Effects of microplastics in soil ecosystems: above and below ground

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Highlights:

- Microplastics may affect important processes in soil ecosystems.
- HDPE, PLA and fibres affected initial growth of perennial ryegrass.
- Plant primary production may be affected by microplastics in soil.
- Earthworm biomass gain was negatively affected by microplastics.
- Microplastics may alter soil structural stability.

Abstract

Environmental contamination by microplastics is now considered an emerging threat to biodiversity and ecosystem functioning. Soil ecosystems, particularly agricultural land, have been recognised as a major sink of microplastics, but the impacts of microplastics on soil ecosystems (e.g. above and below ground) remain largely unknown. In this study, different types of microplastics (biodegradable polylactic acid (PLA), conventional high-density polyethylene (HDPE) and microplastic clothing fibres were added to soil containing the endogeic *Aporrectodea rosea* (rosy-tipped earthworm) and planted with *Lolium perenne* (perennial ryegrass) to assess biophysical soil response in a mesocosm experiment. When exposed to fibres or PLA microplastics, fewer seeds germinated. There was also a reduction in shoot height with PLA. The biomass of *A. rosea* exposed to HDPE was significantly reduced compared to control samples. Furthermore, with HDPE present there was a decrease in soil pH. The size distribution of water stable soil aggregates was altered when microplastics were present, suggesting potential alterations of soil stability. This study provides evidence that microplastics manufactured of HDPE and PLA, and synthetic fibres can affect the development of *L. perenne*, health of *A. rosea* and basic, but crucial soil properties, with potential further impacts on soil ecosystem functioning.

1. Introduction

Microplastics have been found to contaminate a wide range of aquatic environments around the world[1,2] negatively affecting a wide range of organisms and have received much scientific attention over the last decade. There are now studies that have reported microplastics present in soil environments[3,4]. Soils may represent a large reservoir of microplastics[5,6], with sources such as sewage sludge applied as fertiliser[7], fallout from the air[reference8], and in precipitation[9] therefore microplastics may pose a threat to soil biodiversity and ecosystem functioning[10], but there is still a dearth of information[11].

Soil fauna are critical for maintaining a healthy soil[12]. Earthworms are arguably one of the most important and are considered key ecosystem engineers[13] and bio-indicators of environmental quality[14]. Through their feeding, burrowing and casting behaviour, earthworms break down organic matter, turn over nutrients and aid in the structural development of soil aggregates[15]. Of particular interest are endogeic species, such as *Aporrectodea rosea*, which are numerically dominant in temperate agroecosystems[16]. Intensive farming can result in reduced soil health, including less organic matter and can lead to deterioration of soil structure[17]. With potentially increasing contamination by microplastics, soil fauna may be exposed to further stress.

Outside of landfills and industrially intense areas, other terrestrial habitats, such as agroecosystems, are likely to be exposed to microplastics[18] manufactured of a myriad of different polymers. In European agricultural land, microplastic loadings have been estimated at between 63,000 to 430,000 tonnes $year^{-1}$ [19], with studies reporting anywhere between 700-4000 plastic particles kg^{-1} of soil[20,21] and, by dry soil weight, up to 7% microplastic fragments has been reported[22] Microplastics are thought to accumulate in soils[23] with sources of microplastic pollution to agroecosystems typically derive from agricultural practices, such as the use of plastic mulches[24] the spreading of sewage sludge[19] and during the irrigation of land[25]. Many plastic items are manufactured from durable polymers, such as polyethylene (e.g. high-density polyethylene), which is not considered biodegradable and can persist in the environment for decades[26] Increasingly, biodegradable polymers, such as polylactic acid (PLA), are becoming a common alternative to conventional agricultural mulches[27], but the degradation of many biodegradable polymers under ambient conditions has proven to be lengthy or incomplete[28,29]. Agricultural land that has not been exposed to the application of plastic mulches and biosolids, may still come into contact with microplastics during the irrigation of crops, with some microplastics bypassing the treatment process at waste water treatment works[7].

Recent research has shown that once in the soil, microplastics can easily be ingested by soil-living organisms, potentially affecting their fitness and survival[23]. To date, the ingestion of

78 microplastics in the soil has been demonstrated in the anecic earthworm *Lumbricus*
79 *terrestris*[30,31,32,33], with earthworms displaying reduced growth rates after 60 days exposure to
80 polyethylene microplastics at concentrations ranging from 0.2 to 1.2% in dry bulk soil[32]. Maaß
81 *et al*. [34] reported that springtails aid in moving microplastic particles through the soil matrix,
82 therefore potentially contributing to the bio-availability of microplastics to the soil food web.
83 Despite this, minimal research has explored the effects of microplastics on other important
84 aspects of the soil environment, including effects on plant development[35,36]. De Souza Machado
85 et al. [37,38] reported that different microplastics can affect several below ground processes[37] such
86 as soil structure and microbial activity, and physiological components of *Alium fistulosum*
87 (spring onion) when grown in presence of microplastics[38] They have proposed a conceptual
88 model that links soil biophysical processes to plant performance, in which microplastics alter
89 several aspects of the soil environment with cascading effects on soil biota, including plants[38].
90 The current knowledge on the impacts of microplastics on soils (physico-chemical
91 characteristics and structure) and its associated biota (above and below ground) currently
92 remains inadequate to fully address the risks to the terrestrial environment.
93 This study, therefore, was set up to assess the above and below ground responses to microplastic
94 contamination of soil ecosystems, using the endogeic earthworm *Aporrectodea rosea* (rosy-
95 tipped earthworm) and soil sown with *Lolium perenne* (perennial ryegrass). The effects of
96 synthetic fibres (acrylic and nylon mixture), and microplastics manufactured of conventional
97 high-density polyethylene (HDPE) or biodegradable polylactic acid (PLA) were assessed using
98 mesocosm systems, providing realistic, but controlled, semi-natural conditions. The experiment
99 tested the hypotheses that the addition of synthetic fibres, HDPE and PLA microplastics to soil
100 would alter the (i) seedling growth and germination of *L. perenne*, (ii) shoot and root biomass,
101 and root/shoot ratio of *L. perenne*, (iii) total chlorophyll, chlorophyll-a and -b contents and
102 chlorophyll a/b ratio of *L. perenne* as a potential stress response, (iv) growth of *A. rosea* and

(v) pH, organic matter content and stability of soil, with regards to soil aggregate distribution and aggregate mean weight diameter.

2. Materials and Methods

2.1 Experimental design and set-up

For this experiment, *L. perenne* was chosen as a model species on the basis that it is one of the most important grass species in temperate regions[39], providing high yields and quality forage throughout a wide range of environmental conditions[40]. The endogeic earthworm *A. rosea* was chosen as a model species because of its natural abundance in grassland ecosystems[41]. The experiment was conducted under laboratory settings at Anglia Ruskin University, Cambridge, United Kingdom and followed an orthogonal, fully crossed design with two factors, "PLANT" and "PLASTIC". The factor "PLANT" had two levels: *Planted* or *Unplanted*; the factor "PLASTIC" had four levels: *Fibres* (synthetic fibres), *HDPE* (high-density polyethylene), *PLA* (polylactic acid) and a control (*Control*). All treatments were replicated five times (n = 5, N = 40).

The mesocosms were created using clean, opaque polypropylene plant pots with a 1.3 litre capacity (height = 13.0 cm, top diameter = 12.5 cm, bottom diameter = 10.2 cm). Each mesocosm was filled with top soil sourced from Westland Garden Health (Dungannon, Northern Ireland). Top soil was chosen to represent similar soil conditions in which *A. rosea* is commonly found. The topsoil was a sandy clay loam soil composed of 18.6 ± 0.7% (mean ± SEM, n =3) organic matter and a pH of 6.9 ± 0.01 (mean ± SEM n = 3). All soil was air dried for 24 hours, before being manually sieved through a 2000 µm mesh to remove any stones and homogenise the soil.

