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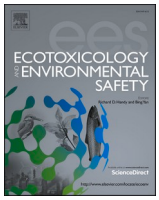

Toxicological impacts of microplastics on virulence, reproduction and physiological process of entomopathogenic nematodes

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ABSTRACT

Microplastics have emerged as significant and concerning pollutants within soil ecosystems. Among the soil biota, entomopathogenic nematodes (EPNs) are lethal parasites of arthropods, and are considered among the most effective biological agents against pests. Infective juveniles (IJs) of EPNs, as they navigate the soil matrix scavenging for arthropod hosts to infect, they could potentially encounter microplastics. Howver, the impact of microplastics on EPNs has not been fully elucidated yet. We addressed this gap by subjecting *Steinernema feltiae* EPNs to polystyrene microplastics (PS-MPs) with various sizes, concentrations, and exposure durations. After confirming PS-MP ingestion by *S. feltiae* using fluorescent dyes, we found that the PS-MPs reduced the survival, reproduction, and pathogenicity of the tested EPNs, with effects intensifying for smaller PS-MPs (0.1–1 μm) at higher concentrations (10^5 μg/L). Furthermore, exposure to PS-MPs triggered oxidative stress in *S. feltiae*, leading to increased reactive oxygen species levels, compromised mitochondrial membrane potential, and increased antioxidative enzyme activity. Furthermore, transcriptome analyses revealed PS-MP-induced suppression of mitochondrial function and oxidative phosphorylation pathways. In conclusion, we show that ingestion of PS-MPs by EPNs can compromise their fitness, due to multple toxicity effects. Our results bear far-reaching consequences, as the presence of microplastics in soil ecosystems could undermine the ecological role of EPNs in regulating pest populations.

1. Introduction

Plastic is omnipresent in our daily lives, offering advantages such as affordability in production, adaptability in shaping, lightweight construction, and long-lasting durability. However, projections suggest that annual plastic waste emissions could soar to 53 million metric tons by 2030 (Borrelle et al., 2020), exacerbating a myriad of environmental challenges on a global scale (Geyer, 2020). Particularly, after entering the environment, common plastic materials undergo mechanical or chemical weathering, leading to the generation of microplastics (MPs) – tiny plastic fragments measuring less than 5 mm in length – which can ultimately find their way into oceans, soils and food chains (Nizzetto et al., 2016). Because of their small size and their widespread occurrence in basically all ecosystems (Amaral-Zettler et al., 2015), MPs have been shown to accumulate in a wide range of organisms (Bouwmeester et al., 2015; Lenz et al., 2016; Mattsson et al., 2018), such as in oysters (Sussarellu et al., 2016), mussels, langoustines (Galloway et al., 2017), fishes (Foley et al., 2018; Cheng et al., 2023), copepods, short crabs, and lungworms (Franzellitti et al., 2019), affecting the animals' growth, reproduction or survival. Such tiny plastic particles could even be absorbed by human body and found in human bloodstream (Leslie et al., 2022). MPs have thus become an increasing concern in terms of environmental pollution worldwide (Thompson et al., 2009; Zeb et al., 2023).

While initial research primarily centered on MPs' presence in aquatic environments, particularly oceans, there is now a growing emphasis on their impact in terrestrial ecosystems (Holterman et al., 2006; Rillig, 2012). For instance, between 300 and 67,500 mg/kg of MPs were detected in Australian industrial soils (Fuler and Gautam, 2016), or up to 55.5 mg/kg of MPs were found in Swiss floodplain soils (Scheurer and

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Bigalke, 2018). Along those lines, it was found that agricultural soils might store more MPs than ocean basins (Nizzetto et al., 2016). MPs incorporated into the soil matrix can then be readily ingested by the soil biota, ultimately affecting their fitness. For example, exposure to high concentrations (28–60% w/w) of MPs caused physical lesions to the mucus membranes and higher genotoxicity in earthworms (Huerta Lwanga et al., 2016; Rillig et al., 2017; Xu et al., 2021). It was found that PS-MPs accumulated in the gut of *Caenorhabditis elegans* nematodes and after subacute exposure, MPs induced oxidative stress by reactive oxygen species (ROS) production, ultimately causing gut damage (Yu et al., 2020). Similarly, it was observed that the lifespan of *C. elegans* was shortened during long-term nanopolystyrene exposure, and the innate immune response was inhibited severely (Qiu et al., 2020). Accordingly, MPs can induce negative fitness consequences for soil organisms that ingest them. Furthermore, the health effects of MPs were also investigated in mammalian model mice, manifested in metabolic and gut microbial disorders (Shi et al., 2022). The transgenerational toxic effects of MPs had been investigated in *C. elegans* (Chen et al., 2021), *Daphnia magna* (Liao et al., 2023), rotifer (Yeo et al., 2023), etc. In addition, MPs increased gene flow in order to cause unintended consequences for microbiome health (Maguire and Gardner, 2023). For example, different MPs enriched the antibiotic resistance genes in sludge digester and promoted horizontal gene flow (Luo et al., 2023). Nevertheless, the effects of MPs on other soil biota remain largely untested. MPs distribution was related to the seasonal occurrence and monsoon promoted the increase of MPs concentration in the lake ecosystems (Warrier et al., 2022). The highly connected networks for transporting plastics among terrestrial, riparian, transitional, benthic and flood plains ecosystems are river systems (Windsor et al., 2019).

Among soil biota, terrestrial nematodes comprise a highly diverse group of species, and play a crucial role in soil ecosystems by enhancing nutrient cycling, promoting decomposition, aiding in soil structure maintenance (Yeates and Bongers, 1999), and by influencing soil community composition and diversity (Ferris, 2010). Among these roundworms, entomopathogenic nematodes (EPNs) are obligate parasites of arthropods, particularly pathogenic to a wide range of insect species in the orders Coleoptera, Lepidoptera, Homoptera and Diptera (Peters, 2013). Due to the soil-dwelling nature of a variety of insect pests affecting crop plants, EPNs have been extensively researched as potential biological control agents in agroecosystems. (Shapiro-Ilan et al., 2015; Koppenhöfer et al., 2020). Moreover, EPNs have been developed as model organisms for toxicity assessment due to their transparent bodies, short life cycles and easy artificial *in vitro* propagation (Guo et al., 2023).

The infective juvenile (IJ) stage of EPN, a stage analogous to the *C. elegans* dauer juvenile, is the only free-living stage within their life cycle, the rest being done inside an insect cadaver. Accordingly, IJs in the soil are developmentally arrested, and they only reinitiate development once they penetrate a suitable host (Lu et al., 2017). IJs are non-feeding stress-tolerant organisms that can persist in the soil over long periods of times up to one year before infecting new hosts (Koppenhöfer and Fuzy, 2009). IJs do not feed, as they possess a sealed mouth and a collapsed pharynx, and have protective sheaths formed by retaining the second stage cuticles (Crook, 2014). Accordingly, it can be hypothesized that the non-feeding properties of IJs would reduce the chance of pollutants, such as MPs, to accumulate in their bodies, hence limiting the potential negative effects of MPs on IJs' fitness. That said, the effect of MPs on EPN IJs has not been fully addressed to date (Prokić et al., 2021).

One way to measure toxicity of MPs on organisms is by measuring oxidative stress and pathological damage. Oxidative stress arises from an imbalance between the ROS production and cellular antioxidant defense system involving antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Papas et al., 2019). SOD is needed for catalyzing the disproportionate production of superoxide anion ($O_2^{\bullet-}$) radicals into hydrogen peroxide (H_2O_2) and oxygen, in order to protect cells from the damage produced by ROS (Houmani et al., 2016). Similarly, CAT can prevent cell damage by catalyzing the conversation of H_2O_2 to H_2O (Gill et al., 2010). Antioxidant enzyme activities induced against MPs have been reported in several organism, such as in the silkworm *Bombyx mori* (Muhammad et al., 2021), *Daphnia pulex* (Liu et al., 2020), zebrafish *Danio rerio* (Lu et al., 2016), or *Karenia mikimotoi* (Zhao et al., 2020). Similarly, transcriptome sequencing (RNA-Seq) has been used to measure the effects of MPs exposure in multiple organisms, such as in *C. elegans* (Lei et al., 2018a), tilapia (Pang et al., 2021), zebrafish (Xue et al., 2022), or *D. pulex* (Liu et al., 2021). Therefore, antioxidant enzyme activities and RNA-Seq could be utilized to explore the effects of MPs exposure on EPNs.

Since previous research has shown that polystyrene microplastic (PS-MPs) can induce negative fitness consequences on both marine (Lu et al., 2016) and terrestial organisms (Wang et al., 2021;Qiu et al., 2020), PS-MPs were selected to evaluate the toxicological impacts MPs on EPNs. Accordingly, we here studied the response of EPN IJs to polystyrene microplastics (PS-MPs) exposure at different sizes, concentrations, and exposure time (Chen et al., 2021; Kim et al., 2019; Lei et al., 2018b). First, we measured the distribution of the green fluorescence-labeled PS-MPs in IJs. Second, we assessed the physiological effects of PS-MP on IJs, including mortality, reproduction and pathogenicity. Third, we measured potential oxidative stress induced by PS-MP via ROS production, antioxidative activities and mitochondrial activity. Finally, we performed transcriptome sequencing to explore the underlying genetic mechanisms of PS-MP toxicity to IJs. Collectively, our study provided a holistic insight into the effects of PS-MPs exposure to EPNs.

