Changes of soil-rhizosphere microbiota after organic amendment application in a *Hordeum vulgare* L. short-term greenhouse experiment

Michael M. Obermeier · Eva-Maria L. Minarsch · Abilash C. Durai Raj · Francois Rineau · Peter Schröder

Abstract

Aims In order to counteract the enduring decreases in the quality of agricultural land, mechanistic studies for a more sustainable agricultural crop production were performed. They aimed to assess the effects of organic amendments in combination with mineral fertilizer on soil-rhizosphere microbiota and their influence on soil health and plant performance.

Methods In a short-term greenhouse experiment, the effects of pelletized spent mushroom substrate, with different combinations of biochar and mineral fertilizer, on agricultural soil and performance of *Hordeum vulgare* L were scrutinized. To evaluate improved soil quality, different soil biological and chemical properties, microbial activity, bacterial diversity and plant performance were assessed.

Results Plant performance increased across all fertilizer combinations. Bacterial β-diversity changed from the initial to the final sampling, pointing at a strong influence of plant development on the rhizosphere with increasing abundances of *Acidobacteria* and decreasing abundances of *Actinobacteria, Chloroflexi*, and *Bacteroidetes*. Microbial activity (FDA), potential enzyme activity and metabolic diversity of the microbial community (BIOLOG) were not affected by the amendments, whereas bacterial community structure changed on family level, indicating functional redundancy. Treatments containing biochar and the highest amount of mineral fertilizer (B_MF140) caused the strongest changes, which were most pronounced for the families *Xanthobacteraceae, Mycobacteriaceae,* and *Haliangiaceae*.

Conclusion Applying organic amendments improved plant performance and maintained soil health, contributing to more sustainable crop production. Nevertheless, long-term field studies are recommended to verify the findings of this short-term experiment.

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Abbreviations

ASV	Amplicon sequence variant
BSA	Bovine serum albumin
B	Biochar
Z	Carbon
Chl	Chlorophyll
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
dw	Dry weight
fw	Fresh weight
FACCE-JPI	Joint Programming Initiative on Agriculture, Food Security and Climate Change
FDR	False Discovery Rate
INTENSE	Intensify production, transform biomass to energy and novel goods and protect soils in Europe
JE Lange	Jakob Emanuel Lange
L	Linné
LAI	Leaf area index
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
MUG	β-glucosidase
MUN	β-N-acetylhexosaminidase
MUP	acid phosphatase
MF	Mineral fertilizer
N	Nitrogen
NA	Not assigned
NMDS	Non-metric multidimensional scaling
OECD	Organization for Economic Co-operation and Development
P	Pellets
PB10	Pellets +10% biochar
PB20	Pellets +20% biochar
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
SMS	Spent mushroom substrate
TDN	Total dissolved nitrogen
TNb	Total nitrogen bound
TPB	Total plant biomass
V	Version

Introduction

Since the beginning of the twentieth century, application of mineral fertilizers has expanded agricultural production and increased yields to feed a rapidly growing world population (Erisman et al. 2008). This agricultural intensification and the continuously increasing need of food, feed, fiber and byproducts exerts tremendous pressure on the Earth's soils and their functioning. Excessive use of mineral fertilizer has been proven to be detrimental for soil microbial biomass, soil habitat functioning, plant species diversity, plant and even human health (Geisseler and Scow 2014; Horrigan et al. 2002). To mitigate these negative effects and to make agriculture more sustainable, the application of organic amendments (OA) obtained via cascading, upgrading and recycling of bio-based products has found raising interest (SCAR-report 2015; Schröder et al. 2018). Its application can influence various physical and chemical soil properties such as nutrient availability, soil aeration, water holding capacity and moisture (Bonilla et al. 2012a; Haider et al. 2016). Moreover, biological properties can be affected as shown for the soil microbial community structure and changes in its quantity, diversity and activity (Albiach et al. 2000; Bonilla et al. 2012b; Schmid et al. 2017).

Organic amendments have been reported to induce various positive but also negative effects on soil health and plant performance (Gómez-Sagasti et al. 2018; Schröder et al. 2018). For instance, application of residues from industrialized mushroom production (spent mushroom substrates; SMS), containing a high proportion of slowly decomposable lignocellulose (Hanafi et al. 2018), has proven positive effects on soil structure, microbial abundance and plant yield (Alvarez-Martín et al. 2016; Meng et al. 2018; Zhang et al. 2012). Additionally, biochar obtained from pyrolysis of organic wastes shows high potential in improving soil water retention, regulating the soil nitrogen cycle and decreasing nitrogen leaching (Haider et al. 2016; Liu et al. 2018; Ulyett et al. 2014). Its porous microstructure seems favorable for the colonization by microorganisms (Lehmann et al. 2011; Palansooriya et al. 2019). Furthermore, biochar incorporation into soil facilitates carbon sequestration and thereby contributes to the mitigation of climate change effects (Matovic 2011). The application of biochar during composting can be used to adjust the C/N ratio of the amendment which later influences soil microbial activity. Combination of different organic amendments with distinct features can improve overall amendment quality and reduce greenhouse gas emissions already during the composting process (Meng et al. 2018; Barthod et al. 2018).

