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

3

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15

16

17

18 **Abstract**

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

24 1.0 %w/w MF for a period of 35 days (in the dark at 15 °C; n = 4 for each treatment). Dried
25 plant litter was applied at the soil surface as a food source for the earthworms. We assessed
26 earthworm vitality through mortality, weight change, depurate production and MF avoidance
27 testing. In addition, we measured stress biomarker responses via the expression of
28 metallothionein-2 (*mt-2*), heat shock protein (*hsp70*) and superoxide dismutase (*sod-1*). Our
29 results showed that exposure and ingestion of MFs (as evidenced by subsequent retrieval of
30 MFs within earthworm depurates) were not lethal to earthworms, nor did earthworms actively
31 avoid MFs. However, earthworms in the MF1.0% treatment showed a 1.5-fold lower cast
32 production, a 24.3-fold increase in expression of *mt-2* ($p < 0.001$) and a 9.9-fold decline in
33 *hsp70* expression ($p < 0.001$). Further analysis of soil and MF samples indicated that metal
34 content was not a contributor to the biomarker results. Given that burrowing and feeding
35 behaviour, as well as molecular genetic biomarkers, were modulated in earthworms exposed
36 to MFs, our study highlights potential implications for soil ecosystem processes due to MF
37 contamination.

38

39

40 Keywords

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

42

43

44 **Capsule**

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

47

48 **1. Introduction**

49

50 Microplastic (MP) pollution, its sources, global extent and impacts, continues to receive
51 much attention (Burns and Boxall, 2018; Horton et al., 2017). Research shows that MPs are
52 easily ingested by aquatic and terrestrial fauna, and may affect their physiology,
53 reproduction and mortality (e.g. Derraik, 2002; Hodson et al., 2017; Huerta Lwanga et al.,
54 2016b; Imhof et al., 2017; Rillig et al., 2017; Wright et al., 2013). Although MPs have been
55 shown to be sorptive for organic and inorganic pollutants, mass balance calculations indicate
56 that ingestion of MPs is unlikely to be a significant exposure route for such pollutants
57 (Hodson et al., 2017; Koelmans et al., 2016). Therefore, while the extent and magnitude of
58 MP pollution is evident, the current and future health implications for humans as well as
59 aquatic and terrestrial ecosystems are open to debate.

60

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

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

77

78 Microfibres in the environment are mainly released from domestic and industrial laundering
79 of synthetic textiles (polyester, polyamide, polypropylene and acrylics), as well as during
80 textile production and disposal (Henry et al., 2019). The environmental input of MFs is likely
81 to rise given the steady growth in synthetic textile production, which increased by 2% in
82 2016, while natural fibres, cotton and wool, remained unchanged (Industry, 2017). Overall,
83 production of synthetic textiles is now 71 million tonnes, compared to 14 million tonnes in the
84 1980s (Industry, 2017). Polyester constitutes the dominant form of MFs in coastal and
85 aquatic systems, reflecting the dominance of polyester production compared to other
86 synthetic textiles (Henry et al., 2019). Although MF exposure has been observed in aquatic
87 organisms (e.g. Mason et al., 2016; Sanchez-Vidal et al., 2018), and MFs have been
88 detected in agricultural soils (Habib et al., 1998; Zubris and Richards, 2005; Zhang and Liu
89 2018; Zhang et al., 2019; Corradini et al., 2019), to date, investigation into MF effects on soil
90 fauna and the potential MF-related ecotoxicological impacts is limited: for example, Song et
91 al., (2019) exposed the land snail (*Achatina fulica*) to polyethylene terephthalate (PET) MFs
92 and reported that MFs had adverse effects on feeding and fitness of the snails.

