



Airborne micro- and nanoplastics revealed at the submicron scale using an optimized Nile Red–confocal microscopy workflow: Implications for inhalation exposure

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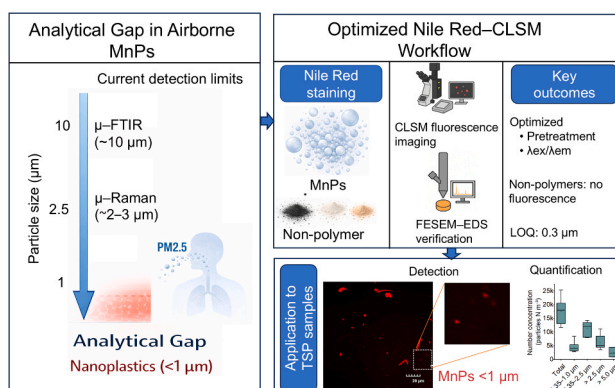
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HIGHLIGHTS

- Optimized Nile Red–CLSM workflow enables direct on-filter detection of airborne MnPs.
- Submicron MnPs quantified down to 0.3 μm .
- Optimized 561/570–670 nm configuration enhances detection efficiency.
- Minimal fluorescence interference from nonpolymeric particles.
- Airborne MnPs concentrations of 10^4 particles Nm^{-3} in suburban environments.

GRAPHICAL ABSTRACT



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ABSTRACT

Airborne micro- and nanoplastics (MnPs) are emerging atmospheric contaminants of increasing concern for inhalation exposure. However, their environmental quantification remains highly uncertain due to limited detection capability in the submicron range and insufficient validation of fluorescence selectivity. Here, we developed and validated an optimized Nile Red–assisted confocal laser scanning microscopy (NR–CLSM) workflow enabling direct on-filter detection of airborne MnPs down to 0.3 μm . An optimized excitation–emission configuration (561/570–670 nm) significantly enhances particle recovery while maintaining high polymer selectivity. Common nonpolymeric particulates, including mineral particles, activated carbon, and plant-derived debris, exhibit negligible fluorescence interference under the optimized conditions. Application to total suspended particulate samples collected in suburban Vietnam revealed MnPs concentrations ranging from 1.2×10^4 to 2.5×10^4 particles Nm^{-3} , with submicron and fine particles (0.3–2.5 μm) constituting the dominant fraction. Comparative imaging further demonstrated that non-optimized fluorescence configuration can substantially underestimate airborne MnPs abundance, particularly in the submicron size range. Optical validation using point

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spread function and full width at half maximum analysis confirmed that the observed submicron fluorescence signals correspond to discrete plastic particles rather than background artifacts. By reducing analytical bias and improving detection reliability, this validated NR–CLSM workflow provides a robust foundation for standardized monitoring and more accurate assessment of inhalation exposure to airborne plastic contaminants.

1. Introduction

Microplastics (MPs) are generally defined as plastic particles smaller than 5 mm, whereas nanoplastics (NPs) are typically considered to be smaller than 1 μm [1–3]. Collectively referred to as micro- and nanoplastics (MnPs), these particles have emerged as contaminants of global concern [4,5].

Despite increasing research attention, the analytical detection and quantitative assessment of MnPs remain highly challenging. Existing methodologies often lack sufficient sensitivity for nanoscale particles, exhibit substantial interlaboratory variability, and are further limited by the scarcity of standardized MnPs reference materials [6–8]. These constraints hinder the development of robust and reproducible analytical workflows, particularly for high-resolution fluorescence imaging and quantitative analysis of airborne MnPs in complex environmental matrices. This limitation is particularly critical for airborne NPs, because smaller plastic particles can penetrate deeply into the human respiratory system following inhalation and may contribute to respiratory toxicity and associated health risks [9]. Moreover, recent evidence indicates that MPs associated with fine particulate matter ($\text{PM}_{2.5}$) can act as carriers of toxic metals into the respiratory system, further emphasizing the importance of accurately detecting and quantifying plastic particles in the smallest size fractions [9]. Consequently, reliable analytical approaches capable of detecting and quantifying MnPs across defined size fractions, particularly in the submicron range, are critically needed to accurately assess the occurrence of airborne MnPs and their associated exposure risks.

Chemical characterization of MnPs commonly relies on Raman spectroscopy, micro-Raman (μ -Raman), confocal Raman microscopy, Fourier-transform infrared (FTIR), and μ -FTIR spectroscopy [6,7,10]. In parallel, imaging techniques such as optical microscopy, digital microscopy, scanning electron microscopy (SEM), fluorescence microscopy, and confocal Raman microscopy are widely used to investigate particle morphology and physical characteristics. Among these approaches, fluorescence-based detection using Nile Red (NR) has received increasing attention because of its operational simplicity, rapid response, and capability to visualize plastic particles in complex environmental matrices [11–13]. However, existing analytical techniques for MnPs remain fundamentally constrained in detecting and characterizing particles in the submicron size range. For example, μ -FTIR spectroscopy is typically limited to particles larger than ~ 10 – $20\ \mu\text{m}$ due to spatial resolution constraints, whereas μ -Raman can extend to the micrometer scale but is often affected by fluorescence interference and limited throughput [10]. Although high-resolution techniques such as confocal microscopy offer improved spatial resolution, their performance remains constrained by diffraction limits and matrix-related fluorescence background [7]. In addition, particle-based analysis of airborne MnPs remains inherently challenging for particles approaching the micrometer scale due to combined sampling, matrix, and analytical constraint [14]. Consequently, the application of fluorescence-based methods, including NR staining, to airborne plastic particles—particularly submicrometer-sized particles corresponding to NPs—remains limited and insufficiently validated [7,8]. Airborne plastics predominantly occur as small solid particles, and their potential health impacts are strongly size-dependent [4,15].

Sample pretreatment introduces additional analytical challenges. Some studies perform direct on-filter analysis using stereomicroscopy combined with FTIR spectroscopy [16–18], whereas others employ chemical digestion followed by flotation or density separation to remove

organic matter [19–21]. However, recovery efficiencies decrease sharply as particle size approaches the submicrometer range [22,23], and additional particle transfer steps from filter surfaces can further reduce recovery for airborne MnPs. Simplified on-filter workflows that minimize organic residues, which may contribute to false-positive NR fluorescence, are therefore critically needed.

Beyond pretreatment challenges, optimization of NR fluorescence is equally critical for improving the detection of submicrometer plastics. NR is typically excited within the blue–green region (450–490 nm), with excitation wavelengths around 470 nm frequently reported to yield high-contrast signals [24–26]. Emission detection has commonly been conducted within narrow green windows (e.g., 515–565 nm), although broader ranges extending into the red region (520–670 nm) have also been reported [27]. While polarity-dependent red shifts in NR emission are qualitatively attributed to its solvatochromic behavior [28], systematic evaluations across polymers spanning a wide polarity range remain limited. Moreover, because NR absorption and emission properties depend on excitation wavelength (λ_{ex}), the fluorescence emission maximum ($\lambda_{\text{em,max}}$), corresponding to emitted fluorescence, may shift with different λ_{ex} values, complicating empirical selection of optimal emission windows [27,29].

Substantial variability also exists in the polymer types, origins, and particle sizes employed in NR-based method development. Many studies have relied on commercial virgin polymers [24,29–31], while commercial reference plastics have been used for confocal laser scanning microscopy (CLSM)-based detection in only a limited number of cases [32]. However, only a limited number of studies have incorporated polymer particles smaller than 2.5 μm , despite the importance of this size range for establishing size-based limits of detection and quantification (LOD/LOQ) and for characterizing fluorescence–size relationships critical to nanoscale analytical performance.

In this study, a Nile Red-assisted confocal laser scanning microscopy (NR–CLSM) method was developed for the detection and identification of airborne plastic particles down to the submicrometer scale. The work focused on determining size-based limits of LOD/LOQ, establishing fluorescence–size relationships, and systematically evaluating fluorescence behavior across polymer particles spanning a broad polarity range representative of environmentally relevant plastic types. Method selectivity was further assessed using multicomponent particle systems representing heterogeneous atmospheric particulate mixtures. The optimized workflow was subsequently applied to total suspended particulate (TSP) samples to enable selective detection and quantitative assessment of airborne MnPs in complex environmental matrices and respirable particle fractions.

Beyond these methodological considerations, environmental plastic materials are highly heterogeneous, comprising diverse polymer types with varying additive compositions and undergoing weathering-induced fragmentation that generates progressively smaller particles. This complexity complicates representative sampling and the distinction between intrinsic polymer fluorescence and additive- or matrix-related effects. To enable controlled and systematic evaluation of fluorescence behavior across polymers with different physicochemical properties, particularly polarity, this study employs well-defined polymer materials as model systems. Importantly, the inclusion of a broad particle size distribution, including a substantial submicron fraction, ensures relevance to environmentally generated particle sizes. Complementary experiments using real plastic materials with different colors were further conducted to assess additive effects on staining behavior and fluorescence response, providing validation under more realistic conditions.

Therefore, the use of pristine polymers as model systems enables controlled method development while maintaining environmental relevance.

2. Materials and methods

2.1. Chemicals and materials

Ultrapure water (HPLC grade; Fisher Scientific, USA) was used throughout the study. Ethanol ($\geq 99.9\%$) and acetone ($\geq 99.8\%$) were obtained from Merck (Germany). Nile Red (NR; FUJIFILM Wako Pure Chemical Corporation, Japan) served as the fluorescent dye for all staining procedures. Quartz fiber filters (Tissuquartz 2500QAT-UP, Pallflex, 8×10 in.) were used as substrates for both laboratory-prepared MnPs samples and environmental TSP samples during staining and CLSM imaging.

Nine types of MnPs representing different polymers were used as reference materials in this study, and each polymer type was analyzed separately. These particles were generated from commercial virgin polymers spanning a broad polarity range, including acrylonitrile-butadiene-styrene (ABS), poly(vinyl chloride) (PVC), low-density polyethylene (LDPE), polystyrene (PS), polypropylene (PP), polycarbonate (PC), poly(vinyl alcohol) (PVA), high-density polyethylene (HDPE), and linear low-density polyethylene (LLDPE).

These MnPs were produced using a dissolution-precipitation approach designed to generate polymer particles with broad size

distributions spanning the nanometer-to-micrometer scale. FE-SEM observations confirmed that the resulting particles covered a wide diameter range (~ 0.03 – $23 \mu\text{m}$), with approximately 80–95% of particles occurring within the respirable size fraction ($< 2.5 \mu\text{m}$), depending on polymer type. The particles predominantly exhibited spherical or near-spherical morphologies with smooth surfaces, which are characteristic of polymer particles generated through solution precipitation. Such particle systems provide suitable reference materials for evaluating fluorescence-based detection performance and for determining size-dependent LOD/LOQ for submicrometer plastic particles. These materials were subsequently used for fluorescence optimization, evaluation of particle size-intensity relationships, and assessment of method selectivity under CLSM conditions. Detailed procedures for particle generation and physicochemical characterization, including recovery yield estimation, are provided in the [Supporting Information](#) (Section S1).

