



## Field application of pure polyethylene microplastic has no significant short-term effect on soil biological quality and function

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1 **Field application of pure polyethylene microplastic has no significant short-term effect**  
2 **on soil biological quality and function**

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25

26 **ABSTRACT**

27 Plastics are now widespread in the natural environment. Due to their size, microplastics (MPs;  
28 defined as particles < 5 mm) in particular, have the potential to cause damage and harm to  
29 organisms and may lead to a potential loss of ecosystem services. Research has demonstrated  
30 the significant impact of MPs on aquatic systems; however, little is known about their effects  
31 on the terrestrial environment, particularly within agroecosystems, the cornerstone of global  
32 food production. Soil biology is highly responsive to environmental perturbation and change.  
33 Hereby, we investigated the effect of pure low-density polyethylene (LDPE) MP loading (0,  
34 100, 1000, or 10000 kg ha<sup>-1</sup>) on soil and plant biological health in a field environment over a  
35 cropping season. Our results showed that MP loading had no significant effect ( $p > 0.05$ ) on  
36 the soil bacterial community diversity (as measured by amplicon sequencing of bacterial 16S  
37 rRNA gene), the size and structure of the PLFA-derived soil microbial community, or the  
38 abundance and biomass of earthworms. In addition, metabolomic profiling revealed no dose-  
39 dependent effect of MP loading on soil biogenic amine concentrations. The growth and yield  
40 of wheat plants (*Triticum aestivum* L., cv. Mulika) were also unaffected by MP dose, even at  
41 extremely high ( $\geq 1000$  kg ha<sup>-1</sup>) loading levels. Nitrogen (N) cycling gene abundance before  
42 and after N fertiliser application on the MP loaded experimental plots showed relatively little  
43 change, although further experimentation is suggested, with similar trends evident for soil  
44 nitrous oxide (N<sub>2</sub>O) flux. Overall, we illustrate that MPs themselves may not pose a significant  
45 problem in the short term (days to months), due to their recalcitrant nature. We also emphasise  
46 that most MPs in the environment are not pure or uncontaminated, containing additives (e.g.  
47 plasticisers, pigments and stabilisers) that are generally not chemically bound to the plastic  
48 polymer and may be prone to leaching into the soil matrix. Understanding the effect of additives  
49 on soil biology as well as the longer-term (years to decades) impact of MPs on soil biological  
50 and ecological health in the field environment is recommended.

51 **Keywords:** Plastic pollution, Metabolomics, Toxicology, Soil quality, Environmental impact

52

## 53 **1. Introduction**

54 The use of plastics is globally ubiquitous due to their low cost, malleability, and durability;  
55 however, inappropriate disposal has led to their progressive accumulation in the environment  
56 (Geyer et al., 2017). To date, much of plastic and microplastic (MPs; particles < 5 mm in size)  
57 pollution research has focused on freshwater and marine systems, where the negative effects  
58 of plastics on organism health and loss of ecosystem function is now becoming well  
59 documented (Avio et al., 2017; Sharma and Chatterjee, 2017). However, plastics are also  
60 rapidly being identified as a threat to terrestrial ecosystems, yet their potential effects remain  
61 largely unexplored (de Souza Machado et al., 2019).

62 In agroecosystems, plastic entry may occur through a variety of pathways, with the  
63 most common including (i) the use, and incorporation of plastic mulch films to improve plant  
64 growth and reduce moisture loss (Huang et al., 2020; Sun et al., 2020; R. Qi et al., 2020); (ii)  
65 the addition of municipally-derived organic fertilisers, digestates or compost (Watteau et al.,  
66 2018); (iii) the application of biosolids (van den Berg et al., 2020); (iv) the accumulation of  
67 slow-release fertiliser coatings (Katsumi et al., 2021) and (v) atmospheric deposition (Allen et  
68 al., 2019) (vi) irrigation from polluted sources (Bläsing and Amelung, 2018). The drive for  
69 food security and sustainable intensification has led to an inevitable increase in plastic loading  
70 to soils globally. For example, the annual input of plastics into agricultural soils is estimated to  
71 be between 63 - 430 and 44 - 300 × 10<sup>3</sup> t in Europe and North America, respectively, and  
72 potentially exceeding 1.3 × 10<sup>6</sup> t annually for China (Jian et al., 2020; Nizzetto et al., 2016a).  
73 Globally, this greatly surpasses the extrapolated annual mass discharge of MPs to ocean surface  
74 waters, predicted to be 9.3 × 10<sup>7</sup> – 2.36 × 10<sup>8</sup> tonnes (Nizzetto et al., 2016b, 2016a, Sebille et

75 al., 2015). Primary MPs (MPs manufactured for a specific application, e.g. clothing  
76 microfibres; de Falco et al., 2019) may be applied through waste streams (i.e. biosolids  
77 application), due to their difficulty of removal (Cole et al., 2011). In contrast, secondary MPs  
78 are formed through degradation and disintegration of larger plastic pieces (Cole et al., 2011;  
79 Rocha-Santos and Duarte, 2015), such as agricultural mulch films (Piehl et al., 2018). Both  
80 primary and secondary MPs are likely to influence the ecology, health and function of soils,  
81 potentially having similar negative effects to those extensively reported in marine ecosystems,  
82 e.g. organismal ingestion leading to oxidative stress and assimilation of endocrine-disrupting  
83 chemicals, and subsequent reduced growth and reproduction, as well as bioaccumulation up  
84 the food chain (Galloway and Lewis, 2016; Kim et al., 2017). Although, bioaccumulation is  
85 likely to be less of an issue comparatively, due to the relatively smaller size of soil-dwelling  
86 fauna.

87         Soil is an extremely valuable and non-renewable resource and provides of range of  
88 ecosystem services, not least the provisioning of food resources (Comerford et al., 2013;  
89 Kopittke et al., 2019). Maintaining soil health and quality is therefore key for agricultural and  
90 anthropogenic sustainability (Hou et al., 2020). Soil quality is often broadly defined as the  
91 capacity of a soil to function (Karlen et al., 1997). Traditional measurements of soil quality are  
92 based on physical or chemical soil properties, with little exploration of soil biology (Bünemann  
93 et al., 2018). However, the fertility and productivity of soil are not simply a function of soil  
94 physical and chemical characteristics, and recently a more holistic view has been proposed  
95 (Rinot et al., 2019). Soil biology is a crucial mediator and driver of many processes linked to  
96 nutrient cycling, plant health, and soil productivity (Lal, 2016). It is highly responsive to  
97 changes in management and environmental conditions and is often associated with functional  
98 change (Lehman et al., 2015). Research has shown that MPs can have significant negative  
99 effects on soil microbial community composition (Guo et al., 2020; Zang et al., 2020; Zhang

100 et al., 2019), enzymatic activities and nutrient cycling (Fei et al., 2020; Huang et al., 2019; Yi  
101 et al., 2021), mesofaunal health (Huerta Lwanga et al., 2016; Lahive et al., 2019; Lin et al.,  
102 2020), plant health (de Souza Machado et al., 2019; Zang et al., 2020), and greenhouse gas  
103 (GHG) emissions (Ren et al., 2020; Sun et al., 2020), all of which will impact the soils ability  
104 to function effectively. However, most studies to date have been laboratory or mesocosm  
105 based, over relatively short sampling periods (weeks) and in many cases at unrealistic MP  
106 doses, which may not accurately reflect processes occurring at the field scale (Fidel et al.,  
107 2019).

108         This field-based study aimed to assess the effect of different quantities (0, 100, 1000,  
109 or 10000 kg ha<sup>-1</sup>) of pure MP loading on the health and function of key soil biological quality  
110 indicators over a cropping season, using a range of commonly used biological indicators, as  
111 well as the novel use of biogenic amine analysis as indicators of metabolism and N cycling in  
112 soil. Loading rates were chosen to represent ‘existing’, ‘normal’, ‘future’, and ‘extreme’ (or  
113 ‘hotspot’) MP loading to soil (Gao et al., 2019; Huang et al., 2020; R. Qi et al., 2020). Pure MP  
114 was chosen as much of the the current literature does not disentangle the effect of pure plastic  
115 from the plastic additives for example, UV stablisers (Stenmarck et al., 2017) and pigments  
116 (Gičević et al., 2020). This study aims to serve as a "negative" control, supporting future  
117 research on these chemicals and helping to exclude confounding effects that could derive from  
118 the particulate nature of the plastic particles. We hypothesised that i) MP loading will have  
119 negative effects on all measured aspects of soil biological quality, ii) higher MP loading rates  
120 will increase the detrimental impact on soil biology, and iii) crop biomass and yields will be  
121 negatively affected by MP loading.

122

## 123 **2. Materials and methods**

124 2.1. Experimental setup

125 The experiment took place at the Henfaes Agricultural Research Station, Abergwyngregyn,  
126 North Wales (53°14'N, 4°01'W). The soil is classified as a sandy clay loam textured Eutric  
127 Cambisol, overlying a glacial till, with a temperate-oceanic climate. The mean annual rainfall  
128 is 1060 mm and the mean annual temperature is 10°C. The site has no previous history of  
129 plastic pollution or application over the last 50 years (Zang et al., 2020). On 18<sup>th</sup> April 2019, a  
130 randomised plot design was established to create 4 independent replicates ( $n = 4$ ) of each  
131 treatment. Each plot (1.4 × 2.85 m) was then treated with LDPE microplastic powder  
132 (RXP1003 natural; Resinex Ltd., High Wycombe, UK), at a rate of 0, 100, 1000, or 10000 kg  
133 ha<sup>-1</sup> by thorough manual mixing with the top 10 cm of soil. This equated to loading rates of  
134 0%, ~0.1%, ~1%, and ~10% (w/w) (soil bulk density = 1040 kg m<sup>-3</sup>;  $n = 4$ ). The microplastic  
135 powder was confirmed to have a very low level of contamination through total carbon (C) and  
136 nitrogen (N) analysis using a TruSpec<sup>®</sup> Analyzer (Leco Corp., Michigan, USA) (Total C,  
137 82.88% ± 0.03%; Total N, 0.03 ± 0.01%;  $n = 5$ ). LDPE was chosen due to its extensive use in  
138 agricultural films (Espí et al., 2006; Rong et al., 2021). Plots were subsequently sown with  
139 spring wheat (*Triticum aestivum* L., cv. Mulika) at a rate of 400 plants m<sup>-2</sup>. In line with the  
140 fertiliser recommendations for wheat, and taking account of the soil's Soil Nitrogen Supply  
141 (SNS) (AHDB, 2018), 120 kg N ha<sup>-1</sup> yr<sup>-1</sup> was applied to the field as NH<sub>4</sub>NO<sub>3</sub> over two  
142 applications, 40 kg N ha<sup>-1</sup> on 3<sup>rd</sup> June and 80 kg N ha<sup>-1</sup> on 3<sup>rd</sup> July (reflecting the late sowing of  
143 the crop). For scanning electron microscopy (SEM), LDPE powder was mounted on adhesive  
144 tape, coated with gold, and imaged at 10 kV (Tescan Vega3 SEM). These SEM images  
145 illustrate the heterogeneous nature of the MP mixture, both in terms of particle size and surface  
146 texture (Fig. 1).

