

Enzyme-driven fluorescence quantification of micro and nanoplastics

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ABSTRACT

Accurate detection of micro- and nanoplastics (MNPs) remains challenging due to analytical limitations, particularly for nanoplastics (<1 μm). Here, we introduce a rapid and simple fluorescence-based method for MNP quantification using a plastic-degrading enzyme. Target plastics are enzymatically depolymerized to generate specific degradation products, which are subsequently quantified via fluorescence measurement. The method enables selective detection of polyethylene terephthalate (PET) and other terephthalate-releasing plastics across a broad size range, from microplastics to nanoplastics. After removal of divalent cations, the assay provides results within 6 h of enzymatic reaction with LODs of 0.43 mg/L and 0.04 mg/L for microplastics (90 μm) and nanoplastics (0.2 μm) PET standards, respectively; concentrations are reported as PET-equivalent mass referenced to these size-matched standards. Quantitative results showed good agreement with orthogonal reference methods — micro-Fourier transform infrared spectroscopy for microplastics and pyrolysis–gas chromatography/mass spectrometry for nanoplastics. This approach provides a practical alternative for rapid MNPs analysis and offers improved accessibility for nanoplastics, supporting its high throughput use in laboratories without specialized instrumentation, for aqueous samples of low matrix complexity.

1. Introduction

Microplastics (MPs, particles <5 mm in size) and nanoplastics (NPs, <1 μm) have become widespread contaminants in aquatic environments, raising concerns about chronic human exposure and long-term ecological risk [1–3]. As plastic debris progressively fragments into smaller particles, its environmental mobility, surface reactivity, and biological accessibility increase, thereby enhancing the likelihood of uptake by aquatic organisms and transfer through food webs [4]. In particular, nanoplastics are of growing concern because their small size may confer greater bioavailability and toxicological relevance [5,6]. Accordingly, reliable analytical methods for the sensitive detection and quantification of plastic particles are essential for environmental monitoring, fate assessment, and risk evaluation.

Conventional microplastic analysis commonly combines visual or fluorescence microscopy for particle screening and counting with micro-FTIR or Raman microscopy for particle-level polymer identification, while pyrolysis–gas chromatography–mass spectrometry (py-GC/MS) provides polymer-specific mass quantification [7–11]. However, microscopy-based approaches require labor-intensive sample preparation and are limited in their ability to reliably identify small particles, whereas thermal methods are destructive and do not retain particle-number, size, or morphology information. These approaches also generally require costly benchtop instrumentation and extensive

workflows involving digestion, density separation, and filtration.

In 2025, FT-IR and Raman methods for microplastic analysis were formally codified in ISO 16094-2:2025, which prescribes sample preparation via filtration and subsequent spectroscopic identification [2]. Because particles smaller than the membrane pores will pass through and not be retained for analysis, and given the spatial resolution limits of Fourier transform infrared spectroscopy (FT-IR) imaging (~10–20 μm) and Raman microscopy (~0.5–1 μm) [7,10,12], the effective lower detection limit of ISO-compliant workflows is roughly $\geq 1 \mu\text{m}$. Thus, despite standardization, these international methods are not capable of reliably detecting nanoplastics (<1 μm).

Fluorescence-based detection strategies have been explored to overcome these limitations. Nile Red staining, for example, allows relatively rapid and low-cost screening, but suffers from non-specific binding to lipids, natural organic matter, and other hydrophobic materials, leading to false positives [8,13]. These drawbacks have spurred interest in more selective, enzyme-assisted methods.

Enzyme-based detection offers polymer specificity because biological catalysts can target defined chemical structures. PET hydrolases can degrade poly(ethylene terephthalate) (PET) into monomers like terephthalic acid, which can then be quantified via fluorescence or other means [14–16]. Such strategies are inherently selective for polyester containing terephthalic acid and, in principle, can detect smaller particles because detection depends on chemical degradation rather than

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optical resolution or filtration.

However, existing enzyme-based fluorescence work has largely been directed at screening for polyester-degrading activity rather than at quantifying plastic particles, and quantitative performance criteria such as working range, detection limit and reproducibility have not been established [14]. The assay described here differs from each of the preceding approaches in the origin of its signal: specificity derives neither from optical identification of individual particles, as in FT-IR and Raman microscopy, nor from hydrophobic partitioning of a dye, as in Nile Red staining, but from a polymer-specific degradation product. This makes the sub-micrometer range accessible at a throughput and instrumentation cost not attainable with spectroscopic or thermoanalytical methods.

Here, we present a fluorescence assay for PET micro- and nanoplastics, based on the enzymatic activity of PET hydrolase (PlastiCUT™), followed by formation of fluorescent 2-hydroxyterephthalic acid (hTPA). The assay uses glass vials, avoids filtration steps, and is read with a conventional microplate reader with a photon multiplier tube (PMT). We demonstrate a linear response in the mg/L range for PET-90 (average 90 µm sized microplastics), and sub-mg/L range for PET-0.2 (average 200 nm sized PET nanoplastics), high selectivity against other types of plastics (PE, PP, TPU, and PS), and compatibility with 96-well high-throughput measurement. The workflow—from gravimetric preparation of standards, buffer/enzyme addition, 6 h incubation, to fluorescence readout—can be completed in one working day with less than 30 min of hands-on time for a set of five to ten samples. This method provides a practical, polymer-specific approach for PET monitoring in drinking water, extending quantification into the sub-micrometer range that ISO-compliant spectroscopic workflows cannot reach.

2. Materials and methods

2.1. Used plastic materials

PET-90 (90 µm, Guangyuan Plastic Raw Materials, China), PET-5 (1–5 µm, Nanochemazone, Canada), PET-0.2 (200 nm, Magerial Science, China), PBT, PETG (10–20 µm, Nanochemazone, Canada), PBAT (Hebei Luozheng Technology, China), PEN (Hebei bonster Tech., China) PP, PE (100 mesh, Magerial Science China), TPU (200 mesh, Guangyuan Plastic Raw Materials, China), PS (100 mesh, Guangyuan Plastic Raw Materials, China), PE nanoparticles (50, 100 and 200 nm, Magerial Science), PS-15 and PA6-15 (1000 mesh, Magerial Science).

