Earthworms ingest microplastic fibres and nanoplastics, but shape affects egestion rate and long-term retention

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Abstract

Microplastic fibres (MPFs) and nanoplastics (NPs) have the potential to be hazardous to soil organisms. Understanding uptake into organisms is key in assessing these effects, but this is often limited by the analytical challenges to quantify smaller-sized plastics in complex matrices. This study used MPFs and NPs containing inorganic tracers (In, Pd) to quantify uptake in the earthworm *Lumbricus terrestris*. Following seven days exposure, tracer concentrations were measured in earthworms and faeces. Earthworms exposed to 500 µg MPFs/g soil retained an estimated 32 MPFs in their tissues, while at 5000 µg MPFs/g earthworms retained between 2 and 593 MPFs. High variation in body burdens of MPFs was linked to soil retention in earthworms and reduced faeces production, suggesting egestion was being affected by MPFs. NPs uptake and elimination was also assessed over a more extended time-period of 42 days. After 1 day, NPs were no longer detectable in faeces during the elimination phase. However, some retention of NPs in the earthworm was estimated, not linked to retained soil, indicating not all NPs were eliminated. MPFs and NPs uptake can be quantified in earthworms and both particle types can be retained beyond the depuration period, suggesting the potential for longer-term accumulation.

Keywords: Plastic, soil, terrestrial, bioaccumulation*, Lumbricus terrestris*

Synopsis: Using metal-doped nanoplastics and microplastic fibres allowed their uptake from soil to be tracked and showed they can be retained in the earthworms, suggesting longer-term accumulation.

1. Introduction

Terrestrial environments are subject to extensive pollution by plastics, prompting concern about their potential negative consequences for soil biodiversity and function, and the overall health of soils.[1] Although macroplastic pollution is more easily visualized in the environment, smaller-sized plastic particles such as nanoplastics (NPs) and microplastics (MPs) are more numerous and of more biological relevance as they can be taken up by organisms. [2, 3] NPs and MPs can enter the terrestrial environment directly as primary plastic materials, for example, from polymer-coated fertilisers.[4] However, it is anticipated that secondary NPs and MPs, generated from the breakdown of larger macroplastic items, are likely to dominate emissions to soils. For example, in agricultural systems, sources include the degradation of plastic mulch films and the application of soil conditioners (sludge and composts) which contain NPs and MPs. [5, 6 7 8] More generally, terrestrial systems will also receive inputs from littering and atmospheric deposition. [9, 10] However, large disparities between plastic inputs are expected between residential, industrial, natural and agricultural areas for different types of plastic pollution, since specific uses of plastics will determine the magnitude of the corresponding emissions.[11]

While early research on plastic effects on soil dwelling organisms showed limited or no effects on life history traits such as survival, growth, or reproduction,[12, 13] there is emerging evidence that ingestion of plastic by some soil organisms has the potential to cause detrimental effects, albeit at high concentrations.[14-16] One reason for these seemingly contradictory findings is that many of the effects of NPs and MPs on soil organisms appear to be mediated by physical parameters, such as particle shape and size, rather than by overt chemically-mediated toxicity. The feeding traits and size of the organism, as well as the characteristics of the particles to which they are exposed, can determine the likelihood of ingestion. For example, larger MPs (fragments), similar in size to the mouthparts of *E. crypticus,* were ingested less compared to MPs much smaller than their mouthparts, which in turn was linked with greater effects on reproduction associated with these smaller MPs.[15] Longer-term studies, or those that investigated biochemical markers of toxicity (e.g. altered gene expression, signs of

oxidative stress, changes in energy metabolism), more consistently demonstrated negative impacts.[14, 16] In soil invertebrates, effects on food intake, cast production and invertebrate biomass have been shown.[13, 17 16] Particle morphology has also proven important in changing soil aggregates, water holding capacity, and microbial diversity and functioning.[18] Therefore, particles of different sizes and/or morphology may impact organisms directly, by affecting life history traits or inducing biochemical stress responses, or indirectly, by changing the soil properties in which the organisms reside.

Microplastic fibres (MPFs) have the potential to cause physical harm while outside of the organism, for example through abrasion[19], or once ingested can cause damage to the intestine and stomach.[16] They may also become trapped in the gut of organisms resulting in lower assimilation of food or reducing egestion of faeces.[13] In many studies, however, only toxicological endpoints were measured and the actual body burden of MPs or MPFs were less frequently assessed, since the latter metric still remains analytically challenging. Analysis of MPFs in soil, organic residues and soil dwelling organisms is an involved process requiring specific sampling, extraction/separation and concentration analysis steps, which collectively makes for a demanding and time-consuming task. For particles below 10 µm, there still are few documented protocols to measure these materials.[20] These analytical challenges of plastic detection and quantification are exacerbated when considering particles of even smaller sizes (e.g. NPs), and thus the impacts of NPs have focused on effects on organisms and to date, few have considered the extent of retention of the particles within soil organisms. [21, 22] However, the study of nanoparticulate matter in terrestrial systems and their impacts is not entirely new, as NPs have been studied in the context of engineered nanomaterials as a representative non-dissolving nanoparticle. It is only recently that their inherent toxicity or potential for adverse effects has been considered from the perspective of plastic pollution. [23] Organisms can easily ingest nano-sized plastics; they have the potential to cross biological barriers and penetrate tissues, and consequently bioaccumulate in tissues, and thus this area remains active in current research investigations.

