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Polyester-derived microfibre impacts on the soil-dwelling earthworm *Lumbricus terrestris*

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Abstract

Microplastic (MP) pollution is everywhere. In terrestrial environments, microfibres (MFs) generated from textile laundering are believed to form a significant component of MPs entering soils, mainly through sewage sludge and compost applications. The aim of this study was to assess the effect of MFs on a keystone soil organism. We exposed the earthworm *Lumbricus terrestris* to soil with polyester MFs incorporated at rates of 0, 0.1 and

1.0 %w/w MF for a period of 35 days (in the dark at 15 °C; n = 4 for each treatment). Dried plant litter was applied at the soil surface as a food source for the earthworms. We assessed earthworm vitality through mortality, weight change, depurate production and MF avoidance testing. In addition, we measured stress biomarker responses via the expression of metallothionein-2 (*mt-2*), heat shock protein (*hsp70*) and superoxide dismutase (*sod-1*). Our results showed that exposure and ingestion of MFs (as evidenced by subsequent retrieval of MFs within earthworm depurates) were not lethal to earthworms, nor did earthworms actively avoid MFs. However, earthworms in the MF1.0% treatment showed a 1.5-fold lower cast production, a 24.3-fold increase in expression of *mt-2* ($p < 0.001$) and a 9.9-fold decline in *hsp70* expression ($p < 0.001$). Further analysis of soil and MF samples indicated that metal content was not a contributor to the biomarker results. Given that burrowing and feeding behaviour, as well as molecular genetic biomarkers, were modulated in earthworms exposed to MFs, our study highlights potential implications for soil ecosystem processes due to MF contamination.

Keywords

Lumbricus terrestris; earthworm; microplastic; biomarker; stress; metallothionein.

Capsule

Microfibre contamination has potential implications for soil ecosystems as exposure of *Lumbricus terrestris* to soil-microfibre mixtures reduced burrowing and altered physiology.

1. Introduction

Microplastic (MP) pollution, its sources, global extent and impacts, continues to receive much attention (Burns and Boxall, 2018; Horton et al., 2017). Research shows that MPs are easily ingested by aquatic and terrestrial fauna, and may affect their physiology, reproduction and mortality (e.g. Derraik, 2002; Hodson et al., 2017; Huerta Lwanga et al., 2016b; Imhof et al., 2017; Rillig et al., 2017; Wright et al., 2013). Although MPs have been shown to be sorptive for organic and inorganic pollutants, mass balance calculations indicate that ingestion of MPs is unlikely to be a significant exposure route for such pollutants (Hodson et al., 2017; Koelmans et al., 2016). Therefore, while the extent and magnitude of MP pollution is evident, the current and future health implications for humans as well as aquatic and terrestrial ecosystems are open to debate.

The potential environmental impacts of man-made plastic-derived microfibers (MFs) as a distinct component of MP pollution are increasingly of concern (De Falco et al., 2018; Henry et al., 2019; Sanchez-Vidal et al., 2018). It is estimated that every year, 0.19 million tonnes of synthetic MFs enter marine systems in discharge from wastewater treatment plants. In terrestrial environments, sewage sludge and compost products are widely used as soil fertilisers and are believed to be important reservoirs of MPs and dominated by high MF contents (Nizzetto et al., 2016; Weithmann et al., 2018; de Souza Macahdo et al., 2018; Ziajahromi et al., 2017; Corradini et al 2019; Zhang et al., 2019). Although wastewater treatment plants efficiently remove 99% of MPs from treated water, MPs are retained in sewage sludge (Li et al., 2018), which is then applied to land. Due to their long thin morphology, MFs are difficult to remove with traditional filtration and sedimentation treatment technologies (Habib et al., 1998; Weithmann et al., 2018; Zubris and Richards, 2005), and once in the soil, they can be persistent; for example, in one study showed MFs could still be

identified 15 years after sewage sludge application (Zubris and Richards, 2005). It has recently been shown that repeated applications of sewage sludge build up a reservoir of MF in agricultural soils (Corradini et al., 2019).

