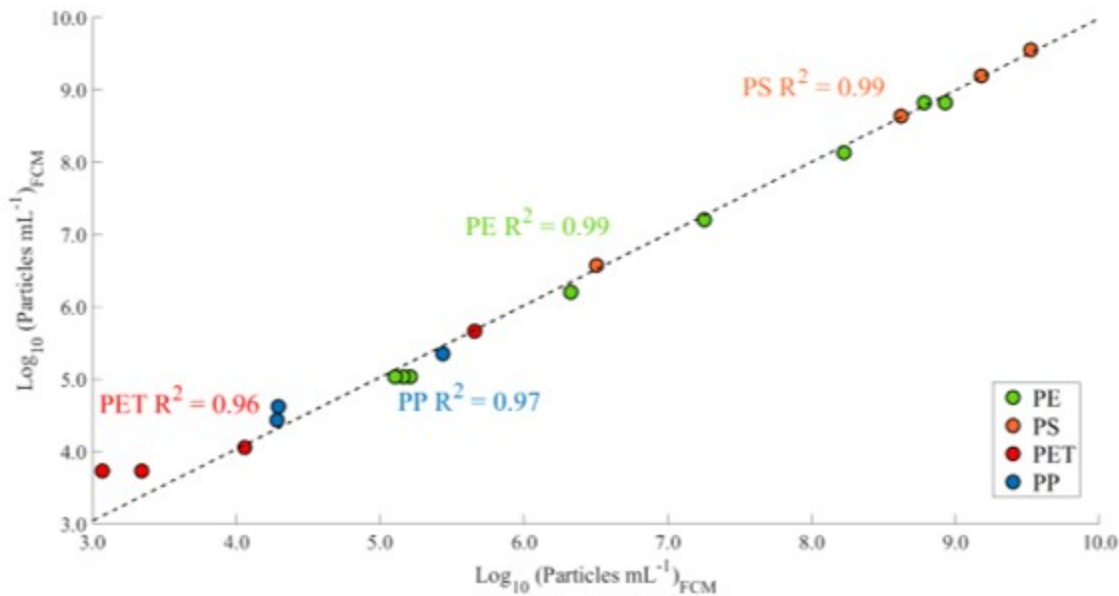


Figure 5

Linear regression of log-transformed particle counts obtained from MFCM and epifluorescence microscopy of PS (4.8-5.8 μm), PE (0.2-9.8 μm), PP (> 20 μm), and PET (< 0.2 μm) at varying concentrations in UP water. The dotted line indicates the overall fit of the regression for MNP measurements ($R^2 = 0.99$).

