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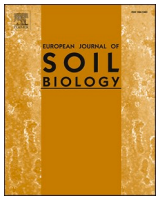

Original article

Pristine and UV-aged polyethylene microplastics' impact on gut microbiome and reproduction of earthworm *Eisenia andrei*

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ABSTRACT

The impact of microplastics (MPs) on soil organisms is still a growing field, yet very little is known about the exposure of ultraviolet (UV) aging of MPs to soil organisms. In this study, we explored the response of the epigeic earthworm *Eisenia andrei* to pristine and UV-aged polyethylene (PE-MP) exposure at a wide range of environmentally relevant concentrations (0, 0.2, 2, 20, 200 and 2000 mg kg^{-1}) in an organic farm soil for 56d and assessed changes in reproduction, ingestion, egestion and gut microbiome. Results showed that exposure to 20 mg kg^{-1} pristine PE-MP significantly increased earthworm reproduction by 39 % but the same concentration decreased reproduction by 29 % when they were exposed to UV-aged PE-MP. Ingestion of PE was verified by staining the whole worm body and their casts after 48h of starvation. The amounts of PE-MP found in the body and the casts were positively correlated with PE-MP concentrations in the soil, however only significantly so with pristine PE-MP. A decline in *E. andrei* gut microbiome alpha diversity and a significantly different community composition were observed in UV-aged PE-MP exposures compared to pristine PE-MP. Relative to the control treatments, Proteobacteria increased up to 135 %, Actinobacteria increased up to 35 %, and Firmicutes decreased up to 38 % under UV-aged PE-MP exposure, whereas Cyanobacteria increased up to 19 times in the pristine PE-MP treatments. These results confirm the negative effect of UV-aged PE-MP on earthworms even at low concentrations and could have important implications in the well-functioning of agricultural soils.

1. Introduction

In the current plastic age, the production and management of plastic and its residues have become unsustainable [1]. In 2021, plastic production reached a global amount of 390.7 Mt [2] and is still rising. The OECD estimates, as of 2019, that globally 22 % of plastics are mismanaged and end up as litter [2]. These staggering numbers result in plastic debris in every environmental compartment on earth [3], characterized by its own biogeochemical cycle [4]. Terrestrial environments are a prominent and growing sink for plastic debris, with increasing concern towards smaller size fractions [5]. Concentrations of microplastics (plastic particles between 1 μm and 5 mm in size [6]) in the soil have been reported globally ranging from 4×10^{-6}%–6.7 % (0.004–67000 mg kg^{-1}) of soil weight [7]. The accumulation and persistence of microplastics in soils pose a potential threat to the pedosphere, the organisms that inhabit it, and the functions they play a role in.

A considerable part of plastic debris present in soils is represented by secondary microplastics, which are formed from macroplastics subjected to weathering and degradation in the environment, predominantly by ultraviolet (UV) irradiation [8–10]. The importance of studying the effects of aged plastic debris/microplastics has been urged [11], because the process of aging is inevitable in the environment and can affect the particle's physicochemistry [12–14]. In addition, weathering processes alter the polymer's interactions with its surroundings biologically, geologically, and molecularly [15–18], altering the severity and mechanisms of toxicity posed to organisms. For example, UV-aged microplastics have been shown to exacerbate toxicity in a wide range of mostly aquatic organisms, from intestinal injury to cellular dysfunction, among other effects [19–22]. In soil organisms, however,

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knowledge of aged microplastic toxicity is more limited. Li et al. [23] reported that the aging process (H_2O_2 or UV) did not change the toxicity of polyethylene microplastics (PE-MPs) in the earthworm *Eisenia fetida* when exposed to 0.1 %, 1 %, and 10 % dw soil, indicated by negligible differences in oxidative stress levels. Whereas oxidative stress was exacerbated in *E. fetida* exposed to field aged LDPE film (0.25 %) [24], and in *E. andrei* exposed to mixtures of plastics (predominantly PE) at 0.1 mg kg^{-1} [25], both in agriculturally sourced soils. Although the effects of MPs at environmentally relevant concentrations on apical endpoints are still variable among test organisms, especially regarding earthworms [26], there is a great consensus when evaluating physiological and biochemical parameters (e.g., oxidative stress biomarkers and gut microbiome composition [15,26]) as they could be considered early indications of microplastic's toxicity.

Comprising the most abundant animal biomass in most terrestrial systems, the ecosystem services earthworms provide, such as litter decomposition, nutrient cycling, and pollutant transformation [27], are mediated by their gut microbiome [28]. Plus, being closely linked to the host's health, the gut microbiota is essential to vital processes in the earthworm body [29,30] and thus can serve as a powerful biological indicator of health [31]. Previous studies have shown that *E. fetida* exposed to pristine polystyrene (PS) nanoplastics at 200 mg kg^{-1} experienced a reduction in diversity and species richness of gut microbiome along with community composition shifts [32]. Similar changes were found in the gut microbiota of *E. fetida* exposed to polystyrene (PS) MPs (0.1–10 μm) at 10 and 100 mg kg^{-1} [33]. These authors also found more negative interactions among gut communities when exposed to larger-sized particles [33]. With regards to aged MPs, H_2O_2 degraded PE-MPs have been shown to intensify gut microbial disruptions where 500 mg kg^{-1} increased pathogenic bacteria and inhibited probiotic bacteria that promote gut homeostasis [34]. In addition, aged farmland residual PE mulch film MPs (0.25 %) showed a notable effect on *Metaphire guillelmi* gut community structure compared to pristine PE-MP exposure resulting in more simplified microbial networks with greater antagonistic interactions [35]. This disruption in mutualistic processes could ripple throughout the soil ecosystem where they reside [15], making the potential risks of altered gut microbial function a pressing matter to investigate.

