Effects of environmentally relevant mixtures of microplastics on soil organisms

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DATA AVAILABILITY
The data underlying this article will be shared on reasonable request to the corresponding author.

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CONFLICT OF INTEREST
None.

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Abstract

Soil ecosystems are considered important sinks for microplastics (MPs). However, the effects of environmentally relevant mixtures of MPs on soil organisms have rarely been assessed. This study aimed to evaluate the chronic effects of a mixture of MPs on two model soil organisms, the earthworm *Eisenia andrei* and the springtail *Folsomia candida*. The MP mixture was composed of polymers and shapes frequently found in agricultural soils amended with sewage sludge, including HDPE and PP fragments, and PES fibres. The organisms were exposed in LUFA 2.2 soil to MP concentrations of 0–1% dry soil for *E. andrei*, and 0–5% for *F. candida*. This study shows that particle ingestion by *E. andrei* was proportional to MP exposure levels, and the size distribution taken up was similar to that observed in the exposure medium, suggesting non-selective uptake behaviour. In contrast, very low ingestion levels of MPs were found for *F. candida*, even at the highest test concentration. No significant effects were found on survival, growth or reproduction of *E. andrei*. However, significant adverse effects were found on the reproductive output (number of juveniles) and juvenile dry weight for *F. candida*, with a reduction of approximately 30% in both endpoints at the highest test concentration, and calculated NOECs of 0.4% and 1%, respectively. These adverse effects may have been caused by changes in soil properties, mobility reduction, and/or the presence of plastic additives, instead of MP uptake. The comparison of MP exposure concentrations in soils obtained from the literature with the threshold concentrations derived for *F. candida* indicates insignificant environmental risks at current exposure levels.

Keywords: soil invertebrates, microplastics, ecotoxicology, uptake, risk assessment

INTRODUCTION

Microplastic (MP) pollution in soil ecosystems has received significantly less attention compared to marine and freshwater environments (Kim et al. 2020; Kumar et al. 2020; Redondo-Hasselerharm et al. 2024; Shi et al. 2024). However, the amount of plastic entering soil ecosystems is believed to surpass that entering the oceans (Möhrke et al. 2022). Poor waste management practices and low recycling rates contribute to the significant accumulation of plastic in soils, where it can fragment into MP-sized particles (1–5000 μm; James et al. 2021; Renault et al. 2024). Moreover, various agricultural practices, such as the use of mulching films, irrigation with reclaimed or polluted water, and the application of sewage sludge, can exacerbate MP pollution in agricultural soils (Hurley and Nizzetto 2018; Briassoulis 2023; Sa'adu and Farsang 2023).

Both sewage sludge and biosolids - sludge that goes under different pre-treatment methods before its application in agricultural fields - have been shown to contain varying MP levels across the world (Harley-Nyang et al. 2023). The application of sewage sludge and biosolids as a soil amendment is a common practice in many countries (Zhang et al. 2020). This practice introduces substantial amounts of MPs into soils, with concentrations increasing after repeated applications (Corradini et al. 2019; Van Den Berg et al. 2020). It is estimated that between 63,000 and 430,000 tons of MPs are added to European soils annually through this practice (Nizzetto et al. 2016; Hann et al. 2021). The highest concentration of MPs reported in European soils amended with sewage sludge was 12.9 mg/kg (0.001%) after four applications (Corradini et al. 2019). However, concentrations as high as 915 ± 63 mg/kg (0.1%) of MPs have been observed in soils near roadways exposed to multiple MP sources (Dierkes et al. 2019).

Research indicates that MP pollution can alter soil structure, bulk density, aggregation, pore size, and water dynamics, as well as influence soil pH (de Souza Machado et al. 2018; Zhang et al. 2019; Wang et al. 2022; Zhao et al. 2021). These physical changes can, in turn, affect the composition and structure of soil microbial communities (Han et al. 2024). Such disruptions to soil properties and microbial communities can cascade into negative effects on soil fauna, compounded by their direct exposure to MPs (Liu et al. 2023). Studies show that soil invertebrates can ingest MPs of various shapes, polymer types, and sizes (ranging from 100 nm to 2800 μm), depending on the species and their feeding traits (Möhrke et al. 2022). MP ingestion by soil invertebrates has been linked to long-term adverse effects, including reduced growth, reproduction, and altered metabolic activity (Jemec Kokalj 2024). For instance, nematode reproduction decreased at soil concentrations exceeding 10 mg/kg (0.001%) of polyacrylonitrile (PAN) fibers smaller than 630 μm (Kim et al. 2020). Similarly, metabolic enzymatic activities in the earthworm *E. fetida* were significantly increased at concentrations equal to and above 2.5 g/kg (0.25%) of polyethylene (PE) fragments measuring 30–50 μm. These metabolic alterations are usually associated with toxic effects like neurotoxicity, inflammation and oxidative stress (Yang et al. 2023).

Most studies on the impact of MPs on soil organisms have focused on single polymers with specific shapes and sizes under (semi-)controlled conditions

(Richard et al. 2024). However, in natural environments, organisms are exposed to diverse MP mixtures that vary in polymer type, shape, and size composition. While the effects of MP mixtures have been investigated for aquatic organisms (Renzi et al. 2019; Stanković et al. 2021; de Ruijter et al. 2023; Martínez-Pérez et al. 2024), research on the impact of environmentally relevant MP mixtures on soil organisms remains limited and requires further exploration (but see Baeza et al. 2020).

The heterogeneity of MPs—variations in shape, polymer type, and size—is expected to influence their bioavailability to soil organisms, potentially resulting in effects that differ from those observed with single MP types (Schell et al. 2022a; Schwarzer et al. 2022). In agricultural soils, fibres and fragments are the most commonly encountered MP shapes (Raza et al. 2022), and the predominant polymers include polyethylene (PE), polypropylene (PP), and polyester (PES) (Schell et al. 2020; Yu et al. 2022). Although MPs span a broad size range (1–5000 µm), smaller sizes are more abundant across most MP types.