Soil microorganisms are major drivers of the biochemical carbon and nitrogen cycle, thereby playing a

crucial role for soil health, its functioning and hence for crop and livestock health (Falkowski et al. 2008). The nitrogen cycle comprises dinitrogen fixation, assimilation into organic nitrogen, mineralization, nitrification subsequent denitrification and anaerobic ammonium oxidation (Kuypers et al. 2018). Its fluxes are defined by adsorption, mineralization, gaseous losses, plant uptake, leaching and microbial N immobilization (Murphy et al. 2000). The latter is driven by the composition of the microbial community and increases with higher C/N ratios of the organic amendments (Heijboer et al. 2016). The chemical composition of organic amendments affects the balance between plant N uptake and soil N retention and is therefore essential for plant growth. Amendments containing complex organic compounds (e.g. lignocellulose) trigger soil extracellular enzyme activity (EEA) to degrade these into lower molecular weight compounds like sugars, amino acids or ammonium (Burns and Dick 2002; Allison and Vitousek 2005). Soil microbial community composition and enzyme activity is hence pivotal for health and fertility of soils and thus to maintain crop performance.

Short-term greenhouse and long-term field experiments have recorded positive as well as negative effects of organic amendments in different cropping and soil systems (Prendergast-Miller et al. 2014; Schmid et al. 2017; Zhao et al. 2016). This highlights the importance of evaluating organic amendments in a holistic approach to reveal and understand the underlying mechanisms. Microbial indicators defining and monitoring soil quality and health are already abundant but the right choice and combination of the various indices is still under debate (Schloter et al. 2018).

For this study the barley cultivar Ella (*Hordeum vulgare* L. cv. Ella) was selected since it showed promising grain weight per plant and grain yield per plot (Surma et al. 2019). To evaluate the effects of organic amendments in combination with mineral fertilizer on soil-rhizosphere microbiota and performance of barley, different biological and chemical indices were used. Soil pH, mineral nitrogen, dissolved organic carbon/nitrogen, microbial biomass carbon/nitrogen, microbial activity and bacterial composition were analyzed to assess soil quality as well as plant morphology together with shoot and root carbon/nitrogen to describe plant performance. Potential soil microbial activity was determined photometrically, and bacterial diversity was analyzed by 16S amplicon sequencing. Working hypotheses were: (1) input of organic amendments maintains soil quality

and improves plant performance, (2) input of organic amendments alone and in combination with mineral fertilizer changes microbial activity and bacterial community structure differently, (3) potential extracellular enzyme activity decreases after addition of mineral fertilizer (or increases by organic amendment addition), and (4) C/N ratios of the amendment combinations influence microbial N immobilization.

Material and methods

Soil and organic amendment characteristics

Soil for the greenhouse experiment was collected at Martlhof, in Ostin am Tegernsee (Bavaria, Germany) from the topsoil (0–20 cm) of a former study site for sustainable field management. A previous study (Obermeier et al. 2020) had focused on crop rotation, following the conversion of neglected grassland, using broad bean (*Vicia faba* L.) and fodder beet (*Beta vulgaris* L.). Average soil pH at the site ranged from 5.2–5.6 and its texture had been classified as clayey loam (28.2% sand, 43.1% silt and 28.8% clay). Solid organic amendments were applied to the pots as pellets (P, PB10 and PB20) and biochar granules (B) listed in Table 1. The pellets were produced by conventional composting and subsequent pelletizing of 50% spent mushroom substrate with 30% bio-rest from biogas production and 20% straw at a temperature of 59 °C and a compost humidity of 20–23% (pers. Comm. Prof. W. Szulc, Warsaw University of Life Sciences, Poland). Spent mushroom substrate was obtained after cultivation of *Agaricus bisporus* (JE Lange) Imbach. Additionally, 10 and 20% biochar was added to the pellets (PB10, PB20). Biochar had been produced from conifers and broadleaf trees through pyrolysis at 800 °C (Martlhof, Germany). It has a pH of 8.5 ± 0.1, organic

Table 1 Chemical composition of organic amendments. Abbreviations for the amendments used within the study, total nitrogen (N_{tot}) and total carbon (C_{tot}) in percentage and C/N ratio

	Organic amendment	N_{tot} [%]	C_{tot} [%]	C/N
P	Pellet	1.48	21.28	14
PB10	Pellet +10% Biochar	1.46	25.28	17
PB20	Pellet +20% Biochar	1.32	30.21	23
B	Biochar	0.23	71.19	310

matter content of $91.9 \pm 5.0\%$ and dry matter content of $81.8 \pm 2.4\%$ (pers. Comm. Prof. E. Maestri, University of Parma, Italy).