2. Materials and methods

2.1. Entomopathogenic nematode preparation

The life cycle of EPN includes the egg stage, four juvenile stages and the adult stage. The third instar juveniles (IJs), particularly, represent a pivotal developmental phase, characterized by heightened mobility and infectivity, making them essential agents in biological pest management strategies across various agricultural systems (Ciche, 2007). Once a host is found and penetrated, IJs release mutualistic bacteria into the insect hemocoel, where they multiply, and ultimately kill the host from sepsis (Dowds and Peters, 2002; Han and Ehlers, 2000). On the other hand, the bacterial soup in the insect hemocoel allows EPNs to grow and reproduce for up to three generations (Lu et al., 2017). Nematode development continues until food resources from the insect cadavers are exhausted, after which nematode development is inhibited and infective larvae accumulate. The IJs will then disperse back into soil in search of new insect hosts. In the soil, IJs can survive for months, during which time they neither feed nor reproduce (Hominick, 1990; Gaugler and Kaya, 1991; Dempsey and Griffin, 2002).

The EPN *Steinernema feltiae* (Rhabditida: Steinernematidae), which can control a broad range of insect pests (Qiu et al., 2008), and that is currently commercially (Labaude and Griffin, 2018), was chosen as model system to assess MPs toxicity. Stock cultures of *S. feltiae* strain were obtained from David Shapiro-Ilan (USDA-ARS Southeastern Fruit and Tree-Nut Research Laboratory, Byron, Georgia, United States of America). Colonies were maintained on last-instar *Galleria mellonella* larvae according to standard protocols (Kaya and Stock, 1997). For all experiments described below, only freshly hatched IJs (maximum one week old) were used.

2.2. Microplastics

Green fluorescent and non-fluorescent polystyrene microplastics (PS-MPs), supplied as 1% (w/v) and 2.5% (w/v) mono-dispersed in deionized water respectively, were purchased from BaseLine Chromtech Research Centre (Tianjin, China). The stock suspensions were diluted

with distilled deionized water to 2, 20, 200, 2000, 200000 μg/L. Untreated PS-MPs were used for toxicity evaluation, while green fluorescent PS-MPs were used to observe PS-MPs intake by EPN IJs. PS-MP solutions were sonicated at 40 kHz for 30 min before use (Qiu et al., 2020). All experiments below were performed at 25 ℃.

2.3. PS-MPs ingestion assay

To measure the ingestion potential of MPs by EPNs, *S. feltiae* IJs were exposed to three different sized (0.1 μm, 1 μm, 5 μm) green fluorescent PS-MPs, at a concentration of 10^5 μg/L, and a control without MPs, for 72 h. For this, IJs were transferred to a 3% agar pad pre-dropped with 20 μL 0.25 mM levamisole and containing the prepared PS-MPs solutions. The distribution of the PS-MPs particles in the nematodes' bodies was observed under a positive fluorescence microscope (ZEISS Axio Imager Z2, Germany) and a laser scanning confocal microscopy (LSCM, Leica TCSSP5, Germany) at excitation wavelength of 488 nm and emission wavelength of 518 nm. LSCM allows acquiring three-dimensional images in order to confirm the uptake of PS-MPs by EPNs, while ruling out the possibility that MP particles were only attached to the EPNs' cuticles. Specifically, LSCM imagery allows combining the xy plane (focal plane) with the z axis (optical axis), ultimately generating a three-dimensional image of the sample. For this, individual IJs were scanned layer by layer in units of 1 μm. For better visualization, darkfield fluorescence images were superimposed with brightfield images.

2.4. Entomopathogenic nematode mortality assay

To assess the potential negative effects of MPs on EPNs, *S. feltiae* IJs suspension was mixed with PS-MPs solutions and added to 24-well tissue culture plates, each well containing 500 μL of PS-MPs solution and 50–60 IJs. The final MP concentrations for exposure were 1, 10, 100, 1000, 10^5 μg/L, and three sizes (0.1, 1 and 5 μm) and a control without MP were tested (Chen et al., 2021; Kim et al., 2019; Lei et al., 2018a). IJs mortality was observed with an inverted biological microscope (Olympus CKX41, Japan) at 24, 48 and 72 hours after exposure. We produced six replicates per treatment for a total N = 3 particle sizes × 6 concentrations × 3 exposure times × 6 replicates = 324 samples. The EPNs in a rigid state were considered as dead if there was no response after being stimulated with an eyebrow needle (Kim et al., 2019). Mortality rate (%) was calculated as the number of dead individuals divided by the total number of individuals in each well and multiplied by 100.

2.5. Entomopathogenic nematode reproduction assay

For assessing the effect of MPs on EPN reproduction, we first produced the mutualistic bacteria solution for EPNs to grow on. For this, bacteria were extracted from the haemolymph of EPN-infected *G. mellonella* larvae, purified on NBTA medium (32 g nutrient agar L^{-1}, 0.025 g bromothymol blue L^{-1}, 0.04 g 2,3,5-Triphenyltetrazolium chloride L^{-1}) at 25℃, and their identity confirmed as they appeared blue-green on the NBTA medium. Next, the TSY liquid medium (40 g tryptic soy broth L^{-1}, 5 g yeast extract L^{-1}) was used the bacteria at 25℃ under constant motion (180 rpm) in a shaker. The cultured bacteria were then supplied as food source to the PS-MPs-exposed IJs on nutrient agar (NA) medium (32 g/L). An aliquot of 400 μL bacterial fluid was added to each NA plate (Zhen et al., 2018). Subsequent to the PS-MPs exposure treatments as described above (N = 3 particle sizes × 6 concentrations × 3 exposure times × 6 replicates = 324 samples), IJs were washed in distilled deionized water three times, after which, 50–60 IJs were inoculated in each plate. Finally, after a four days incubation period at 25℃, EPNs were washed out of the dishes, and the numbers of offspring of all stages, except the eggs, were counted under an inverted biological microscope (Olympus CKX41, Japan) (Zhao et al., 2014).

2.6. Entomopathogenic nematode pathogenicity assay

To measure the effect of PS-MPs on EPN pathogenicity, last-instar *G. mellonella* larvae, individually placed in wells of a 24-well tissue culture plate, were added with approximately 50 PS-MP-exposed IJs in 40 μL water solution and incubated at 25℃ (Han and Ehlers, 2000). Exposure treatments were as described above, but we performed 25 replicates per treatment for a total of 25 replicates × 2 concentrations × 3 particle sizes × 2 exposure times + 1 control) = 325 samples. Insect larvae were checked every four hours and time-to-death was recorded.

2.7. ROS measurement

ROS production was assayed to indicate the level of oxidative stress induced by PS-MPs as described by Qu et al. (2019). The PS-MP concentrations for exposure were at 100 and 10^5 μg/L for two particle sizes (0.1 and 1 μm) and a control without PS-MP. Duration of exposure was 72 hours. Forty IJs per treatment were tested. The PS-MPs exposed EPNs were cultured with 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA, 20 μM) (ID3130, Solarbio, China) for 1.5 h at 20 °C in the dark and then washed three times with sterile water (Yu et al., 2020). The EPNs were immobilized on 3% agarose-padded slides by adding 20 μL 0.25 mM levamisole and then sealed with coverslips. Fluorescent images were collected under a positive fluorescence microscope (ZEISS Axio Imager Z2, Germany) at excitation wavelength of 488 nm and emission wavelength of 525 nm and analyzed by Image J software (the National Institutes of Health, Bethesda, MD, USA). The relative fluorescence intensity was expressed as the ratio of intestinal ROS signal to intestinal autofluorescence, and expressed as relative fluorescence units (RFU).

2.8. Determination of antioxidant enzyme activity

The activity of SOD and CAT enzymes were evaluated to investigate the oxidative stress in EPN IJs induced by PS-MPs. For the exposure treatment, IJs were exposed to PS-MPs of two sizes (0.1 and 1 μm) at concentrations of 100 and 10^5 μg/L and a control without PS-MP. After 72 h of exposures, IJs were washed three times and homogenized in ice-cold PBS (1:10). Homogenates were centrifuged at 12,000× g for 10 min (4 °C), and the supernatants were collected for subsequent assays. The total protein (TP) content in each sample was assayed using the BCA Protein Assay Kit (ADS-W-SP002, Jiangsu Edison Biotechnology, China) according to the manufacturer's instruction. SOD and CAT activities were assayed based on enzyme-linked immunosorbent assay (ELISA) method, using the SOD ELISA Kit (MK530408B, Jiangsu Sumeike Biological Technology, China) and the CAT ELISA Kit (MK530386B, Jiangsu Sumeike Biological Technology, China) according to the manufacturer's instructions, respectively. The output results of TP contents, SOD and CAT activities were measured through reading the absorbance Optical Density (O. D.) using a microplate reader (Thermo Scientific MK3, USA) at 570 nm (TP contents) and 450 nm, for SOD and CAT, respectively.

2.9. Mitochondrial membrane potential (MMP) assay

MMP was determined by flow cytometry (McKinnon, 2018). JC-1 (5, 5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide), a cationic and carbocyanine fluorescent dye, was used to study mitochondrial behavior (Perelman et al., 2012). JC-1 exists as a monomer in the cytoplasm and emits at 530 nm (green fluorescence) while JC-1 monomers accumulate in the intact, negatively charged and hyperpolarized mitochondria and form JC-1 aggregates (red fluorescence) emitted at 590 nm after excitation at 488 nm, that is the basis on which JC-1 is used to distinguish between energized and deenergized mitochondria (Chaoui et al., 2006). Hence, normal cell populations with intact mitochondria and abnormal cell populations with damaged mitochondria can be separated through flow cytometry.