Celite 545 (20 – $100 \mu\text{m}$, Merck, Germany), activated carbon ($< 100 \mu\text{m}$, Merck, Germany), and wood powder (Acacia sp., sieved through a $100 \mu\text{m}$ mesh prior to use) were used as representative nonpolymeric materials to assess potential interference during NR staining. Celite 545 is composed predominantly of silica (SiO_2 , $\sim 89\%$), with minor contributions from CaO ($\sim 6.7\%$), Al_2O_3 ($\sim 1.0\%$), Fe_2O_3 ($\sim 0.5\%$), and alkali oxides ($\text{Na}_2\text{O} + \text{K}_2\text{O}$, $\sim 1.9\%$). Activated carbon is an amorphous carbonaceous material characterized by a high specific surface area, whereas wood powder consists mainly of lignocellulosic components such as cellulose, hemicellulose, and lignin. These materials were

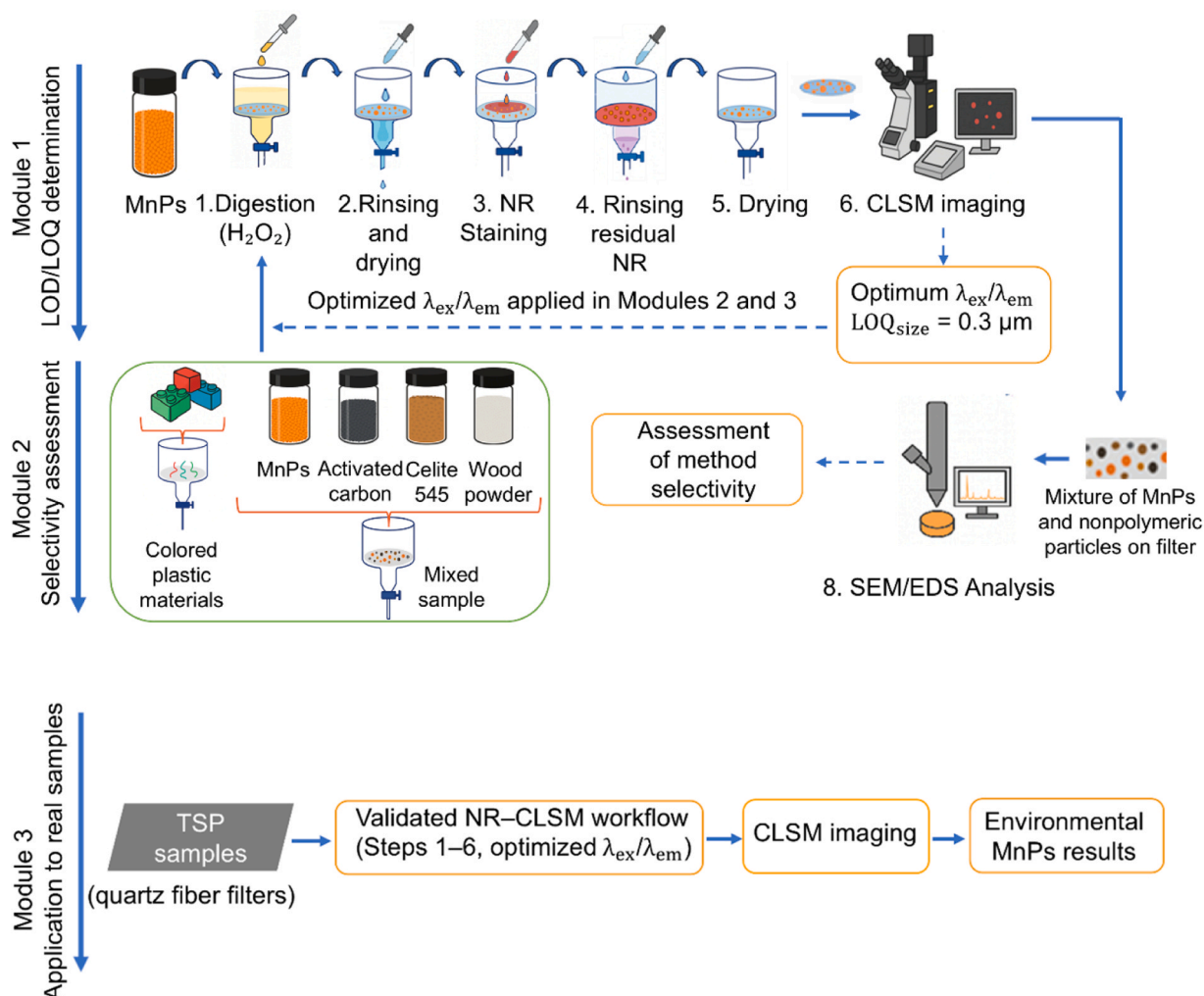


Fig. 1. Workflow of the NR-CLSM method, showing sample preparation, NR staining, CLSM imaging, SEM/EDS confirmation, and application to TSP samples.

selected to represent typical classes of airborne nonpolymeric particulates, including mineral dust, carbonaceous particles, and plant-derived debris that may coexist with plastic particles and potentially generate fluorescence interference. Their inclusion enabled systematic evaluation of NR–CLSM selectivity under environmentally relevant particulate mixtures.

2.2. Sample pretreatment and Nile Red staining protocol

An overview of the NR-assisted CLSM workflow, including sample pretreatment, NR staining, CLSM imaging, FE-SEM/EDS confirmation, and application to TSP samples, is presented in Fig. 1. As the first step of this workflow, all samples were pretreated with 30% hydrogen peroxide (H_2O_2) to remove organic matter prior to NR staining.

Previous studies on airborne and atmospheric dust have applied different H_2O_2 digestion conditions depending on matrix composition, ranging from 70 °C for 1 h to 24 h at room temperature [21,33,34]. Considering the similarity between these matrices and the airborne TSP examined in this work, an incubation temperature of 60 °C for 24 h was selected to ensure efficient oxidation while preserving polymer integrity.

Laboratory-prepared MnPs representing individual polymer types (Section 2.1) were first dispersed in ultrapure water using ultrasonication (ultrasonic bath, Elmasonic, Elma, Germany, 5 min) to promote particle deagglomeration and obtain a transient suspension. The suspension was briefly vortexed prior to deposition onto quartz fiber filters to ensure homogeneous distribution. Environmental total suspended particulate (TSP) samples were collected on the same type of quartz fiber filters. Both laboratory-prepared MnPs and environmental TSP samples were maintained directly in glass filter holders throughout the entire pretreatment and NR staining workflow to minimize particle transfer and associated losses, consistent with previous studies highlighting the challenges of particle-based analysis of airborne MnPs and the importance of minimizing sample handling [14]. During the digestion step, each filter was fully submerged in 30% H_2O_2 . After oxidation, the filters were rinsed three times with ultrapure water followed by ethanol and subsequently dried overnight at 40 °C. This procedure enabled all pretreatment and staining steps to be performed directly on the filter surface, thereby minimizing particle transfer and potential losses of small MnPs.

NR staining was performed exclusively on particles retained on the filters. A volume of 300 μL of NR solution (20 $\mu\text{g mL}^{-1}$) was gently dispensed onto each 16 mm circular filter. The stained filters were incubated at 60 °C for 30 min in the dark to enable uniform dye penetration. The dye concentration and incubation conditions were selected based on the optimized parameters reported previously [29]. After staining, excess dye was removed by rinsing the filters three times with ultrapure water, followed by overnight drying at 40 °C prior to CLSM imaging.

2.3. Confocal laser scanning microscopy

2.3.1. Instrument configuration

NR-stained particles retained on quartz fiber filters were imaged using a confocal laser scanning microscope (CLSM; Olympus FLUOVIEW FV3000, software version 2.6.1.243) equipped with a UPLXAPO 20 $\times$ objective lens (NA = 0.8). XY imaging was performed in unidirectional Galvano mode with a scanning speed of 10 μs per pixel and a spatial resolution of 512×512 pixels. Two digital zoom levels were used for analysis: $\times 4.0$ (0.311 $\mu\text{m/pixel}$) for general surveys and determination of size-based LOD/LOQ, and $\times 15.0$ (0.083 $\mu\text{m/pixel}$) for detailed morphological and fluorescence intensity evaluation.

Fluorescence excitation was performed using two laser lines (λ_{ex} = 488 and 561 nm). For excitation at 488 nm, emission signals were collected within the ranges of 500–570 nm (configuration C1) and 570–670 nm (configuration C2), whereas for excitation at 561 nm,

emission signals were collected within the ranges of 570–620 nm (configuration C3) and 570–670 nm (configuration C4). These detection windows were selected based on the excitation–emission characteristics of NR-stained polymer reference particles to enable effective capture of polymer-dependent fluorescence during CLSM imaging. The use of multiple emission detection windows (Em. DW) allowed systematic evaluation of fluorescence responses under different excitation–emission configurations.

2.3.2. Image analysis

CLSM images obtained from different samples were processed using ImageJ software (version 1.54 g, National Institutes of Health, USA) to extract quantitative parameters related to particle morphology and fluorescence intensity. The extracted data were used to evaluate background fluorescence, staining efficiency, and to determine size-based LOD/LOQ based on particle size and fluorescence intensity. Comparative analyses of CLSM images were performed to assess the influence of excitation and emission wavelength selection (λ_{ex} and λ_{em}) on the fluorescence intensity of individual particles.

2.3.3. Background interference, LOD and LOQ

To determine the LOD and LOQ based on fluorescence intensity (LOD_{int} and LOQ_{int}) and particle size (LOD_{size} and LOQ_{size}) for each polymer type, background fluorescence signals were collected from 10 randomly selected regions without particles in the CLSM images. Each region contained 30 measurement points, yielding a total of 300 background intensity values. The mean and standard deviation (SD) of the background signals were then calculated to estimate the detection thresholds as follows:

$$\text{LOD}_{\text{int}} = \text{mean}_{\text{background}} + 3 \times \text{SD}$$

$$\text{LOQ}_{\text{int}} = \text{mean}_{\text{background}} + 10 \times \text{SD}.$$

LOD_{size} and LOQ_{size} were defined as the smallest particle diameters exhibiting fluorescence intensities $\geq \text{LOD}_{\text{int}}$ and LOQ_{int} , respectively. The relationship between fluorescence intensity (mean gray value) and particle size (expressed as the major-axis length) was established for particles with sizes $\geq \text{LOQ}_{\text{size}}$. Only particles with circularity (Circ.) values of 0.8–1.0 and aspect ratios (AR, defined as the ratio of the major to minor axes of the fitted ellipse) between 1.0 and 1.2 were included, ensuring that only near-spherical particles were analyzed. This morphological constraint excluded fibrous, fragment-like, or irregular particles, as only morphologically homogeneous particles exhibit a positive correlation between particle size and surface area.

2.4. Evaluation of method selectivity

2.4.1. Effect of color additives in colored plastics

Colored plastic toy materials were cut into small fragments with sizes of approximately 200–1000 μm . ATR-FTIR analysis confirmed that the fragments were composed of polypropylene-based polymers (Figure S1). Bright-field images of the unstained fragments were first acquired using a stereomicroscope (Leica M80, Leica Microsystems, Germany) to document their color, morphology, and size.

Following bright-field imaging, the fragments were subjected to the same pretreatment and NR staining protocol applied to airborne MnPs samples. The stained fragments were then examined by CLSM under both excitation wavelengths (λ_{ex} = 488 and 561 nm).