2.2. Preparation of PET standards and water samples

PET microplastics (PET-90) were used as reference material. Empty glass vials were tared on an analytical balance (XP-205, Mettler Toledo; readability 0.01 mg), and the appropriate amount of PET was dispensed to obtain final concentrations of 0, 20, 40, and 80 mg/L in a total volume of 10 mL. The 0 mg/L standard (standard zero) contained enzyme but no PET, whereas the standard blank contained neither PET nor enzyme and consisted of the reaction buffer components only. All weighing steps were performed carefully to minimize static interference, and vials showing any moisture were not used. Water samples (45–50 mL) were collected in 50 mL borosilicate vials. Each sample was treated with an appropriate amount of sodium-form ion exchange resin (DOWEX HCR-S, DuPont, USA) to remove calcium and magnesium ions and was divided into two vials designated as sample test and sample blank. Water hardness was determined by EDTA-based photometric method (HI97735, Hanna Instruments) and is reported as mg/L CaCO₃.

2.3. Reaction composition

The reaction mixture was adjusted to final concentrations of 50 mM sodium phosphate buffer (pH 7.5), 0.75 mM EDTA, and 0.75 mM FeSO₄. A PET hydrolase, PlastiCUT™ (E.C. 3.1.1.- esterase, cat. no. DP-ENZ-

PC01, Decomposition, South Korea) served as the plastic degrading enzyme. The enzyme was added only to sample test vials, resulting in a final concentration of 0.01 mg/mL. Sample blank vials contained identical buffer components but no enzyme, and therefore received an equivalent volume of water. All components were added in a consistent order for every vial, and mixtures were briefly vortexed to ensure homogeneity.

2.4. Enzymatic hydrolysis

Reaction temperature and buffer pH were optimized prior to this work; response was maximal at 70 °C and pH 7.5, and declined on either side (Fig. S1, Table S5). The prepared vials were incubated at 70 °C for 6–24 h in a thermostated water bath. The vials were completely submerged to maintain uniform heating. No shaking was applied during incubation. After the reaction, the vials were removed and cooled under running tap water. The reaction mixtures were used directly for fluorescence measurements without centrifugation or filtration. The fluorescence signal is stable and can be read up to 24 h after the incubation.

2.5. Fluorescence measurement

Supernatants were carefully transferred into flat, black 96-well microplates (SPL) at a volume of 200 µL per well, and five replicate wells were prepared for each condition. Liquid was aspirated from the upper portion of each vial to avoid disturbing settled particles.

Fluorescence intensity was measured using a microplate reader (Hidex Sense). The instrument was operated with a filter set where the excitation wavelength is 320 nm (bandwidth 40 nm) and the emission wavelength of 405 nm (bandwidth 50 nm). Filter settings were selected to encompass the excitation and emission maxima of hTPA (328 and 421 nm). Data were exported in spreadsheet format for further analysis.

2.6. Data processing and quantification

Fluorescence values from the five replicates were averaged for each sample. The corresponding Sample Blank value was subtracted from the Sample Test value to obtain the corrected signal.

Calibration curves were generated by PET-90 and PET-0.2, and sample concentrations were calculated using linear regression. Detection was defined as a corrected signal greater than that of the (standard zero - standard blank) value. Samples falling below the detection threshold were reported as “not detected.” A calibration series is prepared with the same enzyme preparation and measured in the same run as the samples it is used to quantify; calibration is not carried over between runs.

All data processing and statistical analyses were performed using standard analytical procedures. The limit of detection (LOD) was calculated as $3\sigma/S$, where σ is the standard deviation of the sample blank (enzyme-free) signal measured in 5 independent replicates and S is the slope of the calibration curve.

Precision was expressed as the coefficient of variation ($CV = SD/\text{mean} \times 100$), calculated from five replicate wells prepared from a single reaction vial and therefore reflecting the variability of dispensing and fluorescence readout. CVs were determined for signals at each calibration level before blank correction, and are reported as the mean with the observed range across the working range. The linearity of the calibration curve was assessed based on the coefficient of determination (R^2).

2.7. PET-CaCO₃ test material preparation and py-GC/MS analysis

A matrix-matched PET-CaCO₃ test material was prepared by cryo-milling PET-0.2 with CaCO₃ so that both methods could be applied to the same sample, since the PET mass compatible with py-GC/MS is below the reliable weighing limit. PET-0.2 (10 mg) and CaCO₃ (990 mg, Sigma-Aldrich, USA) were weighed on an analytical balance (Sartorius,

Germany) and cryo-milled in an IQ MILL-2070 (Frontier Laboratories, Japan) at 600 rpm, using a program of 20 s milling and 10 s pause for 15 cycles per set, repeated for 15 sets. The resulting powder had a nominal PET content of 10 µg per 1 mg of mixture.

Pyrolysis-GC/MS analysis was conducted to quantify PET in the PET-CaCO₃ test material as the orthogonal reference for the enzyme-fluorescence assay. An MPCS-CaCO₃ standard (Frontier Laboratories, Japan) was used to generate the calibration curve. PET was quantified using benzophenone (m/z 182) as the indicator ion, with m/z 51 and 77 as confirming ions. Single-shot pyrolysis was performed on an EGA/PY-3030D pyrolyzer coupled to a 7890A/5975 GC/MS system (Agilent Technologies, USA) at a furnace temperature of 600 °C, interface 320 °C, and split ratio of 30. Separation used a UA-5 column (30 m × 0.25 mm i. d., 0.25 µm; Frontier Laboratories, Japan) with helium at 1 mL/min; the oven was held at 40 °C for 2 min, ramped at 20 °C/min to 320 °C, and held for 14 min. Spectra were acquired over m/z 29–300.

For recovery determination, 1.0 mg aliquots of the test material were weighed and analyzed in triplicate, and the measured PET mass was expressed relative to the nominal content of 10 µg. The same aliquot size was used for the enzyme-fluorescence assay so that both methods sampled the material at an identical scale.

2.8. µFT-IR analysis

For µFT-IR analysis, 0.1 mg of PET-90 was dispersed in ethanol and filtered through a 20 µm stainless steel mesh. The retained particles were dried and analyzed using a µFT-IR system (iN10, Thermo Fisher Scientific, USA). Particles showing greater than 70% spectral similarity to PET

were counted as PET particles.

3. Results and discussion

3.1. A conceptual reaction mechanism illustrates the principle of enzymatic fluorescence-based microplastic quantification

To establish a proof-of-concept for an enzyme-based quantification strategy, we developed a fluorescence-readout system that measures micro- and nanoplastic concentrations by coupling plastic-degrading enzymes with their characteristic degradation products. Numerous plastic-degrading enzymes have been identified and engineered in recent years [17–21]; however, their application has largely focused on enzyme screening or discovery of novel plastic-degrading microorganisms. In contrast, our approach employs these enzymatic reactions specifically for quantitative detection of micro/nanoplastics, which to our knowledge has not previously been demonstrated. To achieve this, we optimized and fine-tuned each step of the reaction to enable robust and reproducible measurement (Fig. 1a).

