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Original Research Article

Co-exposure of di(2-ethylhexyl) phthalate (DEHP) decreased the submicron plastic stress in soil–plant system

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ABSTRACT

The widespread use of agricultural plastic films has made micro- and nanoplastics (MNPs) and phthalate esters (PAEs) contaminants of emerging concern in agroecosystems. However, the interactive mechanisms underlying their combined pollution in soil–plant systems remain elusive. To fill this gap, this study investigated the interaction between submicron plastics (SMPs, 0.01% and 0.1% w/w) and di(2-ethylhexyl) phthalate (DEHP) in soil–lettuce systems. Contrary to the anticipated synergistic toxicity, DEHP significantly reduced SMP uptake into and by cracked surface cells of lettuce roots (with root concentration factors decreasing by 19%–64%), i.e., DEHP alleviated SMP-induced oxidative stress, as evidenced by reduced levels of reactive oxygen species (−26.8% and −66.7%) and antioxidant enzyme activities (−118% and −128%). Metabolomic profiling revealed that SMP exposure significantly dysregulated multiple metabolic pathways (amino acid, carbohydrate, energy, glycan, lipid, and nucleotide metabolism), while SMP + DEHP co-exposure selectively attenuated these metabolic disturbances, showing enrichment only in glycan biosynthesis/metabolism and suppressing SMP-induced perturbations in other pathways (biosynthesis of secondary metabolites, energy metabolism, and signal transduction). Microbial community analysis showed that high-level SMP exposure significantly diminished bacterial α-diversity and amplicon sequence variant (ASV) richness, whereas DEHP supplementation enhanced those of *Myxococcota* in the soil, potentially counterbalancing SMP-induced microbial dysbiosis. These findings collectively demonstrate that co-contamination by MNPs and plastic additives may produce antagonistic interactions rather than uniformly synergistic effects, and provide a more comprehensive evaluation of the risks of PAEs and MNPs to food security, human health, and ecological environment.

1. Introduction

Agricultural plastic film mulching represents a primary source of micro- and nanoplastics (MNPs) in soils [1]. Through mechanical abrasion and weathering, these films progressively degrade [2], lose flexibility, and fragment into smaller particles, including microplastics (MPs, <5 mm) and submicron plastics (SMPs, <1 μm) or nanoplastics (NPs, <100 nm) [3]. Importantly, plastic films and related products commonly incorporate phthalate esters (PAEs) as plasticizers [4]. Since these additives are not covalently bonded to polymer matrices, they can easily leach into adjacent soils [5]. Among PAEs, di(2-ethylhexyl) phthalate (DEHP) is the most prevalent compound detected in agricultural plastic films, exhibiting a 70% detection frequency with maximum concentrations reaching 28.2% (w/w) [6]. Substantial evidence indicates that exposure to MNPs induces multifaceted toxicological effects, including oxidative stress, cellular damage, compromised barrier integrity (intestinal, air-blood, and placental), immune dysregulation, endocrine disruption, and developmental toxicity [7], with toxicity

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showing an inverse relationship to particle size [8]. Furthermore, PAE plasticizers have been associated with potential carcinogenic, teratogenic, and reproductive health risks [9].

Global agroecosystems are subjected to substantial plastic pollution, with annual inputs estimated at 1.15–2.41 million tonnes of plastic waste [10], inevitably leading to significant contamination by MNPs and PAEs. The agroecosystems of China alone used approximately 2.5 million tonnes of plastic film in 2017, leaving over 0.46 million tonnes as residual waste that released 91.5 tonnes of PAEs during use and degradation, including 18.8 tonnes of dibutyl phthalate (DBP) and 42.2 tonnes of DEHP [11]. Soil investigation revealed substantial regional variability in PAE concentrations (0–1232 mg/kg), with the highest levels occurring in cotton fields of southern Xinjiang, followed by vegetable fields of Guangdong (0.20–33.6 mg/kg) and tobacco plantations of Guizhou (0.84–25.7 mg/kg) [12]. Nationwide surveys showed average macroplastic residues of 103 kg/hm^2 and MP abundances of 4537 particles/kg (dry weight) across Chinese farmland [13], with hotspot areas exhibiting MP concentrations up to 12,292 particles/kg [14]. The widespread contamination of agricultural soils by MNPs and PAEs has raised significant concerns regarding their combined ecological and health impacts.

Of particular concern is the food chain transfer dynamics, where the simultaneous presence of SMPs, NPs [15] and PAEs [16] creates synergistic exposure risks. Plants act as critical biovectors in this process [17], not only absorbing and accumulating both contaminant classes but also potentially enhancing their bioavailability. This dual contamination pathway substantially amplifies human exposure risks through dietary intake, as crops may deliver complex mixtures of MNPs and PAEs directly into the food supply. Current research indicates MNPs may enhance PAE phytotoxicity. Hydroponic studies demonstrate MPs can intensify DBP toxicity in lettuce (*Lactuca sativa*) [18], while foliar exposure experiments reveal that amino-functionalized polystyrene (PS) NPs facilitate PAE uptake in maize (*Zea mays* L.), leading to enhanced photosystem II impairment [19]. Existing evidence suggests MNPs can amplify co-pollutant toxicity through the vector effect [20–22]. Antagonistic interactions have also been documented, exemplified by NPs reducing DBP cytotoxicity in *Streptomyces coelicolor* M145 through adsorption-mediated bioavailability reduction [23]. These interactions are governed by multiple factors, including contaminant ratios, sorption properties, MNP characteristics (crystallinity, morphology), and subsequent PAE degradation dynamics [24]. Notably, current studies primarily examine MNP effects on co-pollutants, while largely overlooking pollutant effects on MNP toxicity, leaving PAE-MNP reciprocal interactions in soil–plant systems poorly characterized.

This study examines the combined effects of SMPs and PAEs on plant SMP uptake, physiological responses, and soil microbial communities, with particular focus on PAE-mediated modulation of SMP stress in soil–plant systems. We hypothesize that: (1) co-exposure induces greater phytotoxicity than individual contaminants, and (2) PAE facilitates SMP accumulation in plants while modifying soil microbiota, collectively influencing plant physiology through metabolic alterations. Given that PS constitutes a significant MP contaminant in agroecosystems due to its widespread use in packaging materials [25], and considering DEHP represents one of the most prevalent PAEs in agricultural soils [12], we chose DEHP and PS SMPs as model contaminants, and conducted controlled pot experiments, then analyzed PS-derived SMP accumulation patterns, growth parameters and photosynthetic pigment levels, antioxidant system responses, and metabolomic profiles of lettuce, and soil bacterial community composition to systematically evaluate their individual and combined phytotoxic effects through root exposure assays, thereby testing the aforementioned hypotheses regarding their environmental impact. This integrated approach elucidates mechanistic interactions between PS SMPs and DEHP, providing essential data for ecological risk assessment of contaminant mixtures in agricultural ecosystems.