Polyethylene (PE) is one of the most common and prevalent polymers in agricultural soils, due to its intensive application in mulching and greenhouse films, and irrigation systems, among others [36]. These uses increase exposure to UV photodegradation, where further fragmentation and accumulation in agricultural soils is inevitable [13,37]. This study assessed *E. andrei* survival, growth, reproduction, and gut microbiome composition after being exposed to pristine and UV-aged polyethylene microplastics (PE-MP) at a wide range of environmentally relevant concentrations (0, 0.2, 2, 20, 200 and 2000 mg kg^{-1}) for 56 days. *E. andrei* is a model organism in soil ecotoxicology [38], due to its sensitivity to a wide range of toxic substances, including microplastics (e.g., Ref. [33]), high reproduction rate, short life cycle, and easy maintenance in the laboratory environment. It was hypothesized that increasing concentrations of PE-MPs added to the soil would negatively impact earthworms, with UV-aged PE-MP inducing more pronounced effects than pristine PE-MP exposures. To the best of our knowledge, this is the first study addressing the ecotoxicity of pristine vs. UV-aged PE-MPs on earthworm reproduction and gut microbiome and serves as a steppingstone for further research in this area, considering the prevalence of photodegraded plastics in the terrestrial environment.

2. Materials and methods

2.1. Microplastics and contamination contingency measures

Polyethylene (PE) particles with an average size of 125 μm (ultra-high molecular weight powder, CAS No. 9002-88-4, density 0.94 g mL^{-1} (at 25 °C)), were obtained from Sigma-Aldrich UK without any pre-treatment, and hence considered as "pristine" in the experimental design. UV-aged PE-MPs were prepared by subjecting the pristine particles to an ultraviolet light schedule of 24 h per day for 16 days in a dark box. Briefly, particles were placed in aluminum trays roughly 15 cm below the UV light and gently stirred twice a day for more consistent exposure. UV-C light (VL-6.LC Vilber, Germany), with a wavelength of 254 nm, was used for irradiation and was monitored twice daily, each with 3 readings for intensity consistency using a lux meter wand (Delta Ohm HD 9221).

The particle size was chosen based on average microplastics size ranges in previous studies that used *E. andrei* as the model organism [39–41]. Particle size distribution of the PE-MP particles was determined by Silva et al. [11]. Briefly, 100 g of PE-MP particles were sieved in a vibratory sieve shaker (mesh pore-sizes: 500, 250, 125, 63 and 32 μm); the majority of the particles fell into the size ranges $^{3}125$ - < 250 μm (50.15 ± 2.35 %) and $^{3}63$ - <125 (39.75 ± 1.75).

All equipment was either acid washed or thoroughly washed with soap and water then dried and additionally wiped down with ethanol (96 %) before and after each use. All solutions used were previously filtrated, and fiberglass filters were previously burnt for 3h at 500 °C. Procedural blanks (n = 4) were considered for contamination assessment. The filtering cup from the glassware filtration apparatus was rinsed with 10 % nitric acid, followed by ultra-pure water to avoid cross-contamination.

2.2. Test soil

Agricultural soil was collected from an organic farm (Herdade do Freixo do Meio, 440ha, 38°42′13.2″ N, 8°19′31.4″ W) in southern Portugal (Montemor-o-Novo, in the region of Alentejo). The soil was sieved with a 5 mm mesh in the field and defaunated by freezing, followed by thawing (3 cycles). Detailed soil physicochemical information (texture, phosphorus, effective cation exchange capacity, pH, humic acids, nitrate, ammonium, C and N) are described by Reis et al. [42]. In brief, the soil presented a sandy loam texture, low pH (average pH of 5.4 ± 0.04 (mean ± SE)), and high organic matter content (4.0 ± 0.7 %). Water holding capacity (WHC; measured according to ISO 11267 [43]) was 35.56 ± 0.55 % (mean ± SE).

2.3. Test organisms

The earthworms *Eisenia andrei* (Oligochaeta: Lumbricidae) Bouché, 1972 were used as model organisms and were obtained from cultures maintained in the laboratory at a temperature of 20 ± 2 °C, 40–50 % humidity and 16h:8h light:dark cycle. The earthworms were acclimated in the experimental soil two weeks prior to the beginning of the incubation period and fed weekly with defaunated cow manure. The individuals selected for the tests were adults (clitellated) with an average fresh weight of 308.2 ± 2.3 mg and had an individual fresh weight between 187 and 507 mg.

2.4. MP exposure

Experimental units consisted of mason glass test jars containing 500 g (dw) of soil and PE-MP particles added with distilled water at a volume corresponding to 40 % of soil water holding capacity (WHC), and thoroughly mixed for approximately 5 min for an even dispersion. Nominal concentrations of 0, 0.2, 2, 20, 200 and 2000 mg PE kg^{-1} of soil dw were prepared for both types of PE-MP treatments (pristine and UV-aged). These concentrations corresponded to 0.00002, 0.0002, 0.002, 0.02 and 0.2 % of dw soil and fall within realistic concentrations found in soil samples [44,45,].

Four replicates were prepared for each PE-MP concentration and control treatments (48 jars in total). Water content and pH were measured at the beginning and end of the incubation period. Controls

were prepared in the same manner with no PE-MP (pristine nor UV-aged) addition; pristine and UV-aged exposures began on different dates resulting in two sets of controls. Exposure followed ISO 11268–2 [38] guidelines. The earthworms (ten clitellate adults per treatment) were washed, dried with paper towels, and weighed before being randomly added to the experimental jars. Each week, water was added by mass loss to every replicate to maintain a constant moisture level; additionally, earthworms were fed weekly with initially 10g fresh weight of cow manure (moistened and homogenized), then increased to 20 g in later weeks by demand.