147

## 148 2.2. Soil sampling and analysis

149 The soil was sampled one, two, and six months following MP addition. On each sampling  
150 occasion, multiple fresh soil cores per plot ( $n = 12$ ;  $\phi = 1$  cm; depth = 0 – 10 cm) were randomly  
151 sampled and homogenised by hand to obtain a representative plot soil sample. Soil pH and  
152 electrical conductivity (EC) were measured on 1:2.5 (w/v) soil-to-distilled water suspensions  
153 by submerging standard electrodes. Within 24 h of soil collection, 1:5 (w/v) soil-to-0.5 M  
154  $K_2SO_4$  extracts were performed. The colorimetric methods of Miranda et al. (2001) and  
155 Mulvaney (1996) were used to determine the nitrate ( $NO_3-N$ ) and ammonium ( $NH_4-N$ )  
156 concentrations in the  $K_2SO_4$  extracts, respectively. Bulk density cores (0 – 5 cm,  $100\text{ cm}^3$ ) were  
157 also collected oven-dried ( $105^\circ\text{C}$ , 24 h) before being weighed. Soil characteristics are  
158 summarised in Table 1. Climatic data from an adjacent weather station for the sampling period  
159 and a timeline of sampling are summarised in Fig. S1.

160

## 161 2.3. Phospholipid fatty acid (PLFA) profiling of the microbial community

162 Soil sampling for PLFA analysis was performed after 2 and 6 months of MP addition. Fresh  
163 homogenised soil samples, collected as described in section 2.2, were subsampled for PLFA  
164 analysis. The subsampled soil was subsequently stored at  $-80^\circ\text{C}$  to prevent lipid turnover.  
165 Lyophilisation was performed using a Modulyo Freeze Dryer (Thermo Electron Corporation,  
166 Waltham, MA, USA) attached to a rotary vane pump (Edwards Ltd., Crawley, UK). Samples  
167 were shipped on dry ice ( $-78.5^\circ\text{C}$ ) to Microbial ID Inc. (Newark, DE, USA) for analysis. The  
168 method of Buyer and Sasser (2012) was used for extraction, fractionation and  
169 transesterification of samples. Analysis was performed on a 6890 gas chromatograph (GC)  
170 (Agilent Technologies, Wilmington, DE, USA) equipped with an autosampler, split–splitless  
171 inlet, and flame ionization detector. The system was controlled with MIS Sherlock® (MIDI,



172 Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis  
173 parameters and standards are as described in Buyer and Sasser (2012).

174

#### 175 *2.4. Biogenic amine extraction and analysis*

176 Biogenic amine extraction was performed 6 months after microplastic addition. Biogenic  
177 amines are a subset of the metabolome, key in the processing and cycling of N, which has  
178 previously been shown to be sensitive to changes in biological quality (Brown et al., 2021;  
179 Withers et al., 2020). On this sampling occasion, additional multiple soil cores ( $n = 5$ ;  $\phi = 1$   
180 cm; depth = 0 – 10 cm) were taken across each plot and homogenised by hand to obtain a  
181 representative soil sample. After collection, samples were transferred (< 1 h) to a -80°C freezer  
182 to quench metabolic amine turnover. Samples were stored and lyophilised as described in  
183 section 2.3. Post-lyophilisation, roots and other debris (e.g. plant litter) were removed and the  
184 samples were then ground using a stainless-steel ball mill (MM200, Retsch GmbH, Haan,  
185 Germany), to aid in the recovery of biogenic amines. The mill was sterilised between samples  
186 by rinsing with deionised water followed by a 70% ethanol solution. Ground soil was  
187 transferred to sterile polypropylene 1.5 ml microfuge tubes and shipped, on dry ice (-78.5°C),  
188 to the West Coast Metabolomics Center (UC Davis Genome Center, Davis, CA, USA) for  
189 untargeted biogenic amine analysis using hydrophilic interaction chromatography electrospray  
190 quadrupole time of flight tandem mass spectrometry (HILIC-ESI QTOF MS/MS).

191 Briefly, extraction consisted of vortexing (~15 s) a 0.4:1 (w/v) soil-to-3:3:2 (v/v/v)  
192 MeCN/IPA/H<sub>2</sub>O solution, before shaking for 5 min at 4°C, centrifuging (2 min, 14000 g) and  
193 recovering an aliquot of the supernatant for analysis. LC/QTOFMS analysis of extracted  
194 aliquots was performed on an Agilent 1290 Infinity LC system (G4220A binary pump,  
195 G4226A autosampler, and G1316C Column Thermostat) coupled to a SCIEX Triple TOF mass

196 spectrometer, total runtime was 16.8 min. Polar compounds are separated on an Acquity UPLC  
197 BEH Amide Column, 13 nm (pore size), 1.7  $\mu\text{m}$  (particle size), 2.1 mm  $\times$  150 mm maintained  
198 at 45°C at a flowrate of 0.4 ml min<sup>-1</sup>. Solvent pre-heating (Agilent G1316) was used. The  
199 mobile phases consist of: (A) Water, 10 mM ammonium formate, 0.125% formic acid and (b)  
200 acetonitrile: water (95/5, v/v), 10 mM ammonium formate, 0.125% formic acid. The gradient  
201 was: 0 min 100% (B), 0-2 min 100% (B), 2-7 min 70% (B), 7.7-9 min 40% (B), 9.5-10.25 min  
202 30% (B), 10.25-12.75 min 100% (B), 16.75 min 100% (B).

203 A sample volume of 1  $\mu\text{l}$  for positive mode and 3  $\mu\text{l}$  for negative mode was used for  
204 the injection. Sample temperature was maintained at 4°C in the autosampler. The mass  
205 spectrometer was operated with gas temperatures set to 300°C and pressure to 345 kPa (curtain  
206 gas (CUR) – 2.4 bar; IonSpray Voltage Floating (ISFV) – 4500 V; declustering potential (DP)  
207 – 10 V; capillary electrophoresis (CE) – 100V). Electrospray ionization (ESI) performed full  
208 scans in the mass range  $m/z$  50–1200. The number of cycles in MS1 was 1667 with a cycle  
209 time of 500 ms and an accumulation time of 475 ms. Data were collected in both positive and  
210 negative ion mode and analysed using MS DIAL, open software for metabolome analysis, as  
211 described in Tsugawa et al. (2015). Final curated results were reported as peak heights, internal  
212 standards were included, however, these were for quality control and peak correction purposes.  
213 Data presented are therefore qualitative and compounds are tentatively identified, as is routine  
214 for untargeted analysis (Gertsman and Barshop, 2018). A full compound list is presented in  
215 supplementary information with standardised reference nomenclature being generated using  
216 RefMet (Fahy and Subramaniam, 2020).

217

218 *2.5 Soil N<sub>2</sub>O flux*

219 A mobile, automated GHG monitoring system, utilising a GC-Electron Capture Detector  
220 (8610C, SRI Instruments, CA, USA), as previously described in Marsden et al., (2018), was  
221 used to monitor nitrous oxide (N<sub>2</sub>O) fluxes from three of the four replicates for each treatment.  
222 Stainless steel chamber bases (50 × 50 cm; 0.25 m<sup>2</sup>) were installed into plots two weeks before  
223 MP application, to which chambers (0.0625 m<sup>3</sup>) were tightly secured. A foam strip on the base  
224 of each chamber ensured a tight seal. Briefly, the automated sampling system provided eight  
225 greenhouse gas flux measurements per 24 h period, per chamber during uninterrupted  
226 measurement. Emissions were monitored for 6 months from installation. However, this  
227 manuscript focuses on the 2-week periods following initial MP loading, to test whether the  
228 background emissions from the soil were perturbed by MP incorporation and the two  
229 subsequent N fertiliser application events, respectively, as these periods were likely to produce  
230 the greatest fluxes (Bell et al., 2015; Cardenas et al., 2019).

231

## 232 *2.6. High-throughput sequencing and quantitative PCR analysis*

### 233 *2.6.1. 16S rRNA gene sequencing*

234 Soil samples for 16S rRNA gene sequencing were collected after 6 months of MP  
235 incorporation. Five soil cores ( $n = 5$ ;  $\phi = 1$  cm; depth = 0 – 10 cm) were taken from each plot  
236 and homogenised by hand to obtain a representative sample. After collection, samples were  
237 passed through a 2 mm sieve and subsequently transferred (< 1 h) to a -80°C freezer for pre-  
238 extraction storage. Genomic DNA was extracted by mechanical lysis from 0.25 g soil per  
239 sample using a DNA Soil Fecal/Soil Microbiome Kit (ZymoResearch, CA, USA). Quality and  
240 concentration of extracted DNA were assessed by agarose gel electrophoresis (AGE) using a  
241 Qubit 4.0 Fluorometer dsDNA BR Assay Kit (Life Technologies, United States). Libraries of  
242 16S rRNA gene amplicons were created using primers for rRNA marker genes (identical to

243 those described in Distaso et al., (2020)), specifically for the V4 region of the 16S rDNA  
244 targeting bacteria and archaea (515F/806R), were prepared as previously described in Fadrosh  
245 et al. (2014). PCR was performed using a ViiA7 qPCR system (Applied Biosystems, MA  
246 USA). Thermocycling conditions were: initial denaturation at 95°C for 3 min, followed by 25  
247 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final elongation at 72°C for 5  
248 min. Purified amplicons were then quantified using the aforementioned Qubit 4.0 Fluorometer,  
249 pooled in equimolar amounts and the final pool was run on the Illumina MiSeq platform  
250 (Illumina Inc., CA).

251

#### 252 2.6.2. *Bioinformatic analysis*

253 The previously described protocols of Fadrosh et al. (2014) and Distaso et al. (2020) were used  
254 to process raw sequencing reads. In total, 214,318 raw sequencing reads were produced.  
255 Briefly, data pre-processing extracted the barcodes from sequences, and subsequently cleaned  
256 primer sequences using tagcleaner. Barcodes and sequences were then re-matched using in-  
257 house python scripts and the resulting filtered reads analysed using QIIME v1.9.1. Erroneous  
258 sequences and Chimeras were removed using quality filtering during demultiplexing, and  
259 ChimeraSlayer, respectively, both were implemented in QIIME. The libraries were  
260 demultiplexed based on the different barcodes. Sequences were then classified into operational  
261 taxonomic units (OTUs) combining *de novo* and reference-based methods (open-reference  
262 OTU generation algorithm) using the SILVA reference database version 132 (Yilmaz et al.,  
263 2014). Here, OTUs were determined using an open-reference OTU picking process, where  
264 reads are clustered against a reference sequence collection and any reads which do not hit the  
265 reference sequence collection are subsequently clustered *de novo*, only OTUs with a minimum  
266 coverage of 20 were included in the analysis. Chloroplast and Mitochondrial reads were

267 removed from the OTU count. Sequencing read files analysed in this study can be accessed  
268 from the National Center for Biotechnology Information (project PRJNA762001).