To address the polydisperse nature of MPs (variations in polymer, shape, and size) in risk assessment, Kooi and Koelmans (2019) developed 3D probabilistic distributions to describe MPs in aquatic ecosystems based on these parameters. Their findings revealed that MP size distributions in the environment follow a power-law distribution, which can be used to extrapolate measurable size fractions (typically 50 or 100 to 5000 µm) to the full MP size range (1–5000 µm). Recently, Redondo-Hasselerharm et al. (2024) applied this concept for the risk assessment of MPs for soil organisms considering different effect mechanisms. Their study showed that soils treated with sewage sludge have a higher likelihood of exceeding MP effect thresholds for soil organisms, and emphasized the need for toxicity studies using MP mixtures to refine risk characterizations.

This study aimed to evaluate the uptake and effects of an environmentally relevant MP mixture on two soil invertebrate species: the earthworm *Eisenia andrei* and the springtail *Folsomia candida*, which differ in body size and feeding traits. The MP mixture tested consisted of high-density polyethylene (HDPE) fragments, PP fragments, and PES fibres, reflecting the shape and polymer distribution of MPs found in agricultural soils recently amended with sewage sludge (following Schell et al. 2022b). The specific objectives of our study were: (1) to characterize MP uptake and egestion in *Eisenia andrei* and MP uptake in *Folsomia candida*; (2) to assess the chronic effects of the MP mixture on survival, growth, and reproduction in these species; and (3) to evaluate the ecotoxicological risks of environmentally relevant MP mixtures based on reported exposure concentrations. We hypothesized that the tested invertebrates would ingest varying proportions of each polymer depending on its size and shape, and that the MP mixture might result in long-term adverse effects based on previous single-polymer toxicity data.

MATERIALS AND METHODS

Test materials

Following the methods described by Martínez-Pérez et al. (2024), HDPE and PP fragments were obtained by cryo-milling pellets (3–5 mm), and fibres were generated by cutting a PES blanket (IKEA, Polarvide), with scissors and using a coffee grinder. To minimize potential chemicals sorbed to the plastic surface, the MPs were washed 3 times using methanol in a shaking table following the protocol of Redondo-Hasselerharm et al. (2018). Once the particles were dried, fragments and fibres were sieved to retain the 50–5000 µm fraction for the experiment. This was done because the analytical method employed here was not sufficiently accurate to identify and count the < 50 µm fraction.

Test organisms

Test organisms were cultured at the Ecology & Evolution section of the Amsterdam Institute for Life and Environment (A-LIFE) of the Vrije Universiteit Amsterdam, the Netherlands. *Eisenia andrei* is an oligochaete from the Lumbricidae family which is mainly found in compost and dung heaps and feeds on decomposing organic matter (Kutuzović & Kutuzović, 2013). *E. andrei* laboratory cultures were kept in boxes with 1:1 ratio of sphagnum peat and potting soil. The pH was adjusted to 6.0 ± 1.0 by adding $CaCO_3$ and humidity was maintained to 50 % of the Water Holding Capacity (WHC). They were fed weekly with horse manure and maintained at a constant temperature of 20 ºC. Animals used in the tests came from synchronized cultures, had masses between 300 and 800 mg, and were adults with a clearly visible clitellum.

Folsomia candida is a springtail with an approximate size between 1.5 and 3 mm. It is spread worldwide, inhabiting soils with high organic matter content, feeding mainly on fungal hyphae and reproducing by parthenogenesis (Thimm et al.,1998). In the laboratory, these organisms were kept in boxes with a base of moistened plaster of Paris containing 10% activated charcoal and were fed with dry baker's yeast (AB|Mauri Netherlands, Dordrecht, the Netherlands). The cultures were kept in a climate room with constant temperature at 16 ºC, 75% air humidity, and a photoperiod of 16h light / 8h dark. For the experiment, age-synchronized 22-day old adults were used.

Experimental design

Single species tests using *E. andrei* and *F. candida* were performed using the standardized natural soil LUFA 2.2 soil (Speyer, German) and a exposure duration of 28 days. The soil had, according to the supplier, a pH (in 0.01 M $CaCl_2$) of 5.6 ± 0.3, an organic carbon content of 1.77 ± 0.56 % and a maximum water holding capacity (WHC) of 43.3 ± 5.1%. The experiments were carried out in climate rooms with controlled conditions of humidity (75%), light (16h light/8h dark) and temperature (20 ºC). For both experiments, the MP mixture was spiked into dry LUFA 2.2 soil. A stainless-steel sieve with a 1 mm pore size was used to spread the MPs on top of the soil, however, the particles between 1 mm and 5 mm were also added. Then, the particles were mixed in with the soil using a stainless-steal spoon. Tweezers were used to prevent fibres from forming big clumps. Demineralized water was added to reach a moisture content equal to 50% of the soil's WHC, and the pH of the initial soil mixture

was measured. To determine soil pH, 6.0 ± 0.1 g of soil were mixed with 24 mL of $CaCl_2$ solution at 0.01M, and shaken in an orbital shaker (Edmund Bühler SM-25) for 2 hours at 200 rpm. After allowing the mixture to settle overnight, the pH was measured using a pH-meter (WTW pH 7110).

The concentration range tested included concentrations found in soils (0.1%; Dierkes et al. 2019) and concentrations that are more than an order of magnitude higher (5%; Table 1). The percentage of each polymer type in the mixture (55% of HDPE, 42.5% of PES and 2.5% of PP) was based on data from Schell et al. (2022b) for agricultural soils amended with sewage sludge. Finally, to allow for comparison with studies reporting the concentration as number of MPs per soil mass, the number of MPs per gram of soil was determined (see *Section 2.4*) for two selected test concentrations (i.e., 0.064 and 0.4%). The obtained relationship between particle number and particle mass was used to extrapolate the rest of test concentrations.

Test with Eisenia andrei - The test with *E. andrei* followed the OECD guideline 222 (OECD 2016a). Ten earthworms were added to each test jar after 24h acclimatization in clean LUFA 2.2 soil. Glass jars (800 mL) were filled with 600 g of moist soil. Then, 8 g of horse manure was added weekly as food resource for the earthworms. Food was provided in small holes made in the soil and covered with soil to limit the growth of fungal hyphae. Lids were not completely closed to allow gas exchange. Moisture loss was avoided by weighing the test jars and adding the corresponding amount of demineralized water weekly. Tests were performed using 4 concentrations and controls (Table 1), each with 4 replicates and 10 adult earthworms per replicate.

