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Combined toxicity of organophosphate flame retardants and polyethylene microplastics on *Eisenia fetida*: Biochemical and molecular insights

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ABSTRACT

Microplastics and organophosphate flame retardants (OPFRs), a subset of organophosphate esters (OPEs), are frequently co-detected in terrestrial ecosystems, raising concerns about their combined ecological risks. In this study, the epigeic earthworm *Eisenia fetida* was exposed to polyethylene microplastics (PEs) (0.5 g kg^{-1}), three chlorinated OPFRs (TCEP (tris(2-chloroethyl) phosphate), TCPP (tris(1-chloro-2-propyl) phosphate), and TDCPP (tris(1,3-dichloro-2-propyl) phosphate), each at 0.002 g kg^{-1}), and their binary mixtures for 7 and 28 days. A suite of physiological, biochemical, and molecular biomarkers, including enzyme activities (SOD, CAT, GST, AChE, ATPases), oxidative damage markers (MDA, 8-OHdG), and gene expressions (*sod, hsp70, tctp*), were evaluated to assess sublethal toxic responses. A time-dependent shift in the correlation between sod transcription and SOD enzyme activity was observed, indicating possible early-stage post-transcriptional regulation and later-stage transcriptional control. The results revealed compound-specific and time-dependent toxicities. TDCPP exhibited the highest individual toxicity, suppressing antioxidant enzymes and disrupting ion transport. Co-exposure with PEs attenuated TDCPP-induced effects, likely via reduced bioavailability. In contrast, PEs enhanced the toxicity of TCPP, especially in oxidative and genotoxic responses. TCEP induced moderate but delayed biochemical changes. This study underscores the dual role of PEs as both carriers and modulators of co-occurring pollutants, and highlights the need for mixture-based risk assessments in soil ecosystems.

1. Introduction

Owing to the mass production of plastics, microplastics have become an emerging threat to marine, freshwater, atmospheric, and terrestrial ecosystems (Rochman and Hoellein, 2020). The exceptional durability and longevity that make plastics so useful in daily life also contribute to their persistent accumulation across nearly all environmental compartments (Rochman, 2018). As soil might be potential global environment reservoirs of microplastics (Hurley and Nizzetto, 2018; Alazaiza et al., 2022), growing research efforts have been devoted to the effects of microplastics on terrestrial environments. In terrestrial systems, microplastics can enter soils through various pathways, including the recycling of sewage sludge, irrigation with contaminated water, long-term application of organic fertilizers, degradation of plastic mulch films, atmospheric deposition, and surface runoff (Weithmann et al., 2018; Evangeliou et al., 2020; Qi et al., 2020; Zhang et al., 2020).

Given the widespread presence of microplastics in soils, their ecological impact on soil biota has become a growing concern (de Souza Machado et al., 2018). Microplastics can be ingested by soil organisms, leading to adverse effects on their health and survival (Xu et al., 2021).

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For instance, Ju et al. (2019) found that microplastics altered gut microbiota composition in collembolans, reduced bacterial diversity, and suppressed reproductive output. The toxicity of microplastics is size-dependent. Our previous studies with earthworms (*Eisenia fetida*) have also demonstrated that soil pollutants such as polycyclic aromatic hydrocarbons (PAHs) can significantly affect growth, bioavailability, and antioxidant defense systems, and that these effects are strongly modulated by pollutant aging in soils (Ma et al., 2012a,b). These findings highlight the importance of using earthworms as sensitive bioindicators to evaluate the fate and biological impacts of soil contaminants.

Given that microplastics rarely occur alone in the environment, their interactions with other co-occurring contaminants—particularly organophosphate esters (OPEs)—are of growing concern (Onoja et al., 2022; Castro-Jiménez et al., 2024; Sun et al., 2024). Among these additives, organophosphate flame retardants (OPFRs) are particularly noteworthy due to their environmental persistence and toxicity (Shasha et al., 2017). These substances often coexist with microplastics in contaminated environments and may interact synergistically, leading to enhanced or altered toxicological effects on terrestrial organisms (Ali et al., 2024; Yin et al., 2024). For instance, Tanaka et al. (2013) dissected short-tailed shearwaters (*Puffinus tenuirostris*) from the northern North Pacific and detected high levels of polybrominated diphenyl ethers (PBDEs) in both the stomach contents and abdominal fat, largely originating from ingested plastic debris. Similarly, Browne et al. (2013) reported that microplastics and associated additive chemicals transferred to the gut tissues of the lugworm (*Arenicola marina*) impaired physiological functions linked to health and biodiversity. Syberg et al. (2015) further suggested that chemical pollutants may enter cells via microplastic-mediated carrier effects, increasing intracellular exposure.

Therefore, it has been demonstrated that the combined exposure of microplastics and chemical pollutants can significantly enhance bioaccumulation and toxic effects in exposed organisms (Koelmans, 2015; Zhang et al., 2018; Sun et al., 2022). Such interactions may lead to synergistic or additive toxicity through multiple biological pathways (Kim et al., 2024; Xue, 2024). However, most of these findings are derived from aquatic environments, especially marine systems, whereas evidence from terrestrial ecosystems—particularly involving soil invertebrates—remains limited. Chlorinated alkyl OPFRs, including TCEP (tris(2-chloroethyl) phosphate), TCPP (tris(1-chloro-2-propyl) phosphate), and TDCPP (tris(1,3-dichloro-2-propyl) phosphate), are among the most frequently detected and abundant OPFRs in environmental matrices such as air, water, sediments, and biota, and even in human samples such as hair, breast milk, and semen (Hoffman et al., 2014; Li et al., 2014; Abdallah et al., 2015; Liu et al., 2015). These compounds exhibit multiple toxicities—including neurotoxicity, reproductive toxicity, genotoxicity, carcinogenicity, and endocrine disruption—and show strong resistance to degradation in the environment (Rodil et al., 2012). Our recent research on earthworms has further revealed that exposure to Cl-OPEs (TCEP, TCPP, and TDCPP) can induce oxidative stress, immune defense responses, and metabolic disturbances, with TCPP and TDCPP showing higher toxicity than TCEP (Gao et al., 2022). Due to their persistence, bioaccumulation potential, and toxicity, the EU and the US have listed them as priority pollutants, and they have been categorized as substances of very high concern under EU regulations (Wang and Song, 2024). Therefore, these three OPFRs were selected as representative chlorinated OPFRs in this study, and 7-day and 28-day exposure experiments on *Eisenia fetida* were conducted to evaluate their combined toxicity with microplastics. A range of toxicological endpoints—including enzymatic activities, oxidative stress biomarkers, and mRNA expression levels—were assessed to capture biological responses. The findings aim to advance our understanding of microplastic–OPFRs co-exposure risks in terrestrial food webs, an area that remains poorly understood.