After 28 days of exposure, surviving adults were removed, scored, washed, dried, and weighed. Three adults from each replicate were placed in separate Petri dishes lined with moist filter paper for gut depuration. After 48 h in the dark, adult worms were collected then frozen (−4 °C), while the filter paper with casts were dried in an incubator for further MP quantification described below. For further gut microbiome analysis, three additional adults from each replicate were fixated in 96 % ethanol. Adults were dissected under sterile conditions from the clitellum to the tail and intact gastrointestinal tracts were removed, pooled and separated into 4 eppendorfs (1.5 mL) and frozen (−4 °C).

The experimental soil was incubated once more, without adult earthworms, for 28 additional days to allow the cocoons to hatch. On day 56, the experimental jars were placed in a water bath at 50 °C to coax the juveniles to the soil surface for collection.

2.5. MP quantification and chemical changes during UV-aging

PE-MP extraction and quantification were performed on both the casts and the earthworm bodies adapting the methodology from Silva et al. [11] for the digestion procedure and Prata et al. [46] for PE-MP's quantification. Briefly, dried casts were scraped from the filter paper, where earthworms depurated their guts, into glass beakers where earthworm bodies and casts were digested separately in covered beakers in the oven at 50 °C in a 5 mL solution of nitric acid (63 %) for 3 h, followed by digestion in 5 mL H_2O_2 (30 %) for another 24 h at room temperature under a fume hood to eliminate any biological matter from MP surface. The reaction was terminated with deionized water, and the solution was filtered on 1.2 μm glass fiber filters, followed by incubation for 10 min in 10 mL of acetone, stained for 5 min with 3 drops of Nile Red (0.01 mg mL^{-1} ethanol), then washed again with deionized water. The filters containing suspected microplastics were allowed to dry completely before fluorescent observation using the 470 nm FOCUS LED (SPEX Forensic, USA) with an orange camera lens filter (Standard Pro-Master® Orange Filter) attached to the microscope. Imaging was performed using the Nikon SMZ-U Zoom 1:10 microscope paired with the Nikon Digital Sight DS-L1 camera attachment.

Fourier transform infrared spectroscopy (FTIR Spectrometer Nicolet 6700 from Thermo Nicolet Corp., Madison, WI, USA) was used to analyze the change in the surface functional groups of the PE-MPs (n = 3). FTIR spectra analysis is a valuable tool, and commonly used in microplastic's ecotoxicology, for MP aging assessment [47]. During UV irradiation/aging, FTIR can detect the formation of new functional groups or changes in the intensity of existing ones, indicating i) oxidation with the formation of carbonyl groups (C═O), which is a classic sign of oxidation due to UV exposure; and ii) chain scission, as breaking of polymer chains can change the relative intensities of functional group peaks [48]. The carbonyl index is used to monitor the absorption band of the carbonyl species formed on the surface of a material. In the context of microplastics, it indicates the presence of oxygen-containing functional groups, such as carbonyl (C═O) groups. Carbonyl index was calculated by the area of 1650–1800 cm^{-1} over the area of 1420–1500 cm^{-1} (modified calculation from Almond et al. [49]).

2.6. 16S rRNA gene amplification, sequencing, and bioinformatics analysis

To compare the effects of pristine and UV-aged PE-MP on the gut microbiome of *E. andrei*, 16S rRNA V4–V5 variable regions of the gut bacteria were sequenced by high-throughput sequencing performed on an Illumina Novaseq platform (Novogene, Cambridge, UK). DNA of *E. andrei* gut content was extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with an added initial tissue homogenization step using metal beads in a TissueLyser LT (Qiagen, Valencia, CA). From the 3 adult worms per treatment, a total of 48 composite samples were obtained from combined gut contents (4 per treatment). The quality and concentration of DNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher, USA). Extracted DNA was stored at −20 °C for downstream analysis. Further quality control, PCR, purification, library preparation, and sequencing were performed at Novogene (Cambridge, UK). Forward primer 515F (GTGCCAGCMGCCGCGG) and reverse primer 907R (CCGTCAATCMTTTRAGTTT), labeled with unique barcodes that target the hypervariable V4–V5 region of the bacterial 16S rRNA gene, were selected. PCR amplification was done with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Successful amplification was detected with electrophoresis on 2 % agarose gel; samples with bright bands between 400 and 450bp chosen for further analysis. Then, PCR products were purified with a Qiagen Gel Extraction Kit (Qiagen, Germany).

The same amount of PCR products from each experimental treatment sample were pooled, end-repaired, A-tailed and further ligated with Illumina adapters, then sequenced on an Illumina Novaseq platform (Novogene, Cambridge, UK). DADA2 pipeline [50] was used to infer the amplicon sequence variants (ASVs) present in each sample; a higher accuracy feature sequence analogous to operational taxonomic units (OTUs) [51]. Briefly, paired-end reads were matched to samples and truncated, then merged using FLASH (v1.2.11, http://ccb.jhu.edu/software/FLASH/) [52]. High-quality clean tags were obtained using fastp software [53]. Then, chimeras were identified and removed using Vsearch software. The resulting tags were denoised using DADA2 software with less than 5 abundance sequences filtered out to obtain the final ASVs. Finally, species annotation of each ASV was carried out by the QIIME2's classify-sklearn module [54,55].

