SOIL ECOLOGY LETTERS

SEL | RESEARCH ARTICLE
Volume 6 / Issue 1 / 230184 / 2024
https://doi.org/10.1007/s42832-023-0184-4

Low-density polyethylene microplastics partially alleviate the ecotoxicological effects induced by cadmium exposure on the earthworm *Eisenia fetida*

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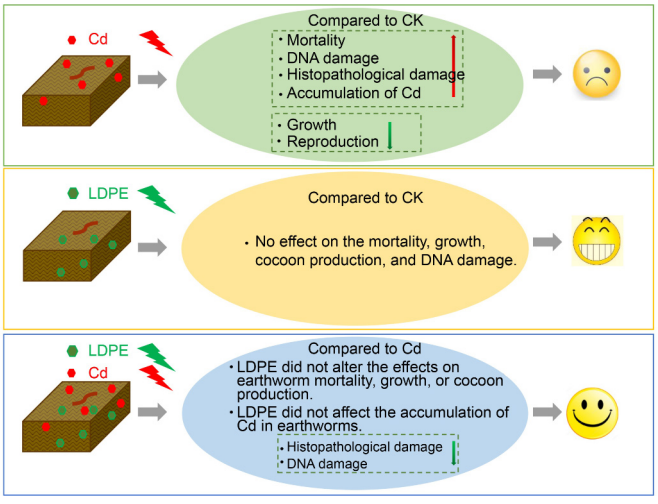
Received February 24, 2023; Revised April 28, 2023; Accepted May 15, 2023

ABSTRACT

- LDPE had no effect on the mortality, growth, and reproduction of earthworms.
- LDPE did not alter the mortality, growth, and reproduction of earthworm caused by Cd.
- LDPE alleviated histopathological damage to earthworms caused by Cd.
- LDPE alleviated DNA damage in earthworm coelomocytes caused by Cd.
- LDPE did not affect the accumulation of Cd in earthworms.



Cadmium (Cd) can accumulate in the food chain, with serious impacts on human health and safety. Microplastics (MPs) such as low-density polyethylene (LDPE) should be considered not only as a single pollutant but also as a carrier of other pollutants. In this study, we investigated the joint effects of 30% LDPE and 313 mg kg^{-1} Cd on mortality, growth, reproduction, microstructure, DNA damage, oxidative stress, and mRNA levels in the earthworm *Eisenia fetida*. We found that 313 mg kg^{-1} Cd inhibited growth and reproduction and damaged the microstructures of the skin and intestine. Meanwhile, LDPE had no effect on the mortality, growth, or cocoon production of earthworms. Moreover, it did not increase the mortality, growth, or inhibition of cocoon production caused by Cd and instead alleviated the DNA damage in coelomocytes caused by Cd treatment. Finally, it did not alter the accumulation of Cd in the worms. These indicators can be used for toxicity safety assessment and soil ecological risk assessment of LDPE and Cd cooccurrence in soil.

Keywords microplastics, *Eisenia fetida*, DNA damage, histopathological damage, heavy metals

1 Introduction

Metal pollution of soils has become an issue of widespread concern worldwide, posing threats to the environment and human health (Rinklebe et al., 2019; Sun et al., 2019; Zhang et al., 2020; Chia et al., 2021). Among heavy metal elements, cadmium (Cd) is a very important pollutant (Satarug et al., 2010). Cd in soil has high mobility and strong biological toxicity, and its level in many agricultural soils has increased (Meharg et al., 2013). It accumulates in the food chain, seriously affecting human health (Kirkham, 2006). Therefore, appropriate methods for evaluating Cd pollution and alleviating its toxicity are needed.

Microplastic (MP) pollution has become an issue of global concern (Alimba and Faggio, 2019). MPs are generally plastic granules, microfibers, plastic particles, foam plastics, and thin films with sizes ranging from 0.2 to 5.0 mm (Ivar do Sul and Costa, 2014). They enter the soil through atmospheric deposition, irrigation water contaminated by MPs, residues

from plastic coverings used in agricultural or horticultural products, and/or deposition of plastic-containing substances such as sludge and feces (Tian et al., 2022). They can be transferred from mussels to crabs, with implications for the health of marine organisms and the wider food web including humans (Farrell and Nelson, 2013). Accidental ingestion of MPs can cause direct damage to an organism (Diepens and Koelmans, 2018). MPs destabilize lipid membranes through mechanical stretching (Fleury and Baulin, 2021). Conventional MPs have been shown to have a negative effect on the growth of earthworms, while biodegradable MPs have weaker negative impacts based on a meta-analysis (Zhu, 2023). However, effects of MPs on earthworms remain controversial. In one study, exposure of earthworms (*Eisenia andrei*) to polyethylene (PE) MPs for 28 days had no significant effects on the survival, number of juveniles and the final weight of adult earthworms (Rodriguez-Seijo et al., 2017). But long-term earthworm PE-MPs intake caused intestinal damage (Cao et al., 2022). The results indicated that the toxicity of PE may be related to the exposure time. Moreover, the protective layer formed by soil particles on plastics were shown to decrease the toxicity of PE-MPs to earthworms (Liu et al., 2022). In another study, no significant effects were reported on the survival, number of juveniles, or weight of adult earthworms after exposure to various concentrations of MPs (Rodriguez-Seijo et al., 2017).

Earthworms are an important component of terrestrial ecosystems (Phillips et al., 2019). They are readily affected by environmental toxins, suggesting that they are a key indicator organism suitable for the ecotoxicological tests and are commonly used for this purpose by the European Union, the Organization for Economic Cooperation and Development (OECD), the International Organization for Standardization, and the Food and Agriculture Organization of the United Nations (Piola et al., 2013; Santadino et al., 2014). *Eisenia fetida* is the standard organism used in such ecotoxicological tests (Shi et al., 2018; Li et al., 2020b; Wang et al., 2022), as it reflects the status of soil pollution and can be used to measure the impacts of soil pollutants, such as pesticides, heavy metals, and MPs (Gu et al., 2017; Lourenco et al., 2011). Oxidative stress occurs and levels of reactive oxygen species increase dramatically under stress, which may lead to cell damage. Numerous antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), can prevent such cell damage (Wang et al., 2018a). The chelation and detoxification of metals in earthworms is mainly accomplished by metallothionein (MT) (Stuerzenbaum et al., 2013). Earthworms can accumulate heavy metals from soil such as Cd and Pb, and store them in intestinal chlorogenic tissue, thereby alleviating heavy metal pollution of soil (Song et al., 2014; Goswami et al., 2016). However, the expression of *MT* in some earthworm species (such as *E. fetida*) cannot be adjusted or downregulated (Goswami et al., 2016).