2. Materials and methods

2.1. Microcosm experiment

The source of SMPs in the experiment was monodisperse PS microspheres with a diameter of 100–200 nm (Fig. 1A) at an initial concentration of 10 mg/mL (w/v). To analyze the size distribution of the purchased monodisperse PS microspheres, a laser particle size analyzer was utilized (Fig. 1B). Standard of DEHP (CAS: 117-81-7) with a purity ≥99.1% was used. All instruments, chemicals, and assay kits used in the experiments can be found in Tables S1 and S2. The soil used for this study was sandy with less than 3% clay, collected from a farmland in Huai'an, Jiangsu Province, China, which mainly comprises yellow cinnamon soil [26]. Soil samples collected at a depth of 0–20 cm were air-dried, ground to pass through a 2 mm nylon sieve, and stored at 25 °C. Table S3 presents an overview of the basic physicochemical properties of the soil.

Lettuce (*Lactuca sativa*) is a globally cultivated leafy vegetable commonly consumed raw and holds significant dietary importance across diverse geographical regions [27]. The study design included a control group (blank soil), a DEHP exposure group (10 mg/kg), two SMP exposure groups (0.01% and 0.1% w/w), and two combined exposure groups (10 mg/kg DEHP + 0.01% w/w SMP and 10 mg/kg DEHP + 0.1% w/w SMP) (Table 1). The exposure concentrations of SMPs and DEHP were selected based on: field survey data estimating macroplastic and MP loads [13,14] and DEHP residue levels [12] in Chinese agricultural topsoils [28] and assuming that for the worst-case scenario, all macroplastics and MPs present would convert at a certain time to SMPs; and (2) using established dose–response relationships from previous phytotoxicity studies on plant–MNP [29,30] and plant–DEHP [31,32] interactions, thus matching methodological consistency with existing research. In detail, methanol was used to dissolve DEHP standard solutions of varying volumes, which were then individually introduced into 600 g of soil. The mixture was thoroughly blended to ensure homogeneity. Initially, DEHP concentrations in the soil were set at 100 mg/kg. To achieve the desired final DEHP concentrations of 10 mg/kg, uncontaminated soil was subsequently added at a ratio of 9:1 to the mixture, then aged for three weeks in a dark environment at 25 °C with 20% relative humidity. To account for potential toxic effects of the methanol, control and the soils intended for treatment with SMPs were exposed to an identical methanol dosage and aging time as that administered to the soils contaminated with DEHP. SMP suspensions were homogenized using an ultrasonic vibration generator for 30 min in a water bath. The suspensions were prepared at concentrations of 0, 500, and 5000 mg/L prior to homogenization. Next, 60 mL of SMP solutions (0, 500, and 5000 mg/L) were applied to the DEHP-contaminated soil and the control soil of 300 g in planters.

All treatments were replicated four times. The shape of the planter was a trapezoidal column with a base diameter of 9 cm and a height of 9 cm. Details of plant culture are described in SI Section 1. Leaves and roots from each parallel group were collected after 25 days, then washed, dried, and stored in the −80 °C refrigerator. The artificial climate chamber was set with light–dark cycle of 16:8 h, temperature cycle of 25:20 °C, and relative humidity cycle of 60%:30%. 10 mL of Hoagland nutrient solution was added each day for each planter. The formula of the modified Hoagland nutrient solution is shown in Table S4.

2.2. Quantification and characterization of SMPs in lettuce

The quantification method for PS SMPs in lettuce adopted the styrene dimer as the characteristic substance [33]. In brief, the fresh leaf and root samples were washed, dried, crushed, ground, and extracted using a series of solvents, then analyzed using pyrolysis coupled with gas chromatography/mass spectrometry (Pyr-GC/MS). More details of quantification and characterization of SMPs and Pyr-GC/MS parameters are shown in SI Section 2 and Table S5. The washing steps likely removed all SMPs loosely attached to the roots; yet, it cannot be fully