EPN IJs were exposed to PS-MPs of two sizes (0.1 and 1 μm) at concentrations of 100 and 10^5 μg/L and a control without PS-MP for 72 h. PS-MPs exposed EPN IJs were washed three times and homogenized in PBS in order to obtain the experimental cell suspensions. Cell concentrations were adjusted to 5×10^5 cells/mL after performing cell counting on a hemocytometer. Cells were then incubated in PBS with JC-1 (Sigma-Aldrich) monomer at 10 μg/mL for 20 min at 37 °C in the dark. JC-1 labeled cells were next washed with PBS three times and immediately analyzed under flow cytometry (BD FACS Calibur, USA, equipped with a 488 nm argon excitation laser). Red fluorescence derived from the mitochondrial JC-1 aggregates and green fluorescence derived from cytoplasmic JC-1 monomers were analyzed on the FL-1 and FL-2 channels, respectively.

2.10. mRNA sequencing and transcription profiling analysis

IJs were exposed to PS-MP concentrations of 100 and 10^5 μg/L of two sizes (0.1 μm and 1 μm) PS-MP and a control without PS-MP for the exposure duration of 72 h. Each treatment contained four replicates and each replicate contained 100 mg of IJs. A total of 20 samples were prepared for RNA-seq. RNA extraction, sequencing and gene assembly methods are described in supplementary material, Methods S1.

Next, we performed differentially expressed genes (DEGs) and functional enrichment analyses. Following annotation, FPKM (fragments per kb per million reads) and read count values for each unigene was calculated using RSEM (v1.3.1) (http://deweylab.biostat.wisc.edu/rsem/) (RSEM 2011). The DEGs between the control and the PS-MP treated group were determined by the DESeq (v1.24.0) (http://bioconductor.org/packages/stats/bioc/DESeq2/). A fold change (FC) $\geq$2 and P value <0.05 was used as the criteria to determine the DEGs. To visualize the overall distribution of DEGs, volcano plots were created in R 4.2.0. To inspect the functions of DEGs, GO enrichment and KEGG pathway enrichment analyses were performed for the DEGs in Majorbio platform (www.majorbio.com) and R 4.2.0. The thresholds for significant enrichment of GO or KEGG with DEGs were set as Q value <0.05, which was obtained by correcting the P value by false discovery rate (FDR) method.

2.11. Statistical analysis

Three-way analyses of variance (three-way ANOVAs) were conducted to determine the interactive effect of PS-MPs size, concentration and exposure duration on EPNs' mortality, reproduction, and pathogenicity. Two-way ANOVAs were performed for testing the effect of PS-MPs size and concentration on ROS production, antioxidant enzyme activity and flow cytometry analysis (FCA). ANOVAs were carried out using SPSS 21.0 software (SPSS Inc., USA). Figures were created using the *ggplot2* package (Wickham, 2016) in R v. 4.2.0. Data were expressed as mean ± standard error, and significant differences were determined at P<0.05. The data of EPN mortality were arcsine-square root--transformed to achieve normal distribution.

3. Results

3.1. Ingestion of PS-MPs by EPNs

No green fluorescent MP particles were found in *S. feltiae* IJs control group (Fig. 1A), or when IJs were added with 5 μm PS-MPs (Fig. 1B). On the other hand, 0.1 μm and 1 μm PS-MPs were visibly ingested by IJs, as shown in Figs. 1C and 1D. Those small-sized MP particles were visible in the anterior, middle and posterior regions of the nematodes. MP ingestion was further confirmed using laser scanning confocal microscopy, for both 0.1 μm and1 μm (Fig. 2). For instance, the distribution of 0.1 μm particles was visible at Z=13 (Fig. 2B), or at Z=25 (Fig. 2C), the optical section of the middle of the EPN's body, while the distribution of 1 μm particles inside the nematode was observed at Z=0 (Fig. 2D), Z=17 (Fig. 2E), Z=33 (Fig. 2F).

3.2. Effect of PS-MPs exposure on EPN mortality

We found that there was no significant interaction of particle size, concentration or exposure time on EPN mortality ($F_{(20,\ 270)}$=1.114,

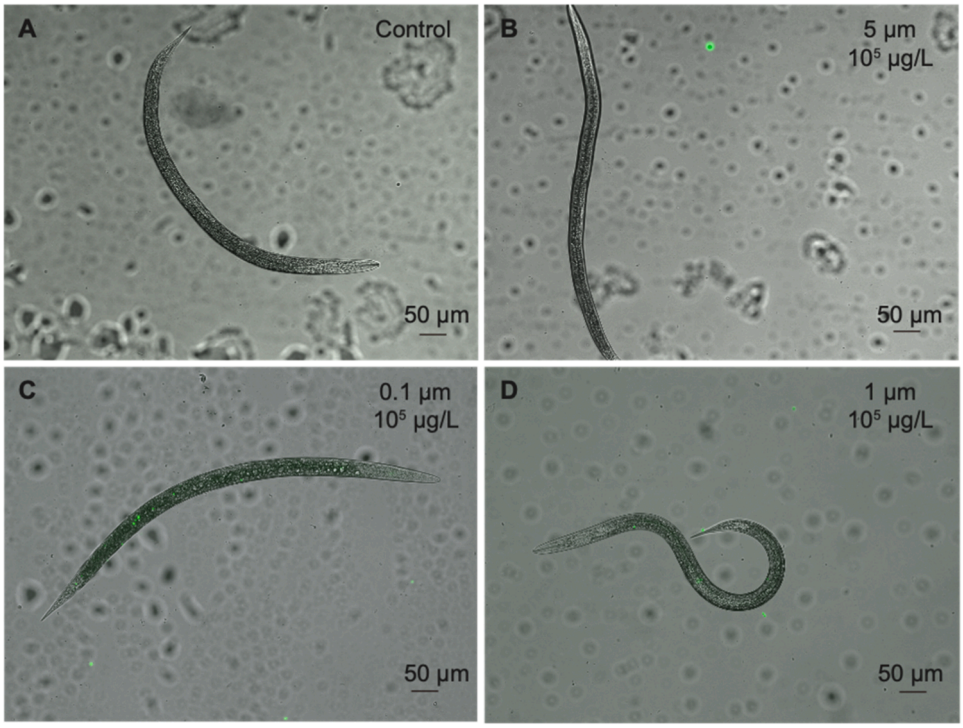

Fig. 1. Positive fluorescence microscope images showing the distribution of green fluorescent PS-MPs in *S. feltiae* IJs for the control group, without PS-MPs (A), or groups added with 10^5 μg/L of 5μm sized PS-MPs (B), 0.1μm sized PS-MPs (C), and 1 μm sized PS-MPs (D).

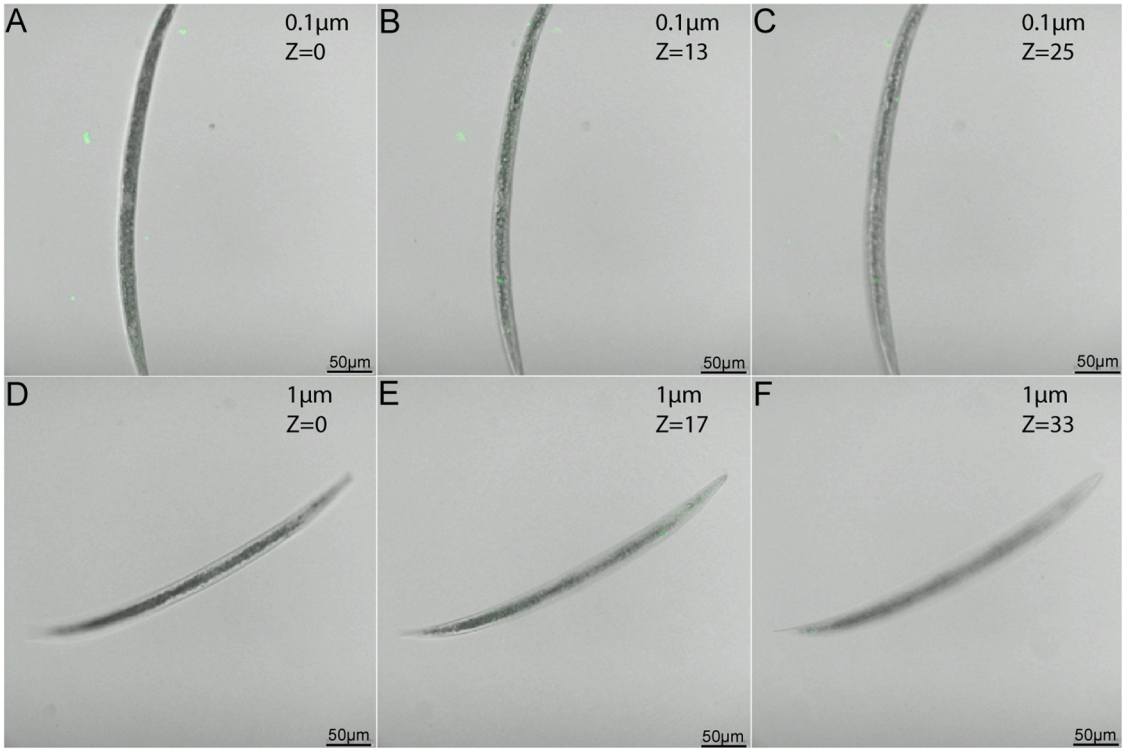

Fig. 2. Laser scanning confocal microscopy images showing the distribution of green fluorescent PS-MPs *S. feltiae* IJs added with 10^5 μg/L of 0.1 μm sized PS-MPs, and at Z=0 (A), Z=13 (B), Z=25 (C), or added with 10^5 μg/L of 1 μm sized PS-MPs, and measured at Z=0 (D), Z=17 (E), Z=33 (F).