The aim of this study was to quantify the uptake of NPs and MPFs in the soil invertebrate *Lumbricus*

terrestris. Earthworms are ecosystem engineers important to soil functioning, and thus their fitness is essential for a healthy soil ecosystem. Measurements of the uptake and retention of plastics in organisms are key to identifying mechanisms of effect and potential for hazard. We have circumvented some of the analytical limitations and challenges posed by these materials, by synthesizing NPs and MPFs containing an inorganic tracer.[24, 25] Metal-doped plastics greatly benefit the assessment of uptake in a laboratory setting, increasing the speed and precision of analysis using standardized techniques for trace metals analysis, allowing measurement of smaller sized particles at lower concentrations than with most currently available plastic detection methods.[26] In this current study, it was possible to accurately assess the mass of NPs and MPFs retained in the body of an earthworm and importantly to determine whether NPs and MPFs were retained in the gut as part of soil aggregates or not. In addition, we assessed the uptake and elimination kinetics of NPs, by measuring body concentrations over a 21-day uptake phase in NP-spiked soil followed by a 21-day elimination phase in clean soil. This approach allowed us to 1) assess the homogeneity of NPs and MPFs in the test soil and quantify true exposure concentrations to the earthworms, 2) quantify uptake and elucidate differences between soils contaminated with NPs or MPFs, and 3) determine the mass and number of plastics that were retained by earthworms after depuration.

2. Materials and methods

2.1 Metal-doped plastic materials

The production steps used in creating the microplastic fibres (MPFs) are described in more detail in SI and Frehland et al 2020.[8] The MPFs were cut to an intended length of approximately 500 µm, corresponding to the length of the MPFs released by textiles when laundering.[27] The MPFs underwent several washing and clean up steps to remove oil residues and metal filings from the cutting process. The MPFs were washed six times in water and detergent to remove the oil residue before being rinsed

five times with water to ensure all detergent was removed. Following the washing steps, the MPFs were placed in water with a magnetic flea and placed on a magnetic stirrer. This was repeated until no more filings were found to collect on the flea. The cleaned MPFs were then dried in preparation for being used in the experiments. A subsample of cut MPFs was observed and measured under a stereomicroscope (Figure S1, S2). Average MPFs length was 633.7 ± 282.8 µm (n=140) and 30 µm in diameter (see SI and Figure S1). The indium content of randomly selected fibres from each spool averaged 0.213 ± 0.005 wt %.

Emulsion polymerization of nanoplastic spheres (NPs) containing entrapped Pd were made in-house and characterized following the protocol described in SI and Mitrano et. al. 2019.[24] The solids content of the stock dispersion content was approximately 11.5% dry weight. The total metal content was 253.6 mg Pd/L and the particle size and electrophoretic mobility was measured with the Malvern Zetasizer (z-average: 187 nm, polydispersity index: 0.04, zeta-potential (derived from the electrophoretic mobility): -43 mV).

2.2 Organisms

The test organism used in this study was the anecic earthworm, *Lumbricus terrestris*. Earthworms were sourced from a commercial supplier (Worms Direct, UK). Adult earthworms (5.5 ± 1.3 g fresh weight) were used in the experiments.

2.3 Short-term MPFs and NPs accumulation assays

Soils were spiked with three different nominal concentrations of MPFs: 50, 500 and 5000 µg MPFs/g dry weight (d.w.) soil; equivalent to 0.11, 1.1 and 11 µg In/g d.w. soil. NPs concentrations were 22, 221 and 2206 µg NPs/g d.w., 0.12, 1.2 and 12 µg Pd/g d.w. These highest concentrations represent the upper limit of the plastic content permitted in compost added to soils as soil conditioner (0.25%

w/w).[28] Soil without any added plastics were also included as a control. There were four replicates for each treatment and the controls. The dried MPFs were added to the dry soil and mixed to create a homogenous distribution (Figure S3). The NPs were added as a dispersion to the dry soil before being mixed thoroughly to ensure homogeneity. The soils were then wet to 50% of their water holding capacity (WHC) and mixed. Soils were distributed to small containers (12 cm diameter, 7 cm height) and held for three days in a temperature-controlled chamber (13 ± 1 ^{o}C) before the earthworms were introduced.

To increase the earthworm's appetite, and encourage burrowing into the soil, each individual was placed on a moist filter paper for 24 hours to void its gut before being introduced to the soil. The fresh weight of each earthworm was recorded and one earthworm was added to each container. The containers were covered with perforated lids, weighed and kept in a temperature controlled room (13 ± 1 ^{o}C with a 12:12 hr light:dark cycle) for the duration of the experiment. After seven days incubation in the soil, the earthworms were gently removed from the soil. They were rinsed, weighed and then placed individually on moist filter paper for 48 hours to allow them void their gut contents. After 24 hours, the filter paper was changed. The faeces produced by the earthworms were collected at the end of the 24 and 48-hour periods and these were pooled for each individual. Following depuration, the earthworms were snap-frozen in liquid nitrogen and freeze-dried in preparation for analysis of In (MPFs exposure) or Pd (NPs exposure).