2. Materials and methods

2.1. Experimental materials and exposure Design

2.1.1. Chemicals

TCEP (98 % purity), TCPP (mixture of isomers, 98 % purity) and TDCPP (96 % purity) (details in Table S1) were purchased from Macklin Biochemical Co., Ltd. (China). Dichloromethane used as the cosolvent of OPFRs, was obtained from Yonghua Chemical Co., Ltd. (China), and dichloromethane-treated soils were set as solvent controls to exclude potential effects of the solvent itself.

Polyethylene (PE) powder was purchased from Huachuang Plastic Chemical Co., Ltd. (China), filtered through 50 μm nylon mesh filters, washed twice with 70 % ethanol and deionized water, and dried at 40 °C for 12 h (Rodriguez-Seijo et al., 2017). The identity of PE was confirmed by FTIR spectroscopy (Nicolet IS5, Thermo Fisher, USA), and its surface morphology was examined by SEM (GeminiSEM 300, Carl Zeiss, Germany) (Fig. S1–S2).

2.1.2. Test substrate and organisms

Artificial soil was prepared following OECD Guideline 222 (OECD, 2004), consisting of 10 % dried sphagnum peat, 20 % kaolin clay (chemically pure; Nanjing Chemical Reagent Co., Ltd.), and 70 % quartz sand (analytically pure; Kemiou Chemical Reagent Co., Ltd.). The components were thoroughly mixed for 15 min using an electric mixer.

Adult *Eisenia fetida* (0.3–0.4 g, with developed clitella) were purchased from a local supplier (Nanjing, China) and acclimated in artificial soil (35 % moisture) at 22 °C (12 h light/12 h dark) for 7 days. Before the experiment, worms were gently rinsed with distilled water and placed on moist filter paper for 12 h to depurate gut contents.

2.1.3. Experiment design and exposure test

The selection of microplastic concentration in this study was informed by the current understanding of microplastic pollution in terrestrial environments. However, standardized methods for microplastic sampling and extraction in soils are still lacking. Similarly, there is no consensus on how to quantify and report microplastic concentrations—whether by number or mass (Dioses-Salinas et al., 2020; Scopetani et al., 2020). To date, only a few studies reported the pollution status of microplastics in terrestrial environments in mass concentration. 0.3–67.5 g kg^{-1} microplastics were detected earlier in an industrial area in Australia (Fuller and Gautam, 2016). Nearly 0.5 g kg^{-1} microplastics were detected in agricultural fields and fruit fields in Northwest China (Zhang et al., 2018).

For OPFRs, the exposure concentration of 0.002 g kg^{-1} for each compound (TCEP, TCPP, and TDCPP) was chosen based on two considerations. First, although field data for OPFRs levels in soils remain limited, environmental monitoring studies have reported concentrations ranging from several μg kg^{-1} to the g kg^{-1} range, with higher levels observed in soil, wastewater treatment plants, and plastic waste treatment area (Wan et al., 2016; Wang et al., 2019; Liao et al., 2020). Second, previous laboratory studies involving soil invertebrates, including earthworms, have used similar g kg^{-1} concentrations to induce measurable sublethal biochemical and molecular responses without causing acute mortality (Jiang et al., 2020; Wu et al., 2022). This ensured that the selected concentration is both environmentally relevant for pollution hotspots and appropriate for mechanistic toxicity assessment under controlled conditions.

Eight experimental groups were established: (1) control group, CK (without PEs and OPFRs); (2) PE group, PE (0.5 g kg^{-1} PE alone); (3) TCEP group, E2 (0.002 g kg^{-1} TCEP alone); (4) TCPP group, P2 (0.002 g kg^{-1} TCPP alone); (5) TDCPP group, D2 (0.002 g kg^{-1} TDCPP alone); (6) TCEP + PE group, $E2^{PE}$ (0.002 g kg^{-1} TCEP with 0.5 g kg^{-1} PE); (7) TCPP + PE group, $P2^{PE}$ (0.002 g kg^{-1} TCPP with 0.5 g kg^{-1} PE); (8) TDCPP + PE group, $D2^{PE}$ (0.002 g kg^{-1} TDCPP with 0.5 g kg^{-1} PE). Each treatment was conducted in triplicate (n = 3).

For each group, 500 g of dry artificial soil was mixed with 10 mL of dichloromethane solution containing OPFRs (0 or 100 μg mL^{-1}) and 0.25 g of PE powder as needed. After thorough mixing, the soil was air-dried under a fume hood to allow solvent evaporation. Moisture was adjusted to 35 % (w/w) using distilled water. Ten pre-selected worms were introduced into each container. Exposure durations were 7 and 28 days, conducted independently to avoid cross-interference. After exposure, worms were removed, weighed, gut-cleared, and snap-frozen in liquid nitrogen.

2.2. Physiological, biochemical, and molecular-level toxicity response analysis

2.2.1. Measurement of enzyme activities and malondialdehyde (MDA) content

Three frozen earthworms per container were homogenized on ice in 9 vol (w/v) of ice-cold normal saline to prepare 10 % tissue homogenates. The homogenates were centrifuged at 3000 rpm for 10 min at 4 °C. Supernatants were collected to determine soluble protein content, MDA levels, and enzyme activities (superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), acetylcholinesterase (AChE), sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase), calcium adenosine triphosphatase (Ca^{2+}-ATPase)) using spectrophotometry (UV 6100, Shanghai Mapada) and microplate reader (Spark, Tecan) with commercial kits (Nanjing Jiancheng Bioengineering Institute, China, Table S2). Results were normalized to protein content and expressed as fold change relative to controls to reduce baseline variability among individuals and facilitate direct comparison across different enzyme activities and treatments.