Prior to filling mesocosms with soil, virgin HDPE (density of 0.95 g cm^{-3}) or PLA (density of 1.2 – 1.3 g cm^{-3}) microplastics or synthetic fibres were thoroughly mixed and homogenised by

hand through the soil in separate containers in bulk. Microplastics were incorporated in such a way as their movement through the vertical soil profile is expected, particularly within agroecosystems where the soil is exposed to management practices, such as ploughing and harvesting[18]. All mesocosms received 1060 g of the sieved soil to reach a dry bulk density of 1.1 g cm^{-3}. As such, mesocosms treated with microplastics received 1 g kg^{-1} dry soil of HDPE or PLA (0.1% w/w), whereas those treated with synthetic fibres received 10 mg kg^{-1} dry soil (0.001 % w/w). Similar to De Souza Machado et al, [38], the volume of fibres at a density 0.1% w/w soil was found to be too large for the mesocosms so less were added than the microplastics. The mean diameter of the microplastic particles was 102.6 µm (range = 0.48-316 µm) for HDPE and 65.6 µm (range = 0.6-363 µm) for PLA (see Green [41]) for more detail on size distribution). Fibres were collected from a standard household washing machine after washing synthetic fabric clothing items (acrylic and nylon) several times at 40°C for 120 minutes with centrifuge steps at 1200 rpm to represent a typical washing cycle but no washing detergent was added. Prior to washing, the filters on the washing machine were thoroughly cleaned to ensure only synthetic fibres were collected. In order to remove any potentially remnant detergent or conditioner the collected fibres were rinsed in 2 litres of water, filtered over Whatman No. 4 filter papers and dried at 30°C[42]. To calculate concentrations of synthetic fibres to add to each mesocosm the amount of fibres was quantified by suspending 1 mg of fibres in 15 of distilled H_2O in a petridish and inspected under a dissection microscope with millimetre graphical paper (n = 5) . The fibres were categorised into the classes: short (< 2 mm; 2290 ± 233 mg^{-1}), medium (< 2-7 mm; 1435 ± 225 mg^{-1}) and long (> 7 mm; 16 ± 4 mg^{-1}). After incorporating the microplastics, each mesocosm was watered to obtain 60% water holding capacity (WHC) determined gravimetrically from separate, dedicated mesocosms: air-dried soil added to the desired bulk density (for each treatment) was saturated with water and weighed when no water leached from the soil at which the soil was at 100% WHC. Mesocosms were allowed to settle for 24 hours before adding *A. rosea*.

Adult *A. rosea* were collected from an urban grassland area in Cambridge, United Kingdom (52°13'27"N, 0°8'2"E) by hand-digging. Mature individuals were kept in a contained moistened sandy loam soil obtained from the collection site. Earthworms were left to acclimatize for 7 days before commencing the study. After a 24-hour depuration period on moist paper towels, 80 adult *A. rosea* were rinsed with distilled water, dried on paper towels and separately weighed. Two individuals were randomly assigned to each mesocosm and once added, all earthworms immediately began to dig into the soil. This gave a density equivalent to of 164 individuals m^{-2}. Despite this density being greater than the average 9 - 56 individual m^{-2} commonly seen for *A. rosea* (e.g. Curry et al. [43]), in some areas densities of *A. rosea* have been recorded as high as 130 - 288 ind. m^{-2} [44]. There were no significant differences between weights of the depurated worms, allocated to the different microplastic treatments at the beginning of the experiment (one-way ANOVA: $F_{7,32} = 2.24$, $P = 0.057$). Diploid *L. perenne* seeds where purchased from Cotswold Grass Seeds Direct and mesocosms in which ryegrass was grown received 200 seeds (approx. 0.36g) giving a density of 27.5 kg ha^{-1}. Seeding rates were chosen based on studies into optimal seeding rates for *L. perenne* (e.g. Lee et al. [45]). *L. perenne* seeds were distributed evenly on the soil surface of each pot and were watered daily with 10 ml of tap water using a spray bottle. The experiment ran for 30 days from 9th July until 7th August 2018 within an air-conditioned laboratory with a mean maximum daily temperature of 21.2°C. Throughout the experiment, mesocosms were watered daily to maintain soil moisture at approximately 60% water holding capacity via adjusting for weight loss from evapotranspiration.

2.2 Germination, growth, above and below ground biomass, and chlorophyll content of Lolium perenne

Seeds were considered germinated by the emergence of radicle which was recorded daily. *L. perenne* growth was also recorded by measuring the length of the shoots at five random points

within each mesocosm. At the end of the experimental period, the abundance of *L. perenne* seeds germinated in each mesocosm was quantified. At the end of the experiment after all other previously described measurements were taken, the mesocosms were destructively sampled by hand and all remaining samples required for analysis were collected. From each mesocosm, shoots were collected by cutting the *L. perenne* shoots to surface level and weighed. A subsample of 2 g was oven-dried at 50^{o}C for 12 hours to gravimetrically determine moisture content. A separate, fresh sample (~0.2 g) required for chlorophyll analysis was collected and stored in falcon tubes in the dark to protect from light at -18^{o}C. Chlorophyll was extracted for 12 hours after adding 10 mL of 90% acetone and chlorophyll-a and -b concentrations were then measured from each sample using a spectrophotometer. Concentrations of chlorophyll in the grass were calculated following equations by Jeffrey and Humphrey[46],(chlorophyll-a: 11.93* λ_{664nm} – 1.93* λ_{647nm} and chlorophyll-b: 20.36* λ_{667nm} – 5.5* λ_{664nm}) and expressed as amount of chlorophyll g^{-1} dry biomass. *L. perenne* roots were carefully hand collected from each mesocosm before washing the roots to remove any remaining soil and drying all roots at 50^{o}C for 12 hours to determine dry mass.

2.3. Measurements of Aporrectodea rosea *and soil.*

Individuals of *A. rosea* were recovered from each mesocosm by hand and rinsed with distilled water before leaving them to depurate the contents of their guts on moist paper towels for 24 hours before recording their weight gravimetrically. A 50 g subsample was oven-dried at 105°C overnight to determine the soil moisture content gravimetrically. The remaining soil from each mesocosm was air dried for 48 hours, before collecting samples required for the analysis of soil pH, organic matter content and soil aggregates. Soil pH was determined at a soil-to-water ratio of 1:5 after mechanically shaking for 1 hour, using deionised H_2O. Soil was settled by centrifugation at 3000 x g for 3 minutes and pH was measured from the supernatant using a Hanna HI 991300 pH meter.

Soil organic matter content was determined by calculating loss on ignition (LOI). From the air-dried soil, a subsample from each mesocosm was oven-dried at 105ºC for 2 hours until constant dry weight was achieved (to remove any moisture), From this, 5 g subsamples were combusted at 550ºC for 12 hours in a muffle furnace and reweighed. The weight loss is proportional to the amount of organic matter within in each sample and were corrected for the carbon added from microplastics[47] assuming complete combustion of the polymers.

Soil water stable aggregate distribution was analysed by collecting a 100 g subsample of air dried soil from each mesocosm. The subsample was then wet sieved to separate the macro and microaggregates[48]. Briefly, soil samples were placed on stack of sieves and slaked by submerging it in water for 2 minutes before sieving. The fractions were categorised by size class into >2000 µm, 2000-1000 µm, 1000-250 µm, 250-63 µm and <63 µm. The sieves were then gently oscillated in an up and down motion by hand for a total of 20 cycles over a 1 min period. Material remaining on each sieve was rinsed into a pre-weighed aluminium weigh boat before oven drying each fraction at 50ºC before reweighing each fraction. The aggregate data were corrected for added microplastics assuming a homogenous distribution within the soil and aggregate profile based on size distribution published in Green (2016). The 100 g soil sample used could contain 100 mg of added HDPE or PLA microplastics, or 1 mg added fibres. The mean weight diameter (MWD) was used as an index of aggregate distribution and was determined for each aggregate portion using the following equation:

$$\mathrm{MWD} = \sum_{i=1}^{n} x_i \, w_i$$

where x_i is the mean diameter of particles in each aggregate fraction and w_i is the proportion of aggregates retained within each fraction after wet sieving.

2.4 Statistical Analysis

All statistical analysis was conducted in R v3.5.1[49] . The data were screened for both normality (Shapiro-Wilk tests and Q-Q plots) and homogeneity of variance using Levene's test from the

car package v3.0-2[50] to check the assumptions for ANOVA. To improve its conformity to these assumptions, transformation of some data was required (specific transformations are stated in the results). Differences in *L. perenne* shoot and root biomass, root/shoot ratio, and chlorophyll content were analysed using a one-way ANOVA with the factor PLASTIC. When the main terms were significant, pairwise comparisons were calculated using Tukey HSD tests to further explore responses. Development of *L. perenne* during the first 30 days was approximated using non-linear least squares (*nls*), assuming a Gompertz model (e.g. Paine et al. [51]):

$$Y = K\left(\frac{M_0}{K}\right)^{e^{-rt}}$$

With Y the germination rate (%) or length (mm) over time (t in days since sowing) and the three unknown parameters M_0, r and K being M_0 = estimated length or germination rate at t = 0 in mm or %, r = the growth rate (day^{-1}), and K = the asymptote or estimated maximum length (mm) or germination rate (%) respectively, using starting values based on observations of the plotted data.