In addition, MPs have high specific surface area, allowing them to absorb other pollutants and thus to act as carriers of pollutants (Holmes et al., 2014; Liu et al., 2020). PE MPs in soil significantly reduce the growth rate of earthworms and cause inflammation and congestion of the intestinal wall (Huerta Lwanga et al., 2016; Rodriguez-Seijo et al., 2017). Intestinal cells of earthworms are damaged by exposure to polystyrene (PS) MPs (Jiang et al., 2020). LDPE decay rate increases and the size of plastics is reduced after passage through the gut of the earthworm *Lumbricus terrestris* (Oligochaeta) (Huerta Lwanga et al., 2018). MPs do not cause significant toxic effects in *E. fetida* under environmentally relevant conditions or enhance the accumulation of hydrophobic contaminants (Wang et al., 2019a). Studies have shown that PS MPs can increase the accumulation of the chemical pollutant polychlorinated biphenyl 105 (PCB105) in the aquatic lugworm (Koelmans et al., 2013). MPs from tires can increase the uptake of heavy metals (Zn, Cd, and Pb) by earthworms (Sheng et al., 2021). MPs and Cd present a risk of co-exposure to soil organisms in Wuxi farmland soils of Taihu Lake area, China (Jiang et al., 2022). MPs are able to increase the accumulation of Cd and strengthen the negative effects of Cd on *E. fetida* (Zhou et al., 2020). PE MPs enhanced the Cd availability which resulted in aggravating the joint toxicity to *E. fetida* (Huang et al., 2021). Cd concentration had antagonistic toxicity effects on *E. fetida* with dependence on PE MPs concentration and MPs particle size under short-term co-exposure, while they showed synergistic effects during long-term co-exposure (Liang et al., 2022). HDPE MPs could act as vectors to increase metal exposure in earthworms, but the associated risk is shown to be unlikely to be significant for essential metals such as Zn that are well regulated by metabolic processes (Hodson et al., 2017). However, data on the joint toxicity of MPs and soil heavy metals on earthworms are still limited. Therefore, it is necessary to systematically analyze the toxicological effects on earthworms of MPs, heavy metal Cd, and co-exposure to LDPE MPs and Cd.

In this study, the effects of LDPE MPs and Cd on *E. fetida* were investigated. The objectives of this study were to explore: (1) whether LDPE MPs and Cd had impact on growth, reproduction, and death; (2) the activities of antioxidant enzymes (SOD, POD, glutathione [GSH], and CAT) and the content of an antioxidant (MDA); (3) possible DNA damage in earthworms' coelomocytes and the mRNA levels of *ANN*, *MT*, *Hsp70*, and *translationally controlled tumor protein* (*TCTP*); (4) possible damage to the epidermis and intestinal tissue of the earthworms and the Cd contents of the soil and earthworms. The results of this study are critical to assessing the synergistic impacts of pollution with MPs and heavy metals on soil fauna.

2 Materials and methods

2.1 Artificial soils, chemicals, and earthworms

Artificial soils were prepared according to OECD guidelines with some modifications (OECD, 2004; Wang et al., 2010): 69% quartz sand, 20% kaolinite clay (chemically pure; Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), 10% peat clay, and 1% calcium carbonate ($CaCO_3$, analytical grade, purity ⩾ 99.0%; Sinopharm Chemical Reagent Co., Ltd). Approximately 2% cow dung (Xilingol grassland, Inner Mongolia, pure natural wind dry block anuric cow dung) was added. The soil pH was adjusted to 6.5 ± 0.5. Then deionized water was used to adjust the soil water content to 20%–30%. Ten earthworms that have adapted to artificial soil were added to each replicate.

The MPs used in this study were LDPE with a particle size of less than 1 mm (Shanghai Plastic Enjoy Trading Co., Ltd, China). Cadmium chloride ($CdCl_2$) was purchased from J&K Scientific (CAS 7790-78-5) (> 99.998% purity). Cd solution was prepared by dissolving $CdCl_2 \cdot 2.5H_2O$ in ultra-pure water, diluting the solution with deionized water, and fully mixing it with artificial soil.

Earthworms *Eisenia fetida* were obtained from Xinyida Earthworm Farm in Hebei Province, China, and weighed 400–600 mg each. They were cultured in artificial soil under laboratory conditions for three days prior to the experiment. The laboratory temperature was 20 ± 2°C and light was provided for 10 h per day.

2.2 Experimental design

2.2.1 Single toxicities of Cd and LDPE

Each plastic container (13.5 cm × 8.5 cm × 6.5 cm) contained 500 g artificial soil (dry weight) with effective Cd concentrations of 0 (control), 200, 600, 1 000, or 1 400 mg kg^{-1}. Cow dung (10 g dry weight) was soaked in deionized water and then added to the test box every 14 days. LDPE was mixed with dry artificial soil at weight ratios of 0.5%, 2%, 5%, 10%, and 60%. Then, 10 earthworms (400–600 mg) were added. The soil was evenly sprayed with deionized water during the experiment to maintain soil moisture of approximately 60%. The survival and weight of earthworms were recorded every 7 days until 28 days.

2.2.2 Joint toxicity of Cd and LDPE

Four treatments were used for this experiment: (1) Control; (2) LDPE: the concentration of LDPE was 30% (w/w); (3) Cd: 313 mg kg^{-1} Cd; (4) LDPE + Cd: the concentration of LDPE was 30% and 313 mg kg^{-1} Cd. The exposure concentration of Cd was 313 mg kg^{-1} (1/3 of the 50% lethal concentration [LC50]), and the concentration of LDPE was 30%.

2.3 Growth, mortality, and cocoon production

On days 0, 7, 14, 21 and 28, the earthworms were removed from the experimental soil, washed with deionized water and wiped on filter paper, and the growth inhibition rate (GIR) and the mortality (M) of the earthworms were calculated as follows (Zhao et al., 2017):

$$\mathrm{M}t = (\mathrm{N}_0 - N_t)/N_0 \times 100\%$$

$$\mathrm{GIR}t = \frac{W_0/N_0 - W_t/N_t}{W_0/N_0} \times 100\%$$

where N_0 and N_t are the survival number of earthworms on day 0 and day t, respectively; W_0 and W_t are the total bodyweight of earthworms on day 0 and day t, respectively; and t refers to the exposure time (days) (Zhou et al., 2020).

Cocoon collection was performed as previously described (Du et al., 2023). On day 28, the soil samples were gently washed with water through a 2-mm mesh sieve, and earthworm cocoons were collected from the remaining peat soil, and the number of cocoons was recorded. The cocoon yield of each treatment was defined as the average number from all repeats in each treatment.

2.4 Histological analysis

On day 28, the earthworms were removed from each treatment, washed, and placed in a Petri dish to clear their intestines for 24 h. Then, 3–5 mm tissue from behind the band was cut, fixed in 10% formaldehyde solution for at least 48 h, and then dehydrated using a series of ethanol concentrations. After xylene treatment, the tissue was embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Finally, the skin and intestinal tissues of the earthworms were observed under a microscope (BX53, Olympus, Japan).

2.5 Comet assay

The DNA damage was performed as described in previous studies, with some modifications (Li et al., 2020b; Yang et al., 2022). The earthworms were put in extrusion buffer (0.25% ethylenediaminetetraacetic acid, 5% ethanol, 95% saline and 1% guaiacol glyceryl, pH 7.3) and they excreted vomited coelomocyte. The coelomocyte fluid was centrifuged at 3 000 r min^{-1} for 3 min; then the supernatant was removed; 1 mL PBS was added, and the mixture was swirled and centrifuged. This process was repeated three times. Then 1 mL PBS was added and swirled to make a suspension. The suspension was preserved at 4°C. Each processed slide was stained with 50 μg mL^{-1} propidium

iodide (C0080; Solarbio, Beijing, China) for 10 min. Fluorescence microscopy (BX53; Olympus, Tokyo, Japan) was used to observe the slides at 200 × magnification. For each treatment, 50 cells were randomly selected and analyzed using CASP software.

