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Microplastic fibers influence Ag toxicity and bioaccumulation in *Eisenia andrei* but not in *Enchytraeus crypticus*

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Abstract

Microplastic fibers (MF) are released from synthetic textiles during washing and end up in the wastewater. Similarly, silver nanoparticles (AgNP), incorporated in textiles as antimicrobial agents, are released in washing machines, also reaching the wastewater treatment plants. Therefore, both MF and AgNP co-exist in the environment and enter the soil compartment mainly via the application of biosolids. Yet, the combined effect of MF and AgNP has not been studied. Here, we assessed the effects of polyester MF on the toxicity of AgNP and $AgNO_3$ to the earthworm *Eisenia andrei* and the enchytraeid *Enchytraeus crypticus*. The organisms were exposed to a range of concentration of AgNP (32, 100, 320, 1000, 3200 mg Ag/kg) and $AgNO_3$ (12.8, 32, 80, 200, 500 mg Ag/kg) in LUFA 2.2 soil in the absence or presence of MF (0.01% DW). Reproduction tests were conducted and the toxicity outcomes compared between soils with and without MF. The exposure to MF caused a decrease in the number of juveniles and changed the biochemical composition of earthworms. Moreover, the presence of MF increased the toxicity of AgNP to earthworm reproduction (EC50 = 165 mg Ag/kg) when compared to AgNP exposure alone (EC50 = 450 mg Ag/kg), but did not alter the toxicity of $AgNO_3$ (EC50 = 40 mg Ag/kg). For enchytraeids, no significant difference in Ag toxicity could be detected when MF was added to the soil for both AgNP and $AgNO_3$. Overall, Ag bioaccumulation was not affected by MF, except for a decrease in earthworm body concentration at the highest Ag soil concentration (3200 mg Ag/kg). Our results suggest that the presence of MF in the soil compartment may be a cause of concern, and that the joint exposure to Ag may be deleterious depending on the Ag form, organism, and endpoint. The present work provides the first evidence that a realistic MF concentration in soil lowers AgNP concentration necessary to provoke reproductive impairment in earthworms. The influence of MF on the risk assessment of AgNP should be considered.

Keywords Chronic toxicity · Earthworms · Enchytraeids · Polyester · FTIR

Introduction

Recently, the contamination of the soil compartment by microplastics has been recognized as an issue of concern. Studies have shown the ubiquitous presence of microplastics in soils, which can vary among sites with different land uses.

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For example, floodplain sites in Switzerland had an average microplastic concentration of 0.0005% dry weight (DW), with the most contaminated site containing 0.0055% (DW) (Scheurer and Bigalke 2018), equivalent to 5 and 55 mg/kg, respectively (Scheurer and Bigalke 2018). In industrial areas in Australia, microplastic concentrations varied from 0.03 to 6.7% (DW) (Fuller and Gautam 2016). Different sources can contribute to the increasing concentration of microplastics in the terrestrial compartment, such as local deposition of waste and atmospheric deposition. Nevertheless, the main source is believed to be the land application of biosolids from wastewater treatment plants (WWTP) (Ng et al. 2018; Horton et al. 2017b).

One major contributor to microplastic pollution is the release of fibers from synthetic textiles (Browne et al. 2011; Horton et al. 2017a). During the washing process, microplastic fibers (MF) can be released from households to wastewater (Sillanpää and Sainio 2017; Pirc et al. 2016; Hernandez et al. 2017). Therefore, MF reaches the WWTP and are also believed to reach soils via biosolids application (Nizzetto et al. 2016). Even their presence can be used as an indicator of sludge application on soil over the years (Zubris and Richards 2005). A study in a Canadian WWTP, for example, showed that 98% of microplastics are retained in sludge with the majority (70%) being represented by fiber-shaped fragments (Gies et al. 2018).

The presence of MF can affect soil properties, including soil water holding capacity (de Souza Machado et al. 2018), bulk density (de Souza Machado et al. 2018), and porosity (Zhang et al. 2019). Another important aspect is the potential of microplastics to act as sorbents for contaminants in soil. The distribution of chemicals in microplastics varies, depending especially on the chemical partitioning coefficients to soil and microplastics as well as the mass of microplastics (Tourinho et al. 2019). With the increasing input of MF to soils, the chances of MF to act as an extra route of exposure to organisms may also increase. Nevertheless, the majority of studies on this topic have been conducted on marine invertebrates (Ribeiro et al. 2019), and there is a lack of studies on the combined effects of MF and chemicals on soil organisms.

Contaminants having a similar pathway to MF are likely to coexist and interact with MF in the environment. This includes silver nanoparticles (AgNP) which are applied to textiles because of their antimicrobial properties (Durán et al. 2007). AgNP are also released during the washing of textiles (Mitrano et al. 2014), ending up in the sludge of WWTP. Therefore, AgNP present a similar pathway as MF, reaching soils via biosolids application (Whiteley et al. 2013). Although predicted environmental concentrations for AgNP reach maximum values in soils in the ng/kg range (Wigger et al. 2015), total Ag concentration in sludge from municipal WWTP can be as high as 200 mg Ag/kg (USEPA 2009). Moreover, AgNP continuously release Ag ions into soil pore water (Diez-Ortiz et al. 2015), and therefore, it is likely that ionic Ag also co-exist with MF.