2.2.2. Measurement of 8-hydroxy-2-deoxyguanosine (8-OHdG)

After 28-day exposure, one earthworm per container was randomly selected for 8-OHdG measurement. Approximately 0.1g of earthworm tissue was used for each assay. Genomic DNA was extracted using Dzup DNA Isolation Reagent (Sangon Biotech, China) following the manufacturer's instructions. DNA concentration and purity were determined spectrophotometrically (A260/A280), and each sample was diluted to the required concentration before the assay. 8-OHdG levels were measured with an invertebrate-specific ELISA kit (Nanjing Jiancheng, China) using a competitive enzyme-linked immunosorbent assay format. Briefly, the standard provided in the kit was serially diluted, and 50 μL of either standard solution or DNA sample was added to each well of a 96-well microplate pre-coated with capture antibody. Then, 50 μL of HRP-conjugate reagent was added to each well, followed by incubation at 37 °C for 60 min. After washing, chromogenic substrates were added sequentially, and the reaction was stopped with the termination solution. The absorbance (OD) at 450 nm was recorded within 15 min using a microplate reader (BioTek, USA). The concentration of 8-OHdG in each sample was calculated from the standard curve and normalized to the DNA content, expressed as ng 8-OHdG per μg DNA. Each measurement was performed in triplicate, and results were expressed as fold change relative to the control group.

2.2.3. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Three frozen earthworms from each container were randomly picked and ground in ceramic mortars with liquid nitrogen. Total RNA was extracted by adding 1 mL of TRIzol reagent (Nanjing Sunshine Biotechnology, China) per 0.1 g of earthworm tissue following the manufacturer's protocol. Then, RNA concentration and purity were determined using Nanodrop 2000 (Thermo Fisher Scientific, USA) by ensuring that A260/A280 were between 1.8 and 2.0. First-strand cDNA was synthesized using HiScriptIIQ RT SuperMix (Vazyme Biotech, China) after removal of genomic DNA by using gDNA wiper and the obtained cDNA was stored at −20 °C until use.

In the present study, *β-actin* was selected as housekeeping gene and *sod*, *hsp70*, *tctp* as target genes. All primers were synthesized by Sangon Biotech (China) and the sequences are listed in Table S3, as well as the corresponding Genbank accession numbers. According to the MIQE Guidelines (Bustin et al., 2009), PCR amplification efficiency of each gene was determined by means of calibration curves (Table S4). Quantitative PCR were performed in a 20 μL reaction system using ChamQ™ SYBR qPCR kit (Vazyme Biotech, China) on StepOnePlus (Applied Biosystems, USA). The 20 μL reaction system included 10 μL of 2 × ChamQ SYBR qPCR Master Mix, 0.4 μL of forward and reverse primers (10 μM), 1 μL of template cDNA and 8.2 μL of ddH_2O. The amplification reactions started with an initial denaturation at 95 °C for 3 min, followed with 40 cycles of denaturation (10 s at 95 °C), primer annealing (30 s at 56 °C) and extension (60 s at 72 °C). Additionally, melting curve analyses were performed after the final round of amplification to exclude the interference of primer-dimers amplified or DNA contaminants (Fig. S3). All samples were analyzed in triplicate and the relative expression levels of target genes were calculated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

2.3. Statistical analysis

All statistical analyses were performed using SPSS 25.0 (IBM Corporation, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to evaluate the statistical differences among each group, after checking for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Correlation analysis of enzyme activity and corresponding mRNA expression level was using Pearson correlation analysis. Differences in relative gene expressions between control and treatments were analyzed using two-tailed T-test. Results were presented as mean ± standard deviations (SD). Differences were considered statistically significant at $p < 0.05$.

3. Results and discussions

3.1. Impacts of OPFRs and microplastics on earthworm growth performance

Body weight loss is a common sublethal response of earthworms to toxicants, serving as a sensitive indicator of physiological stress that reflects both the severity and duration of exposure, as well as the organism's overall metabolic balance (Qi-xing et al., 2006; Travlos et al., 2017; Pochron et al., 2018). In the present study, the slight increase of control earthworms' weight suggested that the soil nutrients were just sufficient to sustain the survival of the earthworms, but insufficient to allow for additional growth. Meanwhile, no significant changes in the body weight of *Eisenia fetida* were observed after 7-day exposure to OPFRs and/or PEs (Fig. S4), suggesting that short-term exposure at the tested concentrations may not immediately impair earthworm growth. However, after 28 days, a significant decrease in body mass was observed in the group exposed to TDCPP alone (D2, $p < 0.05$), indicating that prolonged exposure to TDCPP can disrupt growth or nutrient assimilation.

Interestingly, the combined exposure to TDCPP and PE ($D2^{PE}$ group) alleviated the body weight loss observed in earthworms exposed to TDCPP alone. Earthworms in the $D2^{PE}$ group exhibited significantly higher body weight compared to those in the TDCPP-only group ($p < 0.05$), suggesting a potential antagonistic effect of PE on TDCPP-induced toxicity.

A plausible explanation is that TDCPP may be adsorbed onto the surface of PE particles, thereby reducing its freely dissolved concentration in soil pore water and, consequently, its bioavailability to *Eisenia fetida* (Xuan et al., 2024). Previous studies have shown that TDCPP exhibits higher maximum adsorption capacities (Q_m = 94.02–176.70 mg kg^{-1}) in landfill soils compared to TCEP and TCPP, particularly in humus-rich fractions (Luo et al., 2024; Xuan et al., 2024). Moreover, when the adsorption capacity of microplastics for pollutants exceeds

that of the soil matrix, microplastics can enhance the overall sorption of organic contaminants through a so-called "superposition effect" (Hu et al., 2023). Polyethylene, for instance, may provide additional adsorption sites and hydrogen bonding opportunities (Zhu et al., 2021; Yu et al., 2023), thereby increasing the retention of TDCPP in subsoils. This enhanced sorption may sequester TDCPP from the soil solution, attenuating its direct toxic effects on soil organisms. Taken together, these findings support the hypothesis that the mitigating effect of PE on TDCPP-induced growth inhibition in *Eisenia fetida* is at least partially attributable to MP-mediated adsorption and the resulting reduction in chemical bioavailability.