Microfibres in the environment are mainly released from domestic and industrial laundering of synthetic textiles (polyester, polyamide, polypropylene and acrylics), as well as during textile production and disposal (Henry et al., 2019). The environmental input of MFs is likely to rise given the steady growth in synthetic textile production, which increased by 2% in 2016, while natural fibres, cotton and wool, remained unchanged (Industry, 2017). Overall, production of synthetic textiles is now 71 million tonnes, compared to 14 million tonnes in the 1980s (Industry, 2017). Polyester constitutes the dominant form of MFs in coastal and aquatic systems, reflecting the dominance of polyester production compared to other synthetic textiles (Henry et al., 2019). Although MF exposure has been observed in aquatic organisms (e.g. Mason et al., 2016; Sanchez-Vidal et al., 2018), and MFs have been detected in agricultural soils (Habib et al., 1998; Zubris and Richards, 2005; Zhang and Liu 2018; Zhang et al., 2019; Corradini et al., 2019), to date, investigation into MF effects on soil fauna and the potential MF-related ecotoxicological impacts is limited: for example, Song et al., (2019) exposed the land snail (*Achatina fulica*) to polyethylene terephthalate (PET) MFs and reported that MFs had adverse effects on feeding and fitness of the snails.

The objective of this study was to test the impact of polyester MFs mixed into soil at two concentrations on the litter-feeding earthworm *Lumbricus terrestris*. This is an important anecic earthworm species, creating characteristic middens on the soil surface and permanent vertical burrows within the soil; these formations influence soil structure, water and nutrient availability, and microbial and soil faunal communities. We assessed MF toxicity using a range of approaches: weight change, mortality, avoidance testing; as well as using

biomarkers associated with stress which have been previously used in earthworm exposure studies: metallothionein (*mt-2*), heat shock protein (*hsp70*) and superoxide dismutase (*SOD-1*). Earthworm biomarkers, in particular metallothionein, are sensitive to cadmium (Cd) zinc (Zn), copper (Cu) and lead (Pb) and metal bioavailability is affected by the earthworm gut (Hodson et al., 2017; Sizmur and Hodson, 2009). In addition, textiles like polyester may have a range of additives incorporated during their manufacturing processes, including trace metals, therefore it was deemed important to measure the metal content in MFs as well to determine whether these could affect earthworms. Previous research has indicated that metals like Zn can adsorb onto MP particles (Hodson et al., 2017).

2. Materials and Methods

Soil was collected from the top 20 cm of an arable cambisol (WBR, 2006) in November 2017 at the University of Leeds commercial farm, (53° 51' 44" N 1° 20' 35"W), sieved to < 2 mm whilst field moist and then air-dried at room temperature.

In order to generate a large volume of MFs, polyester microfibres were obtained by manually cutting up the filling of a commercially available cushion. The polyester had been treated to conform to UK fire safety regulations (BS5852), therefore the MFs could have contained a flame retardant (composition unknown). The composition of the fibres was tested by FTIR and has a similar spectrum to other reported analyses of unrefined polyester (Rosu et al 2008; Dholakiya 2012; see Figs. S1&2, Supplementary information). Differences between spectra will be due to variation in analysis conditions and trace amounts of additional additives, such as flame retardants, plasticizers, dyes etc. Average MF length was 361.6 ± 387.0 µm (n = 60 fibres, standard deviation), and diameter was 40.7 ± 3.8 µm (n = 20 fibres, standard deviation) (determined using a ZEISS microscope at 11.2 magnification). A

histogram showing length distributions is given in Fig. S3, Supplementary information. Adult, clitellate, *Lumbricus terrestris* were obtained from Worms Direct (Drylands, Ulting, Near Maldon, Essex, CM9 6QS, UK).

2.1 Earthworm-microfibre exposure experiment

Air-dried soil (300 ± 0.05 g) was added to a clear plastic bag. For microfibre exposure, treatments of 0 (control), 0.1 and 1.0 %w/w (i.e. 0, 0.3 and 3.0 g) were established. The microfibres were added to the soil and then thoroughly mixed to distribute them as evenly as possible. However, the microfibres showed a tendency to clump together (see Fig. S4 Supplementary Information). After mixing, 75 mL of deionised water was added to each bag and thoroughly mixed into the soil to establish a soil water content of 25 %w/w. Individual *L. terrestris* were rinsed with deionized water, depurated for 48 hours on moist filter paper, which was changed every 12 hours (Arnold and Hodson, 2007), weighed again and then added to the soil-microfibre mixtures to create the following treatments: soil + earthworm (control), soil + earthworm + 0.1 %w/w microfibre (MF0.1%), soil + earthworm + 1.0 %w/w microfibre (MF1.0%). *L. terrestris* is a litter-feeding earthworm, therefore, 3 ± 0.05 g litter was added to the surface of each mesocosm as a food source (air-dried, <2 mm sieved, grass-clover litter with a C content of 40.3% and a N content of 2.2% obtained from grass-clover leys established in the arable field that the test soil was collected from). This also ensured that the test results were not confounded by earthworm stress induced by starvation. Four replicates were established per treatment. The plastic bags were sealed with a small air gap left and weighed. The treatments were then placed in a 15 °C controlled temperature room in the dark. Moisture content of the soil was determined by mass loss and deionised water added (0.5 – 1 g) weekly to maintain a constant water content. After 35 days earthworms were removed from the treatments, rinsed in deionised water, weighed, depurated for 24