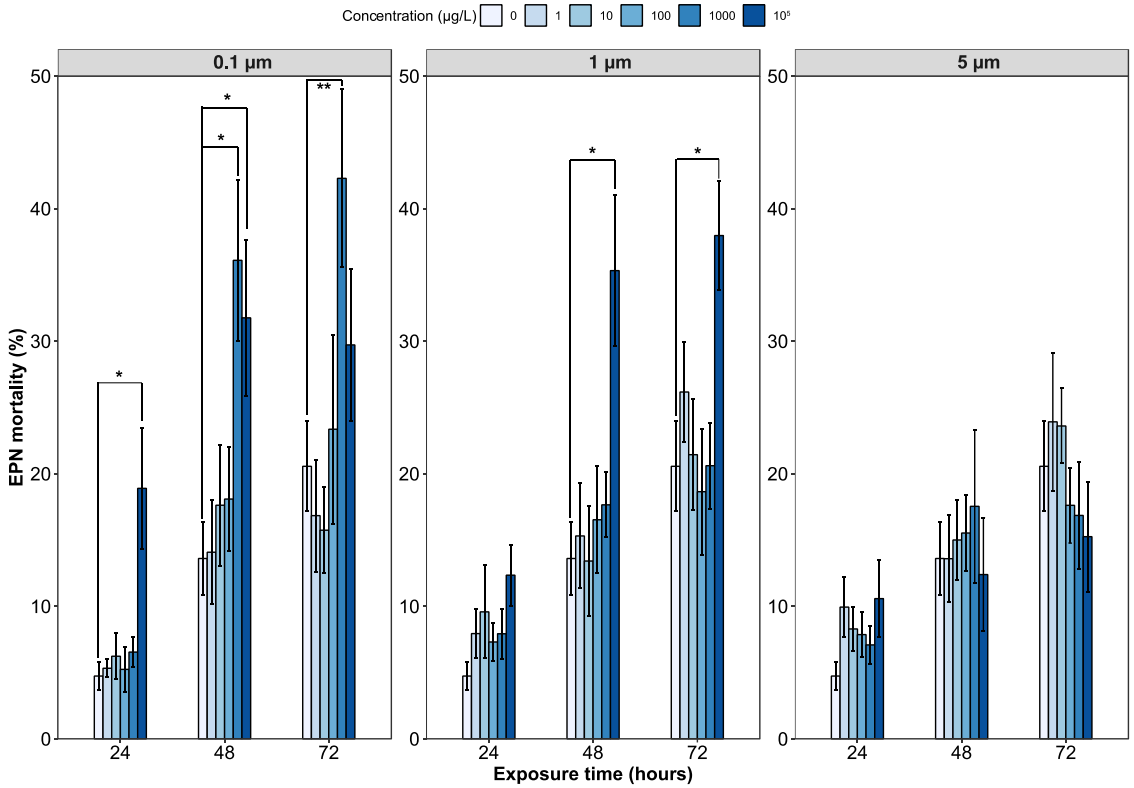

Fig. 3. Mortality (mean± SE, n=6) of *S. feltiae* IJs after being exposed to a range of five different concentrations (0, 1, 10, 100, 1000 and 10^5 μg/L), three different particle sizes (0.1, 1 and 5 μm) and three different exposure times (24, 48 and 72 h) of PS-MPs. A control group without PS-MPs (0 μg/L) was run for each exposure time. $^*P < 0.05$ or $^{**}P < 0.01$ indicates significant differences comparing with the control.

P=0.334). However, we found an interaction effect between particle size and concentration ($F_{(10,\ 270)}$=4.768, P<0.001). Specifically, we found that the PS-MPs at 0.1 μm induced 21.7% and 25.4% higher mortality than the MPs at 1 μm and 5 μm, respectively (particle size effect, $F_{(2,\ 270)}$=4.292, P=0.015), but this effect was contingent on the concentration. Pairwise comparisons showed that the mortality at the concentration of 10^3 μg/L (exposure 48 h or 72 h) and 10^5 μg/L was 54.3%–74.1% higher than the control when the particle size was 0.1 μm (Bonferroni test, P<0.001). When the particle size was 1 μm, the mortality at the concentration of 10^5 μg/L was significantly different from the control after exposing to the PS-MP particle for 48 h and 72 h (Bonferroni test, P<0.001).

Moreover, EPN mortality increased with exposure time, especially for the PS-MP particle sizes of 0.1 μm and 1 μm (Fig. 3 and Table S1). When EPNs were treated with the 5 μm PS-MP particles at the higher concentrations (100, 1000 and 10^5 μg/L), mortality was the same among the three exposure times. The control group at 24 h had the lowest mean mortality rate of 4.7±1.0% (mean ± SE), while 0.1 μm, 1000 μg/L MP-exposed group at 72 h showed the highest mortality of 42.3±6.7% (mean ± SE) (Table S1). After 24 h, higher mortality rates were found at concentration of 10^5 μg/L, where 0.1 μm PS-MP treatment had the highest value (Fig. 3). After 48 h, survival of EPNs in 0.1 μm-1000 μg/L, 0.1 μm-10^5 μg/L and 1 μm-10^5 μg/L MP-exposed groups was significantly negatively affected (Fig. 3). After 72 h, exposure to PS-MPs 0.1 μm (1000–10^5 μg/L) and 1 μm (10^5 μg/L) led to more death, but there was no significant difference in the mortality of 0.5 μm (1–10^5 μg/L) treatment compared to the control group (Fig. 3).

3.3. Effect of PS-MPs exposure on EPN reproduction

Three-way ANOVA showed that there was no significant interaction among the sizes of PS-MP particles, the concentrations and the exposure duration on reproduction ($F_{(20,\ 270)}$=1.110, P=0.339). However, the two-way tests revealed significant interactions between particle size and concentration ($F_{(10,\ 270)}$=4.753, P<0.001), and between concentration and the exposure duration ($F_{(10,\ 270)}$=2.150, P=0.021). Particularly, while we found no difference in the number of EPN offspring in control groups among the three exposure times ($F_{(2,\ 270)}$=2.259, P=0.106), the number of EPN offspring significantly decreased with exposure time, mostly for particle sizes of 0.1 μm and 1 μm (Table S2). A similar decrease only occurred at the concentration of 100 and 10^5 μg/L for the PS-MP particle of 5 μm. After 24 h-MP-exposure, EPNs were found to produce 3.3%–65.6% fewer offspring than that in control group. The number of MP-exposed EPN offspring was 9.3%–71.3% less than control group after 48 h-exposure. After 72 h-exposure to PS-MPs, numbers of offspring declined by 5.5%–78.3% compared to that of control group. Numbers of MP-exposed EPN offspring decreased with increasing MP concentrations and exposure durations (Fig. 4). Compared to the control group, the greatest reduction was found at 0.1 μm, 10^5 μg/L PS-MP after 72 h-exposure. PS-MPs with the sizes of 0.1 and 1 μm inhibited EPN reproduction more strongly than that of 5 μm particles.

3.4. Effect of PS-MPs exposure on EPN pathogenicity

We estimated EPN pathogenicity by measuring survival time (hours) of *G. mellonella* larvae. The longer the survival time of *G. mellonella*, the lower the pathogenicity of EPN. All *G. mellonella* larvae died after being infected with EPNs, but we found variation due to the treatments (Fig. 5, three-way ANOVA for particle size, concentration and exposure duration, $F_{(4,\ 432)}$=2.929, P=0.021). Overall, *G. mellonella* larvae died slowly when EPNs were treated with 0.1 μm PS-MP, and this effect, as this particle size, increased with concentration and exposure time (Fig. 5 and Table S3).