2.4. NPs uptake and elimination experiment

Following the short-term assays, a longer-term assay was set up to assess the uptake and elimination of NPs over an extended period (21 days uptake and 21 days elimination). Based on the outcomes of the above-mentioned short-term NPs assay, a single concentration above the limit of quantification (LOQ) for quantification of Pd in the earthworms was chosen: 464 µg NPs/dry soil (= 2.32 µg Pd/g dry soil). This concentration is equivalent to the permitted plastic content in compost added in a 1:5 ratio

149 to soil. Soils were spiked in the same manner as before. A total of 32 containers were spiked with NPs
150 and individual earthworms added to each as before. Four replicate containers were sampled at each
151 sampling point during the 21-day uptake phase, after 3, 9, 15 and 21 days of incubation. At the end of
152 the 21-day uptake phase, earthworms in the remaining containers were removed from spiked soil,
153 rinsed and transferred to containers with uncontaminated control soil, one earthworm per container,
154 to start the 21-day elimination phase of the experiment. Earthworms were sampled during the
155 elimination phase after 1, 3, 10 and 21 days incubation in the uncontaminated soil, with four replicates
156 sampled per time point. Earthworms sampled during the uptake and elimination phases were allowed
157 to void their gut as in the short-term assay and were preserved in the same manner. Faeces samples
158 were also collected at each uptake and elimination sampling time. Soil samples were collected from
159 the freshly spiked soils (top, middle and bottom of container) and from replicate containers sampled
160 on day 21 of the uptake phase and on day 21 of the elimination phase.

161

162 *2.5 MPFs and NPs detection in organisms, faeces and soil*

163 The sample digestion processes are described in the SI. Elemental analysis was performed by
164 inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies, QQQ 8900) featuring
165 an integrated sample introduction system (ISIS), microMIST spray chamber and nickel cones. A
166 standard calibration was performed on each day of ICP-MS analysis (see SI for details).

167

168 *2.6 Data analysis*

169 The earthworm body concentrations and faeces concentrations were checked for normality using the
170 Anderson-Darling test. Non-normal data was log-transformed where appropriate in order to carry out
171 ANOVA analysis. Significant differences between body burdens at different exposure concentrations
172 were tested using a one-way ANOVA with post-hoc Tukey (Minitab 18).

To establish the likelihood of soil retention in the earthworm to explain measured body burdens in the short-term assays, the total Pd or In in the earthworms and the soil concentration were used to calculate the mass of soil that would need to be retained in the earthworm to result in the measured body burdens:

$$Sr = \frac{Et}{C\exp} \qquad (1)$$

Where *S*r = mass of soil that would need to be retained (g dry weight), *E*t = total mass of Pd or In in the earthworm minus background Pd or In (µg) and *C*exp = measured concentration of Pd or In in the soil minus background Pd or In (µg/g).

Two kinetic models were tested to describe the uptake and elimination of Pd (NPs) in the earthworms' uptake and elimination experiment. These were run using GenStat 19. Model A was a first order one-compartment model, which considers the organism to be one compartment to which the NPs are taken up at a given rate and eliminated at a given rate. Model B was also a first-order one-compartment model but alongside uptake and elimination, it includes an inert fraction. This allows for a proportion of NPs to be stored and not eliminated during the elimination phase.[29] In both cases, the uptake and elimination were fitted simultaneously.

For the uptake phase, the following equation was used in both models:

$$C_{\text{int}} = C_0 + \left(\frac{k_1}{k_2}\right) * C_{\text{exp}} * (1 - e^{-k_2 t}) \qquad 0 \le t \le tn \qquad (2)$$

Where C_{int} = concentration earthworm tissues at time t (µg Pd/g), k_1 = uptake rate constant (g dry soil/g earthworm dry tissue/ day, k_2 = elimination rate constant (d^{-1}), C_0 = Pd concentration in the earthworms at the start of the experiment (µg Pd/g), C_{exp} = exposure concentration (soil, mg Pd/kg dry soil), t = time (days), tn = time where the earthworms were transferred to clean soil (days),

For the elimination phases, two different equations were used in the model, Eq3 in Model A and Eq4 in Model B.[30]

$$C_{\text{int}} = C_0 + \left(\frac{k_1}{k_2}\right) * C_{\text{exp}} * (e^{-k_2 * (t - tn)} - e^{-k_2 t}) \qquad t < tn \qquad (3)$$

$$C_{\text{int}} = C_0 + \left(\frac{k_1}{k_2}\right) * C_{\text{exp}} * (Fi + (1 - Fi) - e^{-k_2 * (t - tn)} \qquad t > tn \ (4)$$

198 *F*i = the fraction (ranging from 0 to 1) that cannot be eliminated and is stored in the body.