MP uptake and egestion in the earthworms was assessed after 28 days of exposure. For this, half of the surviving adults per test jar were immediately frozen at -20 ºC and then freeze-dried, while the rest were kept for 24 hours (in the dark) to allow depuration on petri dishes with a thin water layer. After depuration, the earthworms were washed with tap water, blotted dry with paper, weighed, and frozen. The faeces were also collected for MP analysis.

The evaluated endpoints were survival, growth and reproduction. Survival was assessed as earthworms responding to tactile stimuli with a spatula. Growth was measured as the increase in wet weight. The initial wet weight was measured in 10 randomly selected earthworms included in the test, and the final wet weight was recorded for all adults found. Reproduction was assessed as the number of juveniles per surviving adult. To assess reproduction, after removal of the adults, the soil was returned into the test jars and incubated under the same conditions for 4 additional weeks to allow for the juveniles to emerge. Then, the test jars were placed into a water bath at 60 ºC to expel the juveniles, which emerged at the soil surface after 20–40 min, where they were collected and counted. Additionally, dry weight of adults with their full gut was measured after freeze-drying.

Test with Folsomia candida - The test with *F. candida* was performed following OECD guideline 232 (OECD, 2016b) with some modifications. The test used 100 mL glass jars containing 30 g of moist soil with a few grains of dried yeast added weekly as food resource. Lids were not completely closed to allow air exchange. Soil moisture content was checked weekly by weighing the test jars

and replenishing it as needed to maintain 50% of the WHC. Tests were performed using 6 concentrations and a control (Table 1), each with 4 replicates and 10 adults per replicate.

After 28 days of exposure, 100 mL of demineralized water was added to each jar and all soil was transferred to a larger beaker and gently stirred with a metal spatula allowing all animals float to the water surface. A picture of the water surface was taken using a camara (Nikon, COOLPIX P510) on a tripod to assess adult survival and the number of juveniles. Images were later analysed using the software ImageJ (Rueden et al. 2017). After taking the picture, the springtails were transferred to a black tray using a metallic sieve to separate them from the floating plastic and separate adults from juveniles. Then, the animals were freeze-dried and cleaned from any plastic particles that were attached to their body but not ingested using a stereomicroscope (Wild, M5A) and a glass needle. Finally, all adults from each replicate and all juveniles from each replicate were weighted using an ultraprecision balance (Mettler Toledo, UMT2).

The evaluated endpoints were survival, dry weight and reproduction. Survival was assessed by counting the number of adult organisms recovered from the soil, and reproduction as the number of juveniles per surviving adult. Additionally, total dry weight of adults and juveniles after the exposure period was measured by freeze-drying the recovered sample.

Assessment of microplastic concentrations

MPs were quantified in the test soils containing 0, 0.064, and 0.4 % of MPs in dry soil in both toxicity tests. For each concentration, a sample of the initial soil mixture was dried at 45 ºC. Next, 2 g (0.5 g for the 0.4% concentration) was weighed into 48 mL glass centrifuge tubes. After that, 10 mL of H_2O_2 (30%) was added to each sample by carefully adding 1 mL at a time to control the reaction intensity. Samples were mixed using a vortex and placed into an orbital shaker (Thermo Scientific™ MaxQ™ 4000) at 45 ºC for 48 hours at 120 rpm. Subsequently, the samples were placed into an oven at 50 ºC to evaporate the H_2O_2 for approximately 24 hours. Density separation was performed adding 10 mL of $ZnCl_2$ solution (ρ>1.6 g/cm^3) to the samples. Then, the samples were mixed with a magnetic stirrer for 20 minutes. After this, the samples were centrifuged at 1500 rpm for 20 minutes (Thermo Scientific Megafuge 16). After centrifugation, the supernatant was filtered through nitrate cellulose filters (Scharlau, 47 mm diameter, 0.45 µm pore size) using vacuum filtration. The density separation step was repeated twice.

To extract the MPs ingested by *E. andrei* and retained inside their bodies after depuration, the organisms were washed using tap water to remove particles attached to their bodies, then frozen at -20 ºC and freeze-dried. Each earthworm was transferred to a 50 mL glass beaker and cut into pieces of about 5 mm using metal scissors. Then, 10 mL of H_2O_2 (30%) were added and the beaker was placed on an orbital shaker at 50 ºC for 48 hours at 120 rpm. Afterwards, samples were then filtered over cellulose nitrate membrane filters (Scharlau, 47 mm diameter, 0.45 µm pore size) using a glass filtration unit connected to a vacuum pump. For *E. andrei*, both the egested particles and the particles retained inside their body after the depuration were assessed. To

assess egestion, the collected faeces were transferred to a glass beaker using 5 mL H_2O_2 (30%) and treated following the same procedure for dissolving and filtering as used for the earthworms. To assess the amount of retained particles, the same protocol was used as for earthworms with the full gut.

F. candida adults were dissolved following the modified protocol of Kallenbach et al. (2021). In a 50 mL glass beaker, 5 mL of H_2O_2 (30%) was added to each replicate containing 10 freeze-dried springtails. The pH was adjusted to 5.5 using NaOH, which is the optimum level for the chitinase activity, facilitating enzymatic chitin degradation and therefore exoskeleton breakdown. After pH adjustment, 1.6 mL chitinase (EC 3.2.1.14, ASA Spezialenzyme GmbH, Wolfenbüttel, Germany) (40 U/mL sample) was added and samples were placed into an orbital shaker at 37ºC for 24 h at 120 rpm. Afterwards, the samples were filtered over cellulose nitrate membrane filters (Scharlau, 47 mm diameter, 0.45 µm pore size).

All filters from the different matrices were examined at a 4x magnification using a stereomicroscope (Olympus SZX10). Depending on the MP load of each filter, 100%, 50%, 25% or 12.5% of the MPs in the filter were counted per colour and shape and then measured. The length of the longest axis was measured using a digital microscope camera (Olympus DP21) and the DP2-TWAIN software.