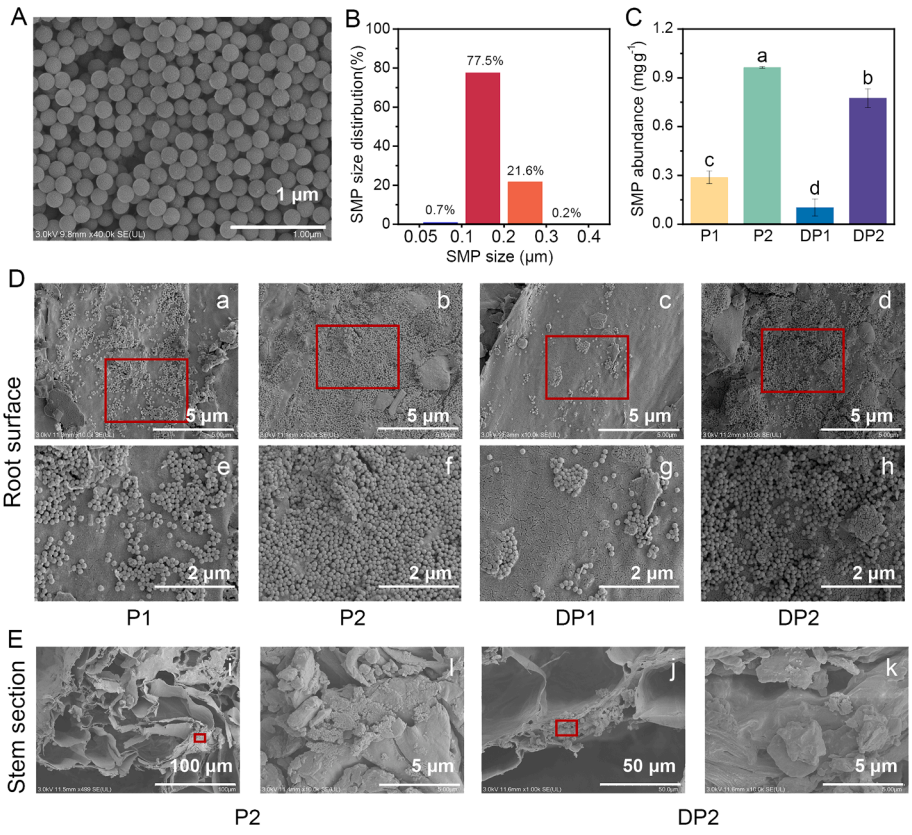

Fig. 1. Scanning electron microscopy (SEM) images (A) and size distribution (B) of the added polystyrene (PS) submicron plastics (SMPs) in ultrapure water. SMP abundance (mg/g dry weight) in roots (C), SEM images of root surfaces (D) and stem cross sections (E) of lettuce grown in soil with different treatments. SMPs are marked with red ovals in the SEM images of the root surface and stem section. e, f, g, h, l, and k show enlargements of the areas indicated by the red squares of a, b, c, d, i, and j, respectively.

Table 1
Experimental design for the microcosm.

Group	Abbreviation	Reagent
Control check	Control	
DEHP	D	10 mg/kg DEHP
Low level of SMPs	P1	0.01% (w/w) SMPs
High level of SMPs	P2	0.1% (w/w) SMPs
Low level of SMPs + DEHP	DP1	0.01% (w/w) SMPs + 10 mg/kg DEHP
High level of SMPs + DEHP	DP2	0.1% (w/w) SMPs + 10 mg/kg DEHP

Note: concentrations were measured by soil dry weight.
DEHP, Di(2-ethylhexyl) phthalate; SMPs, submicron plastics.

excluded that some of the SMPs remained stuck in or at disturbed surface cells, as also discovered by SEM (Fig. 1D).

The surface morphology of SMPs (Fig. 1A) and the accumulation of SMPs on lettuce root and in lettuce stem cross sections were examined at an accelerating potential of 20 kV in high vacuum mode with backscatter detection, and observed by scanning electron microscopy (SEM).

2.3. Analysis of biomass, photosynthetic pigments, and antioxidants

Fresh leaves and roots were washed in distilled water, dried, and then weighed. Since the fibrous roots of lettuce were still young and developing at the time of harvest, it was not possible to completely remove them from the soil. For this reason, the length and weight of the roots were not measured. The total amount of chlorophyll and carotenoids was determined by UV spectrophotometry. Details are described in SI Section 3. Fresh leaf and root samples were promptly frozen using liquid nitrogen and then finely ground into powder. For each sample, 0.20 g of the powder was carefully transferred into a centrifuge tube with 2 mL of extract. Subsequently, the tubes underwent centrifugation at a speed of 8000×*g* for 10 min, all while maintaining a cool temperature of 4 °C. As a result of this process, the supernatants, which contained the desired components, were successfully separated and subsequently stored in 10 mL tubes. To determine the levels of reactive oxygen species [ROS, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2)], as well as to assess the activities of the enzymes superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), specific assay kits (Table S2) were employed according to provided instructions.

2.4. Metabolomics analysis of lettuce leaves and roots

The collected lettuce leaves were stored and processed to extract metabolites using various techniques, including washing, crushing, vortexing, and centrifugation. The resulting supernatant was analyzed using UPLC-MS/MS in both positive and negative modes to study the effects of DEHP and SMPs on lettuce metabolism. Further measurement and data analysis information can be found in SI Section 4. The samples' raw sequence data has been uploaded to the MetaboLights (https://www.ebi.ac.uk/metabolights) (MTBLS12878).

2.5. Analysis of soil microbial community structure

The DNA from the soil was extracted using the PowerSoil DNA Isolation Kit following the provided instructions. Agarose gel electrophoresis was performed to assess the quality of the extracted DNA, while a NanoDrop 2000 UV-Vis spectrophotometer was used to determine DNA concentration and purity. The bacterial 16S rRNA gene V3–V4 hypervariable region was amplified using universal primers 338F and 806R. PCR master mix kit and the extracted DNA were used for the amplification process following the standard PCR protocol. The resulting PCR products were analyzed using agarose gel electrophoresis and sent for Illumina

sequencing to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Details of sequence data processing are provided in SI Section 5. The samples' raw data have been deposited to National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) under the BioProject number PRJNA1308824.

2.6. Statistical analysis

The root concentration factor (RCF) was the ratio of the abundance of SMPs in and on lettuce root relative to that in soil. Data was processed statistically by analysis of variance (ANOVA), and the least significant difference (LSD) post-hoc test was used to detect significant differences among mean values at $p < 0.05$ using SPSS 23.0. The figures were prepared using Origin 9.0.

3. Results and discussion

3.1. DEHP co-exposure inhibited SMP translocation and accumulation

After root exposure to SMPs, large amounts of SMPs were detected in the lettuce roots of P1, P2, DP1, and DP2 treatments (Fig. 1C), and were found attached to the surface of lettuce roots (Fig. 1D). There was a significant difference between the treatment with low SMP additions (P1: 0.29 ± 0.03 mg/g) and the one with additionally added DEHP (DP1: 0.10 ± 0.05 mg/g); likewise, at high SMP loads, the concentration in the lettuce root of P2 (0.96 ± 0.05 mg/g) was significantly higher than in DP2 (0.78 ± 0.06 mg/g) (Fig. 1C). P2 and DP2 treatments showed dense mats of SMPs attached to the xylem in the main veins of lettuce leaves, while the P1 and DP1 treatments did not (Fig. 1E). When relating these results to the overall SMP added, DEHP thus decreased the RCF of SMPs from soil to lettuce roots from originally 0.96–2.88 without DEHP (P1, P2) to 0.78–1.03 after DEHP addition (DP1, DP2).

Significant amounts of SMPs were detected in washed lettuce roots (Fig. 1C), indicating their potential adherence to or embedding within the root surface (Fig. 1D). However, the detection of SMPs in stem tissues (Fig. 1E) suggests that despite their diameter (Fig. 1B) exceeding the plant cell size exclusion limits (<20 nm) [34] by up to five-fold, a portion of SMPs may have successfully penetrated the root system and translocated to aerial tissues. In the apoplastic pathway, the Casparian strip serves as a formidable physical obstacle [35]. However, SMPs are capable of traversing the plasmodesmata (1–100 nm), despite being unable to penetrate the cell wall (<10 nm) [36]. As a result of swelling or structural changes, the pore size of plasmodesmata increases, allowing larger particles to enter the cell [37]. Once in the xylem, the particles may then be transported to above-ground tissues [38]. However, likely due to the retention in soil [39], SMPs accumulated in the above-ground parts of lettuce only at high SMP exposure (0.1% w/w) (Fig. 1E). This is markedly different from the results obtained in hydroponic systems, where at an exposure level of 40 mg/L SMP, the root uptake of plastic particles and their translocation to the aerial parts of lettuce were observed under the influence of transpiration pull [40].