199

200 **3. Results and Discussion**

201 *Considerations for using doped plastics in biota tracer studies*

202 An advantage in using plastics doped with scarce metal tracers is the ability to overcome the
203 background interferences faced when using alternative tracing methods, such as fluorescence. In
204 addition, they avoid the need for complex and extensive extraction procedures that are required for
205 microscopy or spectroscopy-based analyses. Using metal-doped plastics, in particular for smaller
206 microplastics and nanoplastics, makes them traceable in complex matrices and at low concentrations,
207 with effective digestion procedures and standard methods for trace metal analysis being readily
208 available. The background In (MPFs tracer) concentrations in the test soil used in this study was 0.018
209 ± 0.001 µg In/g which is within the range of measured background In concentrations in unpolluted
210 soils.[31] The background earthworm In concentrations were also low, 0.015 ± 0.002 µg In/g d.w. (Figure
211 1). In comparison, the background Pd (NPs tracer) soil concentration were comparatively more
212 elevated, 0.094 ± 0.0026 µg Pd/g d.w (Table 1). Natural background Pd concentrations have been
213 reported to be as low as 0.015 µg Pd/g, but can be as high as 0.1 µg Pd/g, particularly in soils from
214 urban settings where Pd sources include inputs from vehicle catalytic converters.[32, 33] This contrasts
215 with surface waters which usually have concentrations that are <0.022 µg Pd/l. [34] Background Pd
216 concentrations in the earthworms were also elevated, 0.032 ± 0.01 µg Pd/g d.w., even when measured
217 directly from culture, which utilised a different soil matrix (Figure 2). This highlights that although Pd
218 is often considered a scarce metal, its increasing use over the past 20 years has led to elevated levels
219 in the terrestrial environment. Despite this, our accumulation studies demonstrated that uptake of
220 NPs could still be assessed in the earthworms and importantly, NPs could be reliably detected in
221 earthworms using the Pd tracer at environmentally relevant concentrations (> 0.02 % w/w) (Figure 2).

222

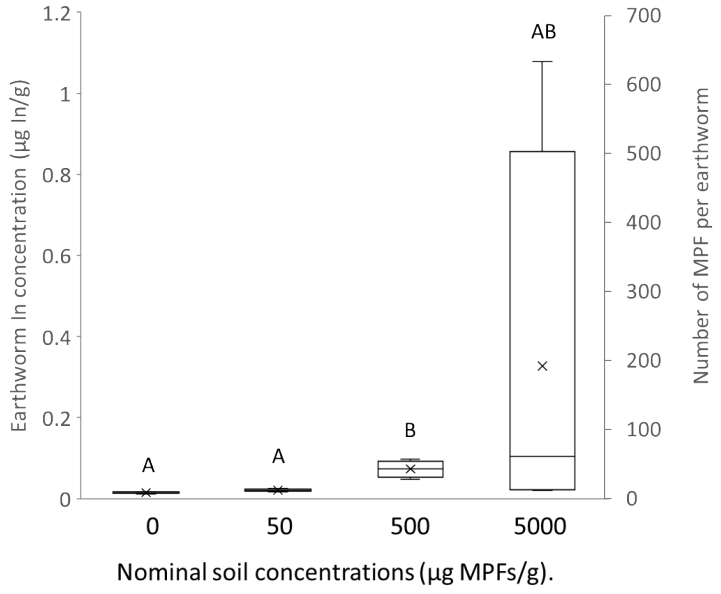

Figure 1: *The concentration of In measured in earthworm tissues (and the corresponding number of MPFs per earthworm, secondary y-axis) following 7 days exposure to three concentrations of In-doped microplastic fibres (MPFs) 50, 500 and 5000 µg MPFs/g (0.11, 1.1 and 11 µg In/g). Earthworms were also exposed in soil not spiked with MPFs (0 µg/g). The x denotes the average concentration and the error bars show the standard deviation (n=4). Different letters indicate treatments that are significantly different from one another.*

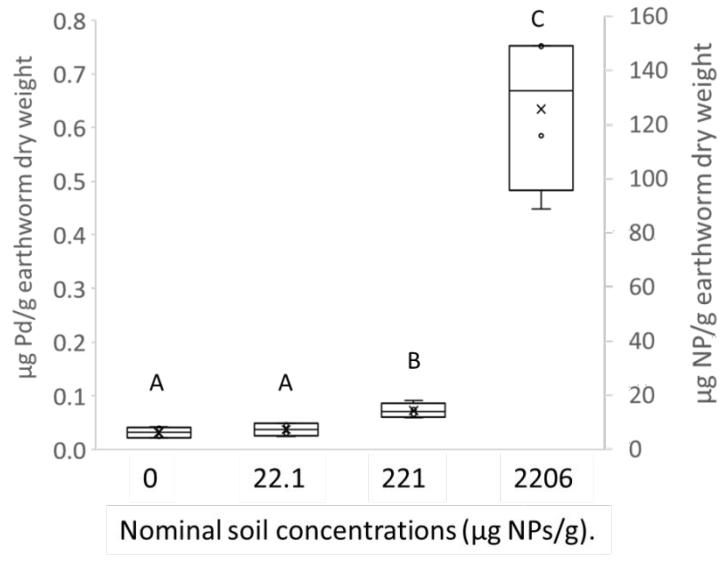

Figure 2: *The concentration of Pd and the corresponding NPs concentration in earthworm tissues following 6 days exposure to three concentrations Pd-doped polystyrene NPs 22.1, 221 and 2206 µg NPs/g (nominal = 0.12, 1.2 and 12 µg Pd/g). Earthworms were also exposed in soil not spiked with plastic (0 µg/g). The x denotes the average concentration and the error bars show the standard*

deviation (n=4). Different letters indicate treatments which are significantly different from the other treatments.