hours and weighed again. Earthworms were then transferred to individual 50 ml centrifuge tubes and stored at -80 °C. The depurates were air-dried and weighed. For each replicate, any remaining visible litter was removed from the surface of the soil; the soil was then homogenized and air-dried.

2.2 Recovery of microfibres from soil and earthworm depurates

Air-dried soils and depurates from the earthworm treatments were ground by hand using a ceramic mortar and pestle to break up aggregates. Microfibres were then retrieved using a density fractionation method, with a 1:5 ratio (w/v) of sample and LST Fastfloat (sodium heteropolytungstates) at a specific density of 1.5 g cm^{-3}. For soils, a subsample of 2.5 g ± 0.05 g was used; for depurates, the whole sample was used (sample size ranged from 0.2 – 0.5 g dry soil). Samples with Fastfloat were placed in 15 ml centrifuge tubes, vortexed for 30 s, then placed on a horizontal shaker for 30 min at 250 rpm. Samples were centrifuged for 20 min at 219 RCF (Hettich Rotanta 460). The supernatant was filtered through Whatman filter paper (#1) and MFs and organic particles associated with the MFs were rinsed off the filter paper into a new 50 ml centrifuge tube using deionised water. The remaining soil sediment was resuspended in Fastfloat, and the procedure repeated. The rinsed particles were left to stand overnight at room temperature, and MFs settled above organic particles. MF fractions were then carefully retrieved using fine forceps and wide-bore pipette tips (1 ml) in deionised water and placed in a 2 ml micro-centrifuge tube and centrifuged for 5 min at 845 RCF (Eppendorf 5424) to further separate MFs and the organic residues. The MFs tended to form a 'ball' layer above organic residues and this was carefully separated and placed on pre-weighed tin foil. The remaining water was visually checked to confirm that all MF had been collected. This process was performed twice. MF samples were then dried for 48 hr at 40 °C. Before recording MF weight, any remaining organic residues were removed using fine forceps under a dissecting microscope (see Fig. S5 Supplementary information).

178

179 2.3 Earthworm avoidance test

180 An earthworm soil-microfibre avoidance test was set up following Langdon et al (2005) and
181 ISO 17512-1:2008. Microfibres were added to sieved (< 2 mm) air-dried soil to give loadings
182 of 0.1 and 1.0 %w/w. The mixtures were moistened to 25% w/w. Test chambers were 17 x
183 17 x 7 cm in size and were divided into two halves by a plastic divider. 870 g of soil was
184 added to each half of the chamber, one half was filled with control soil (with no added MF),
185 the other half with either control soil, soil + 0.1 %w/w microfibers (MF0.1%) or soil + 1.0
186 %w/w microfibre (MF1.0%). The plastic divider was then removed and eight adult clitellate
187 earthworms (*L. terrestris*) were added to each chamber (average mass 5.3 g ± 0.5 g) along
188 the boundary between the two soils. The containers were then covered and left in a
189 temperature-controlled room (15 ºC) in the dark. After 24 hrs, the plastic divider was
190 reinserted into the chambers and the number of earthworms in each compartment
191 determined. There were 5 replicates for each treatment.

192

193 2.4 Stress measurements following MF exposure

194 We used general stress biomarkers as indicators of physiological stress caused by the
195 exposure of earthworms to MFs in soil. Gene expression of the following biomarkers was
196 quantified: metallothionein (*mt-2*), heat shock protein (*hsp70*) and superoxide dismutase
197 (*sod-1*), using glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) as a control for gene
198 expression.

199

200 2.5 RNA extraction

201 Earthworms from the 35-day exposure experiment were thawed on ice. The 3 most posterior
202 segments were discarded, and total RNA was extracted from the 6-7 posterior segments

thereafter. Briefly, the tissue was homogenized to a fine powder in liquid nitrogen with a pestle and mortar. Subsequently, the RNA was extracted by means of a standard Trizol-based method and quantified using a spectrophotometer (Nanodrop ND-1000).