2.6 Biochemical assays

Earthworms were taken from each treatment, their intestines were cleaned, they were frozen in liquid nitrogen, and stored at –80°C. Then the earthworms were ground into powder on ice. Earthworm tissue powder and saline were added to 1.5 mL centrifuge tubes at a 1:9 ratio. The mixture was fully mixed through vortex oscillation and then centrifuged. Then the supernatant was transferred to a new centrifuge tube as a 10% tissue homogenate.

The enzyme activities in earthworm tissues were measured and calculated using kits (Yang et al., 2020). The CAT (Cat # A007-1), POD (Cat # A084-1-1), GSH (Cat # A006-1-1), MDA (Cat # A003-1-2), and SOD (Cat # A001-1-1) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The protein concentration was determined using Bradford's method (Bradford, 1976) with the Super-Bradford Protein Assay Kit (CW0013S, CWBio, Beijing, China).

2.7 Real-time quantitative polymerase chain reaction (qPCR)

Real-time qPCR was performed as described in a previous study (Li et al., 2020b). Earthworms from each treatment were cleaned and placed in a Petri dish for 24 h, frozen in liquid nitrogen, and stored at –80°C. Then they were ground into a powder in liquid nitrogen using a mortar. Each 1.5 mL centrifuge tube contained 0.05–0.10 g earthworm powder and 1 mL trizol (RN0101, Aidlab Biotechnologies, Beijing, China) was added to extract total RNA. The integrity and purity of the RNA were assessed using a Nanodrop fluorometer and agarose gel electrophoresis. The first-strand cDNA was synthesized using the FastKing one-step method (KR118-02, Tiangen Biotech, Beijing, China). Then the cDNA was used for the qPCR assay. The Talent fluorescence quantitative detection kit (SYBR Green, KR118-02 FP 209-02, Tiangen Biotech) and a 96-well PCR plate (PCR-96M2-HS-C, Axygen, Union City, CA, USA) were used for analysis; a qPCR instrument (qTOWER2.2, Analytic Jena, Jena, Germany) was used to read the data.

Primer sequences (Tsingke Biotechnology Co., Ltd., Beijing, China) of five earthworm genes (Table 1) used in this study, namely metallothionein (*MT*), annetocin precursor (*ANN*), translationally controlled tumor protein (*TCTP*), heat shock protein 70 (*HSP70*), and the internal control gene *β-actin*, are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression levels (Huerta Lwanga et al., 2018).

2.8 Determination of Cd contents of earthworms and soil

2.8.1 Cd contents in the soil

The experimental soils for each treatment were collected, air-dried, and sealed. Then, 0.25 g soil samples were added to a digestion tank, and 10 mL aqua regia ($HCl:HNO_3$ = 3:1) was added for digestion overnight. After a cooling program, the digestion tank was transferred to a temperature-controlled heating instrument; acid was driven off by lightly boiling, and the tank was cooled in a ventilation cupboard. Finally, the volume of the solution was adjusted to fill a 50 mL capacity bottle, and the upper clear liquid was transferred to a centrifuge tube. These extracts were stored in a refrigerator at 4°C until testing.

2.8.2 Different Forms of Cd in soil

The continuous extraction method of the Community Bureau of Reference (BCR) was used in this experiment. The soils for each treatment were collected, air-dried, and sealed. For the acid-extractable fraction (B_1), 1.00 g soil samples were weighed in 100 mL centrifuge tubes, 40 mL 0.11 mol L^{-1} glacial acetic acid extract was added, and the mixture was shaken at 20°C for 16 h (250 r min^{-1}), then centrifuged at 5000 r min^{-1} speed for 20 min. The upper supernatant was filtered into a centrifuge tube and stored in a refrigerator at 4°C until testing. For the reducible fraction (B_2), 20 mL ultrapure water was added to the residue remaining after the first step, the mixture was shaken for 20 min, and then it was

Table 1 Sequences of primers used for real-time qPCR.

Gene	GenBank accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-actin	Y09623.1	gttcgaaaccttcaactccc	tggtggtgaagctgtagcct
MT	AJ236886.1	gcaagagagggatcaacttg	caccacagcacccctttcttg
ANN	AB164320.1	tttgtcaacctgtcgctttc	tcgagggcacagaccttgct
TCTP	GU177860	tcgaatatgccctcagcaaa	tggactcgccacagaagagc
HSP70	HQ693698.2	ctgcgtatcatcaacgagccaa	tgtccttcttgtgcttgcgct

centrifuged to remove the supernatant. Then, 0.50 mol L^{-1} $NH_2OH{\cdot}HCl$ was added to 40 mL extract and shaken at 20°C for 16 h (250 r min^{-1}), centrifuged at 5000 r min^{-1} for 20 min, and the upper supernatant was filtered into a centrifuge tube and stored in a refrigerator at 4°C until testing. For the oxidizable fraction (B_3), 20 mL ultrapure water was added to the residue remaining after the second step, the mixture was shaken for 20 min, centrifuged to remove the supernatant, and then 10 mL H_2O_2 was slowly added. Next, the mixture was loosely covered and digested at room temperature for 1 h, with occasional shaking of the centrifuge tube for 3–5 s. Then a water bath was used to heat the tube to 85 ± 2°C for 1 h with occasional shaking in the first 0.5 h. After heating, the lid was opened, solution in excess of 3 mL was removed, 10 mL H_2O_2 was added, the lid was replaced, and digestion was continued for a further 1 h. After which the lid was opened; then the solution volume was reduced by approximately 1 mL, whereafter 50 mL 1 mol L^{-1} ammonium acetate extract was added. The resulting mixture was shaken for 16 h at 250 r min^{-1} at 20°C, and then the supernatant was obtained through centrifugation for 20 min at 5000 r min^{-1}. The supernatant was filtered into a centrifuge tube and stored in a refrigerator at 4°C until testing. The residual fraction (B_4) was calculated using the subtraction method (B_4 = soil total Cd content – $B_1 - B_2 - B_3$).

2.8.3 Determination of total Cd content of earthworms

Determination of the total Cd content of earthworms was performed using HNO_3–H_2O_2 digestion and inductively coupled plasma (ICP) mass spectrometry. The earthworms were removed from the soil and freeze-dried at 105°C for 24 h. Each 0.20 g sample was weighed in a glass tube and 5 mL HNO_3 was added to digest the sample overnight. Open digestion was conducted in a ventilation cabinet with a digestion furnace, and the temperature was raised every 20 min until reaching 180°C, after which digestion continued for 2 h. H_2O_2 was added during the heating process, the digestion process was considered complete after the digestion solution became clear. Ultra-pure water was used to adjust the volume to 50 mL in a volumetric flask, then the supernatants were filtered and stored at 4°C until testing. The instrument used to determine Cd contents was an ICP (Inductively Coupled Plasma) optical emission spectrometer (5110, Agilent Technologies, USA).