One possible way for lettuce roots to absorb SMPs in the soil or from hydroponic solution is through the root tip and the crack entry method, where the lateral roots appear [15]. Crack entry of SMPs can be facilitated by root openings formed during plant aging, as well as after damage by soil-borne pathogens and physical destruction [41]. These root openings can serve as entrances for SMPs in plants. The root apical meristem is highly porous due to active cell division in the root tip, and SMPs can be taken up through the root meristem and then penetrate the cell wall into the xylem, where they can move to the upper part of the plant under the root pressure [15] and accumulate in the main veins of lettuce leaves (Fig. 1E). Upon irrigation, soil pores fill with water and induce buoyancy of SMPs, thus facilitating root uptake. When the density of PS SMPs (1.064 g/cm^3) is less than Hoagland nutrient solution (2.19 g/cm^3), it is theoretically also possible that the floating SMPs migrate toward the soil surface and penetrate directly into the main leaf veins; yet, large numbers of SMPs were found in the cross-section of the main veins of lettuce leaves at locations close to the soil surface (Fig. 1D). Despite existing research efforts, it still remains uncertain whether SMPs have the capability to penetrate the stems and principal leaf veins of lettuce in a direct manner.

Irrespective of the migration mechanism, and in contrast to our main hypothesis, DEHP reduced SMP uptake and subsequent plant performance. The log K_{oc} value for DEHP is in the range of 4.9–5.7 [42], i.e., the majority of DEHP added should stick to the soil. In principle, elevated additions of strongly adsorbing DEHP, could induce an aggregation of soil particles as observed for other soil organic matter constituents [43,44], yet, a high K_{oc} value does not imply that sorption is irreversible. In fact, there might be interactions with dissolved organic matter. Also, PS SMPs might be a stronger bonding partner for DEHP than certain soil constituents, thus allowing a transfer of DEHP from soil surfaces to PS SMPs even at elevated log K_{oc} values. Such interactions may then affect the uptake of SMPs by at least two mechanisms. On the one hand, surface-altered PS SMPs could show increased bonding to soil surfaces and thus enhanced soil retention. On the other hand, the SMPs could agglomerate, increase in size with different materials attached, and change their zeta potential, which are all critical parameters for the uptake of at least nanoparticles by plants, and thus also possibly of larger SMP aggregates [36].

3.2. DEHP mitigates SMP-induced biomass reduction and attenuates oxidative stress in lettuce

Plant growth is the most intuitive indicator of toxicity, and previous studies have confirmed the negative effect of DEHP [45,46] and SMPs [40,47] on plant growth. In this study, as SMP concentrations increased, a detrimental effect on lettuce growth evolved, with larger effects on plant height and leaf weight (Fig. 2A–C). Both the exposure to SMP and DEHP also reduced the contents of chlorophyll and carotenoids in lettuce leaves (Fig. 2D and E). Chlorophyll and carotenoids are crucial for photosynthesis and perform essential functions in this process [48]. A decrease in plant leaf pigment content (Fig. 2D and E) affected the photosynthetic rate, thus decreasing the biomass of lettuce (Fig. 2A–C). Interestingly, the co-exposure of SMP and DEHP exhibited a significantly weaker inhibitory effect on lettuce growth compared to SMP treatment alone (Fig. 2A–E), suggesting that the phytotoxic impacts of SMP and DEHP on lettuce photosynthesis were not synergistic. This finding contrasts with the results in a former report that di-n-octyl phthalate (DOP) (10 mg/kg) exacerbated the adverse effects of PS MPs (<13 μm, 0.5%) on photosynthetic efficiency [49]. This discrepancy may arise from the distinct physicochemical properties of phthalate compounds, which modulate their interactions with MNPs, ultimately leading to differences in the bioavailability effects of DOP and DEHP.

ROS serve as pivotal signaling molecules and master regulators in plants, orchestrating diverse physiological processes spanning cellular metabolism, growth regulation, and developmental programming, while simultaneously mediating adaptive responses to both abiotic and biotic stressors [50]. Under stress conditions, the disruption of cellular redox homeostasis through ROS accumulation triggers profound physiological shifts, wherein the altered redox status not only manifests as visible stress phenotypes but also directly correlates with the phytotoxic consequences of oxygen radical overproduction [51]. Our results demonstrate a dose-dependent accumulation of ROS (O_2^- and H_2O_2) in lettuce tissues under SMP exposure, with two key observations: (1) O_2^- production peaked in SMP-only treatments without DEHP co-exposure (Fig. 2F), and (2) root H_2O_2 levels showed particular sensitivity to SMPs, increasing up to 237% compared to SMP + DEHP combined treatments (Fig. 2G). The elevated ROS content across all exposure groups (SMP, DEHP, and their combinations) triggered compensatory activation of antioxidant enzymes (Fig. 2H–J), reflecting that ROS serve both as oxidative damage markers and signaling molecules initiating defense mechanisms [52]. These findings collectively indicate that SMP