2.6 Reverse transcription

To convert the extracted RNA to cDNA, 4µl M-MLV RT 5x Buffer (Promega), 2µl dNTPs (Promega) (10 mM), 1µl oligo dT (5'-TTT TTT TTT TTT TTT TTT TTV N-3'; 10 µM), 1µl M-MLV RT enzyme (Promega) (200 units/µl) and 1000 ng of extracted total RNA were used per reaction. The mixture was made up to 20 µl with H_2O and placed on a thermal cycler at 42 °C for 60 min followed by 72 °C for 10 min.

2.7 qPCR

Quantitative real-time PCR was performed (Applied Biosystems 7500 Fast Real-Time PCR System) to assess the expression levels of the genes of interest at different conditions (control, MF0.1%, MF1.0%). The assay was carried out in a 96-well plate format and *gapdh* was used as a reference gene. In each well, 5 µl SYBR select master mix (Applied Biosystems), 0.5 µl forward primer (10µM), 0.5 µl reverse primer (10µM), 2 µl H_2O and 2µl of cDNA were added. Each sample was analysed in quadruplicate technical replicates. In addition, to confirm primer specificity, a melting curve analysis was implemented. Lastly, the $2^{-\Delta\Delta Ct}$ Livak method (Livak and Schmittgen, 2001) was applied to determine the relative gene expression. Primer (Sigma Aldrich) sequences used are given in Supplementary Information (Table S1).

2.8 Trace metal analysis of soil and MF polyester

227 Air-dried soil and MF samples were analysed for metal content. Soil samples 1.5 ± 0.05 g,
228 soil reference material (1.5 ± 0.05 g; Loamy sand 4, Flukar Lot 020829) and MF samples
229 (0.15 ± 0.05 g) were digested, in triplicate, in aqua regia in 100 ml digestion tubes at 140 °C
230 for 2.5 hr (BS 7755). Metal concentrations were measured by ICP-OES spectrometry
231 (ThermoFisher) using a certified multi-element standard (Merck). Samples were analysed for
232 a range of metals including zinc (Zn), cadmium (Cd), copper (Cu) and lead (Pb) (see Table
233 S2 Supplementary Information) and results compared to industry standard limits in the
234 OEKO-TEX Standard 100 certification for textile processing and products.

235

236 2.9 Statistical analyses

237 Data are presented as means ± standard deviation. Unless otherwise stated, n = 4 for the
238 exposure experiment means and n = 5 for the avoidance test means. One-way ANOVAs
239 were used to test for significant differences in MF treatments between the average weight of
240 depurated earthworms and mass of MFs retrieved from soil and depurate samples. A
241 Wilcoxon signed rank test was used to compare depurated earthworm weight before and
242 after exposure. T-tests were used to determine the difference in MF content recovered from
243 the two treatments in soil or depurate samples. In the biomarker data, two samples were
244 removed (one control, one exposed) which contributed to inter-sample variation. Differences
245 in qPCR data between control and exposure treatments were then compared using 2-tailed
246 t-tests.

247

248

249 **3. Results and Discussion**

250

251 3.1 Earthworm survival and depurate production

No mortality was observed over the duration of the MF exposure experiment. The average mass of the earthworms at the start of the experiment (after depuration) was 6.18 ± 0.86 g (n = 12) and earthworm biomass was similar between treatments (One-way ANOVA, $p = 0.52$). At the end of the experiment there were no significant differences in earthworm mass between treatments (One-way ANOVA, $p = 0.33$). The average earthworm mass was 7.57 ± 0.76 g (n = 12), significantly greater (Wilcoxon signed Rank Test, $p \leq 0.001$) than at the start of the experiment. By the end of the experiment all of the litter added to the earthworm-present treatments had disappeared from the soil surface. There was a trend for depurate weights to decline with increasing MF content (Fig. 1A), however, this difference was not significant (one-way ANOVA, $p = 0.34$). This is due to the variation in the mass of depurate produced by the MF1.0%-exposed earthworms. The masses of the depurate were 0.46, 0.27, 0.23 and 0.86 g. Removal of the 0.86 g sample resulted in a significant difference (One-way ANOVA, $p \leq 0.01$) being calculated for the depurate masses with the mass of depurate from the MF1.0% treatment being less than that from the other treatments. In the avoidance test, no avoidance or mortality was observed and all 8 earthworms were recovered from each test chamber (see Table S3 in Supplementary Information). In the only other study of which we are aware in which terrestrial organisms were exposed to MFs, Song et al., (2019) also reported evidence of MF ingestion by the land snail *A. fulica* which did not cause mortality but altered snail physiology.