Fluorescence images were directly compared with the corresponding bright-field images to evaluate the effects of colorants and polymer additives on NR emission intensity, staining uniformity, and particle detectability. This analysis was performed to assess the selectivity of the NR-assisted CLSM method when applied to real-world colored plastic materials.

2.4.2. Selectivity against nonpolymeric interferences

Polystyrene (PS) based MnPs were mixed with activated carbon, wood powder, and Celite 545 in ultrapure water. The PS MnPs used in this experiment exhibited a broad size distribution spanning the sub-micron to micrometer range, with the majority of particles occurring below 2.5 μm . The resulting suspensions were gently dispensed onto quartz fiber filters to form composite deposits, while single-material controls for each component were prepared using the same procedure. After air drying, all samples were digested with 30% H_2O_2 and stained with NR following the workflow outlined in Fig. 1.

For the mixed samples, a small representative segment of the same filter, approximately 1.0×1.5 mm, was carefully excised and subjected to sequential CLSM and FE-SEM/EDS analyses. The filter segment was first examined by CLSM to evaluate fluorescence selectivity, and multiple images were acquired from different positions within the segment to ensure representative observation. The identical specimen was then sputter coated with a thin platinum layer and analyzed by FE-SEM/EDS to characterize particle morphology and elemental composition. This sequential approach ensured that fluorescence and elemental analyses were performed on the same localized micro area of the filter, enabling reliable differentiation between polymeric and nonpolymeric particles.

2.5. Application of the optimized NR–CLSM method to TSP samples

TSP samples were collected on quartz fiber filters (Tissuquartz 2500QAT-UP, Pallflex, 8×10 in.) using a high-volume air sampler (HV–1000R, Shibata Scientific Technology Ltd., Japan) operating at a flow rate of 1000 L min^{-1} . Sampling was conducted at a suburban site in Xuan Mai, Hanoi, Vietnam ($20^\circ 54.103' \text{ N}$, $105^\circ 34.617' \text{ E}$). A total of seven consecutive samples were collected, each representing a continuous 23.5 h sampling period.

A circular piece (16 mm in diameter) was cut from each TSP-loaded quartz fiber filter and mounted in a glass filtration holder. Sample pretreatment and NR staining were performed as described in Section 2.2. After staining, excess dye was rinsed off, and the sample was dried prior to CLSM observation. The optimized imaging parameters established in the previous investigations were applied for fluorescence detection of MnPs on the filter surface.

CLSM images ($n = 9$) were acquired from predefined positions across the circular filter (16 mm diameter), including one central location and eight positions evenly distributed along a circular path with a diameter equal to two-thirds of the filter diameter. At each predefined position, a single field of view was acquired without repositioning or adjustment, and without preferential selection based on particle presence or morphology, thereby minimizing selection bias. For quantitative analysis, three fields of view (FOVs) were systematically selected from these predefined positions (without bias toward particle presence) for each sample ($n = 7$), ensuring spatial representativeness while maintaining analytical efficiency. In total, 21 FOVs were analyzed across all samples. Imaging was performed using a $20 \times$ objective with a digital zoom of $\times 4.0$, yielding a field of view of $159.1 \times 159.1 \mu\text{m}$. This approach is consistent with area-based subsampling strategies commonly applied in microspectroscopic analyses of MPs, where particle counts obtained from a representative fraction of the filter are extrapolated to the entire sample, provided that spatial representativeness is ensured [35].

Background interference in the TSP samples was assessed by comparing the fluorescence signal distribution with that obtained from the laboratory-prepared MnPs described in Section 2.2.

The number concentration of MnPs in air (C_{MnPs}) was calculated by dividing the total number of MnPs present in the TSP sample by the air volume sampled under standard conditions (25°C , 1 atm), as expressed in Eq. 1:

$$C_{\text{MnPs}} = \frac{N_{\text{MnPs}}}{V_{\text{air}}} \quad (1)$$

where C_{MnPs} (particles Nm^{-3}) is the number concentration of airborne

MnPs at standard conditions, N_{MnPs} is the estimated total number of MnPs in the TSP sample based on CLSM analysis, and V_{air} is the total air volume sampled under standard conditions. N_{MnPs} was obtained by extrapolating the particle counts obtained from the analyzed FOVs to the entire effective filter area, as follows:

$$N_{\text{MnPs}} = \frac{A_{\text{filter}}}{A_{\text{imaged}}} \times N_{\text{counted}} \quad (2)$$

where A_{filter} is the effective filter area, A_{imaged} is the total imaged area (sum of all FOVs), and N_{counted} is the total number of particles detected in the analyzed FOVs.

Morphological analysis of MnPs was performed using ImageJ software to automatically count particles and extract morphological parameters, including the major and minor axes of the fitted ellipse, circularity, and aspect ratio (AR) from CLSM images. All images were converted to 8-bit grayscale and thresholded prior to particle detection. Particle shapes were classified based on a combination of visual inspection of CLSM images and numerical parameters derived from ImageJ analysis. Fibers were defined as particles with a length greater than $5 \mu\text{m}$, a diameter less than $3 \mu\text{m}$, and an aspect ratio (AR) of at least 3 in accordance with the respirable fiber criteria adopted by the World Health Organization [36]. Spherical particles were defined as those with a major-to-minor axis ratio within 10% of unity (1.0–1.1) and circularity ≥ 0.8 . Irregular particles were characterized by rough or nonuniform outlines with circularity < 0.8 and aspect ratio (AR) < 3 . Particles that did not meet these criteria were categorized as fragments.

2.6. Quality assurance and quality control

All glassware was sequentially cleaned with tap water, ultrapure water, ethanol (99.9%), and 10% HNO_3 solution. The glassware was then rinsed with ultrapure water, dried in a clean oven, and wrapped in aluminum foil until use. No plastic materials were used in any step involving chemical reagents, sample preparation, or filtration. All solutions were handled using glass Hamilton syringes ($100 \mu\text{L}$) and quartz fiber filters to completely avoid polymer contamination. Quartz fiber filters were pre-baked at 550°C for 4 h prior to use to remove potential organic contaminants [37].

To minimize contamination, all experiments were accompanied by procedural blanks. If any fluorescence signal was detected in the blanks, all related reagents were immediately replaced. All experiments were conducted in a closed laboratory environment. The preparation of MnPs particles from virgin polymers was performed in a separate room using dedicated equipment to prevent cross-contamination with TSP sample analyses.

2.7. Statistical analysis

Statistical analyses were performed using OriginPro 2025b (Origin-Lab Corporation, Northampton, MA, USA). The normality of the data was verified using the Shapiro–Wilk test. A one-way analysis of variance (ANOVA) was conducted to assess differences in background fluorescence intensity, background noise, and in the calculated LOD and LOQ among the four emission detection windows. When a significant difference was observed ($p < 0.05$), Tukey's honestly significant difference (HSD) post hoc test was applied for pairwise comparisons. Effect size was estimated using eta squared (η^2) according to Cohen's conventional criteria [38]. All data are expressed as mean $\pm$ standard deviation (SD), and statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. CLSM fluorescence characteristics of NR-stained MnPs

3.1.1. Excitation- and emission-dependent CLSM imaging

CLSM imaging revealed pronounced excitation- and emission-

dependent differences in the fluorescence visibility and detection sensitivity of NR-stained MnPs across the evaluated wavelength configurations. Four excitation/emission configurations (C1–C4) were employed for CLSM imaging. CLSM images of all nine polymers are

provided in Figure S2, with five representative polymers spanning different polarity levels shown in Fig. 2.

At $\lambda_{ex} = 488$ nm (C1, C2), fluorescence images revealed pronounced differences between the two configurations. Nonpolar polymers (PP, PS)

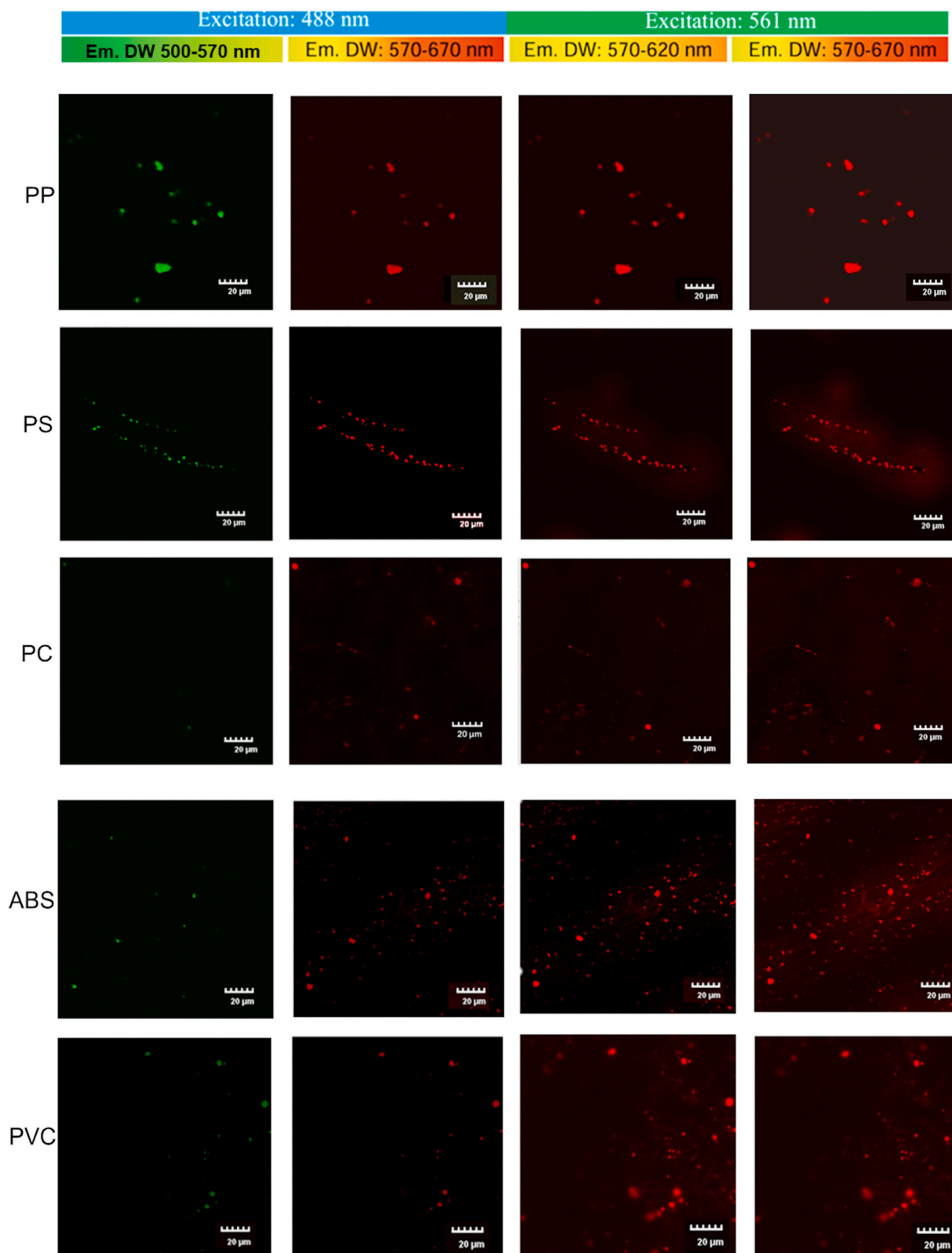


Fig. 2. CLSM images of Nile Red-stained MnPs acquired under four excitation/emission ($\lambda_{ex}/\lambda_{em}$) configurations (C1–C4) for representative polymers with different polarities. Em. DW denotes the emission detection window.

exhibited bright and well-defined fluorescence in both C1 and C2, whereas moderately to highly polar polymers (PC, ABS, PVC) showed weak emissions in C1 but significantly stronger signals in C2. For polar polymers, particle counts in C1 were typically less than half of those in C2, consistent with the red-shifted fluorescence characteristic of these materials.