Alpha values

Due to the expected fragmentation of plastic particles in the environment, Kooi and Koelmans (2019) fitted MPs probability size distributions to a power law function (Equation 1). The exponent of this equation is known as the alpha value (α) and can be used to characterize the size distribution of MPs in the sample. These values were determined to allow comparisons of the MP mixture used here with environmentally relevant mixtures of MPs reported in previous studies, using the extrapolations shown by (Kooi et al. 2021).

The *α* values of the distribution of the MP in the test soil and taken up by *E. andrei* were calculated following the equation:

$$y = b \cdot x^{-\alpha} \qquad \text{(Eq. 1)}$$

where *y* is the cumulative distribution of MPs, *b* is the slope of the frequency distribution plot, and *x* is the particle size (based on the longest axis).

Quality assurance and quality control

Throughout the experiments and sample processing, cotton lab coats were worn, and the use of plastic lab material was avoided. The processing and the filtration of samples was carried out under a laminar flow fume hood. In addition, three blanks were included for each batch of samples processed for MP analysis. Recovery tests were performed to assess the efficiency of the MP extraction from soil, and from *E. andrei* and *F. candida* samples. These were done by adding 20 MPs of each polymer type to 1.0 g of dried soil, one freeze-dried earthworm, or 10 adult springtails. Three replicates were analysed for each test matrix, following exactly the same methods as described above for the exposure assessment. After extraction, all particles recovered were photographed under a stereo microscope (Olympus SZX10) equipped with a digital microscope camera (Olympus DP21). Then, the length of the longest axis

of each particle was measured using the Infinity Analyze software 6.5 (Lumenera Corporation 2015). The calculated recoveries were considered acceptable for the three matrices, with mean recovery values being close to or above 70%, see the Supplementary Material (Table S1).

Data analyses

MPs in initial soil mixture - The number of MPs in the test soil of both species was assessed at concentrations of 0, 0.064 and 0.4%. The number of particles for the rest of the concentrations was estimated by extrapolating these results and subtracting the number of MPs found in the controls. These numbers were used to express the results of the toxicity tests and for the risk assessment.

MP uptake - The number of MPs taken up by the test organisms at the different exposure concentrations were compared using the Kruskal Wallis test followed by the Conover post—hoc test. These tests were chosen as data did not follow a normal distribution (according to Shapiro-Wilk test) or homogeneity of variances (according to Levene's tests).

The MP size range of the initial mixture in the soil, the size range of MPs taken up by *E. andrei,* the size range of MPs retained by this species after 24 hours of depuration, and the size range of MPs egested in their faeces were compared to assess potential differences related to ingestion and digestion processes. To do so, the particle sizes were transformed to relative frequencies using the bins specified in Table S2. Next, Chi-squared tests were performed to compare the size distribution of the MPs in the different matrices. Finally, MP uptake size thresholds for *E. andrei* and *F. candida* were determined as the 90% percentile of the size distribution of ingested particles.

All statistical tests were performed using R studio (R Core Team 2023) with the packages "PMCMRplus" and "car" (Fox, 2023; Mair & Wilcox, 2020), or the software Jamovi (Jamovi, 2024). Differences were considered significant if the calculated p-value was <0.05.

MP effects - The MP effects were described in terms of the calculated No Observed Effect Concentration (NOEC) for all evaluated endpoints. The NOEC was determined using the William's test in R studio (R Core Team 2023) with the package "PMCMRplus" (Mair & Wilcox, 2020). Differences compared to the control were considered significant if the calculated p-values were <0.05.

Risk assessment - Measured environmental concentrations (MECs) of MPs in different soils were extracted from Redondo-Hasselerharm et al. (2024). In their study, MECs were transformed to the size range 1-5000 µm and were classified based on soils subject to different sources of MP pollution, considering background soils (i.e., soils without apparent source of MP contamination), soils treated with sewage in the form of biosolids (treated sludge) or sludge, soils treated with compost, and soils exposed to mulching films. The MECs reported by Redondo-Hasselerharm et al. (2024) were converted to the size range used in this study (50-5000 µm) using a Conversion Factor (CF) calculated with Equation 2.

$$CF = \frac{5000^{1-\alpha} - 50^{1-\alpha}}{X_2^{1-\alpha} - X_1^{1-\alpha}} \quad \text{(Eq. 2)}$$

where x_1 and x_2 are the minimum and maximum values of the size range in the different soil studies, and alpha (α) is the value reported by Redondo-Hasselerharm et al. (2024) for the different MP sources. The re-scaled MECs (i.e., in the range 50-5000 µm) were then compared to the NOECs obtained in this study for the two species tested.

RESULTS AND DISCUSSION

Soil pH and microplastic characteristics in the soil mixture

The initial pH of the soil mixture ranged from 5.48 to 5.98, while in the *E. andrei* and *F. candida* tests, it ranged from 5.47 to 5.60. The addition of microplastics (MPs) did not significantly affect the measured pH values (Table S3).

In the *E. andrei* test, a concentration of 0.064% corresponded to 1.28×10^6 MPs/kg of dry soil, whereas the estimated particle concentration at the highest exposure level (1%) was 2×10^7 MPs/kg of dry soil. Similarly, in the *F. candida* test, the 0.064% concentration contained 1.07×10^6 MPs/kg of dry soil, and the highest exposure concentration (5%) was estimated to contain 8.34×10^7 MPs/kg of dry soil (Table S4).

Measurements of MP particles in the soil mixture revealed a size range of 50–5000 µm. Specifically, HDPE fragments ranged from 50 to 1709 µm, PP fragments from 55 to 555 µm, and fibers from 50 to 4842 µm. The 90th percentile of the MP particle size distribution in the test soil was 252 µm for particles and 1583 µm for fibres (Table S5). The calculated alpha value (α) for the MPs in the soil mixture, considering all polymer types, was 5.14 ± 0.90 (Table S6; Figure S1). This value was slightly higher than the alpha value (α) reported by Redondo-Hasselerharm et al. (2024) (3.38 ± 0.50) for the MP dataset provided by Crossman et al. (2020). In that dataset, 729 particles were measured from soil samples collected in Ontario, Canada, which had been repeatedly amended with sewage sludge. The higher alpha value observed in our study suggests that the sample tested here contained a greater proportion of smaller particles compared to the Canadian samples. This difference may be attributed to the MP generation methods used in our study, which likely resulted in a more extensive breakdown of MPs than typically occurred in natural soils.