Although enzymes capable of degrading polyesters, polyamides, and polyurethanes have been well studied, polymers such as polyethylene and polypropylene lack a single defined depolymerase [20]. Their degradation generally requires initial abiotic oxidation before enzymatic cleavage of the carbon-carbon backbone, resulting in diverse and non-specific degradation products. Therefore, in this study, we focused on polyesters containing terephthalate, one of the most abundant plastics globally and a polymer whose degrading enzymes and hydrolysis products are well characterized [22–24]. Among the available

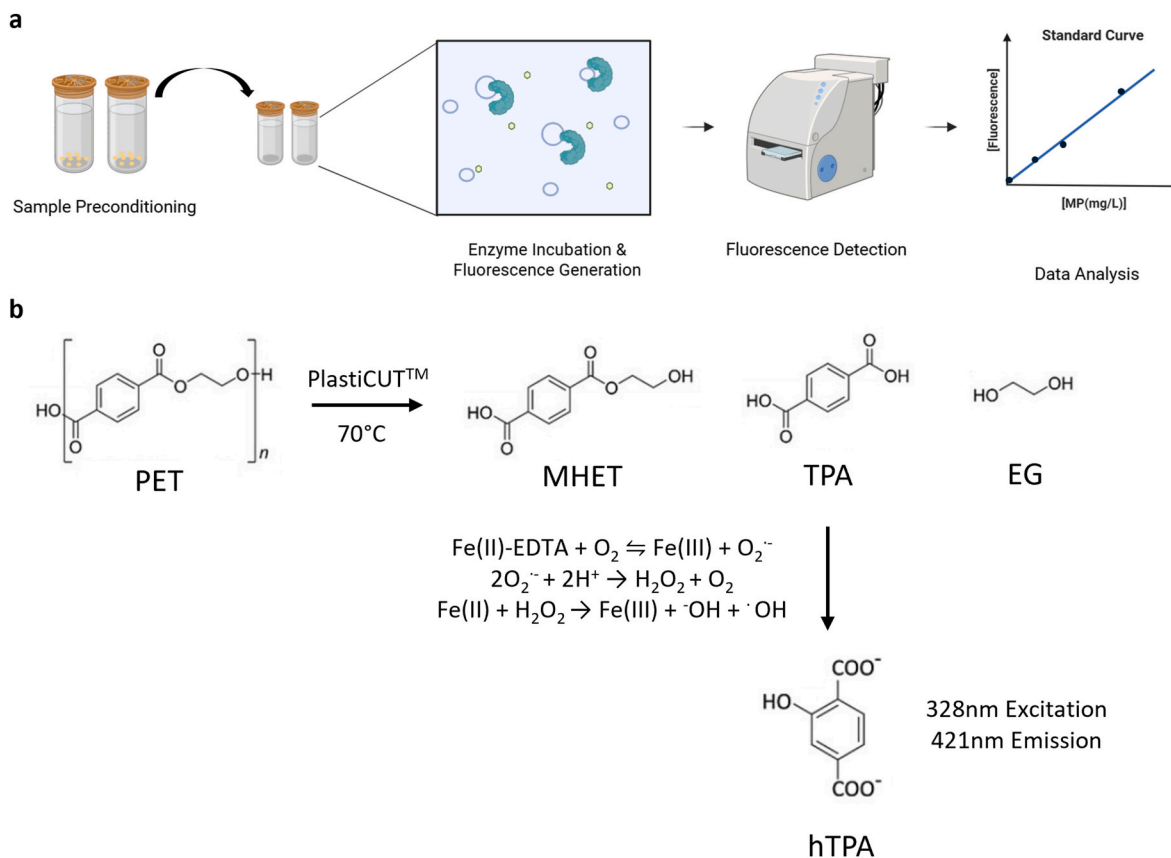


Fig. 1. Overall workflow and mechanism of enzyme-driven fluorescence quantification of micro- and nanoplastics. (a) Analytical workflow. Water samples are collected, pretreated with conditioning resins, and incubated with the enzyme in the reaction buffer. Fluorescence signals are then measured and analyzed to quantify micro- and nanoplastics. (b) Reaction mechanism underlying signal generation. PlastiCUTTM cleaves the ester bonds of PET at 70 °C to release mono(2-hydroxyethyl) terephthalate (MHET), terephthalic acid (TPA) and ethylene glycol (EG). Fe(II) chelated by EDTA undergoes autooxidation to generate hydroxyl radicals, which hydroxylate the aromatic ring of TPA to form 2-hydroxyterephthalic acid (hTPA). hTPA exhibits excitation and emission maxima at 328 and 421 nm, respectively.

degrading enzymes, we employed PlastiCUT™, which hydrolyzes terephthalate containing polyester micro- and nanoplastics and releases terephthalate (TPA) as a major degradation product.

The released TPA was subsequently converted to fluorescent hydroxyl-TPA (hTPA) through Fe(II)-mediated hydroxylation, and the resulting fluorescence intensity was measured [25] (Fig. 1b). By fitting the fluorescence signal to a calibration curve, we quantified the concentration of enzymatically released TPA, from which PET-equivalent concentration was derived. Because the response mainly depends on the enzymatically accessible surface area, results are expressed as PET-equivalent mass concentration referenced to a size-matched standard. For polydisperse samples, the 90 μm and 200 nm calibrations define upper and lower bounds rather than a single absolute value.

3.2. The assay selectively detected TPA-releasing polymers through enzyme-specific hydrolysis

Since the assay employs a defined plastic-degrading enzyme, the detectable signal is intrinsically restricted to polymers that serve as suitable substrates for that enzyme. This substrate specificity, rather than representing a limitation, confers a significant analytical advantage by enabling selective quantification of target microplastics within heterogeneous sample matrices. In the case of PlastiCUT™, PET was readily hydrolyzed, whereas PE, PP, PS, and TPU were not susceptible to enzymatic degradation and consequently gave fluorescence at the blank level (Fig. 2a). Even in instances where partial hydrolysis may occur such as PEN, polymers that do not release TPA as a hydrolysis product do not contribute to the fluorescent output (Fig. 2b).