Differences in relative growth of *A. rosea* was analysed in two ways: using an analysis of covariance (ANCOVA) with the initial wet biomass as a covariate and PLASTIC and PLANT as factors testing the hypothesis that there are differences of biomass between treatments. The second approach involved implementing two-tailed t-tests on each treatment to test whether the difference between initial and final biomass was significantly different from 0%, i.e. an overall growth or reduction for each separate treatment. Soil organic matter content, pH and MWD were analysed using a two-way ANOVA with PLASTIC and PLANT as factors. Transformation of aggregate fraction distribution data ($\sin^{-1}(\sqrt{x})$) did not greatly alter the results for the ANOVA, therefore they were analysed on untransformed data to improve interpretation of the results.

Water stable soil aggregate profiles were compared using a two-way, orthogonal permutational multivariate ANOVA using the *vegan* package v2.5-2[52] with PLASTIC and PLANT as factors, after homogeneity of multivariate variance was checked between centroids with the *betadisper*

function. All permutations were done on Bray-Curtis dissimilarities calculated from square-root transformed data using 9999 permutations of residuals under a reduced model. *Post-hoc* pairwise tests using PERMANOVA were implemented when any of the main terms were significant to further explore sources of differences in a similar fashion. An ordination was calculated to visualise any differences between water-stable aggregate profiles using 2-dimensional non-metric multidimensional scaling (nMDS) plots, generated using 250 iterations or when the lowest 2D stress was reached. This was performed using the *metaMDS* function in *vegan,* whilst employing the *monoMDS* engine. For all tests, statistical significance was assumed at $\alpha = 0.05$.

3. Results

3.1. Germination, growth, above and below ground biomass, and chlorophyll content of Lolium perenne.

On average, 78 ± 1% of the seeds germinated after 30 days, but significantly ($F_{3,16} = 4.13$, $P = 0.024$) fewer seeds germinated when exposed to fibres or PLA, which lead to a 7% and 6% reduction compared to the controls respectively (Table 1a, Figure 1a). The shoot length of *L. perenne* after 30 days ranged between 140 ± 1.1 and 172 ± 9.8 mm, and was significantly ($F_{3,16} = 5.19$, $P = 0.011$) different between microplastic treatments with 19% shorter shoots when PLA was added compared to the control (*Control* vs *PLA*: $P = 0.006$) (Table 1b, Figure 1b). Shoot biomass (dry weight) was on average 28 ± 0.9 g m^{-2} and was not significantly different between any of the treatments (Figure 2a). Dry biomass of the roots ranged between 5.3 ± 1.7 and 9.7 ± 1.3 g m^{-2} and differed significantly ($F_{3,16} = 6.19$, $P = 0.005$) between microplastic treatments (Figure 2b), with *L. perenne* roots exposed to HDPE having 45% more root biomass than those exposed to PLA (*HDPE* vs *PLA*: $P = 0.003$). This corresponded with the dry root:shoot ratio (Figure 2c) which differed significantly ($F_{3,16} = 11.05$, $P < 0.001$) between treatments, with *L. perenne* exposed to HDPE having a 35% greater root/shoot ratio when

compared with all other treatments. There was no significant relationship between germination success and shoot biomass ($F_{1,18}$ = 0.29, P = 0.594) and root biomass ($F_{1,18}$ = 0.16, P = 0.691). The chlorophyll-a content in *L. perenne* shoots after 30 days was 5.41 ± 0.15 mg g^{-1} dry biomass, by this was not significantly different between any treatments (Table 2). Similarly, chlorophyll-b content (on average 4.23 ± 0.16 mg g^{-1} dry biomass) did not significantly differ between the treatments. However, the chlorophyll-a:chlorophyll-b (chla-a:chl-b) ratio, which ranged between 1.09 ± 0.08 and 1.39 ± 0.01, was significantly ($F_{3,16}$ = 11.67, P < 0.001) different between all microplastic treatments compared to the control (Table 2), with shoots grown with fibres, HDPE and PLA having 19%, 21% and 22% greater chla-:chl-b ratios respectively compared to the controls.

3.2. Effects of microplastics on the growth of Aporrectodea rosea

After 30 days of exposure to the microplastics all added individuals of *A. rosea* were recovered (no mortalities). The relative growth of the earthworms was significantly different between microplastic treatments ($F_{3,32}$ = 4.54, P = 0.009), with individuals in soil without added microplastics having gained weight, whereas those with added microplastics having lost weight. There was on average an 5.7 ± 3.1% increase in biomass in the controls compared to the initial biomass at t = 0, and 3.1 ± 1.1% decrease in biomass in the microplastics treatments. (Figure 3). Compared to the initial biomass (testing H_0: $\mu_{final} - \mu_{initial} = 0$), *A. rosea* in the soil containing additional microplastics manufactured of HDPE significantly lost biomass compared to their initial weight ($t_{4,}$= 3.20, P = 0.033) with *L. perenne* present. In the controls, *A. rosea* significantly increased biomass (t_4 = 3.10, P = 0.036) compared to the initial biomass. Furthermore, compared to the other treatments, *A. rosea* in soil with HDPE lost significantly more biomass than the control (*Control* vs *HDPE*: P = 0.007).

3.3. pH, soil organic matter and water stable aggregates

After 30 days of exposure to microplastics the soil pH, ranging between 6.35 ± 0.14 and 6.98 ± 0.03 (Table 3), was significantly different between the microplastics treatments ($F_{3, 32}$ = 21.90, P<0.001), but remained not measurably affected by the presence of *L. perenne*. In particular, the pH of soil when exposed to HDPE was significantly lower than all other treatments (*Control* vs *HDPE*: P<0.001; *Fibres* vs *HDPE*: P<0.001; *PLA* vs *HDPE*: P<0.001), with soil exposed to HDPE being 0.62 units lower than the controls. Soil organic matter content as measured by loss on ignition was on average 17.3 ± 0.4% and was slightly greater in the soil of mesocosms with the addition of *L. perenne*, however this was not significant and was not measurably affected by the microplastics treatments (Table 3).

After correcting for the size faction distribution of the added microplastics, the mean weight diameter (MWD) of water stable soil aggregates ranged between 750 ± 87 µm and 1179 ± 66 µm (Table 3), which was significantly different between the microplastics treatments ($F_{3,32}$ = 7.14, P < 0.001), but remained unaffected by the presence of *L. perenne*. Soils in control treatments had 24%, 35% and 28% greater MWD compared to the fibres, HDPE and PLA treatments respectively (*Control* vs *Fibres*: P = 0.026, *Control* vs *HDPE*: P < 0.001, *Control* vs *PLA*: P = 0.008).

Profiles of water-stable soil aggregates were significantly ($F_{3,32}$ = 6.39, P < 0.001) different between the microplastics treatments (Figure 4), with soil containing any of the added microplastics having significantly different aggregate profiles compared to the controls (*Control* vs *Fibres*: P = 0.018; *Control* vs *HDPE*: P = 0.006; *Control* vs *PLA*: P = 0.006). This was reflected in significant ($F_{3,32}$ = 6.67, P = 0.001) changes in the distribution of large (>2000 µm), medium and small aggregates by the presence of microplastics (Figure 5, Table 4). Soil from the controls had 60% and 53% more large macroaggregates >2000 µm compared to soils with HDPE and PLA respectively (>2000 µm *Control* vs *HDPE*: P = 0.001; *Control* vs *PLA*: P = 0.006). Conversely, microaggregates (250-63 µm) were significantly ($F_{3,32}$ = 7.18, P < 0.001) more present in soil exposed to all types of microplastics when compared to control soil (250-

63 µm *Control* vs *Fibres*: P = 0.016; *Control* vs *HDPE*: P < 0.001). Furthermore, microaggregates (<63 µm) were significantly reduced ($F_{3,32}$ = 12.3, P < 0.001) in soil exposed to HDPE and PLA when compared to soil without added microplastics (<63 µm *Control* vs *HDPE*: P = 0.006; *Control* vs *PLA*: *P* = 0.005) (Figure 5, Table 4).