93

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

100 biomarkers associated with stress which have been previously used in earthworm exposure
101 studies: metallothionein (*mt-2*), heat shock protein (*hsp70*) and superoxide dismutase (*SOD-*
102 *1*). Earthworm biomarkers, in particular metallothionein, are sensitive to cadmium (Cd) zinc
103 (Zn), copper (Cu) and lead (Pb) and metal bioavailability is affected by the earthworm gut
104 (Hodson et al., 2017; Sizmur and Hodson, 2009). In addition, textiles like polyester may have
105 a range of additives incorporated during their manufacturing processes, including trace
106 metals, therefore it was deemed important to measure the metal content in MFs as well to
107 determine whether these could affect earthworms. Previous research has indicated that
108 metals like Zn can adsorb onto MP particles (Hodson et al., 2017).

109

110

111 **2. Materials and Methods**

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

115

116 In order to generate a large volume of MFs, polyester microfibrils were obtained by manually
117 cutting up the filling of a commercially available cushion. The polyester had been treated to
118 conform to UK fire safety regulations (BS5852), therefore the MFs could have contained a
119 flame retardant (composition unknown). The composition of the fibres was tested by FTIR
120 and has a similar spectrum to other reported analyses of unrefined polyester (Rosu et al
121 2008; Dholakiya 2012; see Figs. S1&2, Supplementary information). Differences between
122 spectra will be due to variation in analysis conditions and trace amounts of additional
123 additives, such as flame retardants, plasticizers, dyes etc. Average MF length was $361.6 \pm$
124 $387.0 \mu\text{m}$ ($n = 60$ fibres, standard deviation), and diameter was $40.7 \pm 3.8 \mu\text{m}$ ($n = 20$ fibres,
125 standard deviation) (determined using a ZEISS microscope at 11.2 magnification). A

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

129

130 2.1 Earthworm-microfibre exposure experiment

131

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

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

156

157 2.2 Recovery of microfibrils from soil and earthworm deperates

158 Air-dried soils and deperates from the earthworm treatments were ground by hand using a
159 ceramic mortar and pestle to break up aggregates. Microfibrils were then retrieved using a
160 density fractionation method, with a 1:5 ratio (w/v) of sample and LST Fastfloat (sodium
161 heteropolytungstates) at a specific density of 1.5 g cm⁻³. For soils, a subsample of 2.5 g ±
162 0.05 g was used; for deperates, the whole sample was used (sample size ranged from 0.2 –
163 0.5 g dry soil). Samples with Fastfloat were placed in 15 ml centrifuge tubes, vortexed for 30
164 s, then placed on a horizontal shaker for 30 min at 250 rpm. Samples were centrifuged for
165 20 min at 219 RCF (Hettich Rotanta 460). The supernatant was filtered through Whatman
166 filter paper (#1) and MFs and organic particles associated with the MFs were rinsed off the
167 filter paper into a new 50 ml centrifuge tube using deionised water. The remaining soil
168 sediment was resuspended in Fastfloat, and the procedure repeated. The rinsed particles
169 were left to stand overnight at room temperature, and MFs settled above organic particles.
170 MF fractions were then carefully retrieved using fine forceps and wide-bore pipette tips (1 ml)
171 in deionised water and placed in a 2 ml micro-centrifuge tube and centrifuged for 5 min at
172 845 RCF (Eppendorf 5424) to further separate MFs and the organic residues. The MFs
173 tended to form a 'ball' layer above organic residues and this was carefully separated and
174 placed on pre-weighed tin foil. The remaining water was visually checked to confirm that all
175 MF had been collected. This process was performed twice. MF samples were then dried for
176 48 hr at 40 °C. Before recording MF weight, any remaining organic residues were removed
177 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 °C) 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

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

206

207 2.6 Reverse transcription

208 To convert the extracted RNA to cDNA, 4µl M-MLV RT 5x Buffer (Promega), 2µl dNTPs
209 (Promega) (10 mM), 1µl oligo dT (5'-TTT TTT TTT TTT TTT TTT TTV N-3'; 10 µM), 1µl M-MLV
210 RT enzyme (Promega) (200 units/µl) and 1000 ng of extracted total RNA were used per
211 reaction. The mixture was made up to 20 µl with H₂O and placed on a thermal cycler at 42
212 °C for 60 min followed by 72 °C for 10 min.