We further assessed several PET-like polymers, including PBT, PBAT, and PETG, to evaluate whether enzyme-mediated hydrolysis and subsequent TPA release occurred. PBAT and PETG exhibited clear concentration-dependent increases in fluorescence intensity, consistent with enzymatic generation of TPA. In contrast, PBT showed no dose-dependent response despite containing terephthalate in its backbone, resulting in a profile indistinguishable from that of the TPA-free negative control PEN (Fig. 2b). This suggests that PBT is a poor substrate for PlastiCUT™ under the assay conditions, and that the absence of signal reflects limited enzymatic susceptibility rather than the absence of TPA in the polymer.

These findings demonstrate that the assay exhibits high selectivity toward polymers such as PET, PBAT, and PETG that undergo enzyme-mediated hydrolysis and release TPA. Furthermore, interference from unrelated organic components or from microplastics of non-TPA-releasing polymer types is expected to be minimal.

To confirm that selectivity is retained in the size range where the assay offers its principal advantage, PE was assayed as nanoparticles of

200, 100 and 50 nm alongside PS and PA6 microplastics, each at 10 mg/L, with PET-0.2 as the positive control (Fig. S2). PET-0.2 generated a signal well above blank, whereas none of the non-terephthalate polymers gave a response distinguishable from blank. Selectivity was therefore retained down to 50 nm, as expected for a signal that arises from hydroxylation of released terephthalic acid and is thus determined by polymer chemistry rather than by particle size.

3.3. A one-pot reaction format simplified the workflow and improved assay sensitivity

To streamline the assay workflow, we optimized fluorescence generation by co-incubating phosphate buffer with the Fe-EDTA complex, enabling the reaction to be carried out as a one-pot process. Previous TPA fluorescence assays employing Fe-EDTA chemistry [14,16] typically utilized pH 7.5 phosphate buffer [26] and required a two-step procedure in which the enzymatic hydrolysis was first completed, followed by filtration and subsequent addition of Fe^{2+} and EDTA. In contrast, combining phosphate buffer, Fe^{2+} , EDTA, and enzyme from the outset markedly improved assay sensitivity; the slope of the calibration curve increased by approximately two-fold relative to the two-step method. Linearity extended to ~ 60 mg/L PET-5 in the one-pot format and to ~ 200 mg/L PET-5 in the two-step configuration (Fig. 3).

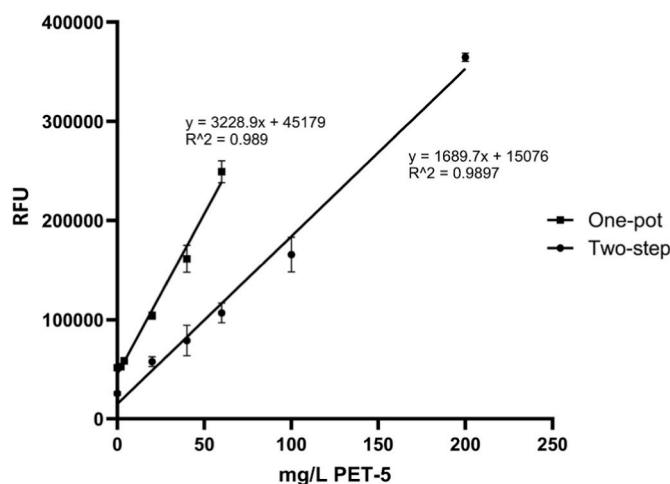


Fig. 3. Comparison of one-pot and two-step fluorescence assay formats for TPA quantification. Calibration curves for the conventional two-step and one-pot formats. The one-pot format showed an approximately two-fold higher slope, indicating improved sensitivity.

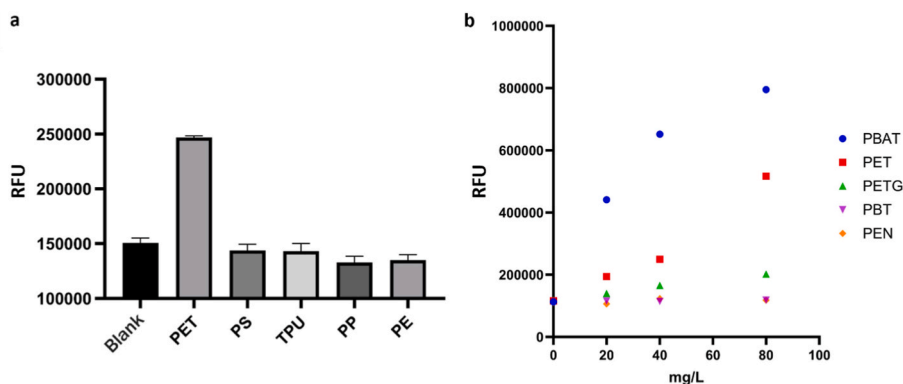


Fig. 2. Specificity of the enzymatic fluorescence assay toward polymers that release terephthalic acid (TPA) upon enzymatic degradation. (a) Polymer powders (100–200 mesh) at 50 mg/L were incubated with PlastiCUT™ for 5 h. Blank represents the negative control without polymer. Error bars represent standard deviation ($n = 5$). (b) Candidate polymers expected to release TPA upon enzymatic degradation (PBAT, PETG, and PBT) were tested together with PEN as a negative control, which does not release TPA.

A further practical advantage of the one-pot reaction concerns sample handling. Microplastics, particularly nanoscale particles, often exhibit intrinsic fluorescence or scatter, necessitating filtration or centrifugation prior to fluorescence measurement to ensure that particulate matter does not interfere with quantification. In the present assay, however, Fe^{2+} added for TPA hydroxylation is partly oxidized and precipitates under the near-neutral phosphate conditions used [26], and the resulting solid aggregates with residual micro- and nanoplastic particles, leaving a clear supernatant.

hTPA is a dissolved small molecule and remains in the aqueous phase. Consistent with this, the one-pot format gave a higher calibration slope than the two-step format, in which the mixture is filtered before Fe^{2+} addition (Fig. 3); sampling the supernatant therefore entails no signal loss. Consistent with this, calibration curves obtained from directly sampled supernatants were linear ($R^2 \geq 0.989$; Fig. 3) and reproducible (mean CV 3.35%, range 1.62–6.85%; Tables S1 and S2). The supernatant can therefore be sampled directly for fluorescence measurement without filtration or centrifugation, simplifying the workflow. Because standards and samples are prepared and processed identically, any residual partitioning of hTPA is systematic and is compensated by the calibration.

The improved sensitivity of the one-pot format is likely attributable to two factors: sustained Fe(II) autoxidation and radical generation in phosphate throughout the incubation [26], and continuous conversion of TPA as it is released, which may relieve product inhibition of the hydrolase [24]. These interpretations are advanced as hypotheses and remain to be tested directly.