At $\lambda_{ex} = 561$ nm (C3, C4), fluorescence appeared more uniform across polymer types. Both nonpolar and polar polymers exhibited stable and well-defined emissions under C3, whereas C4 produced slightly higher background signals due to its broader detection bandwidth. Particle counts obtained from C3 and C4 were nearly identical, confirming that excitation at 561 nm yields more stable fluorescence and higher overall detection efficiency than 488 nm.

The observed CLSM fluorescence behavior of the polymers was consistent with their fluorescence spectral characteristics. Under excitation at 561 nm (C3 and C4), all polymers were clearly visible because their emission maxima fall within the selected detection windows. In contrast, excitation at 488 nm (C1 and C2) produced more pronounced differences among polymer types due to the broader distribution of emission maxima, with nonpolar polymers emitting at shorter wavelengths and polar polymers exhibiting red-shifted emissions.

3.1.2. LOD and LOQ determination

3.1.2.1. Background fluorescence intensity and noise across emission channels. Background fluorescence and noise on quartz filters were evaluated from replicate CLSM measurements (300 randomly selected points per image; nine replicates per configuration), as shown in Fig. 3 (left panels). Both parameters increased markedly under 561 nm excitation compared with 488 nm. Among the four configurations, C3—and especially C4—showed the highest background and noise, whereas C1 provided the most stable baseline. One-way ANOVA confirmed significant effects of excitation–emission configuration on background intensity ($F(3, 32) = 8.64$, $p < 0.001$, $\eta^2 = 0.44$) and noise ($F(3, 32)$

$= 13.23$, $p < 0.001$, $\eta^2 = 0.55$). Tukey's test indicated that C4 differed significantly from C1 ($p < 0.01$). These results demonstrate that background signals and noise increased with excitation wavelength, consistent with enhanced spectral overlap. The 488/500–570 nm window (C1) yielded the lowest background and thus represents the optimal condition for LOD/LOQ determination (see Table S1 for detailed statistics).

3.1.2.2. Uniformity, repeatability and background noise in quartz filter fluorescence. The uniformity and repeatability of the intrinsic background fluorescence of quartz filters were evaluated using one-way ANOVA based on nine replicate CLSM measurements for each configuration. Under 488 nm excitation (C1 and C2), ANOVA revealed statistically significant differences among replicates ($p < 0.0001$, indicating highly significant differences); however, the corresponding effect sizes were moderate ($\eta^2 \approx 0.11$ – 0.12), and the absolute variability remained very low ($CV < 2\%$). These results demonstrate excellent repeatability and spatial uniformity of quartz filter background fluorescence.

At 561 nm excitation (C3 and C4), the effect sizes increased substantially ($\eta^2 = 0.45$ – 0.65), indicating larger differences among replicates. However, this variability predominantly arose from the higher and more fluctuating background fluorescence at longer wavelengths rather than from any methodological inconsistency. Overall, these findings confirm that the NR staining and CLSM imaging procedures were highly reproducible, and that the apparent instability under 561 nm excitation reflected increased spectral overlap and elevated baseline fluorescence inherent to the filter material (see Table S2 for detailed ANOVA statistics).

3.1.2.3. LOD_{int} and LOQ_{int} . Fluorescence intensity-based limits of detection (LOD_{int}) and quantification (LOQ_{int}) varied significantly among the four excitation and emission configurations and across polymer types (Fig. 3; Table S3).

Both parameters differed markedly among configurations ($F(3,32)$

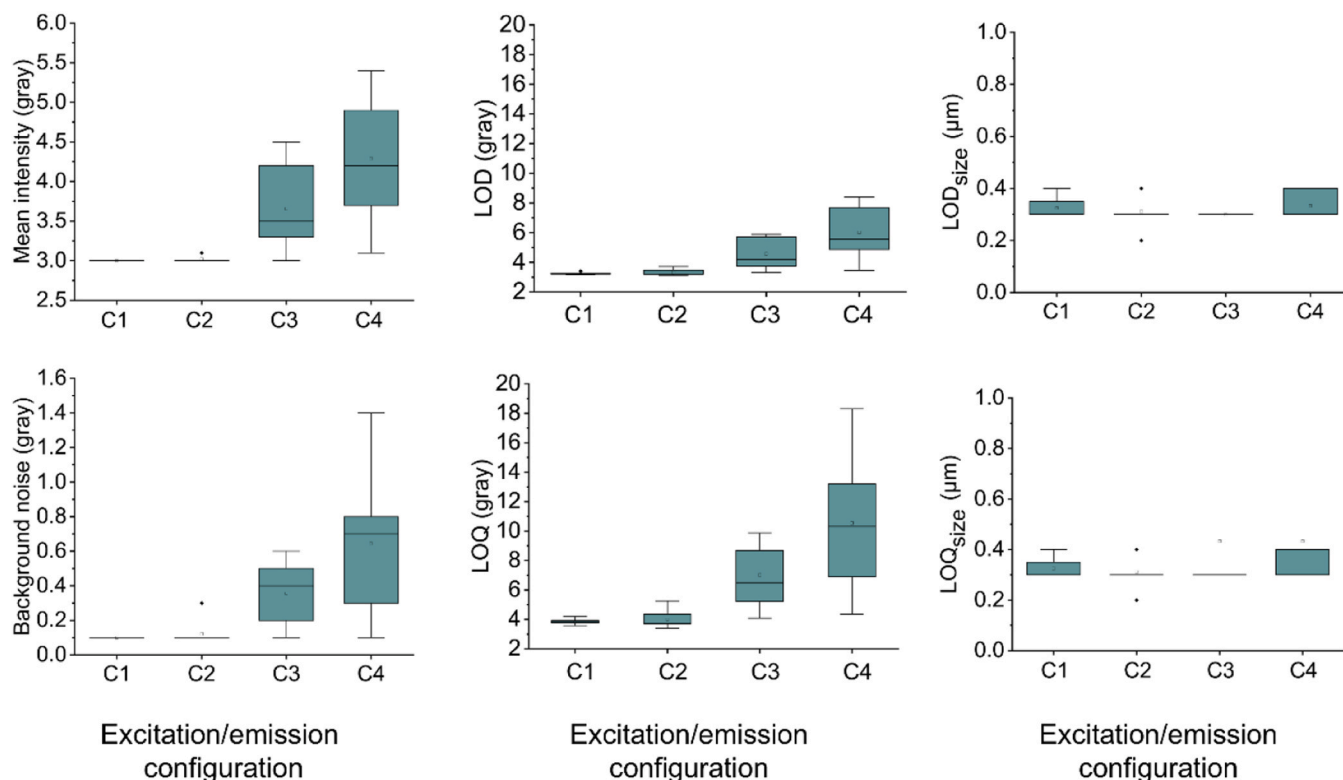


Fig. 3. Summary of background fluorescence intensity, background noise (standard deviation), and the corresponding LOD and LOQ of NR-stained MnPs under four excitation/emission configurations (C1–C4). Numerical values and ANOVA statistics are provided in Tables S1–S3.

$= 14.2$, $p < 0.0001$, $\eta^2 = 0.57$). The lowest and most stable thresholds were obtained under C1, with mean LOD_{int} and LOQ_{int} values of 3.26 ± 0.08 and 3.87 ± 0.20 gray, respectively. Expanding the emission window under C2 resulted in slightly higher thresholds, whereas excitation at 561 nm (C3 and C4) produced larger shifts and increased variability. These results demonstrate that both λ_{ex} and emission bandwidth strongly influence fluorescence sensitivity, with C1 providing the most reliable conditions for intensity-based detection.

3.1.2.4. LOD_{size} and LOQ_{size} . Size-based limits of LOD_{size} and LOQ_{size} were consistently observed in the submicrometer range for most polymers across the evaluated configurations, with polymer-specific exceptions reflecting polarity-dependent fluorescence behavior.

CLSM imaging and ImageJ analysis showed no detectable fluorescence from PVA particles under C1, whereas clear signals appeared under all other configurations. This observation is consistent with the fluorescence spectrum of NR-stained PVA, which exhibits negligible emission within this detection range. The absence of fluorescence arises from the high polarity of PVA, causing the strongest red-shift among all polymers and shifting its emission maximum beyond 570 nm. Consequently, LOD_{size} and LOQ_{size} for PVA could not be determined under C1. Therefore, particle edges could not be segmented in ImageJ, preventing size threshold determination under C1. In contrast, distinct fluorescence images were obtained for the remaining eight polymers across all configurations, as well as for PVA under C2–C4. The corresponding LOD_{size} and LOQ_{size} values are summarized in Table S3 and shown in Fig. 3.

For most polymers, LOD_{size} and LOQ_{size} were identical across all conditions, except for PC at $\lambda_{ex} = 561$ nm, likely due to position-dependent signal loss, limited NR uptake, or photobleaching effects.

Although background fluorescence intensity, noise, and LOD_{int}/LOQ_{int} varied significantly among emission windows, LOD_{size} and LOQ_{size} remained consistent, with a median of approximately $0.30 \mu m$. After excluding two PC outliers, the overall mean $\pm$ SD of LOD_{size} ($= LOQ_{size}$) ranged from 0.30 ± 0.00 – $0.33 \pm 0.05 \mu m$, demonstrating excellent reproducibility.

ImageJ reported particle sizes with one decimal precision; thus, both limits were recorded as $0.3 \mu m$. This threshold is substantially lower than those typically reported for μ -FTIR and μ -Raman spectroscopy [6], underscoring the higher sensitivity of the NR-CLSM approach. Importantly, this result establishes an analytical detection boundary for airborne MnPs at approximately $0.3 \mu m$. Extending the detectable size range into the submicron domain substantially improves the ability to quantify respirable plastic particles in atmospheric samples. The method reliably detected and quantified polymer nanoparticles in the 300–1000 nm range, consistent with the definition of NPs as particles smaller than $1 \mu m$ [6,39,40]. Previous studies have likewise shown that CLSM achieves a spatial resolution of up to 200 nm, allowing accurate visualization of MnPs in biological matrices after proper sample preparation [32].

Nanoplastic particles ($<1 \mu m$) were directly resolved by CLSM (Fig. 4) using a $20 \times$ objective and a $15 \times$ digital zoom (scale bar = $1 \mu m$). These high-magnification observations independently validated the LOQ_{size} established under standard measurement conditions ($20 \times$ objective, $4 \times$ digital zoom).