4. Discussion

4.1. Responses of Lolium perenne *to microplastics in the soil*

Several growth responses of *L. perenne* were altered when plastic fibres and HDPE or PLA microplastics were incorporated into the soil matrix. Fewer seeds germinated compared to soil without added microplastics, especially when fibres were present, and the average shoot length was supressed when microplastics manufactured of PLA were present. The root biomass was greater with HDPE present, which was significantly different from the plants exposed to PLA. There was, however, no significant relationship between germination success and root and shoot biomass. With fewer individuals to compete with for resources such as space, water and nutrients, plants may better utilise these which enables higher levels of biomass for individual plants. There is currently not much information regarding how plants may respond to the presence of microplastics, but recently a study by De Souza Machado et al. [38] reported that *Allium fistulosum* (spring onion) responded to different types of microplastics, demonstrating that root biomass was significantly increased with polyester fibres and polystyrene, but not by HDPE particles suggesting differential responses based either on shape or the type of polymer. In the current study, despite fewer fibres being added compared with HDPE or PLA microplastics, they still had a negative effect on germination of *L. perenne*. It is possible that some shapes may have a stronger effect than others, for example, irregular shaped polyethylene microplastic fragments had a stronger negative effect on the mobility of sheepshead minnows than spherical polyethylene microplastics[53]. In addition, polypropylene fibres had a more toxic

363 effect than polyethylene particles on amphipods[54] and PES fibres had stronger effects on plant
364 growth than HDPE fragments[38], however in these studies the effects of shape cannot be
365 separated from that of polymer type.
366 Bosker et al.[55] reported that polystyrene microplastics lead to a reduced germination of the
367 dicotylodon *Lepididium sativum* (cress) when grown on cellulose filter paper in petridishes.
368 Reduction in germination could possibly be because the particles blocked pores in the seed
369 capsule. The current study had microplastics incorporate within the soil, but it is yet unclear
370 how they may caused reduction in germination success of *L. perenne*. Other plant growth
371 responses have been found when *Triticum aestivum* (wheat) was grown with microplastics[35].
372 When exposed to biodegradable macro- and microplastic residues (polyethylene terephthalate
373 and polybutylene terephtalate), *T. aestivum* also had reduced plant height, shoot biomass and
374 leaf area[35]. Although the exact mechanisms remain unclear, the inhibitory effect of PLA on *L.*
375 *perenne* shoot length as seen within this study could be attributed to potential stress caused by
376 degradation by-products of PLA. When degrading, PLA has been found to be enzymatically
377 degraded via microbes into lactic acid oligomers under controlled conditions[56] , and microbes
378 aid in the degradation when PLA is in the soil. It remains unclear however, via which pathways
379 microplastics incorporated in the soil could affect responses aboveground, but possibly via
380 immobilisation of nutrients by organic compounds originated from degradation. For instance,
381 the degradation of PLA has found to have cytotoxic and genotoxic effects on the
382 monocotylodon *Allium cepa* (onion)[57], where 1 cm^2 particles of PLA were left to degrade in
383 compost for 76 days and the leachate collected. Seeds of *A. cepa* exposed to leachate from PLA
384 treated compost had a lower germination rate and, during development, inhibited cell division
385 rate compared to the control. Degradation products from biodegradable plastics, such as lactic
386 acid, can have effects on growth as was shown for *Lycopersicon esculentum* (tomato) and
387 *Lactuca sativa* (lettuce)[58] with high concentrations of lactic acid resulting in increased shoot
388 biomass while the roots were less with lower concentrations. The study by Martin-Closas et

al.[58], however, did not include a soil medium which likely would contain (micro)biota that utilise lactic acid as carbon source. With great expectations placed on biodegradable plastic alternatives, with the aim of solving the plastic pollution crisis, using PLA in agricultural soils may need to be reconsidered, especially considering such materials are gaining traction as an alternative to conventional plastic mulching[27].

One strategy employed by plants to cope with stressful environments (e.g. reduced water and/or nutrient availability) is by expanding the root system, thereby increasing the root/shoot ratio, as larger and deeper root systems will help to increase the uptake of water and nutrients to maintain vigorous root growth to overcome stress[59,60]. When exposed to microplastics made of HDPE, *L. perenne* displayed significant alterations in root biomass and related to that; root:shoot ratio. Since soil moisture was held consistently throughout the experimental period (and no other edaphic conditions were altered), the increased root biomass and root/shoot ratio of *L. perenne* exposed to HDPE may therefore be indicative of stress[61] caused by the presence of the microplastics. It remains unclear whether the microplastics directly or indirectly caused stress via e.g. physical contact or soil physico-chemical properties. Despite being typically considered as biologically inert[62], the absorption of organic compounds by plants, released from e.g. mulch polymers, has already been identified within various crop plants[63,64]. This could in turn affect the soil microbiota, for example at the rhizosphere scale as also suggested by De Souza Machado et al. [38] altering nutrient dynamics. Furthermore, the addition of microplastics could alter the soil physical properties, including moisture retention and root penetration dynamics. However, the current study did not attempt to distinguish mechanistic physical and chemical (e.g. toxic) effects of the microplastics, and either or both could possibly explain the plant physiological changes.

Although there were no measurable alterations in the chlorophyll-a and chlorophyll-b contents of *L. perenne* exposed to different microplastics, interestingly the chlorophyll a:chlorophyll-b (chl-a:chl-b) ratio was elevated in response to microplastics, regardless of type. The chl-a:chl-

b ratio is a fundamental parameter for photosynthetic activity with deviations often used as an indicator of stress in plants[65]. The increased chl-a:chl-b ratio of *L. perenne* in this study is therefore indicative of a stronger inhibition of chlorophyll b synthesis in response to microplastic addition. Chlorophyll-b is an important pigment responsible for improving the efficiency of photosynthesis (e.g. Katz et al.[66]) and thus plays an important role in primary production of grassland agro-ecosystems. It is possible that due to biophysical changes in the soil, macro- and micronutrient (e.g. magnesium, potassium) availability to the plant was altered, which has cascading effects on the photosynthetic capacity as measured by chlorophyll. It remains unclear if HDPE or PLA microplastics, or fibres alter micronutrient availability, but the recent study by De Souza Machado [38] reported that spring onion leaves had increased N content when polyamide was present in the soil, while polyester fibres decreased it. Polyamide might be a source of nitrogen when degrading (nylon and acrylic also contain nitrogen atoms), whereas polymers such as polyester, PLA, HDPE typically have no nitrogen atoms in their pure form. Differential responses of chlorophyll content in plants based on polymer type have been found in marine primary producers (microalgae) when exposed to microplastics[67,68], with less chlorophyll present when HDPE or PLA microplastics were present. Similar responses have been found in freshwater ecosystems, where polystyrene nanoplastics have been found to reduce chlorophyll content of *Scenedesmus obliquus*, a freshwater microalgae[69], but polyethylene microbeads did not measurably affect chlorophyll content of *Lemna minor* (duckweed), a freshwater plant[70]. This suggest that primary producers respond differently in terrestrial, aquatic (freshwater and marine) habitats, which warrants more investigation given the crucial position of primary producers within ecosystems including soils[10].

4.2 Responses of Aporrectodea rosea *to microplastics in the soil*

The biomass of *A. rosea* was generally reduced when microplastics were experimentally added to the soil, but this was most severe when exposed to HDPE microplastics. This corroborates

findings by Huerta Lwanga et al.[32] who found a reduction in weight of the anecic earthworm *Lumbricus terrestris* when exposed to low-density polyethylene (LDPE) microplastics. Being a geophagous (endogeic) species, the diet of *A. rosea* consists primarily of soil organic matter. The feeding strategy of the species is therefore generally one of high consumption and rapid turnover of large quantities of soil due to low assimilation of nutrients from the poor-quality food material[71]. Despite the current study not directly assessing the ingestion of microplastics, the feeding strategy of *A. rosea* suggests the likelihood is high and the consumption of plastics therefore remains plausible. The response mechanisms of earthworms to microplastics may be comparable to that of aquatic species, such as the polychaete *Arenicola marina* (lugworm) and include the obstruction and abrasion of the digestive tract, thereby limiting the bioavailability and absorption of nutrients, reducing growth and ultimately compromising the survival of the organism[67,72,73]. Being an important component of soil ecosystems, earthworm activity can influence below-ground plant properties, which in turn affects the above ground plant. A study by Arnone III and Zaller[74] demonstrated significantly reduced root density with increased density (activity) of earthworm communities but without any observed responses of the aboveground plant biomass. In the current study the root biomass was greater under HDPE treatments, whilst the earthworm biomass was significantly reduced. The current study did not directly measure the activity of *A. rosea*, rather the formation of water stable aggregates were measured as a proxy for earthworm activity, with more large (> 2000 µm) water stable aggregates being formed with HDPE present. In nature, the burrowing activities of *A. rosea* might distribute microplastics throughout the soil profile as was observed with *Lumbricus terrestris*[31] (an anecic species), which can expose microplatics to other parts of the soil ecosystem. In addition, via ingestion, earthworms can further incorporate microplastics into the soil matrix when expelled as cast, where they can remain inside. Soil aggregates can contain different microbial communities depending on the size of the aggregate[75], also when passed through the gastro-intestinal tract of an earthworm[76]. As such, when incorporated into soil

aggregates, the microplastics might be exposed to altered degradation rates (e.g. protected from external influences or exposed to greater microbially-induced degradation) compared to when loosely in the soil matrix.