3.4. Assay sensitivity increased with decreasing particle size, extending quantification to 200 nm PET in clean matrices

Given that nanoplastics, smaller than 1 μm , may exhibit greater

biological uptake and associated risk than larger particles, we evaluated whether the assay could reliably detect nanoscale PET particles. Notably, this size dependence works in our favor: nanoplastics that poses the greater biological risk is also the fraction our assay detects more sensitively. At the same PET mass concentration, smaller particles have a higher total surface area available for enzymatic hydrolysis. Consequently, smaller particle sizes are expected to produce steeper standard-curve slopes.

Using PET-90 and PET-0.2 as representative micro- and nanoplastic standards, we observed that the slope of the PET-0.2 calibration curve was approximately 8–11 times greater than that of PET-90 (Fig. 4a and b). This trend was also apparent across a third size class: PET-5 (1–5 μm) gave a calibration slope intermediate between those of PET-90 and PET-0.2 (Fig. 3), consistent with an inverse relationship between particle size and fluorescence response.

Under a 6-h incubation period, the limit of detection (LOD) for PET-90 was 0.43 mg/L, whereas the LOD for PET-0.2 was markedly lower at 0.04 mg/L. Both calibration curves exhibited excellent linearity, with R^2 values exceeding 0.99. When slight deviations from linearity were observed, extending the incubation time improved linearity within the tested conditions. This improvement was empirically observed and likely reflects enhanced product accumulation and signal resolution at lower PET concentrations. Coefficients of variation (CV) remained below 5%, with fluorescence values computed as the mean of five technical replicates to account for inherent fluorescence variability.

The dynamic range of PET-90 spanned 0.43–100 mg/L (Fig. 4c), with a noticeable decrease in the slope occurring at higher concentrations (200–500 mg/L). By comparison, PET-0.2 showed a narrower dynamic range of 0.04–17 mg/L, and signal saturation, reflected by a reduced slope, emerged at concentrations between 25 and 50 mg/L (Fig. 4b and d).

Between-run precision was evaluated using five independent

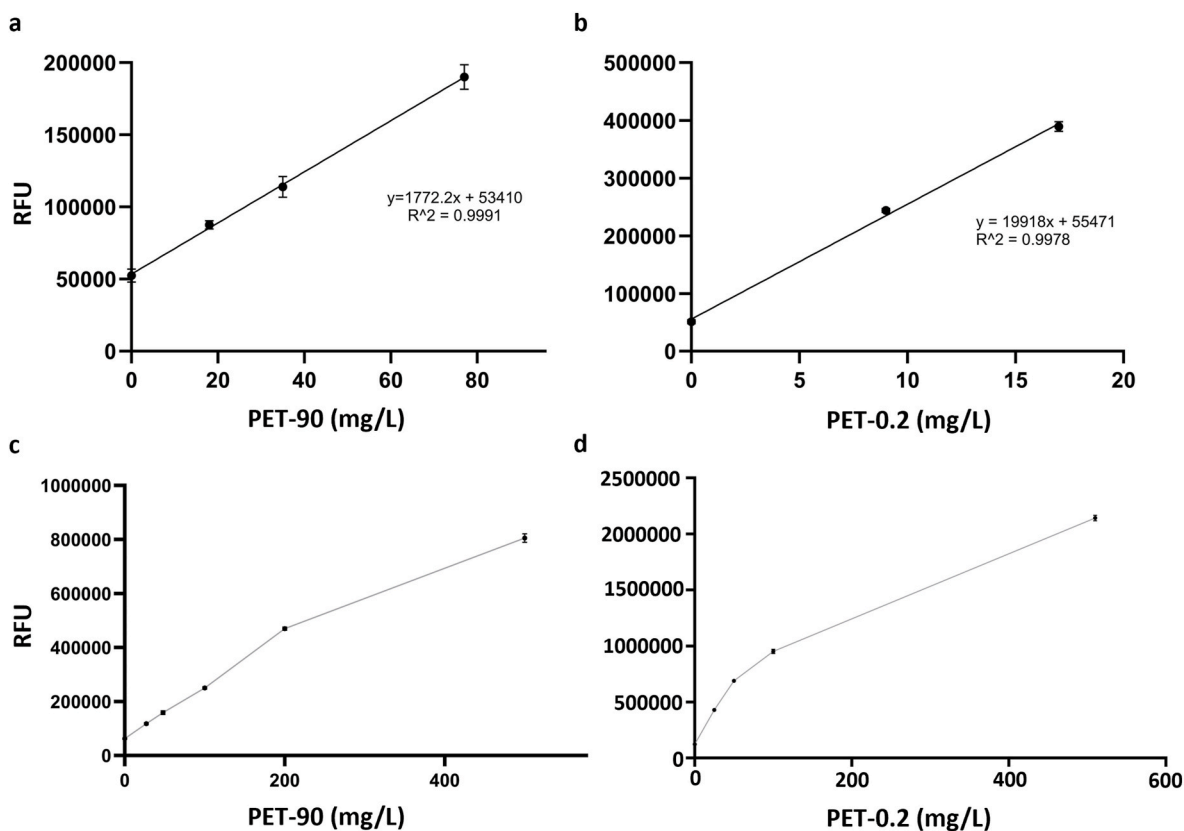


Fig. 4. Calibration curves and dynamic ranges of PET-90 and PET-0.2. (a, b) Standard curves for PET-90 and PET-0.2, respectively. (c, d) Extended concentration ranges for PET-90 and PET-0.2 showing reduced slope and signal saturation at higher concentrations.

calibration series. Three series were prepared on separate days over a three-week period using the same enzyme lot, whereas two series were prepared using independently produced enzyme lots. Across the five runs, calibration-curve slopes ranged from 1975 to 2758 RFU·L·mg⁻¹ (mean 2250 RFU·L·mg⁻¹; CV 15.1%), and R² values ranged from 0.946 to 0.990 (Table S4). The slope values obtained using independently produced enzyme lots fell within the range observed across separate day runs using a single enzyme lot. Because enzyme lot and run conditions were not independently varied in a fully crossed experimental design, the present data cannot isolate the contribution of enzyme lot to the overall between-run variability. Regardless of its source, modest run-to-run differences in reaction performance or RFU readout are accommodated by generating a new calibration series alongside each sample set. Under the standard 6-h incubation condition, runs were accepted only when the corresponding PET-90 calibration series met predefined quality criteria: R² ≥ 0.95 and a run-specific limit of detection of ≤0.65 mg/L.