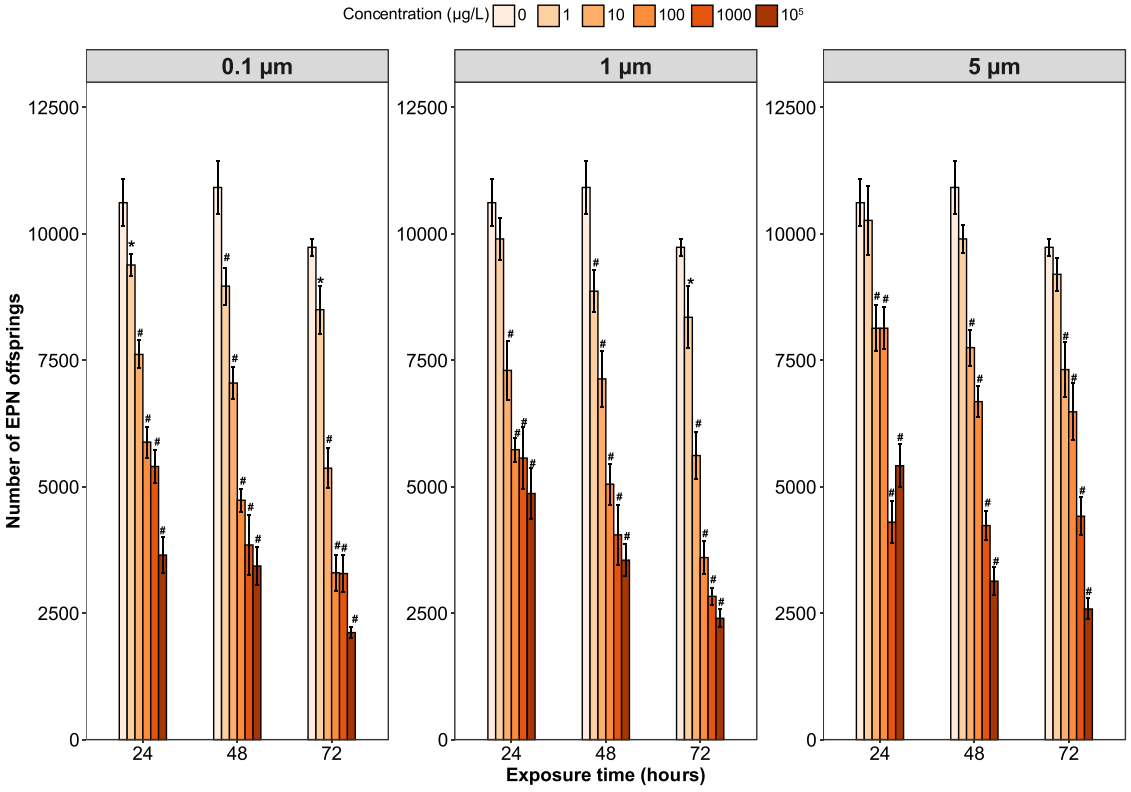

Fig. 4. Number of offspring (mean ± SE, n=6) of *S. feltiae* IJs after being exposed to a range of five different concentrations (0, 1, 10, 100, 1000 and 10^5 μg/L), three different particle sizes (0.1, 1 and 5 μm) and three different exposure times (24, 48 and 72 h) of PS-MPs. A control without PS-MPs (0 μg/L) was run for each exposure time. *P < 0.05 or # P < 0.001 indicates significant differences comparing with the control.

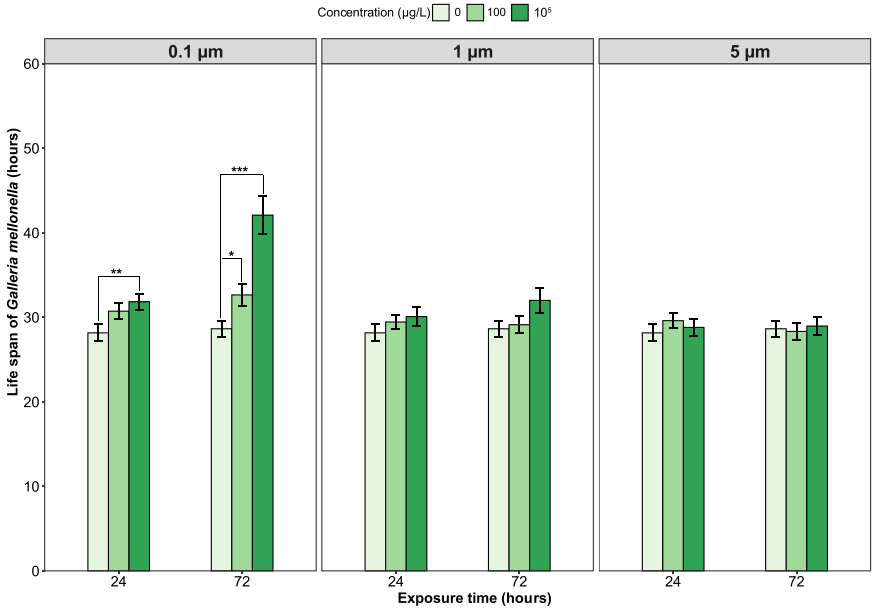

Fig. 5. Time-to-death (mean ± SE, n=25) of *G. mellonella* after being infected with *S. feltiae* IJs, which were exposed to two concentrations (100 and 10^5 μg/L), three particle sizes (0.1, 1 and 5 μm) and two exposure times (24 and 72 h) of PS-MPs. A control without PS-MPs (0 μg/L) was run for each exposure time. $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ indicate significant differences comparing with the control.

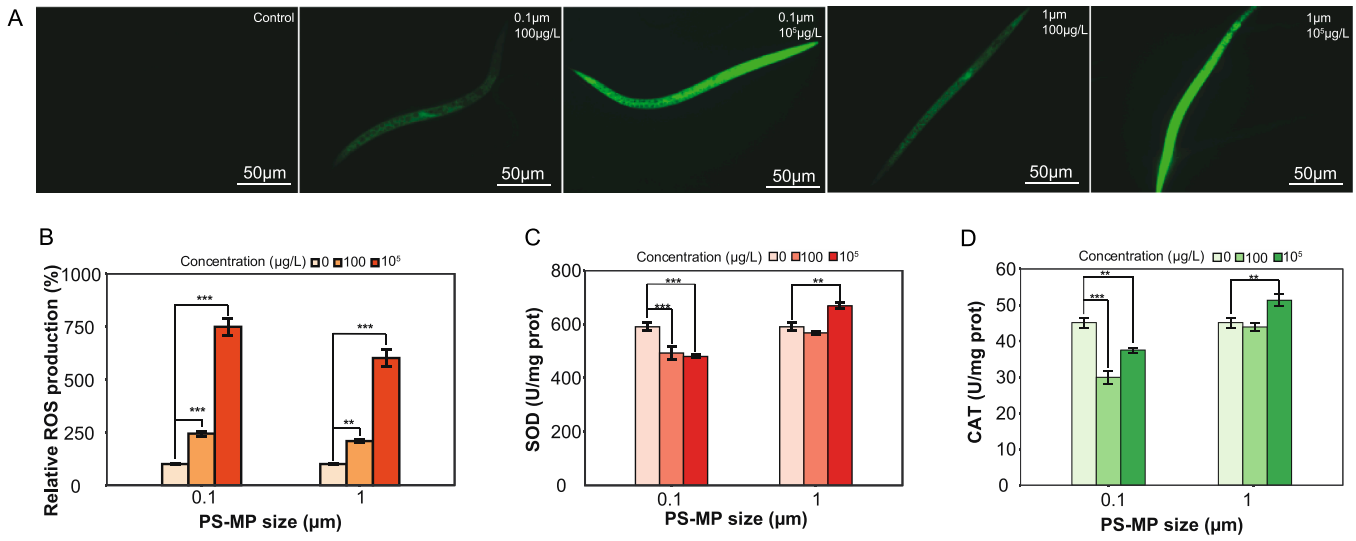

Fig. 6. Fluorescent images of ROS production (A), relative ROS production (B), SOD activities (C) and CAT activities (D) of *S. feltiae* IJs after being exposed to two concentrations (100 and 10^5 μg/L), and two different particle sizes (0.1 and 1 μm) of PS-MPs. The relative fluorescence intensity compared with the control group are presented as mean ± SE (n=40) (B). SOD and CAT activity compared with the control group are presented as mean ± SE (n=3) (C and D). A control without PS-MPs (0 μg/L) was run for each particle size. $^{**}P < 0.01$ or $^{***}P < 0.001$ indicate significant differences comparing with the control.

3.5. Effect of PS-MPs exposure on ROS production in EPNs

Two-way ANOVA showed that the interaction between particle size and concentration was significant ($F_{(2,\ 234)}$=4.956, P=0.008) (Fig. 6). The PS-MP concentration had significant effects on the ROS production at the particle size of 0.1 μm ($F_{(2,\ 234)}$=194.283, P<0.001) and 1 μm ($F_{(2,\ 234)}$=116.438, P<0.001). Both PS-MP concentrations of 10^2 μg/L and 10^5μg/L significantly increased the ROS production compared with the control (Bonferroni test, P<0.001). Fluorescence analyses of EPNs showed that ROS production was dependent of both particle size and concentration (Fig. 6A and Table S4), with ROS activity increasing with concentration, but with the highest values for ROS signals found in EPNs exposed to 10^5 μg/L of 0.1 μm PS-MP (Fig. 6B).

3.6. Effect of PS-MPs exposure on antioxidant enzyme activities

Antioxidant enzyme activities were measured as indirect means for assessing toxic effects of PS-MPs-mediated oxidative stress in EPNs. PS-MPs exposure caused idiosyncratic changes in SOD and CAT activities depending on particle size and concentration (Fig. 6; two-way ANOVA, for SOD ($F_{(2,\ 12)}$=23.563, P<0.001), and for CAT ($F_{(2,\ 12)}$=16.730, P<0.001)). Particularly, SOD activity decreased with increasing concentration at 0.1 μm, but increased with concentration at 1 μm (Fig. 6C and Table S5), and a similar pattern occurred for CAT (Fig. 6D and Table S6).