3.2. Enzymatic activity alterations under OPFRs and microplastic exposure

Enzymatic biomarkers are widely used to assess sublethal toxic effects in soil organisms, reflecting oxidative stress, detoxification capacity, neuromodulation, and energy metabolism (Yang et al., 2012; Tiwari et al., 2016). In the present study, significant alterations in the activities of antioxidant enzymes (SOD, CAT), detoxification enzyme (GST), cholinesterase (AChE), and ATPases were observed following exposure to OPFRs and/or PEs, suggesting compound-specific biochemical disturbances in *Eisenia fetida*.

3.2.1. Effects on antioxidant enzyme activities (SOD and CAT)

Under normal physiological conditions, SOD maintains a dynamic balance to support cellular metabolism and eliminate excess reactive oxygen species (ROS) (Hu et al., 2016; Parelho et al., 2018). In response to oxidative stress, SOD catalyzes the dismutation of superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2), thereby protecting cells from oxidative damage (Koivula et al., 2011; Shao et al., 2019).

The variation in SOD activity under exposure to different OPFRs revealed distinct oxidative stress responses in earthworms. After 7 days of exposure, no significant changes in SOD activity were observed in the TCEP (E2) and TDCPP (D2) groups, whereas a pronounced decrease was found in the TCPP (P2) group, indicating that TCPP exerts stronger acute oxidative toxicity. By day 28, however, both E2 and D2 groups exhibited significantly reduced SOD activity, suggesting cumulative toxic effects and impairment of the antioxidant defense system. This was particularly evident for TDCPP, likely due to its high hydrophobicity ($\log K_{ow} = 3.76$, Table S1) and bioaccumulation potential (Wang et al., 2015).

Further analysis of the microplastic co-exposure groups ($E2^{PE}$, $P2^{PE}$, $D2^{PE}$) revealed distinct patterns of combined oxidative stress responses. On day 7, $P2^{PE}$ and $D2^{PE}$ exhibited significantly decreased SOD activity. This synergistic inhibition aligns with previous studies showing that PEs may enhance pollutant toxicity by increasing reactive oxygen species (ROS) generation or by facilitating chemical transport into tissues (Das, 2023; Zhu et al., 2023). While $E2^{PE}$ remained comparable to the control, indicating limited short-term interactive toxicity between TCEP and PE. By day 28, SOD activity in $E2^{PE}$ was markedly reduced, suggesting delayed oxidative damage induced by TCEP. In contrast, $P2^{PE}$ and $D2^{PE}$ showed a slight recovery in SOD activity compared to day 7, potentially due to physiological adaptation or mitigation of OPFRs toxicity via reduced bioavailability caused by sorption to PEs (Zhang et al., 2020).

The temporal trend of catalase (CAT) activity closely mirrored that of SOD (Fig. 1b), displaying a similar time-dependent response pattern. Generally, CAT, as a key enzyme responsible for the breakdown of hydrogen peroxide, serves as an important indicator of the cell's ability to mitigate H_2O_2-induced oxidative damage (Xu et al., 2022). A concurrent decline in CAT and SOD activities suggests a severely compromised antioxidant defense system, thereby increasing the risk of oxidative injury (Ighodaro and Akinloye, 2018). Further two-way ANOVA confirmed these observations. At day 7, significant *PEs* × *OPFRs* interaction effects were observed for SOD ($F = 3.826$, $p = 0.031$) and CAT ($F = 3.955$, $p = 0.028$). By day 28, the interaction remained significant for SOD ($F = 3.480$, $p = 0.041$) but not for CAT ($F = 0.462$, $p = 0.713$). Across both exposure durations, *PEs* × *OPFRs* interaction was significant for SOD ($F = 3.517$, $p = 0.026$) and marginally significant for CAT ($F = 2.352$, $p = 0.091$). Importantly, interaction analysis with exposure time revealed that *OPFRs* × *Time* had highly significant effects on both enzymes (SOD: $F = 27.511$, $p < 0.001$; CAT: $F = 15.450$, $p < 0.001$), whereas *PEs* × *Time* showed no significant influence (all $p > 0.3$). These findings suggest that the temporal variation in oxidative stress responses was mainly driven by OPFRs rather than PEs. Overall, the time-course changes in CAT activity further support the oxidative toxicity of OPFRs and their combined effects with PEs. Notably, under prolonged exposure conditions, the co-exposure groups exhibited more pronounced inhibition, indicating that PEs may exacerbate oxidative stress by altering the bioavailability of pollutants or triggering synergistic oxidative responses, thereby intensifying the disruption of the antioxidant system in earthworms.

3.2.2. Glutathione S-transferase (GST) and acetylcholinesterase (AChE) response to OPFRs and microplastics

GST is a key detoxification enzyme involved in the metabolism of lipophilic organic pollutants and plays a critical role in cellular defense against oxidative stress (Jaskulak et al., 2021). Its activity is widely recognized as a reliable biomarker for evaluating sublethal toxicity in soil invertebrates (Kaur and Hundal, 2022). In this study, a significant increase in GST activity was observed after 7 days of TCEP exposure in both the E2 and $E2^{PE}$ groups ($p < 0.01$), while no significant changes were detected in the TCPP and TDCPP treatments (Fig. 2a). This transient elevation suggests that GST plays a short-term defensive role in response to TCEP-induced oxidative stress. Yang et al. (2018) also reported that TCEP exerts stronger ecotoxic effects than other OPFRs, which aligns with our observations. In addition, GST activity in the combined exposure group ($E2^{PE}$: TCEP + PE) was slightly lower than in the TCEP-only group (E2), indicating that the presence of microplastics may reduce the bioavailability of TCEP by adsorbing it onto plastic surfaces, thereby attenuating the oxidative stimulus required to induce