2.9 Statistical analysis

All data were classified and plotted using Excel 2019 (Microsoft, Redmond, WA, USA), GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), and analyzed using SPSS 21.0 (IBM Corporation, Armonk, NY, USA). The differences between groups were analyzed using single-factor analysis of variance and the Duncan test. All data are presented as mean ± standard deviation.

3 Results

3.1 LDPE has no effect on the mortality and growth of earthworms exposed to Cd

The 7-day LC50 of Cd for *E. fetida* was 938.75 mg kg^{-1} (Fig. 1A). The growth rate of earthworms did not decrease under exposure to 0.5%, 2%, 5%, or 10% LDPE. The growth rate of earthworms decreased with exposure to 60% LDPE. Compared to the control group, no significant changes in earthworm mortality were seen on days 21 or 28 in the 30% LDPE treatment group, 313 mg kg^{-1} Cd treatment group or LDPE + Cd group on days 21 or 28 (Fig. 1C). Compared to that in the control, the earthworm growth rate in the 313 mg kg^{-1} Cd treatment group was seen to be significantly inhibited on days 7 and 14. The earthworm growth rates in both the 313 mg kg^{-1} Cd treatment group and the LDPE + Cd treatment group were significantly inhibited early in the experiment. But at 21 days and 14 days, respectively, they were equal to that of the control group. The growth inhibition rates measured in all treatment groups were equal to that in the control on day 28 (Fig. 1D). A significant decrease in the cocoon yield of earthworms occurred in the 313 mg kg^{-1} Cd and LDPE + Cd treatment groups on day 28 (Fig. 2). However, the cocoon number showed no response to exposure to 30% LDPE (Fig. 2B and E). LDPE had no effect on the inhibition of earthworm cocoon production caused by Cd.

3.2 LDPE alleviates damage to the microstructure of *E. fetida* caused by Cd

The epidermis, circular muscle, or longitudinal muscle layer of the body wall tissue of earthworms were unaffected by exposure to LDPE throughout the measurement period, up to day 28 (Fig. 3B–B′). However, 313 mg kg^{-1} Cd exposure damaged the epidermis and circular muscle of the body (Fig. 3C–C′). No damage to the body wall was apparent with joint exposure to LDPE + Cd (Fig. 3D–D′), indicating that LDPE alleviated damage to the body wall of the earthworm caused by Cd.

The microstructures of intestinal epithelial tissue and chlorogenic tissue were intact on day 28 of exposure to 30% LDPE. The microstructure of chlorogenic tissue exhibited deformation with exposure to 313 mg kg^{-1} Cd (Fig. 3C″). The microstructures of intestinal epithelial tissue and chlorogenic tissue were intact with exposure to 30% LDPE + 313 mg kg^{-1} Cd on day 28 (Fig. 3D″), indicating that LDPE

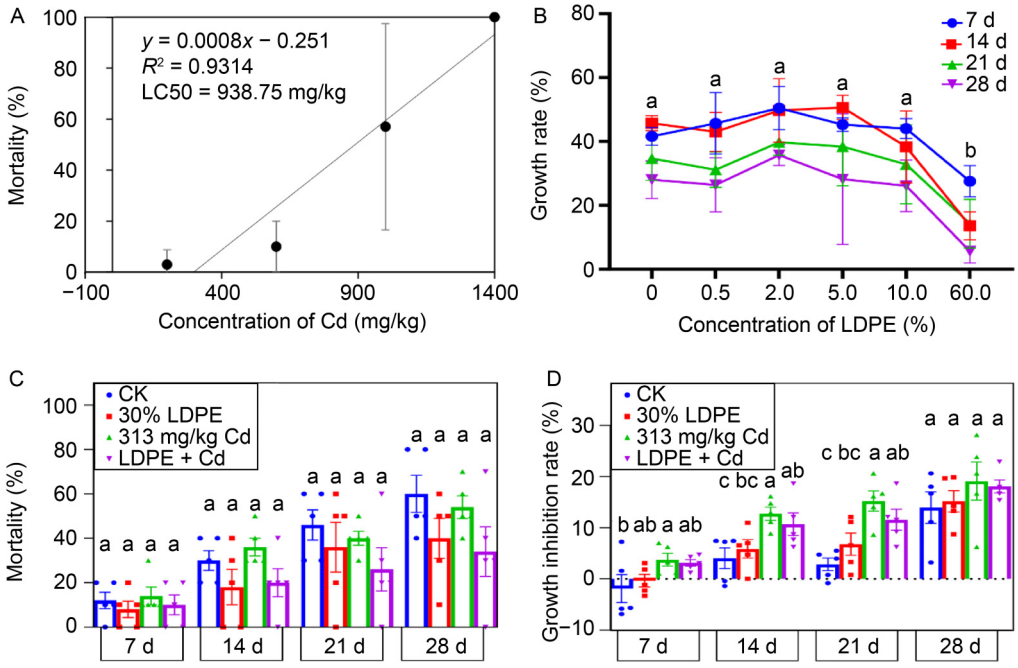


Fig. 1 LDPE has no effect on inhibition of earthworm growth caused by Cd. (A) The mortality of earthworms increased with increasing Cd concentration. (B) The growth rate of earthworms decreased in response to 60% LDPE. (C) Mortality of earthworms showed no significant difference among treatments on days 7, 14, 21, and 28. (D) LDPE (30%) had no effect on the inhibition of earthworm growth caused by 313 mg kg^{-1} Cd. Different letters (a–c) in the graphs indicate significant differences between treatments ($p < 0.05$).

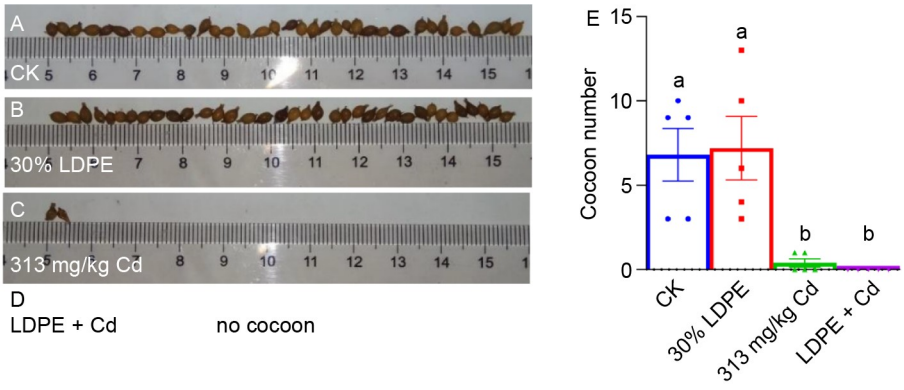


Fig. 2 LDPE (30%) has no effect on the inhibition of earthworm cocoon production cause by 313 mg kg^{-1} Cd. (A) Earthworm cocoons from the control group. (B) The number of earthworm cocoons in the 30% LDPE group was nearly equal to that in the control group. (C) Fewer earthworm cocoons were obtained from the 313 mg kg^{-1} Cd group than the control group. (D) LDPE (30%) did not alleviate the inhibition of earthworm cocoon production caused by 313 mg kg^{-1} Cd. (E) Statistical analysis of A, B, C and D. The data are expressed as mean ± standard deviation ($n = 5$). *$p < 0.05$.

alleviated damage to the intestinal tissue of the earthworm caused by Cd.