In this context, we aim at evaluating the effects of polyester MF on the toxicity of AgNP and the ionic counterpart $AgNO_3$ to the earthworm *Eisenia andrei* and the enchytraeid *Enchytraeus crypticus*, using an environmentally relevant MF concentration of 0.01% DW. Earthworms and enchytraeids have different traits and, therefore, the influence of MF on Ag toxicity may differ between them. Polyester was selected as being the main plastic material used in fiber production (Geyer et al. 2017). Standard reproduction tests were performed with AgNP and $AgNO_3$ in the absence or presence of MF.

It has been previously shown that microplastics can adsorb AgNP and ionic Ag (Khan et al. 2015; Kalčikova et al. 2020; Li et al. 2020), and that micro- and nanoplastics increase the toxicity of Ag to aquatic organisms (Khan et al. 2015; Monikh et al. 2020). This study is, however, the first report on the combined toxicity of Ag and MF to soil invertebrates.

Methodology

Test chemicals

AgNP (99.5% trace metal basis, CAS 7440-22-4) and $AgNO_3$ (≥99% purity, CAS 7761-88-8) were purchased from Sigma-Aldrich. The nanoparticles were provided as nanopowder with particle size <100 nm, coated with polyvinylpyrrolidone (PVP). The nanoparticle morphology was investigated by transmission electron microscopy (TEM), using a JEOL 2200 FS microscope. A dispersion of AgNP in water was deposited on a holey carbon coated Cu TEM grid and dried at room temperature before examination. The TEM images showed that the AgNP have a spherical-like shape (Figure SI1).

To produce the MF, a continuous filament yarn was used. The polyester yarn was transparent in color and had a diameter of 400 µm (Korálky Katlas). The yarns were initially cut in smaller filaments with a length of about 1 mm and subsequently milled in a centrifugal mill (Cryomill by Retsch). Milling conditions were set as 8x45s (cycles - time) with 15 s homogenization between the cycles. The final fibers were analyzed by TESCAN VEGA-3 LMU scanning electron microscopy (SEM).

Microplastic fibers characterization

FTIR analysis was performed to define the composition of polyester (Fig. 1A). SEM images of the MF confirmed the presence of elongated fibers as well as irregular shapes after the cryo-milling process (Fig. 1C). A peak at 1732 cm^{-1} can