3.2 Microfibre retrieval from soil and earthworm depurate samples

Microfibres from the exposure experiment were retrieved from soil and depurate samples. The amount of MFs collected from the soil treated with MF1.0% (1.03 ± 0.18 mg MF g^{-1}) was higher (t-test, $p = 0.006$) than from the MF0.1% treatment soil (0.36 ± 0.25 mg MF g^{-1}) (Fig. 1B). Although 3.3 ± 2.2 mg MF g^{-1} depurate were collected from the MF1.0% treatment and

278 0.45 ± 0.24 mg MF g^{-1} depurate from the MF0.1% treatment (Fig. 1B), the difference
279 between treatments was not significant, even after removing one replicate where 82.9 mg
280 MF g^{-1} depurate was collected from a MF0.1% sample. Microfibre recoveries were highly
281 variable in soil and depurate samples, and less than expected compared to the original
282 loadings in the soil samples, which is most likely an artefact of the significant 'clumping'
283 behaviour of the MFs once they were mixed into the soils. A two-way ANOVA with sample
284 type (soil vs depurate) and treatment (control, MF0.1%, MF1.0%) as factors indicated a
285 significant difference ($p < 0.001$) between treatments, with MF content greater in the
286 MF1.0% treatment (2.2 ± 1.9 mg g^{-1}, n = 8) than in the MF0.1% treatment (0.4 ± 0.2 mg g^{-1},
287 n = 7); however, although across treatments mean MF retrieved in depurates was ~ 3 times
288 greater than that retrieved from soil (2.1 ± 2.2 vs 0.7 ± 0.4 mg g^{-1}, n = 8), this difference was
289 not significant ($p = 0.105$). Likewise, there was no evidence of an interaction between
290 treatment and sample type ($p = 0.358$). The average ratio of MFs in soils:depurates was 1.1
291 ± 0.64 and 0.5 ± 0.45 in the MF0.1% and MF1.0% treatments respectively; however, this
292 difference was not significant ($p = 0.209$). The presence of MFs within earthworm depurates
293 is evidence that the earthworms ingested soil containing MFs as part of their burrowing
294 activities. The similarity of MF contents in depurates and soil suggests the absence of
295 preferential MF ingestion or avoidance by the earthworms. Similar non-avoidance of MPs
296 has been reported before for earthworms (Hodson et al., 2017; Huerta Lwanga et al., 2016a,
297 b; Rillig et al., 2017). As MFs were present in the depurates of the earthworms and
298 concentrations in the depurate and bulk soil were not significantly different, mass balance
299 calculations suggest that no MFs were accumulated in the earthworms. However, given the
300 variability in the concentration data, it is conceivable that MFs also accumulated in the
301 earthworm tissues or were retained in their gut but at concentrations too low to detect. As
302 earthworms were frozen at -80 °C at the end of the experiment for biomarker assessment,
303 we are unable to test for this. The tendency for lower depurate weights in the MF1.0%
304 treatment suggests that although MFs were not lethal, there was some effect on earthworm
305 (burrowing) behaviour (Huerta Lwanga et al., 2016a, b), which may have implications for soil

306 ecosystem services which earthworms like *L. terrestris* provide, such as soil structure,
307 nutrient cycling, soil diversity and soil moisture availability (Blouin et al., 2013). Field
308 observations are required to determine the behaviour of MFs in soils following sludge
309 amendments to confirm whether clumping is also seen "naturally", perhaps enhanced by
310 MFs and organic matter binding together, or whether MFs occur as individual fibres in soil.
311 Currently, there are relatively few studies that determine MF presence in field soils as a
312 result of agricultural plastic use and/or sewage sludge applications (Zubris and Richard,
313 2005; Zhang and Liu 2018; Zhang et al., 2019; Corradini et al., 2019). Zhang and Liu (2018)
314 reported that MFs were mainly found incorporated within soil aggregates (72%) and a
315 smaller proportion was dispersed and found as individual fibres (28%), which suggests that
316 MFs may increase soil aggregation through entanglement of fine particles (Zhang et al.,
317 2019). The clumping phenomena of the polyester microfibres used in the experiment could
318 be overcome in future work by using a cryotome method to prepare MF samples e.g. Cole
319 (2016), or by mixing the MFs in a surfactant before mixing into the soil; however,
320 physiological impacts on test organisms of the residues of any freezing agent or surfactant
321 used would need to be taken in account.