3.3 LDPE alleviates the DNA damage in coelomocytes caused by Cd stress

The coelomocytes of the earthworms in the control group were nearly spherical on day 28 (Fig. 4D). We found there is no DNA damage to coelomocytes under the exposure of 30% LDPE on days 7, 14, 21 or 28 (Fig. 4A′–D′). The tail DNA% values of the control group and LDPE treatment group were less than 5%, and the Olive tail moment (OTM) value was less than 2, indicating no damage (Fig. 4E–F). Notable tailing of coelomocytes was observed with 313 mg kg^{-1} Cd exposure on days 7, 14, 21, and 28 (Fig. 4A′′–D′′). Under 313 mg kg^{-1} Cd stress, the tail DNA% of body cavity cells was 40%–95%, and the OTM value was 25–55, demonstrating a high degree of damage (Fig. 4E–F). In the LDPE + Cd treatment group, the tailing was significantly reduced compared to the Cd treatment group (Fig. 4A‴–D‴). After 7–21 days of LDPE + Cd treatment, the tail DNA% was 55%–57%, and the OTM value was 18–20, (Fig. 4E–F).

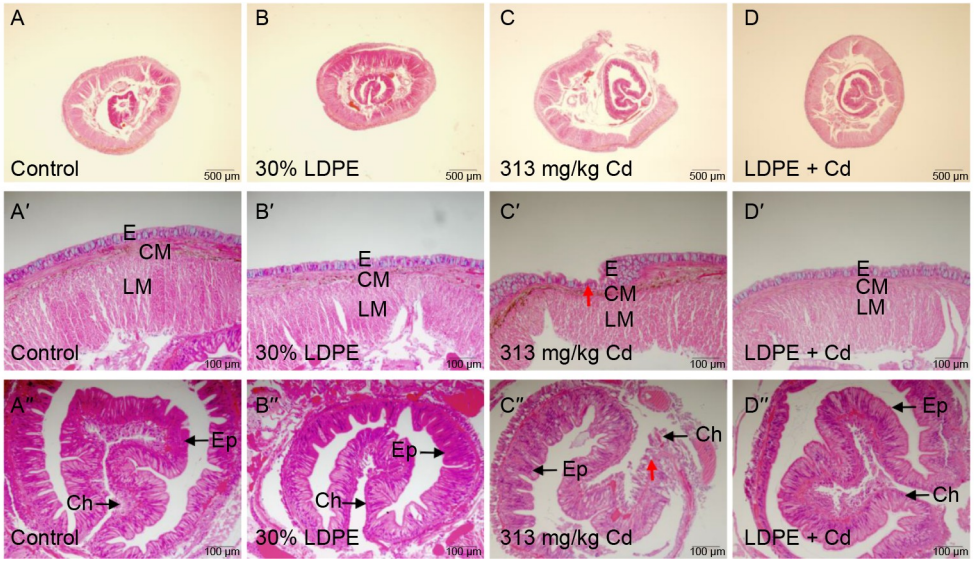


Fig. 3 LDPE alleviates the damage to the microstructure of *E. fetida* caused by Cd. (A–D) The body wall tissues of earthworms exposed to 313 mg/kg Cd were severely damaged (scale bars, 500 μm). (A′–D′) The epidermis of earthworms exposed to 313 mg kg^{-1} Cd was damaged and the circular muscle layer was thinned. E, CM and LM represent the epidermis, circular muscle and longitudinal muscle layers, respectively (scale bars, 100 μm). (A″–D″) The chlorogenic tissue of earthworms exposed to 313 mg kg^{-1} Cd was damaged. Ch and Ep represent the chlorogenic tissue and epithelial tissue of the intestinal tract, respectively (scale bars, 100 μm).

However, the tail DNA% was less than 5% and the OTM value was 0.65 on day 28, showing that no damage was caused and thus indicating that 30% LDPE alleviates the DNA damage in earthworm coelomocytes caused by 313 mg kg^{-1} Cd stress (Fig. 4E–F).

3.4 LDPE can aggravate the inhibition of the antioxidant system caused by Cd

CAT activity of earthworms decreased with exposure to 313 mg kg^{-1} Cd by days 14 and 21. The activity was lower for earthworms treated with 30% LDPE + 313 mg kg^{-1} Cd than in the 313 mg kg^{-1} Cd treatment on days 7 and 21, and then was higher on day 28 (Fig. 5A). POD activity was lower with exposure to 313 mg kg^{-1} Cd on days 14 and 21. LDPE reduced the POD activity driven by 313 mg kg^{-1} Cd on days 7 and 21 but increased the activity caused by 313 mg kg^{-1} Cd by days 14 and 28 (Fig. 5B). GSH activity increased with exposure to 313 mg kg^{-1} Cd by days 7, 21, and 28. LDPE decreased the activity associated with 313 mg kg^{-1} Cd by days 7 and 21 (Fig. 5C). On day 14, MDA activity increased with 313 mg kg^{-1} Cd exposure and LDPE decreased the MDA activity caused by 313 mg kg^{-1} Cd (Fig. 5D). The activity decreased with 313 mg kg^{-1} Cd exposure by days 7 and 21. LDPE increased the SOD activity driven by Cd exposure by days 7 and 14 (Fig. 5E).

3.5 LDPE + Cd increases the mRNA level of *MT*

The mRNA level of *MT* was higher with exposure to LDPE + Cd than in the control and 313 mg kg^{-1} Cd treatment groups on days 7, 14, 21, and 28. On day 14, *MT* gene expression was higher in the Cd treatment than in the control treatment (Fig. 6A). On day 28, the expression of the *ANN* gene was lower in the LDPE + Cd treatment than in the control (Fig. 6B). Compared to the control, the mRNA level of *TCTP* increased with exposure to LDPE + Cd by days 7 and 14. The mRNA level of *TCTP* was higher with exposure to LDPE + Cd than Cd alone on days 7 and 28 (Fig. 6C). The mRNA level of *HSP70* increased with exposure to 313 mg kg^{-1} Cd by day 14. The mRNA level of *HSP70* was higher with exposure to LDPE + Cd than Cd exposure alone on days 7 and 21 (Fig. 6D).

3.6 LDPE does not alter the accumulation of Cd in earthworms

The level of Cd was higher in the acid-extractable fraction (B1) than in the reducible (B2), oxidizable (B3), and residual fractions (B4). It was higher in earthworms treated with Cd and LDPE + Cd than in the CK and LDPE-treated earthworms. However, no difference was found in Cd content between earthworms in the Cd and LDPE + Cd treatments. LDPE did not alter the accumulation of Cd in the worms (Fig. 7).