Experimental setup

Fertilization scheme

Thirteen combinations of organic amendments alone and in combination with mineral fertilizer were tested (Table S1). The initial soil contained 60 kg N ha^{-1} and was fertilized up to 200 kg N ha^{-1}. Organic amendments were applied to reach equal C_{tot} contents and a maximum of 140 kg N_{tot} ha^{-1}, except for treatment PB10N and PB20N. Here, 140 kg N_{tot} ha^{-1} were applied despite the higher C_{tot} content compared to the latter treatments. Calcium ammonium nitrate (CAN, Borealis L.A.T. GmbH, Linz, Austria) containing 27% nitrogen (1:1 nitrate and ammonium) was applied as mineral fertilizer in two conditions. First, to obtain maximum fertilization of 140 kg N_{tot} ha^{-1} (MF140) since due to C_{tot} equality, the treatments containing biochar were short in nitrogen, which was supplied by mineral fertilizer. Second, by adding 50 kg N_{tot} ha^{-1} (MF50) referring to a common fertilization practice according to local farmers. All calculations are in kilogram per hectare and refer to 30 cm soil depth and a bulk density of 1.5 t m^{-3}.

Setup and management

Plants were grown for 8 weeks until the majority reached the first nodal stadium (BBCH 31). Each of the 13 treatments and untreated controls were set up in four biological replicates, resulting in 56 independent pots. Standardized PVC DN 110 pipes (height 0.5 m) were sealed with plugs, drained with 0.7 kg crystal quartz sand (2–3.5 mm) and filled with 4.2 kg soil. To the upper 30 cm soil layer ground solid organic amendments were applied. Four spring barley seeds (*Hordeum vulgare* L. cv. Ella) per pot were sown (DANKO Hodowla Roślin Sp. z o.o., Kościan, Poland). When plants reached the two leaf stadium (BBCH 12), in the second week of the experiment, two seedlings were carefully removed and dissolved mineral fertilizer was applied. Pots were watered twice a week to obtain 60% water holding capacity. Throughout the experiment, pots were randomized and plant growth was supported by sodium-vapor lamps and a ceiling fan. The

management and sampling scheme is given in Supplementary Fig. S1.

Sampling

Sampling was performed in the initial phase (week 1; initial) as well as in week 2 of the experiment, 4, 6 and finally during harvesting (week 8; final). Soil sampling to analyze soil properties and bacterial composition was performed at the initial ($n = 4$) and final sampling ($n = 56$) resulting in 60 independent soil samples. Five sub-samples from each pot were taken in various depths, pooled, homogenized and subsequently sieved (2 mm) for later analysis. Due to the root architecture and extent of the rhizosphere the final soil samples comprised a mixture of rhizosphere and bulk soil and are thus defined as soil-rhizosphere. Soil samples (< 1 g) taken in week 2, 4 and 6 for extracellular enzyme activity measurements were collected from the upper 10 cm in the center of each pot to avoid disturbance of plant development. Soil material for chemical, enzyme and bacterial diversity analysis was stored at 4, −20 and − 80 °C, respectively. Shoots and roots of each pot were harvested separately. The phenological development stage of plants was determined weekly following the BBCH system according to Bleiholder et al. (2001). Chlorophyll content was measured during the second half of the experiment using a Dualex® Scientific Dx4.5 sensor (FORCE-A, Orsay, France).

Soil chemical analysis

Mineral nitrogen (N_{min}), dissolved organic carbon (DOC), and dissolved organic nitrogen (DON) were extracted from soil using 0.01 M CaCl$_2$ (1:4 w/v). Samples were shaken overhead using a horizontal shaker (Reax 2, Heidolph Instruments GmbH, Schwabach, Germany) at room temperature for 45 min and filtered through Whatman® filter type 595 1/2 (GE Healthcare, Buckinghamshire, UK). N_{min} as ammonium (NH_4^+), nitrate (NO_3^-) and total dissolved nitrogen (TDN) were analyzed photometrically following published methods (Obermeier et al. 2020) by continuous flow measurements using an autoanalyzer CFA-SAN Plus 5100 (Skalar Analytic, Erkelenz, Germany). DOC and DON were quantified using a DIMATOC®2000 (DIMATEC, Langenhagen, Germany). The gravimetric soil water content was determined after drying samples at 105 °C for 24 h. Soil pH was assessed in a 1:5 (w/v) dilution

with 0.01 M $CaCl_2$ following OECD guidelines (DIN ISO 10390 2005).

Plant analysis

Immediately after harvesting, barley leaves were scanned on an Epson Perfection 4180 Photo scanner (Epson®, Seiko, Japan) to determine the leaf area index (LAI). The green pixel content was analyzed with MATLAB® (The MathWorks® Inc., Natick, United States). The gravimetric water content of the plant material was determined after drying at 60 °C for 24 h. Total carbon (C_{tot}) and total nitrogen (N_{tot}) content of dried leaves and roots were determined after grinding them in a mixer mill (MM 400, Retsch®, Haan, Germany) and following combustion in an elemental analyzer (Euro EA, Eurovector Srl, Pavia, Italy).