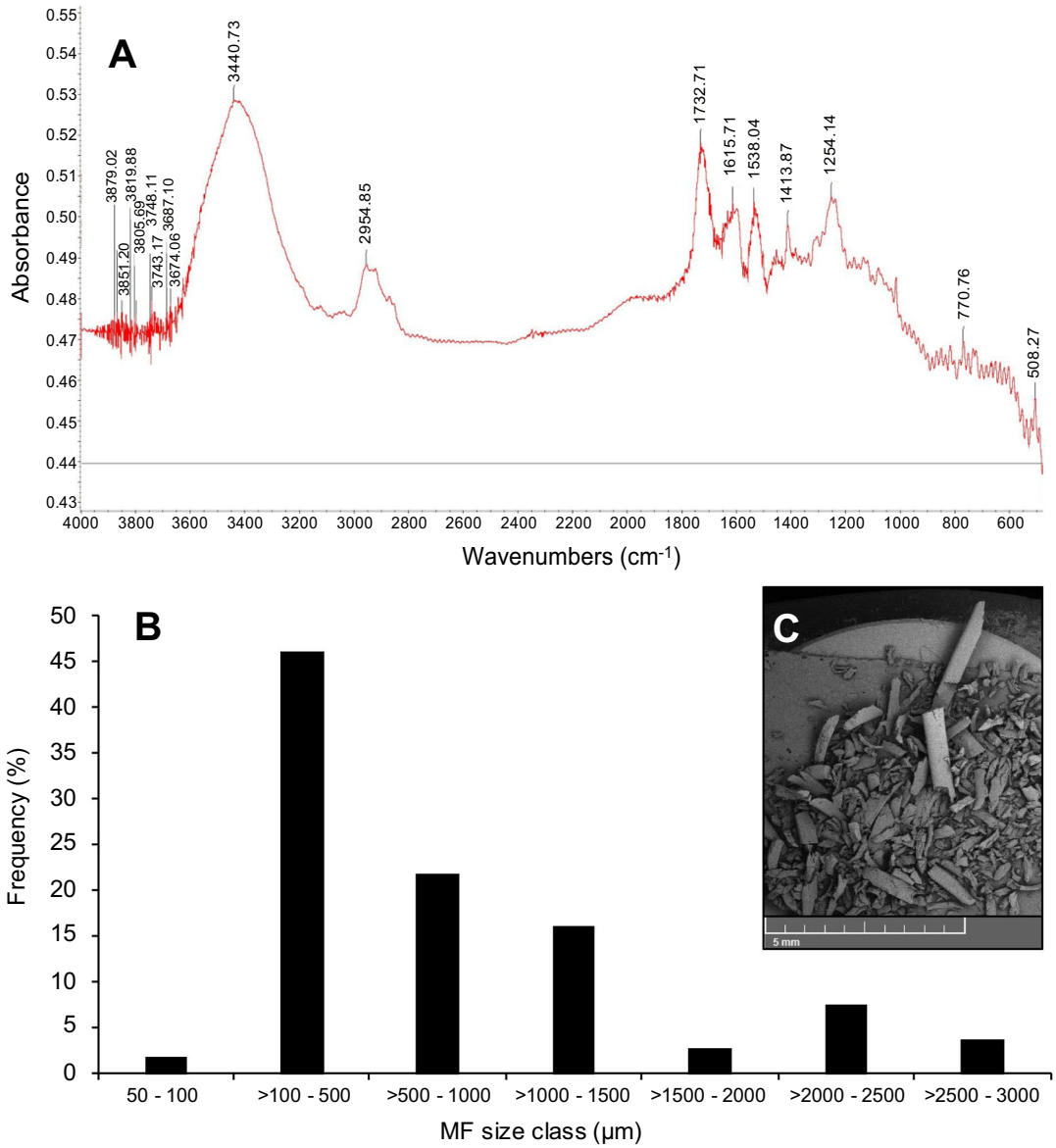

Fig. 1 **A** Fourier Transform Infrared (FTIR) Spectroscopy of polyester microplastic fibers. **B** Percentage of different sizes of microplastic fibers (MF) found in LUFA 2.2 soil spiked at 0.01% w/w. **C** Scanning Electron Microscopy (SEM) image of microplastic fibers after the cryo-milling process

be attributed to the carbonyl group, shown by the C=O vibration. Bands around 1730 cm^{-1} indicate the presence of the ester group, confirming the material as polyester (Dholakiya 2012; Rosu et al. 2008).

MF were successfully found in the 0.1 g samples of spiked soils, indicating a fair distribution of the fibers in soil. Average numbers of MF in the MF control soils of AgNP (±SD; $n = 70$) and $AgNO_3$ (±SD; $n = 60$) experiments were 116 ± 34 and 119 ± 59 fibers/g soil and MF lengths were 820 ± 730 and 770 ± 740 µm, respectively. The size distribution of MF varied from approximately 50 to 3000 µm, with almost half of the fibers having a size between 100 and 500 µm (Fig. 1B).

Test organisms

The earthworm *Eisenia andrei* and the enchytraeid *Enchytraeus crypticus* were cultured at the Vrije Universiteit (Amsterdam, The Netherlands). Earthworms were kept at 20 °C in plastic containers containing a mixture of 1:1 potting soil and peat with a teaspoon $CaCO_3$ to adjust the pH and were fed once a week with horse manure free of any pharmaceuticals. Enchytraeids were kept on agar prepared with soil extract at 16 °C, and fed once a week with a mixture composed of oatmeal, dried yeast, yolk powder and fish oil. The cultures were maintained at a controlled photoperiod of 16 h light/8 h dark, however the animals were

cultured in darkness to avoid any stress caused by light, especially for enchytraeids, which are kept on agar. Only sexually mature animals of similar age were used in the experiments (i.e., totally developed clitellum). Earthworms were acclimated in LUFA 2.2 soil for one day before starting the exposures.

Spiking procedure

The standard natural LUFA 2.2 soil (Speyer, Germany) was used in the toxicity tests and characterized as sandy loam with an organic carbon content of 1.7 ± 0.3%, pH (0.01 M $CaCl_2$) of 5.6 ± 0.4, cation exchange capacity of 9.2 ± 1.4 $cmol_c$/kg and a maximum water-holding capacity (WHC_{max}) of 44.8 ± 2.9 g/100 g. Soil was firstly spiked with different concentrations of AgNP or $AgNO_3$, followed by the addition of MF to half of the Ag spiked soils. The concentrations of Ag were chosen based on the studies by Diez-Ortiz et al. (2015) and Topuz and van Gestel (2017). AgNP were added as dry powder, resulting in nominal concentrations of 32, 100, 320, 1000, and 3200 mg Ag/kg dry soil. For $AgNO_3$, stock solutions were prepared with Milli-Q® water, and added to the soil to reach nominal concentrations of 12.8, 32, 80, 200, and 500 mg Ag/kg dry soil. The soils were manually mixed, and Milli-Q® water was added to reach a moisture content equivalent with 50% of the WHC_{max}. Control soils were also prepared by adding Milli-Q® water at 50% WHC_{max}.

After the addition of Ag, the soils were divided in two parts. One part of the soil was spiked with MF at 0.01% DW. This method was chosen in order to avoid different exposure levels of MF among treatments due to a possible non-homogeneous distribution of the MF in the soil. The soil was again rigorously mixed and left to equilibrate for one or two days before the experiments with earthworms or enchytraeids, respectively. Tests with the single and combined MF and Ag treatments were run simultaneously to ensure a proper comparison. The pH of freshly spiked soils (5 g) was measured in 0.01 M $CaCl_2$ solution (25 mL), after shaking for 2 h and left to settle overnight. Soil samples were kept at room temperature for the quantification of MF in soil or at −20 °C for total Ag analysis.

Toxicity tests

Earthworm reproduction test

The test was adapted from the OECD guideline n° 222 (OECD 2004a). Approximately 400 g moist spiked soil, 10 g horse dung and 10 adult earthworms (individual masses between 250 and 500 mg) were added to 1000 mL glass jars. For controls and treatments, 5 and 3 replicates were used, respectively. Animals were kept at 20 °C and 16 h/8 h light cycle. Water loss was replenished with deionized water weekly, and additional food was given after 2 weeks if necessary. After 4 weeks of exposure, surviving adults were collected from the jars, washed, blotted dry on filter paper and weighted. Worms were placed in Petri dishes with moist filter paper for one day to purge their gut, and then frozen at −20 °C. The soils were incubated for another 4 weeks, and at the end of this period, the number of juvenile earthworms emerging from the soil after incubation in a water bath at 60 °C was recorded.