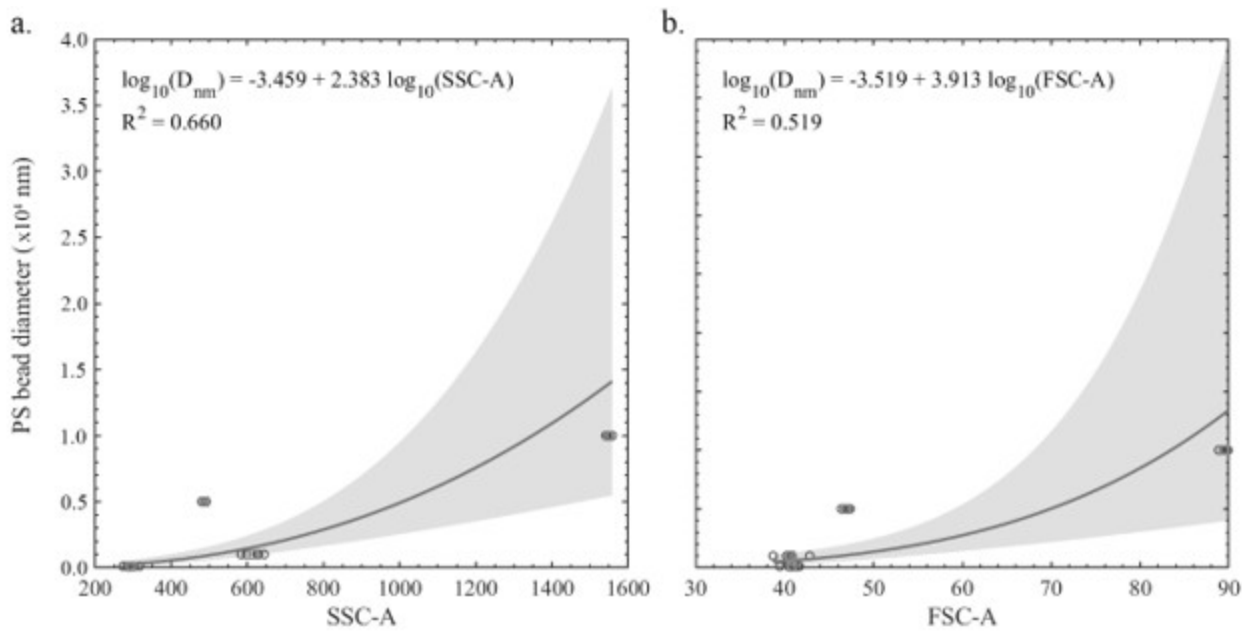


Figure 6

Polystyrene (PS) bead size calibration. (a) SSC-A-based calibration plotted in log-log space using replicate geometric mean SSC-A values and the fitted regression used to convert scatter to PS-equivalent diameter. Shaded region denotes the 95% confidence interval of the mean regression. (b) FSC-A-based calibration plotted in log-log space and included for comparison only; FSC-A exhibits a compressed dynamic range and poor discrimination between 100 and 1000 nm beads, demonstrating that FSC-A is not a suitable primary size proxy for the Skittles method.

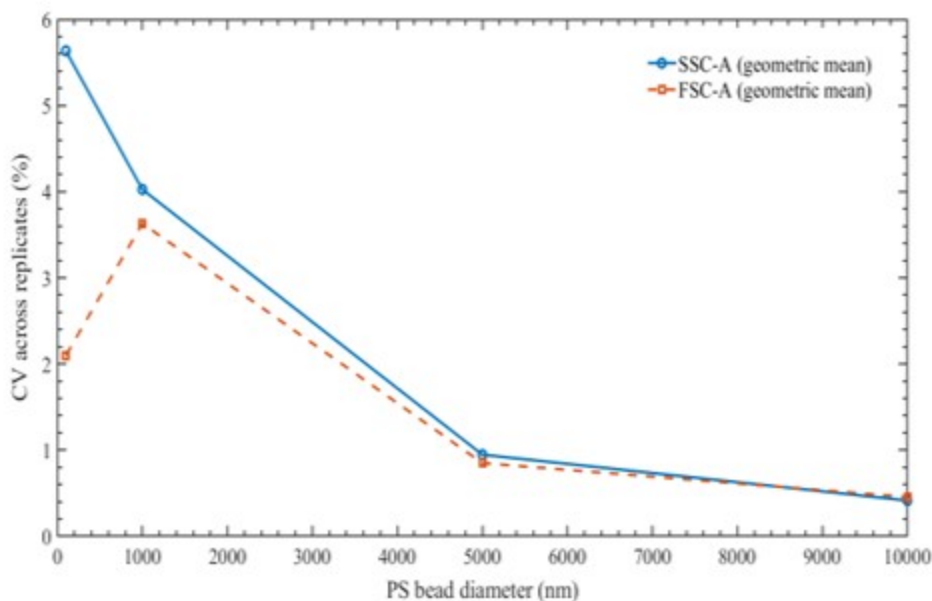


Figure 7

Coefficients of variation (CVs) of geometric mean SSC-A and FSC-A across replicate measurements for polystyrene bead size standards. SSC-A shows consistently low variability across sizes, whereas FSC-A

exhibits reduced sensitivity at smaller particle sizes.