3.5. Reaction kinetics revealed time dependent increases in assay sensitivity for both PET-90 and PET-0.2

To determine the optimal reaction time, the slopes of the standard curves were compared across different incubation times (2, 4, 6, 8, 12, 24, and 48 h) within the relevant concentration ranges. Based on the dynamic ranges identified above, PET-90 was evaluated over 0–80 mg/L, whereas PET-0.2 was evaluated over 0–20 mg/L.

For both PET-90 and PET-0.2, the response slope increased logarithmically from 2 to 12 h and approached saturation after 24 h (Fig. 5a and b). Although 24 h provided the highest analytical sensitivity, 4–6 h was identified as a practical minimum incubation time for rapid detection of micro- and nanoplastics. A 24 h incubation can be applied when higher sensitivity is required. Notably, the fluorescence signal remained stable over the tested time period, indicating that the measured fluorescence signal did not appreciably decay during the assay.

For PET-90, incubation at 70 °C for 6 h yielded a slope of 2,534, which increased to 11,131 after 24 h, a 4.4-fold gain. The coefficient of determination (R²) exceeded 0.99 from 4 h onward. For PET-0.2, the slope increased from 12,091 at 6 h to 52,142 at 24 h, a 4.3-fold gain. The comparable fold-increase observed for two size classes differing by more than two orders of magnitude indicates that the extent of conversion at a given incubation time is governed by the reaction conditions rather than by particle size.

3.6. Removal of divalent ions by a sodium-form ion exchange resin restored assay sensitivity under hard-water conditions

Because drinking water contains dissolved ions over a wide range of hardness levels, the effect of water hardness on assay sensitivity was evaluated under realistic sample conditions. Hardness, primarily

attributable to Ca²⁺ and Mg²⁺, interfered with hTPA quantification in the one-pot reaction through interactions with phosphate, iron species, and EDTA.

Specifically, the formation of insoluble phosphate precipitates by Ca²⁺ and Mg²⁺ ions depleted the available phosphate, thereby reducing the sensitivity of the fluorescence-based detection of TPA [26,27]. In addition, these divalent ions compete with Fe(II) for EDTA, limiting Fe (II) stabilization and consequently reducing the oxidation efficiency of TPA to hTPA [28]. Even in a two-step format, Mg²⁺ suppressed the response, whereas Ca²⁺ interfered only with fluorescence generation — a pattern consistent with enzyme inhibition by Mg²⁺ and phosphate depletion by Ca²⁺. These results indicate that divalent ions reduce assay sensitivity at both the enzymatic reaction and fluorescence detection stages.

To remove these interfering ions, a sodium-form ion exchange resin was introduced. Na⁺ did not adversely affect either the enzymatic depolymerization reaction or the fluorescence assay. Although resin treatment increased the background fluorescence signal, this effect was corrected by measuring a sample blank without enzyme for each sample and subtracting the corresponding blank signal.

The effect of water hardness on assay sensitivity was first evaluated by diluting water with an initial hardness of 200 mg/L CaCO₃ using DDW and examining the slope of the PET-90 standard curve after 5 h of enzymatic incubation. The slope progressively decreased with increasing water hardness (Fig. 6a).

To validate the resin treatment, CaCl₂·H₂O was dissolved in DDW at 147 mg/L, resulting in a measured hardness of approximately 78 mg/L CaCO₃. After overnight treatment with the sodium-form ion exchange resin at room temperature, the hardness decreased to approximately 17 mg/L CaCO₃. PET-90 standard curves generated using DDW and resin-treated water showed that the reduced-hardness condition nearly fully restored the calibration slope to the DDW level (Fig. 6b and c).

3.7. The enzyme–fluorescence assay showed performance comparable to μFT-IR for microplastics and to py-GC/MS for nanoplastics

To benchmark the performance of the enzyme–fluorescence assay, comparative analyses were conducted against μFT-IR and pyrolysis–GC/MS, with experimental designs tailored to the technical constraints of each method.

Because μFT-IR quantifies microplastics by particle counting and is limited to particles larger than 20 μm [7], PET particles with an average size of 90 μm were used. As a reference, 0.01 mg of PET-90 was weighed using an ultramicrobalance, and the corresponding particle count was determined. Across three measurements, 0.01 mg contained an average of 35.33 particles, corresponding to 353.3 particles per 0.1 mg. For μFT-IR analysis, 0.1 mg of PET-90 was analyzed, counting particles with >70% spectral similarity to PET. In parallel, an equivalent mass was analyzed using the enzyme–fluorescence assay, and the resulting

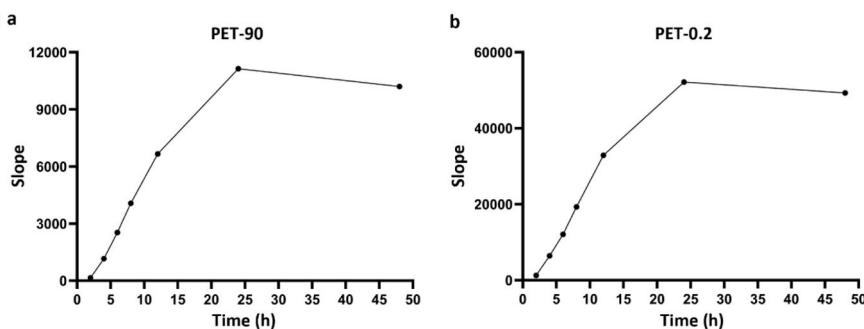


Fig. 5. Effect of incubation time on the calibration slope of PET-90 and PET-0.2. (a) Calibration slope of PET-90 measured at different incubation times (2, 4, 6, 8, 12, 24, and 48 h) over the concentration range of 0–80 mg/L. (b) Calibration slope of PET-0.2 measured at different incubation times (2, 4, 6, 8, 12, 24, and 48 h) over the concentration range of 0–20 mg/L.