Enchytraeid reproduction test

The test was adapted from the OECD guideline n° 220 (OECD 2004b). Ten adult enchytraeids were added to 100 mL glass test jars containing approximately 25 g moist soil and 2 g oatmeal as food. Four replicates were used per treatment and control. The animals were kept in the same conditions as described for the earthworms. Water and food were replenished weekly. After 3 weeks, adults were collected from soil, washed in distilled water and frozen at −20 °C. Bengal rose and ethanol were added to the soils. After incubation at 4 °C, the juveniles were collected from the soils by sieving and placed in a tray. Pictures were taken and further treated using Photoshop C6 (Adobe) for counting the number of juveniles.

Quantification of microplastic fibers in soil

Control soil samples were checked for the presence of MF, using a method adapted from Selonen et al. (2020). Briefly, MF were isolated from the soil samples (0.1 g) manually using tweezers, at the same time counted and placed onto filter paper (Whatman, 934-AH™). The length of fibers was estimated from stereo microscope Leica MZ FLIII (Leica, Germany) images using Axio Vision 4.8.2 Software.

Total Ag analysis

For total Ag analysis, soil samples ($n = 2$) were dried for 8 h at 70 °C and weighted (approximately 130 mg) and earthworms ($n = 3$ per replicate) were freeze-dried for 48 h and individually weighted. Soil and earthworm samples were digested in 2 mL of a mixture of concentrated HCl (Merck Emparta, purity 37%) and HNO_3 (Merck Emsure, purity 65%) (4:1, v/v) for 7 h in an oven (Binder ED 53) at 140 °C, using tightly closed Teflon containers. After digestion, the soil and earthworm samples were taken up in 10 or 6 mL of demineralized water, respectively, and analyzed by flame Atomic Absorption Spectrometry (AAS; PerkinElmer AAnalyst 100). Certified reference material DOLT-4 was used to ensure the accuracy of the analytical procedure. Ag concentration in the reference material was 85 ± 24% (mean ± SE) of the certified value.

For extracting soil pore water, at the beginning of the exposures soil samples were saturated with Milli-Q® water. After 7 days of equilibration, samples were centrifuged at 2000 RCF for 25 min and filtered over a 0.45 um filter. Filters were pre-treated with 0.1 M $Cu(NO_3)_2$ to avoid Ag sorption (Cornelis et al. 2010). Samples were analyzed by flame AAS (Agilent 280 fs AAS).

The enchytraeids were freeze-dried for 24 h and weighted individually. The samples were digested in 300 µL of a mixture of HNO_3 (67–69%; Fisher Chemical Optima Grade) and $HClO_4$ (70%; JT. Baker Ultrex II Ultra Pure Reagent), in the ratio 7:1 and evaporated to dryness. The residues were taken up in 0.5 mL 1 M HCl. Silver content was determined by graphite furnace AAS (PerkinElmer, Singapore). Recovery of Ag in the reference material (DOLT-4) was 80 ± 1.9% (mean ± SE) of the certified value.

Fourier Transform Infrared (FTIR) Spectroscopy

MF was characterized by FTIR spectroscopy using a Nicolet 6700 (Thermo Nicolet Corp., Madison, WI). The samples were homogenously crushed with potassium bromide salt (KBr) using mortar and pestle and make them into disks (using a hydraulic press). In order to assess any biochemical modification caused by MF and Ag exposure, freeze-dried earthworms exposed to control, MF, and the combination of MF with AgNP (3200 mg Ag/kg) or $AgNO_3$ (200 mg Ag/kg) were also analyzed, prepared in the same manner as the MF. The analysis was carried out at a 4 cm^{-1} resolution within the 4000–400 nm range. The OMNIC 8 software was used for spectral acquisition.

Data analysis

Performances of the controls from the tests with and without MF were compared by a *t*-test. LC50s and EC50s were calculated using Probit analysis and a 3-parameter logistic curve, respectively. The EC50s between treatments were compared by a generalized likelihood ratio (GLR) test. A *t*-test was applied to compare Ag body concentrations in worms exposed in the absence or presence of MF, within the same Ag exposure level. Statistical tests were performed in SPSS Statistics 24 (IBM Corporation).

Results

Ag content in soil and pore water

Soil pH_{CaCl2} varied from 5.4 to 5.8 among treatments, with no influence of Ag concentration, Ag form or MF presence. Total Ag concentrations in soil overall were between 72 and 100% of the nominal concentrations (Table SI1). The low recovery (<20%) observed in soil spiked with AgNP at 3200 mg Ag/kg probably is an artefact as porewater concentrations (Table SI2) and responses of the test organisms (body Ag concentrations in earthworms and enchytraeids in Fig. 3, and earthworm avoidance behavior in Figure SI2) were consistent with a dose-related pattern. All toxicity results are expressed using the nominal concentrations.

Porewater concentrations were lower than the detection limit (0.01 mg Ag/L) in AgNP spiked soils up to 100 mg Ag/kg (Table SI2). Above this concentration, Ag was detected but still low porewater values (0.05 mg Ag/L) were observed even at 3200 mg Ag/kg, which were similar in soils with or without MF. For $AgNO_3$, porewater concentration in control soil and soil spiked at 12.8 mg Ag/kg were lower than the detection limit, while values close to the detection limit (0.01 to 0.03 mg Ag/L) were observed in soils spiked up to 80 mg Ag/kg (Table SI2). At nominal concentrations of 200 and 500 mg Ag/kg, porewater concentrations were 0.29 and 13.6 mg Ag/L, respectively. When MF were added to these soils, Ag porewater concentration slightly decreased to 0.27 and 10.6 mg Ag/L, respectively.