4 Discussion

MPs are a global threat to soil health due to their direct

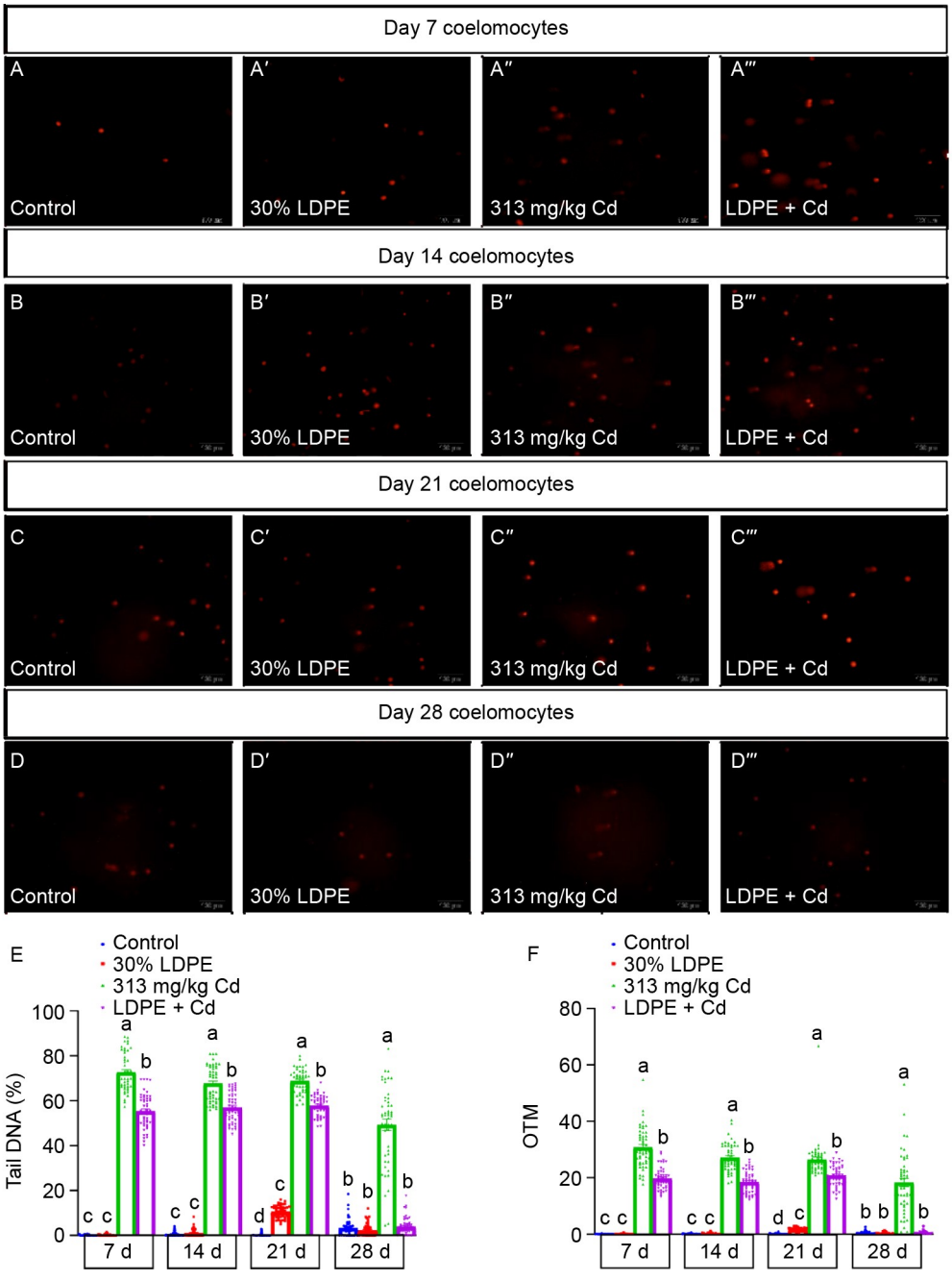


Fig. 4 LDPE can alleviate the DNA damage caused by Cd in earthworm coelomocytes. (A–A‴) On the day 7, Cd treatment and LDPE + Cd treatment was seen to have caused DNA damage to coelomic cells. (B–B‴) On day 14, Cd treatment and LDPE + Cd treatment was observed to have caused DNA damage to coelomic cells. (C–C‴) On day 21, Cd treatment and LDPE + Cd treatment was seen to have caused DNA damage to coelomic cells. (D–D‴) On day 28, LDPE alleviated the DNA damage caused by Cd treatment in coelomic cells (E). Control, 30% LDPE, 313 mg kg^{-1} Cd, and LDPE + Cd treated soils on days 7, 14, 21 and 28 (E). OTM values of the control, 30% LDPE, 313 mg kg^{-1} Cd and LDPE + Cd treated soils on days 7, 14, 21 and 28 (F). All data are expressed as mean ± standard deviation (n = 50), $p < 0.05$.

impacts on soil animals. Earthworms are widely considered model soil animals and are used in studies of ecological toxicity to assess the effects of soil pollutants such as heavy metals, nanomaterials, MPs, and various combinations of pollutants (Li et al., 2020a; Li et al., 2020b; Zhou et al., 2020; Huang et al., 2021; Sheng et al., 2021). However, the impact of MP exposure alone and co-exposure to MPs and Cd on earthworms remains unclear. In this study, we found that 30% LDPE did not change the survival or growth of earthworms. This finding is consistent with previous research showing no effect on the survival or weight of earthworms from MPs (Rodriguez-Seijo et al., 2017; Wang et al., 2019a). The indirect effect of LDPE on earthworms under long-term action is worthy of further study. The protective