Microplastic uptake

The survival rate of adults from both species (*E. andrei* and *F. candida*) across all tested concentrations was 90–100% (Figure S2A). Consequently, the uptake of MPs by these species could be determined. In the controls, *E. andrei* individuals contained a small number of MPs in their bodies (mean: 1.1 ± 3 MPs per individual; n = 19). However, these levels were significantly lower than those found in earthworms exposed to MPs (Figure 1A). For *E. andrei* individuals exposed to 0.064% and 0.4% MP concentrations in the soil, the number of MPs taken up was 85 ± 10 MPs and 613 ± 84 MPs per individual (mean ± SD), respectively, indicating that the uptake was directly related to the exposure concentration.

The faeces of *E. andrei* after 24 hours of depuration showed similar patterns, with very low MP concentrations in the controls and significant differences in the

number of MPs between the controls and the exposed groups (p-values: 0.041 and <0.001, respectively). At the 0.064% and 0.4% exposure levels, the MP counts in the faeces were 75 ± 9 MPs and 612 ± 141 MPs per individual, respectively (Table S7).

The maximum size of MPs taken up by *E. andrei* (90th percentile) was 275 µm for HDPE fragments, 325 µm for PP fragments, and 1583 µm for PES fibres (Table S5). This confirms that the full MP size range tested here was bioavailable to *E. andrei*. The maximum ingestible MP size reported for a closely related species, *Eisenia fetida*, was 1660 µm (Li et al. 2021), which closely matches the 90th percentile size of fibres taken up by *E. andrei* in our study.

Chi-squared tests comparing the MP size distributions in the soil, the distribution of ingested MPs, the distribution of retained MPs in the organisms after 24 hours of depuration, and the MP distribution in the feces showed no significant differences (p-values between 0.98 and 1; Figures 2–4). Furthermore, the calculated α-value for the MPs taken up by *E. andrei* (5.69 ± 1.08; Table S6) is very close to that calculated for the initial soil mixture (5.14 ± 0.90), indicating that both size distributions were very similar. These results show that *E. andrei* can ingest all particles within the tested size range without any preferential uptake. Furthermore, our results indicate that *E. andrei* can egest particles relatively quickly (approximately 80% within 24 hours) without preferential retention or breakdown inside the organisms.

In the case of *F. candida*, the number of MPs taken up in the control group was 0.03 ± 0.08 MPs per individual (mean ± SD, n = 6). At the highest exposure concentration (2.5%), the average number of MPs in exposed springtails was 0.26 ± 0.27 MPs per individual (Table S7). However, no significant differences were observed between the control and any of the tested concentrations (Figure 1B). Overall, the number of MPs taken up by *F. candida* was minimal, with only 11 MPs detected across all 278 adults analyzed.

Kim and An (2020) investigated MP uptake behavior in *F. candida* using particles within a size range of 0.47–300 µm. They reported a maximum ingestible size of 42 µm for this species, noting that particles as small as 66 µm were already too large for ingestion. This suggests that the MP size range tested in our study was beyond the ingestion capacity of *F. candida*. Consequently, any potential effect observed in our study would likely result from mechanisms other than direct MP ingestion.

Microplastic effects

The results of our study showed no significant effects on any evaluated endpoints for *E. andrei* across the tested concentrations (0–1% dw) (Figure S1). However, a slight decrease in earthworm reproduction was observed at concentrations of 0.4% and 1% (Figure S2E), although no clear dose-response relationship could be established. Similarly, no significant differences in survival, growth, or reproduction were observed in a recent study by Forsell et al. (2024), which tested the toxic effects of two types of polymers on *E. andrei* at concentrations up to 5% of soil. These polymers included polyethylene (PE) fragments, with particle sizes below 3600 µm (median particle size of 57 µm),

and polybutylene adipate terephthalate (PBAT) fragments, with particle sizes below 500 µm (median particle size of 147 µm).

Other studies have also reported no effects on survival or biomass of *E. andrei* at concentrations up to 0.1% for PE fragments ranging from 250 µm to 1000 µm (Rodriguez-Seijo et al. 2017). Conversely, Quigley et al. (2024) reported negative effects on reproduction and growth in *E. andrei* at a concentration of 0.002% (20 mg/kg). In that study, agricultural soil collected from an organic farm was spiked with weathered PE particles ranging from 32 µm to 500 µm, resulting in a 29% reduction in juvenile numbers. This suggests that smaller MP particle sizes, coupled with the effects of weathering, may have a more pronounced detrimental impact on this species.

For the *F. candida* test, no significant differences in survival or adult weight were observed at any tested concentrations. However, significant differences were observed in reproduction-related endpoints. Based on Williams' test, the NOEC for the number of juveniles and total reproduction (juveniles per surviving adult) was 0.4%. At the highest test concentration (5%), reproduction decreased by 30% in juvenile numbers and 27% in juveniles per adult. For juvenile dry weight, the calculated NOEC was 2.5%, with a 31% reduction at the 5% concentration (Table 2, Figure 5). However, a dose-response model was not be fitted to the data, as the highest test concentration did not result in effects greater than 50% relative to the control (Figure 5).

The number of juveniles counted at the highest test concentrations (2.5% and 5%) may have been underestimated due to the floating behaviour of HDPE and PP fragments, which may have obscured juveniles during image analysis. However, reductions in juvenile numbers at concentrations of 0.4% and 1%, along with variations in juvenile weight (where juveniles were manually separated from plastics), provide stronger evidence for reduced offspring production. Despite these observations, the significant reduction in reproduction was confirmed with a NOEC of 0.4%.