269

### 270 2.6.3. Quantitative PCR of N cycling functional genes

271 Samples for quantitative PCR (qPCR) of N cycling functional genes were collected on the 3<sup>rd</sup>  
272 July (pre-N fertiliser application) and on the 15<sup>th</sup> July (12 days post-N fertiliser application).  
273 On each occasion five soil cores ( $n = 5$ ;  $\phi = 1$  cm; depth = 0 – 10 cm) were taken per plot and  
274 homogenised by hand to obtain a representative sample. After collection, samples were passed  
275 through a 2 mm sieve and subsequently transferred (<1 h) to a -80°C freezer for pre-extraction  
276 storage. Samples were extracted for NO<sub>3</sub>-N and NH<sub>4</sub>-N, as described in section 2.2. DNA was  
277 extracted by mechanical lysis from 0.25 g soil per sample using a DNEASY Powersoil kit  
278 (Qiagen, Hilden, Germany). The quality and concentration of extracted DNA were assessed by  
279 AGE.

280 To obtain the standard curves for qPCR assays, functional genes (urease (*ureC*),  
281 archaeal ammonia oxidation (AOA-*amoA*), bacterial ammonia oxidation (AOB-*amoA*),  
282 complete nitrification (*comammox*), nitrite reductase (*nirK*; *nirS*), nitrous oxide reductase  
283 (*nosZ*) and nitrogenase iron protein (*nifH*)) were amplified using the primers listed in Table S1.  
284 qPCR was performed using a QuantStudio 7 System (Applied Biosystems, Waltham, United  
285 States). The thermocycling conditions for each gene are summarised in Table S1. For each  
286 gene, a high amplification efficiency of 92 – 105% was obtained, the R<sup>2</sup> values were > 0.99  
287 and no signal was observed in the negative controls. The copy numbers for each sample of soil  
288 DNA were calculated based on comparison with the standard curve. qPCR was performed  
289 using a QuantStudio 7 System (Applied Biosystems, Waltham, United States). Results were  
290 subsequently normalised by the extracted DNA concentration for each sample to account for

291 differences in extraction efficiencies within samples and raw results are included in  
292 supplementary information.

293

### 294 *2.7. Earthworm abundance and biomass*

295 Earthworm abundance and weight were assessed after 6 months. Briefly, a 0.018 m<sup>3</sup> (0.3 × 0.3  
296 × 0.2 m) pit was dug in a randomly selected location in each experimental plot. Soil from the  
297 pit was placed into a tray and thoroughly manually sorted, and earthworms collected. All  
298 earthworms were counted (abundance) and weighed (biomass). Abundance is expressed as  
299 individuals m<sup>-2</sup> and biomass as fresh weight biomass m<sup>-2</sup>.

300

### 301 *2.8. Wheat harvest data*

302 Spring wheat was harvested at full maturity, 5 months after sowing. The harvest protocol  
303 consisted of hand cutting, with shears, a 1 × 2.85 m strip, through the centre of each  
304 experimental plot, to remove edge effects. Samples were then dried (85°C, 48 h). For each  
305 harvested sample, ears were removed from stems and each were weighed. Ear and stem weight  
306 were subsequently added to calculate a total wheat biomass dry weight per plot or biomass  
307 yield. Biomass yield was used as it is highly related to grain yield and gives an overall indicator  
308 of plant health (Damisch and Wiberg, 1991). After drying, harvested wheat seeds were  
309 separated, weighed and ground, and subsequently analysed for total C and N using a TruSpec®  
310 Analyser (Leco Corp., St. Joseph, MI, USA) and a C:N ratio calculated.

311

### 312 *2.9. Statistical analysis*

313 All statistical analysis was run using R v 4.0.3 (R Core Team, 2021) unless otherwise stated.  
314 With all graphical analysis being constructed in ‘*ggplot2*’ (Wickham, 2016) unless otherwise  
315 stated. A significance level of  $p < 0.05$  was used for all analyses.

316 Normality and homogeneity of variance of the chemical and physical soil properties of  
317 the treated Eutric Cambisol were assessed using Shapiro-Wilk’s test and Levene’s test,  
318 respectively. For data that did not conform to parametric assumptions even after using  $\log_{10}$   
319 transformation ( $\text{NO}_3\text{-N}$ ,  $\text{NH}_4\text{-N}$ , EC and PLFA Fungal:Bacterial ratio) a Kruskal-Wallis test  
320 (stats package; R Core Team, 2021) was used to assess the similarities between MP treatments  
321 and sampling dates, otherwise a one-way ANOVA (Analysis of variance) was used (for pH,  
322 bulk density and total PLFA biomass). The results for this are summarised in Table 1. A one-  
323 way ANOVA was also used to assess treatment variations in wheat biomass data (total  
324 aboveground biomass, stem and leaf biomass, ear biomass and harvested wheat seed C:N ratio)  
325 and earthworm data (abundance and biomass).

326 The ‘*vegan*’ (Oksanen et al., 2020) and ‘*ggplot2*’ (Wickham, 2016) packages were used  
327 to construct NMDS (Non-metric multidimensional scaling) analysis of the PLFA community  
328 based on Bray–Curtis dissimilarities. All PLFAs detected were used in the analysis, to represent  
329 the whole microbial community. This was followed by computation of an ANOSIM (Analysis  
330 of similarities) to identify differences in dispersion between centroids of groups as determined  
331 by MP loading rate, or time of sampling. Fungal-bacterial ratios and Gram positive to Gram  
332 negative ratios were calculated by summing the FA biomarkers for the respective groups  
333 (summarised in Table S2). Total biomass was calculated by summing the concentration of  
334 PLFAs recovered.

335 Fluxes of  $\text{N}_2\text{O}$  for each chamber were calculated using the methods described in Scheer  
336 et al., (2014). The linear slope of  $\text{N}_2\text{O}$  concentrations over time included either three or four

337 data points. N<sub>2</sub>O fluxes for each two-week period (post-MP and fertiliser application,  
338 respectively) were graphically analysed. Trapezoidal integration was used to calculate  
339 cumulative N<sub>2</sub>O emissions for each treatment, these were tested for significance using for  
340 Kruskal-Wallis tests, after failing parametric assumptions.

341 Bacterial observed OTU richness was tested for significant differences using ANOVA.  
342 The evenness of the 16S community was also calculated using Pielou's evenness (Jost, 2010)  
343 and tested for significant differences using ANOVA. NDMS, followed by an ANOSIM  
344 (Analysis of similarities) was used to test statistically whether there was a significant difference  
345 between groups of sampling units between treatments ( $\beta$ -diversity).

346 N cycling gene abundance, before and after a N fertilisation event was analysed using  
347 mixed effect models with the '*lme4*' package (Bates et al., 2015). We considered MP loading  
348 rate and sampling time and their interaction as fixed effects and individual plots as temporal  
349 random effects. For each variable, residuals from each model were tested for normality,  
350 autocorrelation and heteroscedasticity using graphical tools. For all genes, a log<sub>10</sub> conversion  
351 was found to improve the fitness of all models. An ANOVA was then run on each model to  
352 test treatment effects, significant results were further explored using a Tukey adjusted post-hoc  
353 test using the '*emmeans*' package (Lenth, 2021). Pre- and post- fertilisation soil NO<sub>3</sub>-N and  
354 NH<sub>4</sub>-N concentrations were analysed by ANOVA.

355 MetaboAnalyst v5.0 (Chong et al., 2018; Pang et al., 2020) was used for the analysis  
356 of biogenic amine data. First, normalisation was performed using generalised logarithm  
357 transformation (glog) and Pareto scaling. Normalised data was subsequently used for heatmap  
358 creation (using Euclidean distance and Ward clustering algorithms). One-way ANOVA was  
359 also performed to identify significant differences in compound concentrations between  
360 treatments.



361 Also, we acknowledge that, being a field trial, a high level of representative replication  
362 (i.e., replication with large enough plot sizes) is difficult to obtain, which could potentially  
363 impact the statistical power of the study. However, on calculating the statistical power of the  
364 parametric statistics used here all were  $\geq 0.99$ , with the exception of bacterial OTU evenness  
365 (power = 0.05), thus this result should be interpreted with caution.

366

### 367 **3. Results**

#### 368 *3.1. 16S bacterial community*

369 In total, 7179 bacterial operational taxonomic units (OTUs), were identified across all 16S  
370 rRNA gene reads. There was little variation in the proportional abundance of OTUs between  
371 the different MP treatments with Proteobacteria (Gram-negative) and Actinobacteria (Gram-  
372 positive) being the most abundant phyla (Fig. 2A). There were no significant differences  
373 between bacterial OTU richness ( $F_{(3,12)} = 0.32$ ,  $p > 0.8$ ) (Fig. 2B) or evenness ( $F_{(3,12)} = 1.74$ ,  
374  $p > 0.2$ ) (Fig. 2C) across the different treatments, as tested by ANOVA. Equally, the NMDS  
375 ordination shows no clear separation or divergence in soil bacterial communities between the  
376 MP treatments and the unamended control (Fig. 2D). Lastly, we found no significant  
377 differences in bacterial  $\beta$ -diversity between the treatments, as confirmed by ANOSIM analysis  
378 ( $p > 0.8$ ).

379

#### 380 *3.2. PLFA-derived community*

381 The fungal-bacterial ratio of PLFAs remained similar across all treatments, there was a  
382 significant difference between the 2 months post-application 10000 kg ha<sup>-1</sup> and the 6 months  
383 post-application 0 kg ha<sup>-1</sup> MP loading rates, with the latter having a higher prevalence of

384 bacteria (Table 1). Total PLFA biomass was also similar across all treatments, with a  
385 significant difference between the 2 months post-application 1000 kg ha<sup>-1</sup> and the 6 months  
386 post-application 10000 kg ha<sup>-1</sup> MP loading rates, the latter having a higher PLFA biomass  
387 yield. NMDS analysis was used to show the clustering of all soil-derived PLFA compounds,  
388 under MP treatments, 2 and 6 months after initial MP application (Fig. 3). Overall, the different  
389 MP treatments separated by sampling date, with a clear separation between the 2 and 6-month  
390 points. The PLFA derived community was also more closely grouped at the 6-month sampling  
391 point. Results of the PERMANOVA confirmed that there was no significant difference in  
392 group dispersion between MP loading treatments ( $p > 0.2$ ). There was, however, a significant  
393 difference in group dispersion between sampling times ( $p < 0.001$ ), additionally there was no  
394 interaction effect between MP loading and sampling time ( $p > 0.9$ ).

395

### 396 3.3. N cycling genes

397 The presence and abundances of eight genes involved in the N cycle, specifically *ureC*, *amoA*  
398 (AOA, AOB, and comammox), *nirK*, *nirS*, *nosZ* and *nifH*, (functions are summarized in Fig.  
399 S2), were assayed by qPCR before and after an N fertilisation event. We acknowledge that the  
400 primers used to amplify the functional genes (e.g. *ureC*) do not target all of the community. In  
401 most cases, gene abundance was not greatly affected by either MP loading rate or sampling  
402 time (i.e. pre- and post-N fertilisation) (Fig. 4, Table S3). However, ANOVA showed that there  
403 were significant differences for *nirK* ( $F_{(3,12)} = 4.6, p < 0.05$ ) and *nosZ* ( $F_{(3,24)} = 3.2, p < 0.05$ )  
404 abundance, respectively, by MP loading. For both *nirK* and *nosZ* gene abundance, LMS post-  
405 hoc analysis showed a significant difference between 100 kg ha<sup>-1</sup> and 1000 kg ha<sup>-1</sup> MP loading  
406 ( $p < 0.05$ ). For AOB, ANOVA also showed a significant interaction effect between MP loading  
407 rate and sampling time ( $F_{(3,24)} = 3.5, p < 0.05$ ). LMS post-hoc analysis showed that there were

408 significant differences between 0 kg ha<sup>-1</sup> and 1000 kg ha<sup>-1</sup> MP loading, pre fertilisation ( $p <$   
409 0.05) and between 0 kg ha<sup>-1</sup> MP loading, pre fertilisation, and 10000 kg ha<sup>-1</sup> MP loading post  
410 fertilisation ( $p < 0.05$ ). Concentrations of soil NO<sub>3</sub>-N ( $F_{(1,12)} = 16.6$ ,  $p < 0.01$ ) and NH<sub>4</sub>-N  
411 ( $F_{(1,12)} = 22.0$ ,  $p < 0.01$ ) were significantly higher post-fertilisation (Fig. 4E, F).