Toxicity outcomes

Earthworms

The earthworm reproduction test met the validity criteria according to OECD (2004a); in the control soils, adult survival was 100%, >30 juveniles per vessel were counted, and coefficient of variation for juvenile number was <30%.

In order to assess effects of the MF on survival and reproduction of the test organisms, a comparison was made between control groups from tests performed simultaneously in the absence and presence of MF. Earthworm survival did not differ between groups, with 100% survival in all controls. However, the presence of MF slightly decreased the number of juveniles per test jar. In the AgNP test, juvenile number (mean ± SE; $n = 4$) decreased from 40 ± 3.1 to 35 ± 3.7 when MF was added to the AgNP control soil. This decrease was, however, not significant (*t*-test, $t = 1.0$, $p = 0.31$). In the $AgNO_3$ test, the number of juveniles significantly decreased from 34 ± 1.6 to 22 ± 2.1 with the addition of MF (*t*-test, $t = 3.9$, $p = 0.006$).

The LC50 and EC50 values for the effects of AgNP or $AgNO_3$ in the presence and absence of MF can be found in Table 1 (dose-response curves are shown in Figure SI3). AgNP caused no significant mortality up to 3200 mg Ag/kg. For $AgNO_3$, the LC50 value was higher in the presence than in the absence of MF. The EC50 for AgNP significantly decreased from 450 mg Ag/kg to 165 mg Ag/kg when MF

Table 1 Effects of AgNP and $AgNO_3$ on the survival (LC50) and reproduction (EC50) of the earthworm *Eisenia andrei* and the enchytraeid *Enchytraeus crypticus* after 28 and 21 days of exposure in LUFA 2.2 soil, respectively

Species		AgNP	AgNP + MF	$AgNO_3$	$AgNO_3$+MF
Eisenia andrei	LC50	>3200	>3200	240 (n.d.)	410 (280–780)
	EC50	450 (250–645)	165* (50–285)	38 (27–50)	40 (13–67)
Enchytraeus crypticus	LC50	>3200	3080 (2375–4520)	130 (110–160)	100 (55–200)
	EC50	880 (60–1700)	1020 (450–1600)	90 (63–120)	73 (35–113)

Results for the exposure of Ag alone or combined with 100 mg/kg of polyester microplastic fibers (MF) are presented. LC50s and EC50s are based on nominal concentrations in mg Ag/kg dry soil, and were obtained using Probit analysis and a 3-parameter logistic model, respectively. The 95% confidence intervals are presented in brackets

*Significant difference in EC50 values for AgNP in the absence and presence of microplastic fibers (GLR test, $X^2_{df=1} > 3.84$, $p < 0.05$). n.d.: no confidence interval could be calculated

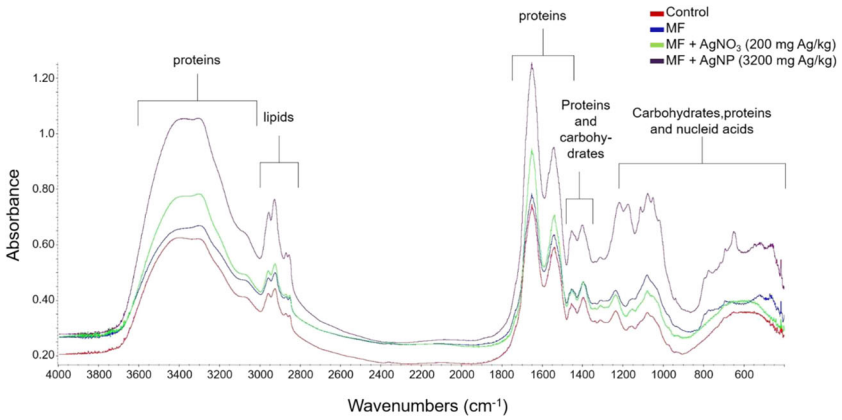

Fig. 2 Fourier Transform Infrared (FTIR) spectra of the earthworm *Eisenia andrei* exposed to control LUFA 2.2 soil, soil spiked with polyester microplastic fiber (MF) alone, and the combination of MF and $AgNO_3$ or AgNP. Bands were described based on Muthukaruppan (2015), Rodríguez-Seijo et al. (2017) and Zohdi et al. (2015)

was added to the soil (GLR test, $X^2_{df=1} = 4.02$, $p < 0.05$). Even though the EC50 for $AgNO_3$ was not affected by MF presence (GLR test, $X^2_{df=1} < 3.84$, n.s.), a total reproductive failure was observed at 200 mg Ag/kg in the absence of MF and at 500 mg Ag/kg in the presence of MF.

The FTIR spectra of earthworms are shown in Fig. 2 (for peak wavenumbers, see Figure SI4). Compared to the control, the intensity of bands increased in worms exposed to only MF and the combination of MF with $AgNO_3$ (200 mg Ag/kg) and AgNP (3200 mg Ag/kg). An increase in proteins (3300, 1650, 1544 cm^{-1}), lipids (2918 cm^{-1}), and polysaccharides such as carbohydrates (1080 cm^{-1}), glycogen and nucleic acids (1100 cm^{-1}), was observed.