In Fig. 4b, the upper-left particle has a diameter of $\sim 0.4 \mu m$ and the lower particle is $\sim 0.3 \mu m$. Their mean fluorescence intensities (14.0 and 13.6 gray, respectively) exceed the highest LOD_{int} value (10 gray) obtained across the four emission detection windows. The corresponding

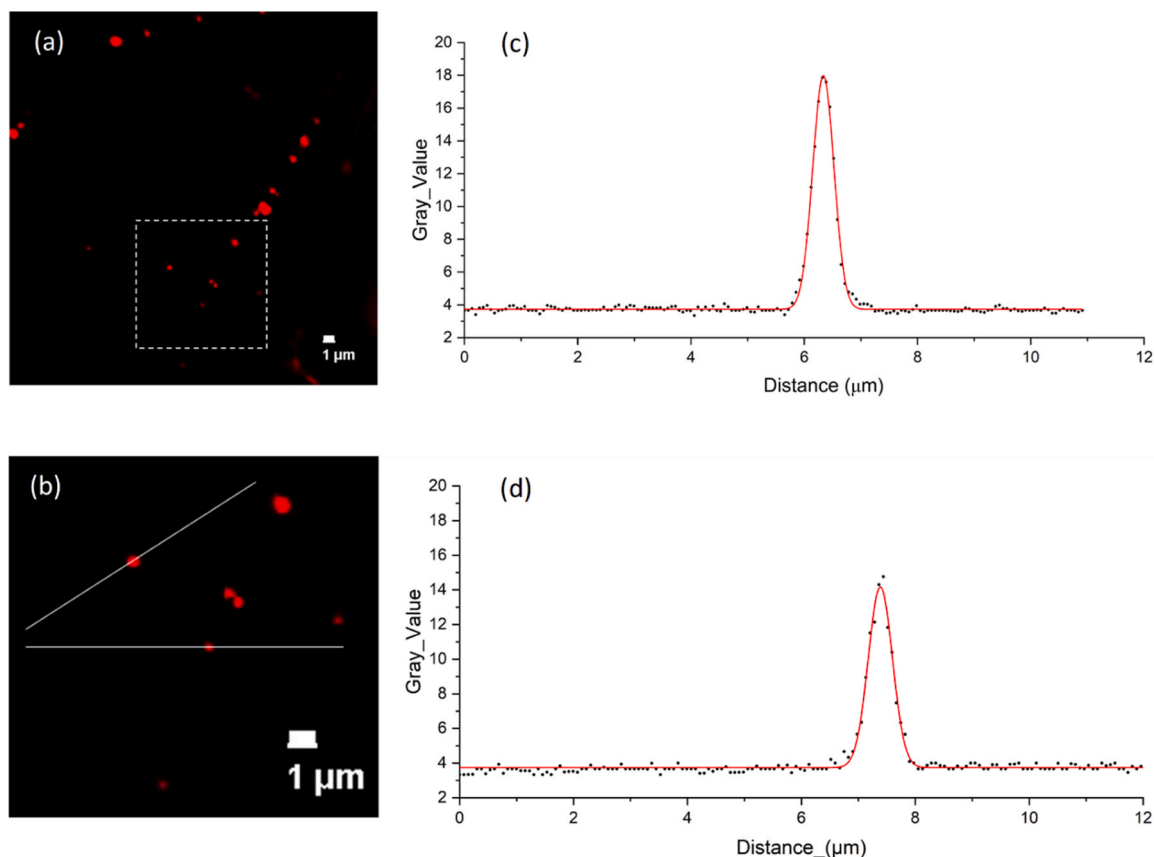


Fig. 4. CLSM images and fluorescence intensity profiles of PVC nanoplastic particles obtained under the C4 detection window. (a) Image acquired using a $20 \times$ objective and $15 \times$ digital zoom (scale bar = $1 \mu m$). (b) Enlarged view of the selected region in (a) showing individual fluorescent particles. (c,d) Line-scan fluorescence intensity profiles and Gaussian fits across particle 1 ($\sim 0.4 \mu m$) and particle 2 ($\sim 0.3 \mu m$), illustrating well-defined diffraction-limited point spread function (PSF) peaks.

line-scan profiles exhibit well-defined point spread function (PSF) peaks above the background baseline (~ 4 gray). These well-defined PSF peaks confirm that the observed signals correspond to discrete fluorescent particles rather than background noise or imaging artifacts. The measured full width at half maximum (FWHM) values (~ 0.4 – 0.5 μm) are comparable to the diffraction-limited lateral resolution of the CLSM system (~ 0.46 μm for $\lambda \approx 600$ nm and $\text{NA} = 0.8$), indicating diffraction-limited fluorescence spots originating from submicron particles acting as point emitters.

Procedural blanks exhibited only background-level fluorescence, with no detectable particle-like signals exceeding the LOQ. The LOQ (gray value: 4.1; range: 3.9–4.5; $\text{RSD} = 6.4\%$) determined from blank measurements was consistent with that established during method development, supporting the validity of the applied LOQ threshold.

3.1.3. Particle determination efficiency across emission windows

The number of detected particles varied substantially across the four measurement configurations C1–C4 (Fig. 5). Configuration C4 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 561/570$ – 670 nm) consistently yielded the highest MnPs counts, particularly for moderately to highly polar polymers (ABS, PC, PVC, PVA), which showed 1.5–2-fold increases relative to the 488 nm-based settings (C1–C2). Weakly or nonpolar polymers exhibited only minor differences among configurations, and particle counts were generally comparable between the two emission windows within each λ_{ex} .

These trends are consistent with the fluorescence emission behavior of NR-stained polymers, which typically exhibit strong emission within the 580–720 nm region. By fully covering this emission range, C4 produces stronger and more distinct particle signals, whereas C1–C2 capture only the short-wavelength tail of the spectra. Although C4 exhibits higher background fluorescence and noise than the other configurations, these factors do not reflect its particle-detection capability. Because LOD_{size} and LOQ_{size} are identical across C1–C4, submicrometer particles (0.3–0.4 μm) remain detectable under all configurations. Under C4,

these particles generate well-defined PSF peaks that are clearly separated from the background baseline. Consequently, C4 provides the highest particle-detection efficiency among all configurations (Figure S3).

The superiority of C4 was further confirmed in real TSP samples collected in Xuan Mai, Hanoi (Fig. 5), where substantially more NR-positive particles were visualized under C4 than under C1. These results indicate that non-optimized fluorescence configurations may lead to systematic underestimation of airborne MnPs abundance, particularly within the submicrometer size fraction.

3.1.4. Correlation analysis between MnPs size and NR fluorescence intensity

A strong positive correlation between MnPs particle size and NR fluorescence intensity was observed for most polymers across the evaluated excitation–emission configurations.

Because PVA exhibited no detectable fluorescence under $\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/500$ – 570 nm, no correlation could be established for this condition. For the remaining polymers, a total of 35 correlations were evaluated under two excitation wavelengths and four emission detection windows. Statistical parameters, including Pearson's r , p -values, slopes, and the number of data points, are summarized in Table S4.

The slopes of the regression equations ($\pm \text{SE}$; Figure S4) revealed positive correlations between fluorescence intensity and particle size. The correlation coefficients (r) ranged from 0.63 to 0.99, with six correlations classified as strong ($r = 0.60$ – 0.79) and twenty-nine as very strong ($r = 0.80$ – 1.00), according to Evans' criteria. The corresponding p -values were < 0.0001 for 29 regressions, < 0.001 for two, and < 0.05 for three.

These statistical outcomes demonstrate that the NR staining process was highly effective for both NPs and MPs. Furthermore, the influence of the quartz fiber filter background on the fluorescence signal—particularly for smaller particles potentially affected by partial

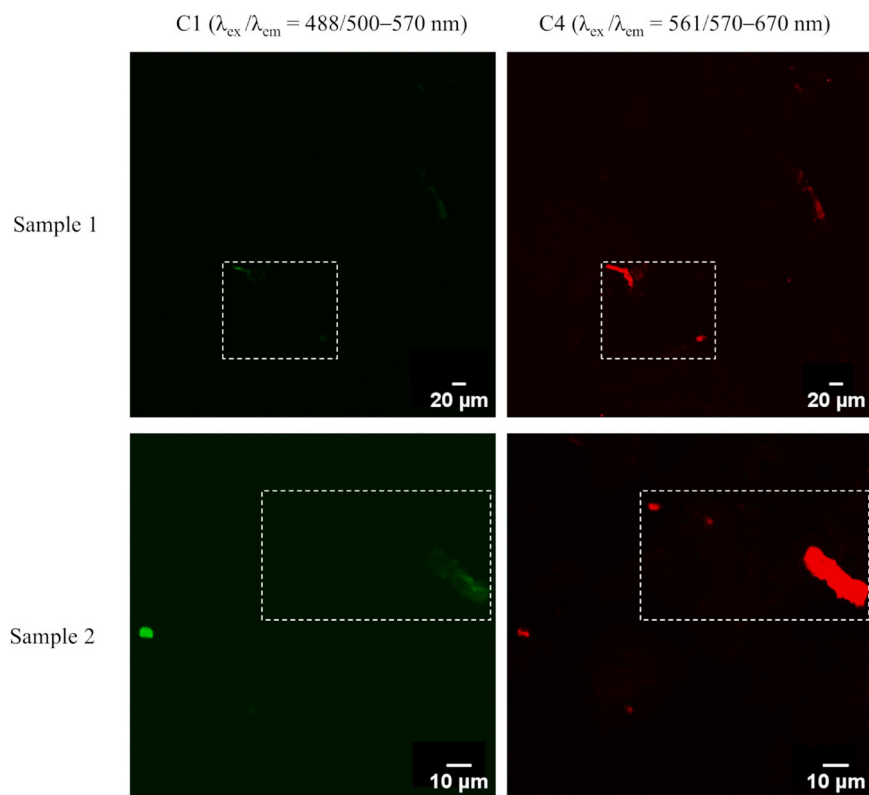


Fig. 5. CLSM images of MnPs in two TSP samples acquired under C1 (488/500–570 nm) and C4 (561/570–670 nm). Dashed boxes highlight representative regions containing NR-positive particles.

masking—was negligible.

However, fluorescence-based methods, as well as other particle-resolved imaging techniques such as μ -FTIR and Raman microscopy, present inherent limitations. Under realistic atmospheric sampling conditions, particularly at high particle loadings, masking effects in fluorescence signals may arise due to particle overlap on the filter surface, where larger particles can obscure smaller ones during observation. In addition, particle aggregation during sample preparation or handling (e.g., filtration, drying, or solvent treatment) may further hinder the discrimination of individual particles. These effects have been reported in size-segregated airborne MnPs sampling, where excessive particle loading induces aggregation and overlap, thereby hindering single-particle analysis and attenuating spectroscopic signals [41]. In this context, the detected fluorescence signal may not accurately represent discrete emitters, leading to uncertainties in particle identification, particularly for smaller particles. Complementary spectroscopic validation (e.g., Raman analysis) may therefore be beneficial for confirming polymer identity in complex environmental samples such as TSP.