412

### 413 *3.4 N<sub>2</sub>O flux*

414 Kruskal-Wallis analysis showed that there were no significant differences between cumulative  
415 N<sub>2</sub>O fluxes for the 2 week period following initial MP application ( $H_{(3)} = 0.74$ ,  $p = 0.9$ ), or the  
416 first ( $H_{(3)} = 4.6$ ,  $p = 0.2$ ) and second fertiliser ( $H_{(3)} = 3.6$ ,  $p = 0.3$ ) application events. Fluxes  
417 over each period are summarised in Fig. 5.

418

### 419 *3.5. Biogenic amines*

420 Untargeted biogenic amine analysis identified a total of 112 tentatively identified compounds.  
421 Of these known compounds detected, none showed statistically significant differences between  
422 treatments. There were no clear grouping or responses within the biogenic amine data (Fig. 6).  
423 The samples were characterised by a wide range of compounds (Fig. S3) but predominated by  
424 amino acids and peptides.

425

### 426 *3.6. Soil properties including inorganic N*

427 Overall, there were no significant differences in soil chemical properties (pH, EC, NO<sub>3</sub>-N and  
428 NH<sub>4</sub>-N) associated with the MP treatment as tested by ANOVA or Kruskal Wallis ( $p > 0.1$ ).  
429 Trends in the data show some natural variation in all soil properties measured throughout the  
430 season (summarised in Table 1).

431

### 432 3.7. Earthworms abundance and biomass

433 Earthworm abundance and biomass were not significantly affected by MP loading. All  
434 earthworms identified in the samples were endogenic. Overall, there were no significant  
435 differences between total earthworm biomass ( $F_{(3,12)} = 0.63$ ,  $p > 0.6$ ) or earthworm abundance  
436 ( $F_{(3,12)} = 0.85$ ,  $p > 0.4$ ; Table 1).

437

### 438 3.8. Plant biomass

439 Plant biomass was not significantly affected by MP loading, however, yields of this field trial  
440 were lower than the typical wheat yields for the year (DEFRA, 2019). There were no significant  
441 differences between total above ground plant biomass ( $F_{(3,12)} = 0.09$ ,  $p > 0.9$ ), stem and leaf  
442 biomass ( $F_{(3,12)} = 0.08$ ,  $p > 0.9$ ), ear biomass ( $F_{(3,12)} = 0.09$ ,  $p > 0.9$ ), or harvested seed C:N  
443 ratio ( $F_{(3,11)} = 0.03$ ,  $p > 0.9$ ; Fig. 7).

444

## 445 4. Discussion

### 446 4.1. 16S bacterial community response to MP addition

447 Soil microorganisms are vital to soil functioning and are considered the most sensitive indicator  
448 of soil quality, due to their ability to rapidly respond to changing environmental conditions  
449 (Bünemann et al., 2018; Lau and Lennon, 2012; Schimel, 2018). Therefore, despite a  
450 significant amount of functional redundancy (Jia and Whalen, 2020), substantial shifts in the  
451 microbial community are likely to represent a change in soil function (Lehman et al., 2015).  
452 This study showed that after 6 months of pure microplastic addition to previously  
453 uncontaminated soil, there was no significant change in the proportional abundance of the

454 bacterial community (Fig 2A), bacterial richness (Fig 2B), evenness, or bacterial community  
455 compositional divergence ( $\beta$ -diversity) (Fig 2D). To contextualise this, a previous study at the  
456 same site, showed significant changes in the microbial community under biochar application  
457 over similar time scales (Jones et al., 2012).

458         Currently, the effect of MPs loading on soil microorganisms is unclear. Our findings  
459 are contradictory to several studies with loading rates equating to  $\leq 5\%$  (lower than the highest  
460 loading rate here of 10%), which observed significant effects of microplastic (e.g. LDPE;  
461 Huang et al., 2019), polyvinyl chloride (PVC; Yan et al., 2020), and combined PE and PVC  
462 (Fei et al., 2020; Seeley et al., 2020)) addition on the soil bacterial community, particularly  
463 richness, evenness, and diversity. However, H. Chen et al. (2020) and Judy et al. (2019) showed  
464 various microplastic additions had no significant effects on the microbial community over short  
465 time periods (70 d and a loading rate of 2% and 9 months and a loading rate of up to 10%,  
466 respectively). Additionally, Ren et al. (2020) reported mixed but largely positive effects of MP  
467 (at a loading rate of 5%) on the microbial community (increase in richness and diversity) in a  
468 fertilised soil over a 30 d period, although the microorganisms may have reacted to the fertiliser  
469 addition and not the MPs. Based on these studies it is evident that the type of plastic  
470 incorporated into the soil will dictate the biological and ecological effects exhibited, therefore  
471 a further study of the effect of different types of plastic, and combinations of plastics are  
472 required to fully understand any impact on soil health.

473

#### 474 *4.2. Effect of MP loading on soil PLFAs*

475 PLFAs give a representation of the living soil microbial biomass and provide a snapshot of soil  
476 community structure and abundance at the time of sampling. NMDS clustering of PLFA  
477 microbial community shows a large amount of overlap between MP loading rates implying

478 community structure had not changed significantly (Fig. 3). This is contrary to previous  
479 microcosm studies that have shown significant shifts in PLFA derived microbial community  
480 even under relatively low levels (from 1%) of MP loading (Zang et al., 2020). MPs are a  
481 recalcitrant C pool and are only likely to become bioavailable as a viable C source over long  
482 time periods (years to decades) with the aid of natural abiotic degradation (hydrolysis, photo-  
483 oxidation or thermal oxidation) (Ángeles-López et al., 2017; Chamas et al., 2020) and to a  
484 lesser extent biological degradation (e.g. earthworms) (Huerta Lwanga et al., 2016). This  
485 biochemical inertness in the short to medium term is unlikely to cause major shifts in microbial  
486 communities. In terms of soil physical properties, MPs have been suggested as a new and  
487 distinct microbial habitat, for example for biofilm colonisation and formation (McCormick et  
488 al., 2014; Zhang et al., 2019), potentially leading to a change in the microbial community.  
489 However, this was not observed in this study as there was no significant community divergence  
490 in MP treatments from control plots in either 16S bacterial community or PLFA derived  
491 microbial community. The SEM (Fig. 1) illustrates that the MP powder used here is not porous  
492 or cavity-containing and therefore may not offer an attractive habitat for microbial colonisation  
493 (Or et al., 2006). Additionally, we would dispute this theory, as studies with biochar, a similarly  
494 recalcitrant C source, have shown that microbial colonisation is very sparse, concluding that  
495 even after several years biochar did not provide a substantial habitat for soil microbes (Quilliam  
496 et al., 2013). However, this requires confirmation with experimental evidence for MPs.

497         Separation between all MP loading treatments groups between the two sampling points  
498 (2 months and 6 months post MP addition) illustrated a distinct temporal shift in the structure  
499 of the microbial community. Seasonal as well as cropping associated shifts in the PLFA  
500 composition in soil have been observed (Duncan et al., 2016; Ferrari et al., 2015; Moore-  
501 Kucera and Dick, 2008). These shifts are generally associated with membrane adaptation to  
502 changing environmental stress levels (for example, temperature, moisture or nutrient

503 availability), resulting in physiological community change (Blagodatskaya and Kuzyakov,  
504 2013; Bossio and Scow, 1998). It is likely the observed change in the soil PLFA community  
505 between sampling points may be due to natural seasonal changes (for example the difference  
506 in soil moisture, illustrated in Fig. S1).

507

#### 508 4.3. Effect of N cycling gene abundance pre- and post- N fertilisation

509 Within agroecosystems, N availability is often considered the predominant limiting factor in  
510 plant growth (Vitousek and Howarth, 1991) and the second most limiting factor after C in  
511 microbial growth (Kuypers et al., 2018; Buchkowski et al, 2015). Microbial uptake,  
512 assimilation, and cycling of mineral and organic N is key to soil function, and as such N cycling  
513 processes (mineralisation, nitrification, and denitrification) have been used as sensitive and  
514 ecologically relevant indicators of soil quality and ecological stability (Bünemann et al., 2018;  
515 Iqbal et al., 2020). Changes in the abundance of the key regulatory functional genes involved  
516 in these processes are likely to indicate changes in soil function. However, there is little  
517 evidence of how MPs could affect soil N cycling (Iqbal et al., 2020). Overall, this study showed  
518 little change in the abundance of N cycling functional genes between pre- and post- inorganic  
519 N addition under all MP loading rates. Genes that did differ significantly in abundances  
520 between treatments were denitrification associated (*nirK* and *nosZ*) and nitrification associated  
521 (AOB *amoA*). For both denitrification associated genes, lower abundances were displayed  
522 within the 1000 kg ha<sup>-1</sup> treatment compared to the 100 kg ha<sup>-1</sup> treatment (Fig. 4C), with no  
523 effects on abundances at either higher or lower MP loading rates. AOB *amoA* gene abundance  
524 was significantly lower than control levels in the 100 kg ha<sup>-1</sup> treatment pre-fertilisation and  
525 10000 kg ha<sup>-1</sup> treatment post-fertilisation. The general trend in N cycling gene abundances  
526 showed variability pre-fertilisation. Post-fertilisation this variability was reduced and gene

527 abundances were more even across all MP loading treatments, while soil inorganic N was  
528 significantly increased post-fertilisation (Fig. 4).

529 N fertilisation has been shown to have a mixed effect on N cycling genes (Tosi et al.,  
530 2020). Effects are highly dependent on the nature of the N source applied (inorganic or  
531 organic), with inorganic sources of N having a much weaker effect than organic sources of N,  
532 as well as the fertilizer duration, crop rotation, and pH (Ouyang et al., 2018). The results of this  
533 study show that there were no large changes in soil N cycling functional genes in the presence  
534 of MP loading. Although there may have been several further factors influencing N gene  
535 abundance, for example when fertiliser was applied the soil was very dry (Fig. S1), preventing  
536 soil biology from accessing the additional N. Equally, as alluded to above, C is the primary  
537 limiting factor for soil microbiology, if the community was already C limited then it is unlikely  
538 that there would be significant growth or change stimulated by N addition. Studies have shown  
539 that MPs have the potential to affect N cycling processes, for example repression of key N  
540 cycling enzymes (e.g. leucine-aminopeptidase and N-acetyl- $\beta$ -glucosaminidase (Awet et al.,  
541 2018; Bandopadhyay et al., 2020)) and N hydrolysis (Huang et al., 2019). However, N cycling  
542 is a key soil function, particularly in agricultural soil, and the longer-term impacts of MPs on  
543 should be explored in more detail.