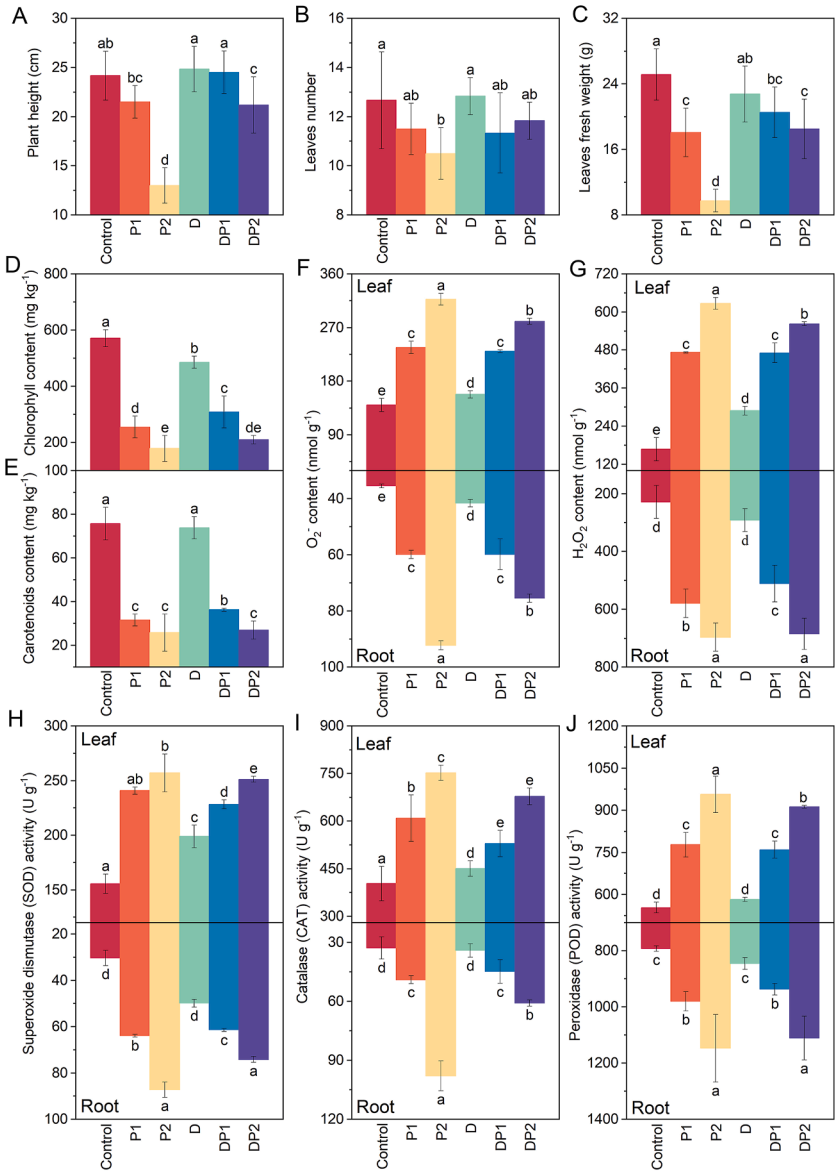

Fig. 2. Plant height (A), number (B), fresh weight (C), total chlorophyll (D), and carotenoids (E) contents of lettuce leaves. The O_2^- (F) and H_2O_2 (G) contents, SOD (H), CAT (I), and POD (J) activities of lettuce leaves and roots under different treatments. Different lowercase letters indicate the significant difference among soils at $p < 0.05$.

and DEHP exposure induces physiological stress in lettuce, disrupting redox homeostasis and activating the antioxidant system as a protective measure against xenobiotic-induced oxidative damage.

SOD serves as the frontline enzymatic defense, catalyzing the disproportionation of O_2^- into H_2O_2 and O_2, playing a pivotal role in detoxifying ROS [53]. Subsequently, H_2O_2 generated as a product of SOD activity is effectively decomposed by both POD and CAT. Within plant systems, POD serves as a crucial H_2O_2-scavenging enzyme, exhibiting plant-specific functionality that is vital for safeguarding chloroplasts and other cellular components against oxidative damage induced by hydrogen peroxide and its derived hydroxyl radicals [54]. Similarly, CAT acts as an essential metabolic sink for H_2O_2, playing an indispensable role in plant stress defense mechanisms [55]. Collectively, SOD [56], CAT [55], and POD [54] form an integrated antioxidant network that mitigates oxidative stress by efficiently removing excess O_2^- and H_2O_2, thereby protecting plants against various biotic and abiotic stressors. Overall, the ROS levels and antioxidant enzyme activities in lettuce leaves and roots across different treatments exhibited the following trend: Control < D < DP1 < P1 < DP2 < P2 (Fig. 2F–J), indicating that the combined toxicity of DEHP and SMPs was lower than that of SMPs alone. Comprehensive comparative analysis revealed that DP1 treatment significantly attenuated oxidative stress in lettuce, reducing total ROS (O_2^- and H_2O_2) accumulation in roots and leaves by 26.8% relative to P1, while decreasing antioxidant enzyme (SOD, POD, and CAT) activity by 118%. Similarly, DP2 treatment demonstrated more pronounced effects, showing 66.7% reduction in total ROS content and 128% decline in antioxidant enzyme activity compared to P2. These findings suggest that DEHP addition mitigated the adverse effects of SMPs on lettuce, particularly under high SMP exposure (Fig. 2). These findings differ from previous investigations examining: (1) lettuce responses to MP-DBP co-exposure [18], where MPs (about 23 μm, 250 mg/L, 500 mg/L, and 1000 mg/L) were shown to amplify DBP (5 mg/L) phytotoxicity and impair photosynthetic function—consistent with our observations, though Gao et al. [18] further demonstrated supra-additive toxicity in combined treatments; and (2) algal systems exposed to MP fibers (<20 μm) with DBP (1 mg/L) [57], which exhibited concentration-dependent interactions ranging from synergistic toxicity at high MP concentrations (100 mg/L) to antagonistic effects at lower

doses (1 mg/L). The observed discrepancies in experimental outcomes may be attributed to multiple factors, including variations in contaminant concentrations and MNP particle sizes. However, it is crucial to emphasize the distinct phytotoxic responses between hydroponic and soil–plant systems, with the latter representing a more complex and ecologically relevant matrix. Our findings specifically address this knowledge gap by elucidating how the intricate interactions within the soil environment modulate the combined phytotoxic effects of these contaminants.

3.3. Metabolic reprogramming in lettuce under DEHP and SMP co-exposure

Through orthogonal partial least-squares discriminant analysis (OPLS-DA) screening (VIP > 1, $p < 0.05$), treatment-specific differential metabolites were identified in both lettuce roots and leaves (Fig. 3). Notably, root tissues exhibited a significantly higher abundance of differential metabolites than leaves (Fig. 3). Metabolic profiling revealed a concentration-dependent response, with elevated SMP exposure leading to a marked increase in differential metabolite accumulation (Fig. 3). Interestingly, DEHP co-exposure attenuated these metabolic perturbations, yielding fewer differential metabolites with diminished expression changes compared to SMP-only treatments (Fig. 3). These metabolic alterations were functionally correlated with physiological modifications in lettuce. Multivariate analysis, coupling both principal component analysis (PCA) and OPLS-DA, elucidated treatment-specific metabolic patterns. PCA score plots demonstrated that SMP stress induced more pronounced metabolic shifts in roots (Fig. S1A) than in leaves (Fig. S1B), where treatment clusters showed considerable overlap, suggesting limited treatment effects on foliar metabolites. In contrast, supervised OPLS-DA achieved superior group discrimination (Figs. S2, S3), with tight clustering of replicates within treatments and clear separation between treatments. This distinct metabolic fingerprinting indicates fundamentally different physiological impacts between SMP-only exposure and combined SMP-DEHP treatments on lettuce growth.