Soil microbial analysis

Microbial biomass

Microbial biomass carbon (MBC) and nitrogen (MBN) were determined after chloroform fumigation of 5 g fresh soil with ethanol-free chloroform in a desiccator for 24 h (Brookes et al. 1985; Vance et al. 1987). Extraction and measurement of DOC and DON was performed as described above for soil chemical analysis (Joergensen 1995). MBC and MBN were calculated using k_{EC} 0.45 and k_{EN} 0.54 (Joergensen and Mueller 1996), respectively.

Microbial activity

Potential extracellular enzyme activities (EEA) were determined according to Pritsch et al. (2005). In short, methylumbelliferone (MU)-labeled substrates (Sigma-Aldrich, St. Louis, United States) in opaque 96-well plates (VWR™, Darmstadt, Germany) were used, and 50 μl soil suspension (400 mg soil in 40 mL sterile Milli-Q water mixed for 15 min and 22–25 μm filtered) was incubated in triplicates with 100 μl of the respective substrate saturation solution (see Pritsch et al. 2004). The substrate concentration and incubation time for each substrate/corresponding enzyme was determined in a pre-experiment as follows: MU-β-D-glucopyranoside/β-glucosidase (MUG, EC 3.2.1.21) 600 μM and 60 min, MU-N-acetyl-β-D-

glucosaminide/β-N-acetylhexosaminidase (MUN, EC 3.2.1.52) 100 μM and 60 min and MU-phosphate/acid phosphatase (MUP, EC 3.1.3.2) 600 μM and 40 min. The enzyme reaction was stopped by adding 100 μl 1.25 M Tris buffer (pH > 10) and the plate was centrifuged for 3 min at 2420 rpm. Fluorescence was measured 20 min after reaction termination at excitation 365 nm and emission 450 nm wavelengths using a SpectraMax® Gemini™ EM microplate reader (Molecular Devices, Ismaning, Germany). A MU calibration curve (0, 1, 2, 3, 4, 5, 6 and 7 μM MU in Milli-Q water) and a soil quenching control (4 μM MU in soil suspension) were performed for each run. The maximum activity is expressed in picomol MU per gram dry soil per hour (pmol MU g^{-1} dw h^{-1}) according to German et al. (2011).

16S sequencing library preparation

DNA for sequencing was extracted from 500 mg soil using the FastDNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, United States). Negative controls were introduced using empty extraction tubes. Quantification of extracted DNA was done by Qubit™ 4 Fluorometer and Qubit™ ds DNA Broad Range (BR) Assay Kit (Invitrogen™, Waltham, United States). Quality was assessed in a NanoDrop™ 1000 spectrometer (PeQlab Biotechnoloy, Erlangen, Germany). The 16S rRNA gene was amplified in the V1-V2 region using the primers S-D-Bact-0008-a-S-16 (5'-AGAGTTTG ATCMTGGC-'3) and S-D-Bact-0343-a-A-15 (5'-CTGCTGCCTYCCGTA-'3) (Klindworth et al. 2012). Therefore triplicated PCR reactions were performed using the NEBNext High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, United States) and 5 pM of each primer, 3% bovine serum albumin (BSA), molecular grade water and 1 ng of extracted DNA. The PCRs were performed with the following program (see Obermeier et al. 2020): initial denaturation (98 °C, 30 s), followed by 28 cycles of denaturation (98 °C, 10 s), annealing (60 °C, 30 s) and elongation (72 °C, 30 s) and ended with a final elongation (72 °C, 5 min). PCR controls were performed under the same conditions and the quality of amplicons was visually assessed on a 1% agarose gel. Pooled samples of the three independent PCR reactions were purified using an Agencourt® AMPure® XP kit (Beckman Coulter Inc., Webster, United States) according to the manufacturers protocol for 96-well plates and with 1.3X the volume of the

sample for the beads. Quantification and quality of purified amplicons was assessed with a Fragment Analyser™ (Advanced Analytical Technologies GmbH, Heidelberg, Germany) using the DNF-473 Standard Sensitivity NGS Fragment Analysis Kit (1–6000 bp). The amplified and purified DNA of each sample was indexed using 10 ng and the Nextera® XT Index Kit v2 (Illumina Inc., San Diego, United States) with NEBNext High-Fidelity 2X Master Mix and molecular grade water resulting in 60 amplicon libraries. The indexing PCR program comprised an initial denaturation (98 °C, 30 s), followed by 8 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 30 s) and elongation (72 °C, 30 s) and ended again with a final elongation (72 °C, 5 min). The indexed amplicons were checked and purified as described above. For sequencing on a MiSeq System with a read length of 2*300 bp (Illumina Inc., San Diego, United States) the amplicons were diluted to 4 nM, pooled equimolar and 11 pM DNA was loaded. The MiSeq Reagent Kit v3 (600 cycles) was used according to the manufacturer's protocol for paired-end sequencing and as a spike-in PhiX (Illumina Inc., San Diego, United States) was used as positive control.