2.7. Statistical analyses

Effects on survival, growth, reproduction, gut microbiota phyla and family abundances, and PE-MP particles found in worm body and casts were analyzed using a two-way ANOVA with PE-MP type (pristine and UV-aged) and concentration level as fixed factors, followed by a post-hoc LSD test. Change in carbonyl index between pristine and UV-aged PE-MP was analyzed using an unpaired *t*-test. Normality was tested using the Shapiro-Wilk test, and homogeneity of variances was tested using Levene's test. Tests were performed using GraphPad Prism 9. Correlation analyses were performed on the PE-MP count found in the body and casts of the earthworms using the Pearson correlation coefficient or Spearman rank correlation coefficient, depending on the normality of the dataset. For microbiome data, QIIME2 software was used to calculate alpha diversity indices and UniFrac distance for beta diversity between treatments. Weighted UniFrac and Unweighted UniFrac distances were used to perform a Principal Coordinate Analysis (PCoA) to explore the differences between microbial communities (beta diversity) [56,57], and visualized using the R "ggplot2" package [58]. Differences in microbiome composition between pristine and UV-aged PE-MP and between exposure concentrations were analyzed using Bray-Curtis distance with a PERMANOVA implemented in the "adonis2" function from R "vegan" package. All values are expressed as mean ± standard deviation (SD).

3. Results

3.1. Characterization of pristine and UV-aged PE

FTIR spectra from pristine and UV aged particles presented similar peak distribution, yet there were significantly different carbonyl indices (Fig. S1, Supplementary Material). Carbonyl index significantly increased from pristine to UV-aged PE-MP with values of 0.0078–0.038, respectively ($t = 8.693$; $df = 4$; $p < 0.001$). This formation of new carbonyl species, demonstrated by increasing CI, indicates oxidation and degradation. Distinction in FTIR spectra between pristine and UV-aged PE-MP can be appreciated in the magnified regions used in the calculation of CI (Fig. S2, Supplementary Material).

3.2. Effects of pristine and UV-aged polyethylene on the survival, growth and reproduction of E. andrei

After 28d, there was 100 % survival in all treatments. Likewise, neither pristine nor UV-aged PE-MP had any significant effect on the earthworm biomass compared to the control (Fig. S3).

After 56d of exposure the number of juveniles recorded was significantly different between pristine and UV-aged PE-MP (Fig. 1) ($F(1, 36) = 5.629$; $p < 0.05$), yet increasing concentrations did not impact reproduction. Earthworm reproduction increased under pristine PE-MP exposures of 2 mg kg^{-1} and above, but it decreased under UV-aged PE-MP exposures, apart from a slight increase of 0.77 ± 13.4 % in reproduction at 2 mg kg^{-1} UV-aged PE-MP observed when compared to the control. The difference in reproduction between pristine and UV-aged PE-MP was particularly evident at the concentration 20 mg kg^{-1}, where the pristine PE-MPs treatments had 39.3 ± 7.3 % more juveniles ($p < 0.05$) and those with UV-aged PE-MPs registered 21.9 ± 3.8 % less juveniles ($p < 0.05$) compared to the respective controls.

Validity of *E. andrei* reproduction tests based on ISO 11268–2 [38] standard was confirmed and further described in Supplementary Material.

3.3. PE-MP concentration in E. andrei body and casts

Both pristine and UV-aged PE-MP particles were found in earthworm bodies and casts at all exposure concentrations (Fig. 2), confirming both ingestion and egestion. However, the two PE-MP types did not differ in the number of particles recovered. Nevertheless, the number of particles in the bodies and casts increased with increasing PE-MP concentrations in the soil (Fig. 2), yet only pristine PE-MP particle concentrations showed a significant positive correlation with particles found in the bodies and the casts (Spearman $r = 1$, $p < 0.05$ in both cases). This positive trend was not significant in the case of UV-aged PE-MP (Pearson $r = 0.6954$, and Spearman $r = 0.8208$, respectively, $p > 0.05$).

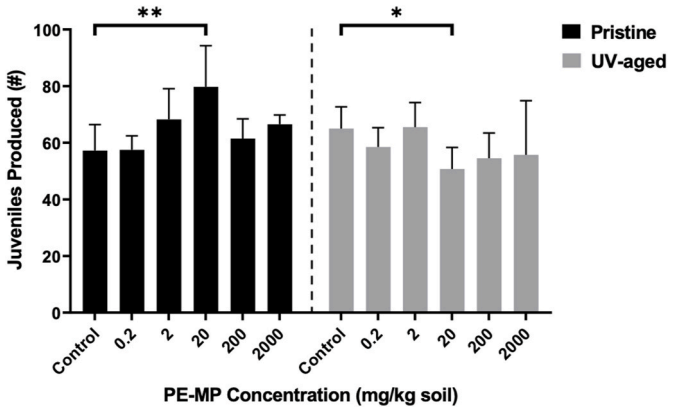

Fig. 1. *E. andrei* reproduction as indicated by the number of juveniles produced after 56 days of exposure to pristine or UV-aged polyethylene microplastics in natural soil. Data are expressed as mean (juvenile #) ± standard deviation (SD) ($n = 4$). Significant differences to control (two-way ANOVA): * ($p < 0.05$); ** ($p < 0.01$).

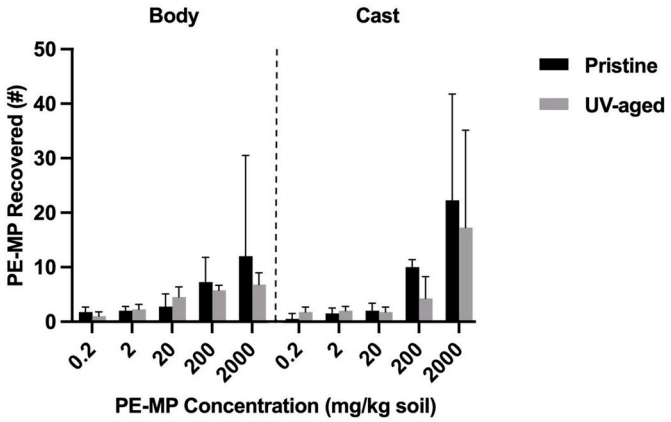

Fig. 2. Concentration of PE-MP particles present in the body and casts of *E. andrei* exposed to pristine or UV-aged polyethylene microplastics after 48h of depuration. Data are expressed as mean number of particles (PE-MP #) ± standard deviation (SD) ($n = 4$).

