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ABSTRACT

Plastics are now widespread in the natural environment. Due to their size, microplastics (MPs; defined as particles < 5 mm) in particular, have the potential to cause damage and harm to organisms and may lead to a potential loss of ecosystem services. Research has demonstrated the significant impact of MPs on aquatic systems; however, little is known about their effects on the terrestrial environment, particularly within agroecosystems, the cornerstone of global food production. Soil biology is highly responsive to environmental perturbation and change. Hereby, we investigated the effect of pure low-density polyethylene (LDPE) MP loading (0, 100, 1000, or 10000 kg ha^{-1}) on soil and plant biological health in a field environment over a cropping season. Our results showed that MP loading had no significant effect ($p > 0.05$) on the soil bacterial community diversity (as measured by amplicon sequencing of bacterial 16S rRNA gene), the size and structure of the PLFA-derived soil microbial community, or the abundance and biomass of earthworms. In addition, metabolomic profiling revealed no dose-dependent effect of MP loading on soil biogenic amine concentrations. The growth and yield of wheat plants (*Triticum aestivum* L., cv. Mulika) were also unaffected by MP dose, even at extremely high (≥ 1000 kg ha^{-1}) loading levels. Nitrogen (N) cycling gene abundance before and after N fertiliser application on the MP loaded experimental plots showed relatively little change, although further experimentation is suggested, with similar trends evident for soil nitrous oxide (N_2O) flux. Overall, we illustrate that MPs themselves may not pose a significant problem in the short term (days to months), due to their recalcitrant nature. We also emphasise that most MPs in the environment are not pure or uncontaminated, containing additives (e.g. plasticisers, pigments and stabilisers) that are generally not chemically bound to the plastic polymer and may be prone to leaching into the soil matrix. Understanding the effect of additives on soil biology as well as the longer-term (years to decades) impact of MPs on soil biological and ecological health in the field environment is recommended.

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Experimental setup

The experiment took place at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). The soil is classified as a sandy clay loam textured Eutric Cambisol, overlying a glacial till, with a temperate-oceanic climate. The mean annual rainfall is 1060 mm and the mean annual temperature is 10°C. The site has no previous history of plastic pollution or application over the last 50 years (Zang et al., 2020). On 18th April 2019, a randomised plot design was established to create 4 independent replicates (n = 4) of each treatment. Each plot (1.4 × 2.85 m) was then treated with LDPE microplastic powder (RXP1003 natural; Resinex Ltd., High Wycombe, UK), at a rate of 0, 100, 1000, or 10000 kg ha^{-1} by thorough manual mixing with the top 10 cm of soil. This equated to loading rates of 0%, ~0.1%, ~1%, and ~10% (w/w) (soil bulk density = 1040 kg m^{-3}; n = 4). The microplastic powder was confirmed to have a very low level of contamination through total carbon (C) and nitrogen (N) analysis using a TruSpec® Analyzer (Leco Corp., Michigan, USA) (Total C, 82.88% ± 0.03%; Total N, 0.03 ± 0.01%; n = 5). LDPE was chosen due to its extensive use in agricultural films (Espí et al., 2006; Rong et al., 2021). Plots were subsequently sown with spring wheat (*Triticum aestivum* L., cv. Mulika) at a rate of 400 plants m^{-2}. In line with the fertiliser recommendations for wheat, and taking account of the soil's Soil Nitrogen Supply (SNS) (AHDB, 2018), 120 kg N ha^{-1} yr^{-1} was applied to the field as NH_4NO_3 over two applications, 40 kg N ha^{-1} on 3rd June and 80 kg N ha^{-1} on 3rd July (reflecting the late sowing of the crop). For scanning electron microscopy (SEM), LDPE powder was mounted on adhesive tape, coated with gold, and imaged at 10 kV (Tescan Vega3 SEM). These SEM images illustrate the heterogeneous nature of the MP mixture, both in terms of particle size and surface texture (Fig. 1).

2.2. Soil sampling and analysis

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

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

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

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

2.4. Biogenic amine extraction and analysis

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

Briefly, extraction consisted of vortexing (~15 s) a 0.4:1 (w/v) soil-to-3:3:2 (v/v/v) MeCN/IPA/H_2O solution, before shaking for 5 min at 4°C, centrifuging (2 min, 14000 g) and recovering an aliquot of the supernatant for analysis. LC/QTOFMS analysis of extracted aliquots was performed on an Agilent 1290 Infinity LC system (G4220A binary pump, G4226A autosampler, and G1316C Column Thermostat) coupled to a SCIEX Triple TOF mass

spectrometer, total runtime was 16.8 min. Polar compounds are separated on an Acquity UPLC BEH Amide Column, 13 nm (pore size), 1.7 µm (particle size), 2.1 mm × 150 mm maintained at 45°C at a flowrate of 0.4 ml min^{-1}. Solvent pre-heating (Agilent G1316) was used. The mobile phases consist of: (A) Water, 10 mM ammonium formate, 0.125% formic acid and (b) acetonitrile: water (95/5, v/v), 10 mM ammonium formate, 0.125% formic acid. The gradient 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 30% (B), 10.25-12.75 min 100% (B), 16.75 min 100% (B).