Processing of the sequencing data

Primer and adapter removal of the de-multiplexed raw data obtained from the MiSeq system were performed with the AdapterRemoval software V. 2.1.7 (Lindgreen 2012), and amplicon sequencing errors were corrected using the model-based approach of the R package DADA2 V 1.8.0 (Callahan et al. 2016). Ten quality plots of forward and reverse reads were investigated, and accordingly quality filtering with a maximum expected error of three and a minimum read quality of two was performed. Forward and reverse reads were trimmed at 10 and 250 bp and 10 and 200 bp, respectively. Remaining contaminations by PhiX sequences were removed during quality filtering. Error modelling of reads and denoising of data was performed. This step comprised merging of paired-end reads and generation of a raw amplicon sequence variants (ASV) table and a chimera-cleaned sequence table. Finally, the ASVs were taxonomically annotated against the SILVA database V. 132 (Quast et al. 2013). Raw sequence data was imported into R V. 3.5.2 (R Core Team 2018). Filtering was performed by removing reads of negative controls, ASVs not assigned to bacteria and archaea as well as

ASVs assigned to chloroplasts, mitochondria and ASVs singletons. The filtered sequence data was subsampled to the lowest read count over all samples using the rarefy function of the vegan package V. 2.5–4 (Oksanen et al. 2019). The raw nucleotide sequence data are available in the NCBI Sequence Read Archive (SRA) (Leinonen et al. 2010) under the BioProject accession number PRJNA540756.

16S sequencing data

Sequencing followed previously established procedures (Obermeier et al. 2020) and resulted in a total of 9.22 million raw reads of which 6.81 million (73.9% of total raw reads) remained after quality filtering, 6.33 million (68.6% of total raw reads) after denoising of forward and reverse reads, 5.15 million (55.8% of total raw reads) after merging and 5.10 million (55.3% of total raw reads) after chimera removal. The clearing of negative control reads, ASVs not assigned to bacteria or archaea (eukaryota and NA), ASVs assigned to chloroplasts or mitochondria and ASV singletons resulted in 5.04 million reads (54.6% of total raw reads). According to the rarefaction curves (Supplementary Fig. S5) all samples showed sufficient coverage of the bacterial community and subsampling resulted in 45,750 reads per sample. The final sequence data contained 2.74 million reads (29.8% of total raw reads) with 15,087 ASVs (97.1% of 15,531 raw ASVs). In total 33 phyla, 86 classes, 162 orders, 228 families, 463 genera and 61 species were aligned.

Statistical analysis

Statistical analysis of soil, plant and microbial data was conducted using R V. 3.5.2. One-way independent ANOVA ($p < 0.05$) and Kruskal-Wallis tests for not normal distributed data were performed using basic R functions to find differences between variants, following the procedure of Obermeier et al. (2020). The term variant includes the initial soil and the final soils comprising all treatments and controls. Normality of data and homogeneity of variance within each group was assessed by Shapiro-Wilk test and Bartlett test, respectively. Pearson and Spearman rank correlation tests for normal and not normally distributed data, respectively, were used to analyze shared variation between measurements to reveal correlations between soil, plant and microbial data. Shared variation was calculated as the

coefficient of determination (r^2) and is given in percentage (Field et al. 2012). Multiple comparisons of soil, plant and microbial data were performed using Tukey's honestly significant difference (HSD) post-hoc test in conjunction with a multivariate ANOVA ($p < 0.05$) using the agricolae package V. 1.3.0 (De Mendiburu 2014).

Subsampled sequencing data was analyzed using the phyloseq package V. 1.24.2 (McMurdie and Holmes 2013). To reveal effects of the different treatments on the bacterial composition bacterial α-diversity was calculated based on Shannon diversity index as well as Pielou's evenness index for species evenness. Bacterial β-diversity was analyzed by ordination using a multivariate principal coordinate analysis (PCoA) method based on Bray-Curtis dissimilarities. To confirm the results of the PCoA analysis a permutational multivariate analysis of variance (PERMANOVA) was performed using the vegan package. Relative abundances and standard deviations on different taxonomic levels were analyzed to indicate effects of different variants as well as the homogeneity of the biological replicates. In addition to the multiple comparisons performed with Tukey's post-hoc test, significant differences among treatments (FDR < 0.05) were checked using edgeR and DESeq2 analyses (Chong et al. 2020; Dhariwal

et al. 2017). Associative relationships of plant and soil parameters to the most abundant bacterial families were identified using univariate ANOVA ($p < 0.01$).