322

323

324 3.3 Markers of stress in earthworms following exposure to microfibres

325 Sequence data from the earthworm *L. terrestris* are limited, thereby restricting the availability
326 of suitable biomarkers of exposure. Responses to general stress were assessed using the
327 following biomarkers: metallothionein *(mt-2)*, heat shock protein 70 (*hsp70)* and
328 superoxidase dismutase (*sod-1)*. One replicate outlier was removed from the MF0.1%
329 dataset as the sample did not meet the quality control threshold; this sample was not related
330 to the outlier in the depurate MF0.1% dataset. Significant dose-dependent changes in gene
331 expression (Fig. 2) were found in *mt-2*, which increased 9.6 (± 0.8) fold in the MF0.1%

treatment ($p < 0.001$) and 24.3 (± 8.4) fold in the MF1.0% treatment ($p < 0.001$) compared to the control. *Hsp70* expression was 9.9 (± 3.3) fold lower in earthworms exposed to MF1.0% compared to the control ($p < 0.001$). Although *sod-1* expression was elevated following the MF treatments, the difference compared to the control was not statistically significant.

3.3.1 Trace metal contents in soil and microfibre samples

Digestion of MF samples indicated that metal concentrations were either below detection limits or below limits set by the OEKO-TEX Standard 100 for textile materials (see Table S2 Supplementary Information). Comparisons of the metal contents of the exposure soil and the MFs, despite the higher detection limits for the MFs, indicate that the presence of the MFs in the soil cause a negligible, if any, increase in the potential for exposure to metals by the earthworms. Therefore it seems likely that an increase of metal content was not the cause of the observed change in *mt-2* and *hsp70* expression, but perhaps supports the notion that MFs induce a general stress response (Imhof et al., 2017).

3.3.2 Application of stress biomarkers in microplastic exposure studies

Other studies have shown that MPs can induce oxidative stress in aquatic organisms (Browne et al., 2013; Imhof et al., 2017; Lei et al., 2018), mice (Deng et al., 2017), nematodes (Lei et al., 2018) and the earthworm *Eisenia fetida* (Rodríguez-Seijo et al., 2018). This is the first result showing the effect of MFs on the earthworm *L. terrestris*. Studies on other pollutants have revealed that *E. fetida* is typically more tolerant of pollutants compared to other earthworm species (Langdon et al 2005; Pelosi et al 2013). Rodríguez-Seijo et al., (2018) exposed *E. fetida* to microplastic particles at a range of concentrations, up to 1000 mg kg^{-1} soil (equivalent to 0.1 %w/w) for 28 d and assessed oxidative stress using enzymatic

assays. As in our study, there were no mortality effects. They reported a reduction in catalase activity at low MP content (125 mg kg^{-1}), but an increase in activity of glutathione S-transferase and lactate dehydrogenase, and increased thiobarbituric acid reactive substances at the higher MP levels (> 500 mg kg^{-1}). Differences in earthworm species and behaviour, contaminant and biomarker selections make direct comparison between our study and that of Rodríguez-Seijo et al., (2018) difficult. In another example, Song et al., (2019) reported that MF exposure to the land snail (*A. fulica*) caused a reduction in total antioxidant capacity and glutathione peroxidase activity but increased the levels of malondialdehyde, indicating a MF effect on oxidative stress. Therefore, it is clear that microplastics and microfibres affect the physiology of soil organisms and warrant further investigation. Metallothionein can act as a biomarker of metal exposure, but also the release of reactive oxygen species (ROS) (Andrews, 2000; Formigari et al., 2007; Hidalgo et al., 2001; Tamai et al., 1993). Given the absence of a metal stressor, it is possible that ingestion of MFs, which increased at higher MF exposure, led to ROS production in the earthworms. HSP70 is regarded as a chronic stress biomarker and high expression levels can be maintained after exposure (Rhee et al., 2009). In our study, *hsp70* expression was down-regulated at the high MF exposure, which aligns well with the *hsp70* response observed in *Daphnia spp.* following MP exposure (Imhof et al., 2017) and also in the intertidal copepod *Tigriopu japonicus* exposed to 4-nonylphenol and 4-t-octylphenol (Rhee et al., 2009). The authors suggest that down-regulation of *hsp70* is an indicator of stress. The MF data revealed no statistically robust change in *sod-1* expression, indicating that this antioxidant was not a key target in the MF response, at least not at the 35-day exposure time-point.