In laboratory studies using soil organisms, plastics have often been spiked in food or liquid media, to guarantee ingestion or to reduce the experimental effort. [16, 21, 35, 36] Although this can give more controlled exposures, earthworms live in intimate contact with, and ingest, soil which means using spiked soil provides a more realistic route of exposure. Where plastics have been dosed to a soil matrix, large variation in exposure concentrations have sometimes been observed, particularly in the case of MPFs, where validation of the dosing has been challenging or heterogeneous distributions have been observed visually in spiked soil. [13, 19] High variation in spiking can preclude confident interpretation of bioaccumulation data. For example, when assessing the retention of plastics in biota, it is necessary for the concentration in the exposure media to be as homogenous as possible so that accumulated plastic in the organism can be distinguished from plastic associated with any soil residues retained in the gut. In this study, it was possible to confirm the homogeneity of our spiking by evaluating the variation in the recovery of In and Pd from the soil, when samples were collected randomly from the spiked batches of soil (Table 1, Figure S3). The coefficient of variance in the spiked soil concentrations in the MPFs exposure was between 2 and 20 times lower when compared with other MPFs soil bioaccumulation studies. [13, 19] Similarly, the coefficient of variance in NPs concentrations in spiked soils was below 10%. This confirmed that the spiking procedure was reliable, achieving reproducible spiking with consistent exposure across replicates. The recovery rate of MPFs from the soil was 102-115% of the nominal concentrations (Table 1). In the short-term accumulation assay, the spiked concentrations of NPs in the soils were mostly lower compared to the nominal concentrations, with exposure concentrations measuring between 47.6% and 70.1% of the nominal concentrations (Table 1). . The resultant NPs concentrations were then calculated as 29.2, 137 and 1566 µg NPs/g d.w., respectively (Table 1).

Table 1: The nominal microplastic fibre (MPFs) and nanoplastics (NPs) mass concentration in soil, the corresponding nominal In and Pd concentration, the measured In and Pd concentrations in the soils and corresponding actual MPFs and NPs mass concentrations in soils spiked at three different concentrations of microplastic fibres or nanoplastics. The % recovery rate is the measured soil concentration as a percentage of the nominal soil concentration. The concentration of In and Pd measured in earthworm faeces. All data show mean ± standard deviation. Faeces concentrations marked with ¥ indicate where faeces concentrations were significantly lower compared to the soil concentrations in that treatment.

Microplastic fibre exposures (MPFs)					
Nominal MPFs concentration (µg MPFs /g dry weight soil)	**Nominal In concentration** (µg In/g dry weight soil)	**Measured In concentration** (µg In/g dry weight soil)	**Actual MPFs concentration*** (µg MPF/g dry weight soil)	**% recovery rate**	**Measured In concentration in faeces** (µg In/g dry weight faeces)
0	0	0.018 ± 0.002	0	NA	0.016 ± 0.001
50	0.11	0.141 ± 0.033	65.9	115	0.110 ± 0.034
500	1.1	1.13 ± 0.024	528.8	104	0.881 ± 0.031¥
5000	11	10.9 ± 0.671	5107	102	9.821 ± 0.497¥
Nanoplastic particle exposures (NPs)					
Nominal NPs concentration (µg NPs/g dry weight soil)	**Nominal Pd concentration** (µg Pd/g dry weight soil)	**Measured Pd concentration** (µg Pd/g dry weight soil)	**Actual NPs concentration*** (µg NPs/g dry weight soil)	**% recovery rate**	**Measured Pd concentration in faeces** (µg Pd/g dry weight faeces)
0	0	0.094 ± 0.006	0	NA	0.149 ± 0.027
22.1	0.12	0.146 ± 0.016	29.2	47.6	0.182 ± 0.039
221	1.2	0.686 ± 0.027	137	53.7	0.645 ± 0.052
2206	12	7.83 ± 0.586	1566	70	5.908± 1.135¥

*Based on measured In/Pd concentration in the soil

Earthworms ingest and retain MPFs and NPs but variation in the body burden is greater at higher MPFs concentrations in soil

Based on the variation in background In concentration of the earthworms, and the In content in the MPFs, the LOQ for measuring In (and therefore MPFs) in the earthworms was calculated as 0.039 µg In/g d.w., equivalent to 23 MPFs in an average-sized earthworm. For earthworms exposed to 500 and

295 5000 µg MPFs/g d.w. soil, this limit was exceeded, with earthworms retaining an estimated average
296 of 32 ± 9 MPFs and 180 ± 280 MPFs per earthworm, respectively (Figure 1). Earthworms exposed in
297 the highest MPFs treatment displayed large variations (155% variance) in body burdens compared to
298 earthworms from the lower MPFs treatments (16-28% variance). Excluding the highest MPFs
299 treatment, and associated large variation, from the dataset showed there were significantly higher
300 body burdens in earthworms exposed at 500 µg MPFs/g d.w. compared to the control and the lowest
301 MPFs exposure ($F=58.1$, $P<0.05$).

302 For the short-term NPs bioassay, the LOQ for measuring Pd above background in the earthworms was
303 comparatively higher, 0.103 µg Pd/g d.w., equivalent to 16.5 µg NPs /g d.w. This concentration was
304 exceeded in earthworms exposed in the two highest NPs treatments and there was a significant
305 increase in Pd body burdens with increasing soil concentration compared to the control (Figure 2).
306 Earthworms exposed to the highest treatment reached tissue Pd concentrations that were equivalent
307 to 121 ± 29 µg NPs/g d.w compared to 34 µg NPs/g d.w in the lower treatment. This corresponds to
308 an average number of NPs retained in the earthworms being 2.04×10^{10} NPs and 7.54×10^{9} NPs,
309 respectively. In contrast with the MPFs exposure, variation in body burdens was less for the NPs
310 exposed worms (between 6 and 23% variance across treatments).