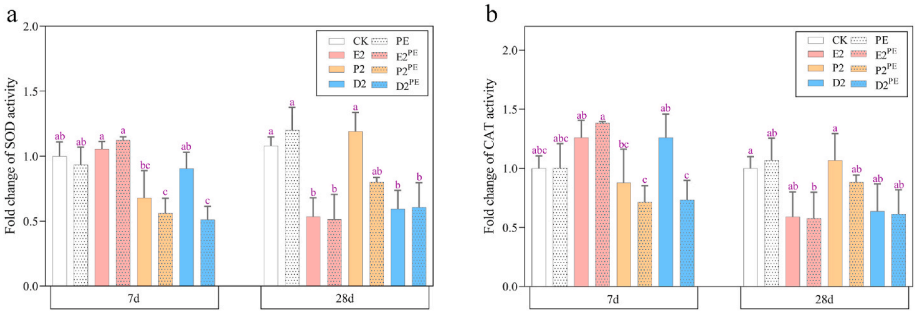

Fig. 1. Fold change of SOD activity (a) and CAT activity (b) in *Eisenia fetida* exposed to PEs and OPFRs (E2, P2 and D2 refer to 0.002 g kg^{-1} TCEP, TCPP and TDCPP, respectively. PE concentration: 0.5 g kg^{-1}. $E2^{PE}$: TCEP + PE; $P2^{PE}$: TCPP + PE; $D2^{PE}$: TDCPP + PE). Values are presented as means ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

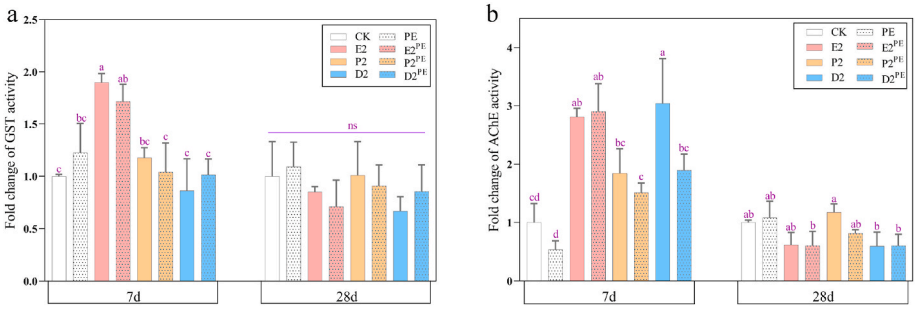

Fig. 2. Fold change of GST activity (a) and AChE activity (b) in *Eisenia fetida* exposed to PEs and OPFRs (E2, P2 and D2 refer to 0.002 g kg^{-1} TCEP, TCPP and TDCPP, respectively. PE concentration: 0.5 g kg^{-1}. $E2^{PE}$: TCEP + PE; $P2^{PE}$: TCPP + PE; $D2^{PE}$: TDCPP + PE). Values are presented as means ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$) and "ns" means no significant change.

GST. However, this elevated GST activity was not sustained at day 28, suggesting that the detoxification response was either completed or that prolonged exposure suppressed the enzymatic system due to cumulative toxicity.

AChE activity was also evaluated to assess potential neurotoxic effects induced by OPFRs and/or PEs. In earthworms, AChE is the predominant form of cholinesterase and is closely associated with the axons of the cholinergic nervous system, actively participating in neurosignaling pathways (Gu et al., 2021). Therefore, AChE activity serves as a mechanism-specific biomarker for evaluating neurotoxicity (Gao et al., 2019). In the present study, neurotoxic responses were assessed in the earthworm nervous system on both day 7 and day 28 of exposure. After 7 days, an overall upward trend in AChE activity was observed across most treatment groups, with a particularly significant elevation in the TCEP + PE group ($E2^{PE}$, $p < 0.001$; Fig. 2b), suggesting that TCEP may induce neural excitation or compensatory cholinergic activity. Here, $p < 0.001$ indicates that the probability of this difference occurring by chance is less than 0.1 %, representing a highly significant difference compared with the control group. This stimulatory response may also reflect that the exposure concentrations of OPFRs and PEs used in this study were below the threshold for overt neurotoxicity (e.g., LC_{50}), and therefore did not result in severe cholinesterase inhibition. This finding contrasts with the results reported by Wang et al. (2015), where earthworms exposed to high concentrations (0.05–0.1 g kg^{-1}) of the neonicotinoid pesticide guadipyr exhibited a significant decrease in AChE activity as early as day 3, indicating rapid neurotoxicity. However, in their study, AChE activity gradually recovered to baseline levels over time. The discrepancy may stem from differences in chemical structure, mode of action, or exposure concentration between guadipyr and OPFRs (Qi et al., 2018; Yang et al., 2018). By day 28 in the present study, AChE activity in all groups returned to near-control levels, suggesting the activation of repair mechanisms or physiological adaptation under prolonged low-level exposure (Qiao et al., 2019; Yin et al., 2024).

3.2.3. *Disruption of ion-transport ATPase activities (Na^+/K^+-ATPase and Ca^{2+}-ATPase)*

To further investigate the neurotoxic effects of OPFRs and/or PEs on *Eisenia fetida*, we examined the activity of two key ion-transport enzymes: Na^+/K^+-ATPase and Ca^{2+}-ATPase. As shown in Fig. 3a, exposure to TCPP and TDCPP, as well as their mixtures with polyethylene microplastics (PE + TCPP and PE + TDCPP), significantly inhibited Na^+/K^+-ATPase activity after 7 days. This inhibition may impair the maintenance of osmotic pressure and interfere with the propagation of nerve impulses (Benarroch, 2011; Huang et al., 2024).