Enchytraeids

The validity criteria was met in the enchytraeid reproduction test (OECD 2004b): adult survival was >80% and number of juveniles was >25 per vessel with a coefficient of variance <50% in the control groups.

Enchytraeid survival and juvenile numbers did not differ between control groups exposed in the absence or presence of MF (*t*-test, $p > 0.05$) although MF seemed to cause a slight decrease in juvenile numbers. Control groups in the AgNP and $AgNO_3$ tests produced (±SE; $n = 4$) 916 ± 240 and 891 ± 88 juveniles per vessel, respectively, while controls in the presence of MF produced 834 ± 23 and 758 ± 82 juveniles per vessel, respectively.

The LC/EC50 values and dose-response curves for Ag toxicity to enchytraeids are shown in Table 1 and Figure SI5, respectively. In the presence of MF, enchytraeid mortality caused by AgNP slightly increased, with an LC50 of 3080 mg Ag/kg (Table 1). No difference in EC50 for AgNP was observed in the presence or absence of MF (GLR test, $X^2_{df=1} < 3.84$, n.s.). For $AgNO_3$, MF presence did not affect the results for survival or reproduction, with no differences between LC50 and EC50 values (GLR test, $X^2_{df=1} < 3.84$, n.s.).

Ag bioaccumulation

Body Ag concentrations of worms exposed to AgNP and $AgNO_3$ can be found in Fig. 3. In general, the enchytraeids accumulated higher Ag levels than the earthworms, but in both species body Ag concentrations increased with increasing exposure levels in the soil. Overall, the presence of MF did not affect Ag uptake in both worm species (*t*-test, $p > 0.05$). Only in earthworms exposed to the highest concentration of AgNP (3200 mg Ag/kg), the average Ag body concentration significantly decreased from 130 µg/g in the Ag treatment to 60 µg/g in the combination of AgNP and MF (*t*-test, $p < 0.05$).

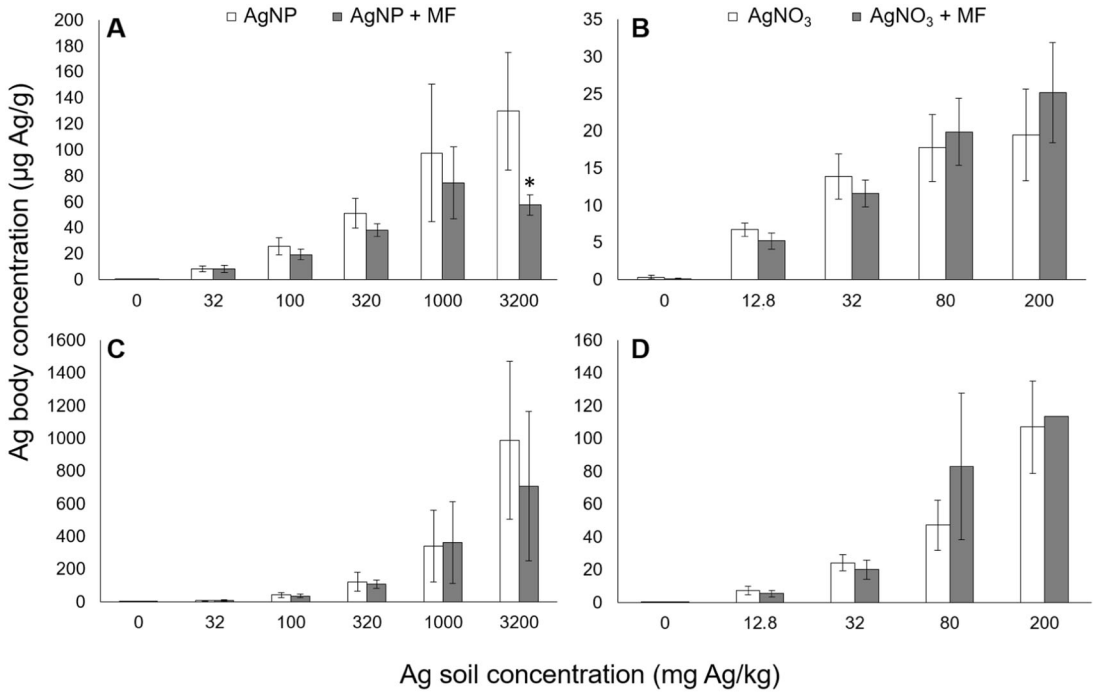

Fig. 3 Ag body concentrations (mean ± SD) in the earthworm *Eisenia andrei* (A and B) and the enchytraeid *Enchytraeus crypticus* (C and D) exposed to AgNP (left) and $AgNO_3$ (right) in LUFA 2.2 soil for 28 and 21 days, respectively. Organisms were exposed to these Ag forms alone (white columns) and in the combination of Ag and polyester microplastic fibers (MF) (gray columns). Asterisk represents differences in Ag body concentrations between worms exposed to only Ag and the combination of Ag and MF within the same Ag exposure level (*t*-test, $p < 0.05$)

Discussion

Microplastic fiber influence on Ag toxicity

The role and mode of action of the combined exposure to microplastics and contaminants is not fully understood (Ribeiro et al. 2019). In order to increase understanding on this topic, the influence of MF, at a realistic concentration, on the toxicity of AgNP and $AgNO_3$ to earthworms and enchytraeids was assessed. For that, we compared EC50 values for Ag alone and in combination with MF. For reasons of time constraints, it was not possible to test different concentrations of MF, we therefore selected a field-relevant MF concentration.