311

312 *Concentrations of MPFs in the earthworm faeces and soil help us interpret the MPFs concentrations in*
313 *the earthworms*

314 Assessment of ingestion by earthworms can be problematic due to their immersion in soil, as well as
315 the soil itself acting as their food source in the exposure. Earthworm depuration, even for extended
316 periods (> 48 hours), does not always successfully result in full clearance of soil from the gut. [37] Thus,
317 it is possible that soil being retained in the gut is accounting for the high variation in body burdens,
318 particularly in the MPFs treatments. If it is assumed the earthworm In concentrations were the result
319 of soil still residing in the gut following depuration, using the soil and earthworm In concentrations,

the amount of soil that would need to be retained in the gut was estimated, *Sr* (Equation 1). It was estimated that 17.1 ± 26.5 mg d.w. soil was remaining in the earthworm gut in the highest MPFs treatment and 30 ± 8.1 mg d.w. soil in their gut (= 30 MPFs), in the lower treatment (500 µg/g) (Table S1). These soil masses are between 3% and 5% of the earthworm whole body weight. There was also a trend showing a decrease in the amount of faeces produced (normalised to the weight of the earthworm) with increasing MPFs concentration in the soil, further suggesting some soil retention in the gut ($F=7.17$, $P<0.05$) (Figure 3). In the highest treatment, there was high variation (88% variance) in the amount of faeces produced between replicates, although the mean was consistent with the downward trend. This is in line with the large variation in body burdens for exposed earthworms (Figure 1). A similar study assessing MPFs ingestion and egestion in *L. terrestris*, found a comparable trend for the lowered production of faeces, although with higher MPFs concentrations in the soil (1% MPFs w/w compared to 0.1% MPFs w/w). [13]

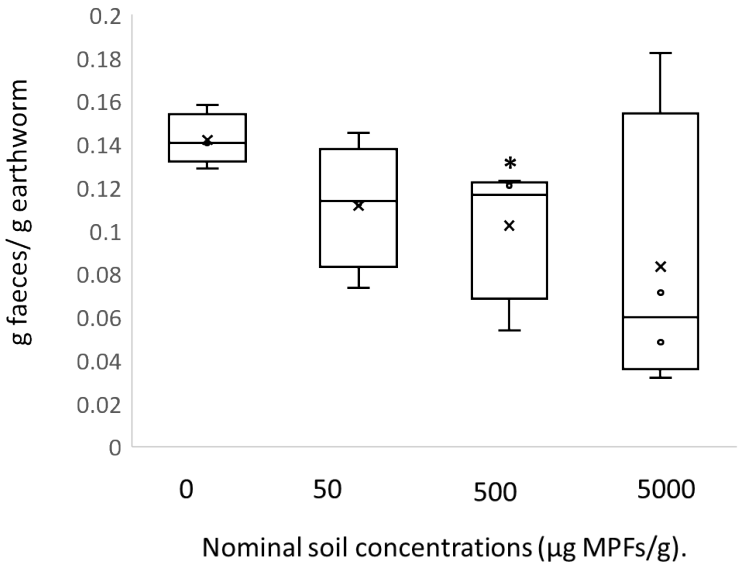

Figure 3: *The biomass of faeces produced per gram earthworm (all dry weight) during 48 hours depuration following 7 day exposures to three concentrations of In-doped microplastic fibres (MPFs) 50, 500 and 5000 µg MPFs/g (0.11, 1.1 and 11 µg In/g). Earthworms were also exposed in soil not spiked with MPFs (0 µg/g). The x denotes the average concentration and the error bars show the standard deviation (n=4). * indicate treatments which are significantly different from the control.*

345 Avoidance of MPFs-spiked soil was not observed in this study or in other similar soil studies, [13] [38] but
346 it is possible that reduced or irregular consumption of soil could also explain some of the variation in
347 body burdens in the highest MPFs treatment. Reduced ingestion or filtration of food has also been
348 observed in other organisms when spiked with MPFs due to plastic particles creating a feeling of
349 satiation or aversion of the food, which could be responsible for lower egestion. [16, 39-41] There was no
350 significant change in worm weight over the 7-day exposure; regardless of MPFs loading treatments
351 ($P>0.05$), although indeed this would not be expected due to the short test duration. The presence of
352 large numbers of MPFs in the earthworms would seem to be more consistent with ingestion and
353 retention by the earthworms. The trend for reduced faeces production suggests that egestion is being
354 impacted by the presence of the large numbers of MPFs in the soil, with clearance of soil from the gut
355 being impeded in some way. Finally, the concentration of In in the faeces of the earthworms was
356 compared with the soil concentrations for each treatment. This revealed significantly lower MPFs
357 concentrations in the faeces compared to the soil for the two highest MPFs treatments, indicating
358 retention of some fibres from the soil within the worms that is not egested with the rest of the soil
359 material (Table 1). The doped MPFs made it possible to look in detail at the ingestion and egestion of
360 MPFs by the earthworms and provide support for the conclusion that MPFs are being retained in the
361 earthworm guts at higher MPFs soil concentrations, regardless of the extent of soil retention in the
362 gut.