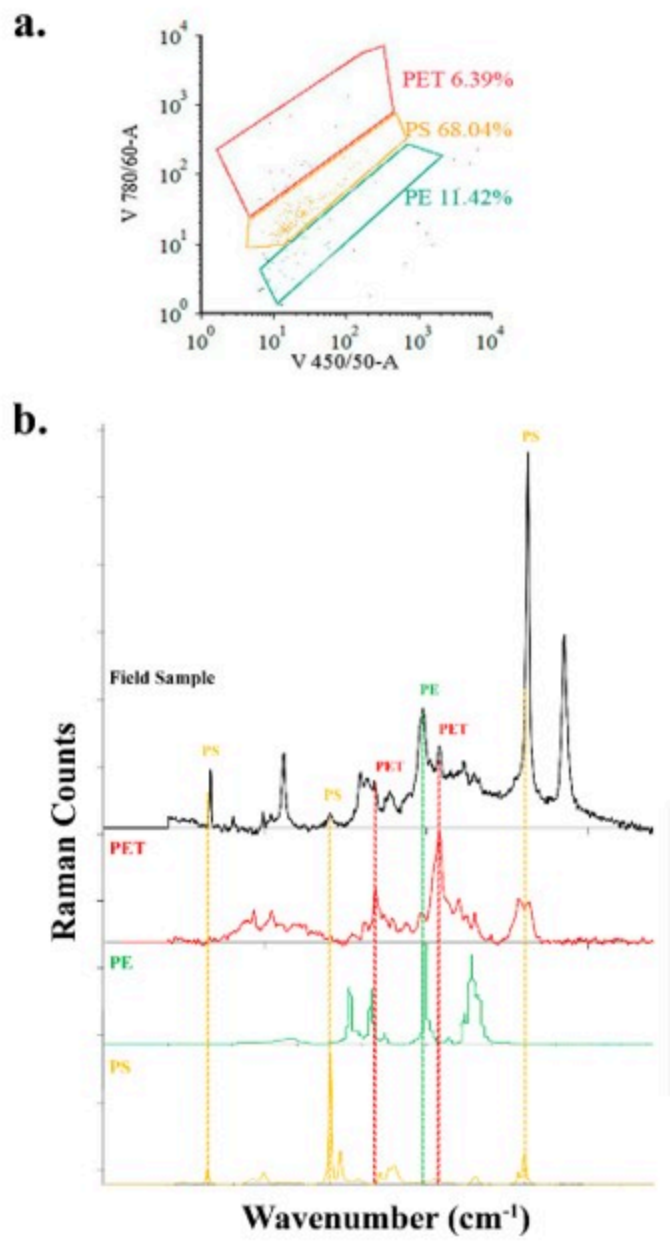


Figure 8

MFCM Bivariate cytogram (top) and Raman wavelength shifts (right) of DANS-stained aquatic samples from Pass Christian Station Harbor area on the MS coast, along with Raman wavelength shifts from reference MNPs. (middle).

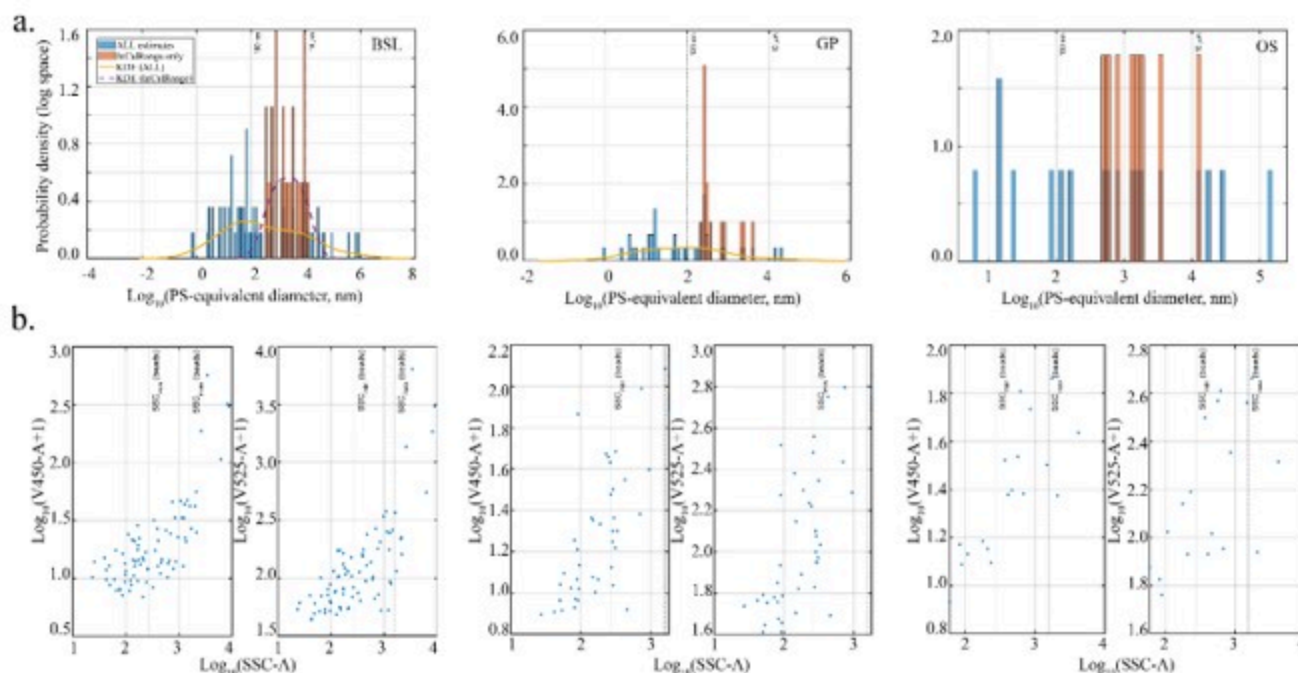


Figure 9

Evaluation of side scatter (SSC)-based PS spherical-equivalent size determination for putative polystyrene (PS) particles detected in tap water samples collected along the Mississippi Gulf Coast: Bay St. Louis (BSL; $N=79$; left), Gulfport (GP; $N=49$; center), and Ocean Springs (OS; $N=18$; right). Quantitative size inference was restricted to events within the bead-calibrated SSC range corresponding to spherical PS particles with diameters of 100–10,000 nm (bounds indicated by dotted lines). (a) SSC-based PS spherical-equivalent size distributions are shown for all detected events (blue) and for the subset of calibration-bounded events (orange). Kernel density estimates (KDEs) are shown only for datasets with $N \geq 20$. (b) Fluorescence intensity plotted as a function of SSC-A, demonstrating a linear relationship across all samples and indicating coherent fluorescence staining of PS particle populations spanning a continuous size spectrum.

Supplementary Files

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