Biomarker results can be characterized by experimental variation, for example, Rodriguez-Seijo et al (2018; 2017) showed biomarker responses in one study but not in another in response to MP exposure. Differences may be due to (inter)species and/or contaminant specific differences and the variation in the exposure time to, and the concentration of, the

MPs. Indeed, studies have shown that the induction of antioxidants in earthworms is variable and gene-specific (e.g. *mt1* vs *mt2,* or *hsp10* vs *hsp70*) and this is a function of duration of exposure (e.g. hours, days or weeks), contaminant concentration (low vs high) and the form of toxin/contaminant (e.g. *mt* is more responsive to Cd than Cu (Fisker et al., 2016)). Therefore, differences in exposure times and biomarker type need to be taken into account when comparing this study to other published data. Furthermore, the activity of *mt* in earthworms is not fully understood: for example, earthworms lack the metal transcription factor (MTF-1) found in higher organisms (Höckner et al., 2015); and Owen et al (2008) observed that *mt* expression in the earthworm *Lumbricus rubellus* was induced in response to Cd, but not fluoranthene or atrazine. Therefore, further work is required to determine the transcriptional response cascade of the earthworm's response to MFs.

It is conceivable that the earthworms were affected by secondary MF effects, such as exposure to chemicals grafted onto MFs during manufacturing processes e.g. flame retardants, antimicrobials and plasticisers (e.g. phthalates) which are known to affect earthworms (Browne et al., 2013; Du et al., 2015; Ruan et al., 2009). However, it is challenging to fully characterise all chemical additives in commercially produced materials, and in the case of earthworms, there are no flame retardant biomarkers available. Therefore, it is important to use a suite of biomarkers to generate a better understanding of MF exposure.

3.4 Environmental exposure: were microfibre application rates realistic?

There are a growing number of terrestrial MP surveys which specifically record microfibre contents in soil samples: e.g. Zubris and Richards (2005), Zhang and Lui (2018), Liu et al., (2018), Zhou et al., (2018), Zhang et al., (2019), Corradini et al., (2019). These studies were single time points and used a combination of extraction and microscopic methods to retrieve

and analyse the microfibres. Zubris and Richards (2005) focused on sewage sludge and agricultural soils where sludge had been applied. Fibre counts ranged from 0.0 to 1.2 g^{-1} soil, however, total number of samples was not reported, nor were further details on length, fibre type or diameter. The study by Zhang and Lui (2018) retrieved total microplastic particles from 50 soil samples within the Chai River valley (China). On average, they retrieved 18760 microplastic particles kg^{-1} soil and the dominant (82%) size range of all microplastics was 0.05 - 2 mm. Of this total count, 92% of MP were identified as microfibres (17259 MF kg^{-1} soil; 17 MF g^{-1} soil). The dominance of microfibres in soil samples as the main form of microplastic was also reported by Zhang et al., (2019) and Corradini et al., (2019). Based on the Zhang and Lui (2018) length range of 0.05 – 2 mm, we estimate this is equivalent to 0.0001 – 0.0007 %w/w polyester MF in soil (using a diameter of 40 µm (this study) and a density of polyester of 1.38 g cm^{-3}). Therefore, the exposures used in this study of 0.1 and 1.0MF% were 1000 and 10000 times greater than soil levels currently available, which when combined with our study results, suggests that the environmental risk to earthworms is low. However, Fuller and Gautam (2016) reported soil microplastic contents of 7 %w/w and as microfibres dominate MP contamination in soil, our highest treatment is not unrealistic. This highlights the urgent need for more studies to quantify microplastic and microfibre contents in soils globally in order to critically assess the environmental risk of terrestrial MP pollution.

4. Conclusions

Earthworms are highly likely to be exposed to MFs as they burrow through and ingest soil, and when they are used to accelerate composting processes. We have shown that microfibres, as a distinct component of MP pollution, can be ingested by *L. terrestris* individuals. This occurred as they ingested soil during burrowing, as the MFs were not incorporated into the surface litter provided which is the primary food source for this species.

Although not fatal over 35 days, ingestion of MFs at 0.1 and 1.0 %w/w application rates did cause transcriptional responses related to general stress and there was evidence of a change in casting behaviour as depurate production decreased in the high MF treatment. Given that there was no evidence for avoidance of MFs in the soil, this may have implications for earthworm survival and fitness, and could potentially disrupt key ecosystem services provided by earthworms (Blouin et al., 2013). However, the MF exposure rates used in the study are significantly greater than those reported from field observations in the literature. Further work is now urgently required to (1) accurately quantify microfibres in global terrestrial environments; (2) use longer exposure times; and (3) determine MF cumulative effects, in order to comprehensively assess the environmental risk of microplastic and microfibre pollution.