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

2.5 Soil N_2O flux

A mobile, automated GHG monitoring system, utilising a GC-Electron Capture Detector (8610C, SRI Instruments, CA, USA), as previously described in Marsden et al., (2018), was used to monitor nitrous oxide (N_2O) fluxes from three of the four replicates for each treatment. Stainless steel chamber bases (50 × 50 cm; 0.25 m^2) were installed into plots two weeks before MP application, to which chambers (0.0625 m^3) were tightly secured. A foam strip on the base of each chamber ensured a tight seal. Briefly, the automated sampling system provided eight greenhouse gas flux measurements per 24 h period, per chamber during uninterrupted measurement. Emissions were monitored for 6 months from installation. However, this manuscript focuses on the 2-week periods following initial MP loading, to test whether the background emissions from the soil were perturbed by MP incorporation and the two subsequent N fertiliser application events, respectively, as these periods were likely to produce the greatest fluxes (Bell et al., 2015; Cardenas et al., 2019).

2.6. High-throughput sequencing and quantitative PCR analysis

2.6.1. 16S rRNA gene sequencing

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

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

2.6.2. Bioinformatic analysis

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

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

2.6.3. Quantitative PCR of N cycling functional genes

Samples for quantitative PCR (qPCR) of N cycling functional genes were collected on the 3rd July (pre-N fertiliser application) and on the 15th July (12 days post-N fertiliser application). On each occasion five soil cores (n = 5; ø = 1 cm; depth = 0 – 10 cm) were taken per plot and homogenised by hand to obtain a representative sample. After collection, samples were passed through a 2 mm sieve and subsequently transferred (<1 h) to a -80°C freezer for pre-extraction storage. Samples were extracted for NO_3-N and NH_4-N, as described in section 2.2. DNA was extracted by mechanical lysis from 0.25 g soil per sample using a DNEASY Powersoil kit (Qiagen, Hilden, Germany). The quality and concentration of extracted DNA were assessed by AGE.

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

differences in extraction effieciencies within samples and raw results are are included in supplementary information.

2.7. Earthworm abundance and biomass

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

2.8. Wheat harvest data

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

2.9. Statistical analysis

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

Normality and homogeneity of variance of the chemical and physical soil properties of the treated Eutric Cambisol were assessed using Shapiro-Wilk's test and Levene's test, respectively. For data that did not conform to parametric assumptions even after using $\log_{10}$ transformation (NO_3-N, NH_4-N, EC and PLFA Fungal:Bacterial ratio) a Kruskal-Wallis test (stats package; R Core Team, 2021) was used to assess the similarities between MP treatments and sampling dates, otherwise a one-way ANOVA (Analysis of variance) was used (for pH, bulk density and total PLFA biomass). The results for this are summarised in Table 1. A one-way ANOVA was also used to assess treatment variations in wheat biomass data (total aboveground biomass, stem and leaf biomass, ear biomass and harvested wheat seed C:N ratio) and earthworm data (abundance and biomass).

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

Fluxes of N_2O for each chamber were calculated using the methods described in Scheer et al., (2014). The linear slope of N_2O concentrations over time included either three or four

data points. N_2O fluxes for each two-week period (post-MP and fertiliser application, respectively) were graphically analysed. Trapezoidal integration was used to calculate cumulative N_2O emissions for each treatment, these were tested for significance using for Kruskal-Wallis tests, after failing parametric assumptions.

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

N cycling gene abundance, before and after a N fertilisation event was analysed using mixed effect models with the '*lme4*' package (Bates et al., 2015). We considered MP loading rate and sampling time and their interaction as fixed effects and individual plots as temporal random effects. For each variable, residuals from each model were tested for normality, autocorrelation and heteroscedasticity using graphical tools. For all genes, a $\log_{10}$ conversion was found to improve the fitness of all models. An ANOVA was then run on each model to test treatment effects, significant results were further explored using a Tukey adjusted post-hoc test using the '*emmeans*' package (Lenth, 2021). Pre- and post- fertilisation soil NO_3-N and NH_4-N concentrations were analysed by ANOVA.

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

Also, we aknowledge that, being a field trial, a high level of representative replication (i.e., replication with large enough plot sizes) is difficult to obtain, which could potentially impact the statistical power of the study. However, on calculating the statistical power of the parameteric statistics used here all were ≥ 0.99, with the expection of bacterial OTU evenness (power = 0.05), thus this result should be interpreted with caution.

3. Results

3.1. 16S bacterial community

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

3.2. PLFA-derived community

The fungal-bacterial ratio of PLFAs remained similar across all treatments, there was a significant difference between the 2 months post-application 10000 kg ha^{-1} and the 6 months post-application 0 kg ha^{-1} MP loading rates, with the latter having a higher prevalence of

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

3.3. N cycling genes

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

significant differences between 0 kg ha^{-1} and 1000 kg ha^{-1} MP loading, pre fertilisation ($p < 0.05$) and between 0 kg ha^{-1} MP loading, pre fertilisation, and 10000 kg ha^{-1} MP loading post fertilisation ($p < 0.05$). Concentrations of soil NO_3-N ($F_{(1,12)} = 16.6$, $p < 0.01$) and NH_4-N ($F_{(1,12)} = 22.0$, $p < 0.01$) were significantly higher post-fertilisation (Fig. 4E, F).

3.4 N_2O flux

Kruskal-Wallis analysis showed that there were no significant differences between cumulative N_2O fluxes for the 2 week period following initial MP application ($H_{(3)} = 0.74$, $p = 0.9$), or the first ($H_{(3)} = 4.6$, p = 0.2) and second fertiliser ($H_{(3)} = 3.6$, $p = 0.3$) application events. Fluxes over each period are summarised in Fig. 5.

3.5. Biogenic amines

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

3.6. Soil properties including inorganic N

Overall, there were no significant differences in soil chemical properties (pH, EC, NO_3-N and NH_4-N) associated with the MP treatment as tested by ANOVA or Kruskal Wallis ($p > 0.1$). Trends in the data show some natural variation in all soil properties measured throughout the season (summarised in Table 1).