363

364 *NPs uptake in the earthworms*

365 Studies assessing uptake of NPs in organisms are less common compared to micron-sized plastics,
366 particularly those studies quantifying uptake from complex matrices such as soil, largely due to the
367 analytical challenges associated with detecting NPs in tissues. The majority of studies have used
368 fluorescently-labelled NPs which can be prone to artefacts of the dissociation of the fluorescent tag
369 leading to sometimes erroneous conclusions about NP absorption. [42] This study is the first to our

knowledge which has been able to use realistic exposures (i.e. in soil at relatively low concentrations) to assess uptake of NPs to soil organisms and understand their potential to be retained in tissues.

The size and shape of the NPs compared to the MPFs means they are less likely to interfere with egestion. They are, however, more likely to be incorporated into tissues due to their small size. The mass of faeces produced by earthworms exposed to NPs in the short-term assay did not vary significantly with increasing soil NPs concentrations (Figure S4). The estimated mass of soil that would need to be retained in the earthworm to explain the tissue Pd concentrations were > 40 mg d.w. (> 7-8% of their body weight). This seems unlikely given smaller soil masses that were estimated for the MPFs. Instead, it is likely that there are some NPs being retained within the gut, independent of soil retention, or even in the tissues. In the highest NPs treatment, the faeces concentrations of NPs were significantly lower compared to the soil concentrations ($P<0.05$) supporting the retention of NPs in the earthworms.

Longer-term uptake and elimination of NPs in earthworms

To assess NPs uptake in more detail, and over a longer timescale than 7 days, the longer-term NPs assay allowed the uptake and elimination kinetics of NPs in earthworms to be determined at a relatively low exposure concentration (464.2 μg NP/g d.w. = 0.046% w/w). The Pd concentration in the earthworm tissues increased as a result of exposure but tissue and faeces concentrations were also highly variable, with an average 50% variance among replicates (Figure 4a). The faeces collected from the exposed earthworms had Pd concentrations that were above background soil concentrations, and slightly lower compared to the spiked Pd concentration in the soil during the uptake phase (Figure 4b). When the earthworms were transferred to clean soil, after 24 hours the concentration of Pd in the faeces was comparable to background soil concentrations, which indicated that earthworms did not egest the NPs over an extended period of time (Figure 4b).

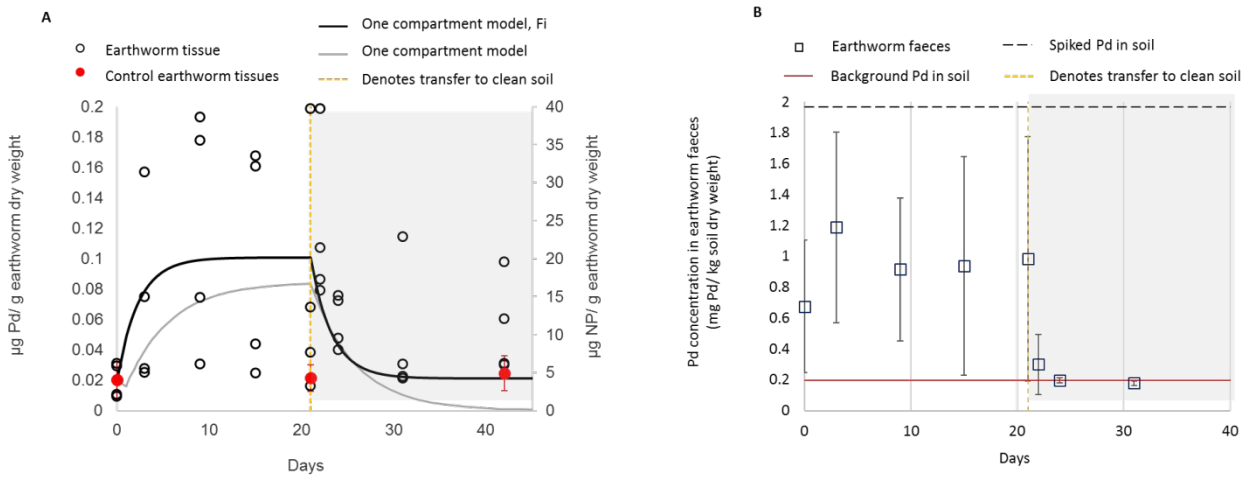

Figure 4: *The concentration of Pd in earthworm tissues (A) and earthworm faeces (B) exposed for 21 days to a single concentration of Pd-doped NPs, 464 mg NP/kg (1.97 mg Pd/kg). The earthworms were also exposed in soil not spiked with plastic (control earthworms). Following 21 days exposure, earthworms were transferred to clean soil and the tissue and faeces concentrations measured during the elimination period. In (A) the one-compartment model fit (Model A = grey solid line) and the one compartment model with the inert fraction (Fi) (Model B = black solid line) are shown along with the concentration in the control earthworms (mean ± standard deviation). In (B) the mean faeces concentrations ± standard deviation are shown along with the Pd concentration in the soil during the uptake phase and the background concentration of Pd in the soil. The vertical yellow line indicates where the earthworms were transferred to clean soil.*

The kinetic parameters obtained by fitting Model A (one-compartment model) and Model B (one-compartment model with an inert fraction) are in Table S2. Including an inert fraction as a parameter in the model (Model B) increased the uptake rate (k_1) and in particular the elimination rate (k_2) (0.432 ± 0.312 d^{-1}), which reflects that NPs were eliminated from the earthworms quickly. Although the inert fraction was small (*Fi*=0.015), it still suggests that not all of the ingested NPs were completely egested by the earthworms, or egestion was too low to be detectable after more than one day in clean soil. These measurements are limited by detection limits for analysing Pd in the earthworms which means that if NPs were present in the earthworms in a concentration < 5 µg NP/g earthworm d.w., they would not be detected. The half-life the NPs in the earthworms was determined to be 1.6 days. This timescale of elimination (1 - 2 days) has also been observed for small microplastics (< 10 µm) in other organisms such as fish and mussels previously. [43 44]

What does this mean for assessing plastic accumulation in organisms in the environment?