3.4. Effect of PE-MP on E. andrei gut microbiota

After assembling and filtering, a total of 2,822,147 clean reads were acquired from 48 samples, ranging from 43,787 to 79,339 per sample and with an average of 58,795 reads. After denoising, 2,6034 ASV's were obtained in each sample. The total amount of ASVs detected across all samples was not significantly different between treatments. Firmicutes (42.09 %), Actinobacteriota (28.69 %), Proteobacteria (12.54 %), Bacteroidota (7.78 %) and Planctomycetota (4.72 %) were the predominant bacterial phyla, comprising 95.82 % of the total microbiome community abundance among the control treatments.

A significant decline in alpha diversity of the gut microbiome occurred in UV-aged PE-MP exposure compared to pristine treatments, according to Chao1 index ($F(1, 36) = 6.679$; $p < 0.05$), however there were no significant differences between the different exposure concentrations ($F(5, 36) = 1.672$; $p > 0.05$).

Fig. 3a shows the ten most abundant phyla and their trends in relative abundance when exposed to increasing concentrations of pristine or UV-aged PE-MP. It can be seen that exposure to UV-aged PE-MP resulted in a larger increase of Actinobacteria compared to the pristine exposed worms ($F(1, 36) = 19.58$; $p < 0.001$), despite not being correlated with the concentration of PE-MPs in the soil. Actinobacteria increased absolute abundance by 0.81–23.74 % under the majority of pristine PE-MP exposures compared to the control, except for slight decreases of 1.7 ± 20.16 % and 7.12 ± 22.78 % at 2 and 2000 mg kg^{-1}, respectively. In contrast, exposure to UV-aged PE-MP increased Actinobacteria absolute abundance by 5.17–35.28 % compared to the control.

Firmicute absolute abundance remained relatively unchanged in the pristine PE-MP exposures, but decreased by 16.76–37.89 % in response to UV-aged PE-MP by showing a relatively steady decline at higher concentrations. This led to Firmicute abundance being the bacteria phyla that showed the most drastic changes between pristine and UV-aged exposures compared to any other phylum ($F(1, 36) = 65.05$; $p < 0.001$).

Proteobacteria exhibited a modest increasing trend when exposed to pristine PE-MP, but a more marked increase in absolute abundance when exposed to UV-aged MPs, with the highest increase of 135.47 ± 130.3 % at 2000 mg kg^{-1} ($p < 0.001$), resulting in PE-MP concentration being a significant factor ($F(5, 36) = 3.984$; $p < 0.05$).

Cyanobacteria showed the largest increase of any phyla with 19

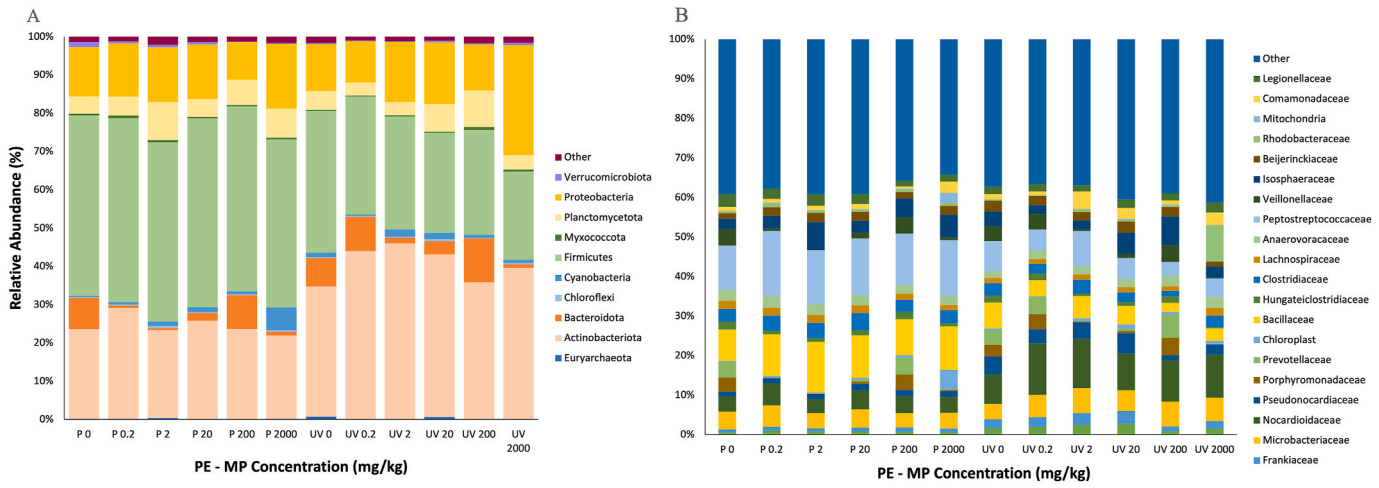

Fig. 3. Relative abundance of *E. andrei* gut microbiota at phylum (A) and family (B) levels present in the earthworm gut among different concentration (mg kg^{-1}) and treatments (prefix "P" signifies pristine and "UV" signifies UV-aged PE-MPs). Stacked bar plot of the top 10 microbial phyla (A) and top 21 families (B) with the largest mean relative abundance (%) in earthworm gut; categories outside of this were grouped into "Other".

times the absolute abundance compared to the control at 2000 mg kg^{-1} exposure to pristine PE-MP ($p < 0.05$).