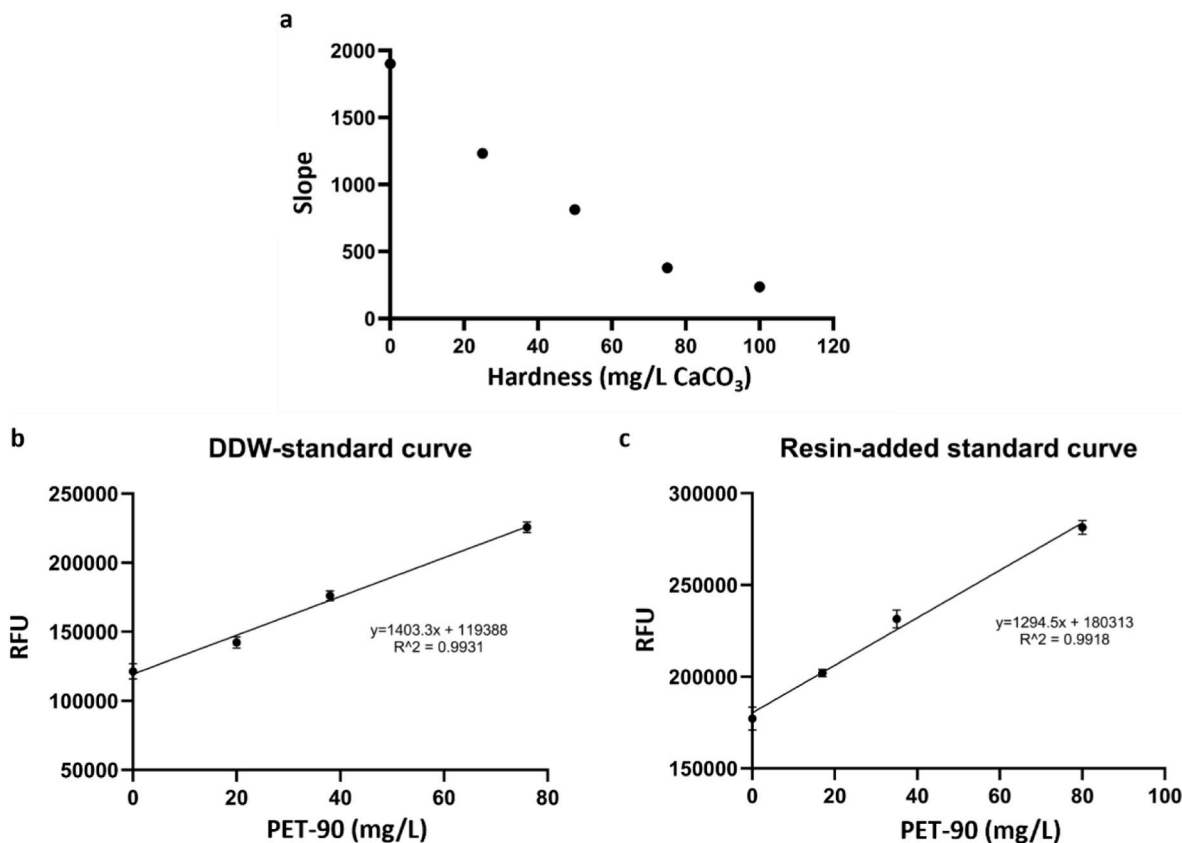


Fig. 6. Water hardness reduced assay sensitivity, which was restored by sodium-form ion exchange treatment. (a) Correlation between water hardness and the slope of the PET-90 standard curve after 5 h of enzymatic incubation. (b, c) PET-90 standard curve generated in DDW and resin-treated water, in which the measured hardness was reduced from approximately 78 to 17 mg/L CaCO₃, showing recovery of the calibration slope.

concentration was converted to particle number using the ultra-microbalance count.

The reference count determined by ultramicrobalance was 353.3 particles per 0.1 mg. μ FT-IR detected 293 particles per 0.1 mg, corresponding to 83% agreement. The enzyme–fluorescence assay gave 383 ± 86 particles per 0.1 mg (mean $\pm$ SD, $n = 3$), corresponding to 92% agreement for the mean; the measured range encompasses the reference count, indicating no evidence of systematic bias relative to the reference, although the replicate-to-replicate variability exists (Fig. 7a).

Unlike spectroscopic particle-counting approaches, py-GC/MS quantifies polymer mass through pyrolysis products and is therefore independent of particle size or optical resolution, making it the appropriate orthogonal reference for validating nanoplastic quantification. Because py-GC/MS accommodates only a small sample mass, the PET content required lies below the reliable weighing limit; a matrix-matched test material was therefore prepared by cryo-milling PET-0.2 with CaCO₃ to a nominal 10 μ g PET per 1 mg powder (Section 2.7). Both methods were applied to 1.0 mg aliquots of this material ($n = 3$ each), so

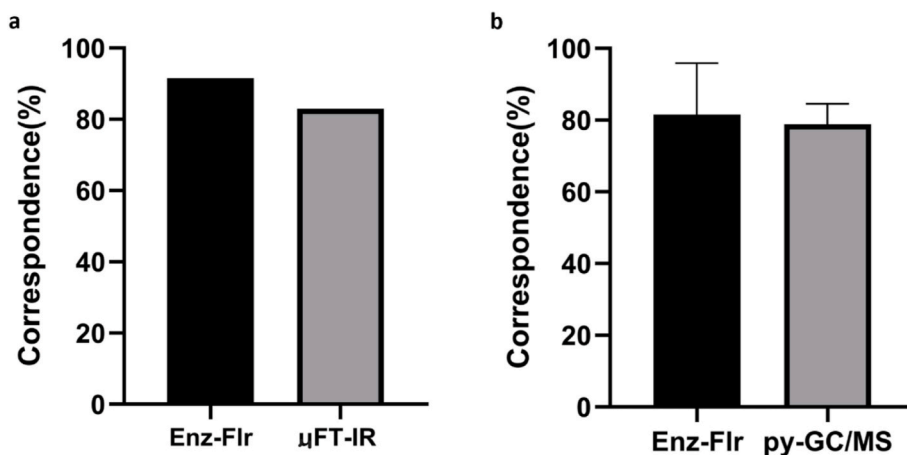


Fig. 7. Comparison of the enzyme–fluorescence assay with μ FT-IR and py-GC/MS. (a) Comparison of particle counts for PET-90 (0.1 mg) measured by μ FT-IR and the mean of enzyme–fluorescence assay ($n = 3$). Particle counts from the fluorescence assay were converted from mass using a mass-to-count ratio. Agreement with the reference value is shown. (b) Comparison of PET quantification in the PET–CaCO₃ test material by py-GC/MS ($n = 3$) and the enzyme–fluorescence assay ($n = 3$). Values are expressed relative to the nominal PET content.

that each sampled the mixture at an identical scale.

Relative to py-GC/MS, the enzyme–fluorescence assay recovered 103% of the PET determined by the reference method (81.5% and 78.9% of the nominal content, respectively; Fig. 7b). Because the nominal content of a matrix-diluted powder carries its own uncertainty, the two methods are compared to each other rather than to that value.