Results

Plant performance

Aboveground plant biomass was different among treatments within the experiment (F(13,42) = 3.60, $p < .001$, $\omega = .61$). All treatments resulted in an increase in total plant biomass (TPB) compared to controls (Fig. 1). No significant difference was observed when OA had been applied alone compared to OA with MF (e.g. PB10 to PB10_MF140). Fertilization with pellets (P) alone and in combination with mineral fertilizer (P_MF50) resulted in the highest TPB significantly different to controls. In contrast, some treatments containing biochar (e.g. PB20_MF140, B_MF140, PB20_MF50 and B_MF50) resulted in a lower increase of TPB exhibiting a negative correlation with a small shared variation of 6% (Supplementary Fig. S2). In addition, a positive correlation with 87% shared variation for TPB and the leaf area index (LAI) as well as a negative correlation with 23% shared variation of TPB with nitrate-N were

Fig. 1 Total plant biomass (TPB) in g *dw* given for the 14 treatments (*n* = 8). Gray dashed line marks median of the control. Different letters (a, b, and c) indicate significant differences (*p* < 0.05) calculated with multivariate ANOVA (Tukey's post-hoc test)
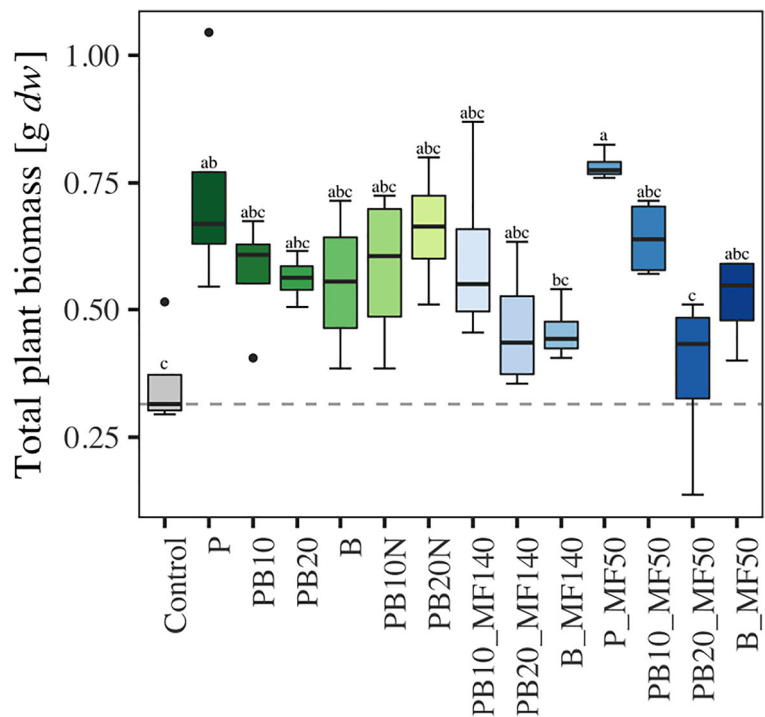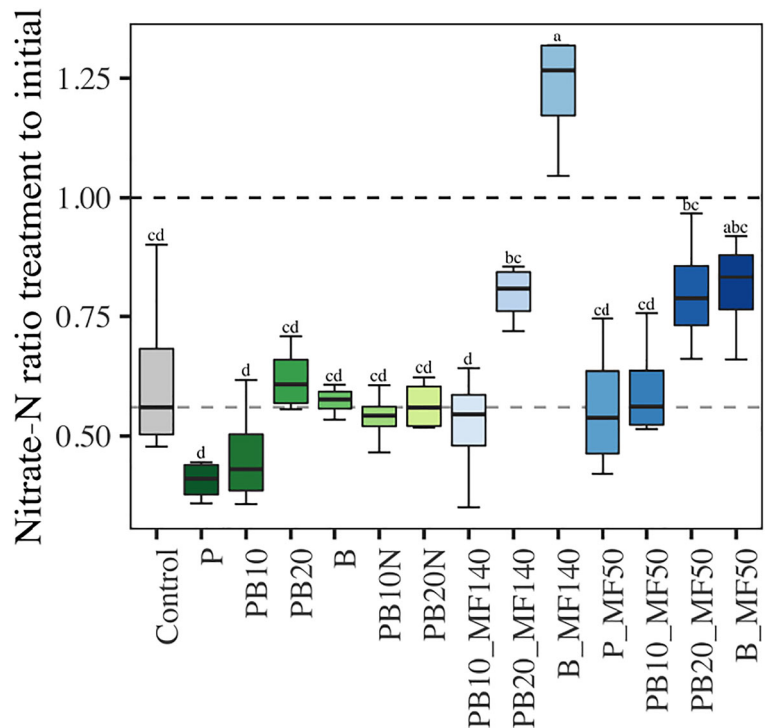
observed (Supplementary Fig. S2). Chlorophyll content, total carbon and nitrogen content of shoots and roots did not differ within the experiment (Supplementary Table S2).