544

#### 545 *4.4 Effect of MP loading on soil N<sub>2</sub>O flux*

546 N<sub>2</sub>O is a potent greenhouse gas, with a global warming potential (GWP) 298 times larger than  
547 carbon dioxide (CO<sub>2</sub>) and it is a stratospheric ozone-depleting substance (Stocker, 2014). In  
548 soil, it is primarily produced by the biological pathways of nitrification and denitrification. As  
549 such it can be used as a functional indicator of soil biological quality at an ecosystem processes  
550 scale (Bünemann et al., 2018). Therefore, understanding whether MP addition influences soil



551 N<sub>2</sub>O fluxes will be key to understanding their overall environmental impact. It has been shown  
552 that MPs may reduce soil N<sub>2</sub>O emissions by inhibiting the microbial phyla associated with N  
553 cycling genes (Ren et al., 2020; Rillig et al., 2021), although results vary depending on the type  
554 of MP applied and environmental conditions (Shen et al., 2020; Sun et al., 2020).

555 While chambers in this study included plant and soil, the plant contribution of N<sub>2</sub>O is  
556 minimal (Chang et al., 1998), therefore we focussed on the soil contribution. Here, N<sub>2</sub>O flux  
557 from the soil after MP and fertiliser applications, respectively, were very low (Fig. 5). N<sub>2</sub>O  
558 fluxes are commonly observed after fertiliser application (up to 250 µg N<sub>2</sub>O-N m<sup>-2</sup> h<sup>-1</sup>; Carswell  
559 et al., 2018), however, we observed none. Equally, there were no differences between fluxes  
560 between MP loading levels (Table S4). However, it is difficult to attribute this low flux directly  
561 to the microplastic application, particularly as control plots also exhibited small fluxes.  
562 Notably, much of the sampling period was dry (Fig. S1), this is likely to have suppressed N<sub>2</sub>O  
563 emission, as water filled pore space (WFPS) was too low to allow the development of the  
564 anaerobic ‘hotspots’ required for N<sub>2</sub>O production (via denitrification) and emission (Barrat et  
565 al., 2020; Dobbie and Smith, 2001). We therefore recommend further field-based measurement  
566 of MPs effect on N<sub>2</sub>O and other GHGs (particularly CO<sub>2</sub> and methane (CH<sub>4</sub>)), under a range  
567 of climatic conditions and soil types.

568

#### 569 *4.5. Biogenic amines as effected by MP loading*

570 BAs are low molecular weight organic bases synthesised by prokaryotes and eukaryotes in the  
571 soil, mainly through decarboxylation of amino acids or amination and transamination of  
572 aldehydes and ketones. In a food context, BAs are often seen as undesirable due to their  
573 potentially toxic properties (Mah et al., 2019), in this sense they are potential food quality  
574 indicators (Ruiz-Capillas and Herrero, 2019). However, there is also evidence that BAs have a

575 role in quorum sensing in the gut between bacteria and host organisms (Hughes and Sperandio,  
576 2008; Sudo, 2019).

577         There has been little exploration of BAs in the soil system specifically. But it is  
578 generally understood that increased N availability in the soil will increase the number of BAs  
579 synthesised both by soil biota and plants (Pérez-Álvarez et al., 2017). Equally, homospermidine  
580 biosynthesis has been proposed as a stress regulator in rhizobia (Fujihara, 2009). In this study,  
581 one of the first to profile the soil BAs, we found no significant change in the BA amine profile  
582 of soil applied with MPs compared to control values, 6 months after initial MP application (Fig  
583 5, Fig. S3). A large range of compounds were extracted, many of which have putative functions  
584 including 5'-methylthioadenosine, an inhibitory by-product of methionine metabolism, which  
585 can be processed to salvage biogenically available sulphur (North et al., 2017). As well as  
586 abscisic acid, a plant hormone that regulates many aspects of plant growth, including  
587 development, maturation, and stress response (Nambara, 2016) and CcpA, which is a core  
588 transcriptional regulator in the control of catabolism in Gram-positive bacteria (Carvalho et al.,  
589 2011). However, due to the variability in response to MP loading and between replicates (Fig.  
590 6), further research is required to understand the role BAs may play in both quorum sensing  
591 and stress regulation in the soil system, as well as their spatial homogeneity.

592

#### 593 *4.6. Effect of MP on earthworms*

594 Earthworms are key representatives of soil fauna in relation to soil health, performing an  
595 important role in the formation and maintenance of soil fertility and structure, as well as being  
596 a major contributor to invertebrate biomass in soil (Blouin et al., 2013). Therefore,  
597 understanding the risks that MPs may pose to their health, abundance, and functioning within  
598 the agroecosystem is a priority. Earthworms have been shown to transport MPs throughout the

599 soil profile either through adhesion to the exterior of the earthworm body (Rillig et al., 2017b)  
600 or egestion of smaller MP particles (Huerta Lwanga et al., 2016). Our study found that there  
601 were no significant differences in earthworm abundance or biomass after 6 months of MP  
602 incorporation into the soil (Table 1), however, we did not measure egestion or adhesion. This  
603 result is inconsistent with much of the existing literature on earthworm exposure to MPs in soil,  
604 with several studies reporting negative effects on earthworm physiology (e.g. skin damage,  
605 induction of oxidative stress, loss of body weight, reduction in growth, mortality), although  
606 experiments were all laboratory or mesocosm based, over short time periods (< 60 days) and  
607 at maximum loading rates ranging from 1% to 60% (Boots et al., 2019; Cao et al., 2017; Y.  
608 Chen et al., 2020; Huerta Lwanga et al., 2016; Judy et al., 2019; Rodríguez-Seijo et al., 2019).  
609 MP loading rates in the aforementioned experiments ranged from 0.01% to 2% (w/w). Here we  
610 added MPs at the rates of 0%, ~0.1%, ~1% and ~10% (w/w), while earthworm health was not  
611 directly measured, a lack of change in earthworm abundance or biomass suggests that  
612 earthworm health had not diminished significantly, even at high MP loading. By proxy, this  
613 also suggests that earthworms do not actively avoid areas of microplastic contamination in the  
614 field, as in this study there were no barriers to earthworms leaving the MP loaded plots.

615         With this, it must be noted that this study only incorporated MPs into the top 10 cm of  
616 soil, therefore exposure of earthworms to MPs will likely depend on their ecotype, with  
617 endogenic earthworms likely to have higher exposure rates than the deeper dwelling anecic  
618 earthworms. As MPs are moved through the soil profile over time it is likely that the full extent  
619 of the impact on earthworms will be clearer. Equally, the longer-term (years to decades) impact  
620 of MPs is likely to be more severe than the short term. As MP particles degrade and fragment,  
621 they will become more ingestible to macrofauna and microfauna, although it is likely that the  
622 MP powder added in this study was already small enough to be digestible, possibly leading to  
623 greater mortality in soil-dwelling fauna (Lahive et al., 2019). Likewise, earthworms live several

624 years, therefore it is likely that this study captures only a snapshot of the earthworm lifecycle.  
625 Longer term monitoring is required to establish trends in earthworm health.

626

#### 627 *4.7. Crop health as affected by MP loading*

628 The ability to effectively grow healthy crop plants is a key ecosystem service provided by the  
629 soil in an agroecosystem context, underpinning human health and nutrition (Power, 2010).  
630 However, data on the effect of MP loading on crop yield and health is limited. MPs have the  
631 potential to affect plants in several ways; altering the soil structure, immobilising nutrients,  
632 contaminant transport, or adsorption and direct toxicity (Rillig et al., 2019). Several short-term  
633 laboratory studies have shown the negative effect of MPs on plant health and biomass at loadin  
634 rates ranging from 0.2 to 2% (de Souza Machado et al., 2019; Y. Qi et al., 2020; Zang et al.,  
635 2020). The results of this field study are contradictory to these studies, suggesting that MPs,  
636 even at extremely high loading rates, have no significant effects on the aboveground, ear  
637 biomass, or C:N ratio of the harvested seed of *T. aestivum* over one cropping season. However,  
638 the effect of MPs on root biomass and rooting structure was not measured in this study, though  
639 it is likely that the aboveground biomass would be affected if root growth characteristics were  
640 altered by MPs, as a high proportion of wheat roots are found within the top 10 cm of soil (Li  
641 et al., 2011).

642

#### 643 *4.8. Implications and future research direction*

644 Most existing data on MPs is based on laboratory or mesocosm based experiments. While these  
645 data are useful, field studies better represent real-world conditions. Longer-term (years to  
646 decades) datasets are required to obtain a more comprehensive understanding of the effect of  
647 MPs on soil physiochemistry as well as soil biology and plant health. The study of extremely

648 high MP loading rates may also be useful to understand future effects of MP on soil, if  
649 continuous loading occurs (e.g. repeated use of plastic mulch films). Generally, it is  
650 recommended that loading rates for future MP studies should reflect realistic loading rates in  
651 soil to accurately reflect a perturbed system. Even in heavily mulched soil MP loading rarely  
652 exceeds 325 kg ha<sup>-1</sup>, although this is likely to increase as MPs continue to be added to the soil  
653 (Huang et al., 2020), although little data explicitly reporting loading rates is available, with  
654 many studies choosing to report as items kg<sup>-1</sup> (Büks and Kaupenjohann, 2020).

655         It must also be noted that the potential negative impacts of (particularly conventional)  
656 MPs on soil and ecosystem health are likely to increase over time as their decomposition rates  
657 are extremely slow relative to the rate of entry to the system, leading to a progressive  
658 accumulation within soil (Rillig, 2012; Rillig et al., 2017a), potentially becoming persistent  
659 organic pollutants. Equally, while biodegradation is possible to a small extent, it is likely MPs  
660 relative recalcitrance means that microbes will prefer less energetically expensive C sources,  
661 and therefore, biological, co-metabolic, break-down of plastic is unlikely to occur to any great  
662 extent in field soils (Ng et al., 2018). That is what our data suggests, i.e. that if there are no  
663 additives, once a biofilm has formed on the outside, pure MPs are no different from an inert  
664 sand particle. However, this study is also limited in respect the the size and shape of MPs  
665 applied to the soil, which may not be typical of primary or secondary MPs typically applied to,  
666 or found in, soils, which in the case of mulch films are more likely to be thin films or peices as  
667 opposed to individual particles applied here (Huang et al., 2020).

668         This study applied pure MP LDPE powder, with very low levels of contaminants and  
669 additives present. The chemical formulation of MP entering agricultural soils, however, is  
670 expected to vary widely due to their origin (e.g. mulch film, biosolids) giving rise to variable  
671 amounts of additives (co-pollutants) such as plasticisers (generally low-volatility, insoluble and  
672 chemically stable; Campanale et al., 2020), colourants and pigments (inorganic pigments

673 containing heavy metals or organic pigments including various chromophoric families that are  
674 potentially carcinogenic and mutagenic; Gičević et al., 2020; Völz, 2009), ultraviolet (UV)  
675 stabilisers (inorganic or organic cadmium, barium, or lead salts; Stenmarck et al., 2017) or  
676 other polymers (Steinmetz et al., 2016). Generally, additives are not chemically bound to the  
677 plastic polymer and subsequent leaching of these additives may pose more of a hazard to soil  
678 ecology (particularly microorganisms) than the relatively recalcitrant MP themselves,  
679 particularly in the short term (days to years). The exchange and effects of additives or  
680 contaminants between plastic particles and the surrounding soil environment and the  
681 subsequent effect on soil function (e.g. enzyme inhibition) is a key area for future terrestrial  
682 plastics research.