The sensitivity of the method was assessed from the slope of the correlation equations and their standard errors (SE). As shown in Figure S4 and the corresponding dataset, the slopes for all polymers excited at $\lambda_{\text{ex}} = 561$ nm were either greater than or comparable to those obtained at 488 nm, indicating an overall higher fluorescence response under 561 nm excitation. For instance, the slope for PS was 13.53 ± 2.15 at C4, compared with 3.91 ± 0.63 at C2.

Polar polymers such as PVA, ABS, PC, and PS exhibited markedly higher fluorescence sensitivity than weakly or nonpolar polymers (PP, HDPE, and LLDPE) under $\lambda_{\text{ex}} = 561$ nm. This difference was most pronounced within the 570–670 nm emission window, where most examined polymers showed slopes comparable to or greater than those obtained in other detection ranges.

These quantitative results are consistent with the CLSM image observations and the red-shift behavior observed in the fluorescence spectra, corroborating the strong influence of polymer polarity on fluorescence response.

Since all fluorescence emission maxima of the polymers fall within 608–626 nm, the optimized condition for MnPs observation is excitation at C4. To minimize background interference, the detection bandwidth can be narrowed according to the instrument configuration, provided that the 600–630 nm region remains fully covered. This configuration offers an optimal balance between sensitivity and selectivity, making it broadly applicable for MnPs detection across diverse environmental matrices.

3.1.5. Method selectivity

3.1.5.1. Effect of color additives on fluorescence staining. Toy plastic samples with different intrinsic colors (white, blue, red, turquoise, and orange) were examined under a stereomicroscope prior to NR staining (Fig. 6a).

After staining, all samples exhibited strong fluorescence under CLSM at both excitation–emission configurations: C4 (Fig. 6b) and C1 (Fig. 6c).

The fluorescence micrographs showed that neither the coloration nor the presence of pigments noticeably influenced the staining efficiency or emission behavior of NR. These results confirm that the NR–CLSM method maintains high selectivity and remains reliable for detecting commercial plastic materials containing color additives. This consistency indicates that dye–polymer interactions dominate the fluorescence response, whereas optical absorption or fluorescence quenching by pigments plays a negligible role under the tested conditions. Because environmental MPs typically originate from commercial plastic products containing pigments and additives, these findings further support the applicability of the NR–CLSM method for detecting MnPs in real environmental samples.

3.1.5.2. Evaluation of false-positive signals from nonpolymeric materials. FE-SEM examination of the mixed sample containing PS-derived MnPs, Celite 545, activated carbon, and wood powder deposited on quartz fiber filters (Fig. 7c–f) revealed a dense fibrous network originating from the filter substrate, numerous small spherical particles (<5 μm), and several larger irregularly shaped particles (>20 μm). The irregular

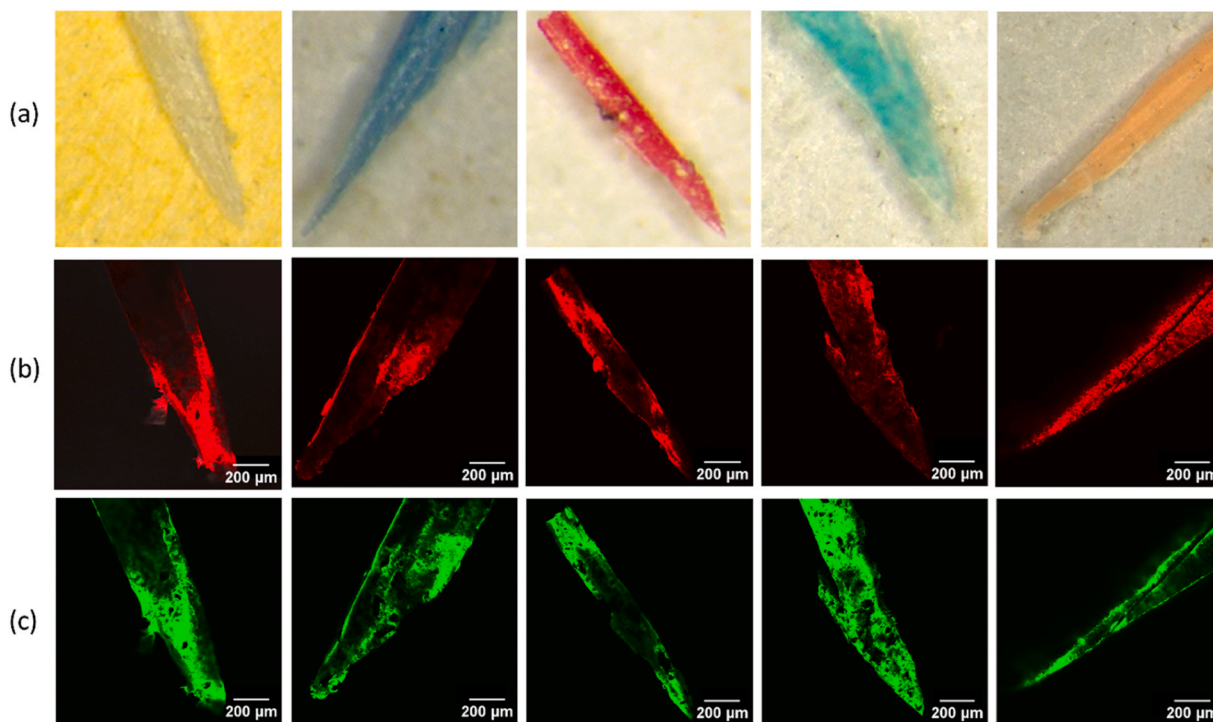


Fig. 6. Toy plastic samples with different intrinsic colors: (a) stereomicroscope images before NR staining, (b) CLSM images after staining at C4, and (c) CLSM images after staining at C1. All samples exhibited strong and uniform fluorescence regardless of their original color.

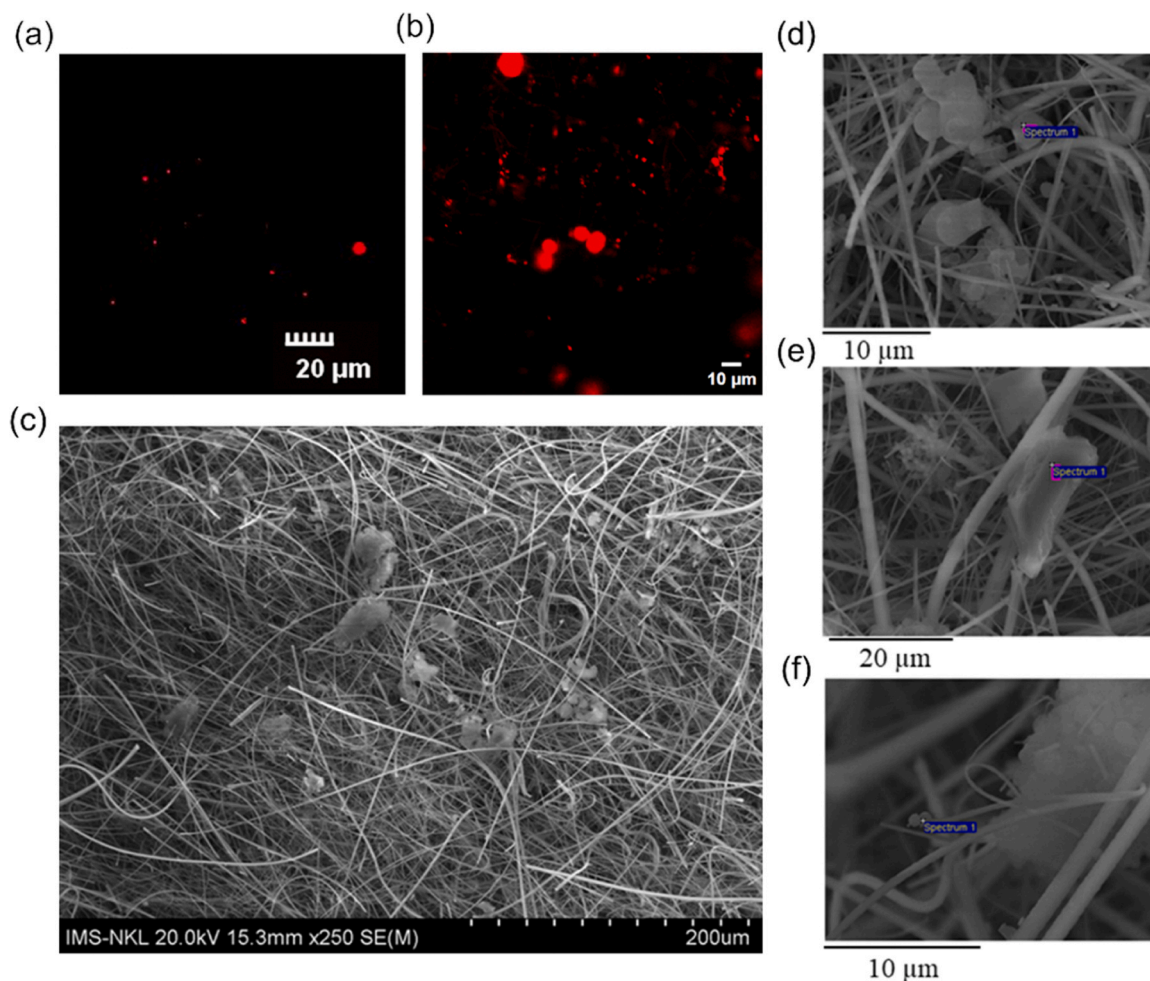


Fig. 7. Representative CLSM (a, b) and SEM (c–f) images of a mixed sample containing MnPs, Celite 545, activated carbon, and wood powder, used to assess potential false-positive fluorescence from nonplastic materials. Additional images are provided in [Figs. S5 and S6](#).

particles were predominantly associated with nonpolymeric materials, whereas spherical particles were distributed across the filter surface and frequently attached to the fibers.

In the corresponding CLSM images acquired from the same filter regions ([Fig. 7a, b](#); full dataset in [Figure S6](#)), all NR-positive signals exhibited spherical or ovoid morphologies consistent with the PS particles introduced in the mixture. Notably, no fibrous or elongated NR fluorescence was observed, confirming that the quartz fiber filter itself did not contribute detectable background fluorescence under the applied excitation and emission conditions.

A quantitative comparison of particle size distributions obtained by CLSM and FE-SEM showed good agreement for particles smaller than 5 µm, with 96.8% and 94.1% of detected particles falling within this size range, respectively. However, within the < 2.5 µm fraction, CLSM detected a lower proportion of particles compared to FE-SEM (64.3% versus 80.9%). This discrepancy is attributed to the limited optical resolution of CLSM and partial convolution of fluorescence signals from small particles attached to larger aggregates or filter fibers, effects that were readily resolved in FE-SEM images.

These results demonstrate that while NR-assisted CLSM provides high selectivity with negligible false-positive signals from nonplastic materials, it may partially underdetect the smallest MnPs particles in heterogeneous mixtures due to optical diffraction and signal overlap. Importantly, this limitation affects detection efficiency rather than selectivity and can be mitigated through complementary FE-SEM imaging.