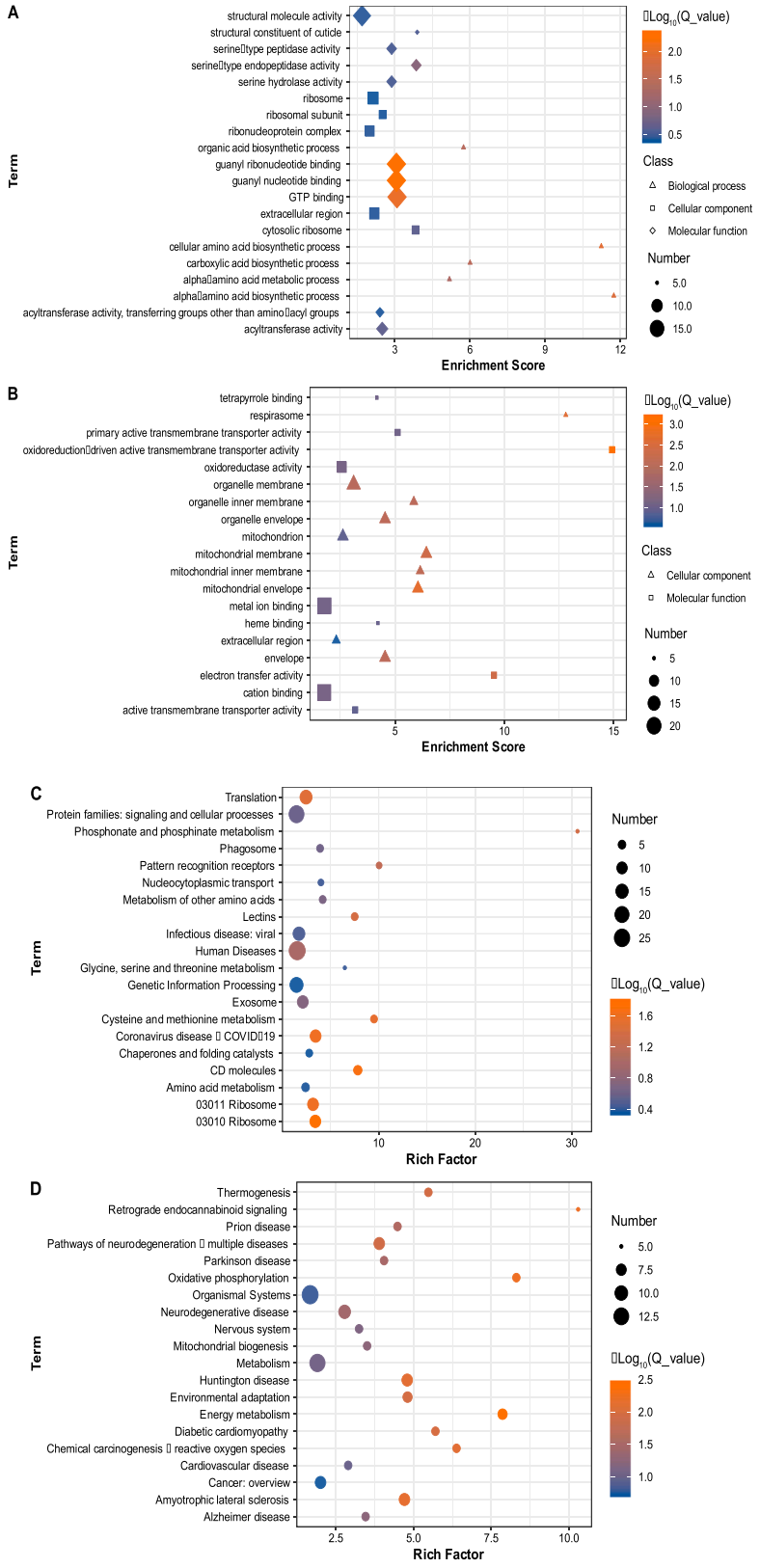

Fig. 7. GO terms enrichment and KEGG enrichment of DEGs in entomopathogenic nematodes *S. feltiae* exposed to PS-MP, relative to the control. (A) GO enrichment analysis with all up-regulated DEGs. (B) GO enrichment analysis with all down-regulated DEGs. (C) KEGG enrichment analysis with all up-regulated DEGs, (D) KEGG enrichment analysis with all down-regulated DEGs. (E) KEGG enrichment with 19 common DEGs in the two treated group with PS-MP at concentration of 10^5 μg/L. (F) Effect of PS-MPs exposure on OXPHOS-related gene expression in *S. feltiae*. COX1: cytochrome c oxidase subunit I; COX2: cytochrome c oxidase subunit II; ND1: NADH dehydrogenase subunit 1; ND5: NADH dehydrogenase subunit 5.

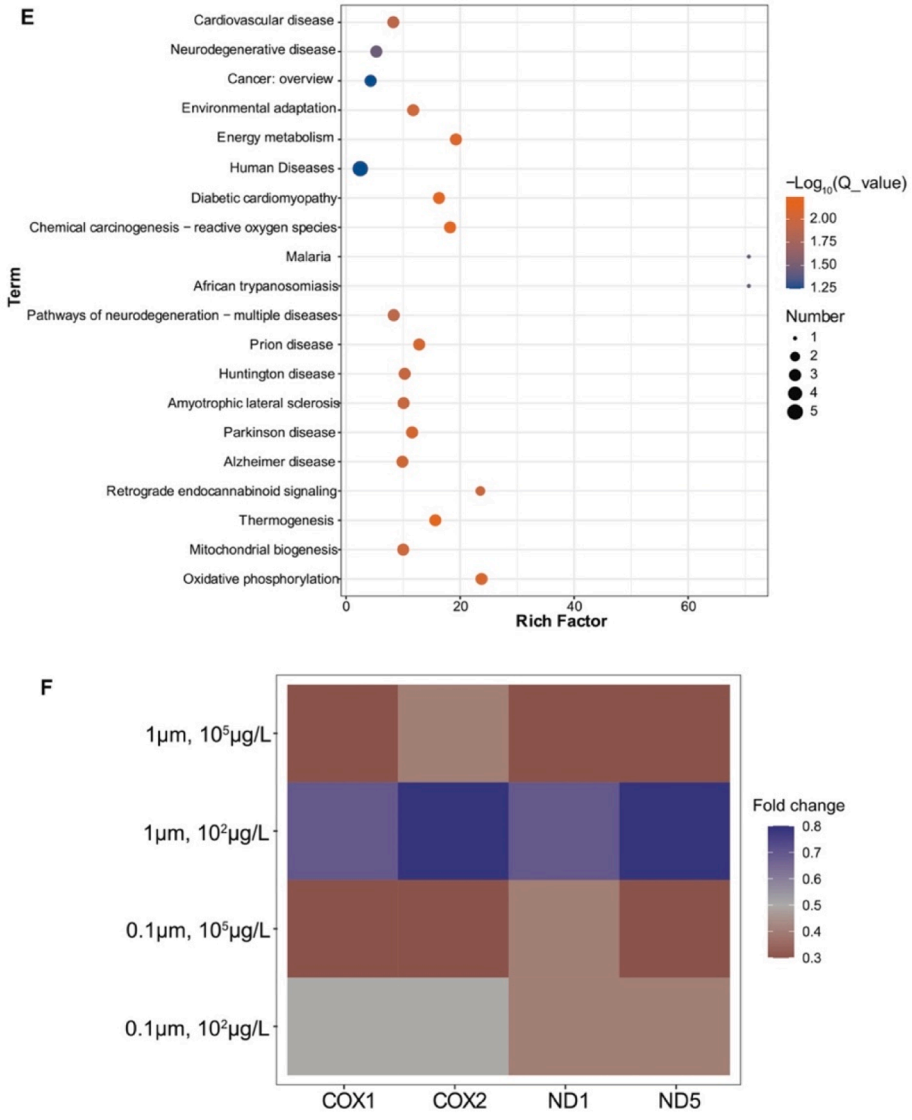

Fig. 7. (*continued*).

3.7. Effect of PS-MPs exposure on MMP of EPN cells

Mitochondrial red fluorescence of JC-1 aggregates and cytoplasmic green fluorescence of JC-1 monomer were analyzed by FCA. As shown in Fig. S1A, the amount of mitochondrial depolarization was determined by the proportion change in JC-1 labeled fluorescence from red to green, in which the upper right (UR) quadrants represent the intact fraction labeled as JC-1 red, while the lower right (LR) quadrants represent the damaged fraction labeled as JC-1 green. Higher ratios of JC-1 monomers indicate lower MMP. We found an interactive effect of particle size and concentration on FCA ($F_{(2, 6)}$=18.241, P=0.003) (Fig. S1B). The particle size ($F_{(1,6)}$=22.897, P=0.003) had significant effects on the ratio of JC-1 monomers. PS-MPs of 1 μm markedly induced JC-1 monomers 7.95% and 5.83% higher than those of control group (P<0.001) and 0.1 μm (P<0.001) group, respectively (Table S7). At higher concentration of 10^5 μg/L, PS-MPs of 0.1 μm (P<0.001) and 1 μm (P<0.001) caused JC-1 monomers 12.71% and 13.17% higher than control group, respectively. Lower MMP induced by PS-MPs was concentration dependent ($F_{(2, 6)}$=295.277, P<0.001), which increased with the increasing dose.