4.3 Effects of microplastics on soil physico-chemical properties

Lowered soil pH when HDPE microplastics where present has also been reported by Bandow et al.[77], who found that HDPE pellets decreased pH of soil eluates after 6 and 12-week exposure to photo- and thermos-oxidative conditions. Given that the microplastics were incorporated in the soil, photo-oxydation was very minimal and probably only limited to those on the surface. HDPE is relatively resistant to biological degradation[78] and may degrade slowly (decades) when microbial biofilms colonise the particles. It could be that the HDPE particles altered the cation exchange in the soil and allowed free exchange of protons in the soil water, given their relatively large, possibly reactive, surface area. The current study, however, was not designed to determine degradation rates not the products of the added microplastics. Further research is required to fully understand the mechanism of how HDPE may be able to change the pH of soil. Nevertheless, alterations within soil pH affect soil microbial communities and the microbial communities of the rhizosphere, which differ from those in the bulk soil, by directly interfering with the diversity and composition of soil microorganisms[79,80]. Recent research[81] has shown that the addition of polypropylene microplastics to soil at 28% w/w altered microbial activity as measured by hydrolysis of fluorescein diacetate and nutrient content of dissolved organic matter was higher than when no microplastics were added. Similarly, De Souza Machado et al.[37,] reported in similar studies that soil microbial activity responded to the addition of microplastics, depending on the added concentration. The activity of microorganisms play an important role in the decomposition of soil organic matter and the cycling of major nutrients, vital for plant growth and root development[82], and shifts in functional processes, including microbially driven processes, can affect these. Further investigation into the structure and

493 diversity of the soil microbial community is therefore necessary to fully understand effects of
494 microplastics on the soil-plant environment.
495 The addition of HDPE or PLA microplastics resulted in significantly fewer macroaggregates
496 (>2000 µm) and altered waterstable aggregate profiles. Altered size fractionation has been
497 reported in the recent study by De Souza Machado et al [38], who found that different types of
498 microplastics with fewer water stable aggregates when polyamide and polyester fibres were
499 present. It is possible that the added microplastics changes the physical characteristics of the
500 receiving soil, for example enhances of reduces the cohesion between aggregate forming
501 particles. Zhang and Liu[4] reported that 72% of microplastics in soil samples were associated
502 with soil aggregates, with fibrous plastics particles found the most in microaggregates.
503 Incorporation of microplastics into the soil matrix (i.e. within soil aggregates) can be facilitated
504 by soil fauna, especially geophagous bioturbating earthworms, such as *A. rosea*. Changes in
505 aggregate profiles may be attributable to earthworm activity in the current study, albeit activity
506 was only measured via the creation of large macroaggregates (> 2 mm, given that the soil was
507 sieved prior) as a proxi. Less bioturbation could be a result from reduced activity of *A. rosea*,
508 including burrowing and feeding. It remains unclear if this is related to the reduction of
509 earthworm biomass, given that the animals significantly lost weight with added HDPE, but not
510 PLA (where they neither significantly lost or gained weight). This is supported by studies into
511 the marine worm *Arenicola marina*, which found the addition of a variety of microplastics to
512 sediment to reduce feeding activity[55,66], with such reductions being attributed to the dilution of
513 food and the ingestion of microplastics, resulting in prolonged gut residence[83]. In this study,
514 food availability in terms of organic matter content proved to be unaltered by the addition of
515 HDPE, PLA and synthetic fibre microplastics, suggesting other mechanisms, such as prolonged
516 gut residence of the microplastics may be altering the feeding activity of *A. rosea.* As important
517 ecosystem engineers, the potential reduction in feeding activities displayed by *A. rosea* exposed
518 to microplastics, highlights the needs for in-depth investigation to fully understand the

519 underlying mechanisms, which may have ultimately lead to the supressed growth displayed by
520 worms in this study.
521 Moreover, the addition of HDPE and PLA microplastics may have interfered with the stability
522 and formation of larger macroaggregates through direct alterations within the binding
523 mechanisms of the soil. Soil aggregate stability is a widely recognised indicator of soil health
524 and is measured as the vulnerability of soil aggregates to external destructive forces, such as
525 rainfall and wind erosion[17,84]. The hierarchical theory of aggregation suggests that
526 microaggregates bind together to form macroaggregates, with the bonds within
527 microaggregates being stronger than the bonds between microaggregates[85,86]. The reduction of
528 large (>2000 µm) macroaggregates and the increase in 250-63µm microaggregates observed
529 within soils exposed to microplastics may therefore be indicative of alterations within the bonds
530 between microaggregates opposed to the bonds within microaggregates, which are stronger.
531 Stability of the soil structure is fundamental for productive soils that can support plant and
532 animal life, through its influence on the transport of water, gas and nutrients[87]. Changes in soil
533 aggregate profiles can result in alterations on soil carbon processes and related nutrient
534 availability in micro-and macroaggregates[86] with potential cascading effects on other
535 biophysical processes in the soil[37,38] . The results of this study therefore highlight the need to
536 further address the potential impacts of microplastics on the structure of the soil environment.
537 It should be noted, however, that the soil substrate used in the current study was likely not
538 devoid of microplastics given that top soils can be sourced from different locations and merged.
539 Using control samples with the same soil (after thorough homogenisation) allows comparing
540 treatment effects whilst acknowledging that there could have been other microplastics present
541 via the substrate, This, however, does not invalidate the findings of the current study.
542
543 *4.4 Wider implications and recommendations*

This study provides further evidence for several potentially detrimental effects of microplastics in terrestrial ecosystems, using a model system based on *L. perenne*, and *A. rosea.* In agricultural settings, such effects may have implications for the production and quality of crop plants, by directly affecting plant development and altering the soil environment in which they are produced as well as having potential implications for human health through the accumulation of microplastics and harmful compounds in the tissues of plants. As the application of biosolids as fertiliser, plastic mulching and irrigation with environmental water continues, the concentration of microplastics in soil is expected to increase. Future studies should therefore focus on addressing the effects of microplastics on soil-plant systems, soil organisms and soil properties at varying concentrations as well as using a realistic mixture of polymers to understand the extent of the effects of microplastics in the soil environment. More research is needed on the mechanistic pathways in how (micro)plastics affect organisms, utilising a holistic approach seeing soil as a matrix of interacting biotic (e.g. soil structure) and abiotic (e.g. earthworms) components to understand the effects observed on soil flora and fauna both in natural and agro-ecosystems.

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Contributions

BB, DSG designed the experiment analysed the data and wrote the manuscript. CWR collected and analysed the data and contributed to the manuscript.

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Tables

Table 1. Coefficients *K*, M_0 and *r* from Gompertz model for a) germination rate (%) and b) shoot growth (mm) of *L. perenne* when exposed to different types of microplastics. Data are averages (± SEM, n = 5) of coefficients from models fitted to each separate replicate. Superscript letters indicate significant differences between the microplastic treatments. All individual coefficients were significantly different from 0 at $P < 0.001$.

a)

PLASTIC	*K* (%)	M_0 (%)	*r* (day^{-1})
Control	81 ± 1.3^{a}	1.75 ± 0.10	4.44 ± 0.17
Fibres	74 ± 1.6^{b}	1.40 ± 0.12	4.39 ± 0.11
HDPE	79 ± 2.2^{ab}	1.77 ± 0.14	4.59 ± 0.09
PLA	75 ± 0.9^{a}	1.56 ± 0.17	4.50 ± 0.20
ANOVA			
PLASTIC	$F_{3,16} = 4.13$, $P = 0.024$	$F_{3,16} = 1.65$, $P = 0.217$	$F_{3,16} = 0.357$, $P = 0.785$

b)

PLASTIC	*K* (mm)	M_0 (mm)	*r* (day^{-1})
Control	172 ± 9.8^{a}	4.96 ± 0.49	7.08 ± 0.39
Fibres	$156 \pm 6.1^{a,b}$	4.27 ± 0.41	6.79 ± 0.22
HDPE	$158 \pm 4.4^{a,b}$	4.38 ± 0.26	6.79 ± 0.12
PLA	140 ± 1.1^{b}	3.59 ± 0.14	6.26 ± 0.09
ANOVA			
PLASTIC	$F_{3,16} = 4.50$, $P = 0.018$	$F_{3,16} = 2.53$, $P = 0.094$	$F_{3,16} = 2.10$, $P = 0.140$

847 **Table 2.** Chlorophyll-a and –b content (mg g^{-1} dry biomass) and ratio between chlorophyll-a
848 and -b in *L. perenne* shoots after 30 days exposure to different types of microplastics. Data are
849 averages (± SEM, n = 5). Included are ANOVA results with superscript letters indicating
850 significant differences between the microplastic treatments.