Similarly, reductions in Ca^{2+}-ATPase activity were also observed in the same treatment groups on day 7 (Fig. 3b), indicating a compromised ability of cells to actively transport Ca^{2+} ions out of the cytoplasm. This may result in intracellular Ca^{2+} overload, increased osmotic pressure, and disruption of cellular integrity (Zhang et al., 2016). One possible explanation for this enzymatic inhibition is the oxidative stress induced by OPFRs and/or PEs. Ca^{2+}-ATPase is a sulfhydryl-rich enzyme that is highly sensitive to oxidative damage caused by reactive oxygen species (ROS) (Burlando et al., 2004; Zhang et al., 2016). Excess ROS can suppress its enzymatic activity, disrupting calcium homeostasis and impairing essential physiological functions—ultimately leading to cellular dysfunction or even apoptosis (Viarengo and Nicotera, 1991; Zhang et al., 2016).

By day 28, both Na^+/K^+-ATPase and Ca^{2+}-ATPase activities remained inhibited across most treatment groups, suggesting that prolonged exposure to OPFRs and/or PEs may induce sustained ion-transport dysfunction (Oluah et al., 2020). Such disturbances could contribute to broader physiological stress responses, particularly affecting the nervous and muscular systems of earthworms (Boeri et al., 2017).

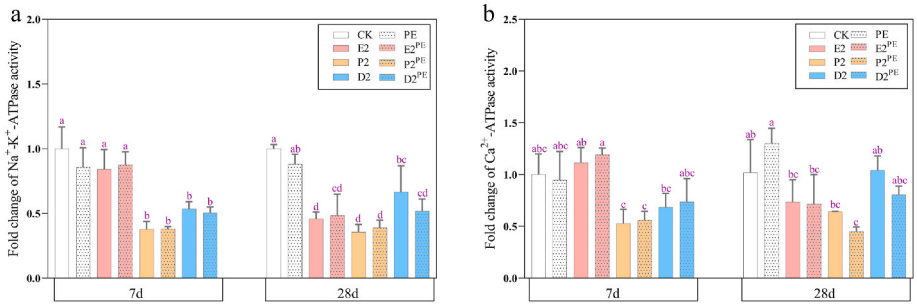

Fig. 3. Fold change of Na^+ -K^+-ATPase activity (a) and Ca^{2+}-ATPase activity (b) in *Eisenia fetida* exposed to PEs and OPFRs (E2, P2 and D2 refer to 0.002 g kg^{-1} TCEP, TCPP and TDCPP, respectively. PE concentration: 0.5 g kg^{-1}. $E2^{PE}$: TCEP + PE; $P2^{PE}$: TCPP + PE; $D2^{PE}$: TDCPP + PE). Values are presented as means ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

3.3. Oxidative damage and gene expression dynamics under combined exposure

3.3.1. Oxidative damage induced by OPFRs and microplastics

To further elucidate the cellular and molecular mechanisms of toxicity, we evaluated oxidative damage biomarkers [malondialdehyde (MDA), 8-hydroxy-2′-deoxyguanosine (8-OHdG)] and the transcriptional expression of stress-related genes (*sod*, *hsp70*, *tctp*) in *Eisenia fetida* after 7- and 28-day exposures. Notably, MDA was measured at both time points, while 8-OHdG was assessed only after 28 days.

Lipid peroxidation, indicated by MDA content, exhibited both compound-specific and time-dependent responses (Fig. 4a). After 7 days, exposure to TCEP alone (E2) significantly elevated MDA levels ($p < 0.05$), suggesting the early onset of oxidative membrane damage. In contrast, TCPP and TDCPP did not significantly alter MDA levels at this stage. Interestingly, co-exposure with PEs resulted in a marked decrease in MDA in the TDCPP + PE group ($D2^{PE}$), possibly due to reduced bioavailability of TDCPP via sorption onto polyethylene surfaces, as previously reported in soil matrices (Tourinho et al., 2019). After 28 days, both TCPP and TDCPP alone significantly increased MDA levels ($p < 0.05$), but these effects were alleviated in the presence of PEs, particularly in $D2^{PE}$. This pattern suggests potential antagonistic effects of PEs under prolonged exposure conditions.

DNA oxidative damage, as measured by 8-OHdG, further confirmed the presence of oxidative stress. After 28 days, elevated 8-OHdG levels were detected in all treatment groups; however, only TCPP and TCPP + PE (P2, $P2^{PE}$) induced statistically significant increases ($p < 0.01$, Fig. 4b). These findings reinforce the genotoxic potential of TCPP, potentially amplified by PEs, which is consistent with prior report of synergistic DNA damage caused by microplastics and hydrophobic organic contaminants (Liu et al., 2025).

3.3.2. Transcriptional responses of antioxidant and stress-related genes

To explore underlying molecular mechanisms, we assessed the transcriptional responses of three key stress-related genes: *sod* (encoding superoxide dismutase), *hsp70* (encoding heat shock protein 70), and *tctp* (translationally controlled tumor protein). These genes are involved in oxidative defense, protein homeostasis, and apoptosis regulation, respectively (Yin et al., 2024). Among them, we emphasized *sod* in the main text because superoxide dismutase represents the first line of defense against oxidative stress and is widely recognized as a particularly sensitive and reliable biomarker for pollutant-induced redox imbalance in soil invertebrate (Xu et al., 2021).

As shown in Fig. 5, the results of relative expression levels of *sod*, *hsp70*, and *tctp* are presented, and the corresponding two-tailed *t*-test results are summarized in Table S5 *sod* expression was significantly downregulated in both TCEP and TCEP + PE groups at both time points, indicating suppression of antioxidant gene activation. In contrast, *sod* expression was initially upregulated by TCPP and TCPP + PE at day 7, but declined substantially by day 28, implying a transient adaptive response followed by potential exhaustion of antioxidant defenses. A similar trend was observed in the PE-alone group, highlighting the chronic oxidative pressure imposed by microplastics, even in the absence of chemical additives.