Soil characteristics

Soil chemical analysis showed differences for nitrate-N $(F(14,45) = 12.71, p < .001, \omega = .86)$, TDN $(F(14,45) = 10.03, p < .001, \omega = .82)$ and DON $(chi^2(14) = 32.77, p = .003)$. A significant decrease of up to 50% in nitrate-N between the initial soil and the final soil samples was observed (Fig. 2). In contrast, treatment B_MF140 led to an increase in nitrate-N compared to the initial soil and resulted in the only significant difference compared to controls. In general, treatments containing higher amounts of biochar and MF (PB20_MF140, B_MF140, PB20_MF50, and B_MF50) resulted in higher nitrate-N in the final soil mixture compared to treatments with only low amounts of biochar and MF (e.g. P, PB10, PB10_MF140, P_MF50, and PB10_MF50). A positive correlation with 31% shared variation for the amount of biochar applied to nitrate-N was found (Supplementary Fig. S2). DON and TDN showed a shared variation of 61% $(r = 0.78, p < .001)$

and its ratios reflected the variability seen for nitrate-N but without significant decrease (Supplementary Fig. S3). Similar to nitrate-N, treatment B_MF140 resulted in a significant increase compared to both, control and initial soil. Other soil quality parameters, including pH_{CaCl2} with on average 5.1 ± 0.13, which was lowest in treatment B_MF140 (5.0 ± 0.08) as well as DOC and ammonium-N remained stable within this experiment (Supplementary Table S2).

Soil microbial activity

Microbial biomass carbon and nitrogen were on average 587.6 ± 85.6 μg g^{-1} dw and 62.1 ± 20.5 μg g^{-1} dw, respectively. Due to the high variability within replicates no significant difference between treatments, controls or initial soil was observed. With 10.3 ± 3.4 the microbial biomass C/N ratio remained constant within the experiment (Supplementary Table S2).

The potential overall microbial activity analyzed by FDA hydrolysis did not differ between treatments and controls. Carbon metabolization was different regarding average well color development (AWCD) between some treatments (highest for P_MF50 and lowest for P and PB20_MF140) but not compared to controls

Fig. 2 Soil nitrate-N ratio of the 14 treatments (n = 4) compared to the mean of the initial soil. Black dashed line marks ratio of 1.0 and indicates no difference. Gray dashed line marks median of the control. Different letters (a, b, c, and d) indicate significant differences (p < 0.05) calculated with multivariate ANOVA (Tukey's post-hoc test)

(Supplementary Fig. S4). Carbon metabolic functional diversity and evenness, presented as Shannon and Pielou's evenness index, were not significantly different between treatments including controls (Supplementary Table S3).

The maximum potential activities of β-glucosidase, acid phosphatase and β-N-acetylhexosaminidase were not significantly different between treatments including controls within the four sampling time points (d0, d14, d28, and d42) (Supplementary Table S3). However, significant differences were observed across all treatments for the average maximum potential activity of the three enzymes regarding sampling time (Fig. 3). Highest activity was measured in week 4, 14 days (d14) after mineral fertilizer application and lowest activity in week 8 (d42).

Bacterial community structure

Diversity analysis

Bacterial α-diversity (Shannon) and species richness were not significantly different throughout the experiment (Supplementary Fig. S6). However, a significant effect was observed for Pielou's species evenness as shown in Fig. 4 ($F_{(14, 45)} = 3.66$, $p < .001$, $\omega = .62$). Highest evenness was observed for the initial soil and lowest for treatment B_MF140. Controls showed high variance and did not differ significantly from treatments and the initial soil. None of the diversity measurements revealed any correlation with soil and plant parameters.

The observed difference in evenness was also explained by bacterial β-diversity assessed with PCoA and sampling as major factor (Fig. 5). The factor sampling comprised the two groups initial and final, with the latter clustering the fourteen different treatments including the control. The factor was proven to be a significant determinant of bacterial community structure in a permutation test ($F_{(1, 58)} = 4.48$, $p < .001$).