At family and genus level, it was observed that absolute abundance of the family Bacillaceae decreased when exposed to UV-aged PE-MP, but increased in pristine PE-MP exposures ($F(1, 36) = 68.25$; $p < 0.001$). The absolute abundance of *Aeromonas* was significantly different between pristine and UV-aged PE-MPs ($F(1, 36) = 6.89$; $p < 0.05$) and among concentrations ($F(5, 36) = 8.5$; $p < 0.001$), as a result of an abrupt increase in numbers at the highest concentration of UV-aged PE-MP whereas abundance was virtually nonexistent in other concentrations and pristine exposure. More bacterial family results can be found in the Supplementary Material.

The results of the Principal Coordinate Analysis (Fig. 4) showed a clear distinction between samples exposed to UV-aged PE-MP and those exposed to pristine PE-MP. The first two principal components (Fig. 4a) explained 49.11 % and 14.87 % of the variation between the treatments (weighted UniFrac distance), respectively. The unweighted UniFrac distance calculated for the first two components of the PCoA explained 21.2 % and 5.6 % of the variation (Fig. 4b), and indicated the existence of important differences in gut microbial communities when the worms were exposed to the highest concentration of UV-aged PE-MP (2000 mg kg^{-1}) compared to those in the pristine PE-MP and control treatments. The PERMANOVA, using Bray-Curtis distance, confirmed these differences in community structure of *E. andrei* gut microbiome between pristine and UV-aged PE-MP exposures ($df = 1$; $F = 5.6896$; $R2 = 0.10260$; $p < 0.05$).

4. Discussion

4.1. Contrasting effects of pristine PE-MP and UV-aged PE-MP on E. andrei reproduction

Pristine PE-MP exposure at concentrations of 2 mg kg^{-1} and above stimulated reproduction in earthworms, whereas UV-aged PE-MP at 20 mg kg^{-1} and above inhibited it. This opposite response was most pronounced at environmentally relevant concentrations of 20 mg kg^{-1} for both PE-MP types. Various studies have found that MP exposures can hinder reproduction in earthworms [59–63], especially at unrealistically

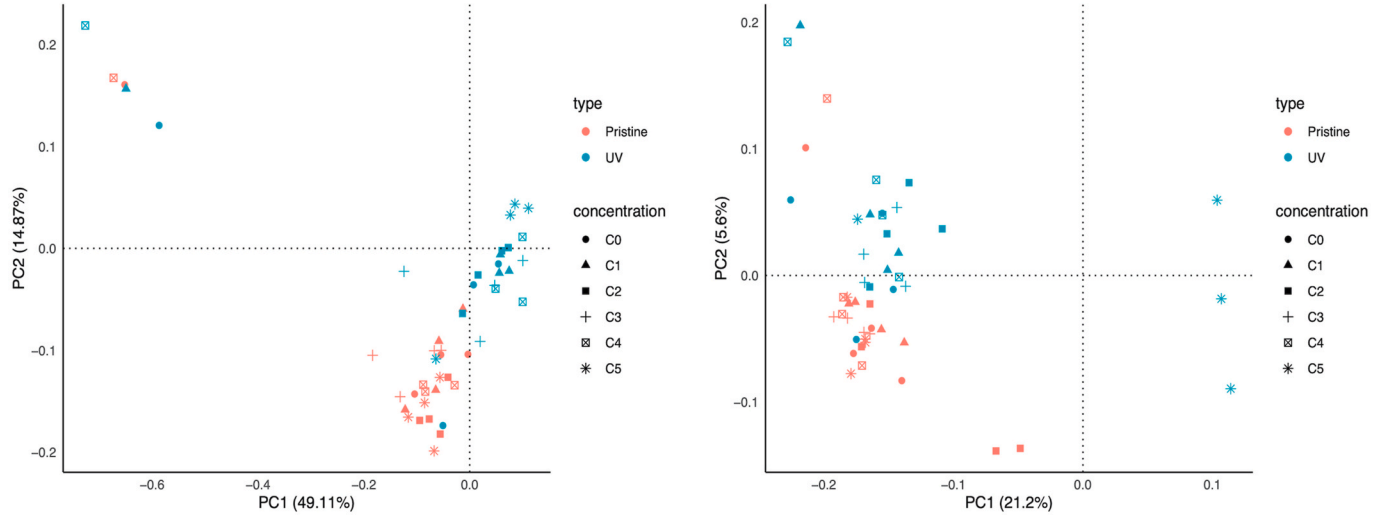

Fig. 4. Principal Coordinate Analysis (PCoA) based on Weighted UniFrac distance (a) and Unweighted UniFrac distance (b) showing the distribution pattern of *E. andrei* gut microbiome among different polyethylene microplastic treatments (0 mg kg^{-1} (C0), 0.2 mg kg^{-1} (C1), 2 mg kg^{-1} (C2), 20 mg kg^{-1} (C3), 200 mg kg^{-1} (C4), 2000 mg kg^{-1} (C5)). Data are based on 16S rRNA amplification and high-throughput sequencing. The microbiome of each sample is shown as a point. The percentages of the axis represent the percentage of variation explained.