In this study, the differential metabolites screened mainly mapped to the following Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Under SMP treatment (P1, P2), the enriched metabolism pathways included amino acid metabolism, biosynthesis of other secondary metabolites, carbohydrate metabolism, energy metabolism, global and overview maps, glycan biosynthesis and metabolism, lipid metabolism, membrane transport, metabolism of other amino acids, nucleotide metabolism, signal transduction, and translation (Fig. 4A). It illustrated that SMP exposure significantly altered metabolite expression of amino acid, carbohydrate, energy, glycan, lipid, and nucleotide, causing the original dysfunction of those regulatory pathways. SMP + DEHP co-exposure resulted in distinct metabolic responses, failing to induce enrichment in specific pathways: biosynthesis of other secondary metabolites, energy metabolism, global and overview maps, metabolism of other amino acids, signal transduction, and translation, while inducing the enrichment of glycan biosynthesis and metabolism (Fig. 4A). The results showed that DEHP had an obvious inhibitory effect on SMP stress-induced metabolic disorders within lettuce roots and leaves.

KEGG pathway enrichment analysis identified distinct tissue-specific regulation patterns in glycerophospholipid metabolism, with significant downregulation observed in root tissues under P1 and DP2 treatments, while leaf tissues exhibited pronounced upregulation under P2 and DP1 exposure conditions (Fig. 4B), demonstrating opposing metabolic responses between belowground and aerial plant organs to differential nutrient treatments. Glycerophospholipids are fundamental components of cell membranes, crucial for cell structure, signaling, and function [58]. Modulation of membrane lipid composition under varying environmental conditions is an important part of plant stress adaptation [59]. Glycerophospholipid metabolism is a metabolic pathway that is commonly altered in response to stress in plants, which was observed in the response of *Lactuca sativa* leaves to SMP stress [40], *brassica juncea* roots to cadmium stress [60], *Cucumis sativus* seedling leaves and cotton (*Gossypium barbadense* and *Gossypium hirsutum*) [61] to drought stress, and *Brassica campestris* to cold stress [62]. Another metabolic pathway that plays an important role in regulating plant growth, development and tolerance to environmental stresses [63] is the ABC transporters, which was also simultaneously enriched both in lettuce roots and leaves under P1 and P2 treatments, while only in lettuce leaves under DP2 treatment (Fig. 4B). The range of processes in which members of the various subclasses of plant ABC transporters have been implicated encompasses polar auxin transport, lipid catabolism, xenobiotic detoxification, disease resistance, and stomatal function [64]. Notably, the ABC transporter pathway exhibited upregulation in lettuce roots under the P2 treatment and in leaves under both P1 and P2 treatments. DEHP mitigated the

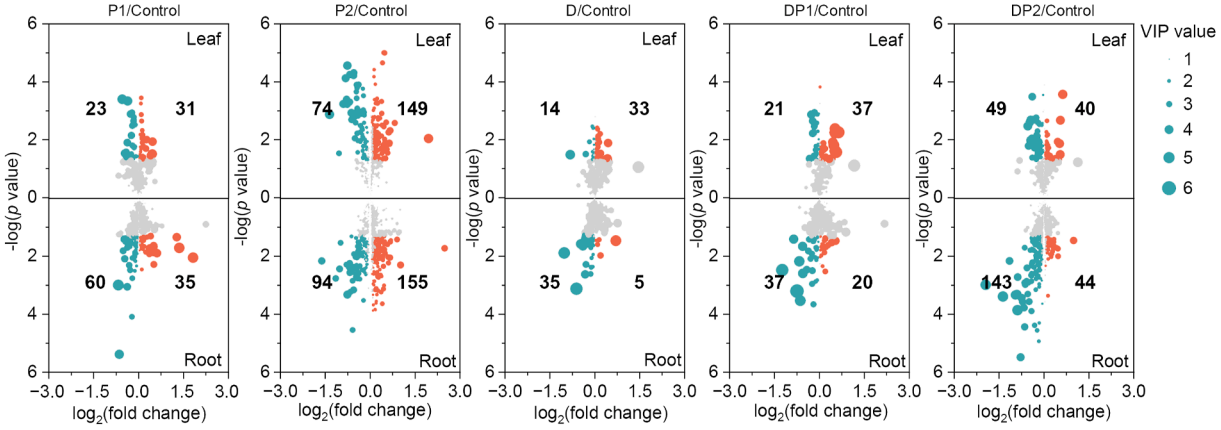

Fig. 3. Volcano map of differential metabolites in the lettuce roots and leaves under P1, P2, D, DP1, and DP2 treatments compared to control. The horizontal axis represents the fold change (FC) in metabolite expression between the two groups, while the vertical axis represents the statistical significance of the differential expression. Higher values indicate more significant differential expression. Both the horizontal and vertical coordinates are logarithmically transformed. Each point in the plot represents a specific metabolite, with the size of the point indicating the Variable Importance in Projection (VIP) value. VIP value indicates the impact and explanatory power of metabolite accumulation differences on sample group classification. Orange points denote significantly upregulated metabolites, green points denote significantly downregulated metabolites, and gray points represent metabolites with non-significant differences.