The exposure to MF alone also affected the reproduction of earthworms and enchytraeids, as shown by the decrease in reproduction in the MF controls. A significant decrease in number of juveniles of *E. crypticus* exposed to polyester fibers (4000–24,000 µm in length) in LUFA 2.2 soil at 0.5% (w/w) has been observed (Selonen et al. 2020). And a dose-related decrease in reproduction of *E. crypticus* exposed to nylon particles (13–150 µm in length) has been reported, with an EC50 of ~100 g/kg (10% w/w) (Lahive et al. 2019). Our study, however, is the first indication of microplastic effects on earthworm reproduction. Two previous studies have found no effect of polyethylene microplastics on the reproduction of *E. andrei* (Rodríguez-Seijo et al. 2017) and *Lumbricus terrestris* (Huerta Lwanga et al. 2016). What the reason is of the decreased juvenile numbers in the MF only exposed earthworm groups remains unclear, and needs further investigation.

The presence of MF in soil significantly increased the toxicity of AgNP to earthworm reproduction. A nano-specific effect is suggested, since the toxicity of $AgNO_3$ (used as the ionic counterpart) was not affected by MF presence. Although the mechanisms behind these results remain unclear, it can be speculated that AgNP and MF acted in an additive/synergistic way. The dissolution of Ag ions into soil pore water is considered the main cause of toxicity to earthworm reproduction, which can affect cocoon production (Shoults-Wilson et al. 2011a; Van der Ploeg et al. 2014) and juvenile survival (Diez-Ortiz et al. 2015; Schlich et al. 2013). The effects of microplastics on soil organisms, on the other hand, are mostly related to physical damages. Besides food dilution (Huerta Lwanga et al. 2016), the ingestion of microplastics can result in gastrointestinal tissue damage in earthworms (Baeza et al., 2020; Rodríguez-Seijo et al. 2017). External lesions are also possible when earthworms are exposed through dermal contact (Baeza et al., 2020). At the cellular level, microplastics can induce increased oxidative stress (Prendergast-Miller et al. 2019; Zhou et al. 2020) and DNA damage

(Jiang et al. 2020). It is most likely that all these subtle effects could affect energy allocation and, therefore, have some impact on reproduction. To our knowledge, this is the first report on the combined effects of microplastics and contaminants on earthworm reproduction. Previous studies have mainly assessed effects on earthworm bioaccumulation, survival and/or weight change (Hodson et al. 2017; Gaylor et al. 2013; Wang et al. 2019a, 2019b; Zhou et al. 2020). Reproduction is a more sensitive endpoint than survival and weight change. Therefore, we encourage more research on the joint effects of microplastics and contaminants in earthworms, using reproduction as an endpoint.

The FTIR analysis of organisms can be used as a biomarker for assessing toxicity (Aja et al. 2014). Metal (Aja et al. 2014; Andre et al. 2010; Muthukaruppan 2015) and microplastic exposures (Rodríguez-Seijo et al.; 2017) have been found to change the biochemical profile in earthworms. In our study, the highest intensity in the peaks from the FTIR spectra was found in earthworms exposed to the combination of MF and AgNP (3200 mg Ag/kg) (Fig. 2). These peaks mostly indicated an increase in protein (3600–3000 cm^{-1}) and lipid contents (3000–2800 cm^{-1}). In addition, other less intensive changes were observed (mainly between 1230 and 550 cm^{-1}), suggesting changes in carbohydrates, proteins and nucleic acids (Zohdi et al. 2015). Overall, these alterations can be related to the production of immuno-protective molecules as a stress-response mechanism in the earthworms (Rodríguez-Seijo et al. 2017). Moreover, the increase in protein levels could be an indicator of metal binding proteins (i.e., metallothionein). Metallothionein is induced in response to AgNP and $AgNO_3$ exposure in earthworms (Hayashi et al. 2013; Patricia et al. 2017; Tsyusko et al. 2012). And more recently, polyester MF were also found to increase the metallothionein expression in the earthworm *L. terrestris* (Prendergast-Miller et al. 2019).

Unlike the results for earthworms, the presence of MF in soil had little or no influence on Ag toxicity to enchytraeids. This discrepancy between the two species could be explained by, but not limited to, exposure time and MF size. First, the longer exposure time could have increased the possibility of detecting any effect caused by the interactions between Ag and MF in the earthworm reproduction test. In addition, earthworms are able to ingest larger particles in comparison to enchytraeids (Coleman andWall 2015), and therefore, earthworms were able to ingest a broader range of MF sizes. Lahive et al. (2019) showed the importance of particle size on the ingestion and toxicity of microplastics in *E. crypticus*. Enchytraeids ingested greater quantities of smaller (10–20 µm) than of larger microplastic particles (60–150 µm), which resulted in a higher toxicity of the smaller particles towards enchytraeid reproduction. As the particle size of MF in our exposures ranged from 50 to 3000 µm, we can assume that the enchytraeids ingested less MF than the earthworms.