EDS spectra ([Figure S5](#)) were acquired from representative regions of

interest to determine the elemental composition of materials exhibiting distinct morphologies. The summarized results for six morphology-defined particle groups are provided in [Figure S7](#). Integration of FE-SEM morphology with supporting EDS analysis enabled clear material assignment in the mixed system: spherical particles were identified as PS particles, fibrous structures as the quartz fiber filter substrate, and irregular particles as nonpolymeric materials, including activated carbon, wood powder, and mineral-derived Celite 545. Detailed EDS compositional analyses for each morphology-defined group are provided in the [Supporting Information](#).

The combined evidence from CLSM fluorescence, FE-SEM imaging, and EDS compositional analysis confirms that the proposed experimental workflow exhibits strong selectivity for identifying MnPs in complex mixtures. The false-positive risk from nonpolymeric materials, including carbon-rich substances such as activated carbon and wood powder, is minimal. Accordingly, this workflow is suitable for reliable NR staining and direct detection of plastic particles on filter surfaces after the removal of natural organic matter.

3.1.6. Effect of H₂O₂ pretreatment on fluorescence sensitivity and polymer stability

Although oxidative digestion using H₂O₂ has been widely applied to remove organic matter prior to fluorescence staining, its impact on fluorescence-based detection sensitivity has rarely been systematically evaluated [[24,26,27](#)].

Oxidative treatment may induce condition-dependent modifications in polymer properties, particularly for polymers more susceptible to

oxidation due to their chemical structure and hydrophilicity (e.g., PVA). Such effects are typically more pronounced under aggressive conditions (e.g., elevated temperatures or UV-assisted oxidation), which were not applied in this study. In this context, PVA was intentionally included to extend the range of polymer polarity examined, enabling evaluation of polarity-dependent fluorescence behavior of NR. Although surface modifications could, in principle, affect dye-polymer interactions, NR staining is governed by non-covalent interactions and is therefore less sensitive to minor changes.

The results indicate that H_2O_2 pretreatment did not compromise fluorescence sensitivity. The LOD_{size} and LOQ_{size} values ($\sim 0.3 \mu\text{m}$) remained within the submicron range across all polymers, with no polymer-specific deviations. Strong size-intensity correlations, together with high particle-detection efficiency and negligible interference from nonpolymeric materials, confirm the reliability of the method. The peak intensities and corresponding FWHM values derived from Gaussian-fitted fluorescence profiles for particles approaching the LOQ_{size} further support the high sensitivity of the method.

Overall, H_2O_2 pretreatment effectively removes interfering organic matter while preserving fluorescence-based detection performance.

3.2. Evaluation and application to TSP samples

3.2.1. Background fluorescence and method selectivity in TSP samples

Application of the optimized fluorescence configuration (C4) to ambient TSP samples demonstrated low and stable background signals comparable to those of blank quartz filters. Across seven ambient TSP samples, the mean background fluorescence intensity was 3.5 ± 0.3 gray (range: 3.3–4.1), and the corresponding background noise was 0.6 ± 0.2 gray (range: 0.3–1.0). These values fall within the ranges obtained for nine blank filters used to establish the LOD and LOQ (Fig. 3, left), indicating that residual materials remaining after H_2O_2 digestion did not measurably increase background fluorescence relative to the blanks.

While these results indicate effective removal of interfering organic matter, it should be noted that oxidative digestion using H_2O_2 may induce minor changes in particle size and surface morphology, particularly for smaller particles, due to surface oxidation and material loss, as reported in previous studies [14]. However, overall, these effects are limited, with particle morphology largely preserved and only minor mass changes observed, while the chemical structure of polymers remains largely unaffected [42–45].

To further assess method selectivity in real samples, identical regions of interest (ROIs) were imaged under two CLSM excitation/emission settings after NR staining. Under the NR emission channel (C4), NR-positive particles were clearly observed as bright red fluorescence, whereas under the control channel ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/480\text{--}495 \text{ nm}$), which was intentionally configured to capture short-wavelength scattering and autofluorescence near the excitation line, the majority of particles exhibited weak or negligible signals, with only limited spatial overlap observed. Representative CLSM images are provided in Figure S8. Collectively, these results demonstrate that the optimized fluorescence protocol maintains high spectral selectivity in complex environmental matrices, enabling reliable discrimination of NR-positive MnPs from nonpolymeric particles.

3.2.2. Characterization of MnPs in TSP samples

To demonstrate the applicability of the optimized NR-CLSM method under real environmental conditions, a limited set of TSP samples ($n = 7$) was analyzed as a proof-of-concept field application. The optimized analytical protocol was applied to TSP samples collected in Xuan Mai, a suburban area of Hanoi, Vietnam. The results revealed the clear presence of both NPs and MPs in the samples. Given the method's LOQ_{size} of $0.3 \mu\text{m}$, NPs within the $0.3\text{--}1.0 \mu\text{m}$ range were successfully visualized by CLSM, along with numerous larger plastic particles. These findings provide direct microscopic evidence for the occurrence of airborne NPs in the suburban atmosphere of Hanoi and, to the best of our

knowledge, represent one of the first reports of airborne NPs in Vietnam. Representative CLSM images of MnPs identified in several TSP samples are shown in Fig. 8.

3.2.2.1. Morphology and size distribution. CLSM images (Fig. 8) revealed that the MnPs collected from the sampling site exhibited diverse morphologies, including fibers, fragments, spheres, and irregular forms. Particle size analysis was performed using ImageJ, with a size-based LOQ of $0.3 \mu\text{m}$. Particle boundaries were delineated using user-defined thresholding, followed by automated calculation of size parameters based on pixel-derived metrics. While the size calculation is automated and reproducible, boundary delineation depends on threshold selection and may be influenced by particle overlap effects, particularly for small or aggregated particles. The observed particle sizes ranged from 0.3 to $35.5 \mu\text{m}$. Among the 274 analyzed particles, fragments constituted the largest fraction (66.5%, $n = 117$), followed by spherical particles (21.8%, $n = 58$), fibers (9.0%, $n = 24$), and irregular shapes (5.6%, $n = 15$).

The predominance of fragment-type MnPs observed in this study is consistent with previous reports [1,46,47]. This observation is further supported by recent studies reporting that fragments account for a dominant proportion of airborne MPs, often exceeding 80% in urban environments (e.g., 87.4% in Seoul and 88.2% in Chinese megacities) [46,47]. Such fragment-dominated morphology is commonly associated with secondary MPs generated through the degradation of larger plastic materials [46].

However, the relatively high proportion of spherical particles (21.8%) contrasts with most earlier findings, in which this morphology was rarely reported in substantial abundance [46]. This discrepancy may be attributable to the detection limits of conventional analytical methods, which typically identify particles larger than $10 \mu\text{m}$ [6]. In contrast, the spherical particles identified in this study were predominantly within the nanometric size range ($0.3\text{--}1.0 \mu\text{m}$). With an LOQ_{size} of $0.3 \mu\text{m}$, this work extends the detection boundary into the nanoplastic regime and provides direct microscopic evidence for the presence of airborne nano-sized plastic particles in Vietnam. Such enhanced detection sensitivity may also influence the observed morphological composition of MnPs in atmospheric samples.

3.2.2.2. Number concentration of MnPs. The number concentration of MnPs ($>0.3 \mu\text{m}$) in ambient air at the sampling site ranged from 11,691 to 25,419 particles Nm^{-3} , with a mean value of $17,595 \pm 4589$ particles Nm^{-3} ($n = 7$). Within this range, the smallest size fraction ($0.3\text{--}1.0 \mu\text{m}$) accounted for 2807–8578 particles Nm^{-3} , corresponding to a mean concentration of 4491 ± 1974 particles Nm^{-3} . The $0.3\text{--}2.5 \mu\text{m}$ fraction exhibited the highest abundance, with concentrations ranging from 7953 to 14,270 particles Nm^{-3} and an average of $11,431 \pm 2600$ particles Nm^{-3} .

For particles in the $0.3\text{--}10 \mu\text{m}$ size range, the mean concentration was $13,304 \pm 6491$ particles Nm^{-3} (range: 392–19,186 particles Nm^{-3}), indicating that the vast majority of detected MnPs occurred within this fine-to-coarse particle size range. Larger particles ($>2.5 \mu\text{m}$) were present at concentrations of 3597–11,148 particles Nm^{-3} , with a mean value of 6164 ± 2694 particles Nm^{-3} . The coarsest size fraction ($\geq 5.0 \mu\text{m}$) contributed the least, ranging from 892 to 4462 particles Nm^{-3} and averaging 2643 ± 1570 particles Nm^{-3} (see Fig. 9).

Overall, the concentrations measured in this study were higher than those reported in previous works, which can largely be attributed to differences in analytical approaches and detectable particle size ranges. For example, Zhu et al. [47] reported mean concentrations of 282 ± 127 particles m^{-3} (range: 104–650 particles m^{-3}) across five Chinese cities using NR staining combined with fluorescence stereomicroscopy, where the smallest detectable particle size was $5.9 \mu\text{m}$. Li et al. [48] identified airborne microplastic fibers at concentrations of up to 5700 fibers m^{-3} by scanning electron microscopy (SEM), with a minimum detectable size of

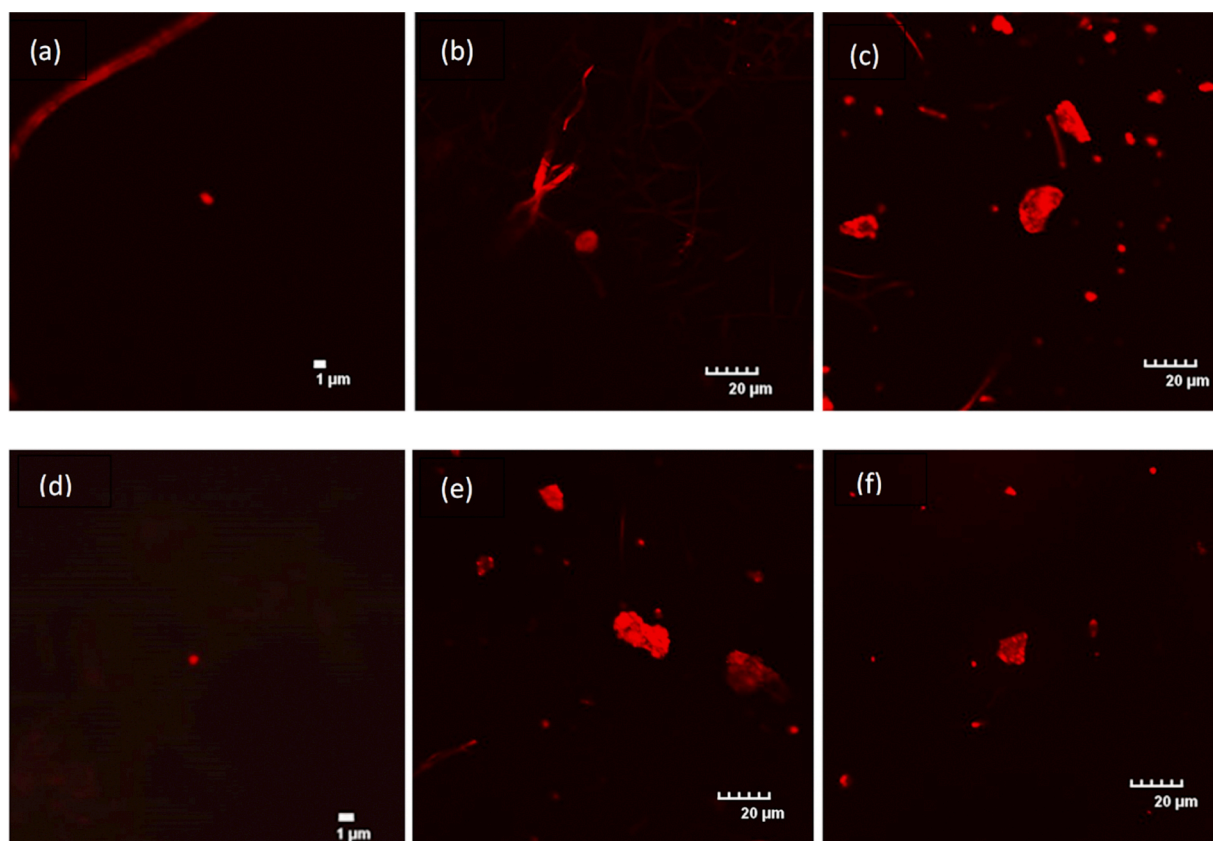


Fig. 8. CLSM images of real TSP samples showing the presence of MnPs under different observation conditions. (a, d) Acquired with a 20 × objective and 15 × digital zoom (scale bar = 1 μm), clearly visualizing NPs (<1 μm). (b, c, e, f) Acquired with a 20 × objective and 4 × digital zoom (scale bar = 20 μm), showing larger MnPs with diverse morphologies.