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Supporting Information. SI contains: Primer sequences for the selected biomarkers; Metal concentrations in soil and polyester microfibre samples**;** Earthworm-soil-MF avoidance test results; FTIR of polyester microfibre sample; Soil-microfibre images

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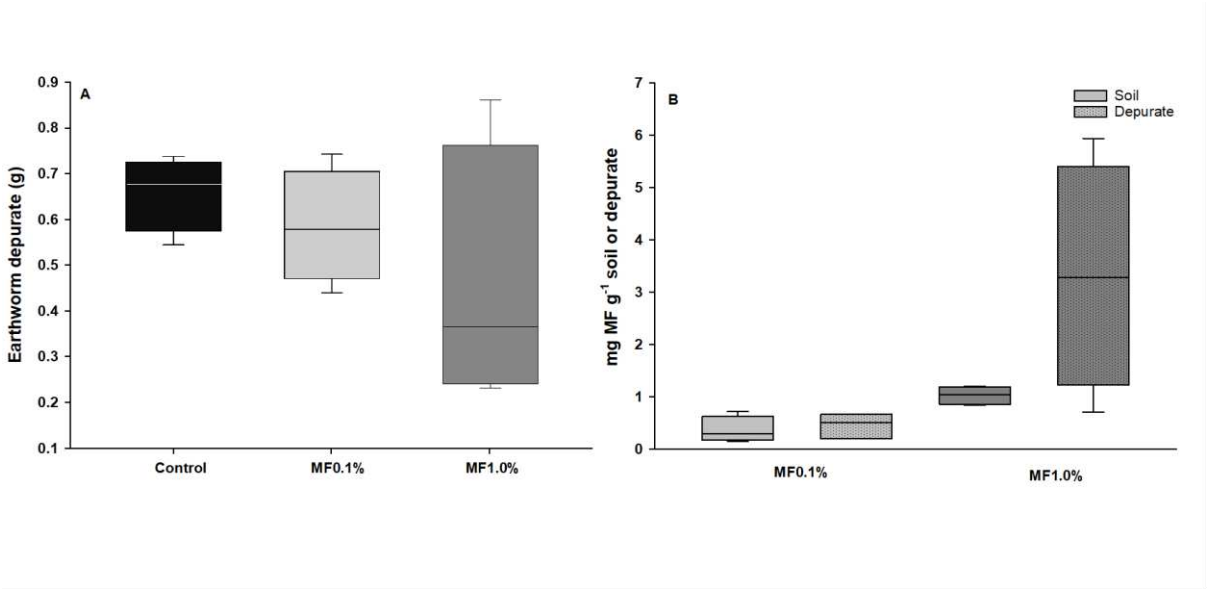

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608 Figure 1. A: Mean mass of depurate produced by earthworms from the different soil
609 treatments (n = 4 in each treatment); B: mean mass of MFs recovered from soil (n = 4 in
610 each treatment) and depurate samples (n = 3 in MF0.1% treatment; n = 4 in MF1.0%
611 treatment). Error bars show standard deviations. Note that one replicate is not included in
612 the MF0.1% depurate, where 82.9 mg g^{-1} MF was recovered.

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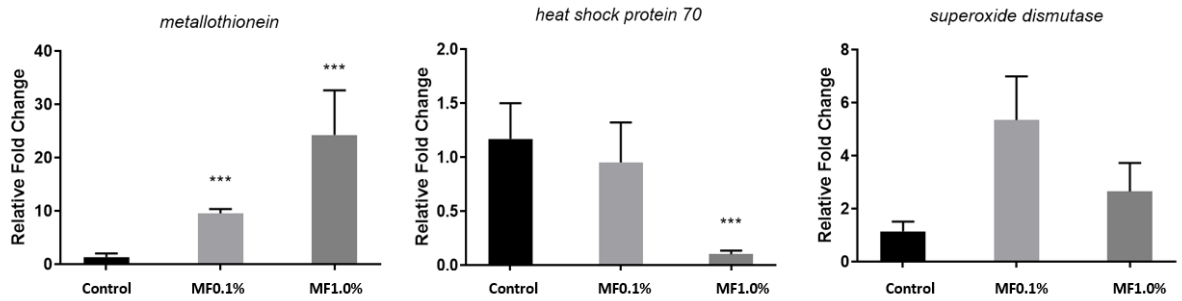

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620 Figure 2. Relative changes in antioxidant gene expression following earthworm exposure to
621 MFs over 35 days. *** denotes significant difference ($p < 0.001$) in expression compared to
622 the control treatment.

623