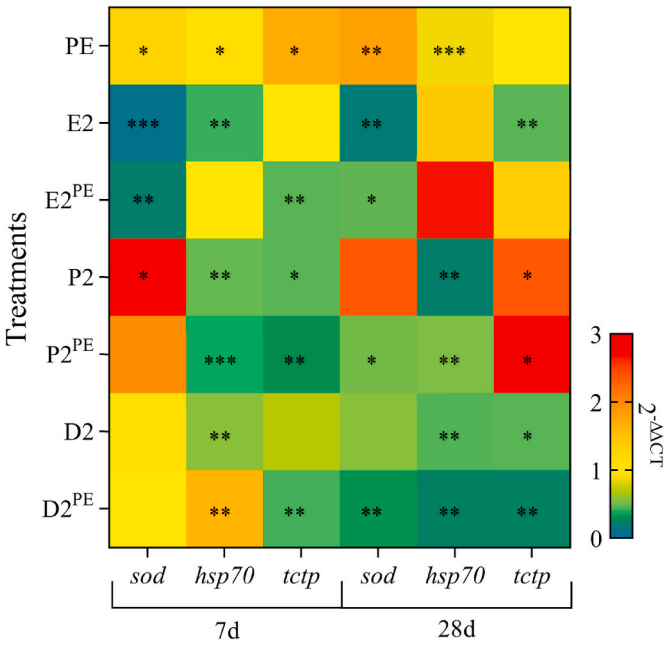

Fig. 5. Relative gene expressions of *sod*, *hsp70* and *tctp* in *Eisenia fetida* exposed to PEs and OPFRs (E2, P2 and D2 refer to 0.002 g kg^{-1} TCEP, TCPP and TDCPP, respectively. PE concentration: 0.5 g kg^{-1}. $E2^{PE}$: TCEP + PE; $P2^{PE}$: TCPP + PE; $D2^{PE}$: TDCPP + PE). Values are relative to control groups using $2^{-\Delta\Delta CT}$ method. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, relative to the control group.

To further examine the transcriptional regulation and functional outcomes of antioxidant responses, Pearson correlation analysis was conducted between *sod* mRNA expression and SOD enzyme activity at both exposure time points. A significant negative correlation ($r = -0.63$, $p < 0.01$) was observed on day 7, indicating that higher gene transcription levels were associated with lower enzyme activity at this stage. This suggests a potential temporal disconnect between molecular regulation and enzymatic functionality during the early oxidative stress response, where transcriptional upregulation may precede the accumulation of functional proteins (Baihetiyaer et al., 2023; Xu et al., 2024). This discrepancy may reflect post-transcriptional delays or oxidative inactivation of the SOD enzyme. By contrast, a significant positive correlation ($r = 0.76$, $p < 0.01$) was found on day 28, suggesting a more coordinated and adaptive transcription-to-function relationship under prolonged exposure. These findings underscore the dynamic nature of antioxidant regulation in *Eisenia fetida* and highlight the

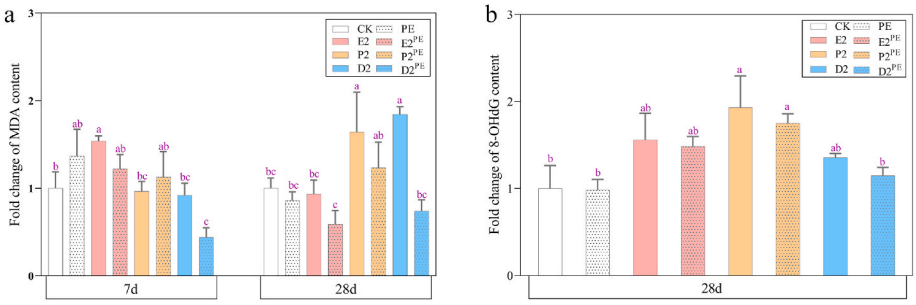

Fig. 4. Fold change of MDA level (a) and 8-OHdG level (b) in *Eisenia fetida* exposed to PEs and OPFRs (E2, P2 and D2 refer to 0.002 g kg^{-1} TCEP, TCPP and TDCPP, respectively. PE concentration: 0.5 g kg^{-1}. $E2^{PE}$: TCEP + PE; $P2^{PE}$: TCPP + PE; $D2^{PE}$: TDCPP + PE). Values are presented as means ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

importance of integrating both molecular and enzymatic indicators in ecotoxicological assessments.

Hsp70 is a highly conserved molecular chaperone that plays a critical role in maintaining cellular homeostasis by assisting in protein folding, preventing aggregation of misfolded proteins, and promoting cellular recovery under stress conditions (Xu et al., 2015). Due to its crucial protective functions, *hsp70* is frequently used as a biomarker in environmental toxicology (Wang et al., 2015). In the present study, *hsp70* expression exhibited compound- and time-specific trends. After 28 days of exposure, significant downregulation was observed in both TCPP and TDCPP groups, irrespective of PE co-exposure, suggesting sustained proteotoxic stress that overwhelmed the protective capacity of the *hsp70* system. In contrast, a notable upregulation of *hsp70* was detected in the TDCPP + PE group on day 7 ($p < 0.01$), which may reflect an early compensatory response aimed at mitigating acute cellular stress—an effect that was not sustained over time. Similarly, a mild but significant induction of *hsp70* was also observed in the PE-alone group at day 7, indicating that microplastics alone can trigger transient proteotoxic stress in *Eisenia fetida*.

Tctp is a highly conserved protein implicated in cell proliferation, survival, and apoptosis regulation (Telerman and Amson, 2009). In the present study, *tctp expression* was significantly upregulated in the PE-alone group on day 7, indicating that microplastic exposure alone may transiently activate growth-related signaling pathways in *Eisenia fetida*. However, *tctp expression* was generally suppressed in most OPFRs-treated groups, suggesting inhibition of cellular proliferation or disruption of normal cell cycle regulation. Notably, after 28 days of exposure, TCPP and TCPP + PE treatments significantly elevated *tctp expression*, potentially reflecting a delayed compensatory mechanism aimed at restoring cellular homeostasis under prolonged toxicant stress. Similar upregulation of *tctp* has been observed in previous studies, such as Wang et al. (2015), where high doses of naphthenic acids induced *tctp expression* in *Eisenia fetida*, raising concerns about possible links to carcinogenic processes, underscoring the relevance of *tctp* as a sensitive indicator of chronic toxic effects in terrestrial invertebrates.