683 It is also important to state that the majority of published literature on MPs does not  
684 state the purity of the plastics, MP used and the type (and concentration) of aforementioned  
685 additives incorporated. Reporting of this information is highly recommended in future  
686 literature, due to the potential varying effects on the soil environment as well as toxicity to soil  
687 ecology, which may significantly affect the results, particularly of biological studies.

688

## 689 **5. Conclusions**

690 This study demonstrated that the application of pure LDPE MP powder to a field site with no  
691 previous history of plastic pollution or application had no significant effect on soil biological  
692 health or function over one growing season (6 months). In this regard, we reject hypotheses i,  
693 ii and iii, as there were no significant changes in biological quality, crop biomass, or yield with  
694 MP loading; equally no effect of loading rate was observed. In conclusion, MPs themselves  
695 may not pose a significant problem, at least in the short term (days to years) due to their  
696 recalcitrant nature. Further work should be undertaken focusing on the effect of additives and

697 contaminants on soil function and plant health, as well as the longer-term (years to decades)  
698 effects of MP incorporation to soil, in a field context.

699

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712

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1118 **Figure and table captions**

1119 **Fig. 1** Scanning electron micrographs of microplastic particles before incorporation into the  
1120 soil. The images were taken across a range of magnifications (A – 20  $\mu\text{m}$ ; B – 50  $\mu\text{m}$ ; C –  
1121 100  $\mu\text{m}$ ; D – 200  $\mu\text{m}$ ; E – 200  $\mu\text{m}$ ; F – 500  $\mu\text{m}$ ). Images illustrate the heterogeneous nature of  
1122 particle size and surface texture within the powder sample.

1123 **Fig. 2** 16S rRNA gene sequenced bacterial community in response to different microplastic  
1124 doses ( $n = 4$ ). A) Proportionate abundances of major phyla within each microplastic loading  
1125 rate. B) Boxplot of observed bacterial OTU richness against microplastic loading rate ( $n = 4$ ).  
1126 C) Boxplot of bacterial OTU evenness against microplastic loading rate ( $n = 4$ ). D) Non-  
1127 metric multidimensional scaling (NMDS) ordination plot of bacterial OTU community  
1128 composition across microplastic loading rates.

1129 **Fig. 3** NMDS plot of the PLFA profile for each microplastic soil treatment. Ellipses represent  
1130 95% confidence intervals for each treatment.

1131 **Fig. 4** N cycling gene soil abundances pre- and post-N fertiliser application ( $n = 4$ ). A)  
1132 Urease-associated gene *UreC*, B) Free N fixation associated gene *nifH*, C) Nitrification-  
1133 associated genes, the *amoA* gene of; i) AOA, ii) AOB, iii) *comammox*, D) Denitrification-  
1134 associated genes; i) *nirK*, ii) *nirS*, iii) *nosZ*, E) Soil nitrate, F) Soil ammonium. All genes  
1135 abundances were normalised by extracted DNA quantities to account for differences in  
1136 microbial biomass and transformed by  $\log_{10}$ . Soil nitrate and ammonium are reported by dry  
1137 soil weight ( $n = 4$ ).

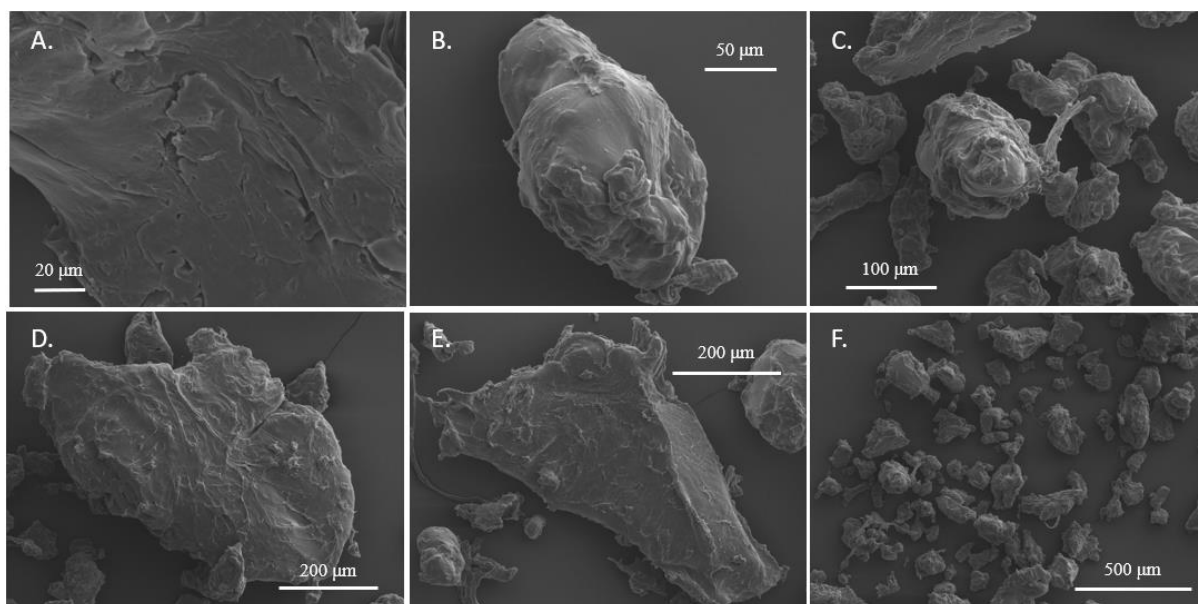
1138 **Fig. 5**  $\text{N}_2\text{O}$  fluxes from soil upon; A) initial MP loading, B) N fertilisation event one (40 kg N  
1139  $\text{ha}^{-1}$  equivalent), C) N fertilisation event two (80 kg N  $\text{ha}^{-1}$ ), by MP loading treatment. In each  
1140 panel, the line represents the mean flux ( $n = 3$ ) and the shaded area represents the upper and  
1141 lower bounds of the SEM.

1142 **Fig. 6** Influence of microplastic application rate on the biogenic amine (BA) concentration in  
1143 soil. Heatmap showing expression profiles of soil treatments based on the top 50 most  
1144 significant known BAs identified by ANOVA ( $p < 0.03$ ). BAs are clustered using Euclidean  
1145 distance and Ward linkage. Data was normalised using a  $\log_{10}$  transformation and Pareto  
1146 scaling. The colour of samples ranges from red to blue, indicating metabolite concentration z-  
1147 score; numbers 3 to -3 on the scale bar indicate the number of standard deviations from the  
1148 mean.

1149 **Fig. 7** Effect of microplastic application rate on above-ground wheat biomass ( $n = 4$ ). A)  
1150 Total above-ground biomass, B) Stem and leaf biomass, C) Ear biomass and D) Seed C:N  
1151 ratio.

1152 **Table 1.** Influence of microplastic dose rate and time since application on soil properties. The  
1153 soil was sampled one, two or six months post microplastic application. Results are expressed  
1154 on mean dry soil weight basis  $\pm$  SEM ( $n = 4$ ). Letters denote significant differences between  
1155 treatments ( $p < 0.05$ ).

1156 **Figure 1**



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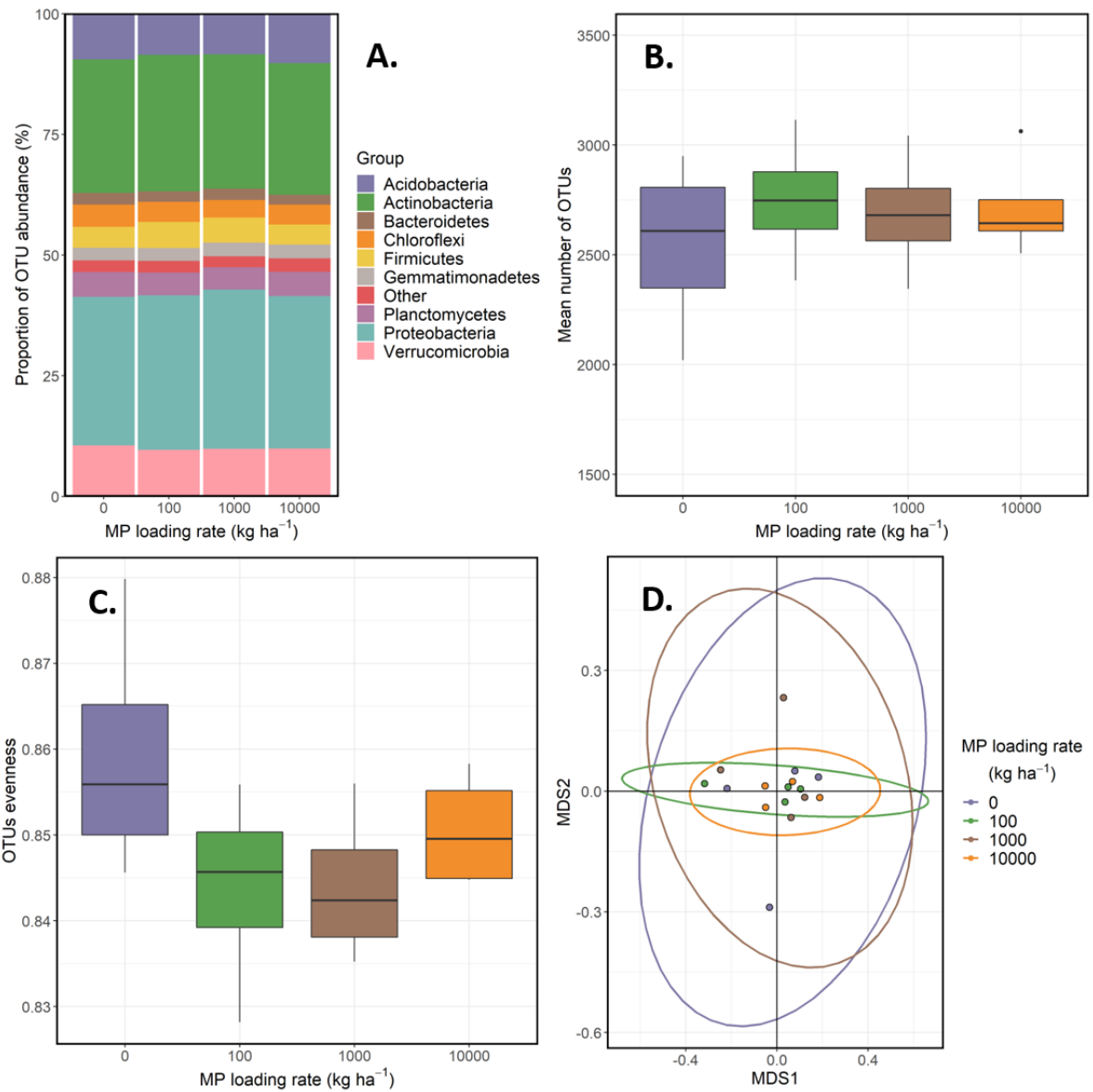
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1170 **Figure 2**