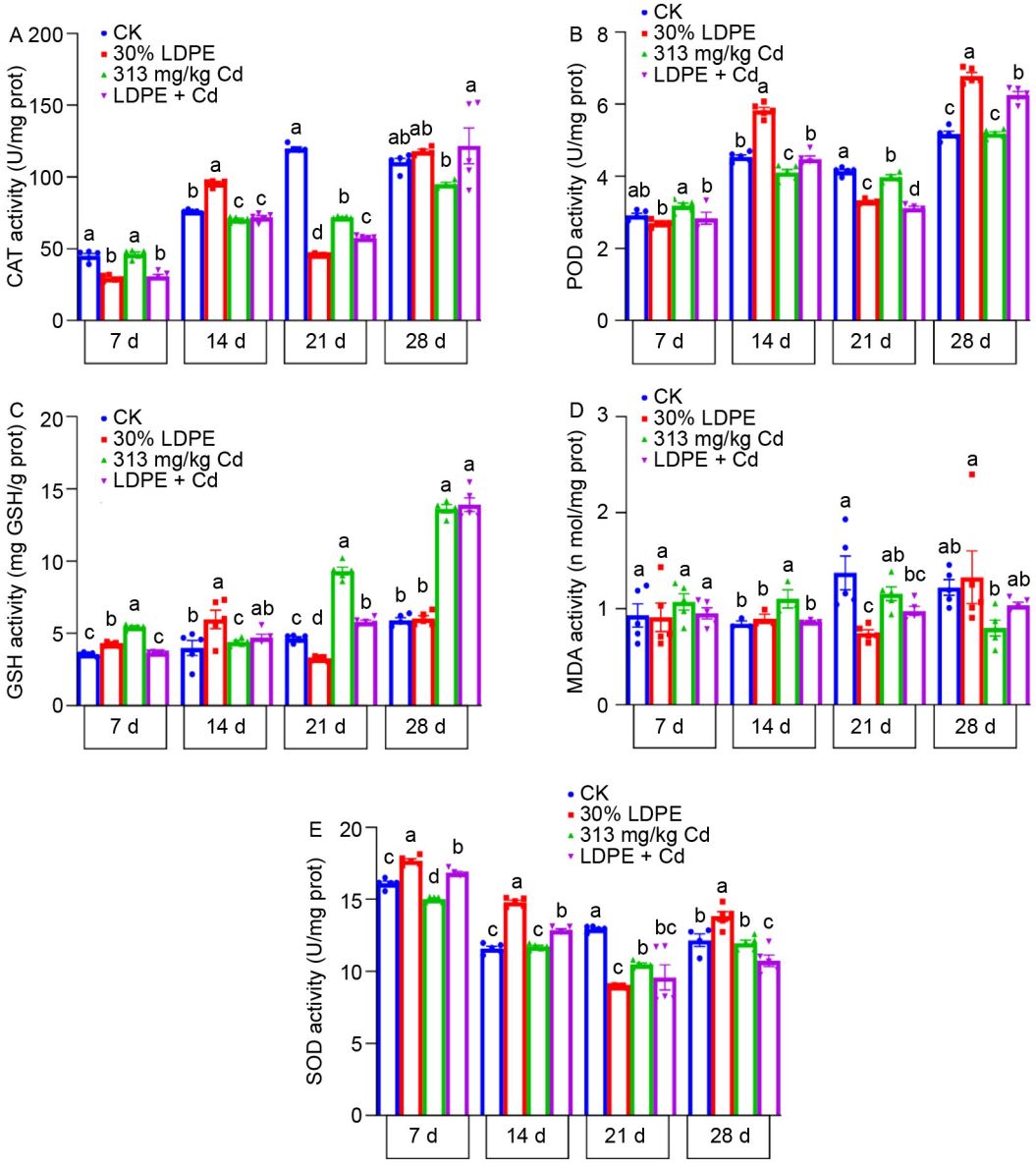


Fig. 5 LDPE may aggravate the inhibition of the antioxidant system caused by Cd. (A) On days 7 and 21, LDPE aggravated the decrease in CAT activity caused by Cd. (B) POD activity was lower in the Cd treatment than the control on the days 14 and 21. (C) To alleviate the strong toxicity of Cd, earthworms were stimulated to produce more GSH. (D) MDA activity in the Cd treatment was higher than in the control on day 14. (E) The presence of LDPE strengthened the inhibition of SOD activity caused by Cd by day 28. (n = 5), $p < 0.05$.

layer formed by soil particles on plastics decreases the toxicity of PE-MPs to earthworms (*Eisenia fetida*) (Liu et al., 2022). Also, when the LDPE was added to 60%, negative effects occurred. So, the dosage of LDPE plays an important role. LDPE may change the earthworm behavior of feeding selectivity or increase the immune system responses (Rodriguez-Seijo et al., 2017). The growth of earthworms was significantly inhibited with exposure to 313 mg kg^{-1} Cd, but compared to that treatment, the growth of those treated with combined 313 mg kg^{-1} Cd and 30% LDPE was not affected, indicating that LDPE does not alter the growth inhibition of earthworms caused by Cd exposure. The cocoon production of earthworms did not respond to exposure to 30% LDPE alone. On the other hand, treatment with 313 mg kg^{-1} Cd alone inhibited cocoon production. Combined treatment with 30% LDPE and 313 mg kg^{-1} Cd inhibited cocoon production to the same extent as 313 mg kg^{-1} Cd alone, indicating that 30% LDPE did not increase the toxic effect on cocoon production caused by 313 mg kg^{-1} Cd. However, this result is not consistent with previous research, showing that joint treatment with MPs and Cd increased the level of toxicity to earthworms (Zhou et al., 2020; Huang et al., 2021). This difference may be related to differences in the types and sizes of MPs among studies.

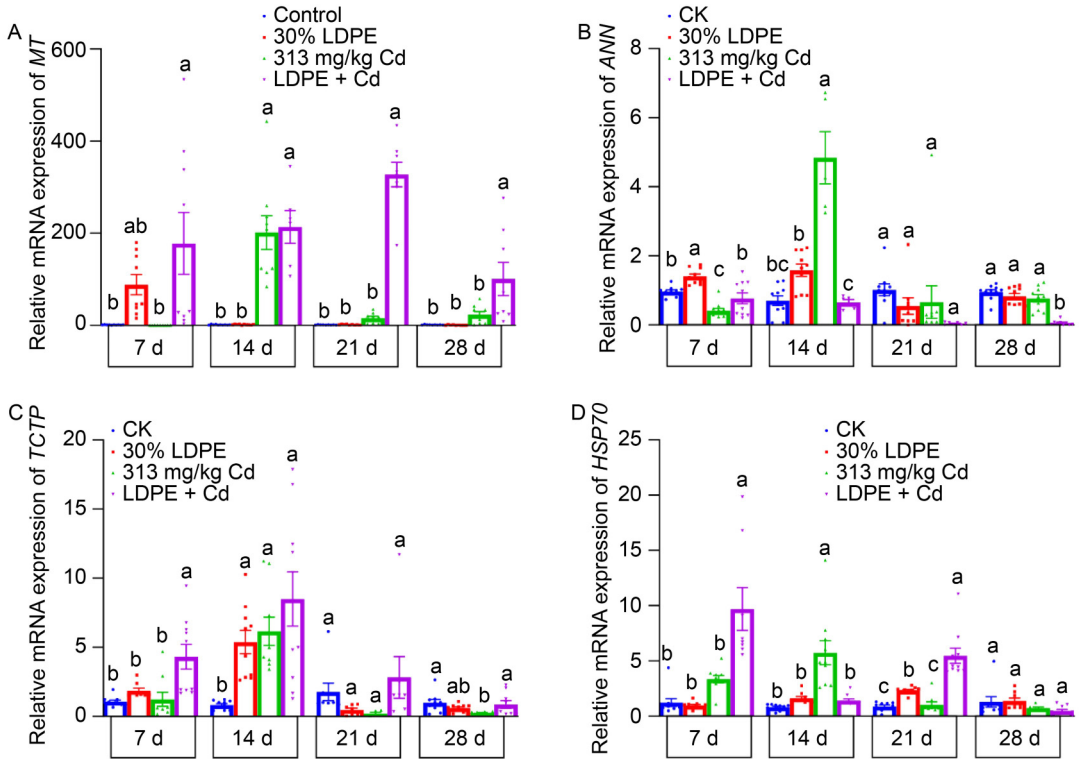


Fig. 6 The mRNA level of *MT* increases with coexposure of earthworms to LDPE and Cd. (A) The LDPE + Cd treatment increased *MT* expression in earthworms. (B) This result suggests that Cd and LDPE can reduce the expression of *ANN*. (C) *TCTP* expression was higher in the LDPE + Cd treatment than the Cd treatment on the days 7 and 28. (D) The *HSP70* mRNA level was higher in the LDPE + Cd treatment than the Cd treatment on days 7 and 21. Data are presented as mean ± standard deviation (n = 5), $p < 0.05$.