A multigenerational study performed with agricultural plastics (PBAT, LLDPE) at concentrations up to 5% did not find any significant effects on *F. candida* (Van Loon et al. 2024). However, other studies have reported effects on *F. candida* reproduction at low MP concentrations. For example, Zhu et al. (2018) observed reproduction effects at 0.1% after 56 days of exposure to commercial polyvinyl chloride (PVC) particles sized 80–250 µm. Similarly, Selonen et al. (2021) found a 38% reduction in *F. candida* reproduction at 1.5% exposure to tire particles (<180 µm, median size <10 µm). Both PVC and tire debris are known to contain high levels of plastic additives, which may play a critical role in these effects (Jang et al. 2021; Meng et al. 2021). In our study, the MP mixture was pre-cleaned with methanol, but the possibility of a small fraction of plastic additive release during the experiment cannot be completely excluded (Ügdüler et al. 2020).

Given to the lack of significant MP ingestion, the observed effects in *F. candida* may be due to mechanisms other than ingestion and subsequent blockage of the digestive tract. Kim and An (2019) suggested that MPs can hinder springtail mobility by obstructing soil pores at concentrations above 0.0008% (8 mg/kg). Additionally, Maaß et al. (2017) reported that MPs smaller than 200 µm can

attach to the body of *F. candida*, potentially impairing movement or, in the case of longer fibres, causing entanglement. Ju et al. (2019) found avoidance behaviour of *F. candida* exposed to PE beads (32% below 50 µm and 43% between 200 and 500 µm) at concentrations of 0.5% and 1%, and a 70% reduction in reproduction at a concentration of 1%. These findings indicate alternative mechanisms that may underlie the observed reduction in springtail reproduction, warranting further investigation in follow-up studies. Future research should encompass analyses of soil structure, examination of plastic leachates, and measurements of organism movement.

Risk assessment

Figure 6 illustrates the cumulative frequency distribution of MECs for soils contaminated with various MP sources—background levels, biosolids, compost, and mulching film—rescaled to the 50–5000 µm size range, as reported by Redondo-Hasselerharm et al. (2024). The highest environmental concentration (8×10^4 particles/kg dry soil) is approximately two orders of magnitude lower than the lowest NOEC derived in our study for springtail juvenile numbers and reproduction (6.67×10^6 particles/kg dry soil). Based on this, it can be concluded that environmentally relevant MP mixtures at current concentrations do not pose an immediate risk of adverse effects on the test species.

However, it is important to note that for *F. candida*, the mechanisms of food dilution (excluding particles larger than their mouth opening) and tissue translocation (considered only for particles smaller than 83 µm), as outlined by Redondo-Hasselerharm et al. (2024), were not accounted for in our study. For this species, MP concentrations in the lower size range (<50 µm) or nanoplastics may be more relevant for assessing potential risks such as digestive tract blockage or accumulation in tissues and organs. Therefore, further long-term toxicity studies and risk assessments focusing on smaller plastic particles are recommended, particularly for small soil arthropod species. This is especially critical considering the routine application of sewage sludge and other MP-contaminated materials in soil ecosystems, which is likely to lead to increased MP exposure concentrations over time (Yang et al. 2024).

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Figures

Figure 1. Number of microplastics (MPs) with a size between 50 and 5000 µm taken up by *Eisenia andrei* individuals (A) and *Folsomia candida* individuals (B) after 28 days of exposure in LUFA 2.2 soil spiked with an environmentally relevant mixture of MPs (see Table 1). The numbers are expressed as means, and the error bars represent the standard deviation. Concentrations that do not share the same letter are significantly different according to the Conover test ($p < 0.05$). Please note that the y-axis of A is displayed on a logarithmic scale.

Figure 2. Size class distribution of high-density polyethylene (HDPE) microplastics (MPs) in the initial soil mixture (A), ingested by the earthworm *Eisenia andrei* after 28 days of exposure (B), in the faeces (C), and retained by the earthworms after 24 hours of depuration (D). The dashed line shows the 90^{th} percentile of the distribution. Small particles (< 50 µm) were not included as part of the analysis.

Figure 3. Size class distribution polyester (PES) microplastics (MPs) in the initial soil mixture (A), ingested by the earthworm *Eisenia andrei* after 28 days of exposure (B), in the faeces (C), and retained by the earthworms after 24 hours of depuration (D). The dashed line shows the 90^{th} percentile of the distribution. Small particles (< 50 µm) were not included as part of the analysis.

Figure 4. Size class distribution polypropylene (PP) microplastics (MPs) in the initial soil mixture (A), ingested by the earthworm *Eisenia andrei* after 28 days of exposure (B), in the faeces (C), and retained by the earthworms after 24 hours of depuration (D). The dashed line shows the 90^{th} percentile of the distribution. Small particles (< 50 µm) were not included as part of the analysis.

Figure 5. Boxplots showing the effects of an environmentally relevant mixture of microplastics (MP; see Table 1) on *Folsomia candida* after 28 days exposure in LUFA 2.2 soil. A) Survival. B) Adult dry weight. C) Number of juveniles. D) Juvenile dry weight. E) Reproduction (number of juveniles per adult). Significant differences compared to the control (William´s test; p-value<0.05) are indicated with an asterisk. Please note that the controls met the validity criteria, with mean adult mortality below 20%, an average of more than 100 juveniles per test unit, and a coefficient of variation in juvenile numbers below 30%.

Figure 6. Cumulative frequency distribution of Measured Exposure Concentrations (MECs) of microplastics (MPs) re-scaled to the 50–5000 µm size range, plotted together with the springtail *Folsomia candida* NOECs for juvenile number and reproduction (purple dashed line), and juvenile weight (green dashed line) derived in this study. Note that for the earthworm *Eisenia andrei* the NOEC was larger than 2×10^7 MPs/kg dry soil for all evaluated endpoints. The legend shows the different MP contamination sources— background levels, biosolids, compost, and mulching film— of the sampling points where the MECs were obtained as described by Redondo-Hasselerharm et al. (2024).

Tables

Table 1. Concentrations of microplastics (MPs) in mass percentage (%) and in g/kg of dry LUFA 2.2 soil used in the toxicity tests with the earthworm *Eisenia andrei* and the springtail *Folsomia candida* in LUFA 2.2 soil. HDPE: high density polyethylene; PES: polyester; PP: polypropylene. The asterisk (*) indicates concentrations only tested with *F. candida*.