high concentrations. However, the majority of research indicates that earthworm reproduction is not significantly impacted [40,62,64–66]. Some of these studies even describe a slight increase in earthworm reproduction exposed to pristine MPs [40,62,64], as observed in this study. Interestingly, a study using agricultural soil reported very minor increases in *E. fetida* reproduction at low concentrations of PE-MP exposure (0.05–2.5 mg kg^{-1}) after 20d, and subsequent measurements showed reproduction to decrease over time and with increasing concentrations, especially at 20 mg kg^{-1} after 60d [67]. In the present study, this concentration showed the greatest increases in reproduction with pristine PE-MP after 56d. Stimulation and hormetic responses to low levels of contamination of MPs have been extensively reported in aquatic organisms [68,69,70] and from this, the reproduction results of *E. andrei* observed here can be interpreted as stimulatory responses to low-risk contamination (i.e., pristine PE-MP exposure) with inversely inhibitory responses when faced with high-risk contamination (i.e., UV-aged PE-MPs). Sun et al. [69] found an overall 31 % increase in reproduction in aquatic organisms exposed to environmentally relevant concentrations of MPs ($\leq$1 mg L^{-1}) regardless of habitat, polymer type, shape, size, exposure period or concentration. They concluded that the stimulation effect acts as a conditioning agent to strengthen biological performance and resistance [71,70]. Furthermore, these MP exposures had inhibitory effects on oxidative stress biomarkers, therefore alleviating oxidative stress, reflecting an adaptive response to maintain redox homeostasis [72]. In agreement with this, a later review [73] of dose responses among a wider range of organisms (22 diverse taxa from aquatic and terrestrial environments) exposed to various types of nanoplastics/microplastics (with differing polymers, sizes, shapes, and concentrations) identified 65 studies that presented hormetic responses in a wide range of endpoints from molecular to individual level. Our results are also supported by previous studies showing an increase in earthworm reproduction (albeit non-significant) after exposure to MP concentrations of low to midrange [40,62,64], which is comparable with increased reproduction seen here in *E. andrei* that was most prominent when exposed to pristine PE-MP up to a mid-level concentration (20 mg kg^{-1}) and subsequently decreased, yet not below the rates observed in the control treatment.

The effects of UV-aged MPs on organisms' reproduction have only been investigated in aquatic media [74–76], and shown to deepen the severity of toxicity, ranging from molecular to individual-level responses [19–22]. For example, nematodes exhibited reproductive toxicity after exposure to pristine and UV-aged PS-MP in an aqueous medium at environmental concentrations (0.1–100 μg L^{-1}); however, significantly more so in UV-aged PS-MPs, which was attributed to DNA damage-induced cell apoptosis [75]. The aged particles used in that study had increased carbonyl indices indicating UV photodegradation causing surface oxidation, and they were substantially dimpled and wrinkled compared to the smooth surface of pristine PS-MPs, creating a larger surface area to potentially interact with cellular membranes [77,78]. Similarly, *Daphnia magna*, when exposed to UV-aged PS-MP showed chronic toxicity in the form of reproductive inhibition, which was linked to smaller-sized particle generation of PS-MPs through the UV-aging process resulting in 3 times the initial pristine concentration of particles [76]. This would reinforce the idea that smaller-sized particles increase specific surface area and biological interaction. In the present study, the carbonyl index increased substantially in UV-aged particles, indicative of oxidation [49], and progressive deterioration [79]. Along with the changing chemical structure, the process of oxidation causes significant changes in the physical properties of the material as well [49].

4.2. UV-aged PE-MP altered E. andrei gut bacterial communities

The UV-aged PE-MP exposure led to decreased alpha diversity (Chao1 index) and to a distinct community when compared to the pristine PE-MP exposure, in agreement with Kim et al. [80]. The stability of the earthworm gut microbiome has been linked to health, pathogen defense, immunity, and metabolism; therefore, any derivation in the community composition can been seen as an indicator of contamination [81,82]. Compared to other microplastic exposures of terrestrial oligochaetes, earthworm gut microbiota richness and evenness was also shown to decrease when exposed to various sizes of PS-MPs (0.1–100 μm, 10–100 mg kg^{-1} soil) [33]. A similar response was observed in the gut microbiome of enchytraeids exposed to 10 % PS nanoplastics (0.05–0.1 μm) in oatmeal [83]. However, in an incubation mesocosm study of natural soil spiked with the equivalent of 340 mg kg^{-1} PE-MP placed on the surface saw no change in bacterial richness, diversity, or community composition in *Lumbricus terrestris* gut microbiota [84].

Aged microplastics have been shown to have more severe impacts on the gut microbiome of other organisms [85–88]. For earthworms, exposure to 500 mg kg^{-1} pristine and H_2O_2-aged PE-MPs caused intestinal microbial disturbances, yet the effects were more severe with aged PE-MP exposure by promoting pathogenic bacteria and inhibiting probiotic bacteria [34]. Similarly, aged farmland residual PE mulch film MPs (0.25 %) impacted *Metaphire guillelmi* gut community structure compared to pristine PE-MP, and showed an enrichment in pathogenic bacteria [35].

Structural and functional damages in the gut have been linked to an imbalance in redox status (i.e. oxidative stress), where free radicals develop on the surface of MPs through photoaging in turn triggering cytotoxicity and greater reactive oxygen species (ROS) produced by the organism [89,90]. In relation to this, Zhu et al. [89] found a strong correlation between the oxidative potential of UV-aged MPs and increased carbonyl index or CI (namely conjugated carbonyl content). Accordingly, Jiang et al. [34] observed intestinal damage, ROS production, and shifted gut microbial communities in *E. fetida* after exposure to H_2O_2 aged PE-MP with increased CI. Similarly, UV-aged PE-MPs in this study, which also had increased CI, could have exerted higher oxidative potential in the gut lumen of *E. andrei* with negative effects on its gut microbiome. Increased oxidative process in the earthworm gut lumen has been associated with shifting dominance towards facultative anaerobes, aerobes and pathogenetic species and away from obligate anaerobes essential to earthworm gut health [91–95].