3.7. Earthworms abundance and biomass

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

3.8. Plant biomass

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

4. Discussion

4.1. 16S bacterial community response to MP addition

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

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

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

4.2. Effect of MP loading on soil PLFAs

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

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

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

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

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

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

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

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

4.4 Effect of MP loading on soil N_2O flux

N_2O is a potent greenhouse gas, with a global warming potential (GWP) 298 times larger than carbon dioxide (CO_2) and it is a stratospheric ozone-depleting substance (Stocker, 2014). In soil, it is primarily produced by the biological pathways of nitrification and denitrification. As such it can be used as a functional indicator of soil biological quality at an ecosystem processes scale (Bünemann et al., 2018). Therefore, understanding whether MP addition influences soil

N_2O fluxes will be key to understanding their overall environmental impact. It has been shown that MPs may reduce soil N_2O emissions by inhibiting the microbial phyla associated with N cycling genes (Ren et al., 2020; Rillig et al., 2021), although results vary depending on the type of MP applied and environmental conditions (Shen et al., 2020; Sun et al., 2020).

While chambers in this study included plant and soil, the plant contribution of N_2O is minimal (Chang et al., 1998), therefore we focussed on the soil contribution. Here, N_2O flux from the soil after MP and fertiliser applications, respectively, were very low (Fig. 5). N_2O fluxes are commonly observed after fertiliser application (up to 250 µg N_2O-N m^{-2} h^{-1}; Carswell et al., 2018), however, we observed none. Equally, there were no differences between fluxes between MP loading levels (Table S4). However, it is difficult to attribute this low flux directly to the microplastic application, particularly as control plots also exhibited small fluxes. Notably, much of the sampling period was dry (Fig. S1), this is likely to have suppressed N_2O emission, as water filled pore space (WFPS) was too low to allow the development of the anaerobic 'hotspots' required for N_2O production (via denitrification) and emission (Barrat et al., 2020; Dobbie and Smith, 2001). We therefore recommend further field-based measurement of MPs effect on N_2O and other GHGs (particularly CO_2 and methane (CH_4)), under a range of climatic conditions and soil types.

4.5. Biogenic amines as effected by MP loading

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

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

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

4.6. Effect of MP on earthworms

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

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

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

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

4.7. Crop health as affected by MP loading

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

4.8. Implications and future research direction

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

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

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

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

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

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

5. Conclusions

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

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

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Figure and table captions

Fig. 1 Scanning electron micrographs of microplastic particles before incorporation into the soil. The images were taken across a range of magnifications (A – 20 μm; B – 50 μm; C – 100 μm; D – 200 μm; E – 200 μm; F – 500 μm). Images illustrate the heterogeneous nature of particle size and surface texture within the powder sample.

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

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

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

Fig. 5 N_2O fluxes from soil upon; A) initial MP loading, B) N fertilisation event one (40 kg N ha^{-1} equivalent), C) N fertilisation event two (80 kg N ha^{-1}), by MP loading treatment. In each panel, the line represents the mean flux (*n* = 3) and the shaded area represents the upper and lower bounds of the SEM.