%	0	0.064	0.16	0.4	1	2.5 *	5 *
g/kg dw	0.00	0.64	1.60	4.00	10.0	25.0	50.0
HDPE	0.00	0.35	0.88	2.19	5.48	13.7	27.4
PES	0.00	0.27	0.68	1.71	4.26	10.7	21.3
PP	0.00	0.02	0.04	0.10	0.25	0.63	1.27

Table 2. No Observed Effect Concentrations (NOECs) for the different evaluated endpoints of the earthworm *Eisenia andrei* and the springtail *Folsomia candida*, exposed for 28 days to a microplastic (MP) mixture. NOECs are expressed in mass percentage (%), g/kg dry soil, and number of MPs per kg dry soil.

Species	Endpoint	NOEC (%)	NOEC (g/kg dry soil)	NOEC (MPs/kg dry soil)
E. andrei	Survival, mass change, number of juveniles, and reproduction	>1	>10	$>2\times10^{7}$
F. candida	Survival	>5	>50	$>8.34\times10^{7}$
	Adult dry weight	>5	>50	$>8.34\times10^{7}$
	Number of juveniles	0.4	4	6.67×10^{6}
	Juvenile dry weight	2.5	25	4.17×10^{7}
	Reproduction	0.4	4	6.67×10^{6}

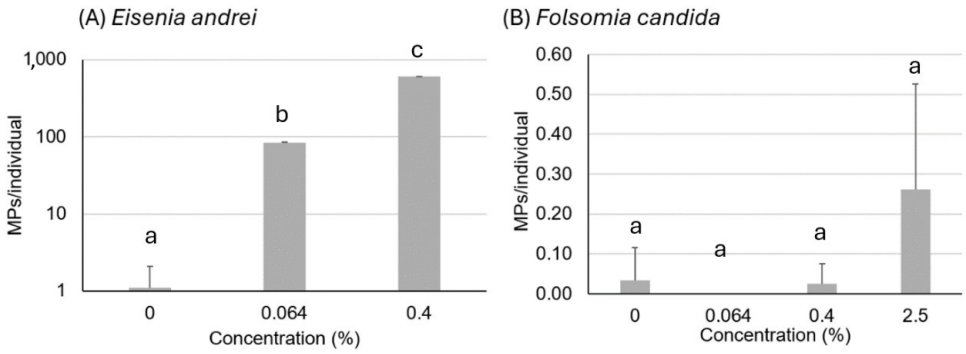

Fig. 1

149x64mm (220 x 220 DPI)

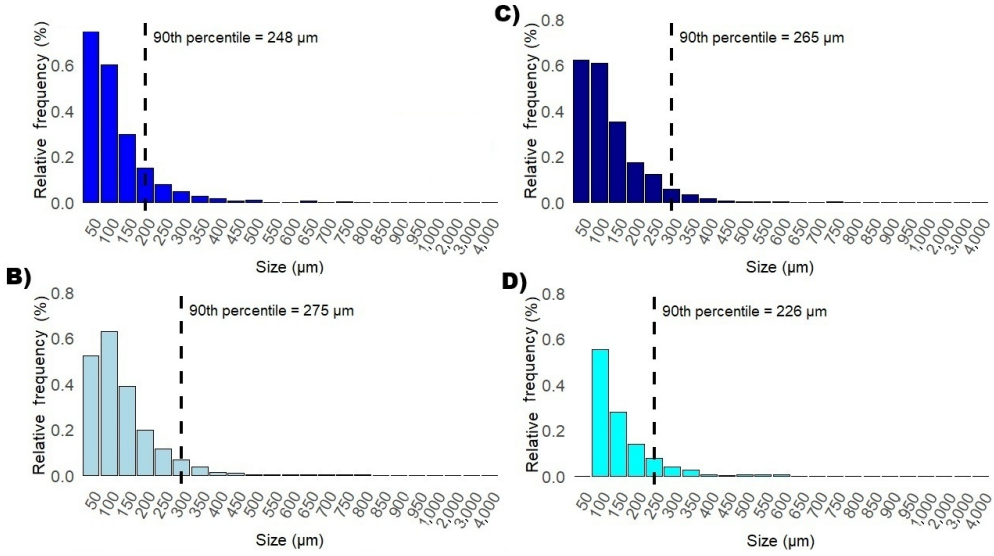

Fig. 2

338x194mm (96 x 96 DPI)

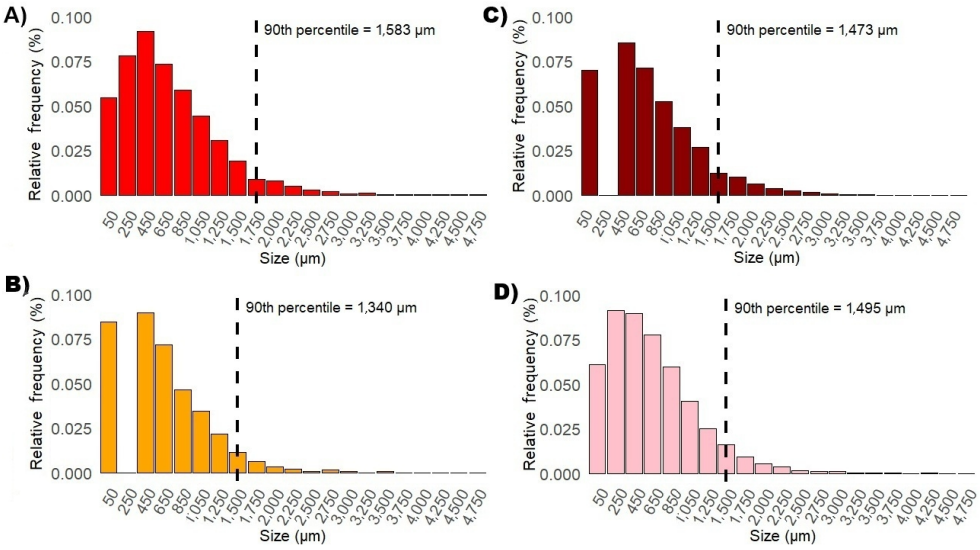

Fig. 3

347x199mm (96 x 96 DPI)