851

PLASTIC	Chl-a (mg g^{-1})	Chl-b (mg g^{-1})	Chl-a:Chl-b
Control	5.04 ± 0.19	4.72 ± 0.39	1.09 ± 0.08[a]
Fibres	5.93 ± 0.36	4.44 ± 0.29	1.34 ± 0.02[b]
HDPE	5.51 ± 0.25	4.02 ± 0.20	1.37 ± 0.01[b]
PLA	5.19 ± 0.32	3.75 ± 0.25	1.39 ± 0.01[b]
ANOVA	Untransformed data	Untransformed data	Arcsine-√ transformed data
PLASTIC	$F_{3,16}$ = 1.86, P = 0.177	$F_{3,16}$ = 2.25, P = 0.122	$F_{3,16}$ = 11.67, P < 0.001

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854

855 **Table 3.** Soil physico-chemical characteristics pH, loss on ignition (LOI) as an approximation
856 for soil organic matter content and mean weight diameter (MWD) of water stable soil
857 aggregates after 30 days of exposure to different types of microplastics with *L. perenne* absent
858 (Unplanted) or present (Planted). Data are means (± SEM, n = 5). Included are ANOVA
859 results.
860

PLANT	PLASTIC	pH	LOI (%)	MWD (µm)
Unplanted	Control	6.96 ± 0.02	16.8 ± 1.5	1124 ± 99
	Fibres	6.84 ± 0.04	17.5 ± 1.3	889 ± 142
	HDPE	6.35 ± 0.14	16.1 ± 0.6	752 ± 46
	PLA	6.94 ± 0.04	16.6 ± 1.0	872 ± 137
Planted	Control	6.98 ± 0.03	19.0 ± 0.7	1179 ± 66
	Fibres	6.84 ± 0.03	19.9 ± 1.6	859 ± 68
	HDPE	6.36 ± 0.17	15.1 ± 0.9	750 ± 87
	PLA	6.94 ± 0.05	17.4 ± 1.2	787 ± 41
	ANOVA	Untransformed data	Untransformed data	√ transformed data
	PLANT	$F_{1,32} < 0.01$, P = 0.961	$F_{1,32} = 1.81$, P = 0.188	$F_{1,32} = 0.03$, P = 0.874
	PLASTIC	$F_{3,32} = 21.9$, P < 0.001	$F_{3,32} = 2.61$, P = 0.069	$F_{3,32} = 7.14$, P < 0.001
	PLANT*PLASTIC	$F_{3,32} = 0.01$, 0.998	$F_{3,32} = 0.92$, P = 0.443	$F_{3,32} = 0.16$, P = 0.923

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862
863

Table 4. Analysis of variance table for the aggregate size class fractions supplementing the result of Figure 5.

Size class	ANOVA	F-value	P-value
> 2000 µm	Plant	0.09[1]	0.768
	Plastic	6.67[2]	0.001
	PxP*	0.29[3]	0.835
2000-1000 µm	Plant	0.03	0.871
	Plastic	2.14	0.115
	PxP	0.99	0.409
1000-250 µm	Plant	0.37	0.547
	Plastic	1.46	0.244
	PxP	0.58	0.635
250-63 µm	Plant	0.03	0.860
	Plastic	7.18	<0.001
	PxP	0.28	0.837
<63 µm	Plant	0.84	0.366
	Plastic	12.28	<0.001
	PxP	0.858	0.473

* Interaction term PLANT x PLASTIC.

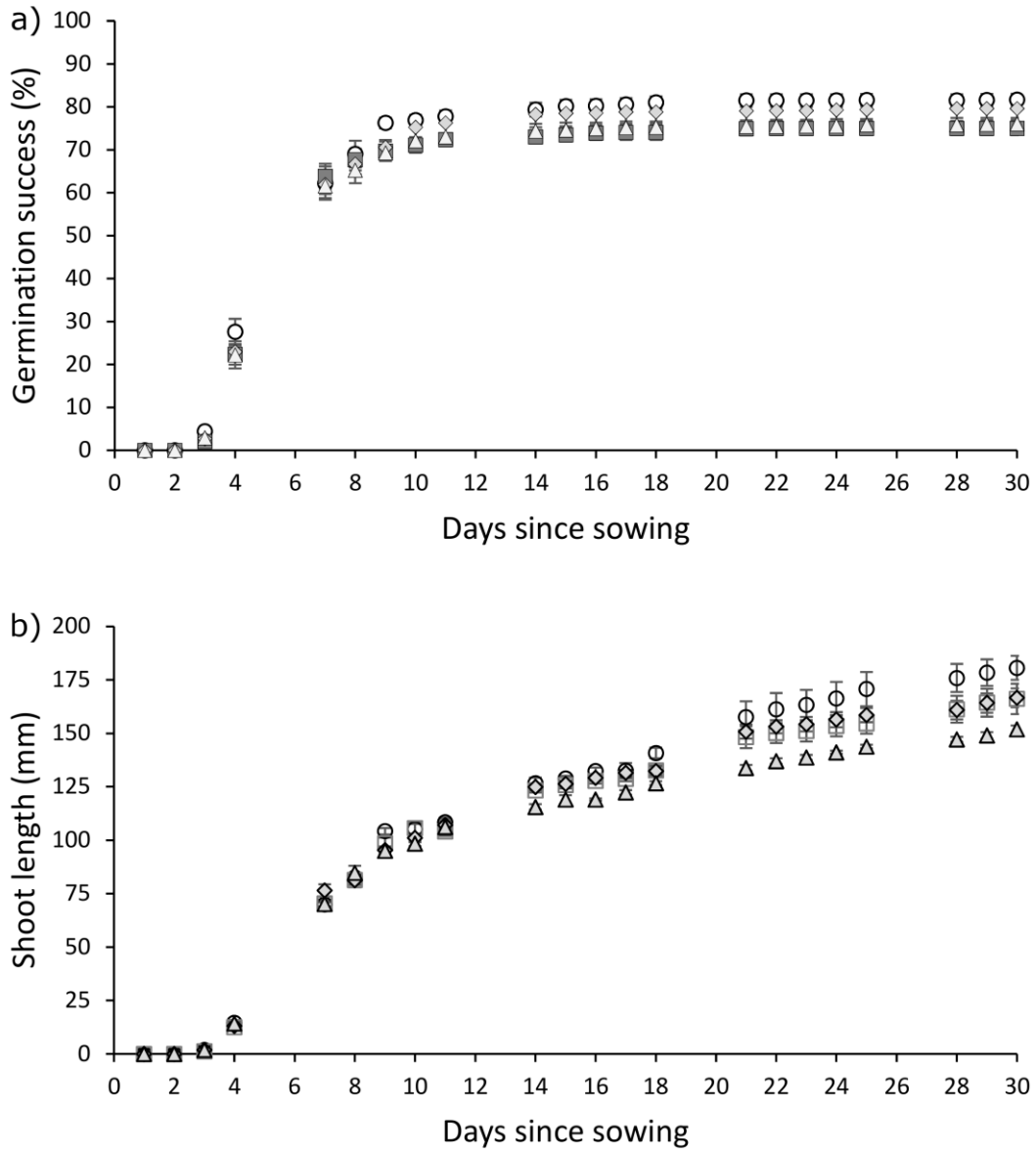
[1] $df_1 = 1$, $df_2 = 32$.

[2] $df_1 = 3$, $df_2 = 32$.

[3] $df_1 = 3$, $df_2 = 32$.