3.8. Transcriptomic response of EPNs to PS-MP exposure

3.8.1. Analysis of differentially expressed genes (DEGs)

The RNA sequencing generated 149.31 Gb of clean data. On average, the GC content of the generated sequences was 49.84%, while the sequence quality score was above Q30 for 92.11% of the reads. The *de novo* assembled transcriptome included a total of 40944 unigenes. A total of 21516 gene transcripts were annotated in the six databases. The BLAST results showed 13914 (64.67%), 9200 (42.76%), 10558 (49.07%), 10894 (50.63%), 11536 (53.62%) and 7142 (33.19%) unigenes that matched the NR, Swiss-Prot, Pfam, COG, GO and KEGG databases, respectively. A total of 728 DEGs were identified, among which 473 DEGs were upregulated and 255 DEGs were downregulated (Fig. S2). The 728 DEGs were compared in EPNs between each of the MP exposure groups and the control group (Fig. S3). In total, 127 (including 74 upregulated and 53 downregulated), 206 (including 116 upregulated and 90 downregulated), 268 (including 209 upregulated and 59 downregulated) and 127 (including 74 upregulated and 53 downregulated) DEGs were identified following treatment with 0.1 μm-100 μg/L, 0.1 μm-10^5 μg/L, 1 μm-100 μg/L and 1 μm-10^5 μg/L, respectively.

3.8.2. Enrichment analysis of DEGs after microplastic exposure

Gene Ontology (GO) terms showed that the several changes for genes coding for cellular components and molecular functions, such as amino acid biosynthetic process, or membrane and nucleotide binding proteins, especially for the 1 μm at 100 μg/L treatment (Fig. S2). No GO terms were significantly enriched using 0.1 μm PS-MP (Fig. S4A and B). Yet, in the treatment with 1 μm and 10^5 μg/L, one GO term (GO: 0031966), relating to mitochondrial membrane structure was significantly enriched (Fig. S4D). GO enrichment showed that the biological processes, including sulfur amino acid biosynthetic process (GO: 0000097), alpha-amino acid biosynthetic process (GO: 1901607), cellular amino acid biosynthetic process (GO: 0008652), sulfur amino acid metabolic process (GO: 0000096), cysteine biosynthetic process from serine (GO: 0006535), and regulation of the Notch signaling pathway (GO: 0045747), were all significantly enriched in EPNs after 72 h of exposure with 100 μg/L of 1 μm PS-MP (Fig. S4C and D). Similarly, molecular functions, including purine nucleoside binding (GO: 0001883), GTP binding (GO: 0005525), purine ribonucleoside binding (GO: 0032550), guanyl ribonucleotide binding (GO: 0032561), guanyl nucleotide binding (GO: 0019001), nucleoside binding (GO: 0001882), ribonucleoside binding (GO: 0032549), and Notch binding (GO: 0005112), as well as cellular components related to hemoglobin complex (GO: 0005833) were also significantly enriched. Furthermore, molecular functions were further explored via up- and down-regulated DEGs (Fig. 7A and B). The analyses showed that the oxidoreduction-driven active transmembrane transporter activity was remarkable inhibited. The mitochondrial membrane and organelle membrane-related GO terms were significantly enriched with the down-regulated DEGs. The top GO terms were GTP binding, guanyl ribonucleotide binding, and guanyl nucleotide binding-proteins, with all up-regulated DEGs.

The KEGG enrichment analysis showed that no pathway was enriched under the 0.1 μm and 100 μg/L treatment (Fig. S5A). Yet, genes related to amino acid metabolism (cysteine and methionine metabolism) were significantly increased in EPNs exposed to 1 μm, 100 μg/L (Q value$<$0.05) (Fig. S5C). Moreover, after exposure to 10^5 μg/L PS-MPs, the number of genes related to oxidative phosphorylation (OXPHOS) and thermogenesis was significantly enriched (Q value$<$0.01) (Fig. S5B and D), while genes related to ribosomes, and retrograde endocannabinoid signaling, were also significantly enriched (Q value$<$0.01) after the 0.1 μm and 10^5 μg/L, and after the 1 μm and 10^5 μg/L treatments, respectively (Fig. S5B and D).

Furthermore, we observed that pathways related to ribosome, cysteine, methionine, translation, phosphonate and phosphinate metabolism were all significantly up-regulated (Fig. 7C and D). However, genes enriched in the pathways of OXPHOS, retrograde endocannabinoid signaling, environmental adaptation, thermogenesis and energy metabolism were significantly down-regulated (Q value $<$0.01) (Fig. 7).

We found 19 common DEGs in EPNs treated with 10^5 μg/L (Fig. S2 and Table S8). The KEGG analysis showed that they significantly enriched the thermogenesis, energy metabolism, oxidative phosphorylation, environmental adaptation, mitochondrial biogenesis and retrograde endocannabinoid signaling pathways (Fig. 7E). The DEGs involved in these pathways code for the cytochrome c oxidase subunit I (COX1), cytochrome c oxidase subunit II (COX2), NADH dehydrogenase subunit 1 (ND1) and NADH dehydrogenase subunit 5 (ND5) (Table S8). The expression of the four OXPHOS related DEGs was inhibited in the four PS-MP treatments (Fig. 7F), while it was significant down-regulated in the two EPN groups treated with 10^5 μg/L.

4. Discussion

In this study, we have presented, for the first time, evidence that entomopathogenic nematodes (EPNs), which are widely used as biological control agents against insect pests, are capable of absorbing PS-MP particles during their infective-juvenile stage. Actually, we expected that IJs would not be negatively affected by PS-MP particles exposure due to their non-feeding characteristics with a sealed mouth and a collapsed pharynx and especially protected by sheaths formed by retaining the second stage cuticles (Crook, 2014). Analogous to investigations revealing the harmful impact of MPs on aquatic organisms (Sussarellu et al., 2016; Galloway et al., 2017; Foley et al., 2018; Franzellitti et al., 2019), we have uncovered similar deleterious effects of PS-MP particles on EPN survival, reproductive capacity, and pathogenic potential. Yet, the influence of PS-MP on EPNs was contingent upon particle size, concentration, and duration of exposure. Exposure of EPNs to PS-MP induced the production of reactive oxygen species (ROS), aberrant antioxidative enzyme activities, and mitochondrial impairment, all factors leading to oxidative stress. The RNA-seq data further revealed enrichment of DEGs within mitochondrial membrane-related terms and oxidative phosphorylation pathways, particularly under high PS-MP concentrations. Hence, our investigation elucidates the detrimental impact of MPs on various facets of EPNs' life history traits, physiological processes, and molecular responses.

EPNs, the ubiquitous insect consumers in soil food webs that are widely present in natural and agro-ecosystems on six continents, had been demonstrated as a key factor in achieving a trophic cascade, where cascade benefits to plants were produced through the effective inhibition of hosts by EPNs (Denno et al., 2008). In the ecological context, interactions between predator and prey produce the trophic cascade that indirectly affects the biomass, abundance or productivity of different trophic link within the food web (Polis et al., 2000; Shurin et al., 2002). Obviously, EPNs could be one of the most important factors in maintaining ecological balance. Once soil pollutants, such as MPs in our study, affected the physiological characteristics of EPNs thus suppressing the effectiveness of EPNs, as a result, trophic cascades related to EPNs might be affected. The adverse impacts of MPs on plankton ecosystems had been investigated to promote algal growth and affect the strength of trophic cascade (Pan et al., 2022). In a pot experiment, different concentrations of polypropylene microplastic pellets (ranging from 0% to 2%, w/w) were added to soil. Results showed that in soils with a 2% microplastic concentration, the abundances of plant parasites, bacterivores, fungivores, and omnivores were reduced by 90.16%, 76.06%, 82.35%, and 100%, respectively, compared to the control group (Yang et al., 2022). Furthermore, a field-based microplastic addition experiment demonstrated significant impacts on the trophic structure of the nematode community (Lin et al., 2020). Disturbance of ecological function caused by MPs is undoubtedly a fatal blow to stability and persistence of ecological environment.

4.1. PS-MP adsorption by EPNs

Contrary to experiments with *C. elegans*, which was shown to ingest PS-MPs (0.1 μm, 1 μm and 5 μm) via feeding (Lei et al., 2018b), it could have been hypothesized that EPNs IJs would not ingest microplastics, since this life stage does not feed with a sealed mouth and a collapsed pharynx and is protected by sheaths formed by retaining the second stage cuticles (Crook, 2014). However, unexpectedly, *S. feltiae* IJs clearly absorbed particles of 0.1–1 μm in size, but not bigger particles, of 5 μm (Fig. 1). One potential explanation for these findings is that while it is expected the mouthparts and the pharynx of IJs are taped up, when compared to the other feeding stages (Lu et al., 2017), it was observed under scanning electron microscopy that these openings are not completely sealed off (Nikdel and Niknam, 2015). Therefore, these tiny openings could still be permeable to small particles sizes, up to 1 μm. Moreover, IJs have other natural pores on their bodies, including transverse slit-like amphidal apertures and anuses, which could also provide an entry door for small MPs particles. That said, future work is needed to study the absorption capacity of the feeding EPNs, while inside the insect host. Indeed, the host insect larva might also pick up MPs during feeding, which in turn could be ingested by the EPNs while feeding upon the bacterial soup inside the insect cadaver. Hence, the

feeding stage of EPNs could potentially take up larger particle sizes, as was shown for *C. elegans* (Fueser et al., 2019). Moerover, EPNs constitute a diverse goup of species, with >125 species in the genus *Steinernema* and >22 species in the genus *Heterorhabditis* (>22). While rather similar in their biology, different EPN species clearly display differences in body size and other traits, making them likely differentially susceptible to MP (Kallali et al., 2024). Hence, our findings should be substantiated with testing additional EPN species (Ridall et al., 2023). In natural environment, MPs pollution had been found to be consumed across trophic levels by over 220 different species, including protists, zooplankton, annelids, cnidaria, amphipods, bivalves, fish, turtles, birds and mammals (Lusher et al., 2017; Bajt, 2021; Prokic et al., 2021). Not only soil nematodes, but some marine nematodes had also been found to be plagued by MPs pollutions (Ridall et al., 2023).