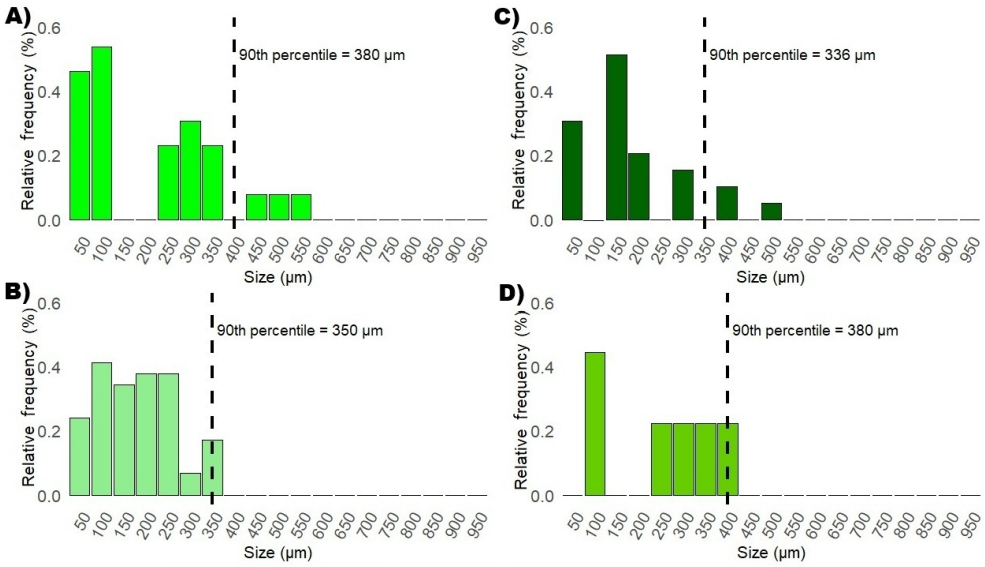

Fig. 4

335x196mm (96 x 96 DPI)

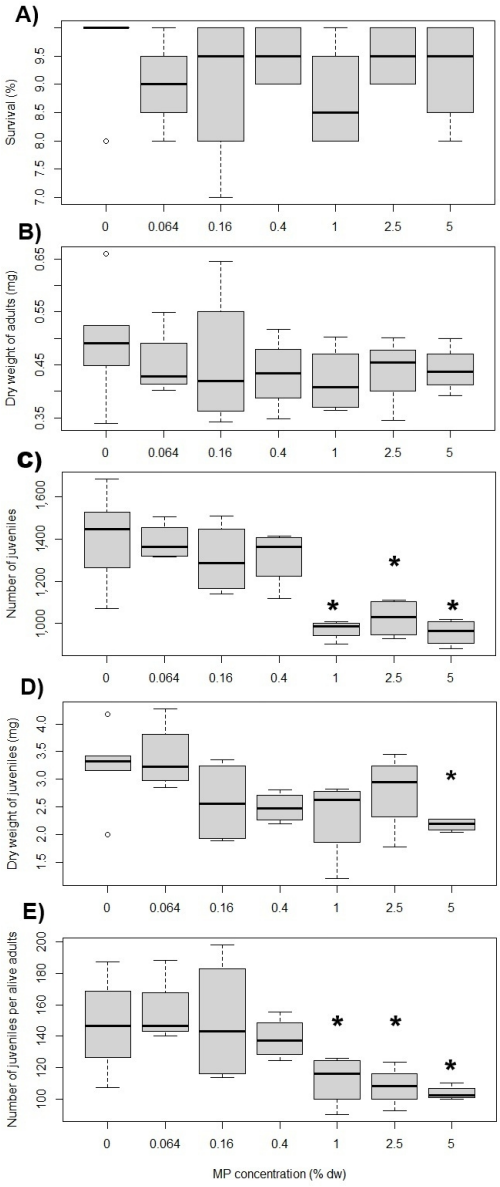

Fig. 5

175x411mm (96 x 96 DPI)

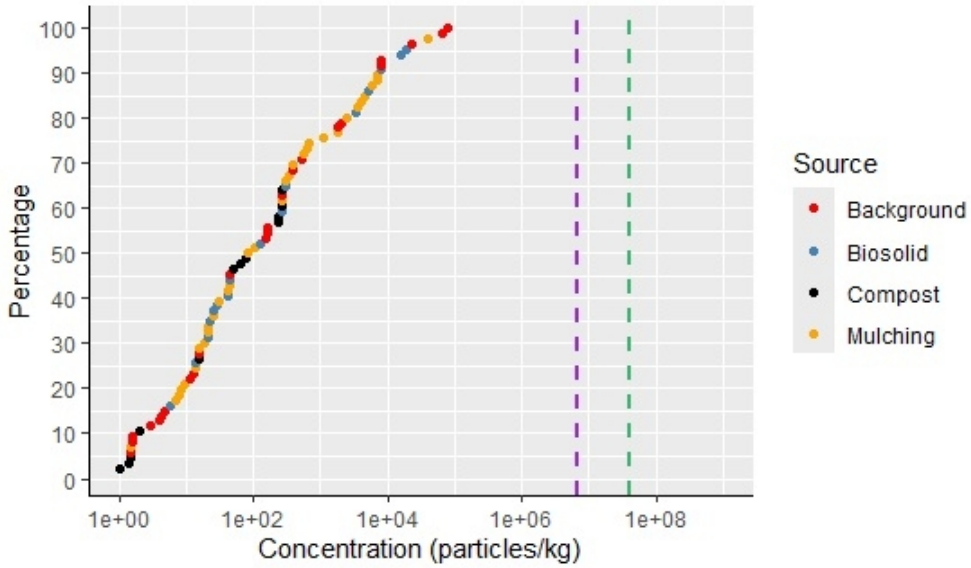

Fig. 6

146x85mm (96 x 96 DPI)