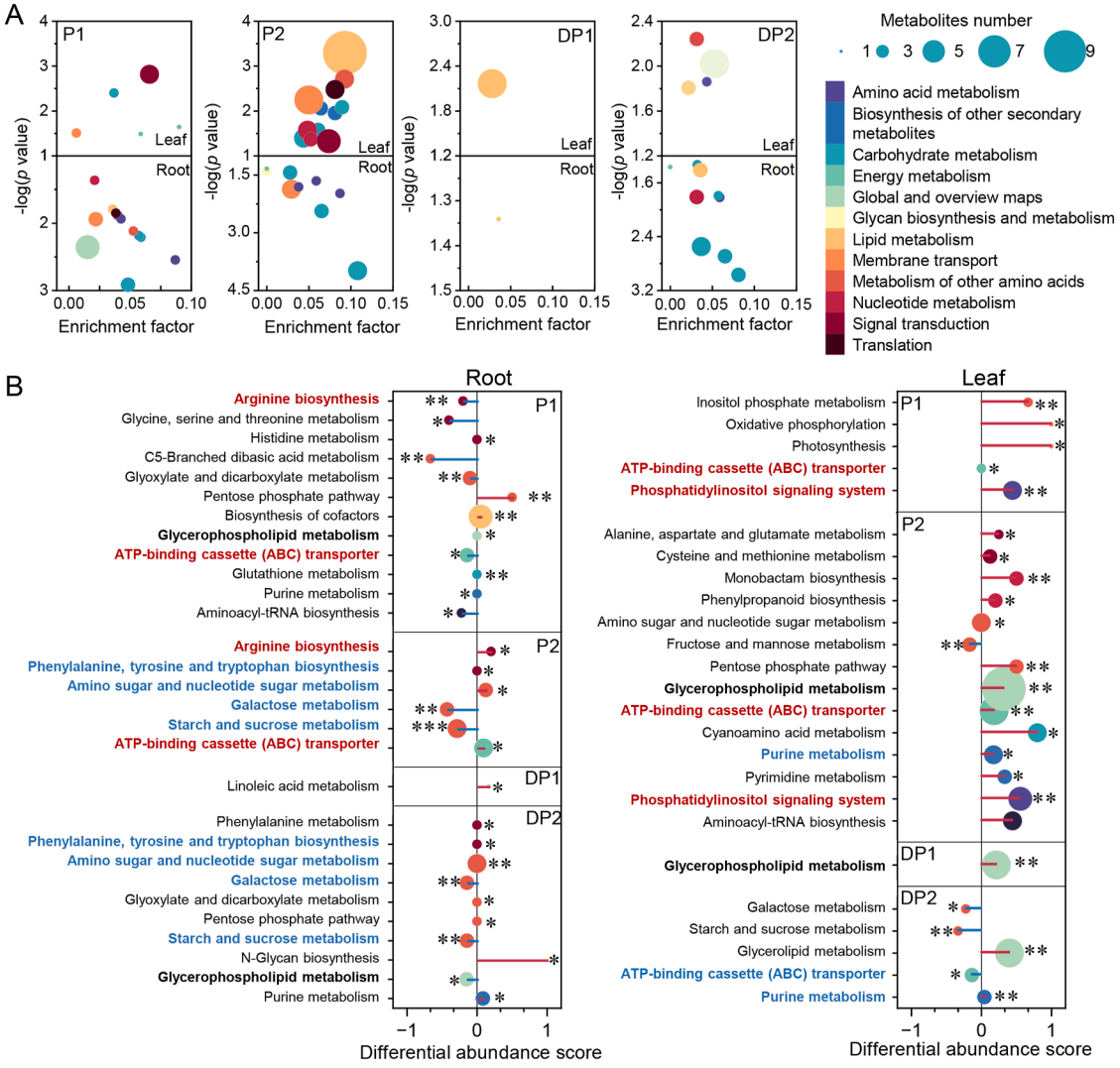

Fig. 4. Enrichment analysis of the differential metabolites in the lettuce roots and leaves under P1, P2, DP1, and DP2 treatments. The bubble size and color represent the number of metabolites and the second category of metabolic pathway, respectively. (A) The abscissa coordinate represents the enrichment factor (EF) of metabolic pathway, EF represents the enrichment rate of metabolic pathways. (B) Differential abundance score represents the overall changes in all metabolites within a metabolic pathway. A positive value indicates an upward trend in the expression of all annotated differential metabolites in the pathway, while a negative value indicates a downward trend. The length of the line segment corresponds to the absolute value of the differential abundance score. Text in red indicates metabolic pathways that appeared in both the P1 and P2 treatments, while text in blue represents pathways present in both P2 and DP2. *, **, and *** indicate the significant meaning of the enrichment results at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

disruption of ABC transporter function induced by SMPs in lettuce roots and leaves at low exposure levels, as well as in roots under high SMP exposure (Fig. 4B). ABC transporters play a pivotal role in the spatiotemporal regulation of secondary metabolites that confer critical protective functions in plant physiological processes [65], potentially explaining their observed upregulation under SMP exposure.

Notably, we observed distinct differences in metabolic pathway enrichment patterns between P1 and DP1 treatments (Fig. 4B), suggesting that DEHP suppresses low-level SMP-induced metabolic modification. Under high SMP exposure conditions (P2, DP2), four metabolic pathways showed consistent enrichment in lettuce roots: phenylalanine, tyrosine, and tryptophan biosynthesis, amino sugar and nucleotide sugar metabolism, galactose metabolism, and starch and sucrose metabolism. In leaves, only purine metabolism was concurrently enriched in P2 and DP2 (Fig. 4B). Comparative analysis revealed that P2 treatment produced more differential metabolites and more significant metabolic alterations in leaves compared to DP2 treatment, while the opposite pattern was observed in roots (Fig. 4B). These results demonstrate that DEHP plays a dual regulatory role: mitigating high-level SMP-induced metabolic dysregulation in leaves while stimulating another metabolic pathway enrichment in root tissues.

3.4. *Rhizosphere bacterial communities under DEHP and SMP co-exposure*

MNPs can significantly disrupt mutualistic interactions between soil bacteria and plant root systems [66]. Notably, bacterial communities exhibit greater sensitivity to DEHP contamination compared to fungal or archaeal populations [67]. This differential responsiveness, coupled with bacteria's pivotal role in nutrient cycling and soil ecosystem functioning, makes them optimal bioindicators for investigating the individual and synergistic impacts of emerging contaminants on soil ecological health. Fig. 5A displays the alpha diversity of the bacterial community, which was assessed using the Shannon, Simpson, and Chao1 diversity index under SMP, DEHP, and their combined exposure. Our analysis revealed that the addition of SMP had a significant impact ($p < 0.05$) on these indices, particularly at high SMP levels (P2, $p < 0.01$). The sequences were clustered into 4343 bacterial amplicon

sequence variants (ASVs). With the increase in SMP levels, the number of ASVs in the soil substantially decreased (Fig. 5B). Among the analyzed bacterial groups, only the facultative Armatimonadota bacteria showed significant responses to both SMP and DEHP exposure. Several phyla, including Proteobacteria, Firmicutes, Bacteroidota, Cyanobacteria, and Sumerlaeot, were significantly influenced by the SMP treatments. Additionally, Gemmatimonadota, Myxococcota, Armatimonadota, Dependentiae, Nitrospirota, and GAL15 exhibited significant alterations specifically under DEHP exposure (Fig. 5C).