213

214 2.7 qPCR

215 Quantitative real-time PCR was performed (Applied Biosystems 7500 Fast Real-Time PCR
216 System) to assess the expression levels of the genes of interest at different conditions
217 (control, MF0.1%, MF1.0%). The assay was carried out in a 96-well plate format and *gapdh*
218 was used as a reference gene. In each well, 5 µl SYBR select master mix (Applied
219 Biosystems), 0.5 µl forward primer (10µM), 0.5 µl reverse primer (10µM), 2 µl H₂O and 2µl of
220 cDNA were added. Each sample was analysed in quadruplicate technical replicates. In
221 addition, to confirm primer specificity, a melting curve analysis was implemented. Lastly, the
222 $2^{-\Delta\Delta C_t}$ Livak method (Livak and Schmittgen, 2001) was applied to determine the relative gene
223 expression. Primer (Sigma Aldrich) sequences used are given in Supplementary Information
224 (Table S1).

225

226 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 \pm 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

252 No mortality was observed over the duration of the MF exposure experiment. The average
253 mass of the earthworms at the start of the experiment (after depuration) was 6.18 ± 0.86 g (n
254 = 12) and earthworm biomass was similar between treatments (One-way ANOVA, $p = 0.52$).
255 At the end of the experiment there were no significant differences in earthworm mass
256 between treatments (One-way ANOVA, $p = 0.33$). The average earthworm mass was $7.57 \pm$
257 0.76 g ($n = 12$), significantly greater (Wilcoxon signed Rank Test, $p \leq 0.001$) than at the start
258 of the experiment. By the end of the experiment all of the litter added to the earthworm-
259 present treatments had disappeared from the soil surface. There was a trend for depurate
260 weights to decline with increasing MF content (Fig. 1A), however, this difference was not
261 significant (one-way ANOVA, $p = 0.34$). This is due to the variation in the mass of depurate
262 produced by the MF1.0%-exposed earthworms. The masses of the depurate were 0.46,
263 0.27, 0.23 and 0.86 g. Removal of the 0.86 g sample resulted in a significant difference
264 (One-way ANOVA, $p \leq 0.01$) being calculated for the depurate masses with the mass of
265 depurate from the MF1.0% treatment being less than that from the other treatments. In the
266 avoidance test, no avoidance or mortality was observed and all 8 earthworms were
267 recovered from each test chamber (see Table S3 in Supplementary Information). In the only
268 other study of which we are aware in which terrestrial organisms were exposed to MFs,
269 Song et al., (2019) also reported evidence of MF ingestion by the land snail *A. fulica* which
270 did not cause mortality but altered snail physiology.

271

272

273 3.2 Microfibre retrieval from soil and earthworm depurate samples

274 Microfibres from the exposure experiment were retrieved from soil and depurate samples.
275 The amount of MFs collected from the soil treated with MF1.0% (1.03 ± 0.18 mg MF g⁻¹) was
276 higher (t-test, $p = 0.006$) than from the MF0.1% treatment soil (0.36 ± 0.25 mg MF g⁻¹) (Fig.
277 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 (\pm 0.8) fold in the MF0.1%

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

336

337

338 3.3.1 Trace metal contents in soil and microfibre samples

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

347

348 3.3.2 Application of stress biomarkers in microplastic exposure studies

349 Other studies have shown that MPs can induce oxidative stress in aquatic organisms
350 (Browne et al., 2013; Imhof et al., 2017; Lei et al., 2018), mice (Deng et al., 2017),
351 nematodes (Lei et al., 2018) and the earthworm *Eisenia fetida* (Rodríguez-Seijo et al., 2018).
352 This is the first result showing the effect of MFs on the earthworm *L. terrestris*. Studies on
353 other pollutants have revealed that *E. fetida* is typically more tolerant of pollutants compared
354 to other earthworm species (Langdon et al 2005; Pelosi et al 2013). Rodríguez-Seijo et al.,
355 (2018) exposed *E. fetida* to microplastic particles at a range of concentrations, up to 1000
356 mg kg^{-1} soil (equivalent to 0.1 %w/w) for 28 d and assessed oxidative stress using enzymatic