Moreover, we cannot ignore the differences in Ag storage between the two species. Enchytraeids did accumulate considerably more Ag than did the earthworms, which indicates a higher storage capacity. Enchytraeidae seem to have a greater capacity to accumulate essential and non-essential metals than Lumbricidae (Santorufo et al. 2012; Tosza et al. 2010; Van Vliet et al. 2006). This species-specific physiological difference could help explaining why AgNP effects differed with and without MF for earthworms but not for enchytraeids.

Microplastic fiber influence on Ag bioaccumulation

In general, no significant difference in Ag bioaccumulation was observed when MF were added to the soil. The only exception was observed in earthworms exposed to the highest AgNP concentration (3200 mg Ag/kg), where the presence of MF decreased internal Ag concentrations. Microplastics can both increase and decrease bioavailable fractions of contaminants, as shown in previous studies. A decrease in porewater concentration of hydrophobic organic compounds (HOCs) led to a decrease in bioaccumulation in *Eisenia fetida* exposed in soil containing polyethylene (PE) and polystyrene (PS) microplastics (≥1% DW) (Wang et al. 2019b). Moreover, the bioaccumulation of arsenic (40 mg As/kg) in the earthworm *Metaphire californica* decreased when polyvinyl chloride (PVC) microplastics (0.2 % DW) were added to the soil (Wang et al. 2019a). The presence of PVC microplastic seemed not to affect the speciation of arsenic in soil, but rather to decrease the proportion of more soluble and bioavailable species in the earthworm gut and tissue. On the contrary, polypropylene microplastics (≥0.03% DW) increased internal Cd concentration in *E. fetida*. Cd concentration in worms increased with increasing microplastic concentration in soil and was positively correlated with microplastic ingestion (Zhou et al. 2020). The authors concluded that Cd adsorbed to the microplastics and desorbed in the gut after ingestion of microplastics by the worms. Taken together, our results and the literature suggest that the influence of microplastic on the bioaccumulation of contaminants is dependent mainly on two processes. The first one is the interactions occurring in soil, which may affect the concentrations of free available fractions in the pore water. The second is the increase or decrease of contaminant uptake in the gut (after microplastic ingestion). These two processes are not only contaminant specific, but also likely to vary with microplastic concentration in the soil. Probably this explains the divergent results for Ag in our study and Cd in Zhou et al. (2020), with the latter one having microplastics concentrations at least three times higher than our test concentration.

The effects of microplastics on bioaccumulation depend on the chemical affinity between microplastics and contaminants (Tourinho et al. 2019). The adsorption of Ag onto microplastics is very likely to occur in aquatic systems in both ionic (Kalčíková et al. 2020; Khan et al. 2015) and nanoparticle forms (Li et al. 2020). However, there is no literature available regarding terrestrial systems yet. From our results, we can conclude that, for a microplastic concentration of 0.01% DW, differences in bioaccumulation could only be observed at an extremely high Ag soil concentration.

Furthermore, Ag porewater concentrations were similar in soils with or without MF (Table SI2). The absence of a strong effect of MF on porewater concentrations could be due to the fact that porewater extraction occurred only a couple of days after spiking. Perhaps this short time period was not enough for detecting a decrease in Ag porewater concentration when adding MF to soils. MF concentration may, however, also have been too low to significantly affect Ag porewater concentrations, which are dominated by the Ag binding to soil organic matter (Levard et al. 2012).

In either case, porewater concentrations could not explain the increase in AgNP toxicity to earthworms with the addition of MF to soil. One possible explanation is that MF altered Ag speciation which increased the concentration of bioavailable and toxic fractions of Ag. It is known that AgNP continuously release Ag ions into the pore water with time (Diez-Ortiz et al. 2015; Van der Ploeg et al. 2014). The sorption kinetics and dissolution rate of AgNP in soil could have been affected by MF, perhaps not leading to a higher porewater concentration but rather to a higher flux of Ag in the pore water, e.g. from the soil through pore water to the animals.

Finally, it should be highlighted that soil invertebrates can store Ag fractions which do not contribute to toxicity (Diez-Ortiz et al. 2015; Tourinho et al. 2016). In fact, many authors have concluded that body concentration is not a good indicator of Ag toxicity in earthworms (Schlich et al. 2013; Diez-Ortiz et al. 2015; Van der Ploeg et al. 2014; Shoults-Wilson et al. 2011b) and enchytraeids (Topuz and van Gestel 2017). Therefore, it is not surprisingly that internal Ag concentrations did not fully explain the toxicity outcomes in our study.

Conclusions

The continuous input of MF to the soil compartment is undoubted. Here, we showed that MF in soil can cause adverse effects on soil invertebrates at a relatively low concentration. These effects included changes in the biochemical composition of earthworms and a slight decrease in the reproductive success of both earthworms and enchytraeids. Moreover, MF presence did affect the toxicity of AgNP and $AgNO_3$. MF may lower the bioavailable Ag fraction due to sorption, which, however, occurred only at high concentrations of Ag in the soil. Nevertheless, it did not result in a decrease in toxicity; the presence of MF, in fact, enhanced the toxicity of AgNP to the earthworms. For enchytraeids, Ag toxicity seemed to be less affected by MF presence, which is probably related to the shorter exposure period together with a lower MF ingestion by the enchytraeids. Based on the present results, we can therefore conclude that MF should be a major concern when considering its effects alone and the combined effects with other chemicals in soils.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files)

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by P. S. T., V. S. S. L. P. T., A. D., R. V., J. C., and A. M. The first draft of the manuscript was written by P. S. T. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

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