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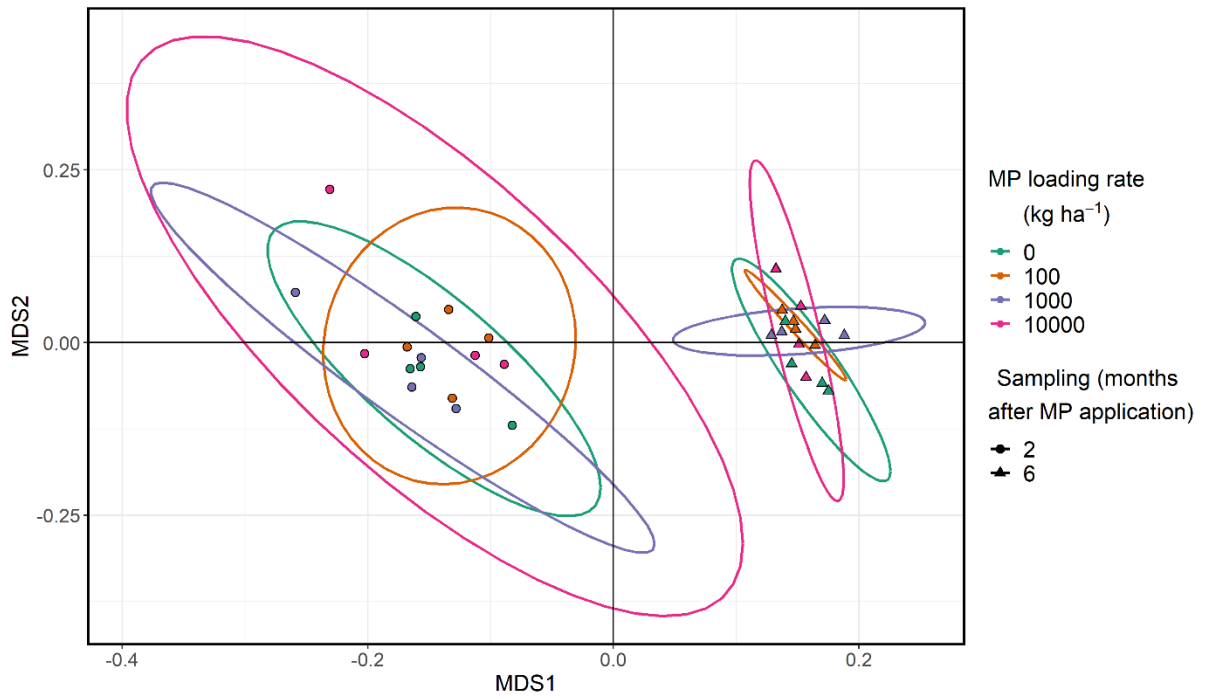
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1177 **Figure 3**



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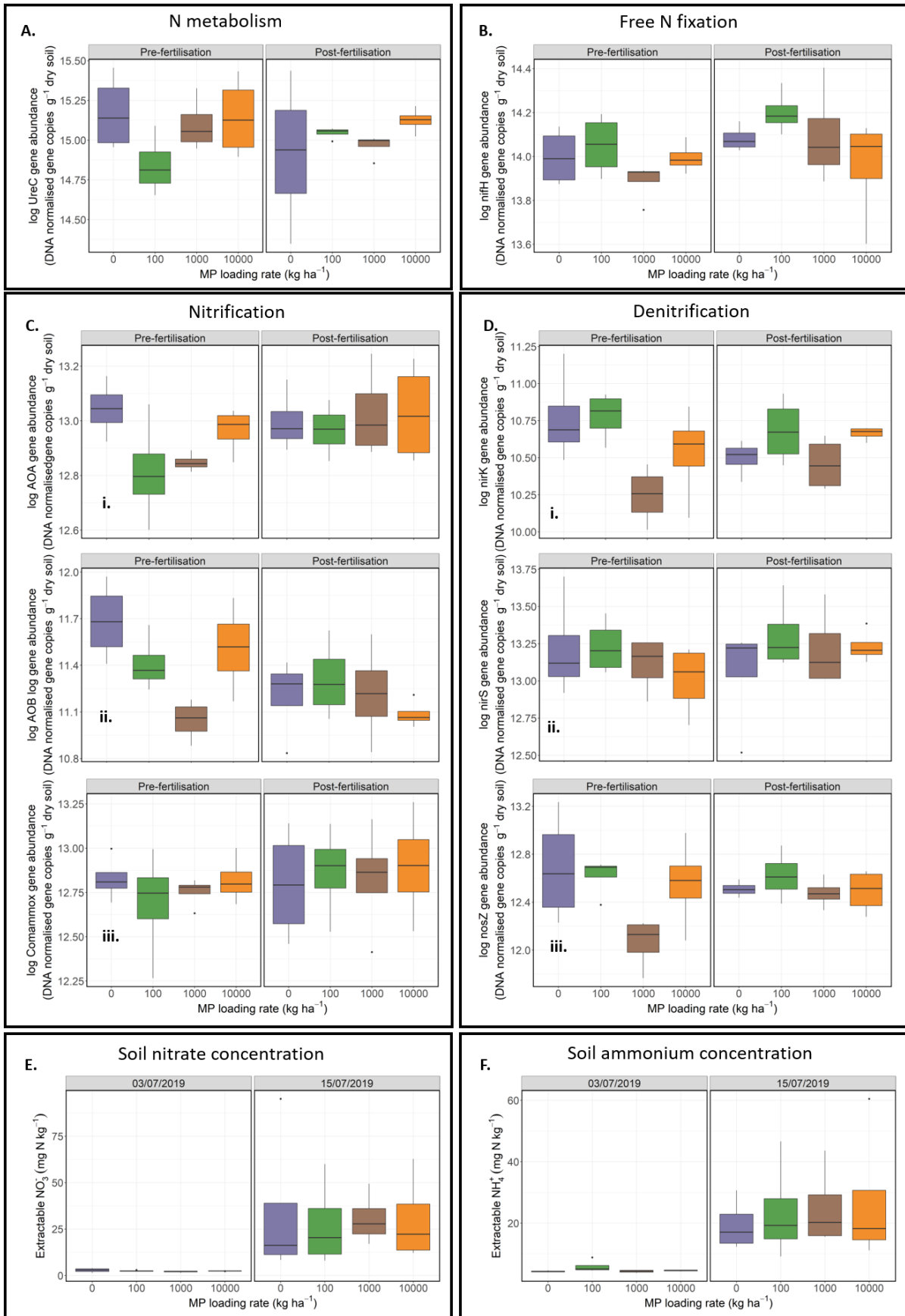
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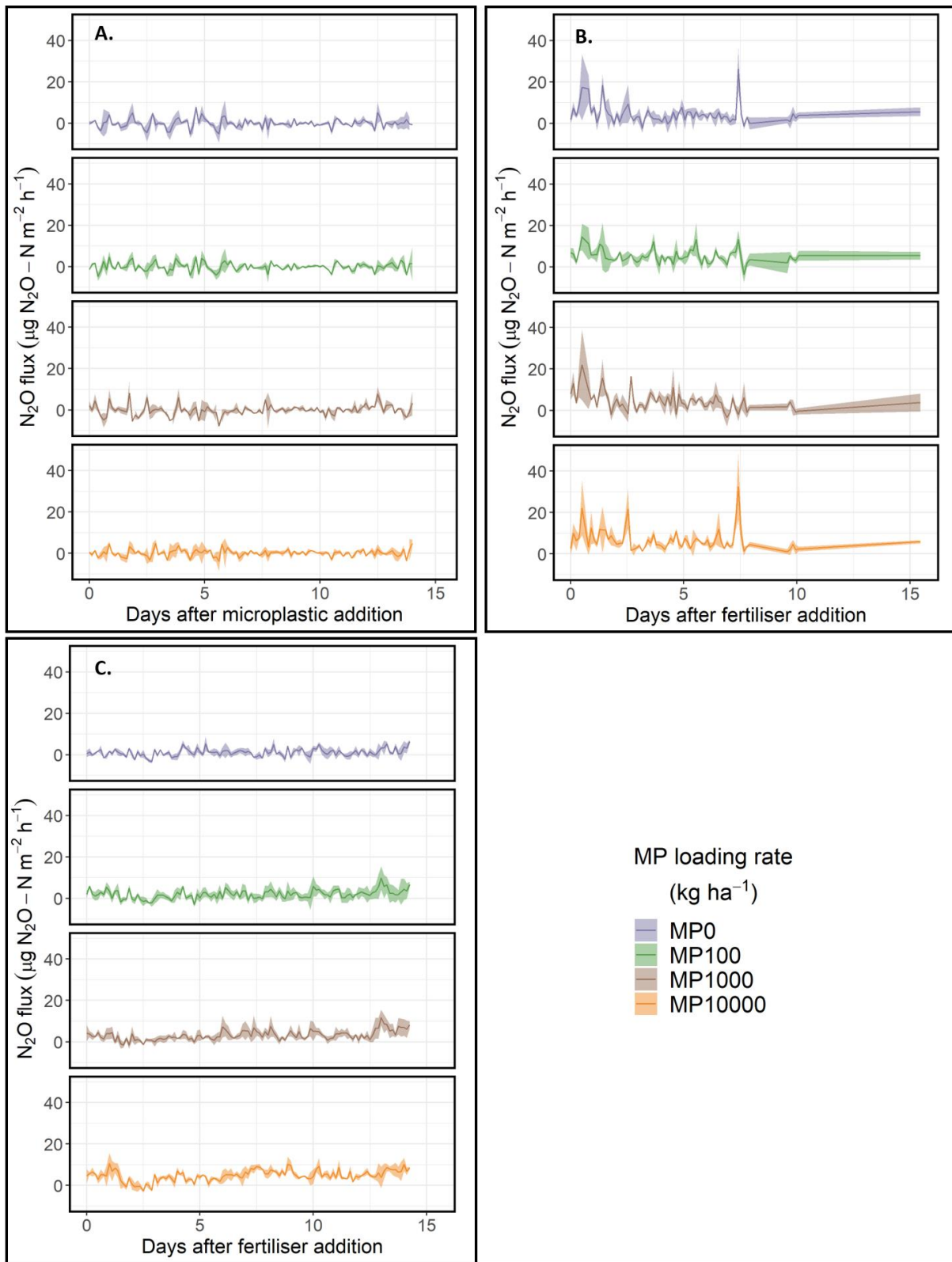
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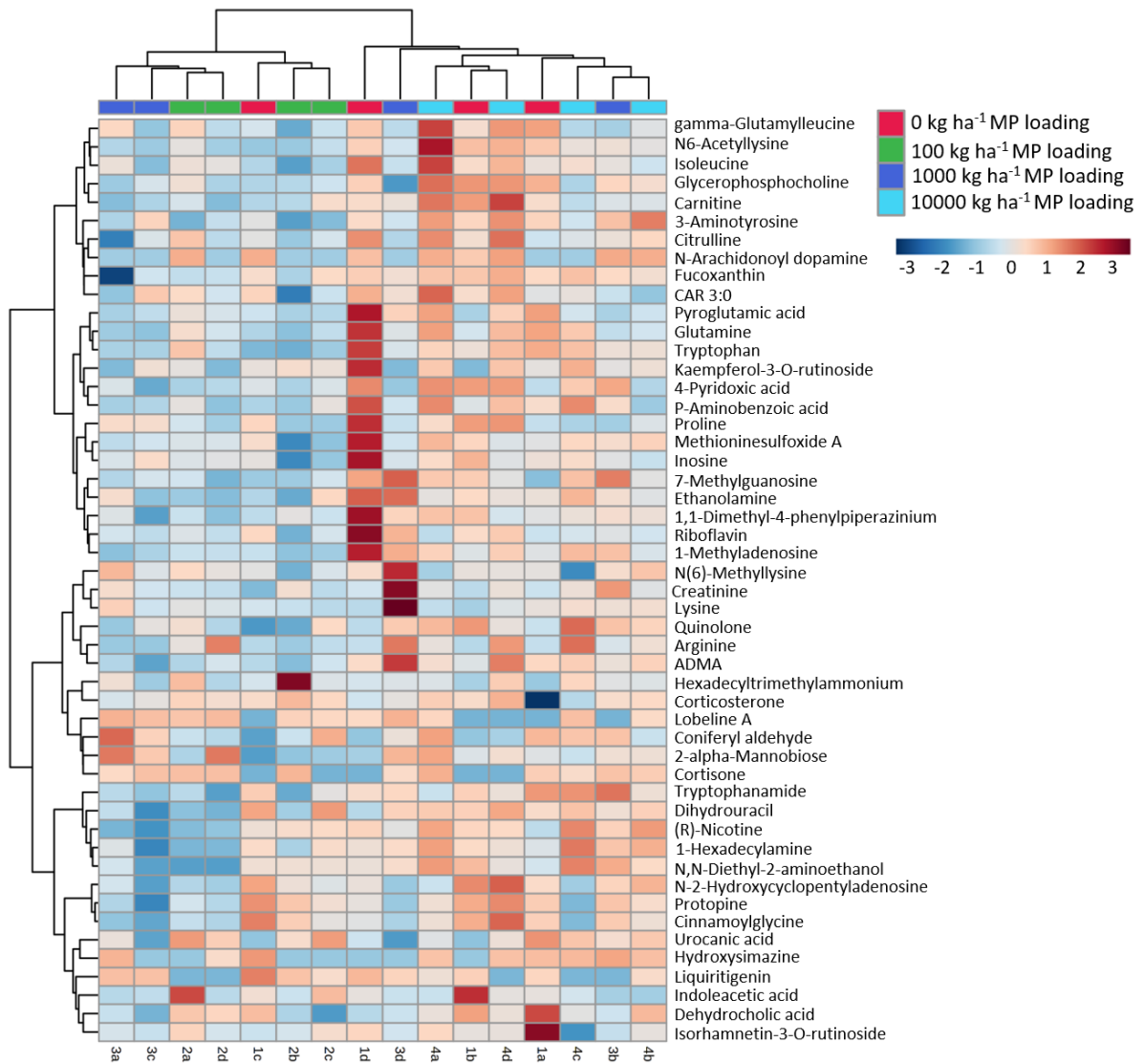
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1192 **Figure 6**