357 assays. As in our study, there were no mortality effects. They reported a reduction in
358 catalase activity at low MP content (125 mg kg⁻¹), but an increase in activity of glutathione S-
359 transferase and lactate dehydrogenase, and increased thiobarbituric acid reactive
360 substances at the higher MP levels (> 500 mg kg⁻¹). Differences in earthworm species and
361 behaviour, contaminant and biomarker selections make direct comparison between our
362 study and that of Rodríguez-Seijo et al., (2018) difficult. In another example, Song et al.,
363 (2019) reported that MF exposure to the land snail (*A. fulica*) caused a reduction in total
364 antioxidant capacity and glutathione peroxidase activity but increased the levels of
365 malondialdehyde, indicating a MF effect on oxidative stress. Therefore, it is clear that
366 microplastics and microfibrils affect the physiology of soil organisms and warrant further
367 investigation. Metallothionein can act as a biomarker of metal exposure, but also the release
368 of reactive oxygen species (ROS) (Andrews, 2000; Formigari et al., 2007; Hidalgo et al.,
369 2001; Tamai et al., 1993). Given the absence of a metal stressor, it is possible that ingestion
370 of MFs, which increased at higher MF exposure, led to ROS production in the earthworms.
371 HSP70 is regarded as a chronic stress biomarker and high expression levels can be
372 maintained after exposure (Rhee et al., 2009). In our study, *hsp70* expression was down-
373 regulated at the high MF exposure, which aligns well with the *hsp70* response observed in
374 *Daphnia spp.* following MP exposure (Imhof et al., 2017) and also in the intertidal copepod
375 *Tigriopus japonicus* exposed to 4-nonylphenol and 4-t-octylphenol (Rhee et al., 2009). The
376 authors suggest that down-regulation of *hsp70* is an indicator of stress. The MF data
377 revealed no statistically robust change in *sod-1* expression, indicating that this antioxidant
378 was not a key target in the MF response, at least not at the 35-day exposure time-point.

379

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

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

395

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

404

405 3.4 Environmental exposure: were microfibre application rates realistic?

406 There are a growing number of terrestrial MP surveys which specifically record microfibre
407 contents in soil samples: e.g. Zubris and Richards (2005), Zhang and Lui (2018), Liu et al.,
408 (2018), Zhou et al., (2018), Zhang et al., (2019), Corradini et al., (2019). These studies were
409 single time points and used a combination of extraction and microscopic methods to retrieve

410 and analyse the microfibrils. Zubris and Richards (2005) focused on sewage sludge and
411 agricultural soils where sludge had been applied. Fibre counts ranged from 0.0 to 1.2 g⁻¹ soil,
412 however, total number of samples was not reported, nor were further details on length, fibre
413 type or diameter. The study by Zhang and Lui (2018) retrieved total microplastic particles
414 from 50 soil samples within the Chai River valley (China). On average, they retrieved 18760
415 microplastic particles kg⁻¹ soil and the dominant (82%) size range of all microplastics was
416 0.05 - 2 mm. Of this total count, 92% of MP were identified as microfibrils (17259 MF kg⁻¹
417 soil; 17 MF g⁻¹ soil). The dominance of microfibrils in soil samples as the main form of
418 microplastic was also reported by Zhang et al., (2019) and Corradini et al., (2019). Based on
419 the Zhang and Lui (2018) length range of 0.05 – 2 mm, we estimate this is equivalent to
420 0.0001 – 0.0007 %w/w polyester MF in soil (using a diameter of 40 µm (this study) and a
421 density of polyester of 1.38 g cm⁻³). Therefore, the exposures used in this study of 0.1 and
422 1.0MF% were 1000 and 10000 times greater than soil levels currently available, which when
423 combined with our study results, suggests that the environmental risk to earthworms is low.
424 However, Fuller and Gautam (2016) reported soil microplastic contents of 7 %w/w and as
425 microfibrils dominate MP contamination in soil, our highest treatment is not unrealistic. This
426 highlights the urgent need for more studies to quantify microplastic and microfibre contents
427 in soils globally in order to critically assess the environmental risk of terrestrial MP pollution.