Accumulation of particulate plastics in organisms in the environment has been assessed more often for aquatic organisms than terrestrial organisms. [45, 46] Typically, analysis is carried out using individuals preserved *in-situ* (i.e. they are preserved as captured and not allowed to void their gut). This could be considered representative of true exposure for organisms in the environment. However, it is also recognised that there can be great heterogeneity in the presence of particulate plastics in the environment and so it is possible that organisms will ingest particulates more randomly compared to other non-particulate chemical pollutants. The distribution of MPFs and NPs in the individual replicate containers of soil were not assessed at the end of the exposure, but it is possible that the distribution was not as homogenous as it was in the beginning due to earthworms turning over the soil, particularly for the MPFs due to their size. This is likely more reflective of a real world scenario where MPFs are found incorporated into soil aggregates to a larger degree as opposed to being freely dispersed. [47] Thus, the likelihood for uptake of MPFs may be more random or stochastic in the environment compared with a carefully controlled exposure, such as the one conducted here. Considering the high variability already observed in body burdens of earthworms exposed to NPs and MPFs under these very controlled exposures, it is likely that predicting MPFs or NPs accumulation and trophic transfer in real environments will be challenging. Better understanding of particulate plastic behaviour in soils and the role and influence of patchiness and heterogeneity in exposure on bioaccumulation kinetics over the longer term could help to provide some insights. [48, 49] However, mechanistic studies allowing for the assessment in controlled conditions gives some power towards making this prediction of uptake of particles and their likelihood to remain in organisms for longer times than either food or soil, which could then be validated in the field.

Another consideration is the size and shape of the particles that are detectable in environmental samples using contemporary analytical techniques for plastics analysis. While there have been

valuable advances in the use of spectroscopic methods (e.g. μFTIR) for plastics identification, a significant amount of work has relied on visual identification and staining of microplastics. This means that detection is constrained by the approach (e.g. visual identification means they must be visible *via* microscope) or limitations of the instrument (e.g. size detection limit). For example, MPFs can be difficult to observe and identify using μFTIR because their width can be close to the limit for detection for the instrument. [50] Consequently, it is very challenging for environmental surveys of biota to detect MPFs, and certainly NPs, which might be present and thus assessing bioaccumulation will be difficult. Alongside this, the potential for an organism to ingest particles will also relate to the interplay between the organism's size, feeding traits and the size and shape of the (plastic) particle. [15, 51] For example, in soil exposures at the same concentrations as in this study (0.5% w/w), MPFs with an average size of 220 μm, found 1-2 MPFs per individual for the small (< 1 cm) earthworm *E. crypticus* (following depuration) and 100-150 MPFs in the relatively larger (~ 2 cm) isopod *P. scaber.* [19] *L. terrestris,* used in this study, are larger again (~ 10-20 cm), with a demonstrated greater capacity to retain more MPFs. This underlines the importance of understanding the role of organism physiology in uptake and retention as well as their functional grouping in the environment, as this can help determine their potential susceptibility to ingest MPs or NPs. The relationship between particle characteristics and characteristics of key species in these functional groups must be understood when aiming to predict the potential for accumulation, trophic transfer and ultimately the impact of plastic pollution on ecosystems. In this study, we were able to determine the number of particles that were retained in the earthworms and link this with responses in earthworm egestion, which could result in altered assimilation longer term.

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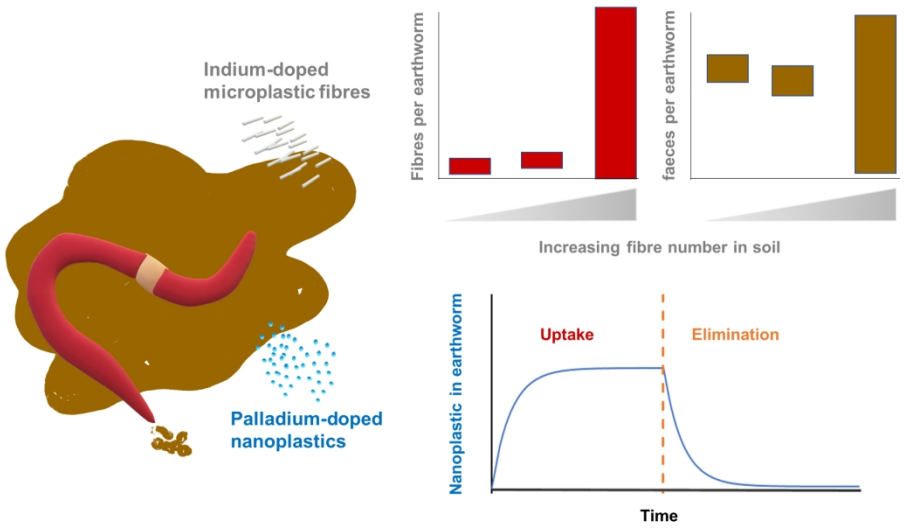

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