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

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

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

Figure 1

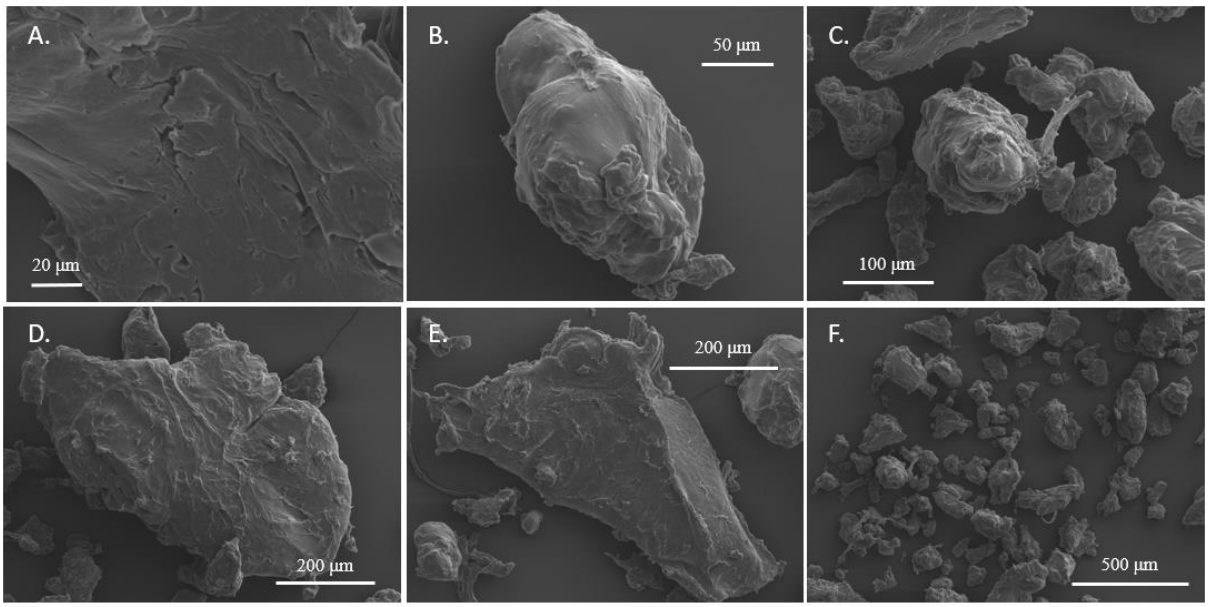

Figure 2

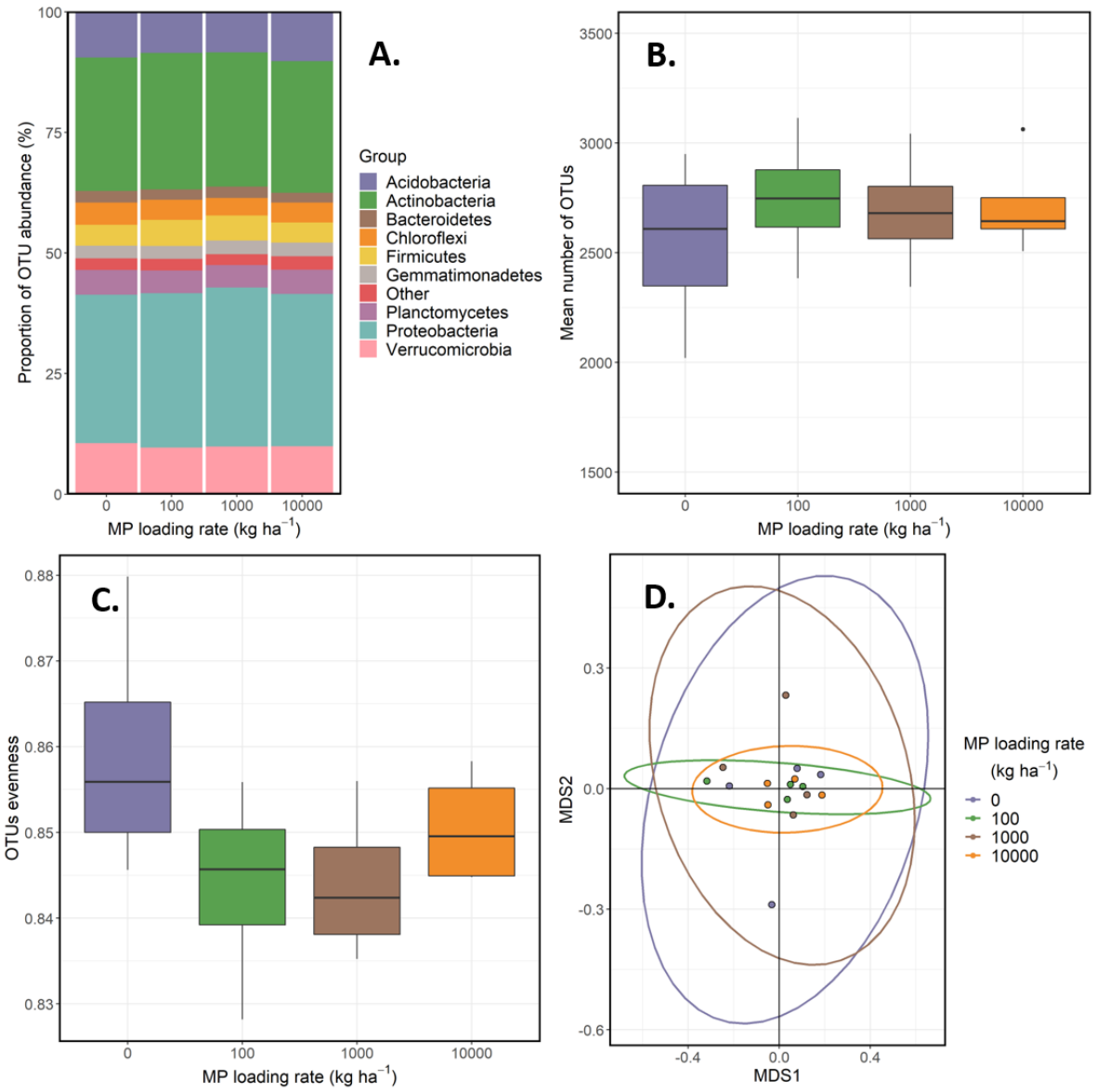
A.
Proportion of OTU abundance (%)
MP loading rate (kg ha^{-1})
Group
Acidobacteria
Actinobacteria
Bacteroidetes
Chloroflexi
Firmicutes
Gemmatimonadetes
Other
Planctomycetes
Proteobacteria
Verrucomicrobia
B.
Mean number of OTUs
MP loading rate (kg ha^{-1})
C.
OTUs evenness
MP loading rate (kg ha^{-1})
D.
MDS2
MDS1
MP loading rate (kg ha^{-1})
0
100
1000
10000