428

429 **4. Conclusions**

430

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

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

447

448

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451 Rebecca Sutton for laboratory assistance.

452

453

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

457

458

459

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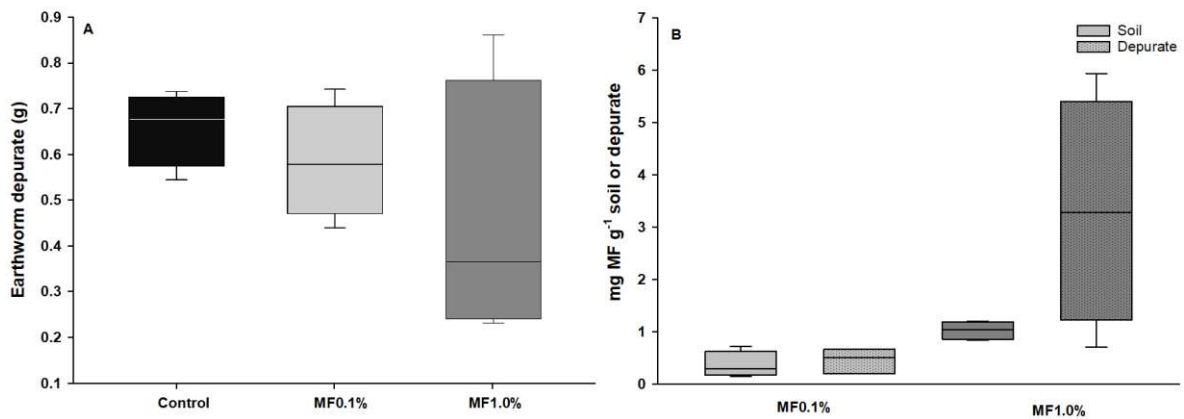
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607

608 Figure 1. A: Mean mass of deplete 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 deplete 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% deplete, where 82.9 mg g⁻¹ MF was recovered.

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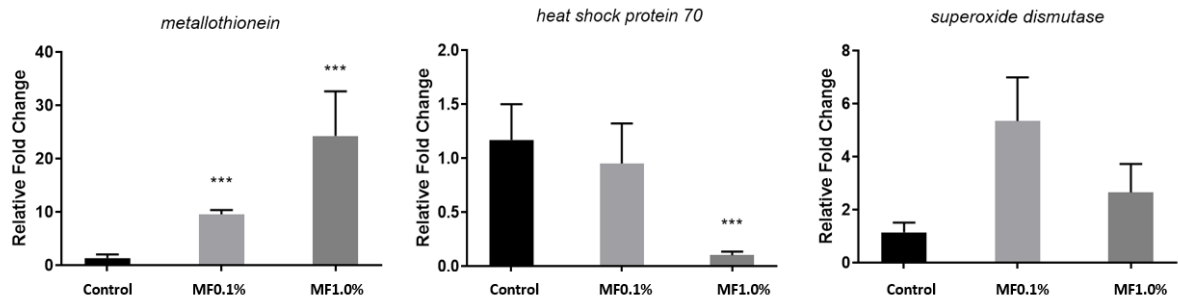
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620 Figure 2. Relative changes in antioxidant gene expression following earthworm exposure to
 621 MFs over 35 days. *** denotes significant difference ($p < 0.001$) in expression compared to
 622 the control treatment.

623