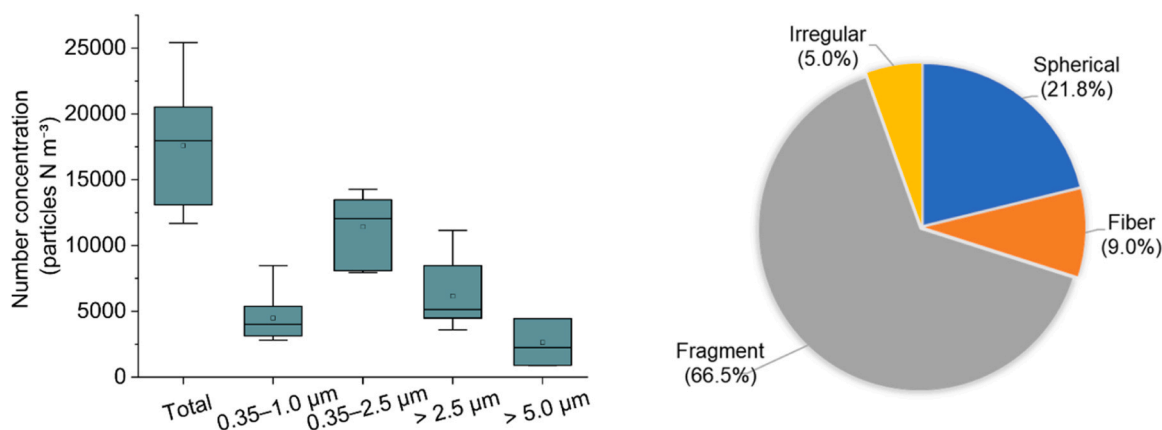


Fig. 9. Number concentrations of airborne MnPs across different particle size ranges (left) and relative abundance of particle morphologies (right) at the sampling site.

5 μm. Much lower concentrations were also reported in Paris, with values of 0.3–1.5 particles m⁻³ [16], and in Seoul, with values of 0.33–1.21 particles m⁻³ [46], where analyses were similarly restricted to larger particle sizes. Several methodological factors may account for these differences. First, many earlier investigations relied on multi-step pretreatment procedures, including density separation and sequential filtration, which can result in incomplete recovery of smaller particles [22,23]. In addition, when fibrous filters are used for sample collection, fine particles may be retained within or lost from the filter matrix during extraction. For example, Levermore et al. [49] analyzed spiked PM₁₀ samples without density separation or chemical digestion and directly

applied Raman spectral imaging to the particulate matrix, reporting airborne microplastic concentrations of up to $\sim 2.5 \times 10^3$ particles m⁻³.

Second, the analytical techniques commonly used in earlier studies, including stereomicroscopy, fluorescence microscopy, and SEM, are typically restricted to particles in the micrometer size range (≥ 5 –6 μm) [6,50]. Although Raman-based spectral imaging has demonstrated theoretical and experimental capability for submicrometer analysis, the smallest validated detections have remained at approximately 2 μm [49]. When applied to ambient particulate samples, the smallest spectroscopically confirmed plastic particle measured 4.7 μm [49]. In contrast, the NR-CLSM approach developed in this study enabled

reliable detection of MnPs down to 0.3 μm , extending into the sub-micrometer size range (0.3–1 μm).

Importantly, particle number concentrations are inherently dominated by the smallest size fractions, with particle abundance increasing markedly as particle size decreases. In this study, particles in the 0.3–1.0 μm range accounted for approximately 25.5% of the total particle count, while particles smaller than 2.5 μm contributed about 65% of the total number concentration. This size-dependent distribution explains why studies restricted to larger particle sizes (e.g., >5–10 μm) are likely to substantially underestimate total particle numbers.

Building on this, the size binning strategy was designed to align with the lower size detection limits reported in previous studies, thereby enabling comparison under comparable size conditions. When evaluated on this basis, the particle number concentrations obtained here are broadly consistent with those reported in earlier works within corresponding size ranges. Specifically, concentrations in the coarse particle fractions (>2.5 μm or >5 μm) are of the same order of magnitude as those reported using SEM [48], and Raman spectral imaging [49].

In addition to methodological factors, environmental conditions and local emission sources may also contribute to the observed concentrations, particularly in relation to traffic-related emissions and the resuspension of road dust in urban environments.

To ensure that these observations were not influenced by contamination during sampling and analysis, field blanks exhibited only background-level fluorescence comparable to procedural blanks and consistently below the established LOQ threshold, indicating negligible contamination during sampling, transport, and analysis.

From an environmental exposure perspective, these findings are particularly relevant in the context of inhalation. Notably, MnPs in the < 2.5 μm size fraction accounted for approximately 65% of the total detected particles. This predominance of fine and submicrometer particles is particularly relevant, as these size ranges are associated with inhalable particulate matter (e.g., $\text{PM}_{2.5}$) and enhanced deposition within the respiratory tract. Previous studies have shown that airborne MnPs in this size range can penetrate deeply into the respiratory system and reach the alveolar regions [51]. Deposition behavior is strongly size-dependent, with particles below 2.5 μm exhibiting increased penetration and retention in the lower respiratory tract. Moreover, MPs associated with fine particulate matter have been reported to act as carriers of co-occurring contaminants (e.g., heavy metals), potentially amplifying adverse health effects [52]. In this context, the ability of the NR–CLSM workflow to detect submicrometer particles provides a clear analytical advantage for the assessment of inhalable MnPs in ambient air.

These methodological advances underscore the strong potential of CLSM-based fluorescence analysis for investigating airborne MnPs, particularly for capturing particle populations that are largely inaccessible to conventional microscopic techniques.

3.3. Broader applicability and future perspectives

The validated NR–CLSM workflow developed in this study is not limited to total suspended particulate (TSP) samples. Because organic digestion, NR staining, and fluorescence imaging are performed directly on the same filter surface, the method can be readily adapted to finer atmospheric fractions (e.g., $\text{PM}_{2.5}$ and $\text{PM}_{1.0}$) collected on either fibrous or membrane filters. Moreover, following appropriate pretreatment and filtration to transfer particulate matter onto filters, this on-filter workflow can be extended to aqueous and solid environmental matrices, including surface water, wastewater, soil, and sediment, thereby enabling MnPs screening across multiple environmental compartments.

This integrated on-filter strategy minimizes sample handling and particle loss while maintaining high selectivity and submicrometer detection capability, supporting more comprehensive and comparable monitoring of MnPs. Future work should focus on optimizing oxidation reagents and digestion conditions (e.g., reagent type, temperature, and

incubation time) to accommodate the organic matrix characteristics of different sample types, while preserving the core principle of conducting all analytical steps directly on a single filter substrate. Matrix-specific validation, including procedural blanks, recovery experiments, and precision assessments, is recommended to confirm analytical performance under diverse sampling and processing conditions.

While the proposed NR–CLSM workflow offers significant advantages for sensitive and high-throughput screening of MnPs, it does not provide direct chemical specificity for polymer identification. Therefore, complementary spectroscopic techniques, such as Raman or $\mu\text{-FTIR}$, may be selectively applied to a subset of sufficiently large particles within the practical detection limits of these methods for confirmatory analysis.

4. Conclusions

This study established and validated a fluorescence-based Nile Red–CLSM (NR–CLSM) workflow for the selective detection and quantification of airborne micro- and nanoplastics (MnPs). By applying the four optimized excitation–emission configurations to confocal imaging, the method achieved a size-based limit of quantification of 0.3 μm and enabled reliable visualization of submicrometer particles directly on filter surfaces. The workflow demonstrated strong polymer-specific selectivity, remained unaffected by color additives and common non-polymeric particulates, and maintained low background fluorescence without the need for density separation or multi-step sample transfer.

Application of the method to ambient TSP samples revealed MnPs concentrations ranging from 1.2×10^4 to 2.5×10^4 particles Nm^{-3} and provided microscopic evidence of airborne nano-sized plastic particles (<1 μm) in Vietnam. The predominance of nanometric and fine-size particles underscores the necessity of analytical approaches capable of resolving submicrometer plastics in atmospheric environments.

Collectively, this NR–CLSM workflow provides a robust, high-sensitivity on-filter analytical platform for MnPs detection using CLSM and establishes an operational foundation for standardized monitoring, exposure assessment, and future cross-matrix applications of fluorescence-based MnP analysis.

These findings highlight the potential underestimation of inhalation exposure to nanoscale plastics and emphasize the need for standardized analytical protocols in atmospheric monitoring.

Environmental Implication

Airborne micro- and nanoplastics (MnPs) are emerging contaminants in atmospheric particulate matter with potential inhalation risks. This study establishes an optimized Nile Red–confocal laser scanning microscopy method for reliable, direct on-filter detection of submicron plastic particles. Optical validation using point spread function and full width at half maximum analysis improves discrimination of true nanoplastic signals from background fluorescence. Application to ambient samples confirmed respirable MnPs associated with total suspended particles, underscoring the need for standardized analytical approaches to monitor airborne plastics and evaluate human exposure.

CRedit authorship contribution statement

Bui Van Nang: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Nghiem Trung Dung:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Nguyen Thi Thu Hien:** Writing – review & editing, Validation, Supervision, Resources. **Vuong-Hung Pham:** Writing – review & editing, Validation, Resources.

Declaration of Competing Interest

The authors declare the following financial interests which may be

considered as potential competing interests: Bui Van Nang and Nguyen Thi Thu Hien report partially financial support was provided by Vietnamese Government Scholarship (Project 89 for domestic PhD training), Vietnam National University of Forestry (Grant No. LN.QM-2024.1) and the Ministry of Education and Training of Vietnam (Ministerial-level science and technology project, Grant No. B2025-BKA-14). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2026.142564](https://doi.org/10.1016/j.jhazmat.2026.142564).

Data availability

Data will be made available on request.

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