Figure 3

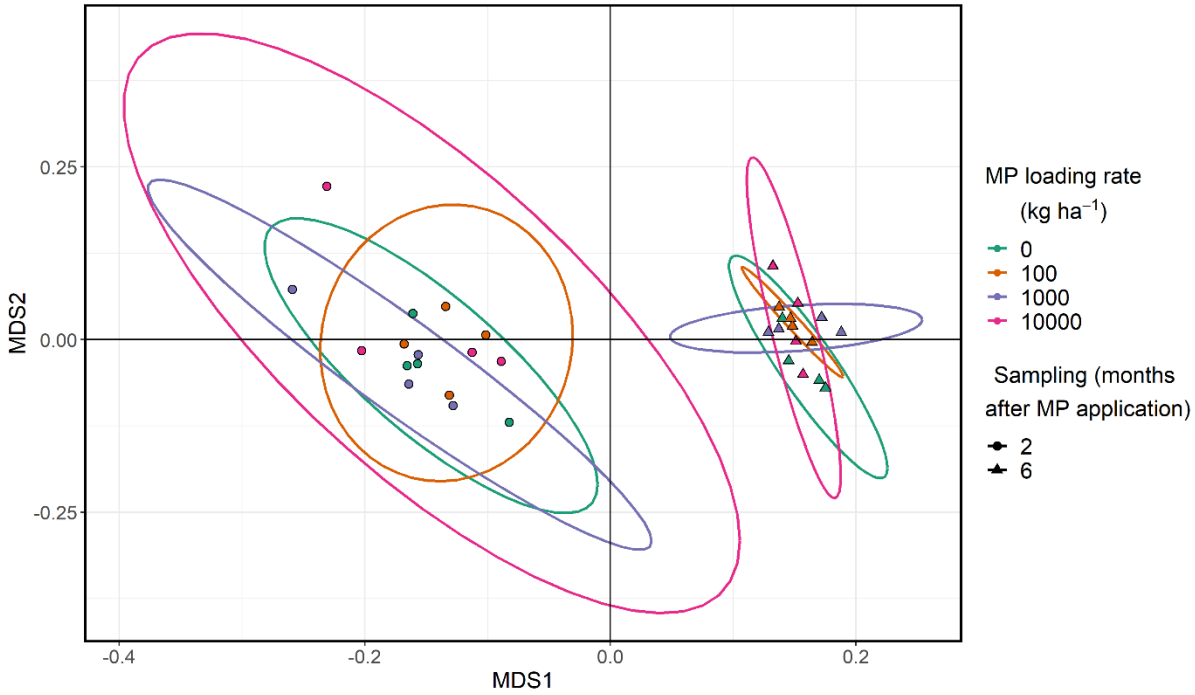
0.25
0.00
-0.25
MDS2
-0.4
-0.2
0.0
0.2
MDS1
MP loading rate
(kg ha^{-1})
0
100
1000
10000
Sampling (months
after MP application)
2
6

Figure 4

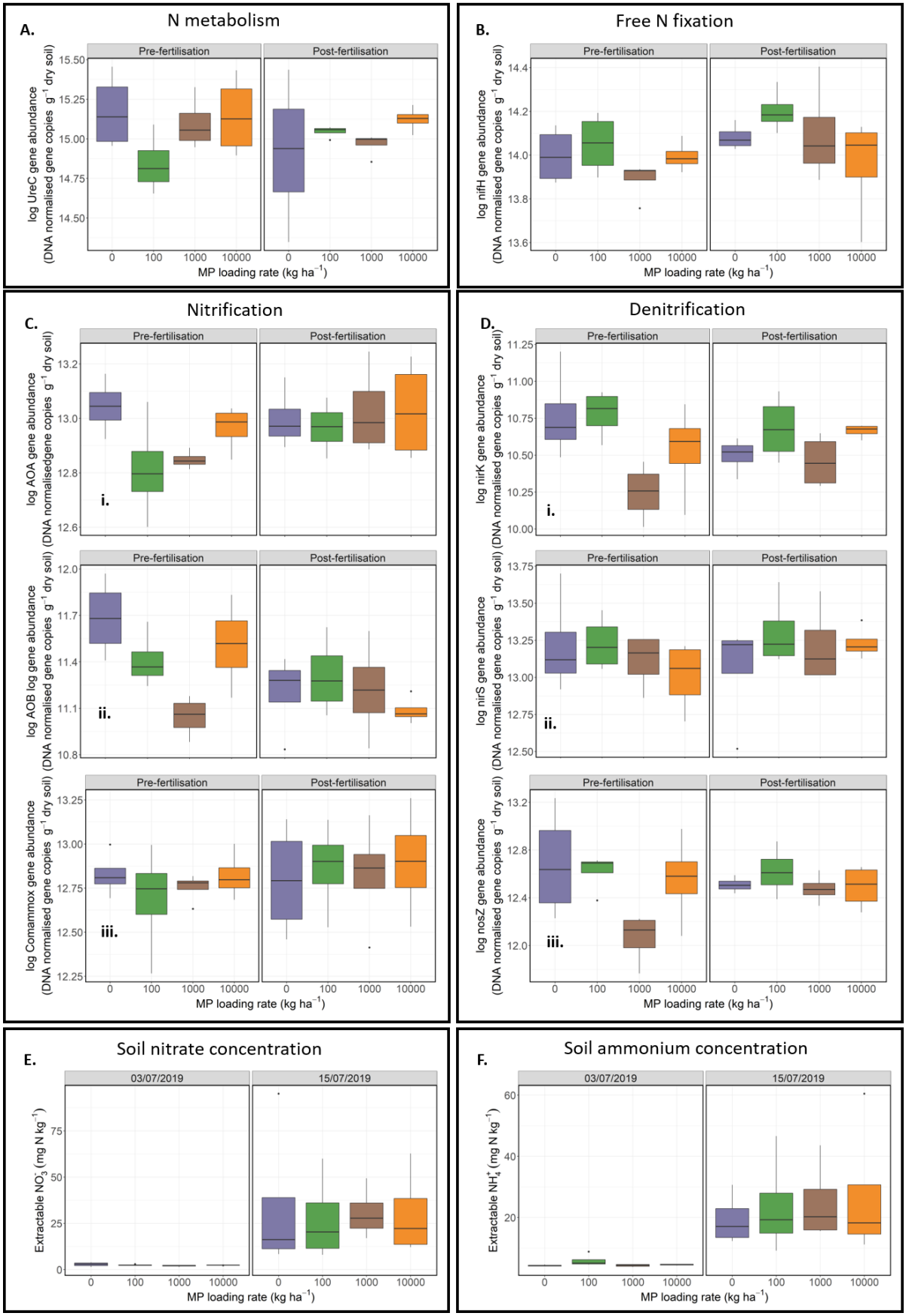
A.
N metabolism
Pre-fertilisation
Post-fertilisation
log UreC gene abundance (DNA normalised gene copies g⁻¹ dry soil)
MP loading rate (kg ha⁻¹)
B.
Free N fixation
log nifH gene abundance (DNA normalised gene copies g⁻¹ dry soil)
C.
Nitrification
log AOA gene abundance (DNA normalisedgene copies g⁻¹ dry soil)
i.
log AOB log gene abundance (DNA normalised gene copies g⁻¹ dry soil)
ii.
log Comammox gene abundance (DNA normalised gene copies g⁻¹ dry soil)
iii.
D.
Denitrification
log nirK gene abundance (DNA normalised gene copies g⁻¹ dry soil)
log nirS gene abundance (DNA normalised gene copies g⁻¹ dry soil)
log nosZ gene abundance (DNA normalised gene copies g⁻¹ dry soil)
E.
Soil nitrate concentration
03/07/2019
15/07/2019
Extractable NO₃⁻ (mg N kg⁻¹)
F.
Soil ammonium concentration
Extractable NH₄⁺ (mg N kg⁻¹)