4.2. Effects of PS-MPs exposure on EPNs mortality

Corroborating the above-mentioned findings, we found that IJs mortality was significantly increased when EPNs were in contact with small (< 1 μm) PS-MPs. This finding thus suggested that the MPs had a negative effect on EPNs fitness. However, even when ingested, different particle sizes could also impose different mortality rates. Accordingly, it was reported that 0.1 μm PS-MP particles at 1–10 mg/m^2 caused lower levels of mortality in *C. elegans* compared to 1 μm particle, while 5 μm-particles were moderate toxic (Lei et al., 2018b). Generally, therefore, it appears, at least when comparing *C. elegans* and *S. feltiae*, that smaller PS-MP particles (0.1 μm or 0.1 μm), at comparatively high concentrations (10^5 μg/L or 10 mg/m^2) tend to impose the highest lethality for nematodes, but clearly, more nematode species would need to be tested to confirm this trend.

Moreover, we observed that mortality increased with exposure time (up to three days in our case), suggesting that the longer the EPNs were in contact with the MPs, the more MPs accumulated in EPNs bodies, ultimately increasing MPs' toxic effects. However, time-dependent morality upon MPs exposure was likely species specific. For instance, honeybees fed for nine days with PS-MPs did not die more than the control group (Wang et al., 2022). Similarly, medical mask microplastics did not cause death of enchytraeids *Enchytraeus crypticus*, woodlice *Porcellio scaber* and mealworms larvae *Tenebrio molitor*, even after 25 days of exposure time (Jemec Kokalj et al., 2022). Hence, we proposed to include longer time points to monitor the long-term effects of MP residues in a wider range of EPN species.

4.3. Effects of PS-MPs exposure to EPN reproduction

PS-MPs also negatively affected reproduction capacity of EPNs, which was dependent on PS-MP size, concentration and exposure duration. Higher PS-MP concentrations and exposure times increased the negative effects of MPs on EPNs reproduction. These results were consistent with what was found in *C. elegans* (Yu et al., 2020). Prolonged exposure time (up to 4.5 days) with 0.1 μm PS-MPs at doses higher than 10 μg/L decreased the brood size of *C. elegans* significantly more than the control treatment (Zhao et al., 2017). The mechanisms driving negative effects of MPs on reproduction need to be elucidated, but some hypotheses could be made. Firstly, MPs affected the general physiology of EPNs (i.e., see above results for increased mortality), in turn also affecting the reproductive capacity. Secondly, the toxic effects on reproduction could be attributed to the permeation of reproductive organs by PS-MP, directly impacting their functions (Zhao et al., 2017). This hypothesis is sustained by the fact that MPs toxicity was shown to be transmitted transgenerationally, at least in *C. elegans* (Zhao et al., 2017), up to five generations post exposure (Chen et al., 2021), indicating that MPs could be transmitted from one generation to another via the reproductive organs. Third, PS-MP exposure might decrease the motivation for reproduction in EPNs, by decreasing the dopamine levels and further causing the dopaminergic neurotoxicity, as it was shown in *C. elegans* (Jewett et al., 2022). In spite of all this, the exact mechanisms underlying the reproductive toxicity of PS-MPs still needs further research.

4.4. Effects of PS-MPs exposure to EPNs pathogenicity

The most applied value of EPNs is their biocontrol potential (Kenney and Eleftherianos, 2016; Torres-Barragan et al., 2011). We found the effects of MPs on EPNs pathogenicity were size-, concentration- and time-dependent, with the lowest pathogenicity after prolonged exposure of 72 h to 0.1 μm particle sizes, and at a concentration of 10^5 μg/L. We postulated that the inhibition of pathogenicity of EPNs by MPs may stem from the negative effects of MPs on intestinal microorganisms, nematode secretions and physiological activities. Firstly, as EPNs work in conjunction with their mutualistic bacteria to kill their host insects, indirect effects of MPs on EPNs' gut bacteria could impair EPN virulence (Lu et al., 2017). For instance, it was shown that honey bees' gut microbiota was altered after bees were exposed to PS-MPs (Wang et al., 2021). Hence, it might be that MPs could also affect the EPNs' intestinal microbiome, ultimately causing diminished pathogenicity. Secondly, some EPNs secretions, such as lethal venom proteins, facilitate EPNs virulence (Lu et al., 2017). MPs exposure might affect the secretion of these proteins, reducing the virulence potential of EPNs. Third, PS-MPs, by generally reducing the physiological activity of EPNs, might also reduce their motility, and penetration force, which were required to effectively enter the insect host. For instance, PS-MPs exposure induced remarkable decreases in body bends and head thrash in *C. elegans* (Chen et al., 2021). All these potential mechanisms for MPs-mediated effects need to be tested in EPNs as well.

4.5. Oxidative stress in response to PS-MP exposure

Excessive accumulation of ROS could instigate oxidative stress, a condition arising from an imbalance between ROS production and detoxification (Schieber and Chandel, 2014; Zorov et al., 2014). We here showed that MPs' exposure could also generate ROS in EPNs, but these effects were size- and concentration-dependent. For instance, following a 72-hour exposure to PS-MPs at a concentration of 10^5 μg/L, the relative ROS levels within EPNs increased by more than six times compared to the control group. These findings aligned with observations in other organisms. For example, more ROS production in methanogenesis-related microorganisms were induced by less than 1 μm sized-particles, compared to 100 μm and 1 mm particles (Li et al., 2022). Similarly, exposure of *C. elegans* to 1 μm PS-MPs at concentrations exceeding 1 μg/L for 72 hours prominently escalated ROS production and led to intestinal damage, with ROS levels rising in tandem with concentration increments (Yu et al., 2020).

Along those lines, we observed that 0.1 μm PS-MPs effectively suppressed EPNs' immune reactions, evident through diminished antioxidant-driven defense mechanisms characterized by reduced SOD and CAT activities. Similarly, PS-MP of 75 nm also suppressed these indicators in *D. pulex* (Liu et al., 2020). In this latter example, inhibition on antioxidant activities was due to oxidative damage and loss of compensation mechanisms caused by the production of a large amount of ROS overwhelming the antioxidant systems (Lv et al., 2018). Therefore, it appears that antioxidant enzyme activity cannot cope rapidly, or efficiently, enough, to compensate for PS-MPs-induced ROS in EPNs.

ROS accumulation resulted in toxic reactions with cellular components like DNA, proteins, and lipid membranes (Schieber and Chandel, 2014). For instance, Nylon microplastic induced oxidative stress in *Microcystis aeruginosa* cyanobacteria, damaging their cellular structure, including cell membranes (Zheng et al., 2022). Similarly, we found that PS-MPs induced higher levels of mitochondrial membrane fragmentation (Liu et al., 2022). ROS are natural byproducts of aerobic energy metabolism occurring via the OXPHOS pathway during the mitochondrial electron transport chains (ETC) (Murphy, 2009). We observed, via

the KEGG enrichment analysis, that the OXPHOS pathway was indeed significantly inhibited after exposure to 10^5 μg/L PS-MPs (Fig. 7F). Hence, this observation might provide a plausible explanation for the enrichment of fragmented membranes, in conjunction with all the down-regulated genes, such as COX1, COX2, ND1 and ND5. Notwithstanding, our study clearly showed that PS-MPs exposure could cause mitochondrial dysfunction in EPNs, a mechanism that might also be linked to the impairment of several life-history traits as discussed above.

5. Conclusions

In summary, the exposure to PS-MPs led to unexpectedly deleterious impacts on key physiological aspects of EPNs, including their survival, reproductive capacity, and infective potential. Altered levels of MMP, increased ROS production, and heightened antioxidant enzyme activities collectively underscored that the detriments arising from PS-MP exposure were a consequence of oxidative stress. Moreover, our transcriptomic investigation revealed the inhibition of the OXPHOS pathway by PS-MPs. Hence, our findings implicate that the toxicity of PS-MPs on EPNs is mediated by a combination of oxidative stress and mitochondrial dysfunction. The effectiveness of EPNs as biocontrol agents could be compromised if soil becomes contaminated with microplastics. This study introduced a fresh vantage point for comprehending the risk potential of microplastics in soil ecosystems, potentially informing future risk assessment studies, particularly in relation to the implementation of EPNs as biological control agents of insect pests. Indeed, EPNs are imbedded in a complex matrix of other nematode clades, representing the entire range of trophic groups, which are also likely to be affected by MPs. Therefore, to comprehensively understand the effects of MPs on the ecological function of EPNs and soil nematode communities, further research in real-world scenarios is warranted.

CRediT authorship contribution statement

Mingge Li: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Bingjun Ha:** Investigation. **Yuchen Li:** Writing – review & editing. **Klaas Vrieling:** Writing – review & editing. **Zhen Fu:** Writing – review & editing. **Qilin Yu:** Writing – review & editing. **Sergio Rasmann:** Writing – review & editing. **Xianqin Wei:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Formal analysis. **Weibin Ruan:** Writing – review & editing, Supervision, Project administration, Methodology, 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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116153.

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