In summary, oxidative damage and gene expression analyses demonstrate that OPFRs, particularly TCPP and TDCPP, can disrupt redox balance and impair cellular homeostasis in *Eisenia fetida*. microplastics frequently amplified these molecular effects, though in certain cases—such as TDCPP + PE—antagonistic interactions were observed. These results highlight the compound-specific and time-dependent nature of microplastic–pollutant interactions and underscore the value of combining biochemical and molecular biomarkers for a more comprehensive ecotoxicological assessment.

3.3.3. Mechanistic insights into divergent PE–OPFRs interactions

The contrasting effects of PEs on TDCPP and TCPP toxicity may be explained by differences in their physicochemical properties and sorption behavior. TDCPP, with a higher hydrophobicity (log $K_{ow} = 3.76$, Table S1) and bulkier molecular structure, is more prone to strong adsorption onto the hydrophobic PE surface. This sorption likely reduces its freely dissolved fraction in soil pore water, lowering its bioavailability and toxicity. Conversely, TCPP (log $K_{ow} = 2.59$, Table S1) exhibits weaker PE sorption, leaving a greater dissolved fraction. In the presence of PEs, the altered physicochemical microenvironment—such as increased surface roughness, microcarrier formation, and changes in soil organic matter interactions—may facilitate TCPP uptake into earthworm tissues. This could enhance oxidative stress and genotoxicity, potentially through synergistic ROS generation from both the chemical and the microplastics. These findings align with previous reports that microplastics–organic pollutant interactions can range from antagonistic to synergistic depending on pollutant hydrophobicity, particle size, and exposure duration (Zhang et al., 2020; Kinigopoulou et al., 2022; Prajapati et al., 2022; Liu and Liu, 2025).

3.4. Implications for ecological risks

This study elucidates the compound-specific ecotoxicological effects of OPFRs and PEs on the soil-dwelling organism *Eisenia fetida*, offering insights into their broader ecological implications. The results reveal that TDCPP exhibits the highest individual toxicity among the three OPFRs tested, significantly impairing earthworm growth, suppressing antioxidant defenses, and disrupting ion-transport ATPase activities. However, when co-exposed with PE, TDCPP-induced toxicity was notably attenuated, likely due to reduced chemical bioavailability caused by sorption to microplastic surfaces. In contrast, TCPP demonstrated an enhanced toxicological effect under co-exposure conditions, especially in inducing DNA oxidative damage, suggesting that microplastics may increase the bioavailability or cellular uptake of certain flame retardants. TCEP exhibited a moderate but time-dependent toxicity pattern, with delayed effects becoming apparent at the molecular and enzymatic levels after prolonged exposure.

Mechanistically, oxidative stress appears to be the predominant toxic pathway for all three OPFRs. TCPP induced acute oxidative damage and triggered strong early antioxidant responses, while TDCPP caused sustained suppression of antioxidant enzymes such as SOD and CAT. All OPFRs also interfered with neural transmission and basal metabolism through the inhibition of Na^+/K^+-ATPase and Ca^{2+}-ATPase activities, indicating potential risks to neuromuscular function. These findings are ecologically significant given the essential roles of *Eisenia fetida* as a keystone soil species and ecosystem engineer. Impaired physiological and biochemical functions in earthworms could translate into reduced soil aeration, altered microbial communities, and disrupted nutrient cycling—ultimately affecting soil health and productivity (Lemtiri et al., 2014; Medina-Sauza et al., 2019).

Importantly, the modifying role of microplastics in mediating OPFRs toxicity complicates traditional single-pollutant risk assessment paradigms. Rather than acting as inert carriers, microplastics may either attenuate or amplify toxicity depending on the physicochemical properties of the co-existing contaminants. This duality introduces new uncertainties into ecological risk evaluations and underscores the need for mixture-oriented and context-specific assessments (Deng et al., 2018; Lu et al., 2023). While the current study provides critical evidence of sublethal toxic effects, several limitations should be acknowledged. First, the exposure durations were limited to 7 and 28 days, which may not fully capture the dynamic progression of toxic responses, especially under long-term environmental conditions. Second, the study employed single fixed concentrations of OPFRs and microplastics, without exploring concentration gradients that are essential for determining dose–response relationships and establishing threshold levels for ecological safety. These constraints highlight the need for future studies incorporating time-series observations, multiple exposure levels, and environmentally relevant field conditions to better validate laboratory findings.

4. Conclusions

This study provides clear evidence that polyethylene microplastics (PEs) are not inert particles but active participants in pollutant dynamics, acting as both carriers and modulators of chlorinated organophosphate flame retardants (OPFRs) in Eisenia fetida. The toxic effects observed were compound-specific and time-dependent: TDCPP exhibited the greatest intrinsic toxicity, TCPP toxicity was amplified when co-occurring with PEs, and TCEP induced delayed biochemical responses. A particularly novel observation was the time-dependent shift in the correlation between sod transcription and SOD enzyme activity, suggesting a transition from early-stage post-transcriptional modulation to later-stage transcriptional regulation. These findings advance our understanding of microplastic–pollutant interactions in terrestrial ecosystems and underscore the need for mixture-based ecological risk assessments. Considering the ecological role of earthworms as key engineers of soil

structure and nutrient cycling, such interactive toxicities may have cascading effects on soil health and ecosystem functioning, highlighting the urgency of incorporating combined-pollutant scenarios into environmental regulations.

CRediT authorship contribution statement

Qingyang Guo: Writing – original draft. **Haixin Shen:** Data curation. **Yongxue Feng:** Data curation. **Minghan Wang:** Methodology. **Yuheng Li:** Investigation. **Xiuxiu Lin:** Software. **Yuxuan Gao:** Writing – review & editing. **Lili Ma:** Funding acquisition. **Xiansheng Liu:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors 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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2025.122992.

Data availability

Data will be made available on request.

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