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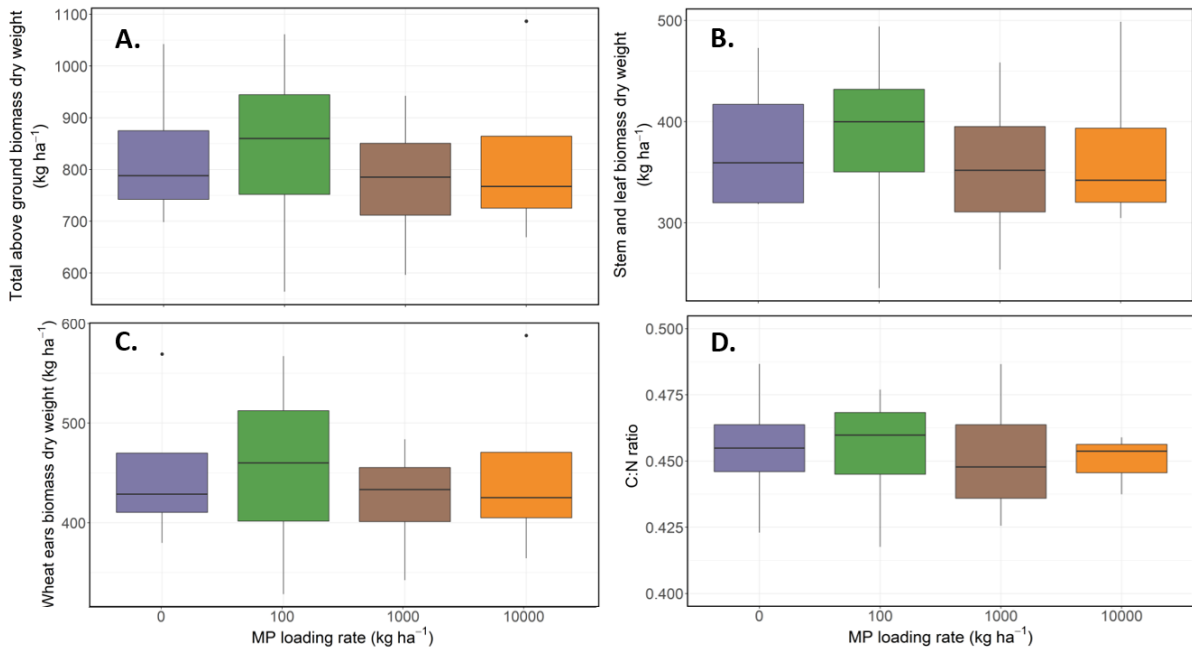
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202 **Table 1.** Influence of microplastic (MP) dose and time since application on soil properties. The soil was sampled one, two or six months post microplastic  
 203 application. Results are expressed on mean dry soil weight basis  $\pm$  SEM ( $n = 4$ ). Letters denote significant differences between treatments ( $p < 0.05$ ).

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MP loading rate (kg ha <sup>-1</sup> )	1 month post-MP application				2 months post MP application				6 months post MP application			
	0	100	1000	10000	0	100	1000	10000	0	100	1000	10000
pH	6.26 $\pm$ 0.04 <sup>a</sup>	6.23 $\pm$ 0.19 <sup>a</sup>	6.26 $\pm$ 0.14 <sup>a</sup>	6.23 $\pm$ 0.10 <sup>a</sup>	6.49 $\pm$ 0.04 <sup>a</sup>	6.34 $\pm$ 0.15 <sup>a</sup>	6.41 $\pm$ 0.12 <sup>a</sup>	6.47 $\pm$ 0.08 <sup>a</sup>	6.27 $\pm$ 0.11 <sup>a</sup>	6.16 $\pm$ 0.26 <sup>a</sup>	6.14 $\pm$ 0.11 <sup>a</sup>	6.09 $\pm$ 0.08 <sup>a</sup>
EC ( $\mu$ S cm <sup>-1</sup> )	129 $\pm$ 38 <sup>a</sup>	91 $\pm$ 13 <sup>a</sup>	123 $\pm$ 24 <sup>a</sup>	96 $\pm$ 22 <sup>a</sup>	37 $\pm$ 1.9 <sup>a</sup>	36 $\pm$ 2.6 <sup>a</sup>	31 $\pm$ 2.3 <sup>a</sup>	31 $\pm$ 3.5 <sup>a</sup>	55 $\pm$ 2.4 <sup>a</sup>	77 $\pm$ 25 <sup>a</sup>	55 $\pm$ 3.9 <sup>a</sup>	51 $\pm$ 2.6 <sup>a</sup>
NO <sub>3</sub> <sup>-</sup> (mg N kg <sup>-1</sup> )	67.4 $\pm$ 21.7 <sup>a</sup>	18.6 $\pm$ 4.6 <sup>a</sup>	33.4 $\pm$ 14.5 <sup>a</sup>	38.3 $\pm$ 0.70 <sup>a</sup>	5.04 $\pm$ 2.60 <sup>a</sup>	4.96 $\pm$ 3.02 <sup>a</sup>	1.86 $\pm$ 0.09 <sup>a</sup>	1.61 $\pm$ 0.14 <sup>a</sup>	10.4 $\pm$ 4.30 <sup>a</sup>	21.9 $\pm$ 9.32 <sup>a</sup>	15.5 $\pm$ 4.1 <sup>a</sup>	10.2 $\pm$ 1.08 <sup>a</sup>
NH <sub>4</sub> <sup>+</sup> (mg N kg <sup>-1</sup> )	57.5 $\pm$ 16.7 <sup>a</sup>	11.0 $\pm$ 5 <sup>a</sup>	22.1 $\pm$ 10.9 <sup>a</sup>	45.8 $\pm$ 1.6 <sup>a</sup>	1.01 $\pm$ 0.06 <sup>a</sup>	1.11 $\pm$ 0.11 <sup>a</sup>	1.13 $\pm$ 0.05 <sup>a</sup>	0.89 $\pm$ 0.06 <sup>a</sup>	2.64 $\pm$ 0.30 <sup>a</sup>	5.36 $\pm$ 2.09 <sup>a</sup>	3.28 $\pm$ 0.88 <sup>a</sup>	3.00 $\pm$ 1.05 <sup>a</sup>
Bulk density (kg m <sup>-3</sup> )					1014 $\pm$ 11 <sup>a</sup>	1065 $\pm$ 27 <sup>a</sup>	984 $\pm$ 30 <sup>a</sup>	977 $\pm$ 31 <sup>a</sup>	1065 $\pm$ 22 <sup>a</sup>	1106 $\pm$ 48 <sup>a</sup>	1092 $\pm$ 44 <sup>a</sup>	1062 $\pm$ 61 <sup>a</sup>
Bacterial/Fungal PLFA ratio					0.11 $\pm$ 0.01 <sup>ab</sup>	0.11 $\pm$ 0.01 <sup>ab</sup>	0.11 $\pm$ 0.01 <sup>ab</sup>	0.14 $\pm$ 0.02 <sup>a</sup>	0.09 $\pm$ 0.00 <sup>b</sup>	0.10 $\pm$ 0.00 <sup>ab</sup>	0.11 $\pm$ 0.01 <sup>ab</sup>	0.10 $\pm$ 0.01 <sup>ab</sup>
Microbial PLFA biomass ( $\mu$ mol PLFA kg <sup>-1</sup> )					174 $\pm$ 11 <sup>ab</sup>	175 $\pm$ 9 <sup>ab</sup>	162 $\pm$ 3 <sup>a</sup>	190 $\pm$ 16 <sup>ab</sup>	199 $\pm$ 6 <sup>ab</sup>	201 $\pm$ 8 <sup>ab</sup>	197 $\pm$ 6 <sup>ab</sup>	218 $\pm$ 12 <sup>b</sup>
Earthworm biomass (g m <sup>-2</sup> )									92 $\pm$ 9 <sup>a</sup>	54 $\pm$ 6 <sup>a</sup>	71 $\pm$ 24 <sup>a</sup>	79 $\pm$ 22 <sup>a</sup>
Earthworm abundance (individuals m <sup>-2</sup> )									26 $\pm$ 5 <sup>a</sup>	13 $\pm$ 2 <sup>a</sup>	24 $\pm$ 13 <sup>a</sup>	20 $\pm$ 6 <sup>a</sup>

EC – electrical conductivity

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