The most significant changes in *E. andrei* intestinal phyla absolute abundance, when exposed to UV-aged PE-MP, was the increasing abundances of Actinobacteria and Proteobacteria and decreasing ones of Firmicutes. Intestinal disorders have been characterized by a decline in bacterial diversity coupled with an increase in Actinobacteria and Proteobacteria [96]. As stated above, the chemical and physical alterations of UV-aged PE-MP might have promoted certain microbial colonization. Actinobacteria are known for their ability to degrade complex organic compounds, specifically with an efficiency in polyethylene-derived MP biodegradation [97], while Proteobacteria encompass diverse bacterial groups with versatile metabolic capabilities [98]. Both have been found to have enriched abundances on the surfaces of PE-MPs in soil [99]. Additionally, the presence of PE in soil has been shown to enrich Proteobacteria and Actinobacteria communities in the soil, and PE weathering favors the abundance of Proteobacteria [100]. Moreover, previous research has shown that field sourced PE-MPs with roughened and indented surfaces served as a suitable microhabitat for Actinobacteria and Proteobacteria, among other phyla [101]. Therefore, earthworms passively ingesting soil with aged-PE MPs would have gradually altered their gut microbial communities reflecting the change in the microbial composition occurring in the soil.

In the current study, there were increases in Proteobacteria absolute abundance with both pristine and UV-aged PE-MP as exposure concentrations rose but much more prominently with the aged material where a 135.5 % increase at the highest concentration was observed, in agreement with previous studies [88]. The increase in Proteobacteria with increasing PE-MP concentrations could also support the idea that gut microbiome analysis is a more sensitive toxicological indicator, as Proteobacterial increases serve as an indicator for gut dysbiosis [102].

Gut microbiome changes in *E. fetida* exposed to PS-MPs were most prominent with Proteobacteria growing more abundant [33,95]. Moreover, network analysis of gut microbial interaction in *E. fetida* revealed that most negative interactions (competition, amensalism, or predation) induced by PS-MP exposure were involved with increasing Proteobacteria, further hastening dysbiosis [103]. A specific genus of Proteobacteria (*Aeromonas*), a facultative anaerobe, was almost nonexistent in *E. andrei* gut microbiome in the present study until it was exposed to the highest concentration of UV-aged PE-MP where it significantly increased absolute abundance with over a 500-fold increase. The genus *Aeromonas* was considered a main contributor to negative interactions in the gut microbiome network of *E. fetida*, mentioned above, after exposure to 100 mg kg^{-1} PS-MPs (10 μm) [103] and caused the most severe intestinal damage, highest oxidative toxicity, immune system and energy metabolism disturbance and increased abundance of antibiotic-resistant genes [95]. This is in line with other studies indicating that increase in facultative anaerobes like *Aeromonas* [94,95], and simultaneously decreasing obligate anaerobes [93] is linked to gut complications.

Firmicutes, on the other hand, are obligate anaerobes and are rich in the oxygen depleted gut of Oligochaeta [104]. Firmicute absolute abundance declined with increasing UV-aged PE-MP exposure concentrations compared to control, but not with pristine PE-MP exposure. A study found that ozone-aged PS-MPs reduced the diversity of intestinal microbes, especially Firmicutes, while interfering with hormones related to energy metabolism in mice, compared to pristine PS-MPs [87]. In the present study, the concurrent increase in Proteobacteria as Firmicutes decreased was most notable during UV-aged PE-MP exposures, suggesting gut dysbiosis [93,105]. In addition, an important genus in the phylum Firmicutes, *Bacillus,* key in organic matter degradation and nutrient cycling [106], was significantly reduced when earthworms were exposed to UV-aged PE-MP compared to pristine. Bacillus suppression has been linked to C and N metabolic disorders [82], and when reintroduced to earthworm coelomic cells it promoted immunity regulation and antimicrobial defense [107]. This indicates that its inhibition by UV-aged PE-MP exposure in the present study possibly hindered *E. andrei* immune defenses and metabolism.

5. Conclusions

Pristine polyethylene microplastics at concentrations of 2 mg kg^{-1} and above induced stimulation of reproduction in *E. andrei* while UV irradiated PE-MPs at 20 mg kg^{-1} and above inhibited reproduction, this dichotomous response was significant at 20 mg kg^{-1} for both PE-MP types. Furthermore, UV irradiated PE-MP exposure to *E. andrei* in soil not only decreased gut microbiome species richness and diversity, but also altered its balance in abundance when compared to the gut microbiome of earthworms exposed to pristine PE-MP, where demonstrably beneficial groups were decreased and potentially opportunistic and/or pathogenetic species were promoted at higher PE-MP concentrations. A positive correlation between gut microbiome function and reproduction has been seen in springtails exposed to PE-MPs [108], and enchytraeids exposed to nano-PS [83] and tire particle MPs [109]. In line with this, Ding et al. [109] confirmed a link between changes in gut bacterial communities and inhibited reproduction, which supports the idea that the gut microbiome is fundamentally tied to reproduction [110]. Other studies exploring the effects of various contaminants have also observed this trend [82,111], with one of them arguing that effects on the gut microbiome precede reproductive inhibition at lower contaminant concentrations [112], possibly being a precursor. This indicates that there could be an interplay between gut microbiome composition and reproduction of earthworms. Gut microbial imbalance can lead to health deterioration, such as increased susceptibility to pathogens and metabolic disorders [113]. Thus, because earthworm gut microbiome is inextricable from the roles they play in the surrounding soil environment, further investigation is necessary assess how the toxicity of UV-aged microplastics could impact ecosystem functioning.

CRediT authorship contribution statement

Elise Quigley: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Ana L. Patrício Silva:** Writing – review & editing, Validation, Resources, Methodology. **Sónia Chelinho:** Supervision, Methodology, Conceptualization. **Luís Cunha:** Writing – review & editing, Methodology. **Maria JI. Briones:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **José P. Sousa:** Writing – review & editing, Supervision, Resources, Project administration, 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. Supplementary data

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