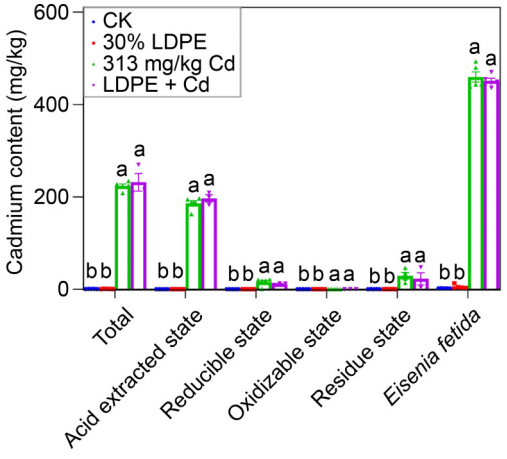


Fig. 7 LDPE does not affect the accumulation of Cd in *E. fetida*. The graph shows the following. Total: the total Cd content of soil (which was lower than the initial amount added to the Cd and LDPE + Cd treatments); acid-extractable fraction, that is, the level of Cd in the acid-extractable fraction; reducible fraction: the level of Cd in the reducible fraction; oxidizable fraction, that is, the level of Cd in the oxidizable fraction; residual fraction, that is, the level of Cd in the residual fraction; (E) the level of Cd in the *Eisenia fetida*. Data are presented as mean ± standard deviation (n = 5), $p < 0.05$.

The body wall and gut showed damage after exposure to 313 mg kg^{-1} Cd. Observation that heavy metal destroy the tissues of the earthworm has previously been reported (Lourenco et al., 2011; Wang et al., 2020; Yan et al., 2021). Here, LDPE alleviated the histopathological damage caused by Cd in earthworms. Comet analysis is an important method for monitoring and detecting genotoxic compounds in earthworms (Gajski et al., 2019). The OTM value can reliably indicate the degree of DNA damage (Song et al., 2009; Wang et al., 2016). In this study, 30% LDPE did not cause DNA damage in earthworm coelomocytes based on the comet assay, indicating that LDPE lacks DNA toxicity to earthworms. Marked tailing appeared in the coelomocytes exposed to 313 mg kg^{-1} Cd, indicating that the earthworms suffered serious DNA damage. Cd also caused serious DNA damage in earthworm in previous studies (Wu et al., 2012; Li et al., 2020a). Compared to 313 mg kg^{-1} Cd stress alone, the combined treatment with 30% LDPE + 313 mg kg^{-1} Cd led to significantly weakened tailing, indicating that LDPE can reduce the toxicity caused by Cd in earthworms.

MT is a low-molecular-weight peptide that is widely expressed in organisms. Numerous studies have confirmed that heavy metal exposure increases the mRNA and protein levels of earthworm MT, activating the expression of proteins involved in detoxification (Dedeke et al., 2016; Wang et al., 2023). In this study, the *MT* mRNA level with exposure to 313 mg kg^{-1} Cd was higher on day 14, but showed no response on days 7, 21, and 28, which may indi-

cate that 313 mg kg^{-1} Cd is too high, causing inhibition of *MT* gene expression by days 7, 21, and 28. Compared to 313 mg kg^{-1} Cd treatment, the *MT* mRNA level in the combined 30% LDPE + 313 mg kg^{-1} Cd treatment was elevated on days 7, 21, and 28. This result suggests that LDPE alleviates the toxicity caused by Cd in earthworms. Previous studies have demonstrated a close relationship between the *ANN* mRNA level and earthworm reproduction, and thus *ANN* can be considered a potential novel reproductive biomarker (Wang et al., 2019b) for use in earthworm toxicity tests. In this study, expression of the *ANN* gene was lower in the 313 mg kg^{-1} Cd and combined 30% LDPE + 313 mg kg^{-1} Cd treatments than in CK on day 28. This pattern suggests that combined LDPE and Cd treatment reduced the expression level of *ANN* mRNA. This finding is consistent with the observed change in the cocoon production rate. Our results indicate that 30% LDPE did not increase the toxicity caused by Cd in earthworms. To the best of our knowledge, this is the first study to show that LDPE and Cd do not have joint toxicity.

Some studies have shown that MPs increase the absorption of Cd (Huang et al., 2021; Zhou et al., 2020). In this study, on day 28, in earthworms treated with 313 mg kg^{-1} Cd or combined 30% LDPE + 313 mg kg^{-1} Cd, we detected CD contents that were higher than those of the CK and 30% LDPE earthworms. This pattern shows that *E. fetida* decreased the content of Cd in the soil because earthworms play an important role in enriching heavy metals, thereby protecting soil health. This finding is consistent with previous research (Wang et al., 2018b). The specific effect of heavy metal adsorption and desorption on MPs is related to the type and size of MP (Sheng et al., 2021). Our results showed that LDPE did not increase the accumulation of Cd in earthworms, indicating that LDPE used in this study may not be the carrier of Cd. The synergistic effects with Cd may be related to the type and size of MPs. The concentration of Cd used in this study was 313 mg kg^{-1}, which is higher than the levels used in other studies, and thus LDPE may have been unable to increase Cd absorption by earthworms because the maximum Cd uptake capacity of *E. fetida* had been reached. LDPE may have alleviated the toxic impacts of Cd on earthworms by altering physical and chemical properties of the soil.

5 Conclusion

To date, few studies have indicated that LDPE MPs do not increase the toxicity of Cd in earthworms, and thus our findings are inconsistent with recently published papers. We found that 30% LDPE had no effect on the mortality, growth, and cocoon production of earthworms. Moreover, LDPE did not increase the mortality, or the inhibition of growth and cocoon production caused by Cd. It alleviated the histopathological damage and DNA damage of coelomocytes to earthworms caused by Cd. The mRNA level of *MT* in earthworms increased with co-exposure to LDPE and Cd. LDPE did not alter the accumulation of Cd in earthworms. Further studies are needed to elucidate why LDPE MPs did not increase the toxicity of Cd in earthworms. The indicators described here can be used for toxicity safety assessment and soil ecological risk assessment of soils contaminated with both MPs and heavy metals.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31172091), the National Key Research and Development Program of China (No. 2018YFC0507204) and The 2115 Talent Development Program of China Agricultural University.

Author contributions

Song Zhang: Investigation, Formal analysis, Data curation, Methodology, Software, Validation, Writing-original draft. Yating Du: Data curation, Methodology, Validation, Software, Writing-original draft. Guangshen Shang: Data curation, Methodology, Validation, Writing-original draft. Kejiao Hu: Writing-original draft. Xing Wang: Project administration, Funding acquisition, Supervision, Conceptualization, Writing-review and editing.

Conflict of interest

The authors declare no competing of interest.

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