Collectively, these comparisons demonstrate that the enzyme–fluorescence assay agrees with μ FT-IR for microplastics and with py-GC/MS for nanoplastics.

3.8. Preliminary application of the enzyme-fluorescence assay to bottled water and PET food container samples

To assess the applicability of the proposed method to consumer-relevant samples, commercially available bottled waters and PET food containers were analyzed after exposure to elevated temperature.

Elevated temperature is an established driver of particle release from PET, attributed to thermal and hydrolytic chain scission at the polymer surface [29,30]. Four bottled water products were heated at 70 °C for 1 h prior to analysis to simulate the elevated-temperature exposure. In parallel, three PET food containers were rinsed with DDW, filled with boiling DDW, and left to stand for 30 min before sample collection. All samples were analyzed by PlastiCUTTM hydrolysis for 9 h, followed by fluorometric detection of the released TPA. Concentrations were corrected by subtracting the signal of the corresponding no enzyme sample blank and expressed as PET equivalent mass using a calibration curve constructed with 200 nm PET NP standards.

PET equivalent concentrations above the LOD were obtained for two of the four heated bottled water samples after 9 h of enzymatic incubation (0.97 and 0.37 mg/L; Table 1) and for two of the three container samples (0.50 and 0.27 mg/L; Table 2). These results indicate that the assay can produce measurable PET derived signals from real consumer samples, supporting its potential as a screening tool for PET release under thermal stress.

3.9. Scope, limitations, and considerations for real samples

A fundamental characteristic of this approach is that it is indirect: the measured signal originates from TPA released by enzymatic hydrolysis, not from the particles themselves. Conversion is incomplete at the 6 h working condition, reaching ~23% of the 24 h calibration slope for both PET-90 and PET-0.2 (Fig. 5); the agreement between these two size classes indicates that the shortfall is systematic and is compensated by identically processed standards. A 24 h incubation is available where higher conversion is required.

The response is further governed by intrinsic material properties, most notably crystallinity. The two reference materials used here were characterized accordingly (Table S3): crystallinity was 44.1% for PET-90 and 41.9% for PET-0.2. This difference likely contributed to their different calibration slopes alongside the difference in particle size. Surface oxidation is expected to act in the same direction, since oxidized surfaces are more susceptible to enzymatic hydrolysis; weathered environmental particles may therefore yield elevated signals relative to virgin reference material.

Taken together, these dependencies mean that the assay meets conventional quantitative criteria against size-matched standards in

Table 1
Detection of nanoplastic signals in commercially available bottled water samples after heating at 70 °C for 1 h.

Bottled water	PET-NPs (200 nm)	LOD
A	0.97 mg/L	0.08 mg/L
B	N.D.	
C	N.D.	
D	0.37 mg/L	

Table 2

Detection of nanoplastic signals in boiling-water extracts from commercially available PET food containers.

Plastic containers	PET-NPs (200 nm)	LOD
E	0.50 mg/L	0.06 mg/L
F	N.D.	
G	0.27 mg/L	

controlled matrices, whereas for samples of unknown provenance it reports a bounded PET-equivalent estimate: the 90 μ m and 200 nm calibrations delimit the range within which the true concentration falls, rather than resolving the underlying size distribution.

Matrix compatibility remains to be established more broadly. Selectivity was assessed here using pure polymer powders, and resin preconditioning addresses the divalent cations that dominate interference in drinking water. In samples containing dissolved organic matter, however, we observed that background fluorescence increased with organic content, which would compromise quantification in environmental samples. Application to such matrices therefore requires a separate pretreatment step such as filtration to remove dissolved organic matter, resuspension in deionized water, and continuation of the assay.

Within this scope, the approach is particularly valuable for the nanoplastic size range, which remains largely inaccessible to conventional analytical techniques. Compared with established methods such as μ FT-IR and py-GC/MS, this approach does not require complex pretreatment or bulky instrumentation, making it accessible to laboratories without specialized instrumentation. Filtration-based methods may remain preferable for complex matrices such as seawater or wastewater, whereas the present assay is well suited for relatively clean matrices such as drinking water and tap water. The microplate format additionally supports higher throughput than sequential spectroscopic or chromatographic analysis. Overall, this work extends fluorescence-based enzymatic assays from qualitative screening to calibrated quantitative analysis of micro- and nanoplastics under defined reaction conditions, and provides a simplified workflow suitable for real-world applications.

4. Conclusions

Despite numerous reported methods for microplastic detection, their practical implementation has been limited by complex workflows, long processing times, and the need for expensive instrumentation. Here, we present a simple and accessible method for micro- and nanoplastic quantification based on enzymatic degradation and fluorescence detection using a microplate reader.

The proposed approach enables quantification by converting plastics into specific degradation products, with fluorescence signals referenced to size-matched calibration curves, yielding PET-equivalent mass concentrations bound by the microplastic and nanoplastic standards. Unlike dye-based fluorescence methods, which depend on non-specific hydrophobic binding, this strategy detects polymer-specific enzymatic degradation products and is therefore selective for terephthalate-releasing plastics. Using PET as a model polymer, microplastics (90 μ m) and nanoplastics (200 nm) were quantified over 0.43–100 mg/L and 0.04–17 mg/L, respectively within 6 h. The performance of this method was comparable to μ FT-IR for microplastics and py-GC/MS for nanoplastics.

Application to environmental matrices will require a pretreatment step to remove dissolved organic matter, and broader matrix validation together with orthogonal confirmation of positive samples remains to be established.

During the preparation of this work, the authors used Claude to improve the readability and language of the manuscript and to assist in reviewing the consistency of the reported data. The authors reviewed and edited the output as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Haemin Jeong: Writing – original draft, Data curation, Conceptualization, Investigation, Methodology, Writing – review & editing, Formal analysis. **Daniel Kim:** Writing – review & editing. **Myounghyun Choi:** Supervision, Project administration, Funding acquisition. **Ah-Ram Kim:** Writing – review & editing, Supervision, Conceptualization, Validation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Haemin Jeong, Daniel Kim, and Myounghyun Choi are employed by Decomposition Co., Ltd., which developed and commercialises PlastiCUT™, the PET hydrolase preparation evaluated in this study, and holds intellectual property related to the assay described herein. Myounghyun Choi is a founder and chief executive officer of the company and holds equity. Decomposition Co., Ltd. provided the enzyme and financial support for this work. Ah-Ram Kim declares no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2026.130659>.

Data availability

Data will be made available on request.

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