872 **Figures**

873



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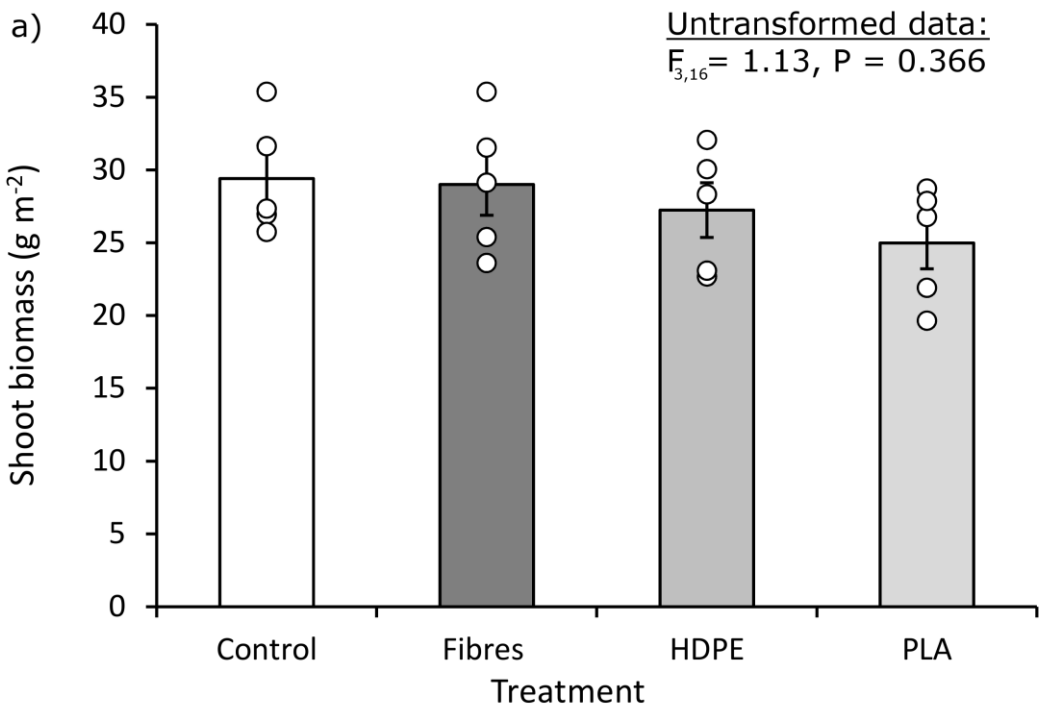
875

876 **Figure 1**. Initial development of *L. perenne* with a) germination rate (%) and b) shoot length

877 (mm) during 30 days of exposure to no added microplastics (○), Fibres (■), HDPE (◆) or PLA

878 (△). Data are means ± SEM, n = 5.

879


a)
Untransformed data:
$F_{3,16}$= 1.13, P = 0.366
Shoot biomass (g m^{-2})
0
5
10
15
20
25
30
35
40
Control
Fibres
HDPE
PLA
Treatment

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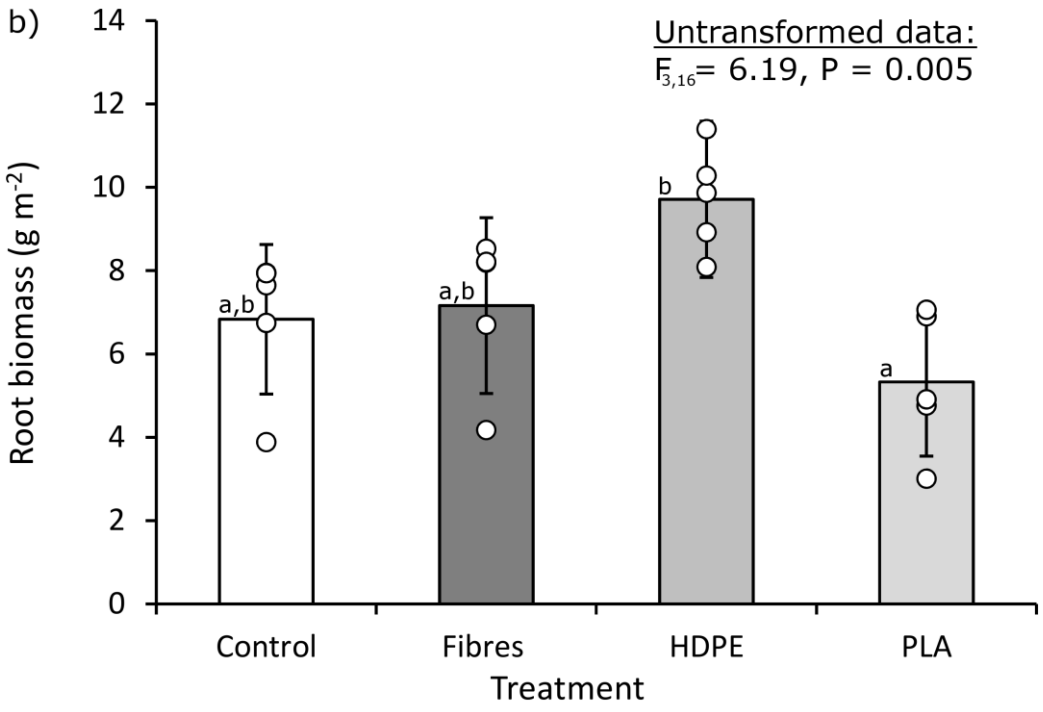
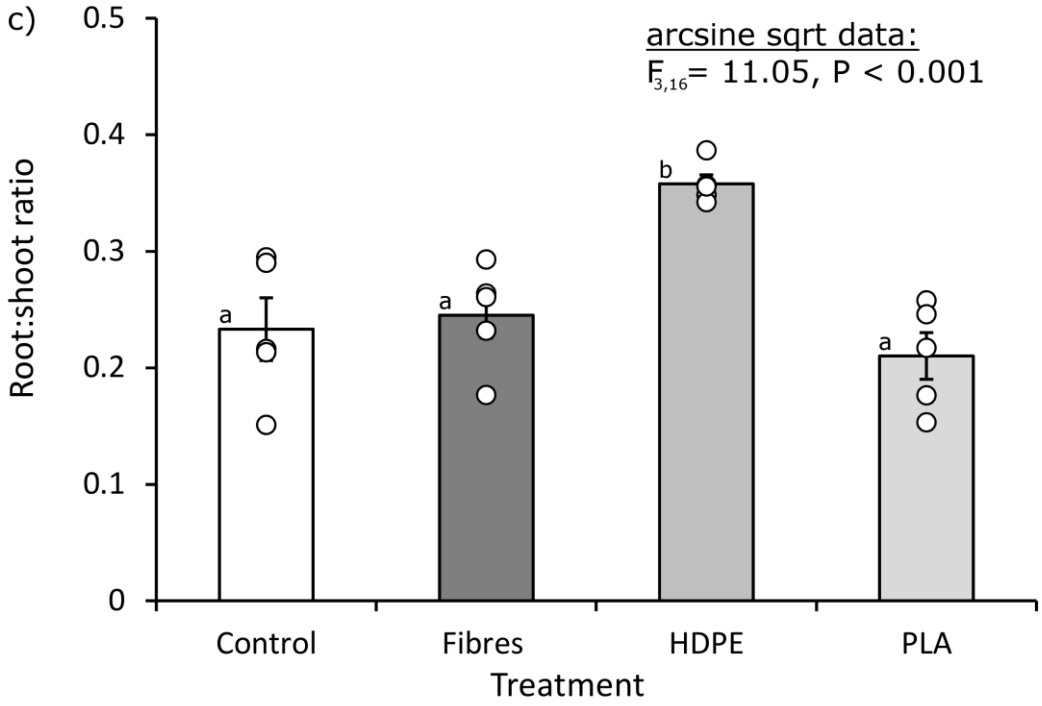

b)
Untransformed data:
$F_{3,16}$= 6.19, P = 0.005
Root biomass (g m^{-2})
0
2
4
6
8
10
12
14
a,b
a,b
b
a
Control
Fibres
HDPE
PLA
Treatment

882



883

884 **Figure 2**. Above and below ground dry biomass (g m^{-2}) of *L. perenne* with a) shoots, b) roots
885 and c) root/shoot ratio after 30 days of exposure to different types of microplastics. Data are
886 means (± SEM, n = 5) and the superimposed dots represent the raw data. Statistical results and
887 associated data transformation are included (ANOVA). Different letters indicate significant
888 differences at $\alpha < 0.05$.