Figure 5

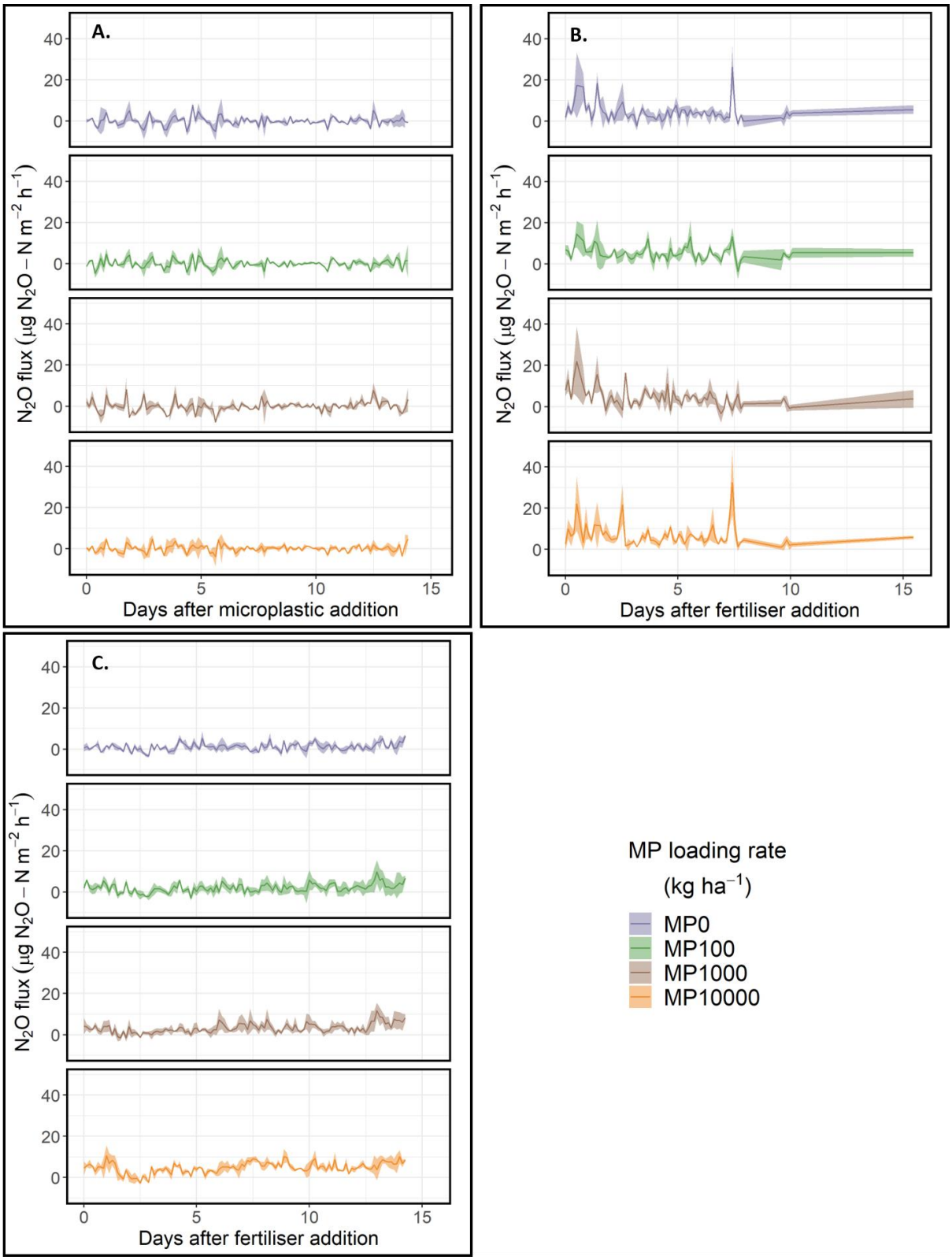
A.
B.
C.
N_2O flux ($\mu g\ N_2O - N\ m^{-2}\ h^{-1}$)
Days after microplastic addition
Days after fertiliser addition
MP loading rate
(kg ha^{-1})
MP0
MP100
MP1000
MP10000