The presence of SMPs can modify the size distribution of water-stable soil aggregates [68], consequently influencing soil bacterial communities [69]. Our results showed that SMPs significantly enhanced the relative abundance of Proteobacteria ($p < 0.001$, Fig. 5C). This finding aligns with previous observations in paddy soils, sediments, and seawater, suggesting that SMPs may consistently promote favorable conditions for Proteobacteria across various environments. Conversely, SMP exposure significantly reduced the relative abundances of Firmicutes, Bacteroidota, Cyanobacteria, and Sumerlaeot in a dose-dependent manner ($p < 0.01$, Fig. 5C). Notably, both Firmicutes [70] and Sumerlaeota [71] are recognized for their stress-resistant properties. The phylum Bacteroidota is a major bacterial group in the plant rhizosphere [72], and Cyanobacteria is an important phylum of bacteria that increase plant tolerance because of their direct action in increasing the availability of water in the soil, improving its physicochemical conditions faced with different abiotic stresses [73]. These results indicate that SMP exposure negatively impacts both soil bacterial stress adaptation mechanisms. Given the intimate relationship between soil microbial communities and plant growth in soil–plant systems [74], such SMP-induced microbial shifts may have important implications for plant development.

DEHP exposure significantly reduced the relative abundance of Gemmatimonadota, Nitrospirota, and GAL15 ($p < 0.05$, Fig. 5C). Gemmatimonadota, commonly found in plant-associated environments and the rhizosphere, plays important ecological roles in organic matter degradation through its ability to utilize diverse organic compounds as carbon and energy sources, thereby contributing to ecosystem stability [75]. Nitrospirota is particularly notable for its essential function in soil nitrification processes [76]. Conversely, DEHP increased the relative abundance of Myxococcota, Armatimonadota, and Dependentiae ($p < 0.05$, Fig. 5C). Notably, Myxococcota, as apex predators in soil microbial food webs, possesses unique predatory capabilities and strong soil adaptability that enable it to regulate microbial ecological networks and suppress plant disease [77]. However, the enrichment of Myxococcota suggests a potential role in mitigating SMP stress, though further genomic analysis is needed to confirm their functional contributions. These results suggest that while DEHP exposure negatively affects the rhizosphere environment and nutrient cycling processes, it may simultaneously alleviate SMP phytotoxicity through specific alterations in bacterial community composition, particularly by enhancing beneficial bacterial phyla. Supporting these findings, previous research has demonstrated that DEHP accumulation in soil can reduce metabolic activity and virulence factors in the plant pathogen *Acidovorax citrulli* [78].

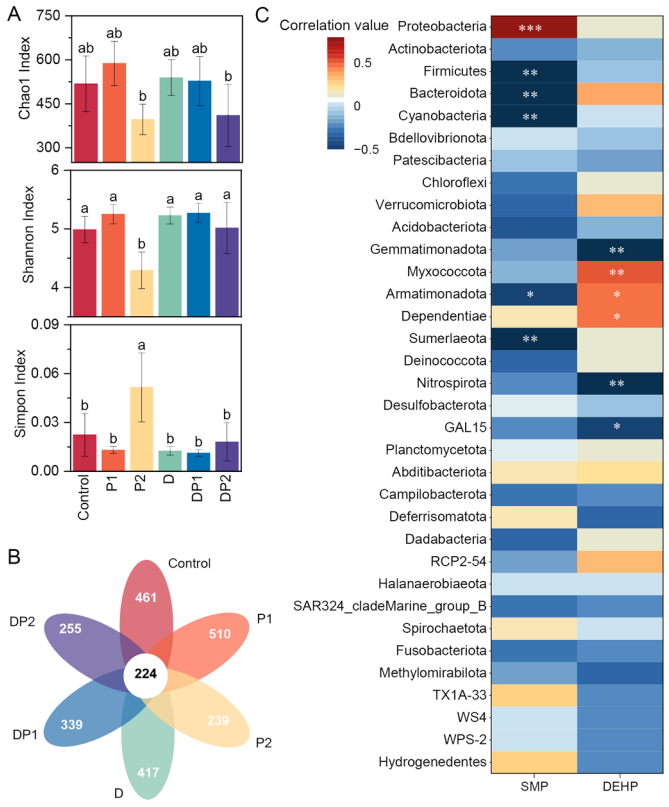

Fig. 5. (A) Alpha diversity index, and (B) Venn diagram of soil bacterial communities on ASV level under different treatments. (C) Heatmap of the relationship of SMP abundance/DEHP concentration and soil bacterial phylum. Different lowercase letters indicate the significant difference among soils at $p < 0.05$. *, **, and *** indicate significant difference between different comparison groups at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

4. Conclusion

MNPs and PAEs, as unavoidable emerging contaminants in agricultural soils, exhibit complex interactions that defy traditional assumptions of additive or synergistic toxicity. Our study demonstrated that DEHP unexpectedly mitigated the phytotoxicity of SMPs by (1) inhibiting SMP uptake by lettuce roots, (2) alleviating metabolic dysregulation in lettuce, and (3) stabilizing soil microbial community structure. These findings challenge conventional contaminant risk assessment paradigms and suggest that the phytotoxicity of SMPs and DEHP is not synergistic but potentially antagonistic. This antagonistic effect underscores the necessity of incorporating contaminant interaction dynamics into ecological risk assessment frameworks, particularly for plastics and their derived pollutants. Future research should focus on evaluating contamination risks under conditions where multiple emerging pollutants coexist and interact, thereby enabling a comprehensive assessment of their ecological and human health risks.

CRediT authorship contribution statement

Yu Wang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fang Wang:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Leilei Xiang:** Writing – review & editing, Methodology. **Maoyuan Liao:** Methodology. **Mingyi Wang:** Methodology. **Yongrong Bian:** Methodology. **Xin Jiang:** Project administration. **Ravi Naidu:** Writing – review & editing. **Matthias C. Rillig:** Writing – review & editing, Funding acquisition. **Wulf Amelung:** Writing – review & editing, Funding acquisition.

Declaration of competing interests

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eehl.2025.100184.

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