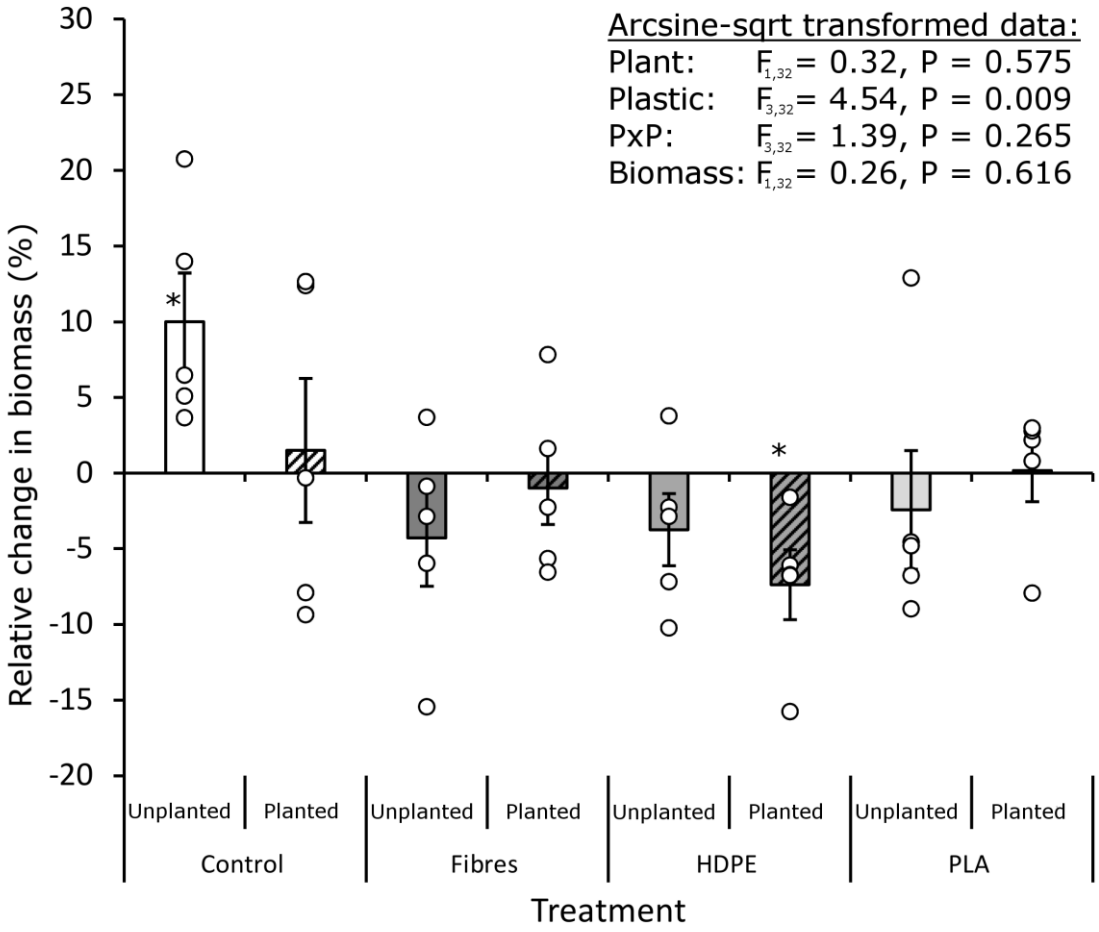
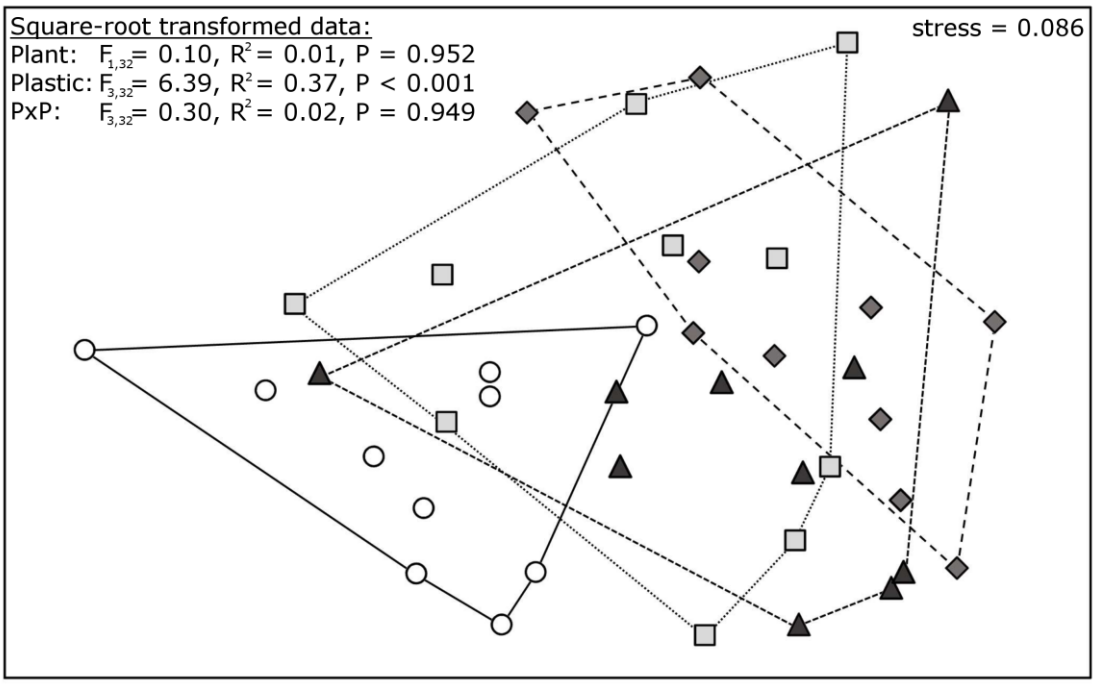


Figure 3. Relative change in biomass (%) of *A. rosea* after 30 days of exposure to different microplastics with *L. perenne* absent (Unplanted: open bars) or present (Planted: hashed bars). Data are means (± SEM, n = 5) and the superimposed circles represent the raw data. Included are results of an ANCOVA with initial biomass ("Biomass") as covariable; "PxP" is the interaction term between PLANT and PLASTIC. *indicates a significant difference from zero change at $\alpha = 0.05$ based on a t-test (H_0: initial – final = 0%).



898

899 **Figure 4**. Non-metric multidimensional scaling ordination of water stable soil aggregate
900 profiles based on Bray-Curtis dissimilarities between samples after 30 days of exposure to
901 different types of microplastics: ○ = control, □ = Fibres, ◆ = HDPE and ▲ = PLA.. Included
902 are the stress level of the ordination and results of permutational multivariate analysis of
903 variance with P-values based on 9999 permutations and R^2 coefficients of explanatory power
904 for each factor. Multivariate variance was homogeneous (*betadisper*: $F_{7,32}$ = 1.65, P = 0.156).

905

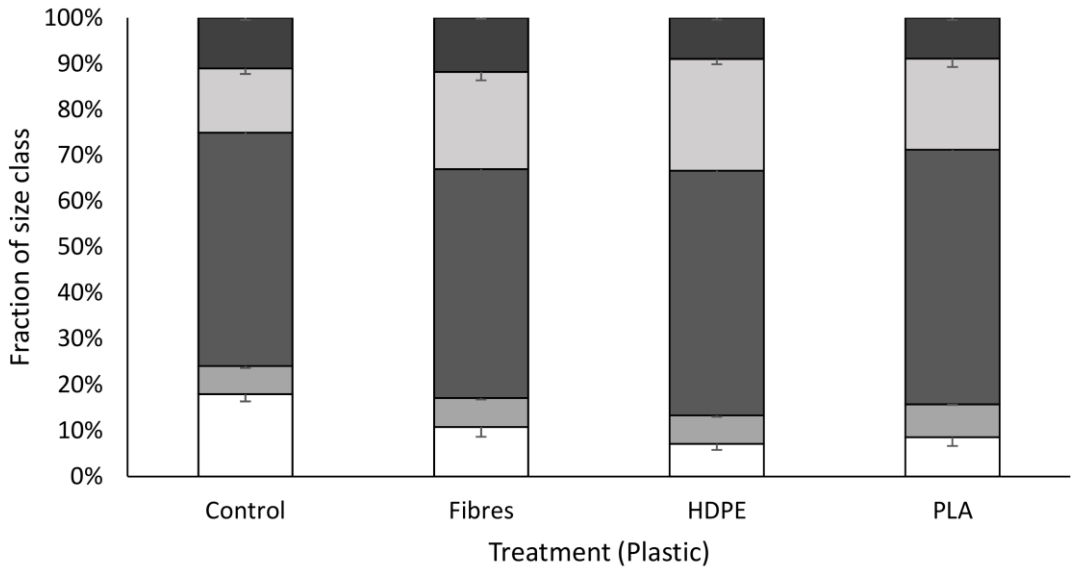


Figure 5. Distribution of water stable aggregate size classes as fractions (% of total) in soils after 30 days of exposure to different types of microplastics, with □ > 2000 µm, ■ = 2000-1000 µm, ■ = 1000-250 µm, □ = 250-125 µm and ■. < 63 µm. Data are means (± SEM, n =10 with observations pooled for factor "PLASTIC").