Figure 6

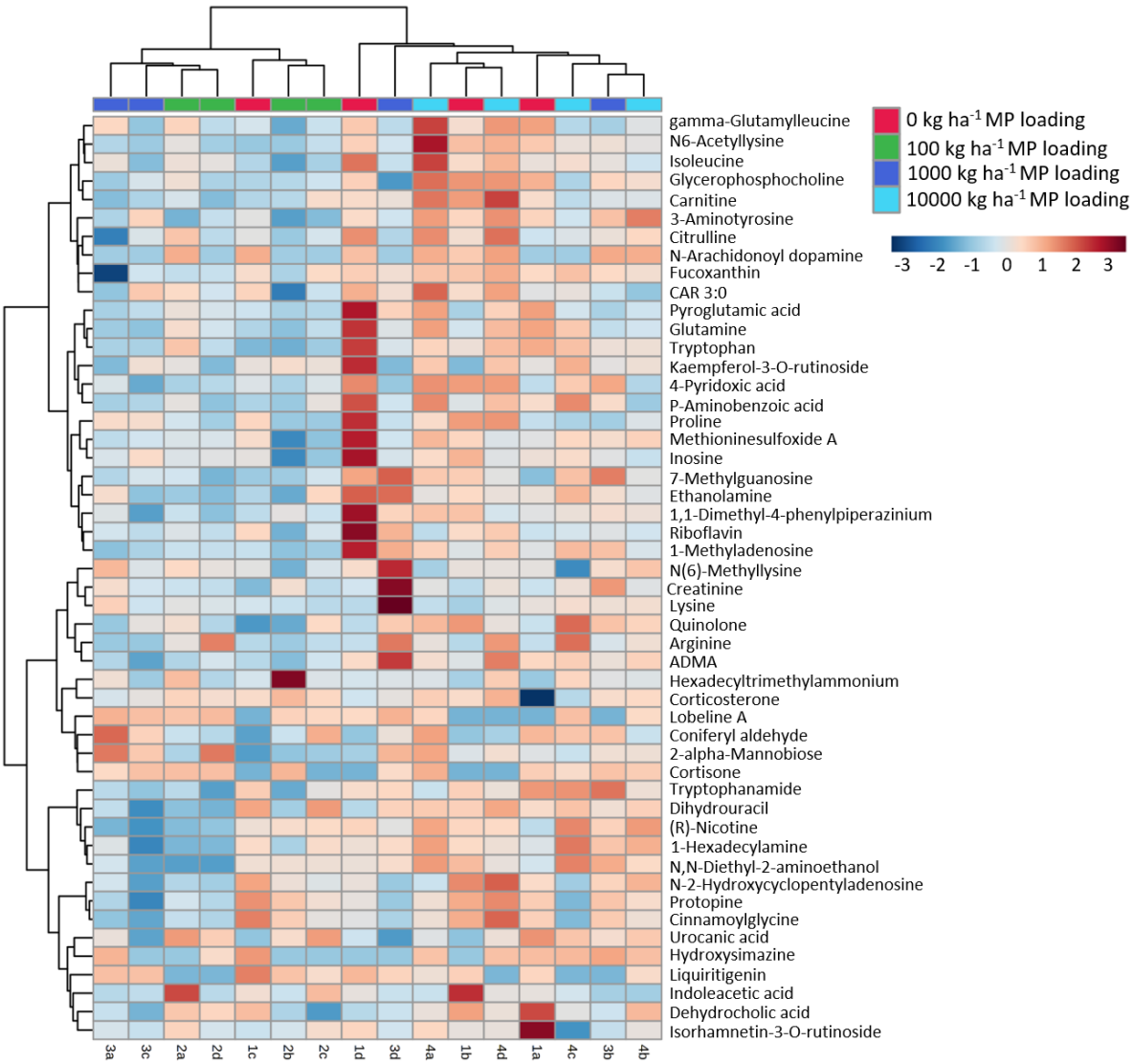
gamma-Glutamylleucine
N6-Acetyllysine
Isoleucine
Glycerophosphocholine
Carnitine
3-Aminotyrosine
Citrulline
N-Arachidonoyl dopamine
Fucoxanthin
CAR 3:0
Pyroglutamic acid
Glutamine
Tryptophan
Kaempferol-3-O-rutinoside
4-Pyridoxic acid
P-Aminobenzoic acid
Proline
Methioninesulfoxide A
Inosine
7-Methylguanosine
Ethanolamine
1,1-Dimethyl-4-phenylpiperazinium
Riboflavin
1-Methyladenosine
N(6)-Methyllysine
Creatinine
Lysine
Quinolone
Arginine
ADMA
Hexadecyltrimethylammonium
Corticosterone
Lobeline A
Coniferyl aldehyde
2-alpha-Mannobiose
Cortisone
Tryptophanamide
Dihydrouracil
(R)-Nicotine
1-Hexadecylamine
N,N-Diethyl-2-aminoethanol
N-2-Hydroxycyclopentyladenosine
Protopine
Cinnamoylglycine
Urocanic acid
Hydroxysimazine
Liquiritigenin
Indoleacetic acid
Dehydrocholic acid
Isorhamnetin-3-O-rutinoside
3a
3c
2a
2d
1c
2b
2c
1d
3d
4a
1b
4d
1a
4c
3b
4b
0 kg ha-1 MP loading
100 kg ha-1 MP loading
1000 kg ha-1 MP loading
10000 kg ha-1 MP loading
-3 -2 -1 0 1 2 3

Figure 7

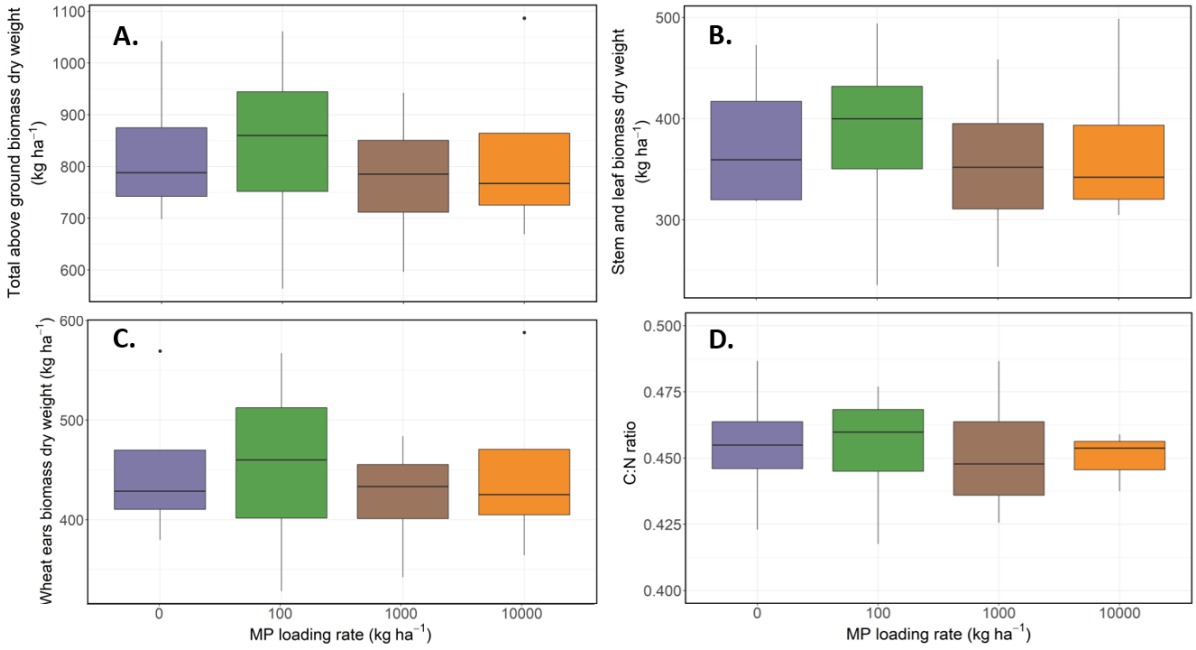
A.
B.
C.
D.
Total above ground biomass dry weight (kg ha^{-1})
Stem and leaf biomass dry weight (kg ha^{-1})
Wheat ears biomass dry weight (kg ha^{-1})
C:N ratio
MP loading rate (kg ha^{-1})
MP loading rate (kg ha^{-1})
0
100
1000
10000

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 application. Results are expressed on mean dry soil weight basis ± SEM (n = 4). Letters denote significant differences between treatments ($p < 0.05$).

MP loading rate (kg ha^{-1})	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± 0.04^{a}	6.23 ± 0.19^{a}	6.26 ± 0.14^{a}	6.23 ± 0.10^{a}	6.49 ± 0.04^{a}	6.34 ± 0.15^{a}	6.41 ± 0.12^{a}	6.47 ± 0.08^{a}	6.27 ± 0.11^{a}	6.16 ± 0.26^{a}	6.14 ± 0.11^{a}	6.09 ± 0.08^{a}
EC (µS cm^{-1})	129 ± 38^{a}	91 ± 13^{a}	123 ± 24^{a}	96 ± 22^{a}	37 ± 1.9^{a}	36 ± 2.6^{a}	31 ± 2.3^{a}	31 ± 3.5^{a}	55 ± 2.4^{a}	77 ± 25^{a}	55 ± 3.9^{a}	51 ± 2.6^{a}
NO_3^- (mg N kg^{-1})	67.4 ± 21.7^{a}	18.6 ± 4.6^{a}	33.4 ± 14.5^{a}	38.3 ± 0.70^{a}	5.04 ± 2.60^{a}	4.96 ± 3.02^{a}	1.86 ± 0.09^{a}	1.61 ± 0.14^{a}	10.4 ± 4.30^{a}	21.9 ± 9.32^{a}	15.5 ± 4.1^{a}	10.2 ± 1.08^{a}
NH_4^+ (mg N kg^{-1})	57.5 ± 16.7^{a}	11.0 ± 5^{a}	22.1± 10.9^{a}	45.8 ± 1.6^{a}	1.01 ± 0.06^{a}	1.11 ± 0.11^{a}	1.13 ± 0.05^{a}	0.89 ± 0.06^{a}	2.64 ± 0.30^{a}	5.36 ± 2.09^{a}	3.28 ± 0.88^{a}	3.00 ± 1.05^{a}
Bulk density (kg m^{-3})					1014 ± 11^{a}	1065 ± 27^{a}	984 ± 30^{a}	977 ± 31^{a}	1065 ± 22^{a}	1106 ± 48^{a}	1092 ± 44^{a}	1062 ± 61^{a}
Bacterial/Fungal PLFA ratio					0.11 ± 0.01^{ab}	0.11 ± 0.01^{ab}	0.11 ± 0.01^{ab}	0.14 ± 0.02^{a}	0.09 ± 0.00^{b}	0.10 ± 0.00^{ab}	0.11 ± 0.01^{ab}	0.10 ± 0.01^{ab}
Microbial PLFA biomass (µmol PLFA kg^{-1})					174 ± 11^{ab}	175 ± 9^{ab}	162 ± 3^{a}	190 ± 16^{ab}	199 ± 6^{ab}	201 ± 8^{ab}	197 ± 6^{ab}	218 ± 12^{b}
Earthworm biomass (g m^{-2})									92 ± 9^{a}	54 ± 6^{a}	71 ± 24^{a}	79 ± 22^{a}
Earthworm abundance (individuals m^{-2})									26 ± 5^{a}	13 ± 2^{a}	24 ± 13^{a}	20 ± 6^{a}

EC – electrical conductivity

1206