Differences in relative abundance between samplings

The six most abundant phyla within the greenhouse experiment were *Proteobacteria, Acidobacteria, Actinobacteria, Gemmatimonadetes, Chloroflexi,* and *Bacteroidetes* in decreasing order (Fig. 6). Together
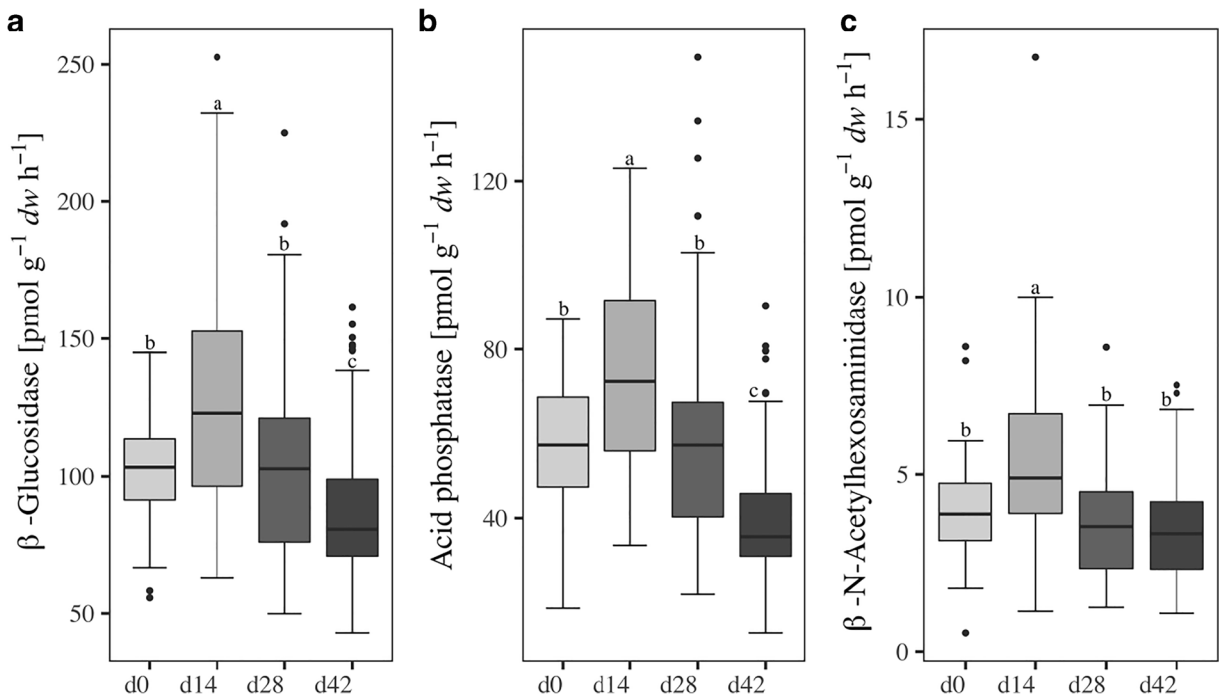

Fig. 3 Maximum potential activity of **a** β-glucosidase, **b** acid phosphatase and **c** β-N acetylhexosaminidase in pmol MU g^{-1}dw h^{-1} on average ($n = 56$) for the different sampling times in days (d0, d14, d28, and d42). Different letters (a, b, and c) indicate significant differences ($p < 0.05$) calculated with multivariate ANOVA (Tukey's post-hoc test)

Fig. 4 Bacterial α-diversity within the experiment illustrated as Pielou's species evenness index calculated for the 15 variants ($n = 4$). Gray line marks median of the initial soil. Different letters (**a**, **b**, and **c**) indicate significant differences ($p < 0.05$) calculated with multivariate ANOVA (Tukey's post-hoc test)
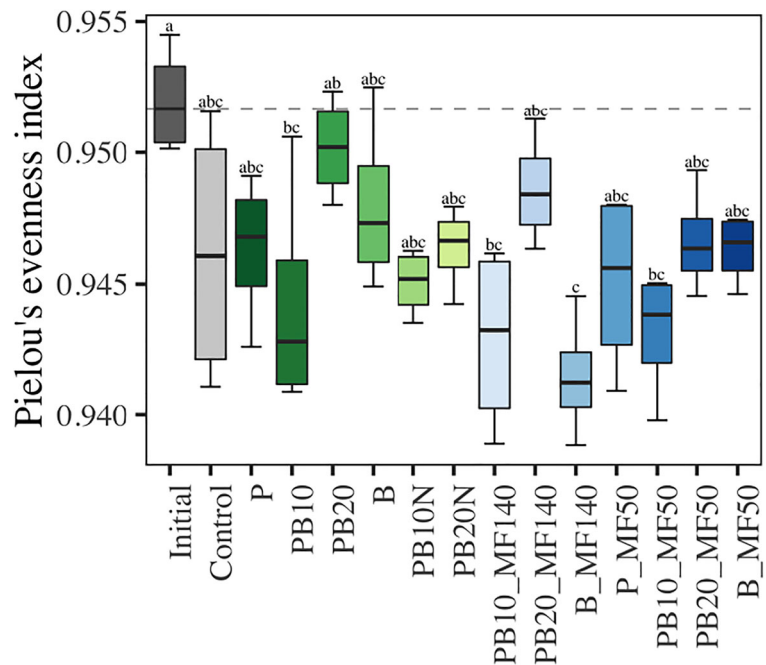
these phyla accounted for $90.4 \pm 9.1\%$ of the total bacterial community structure. With an averaged relative abundance of $36.3 \pm 2.2\%$, *Proteobacteria* was the predominant phylum within the experiment. However, also *Acidobacteria* ($23.0 \pm 2.2\%$), *Actinobacteria* ($19.0 \pm 2.5\%$), *Gemmatimonadetes* ($6.0 \pm 0.5\%$), *Chloroflexi* ($3.1 \pm 1.1\%$) and *Bacteroidetes* ($3.1 \pm 0.6\%$) showed high abundances for both sampling times and across

treatments. Similar to the *β*-diversity analysis the most pronounced differences were found between initial and final sampling. This was mainly driven by the increase of *Acidobacteria* and the decreasing abundances of *Actinobacteria, Chloroflexi*, and *Bacteroidetes* in the final samples. No significant differences among treatments at the final state of the experiment for the six most abundant phyla were observed. Further analyses using
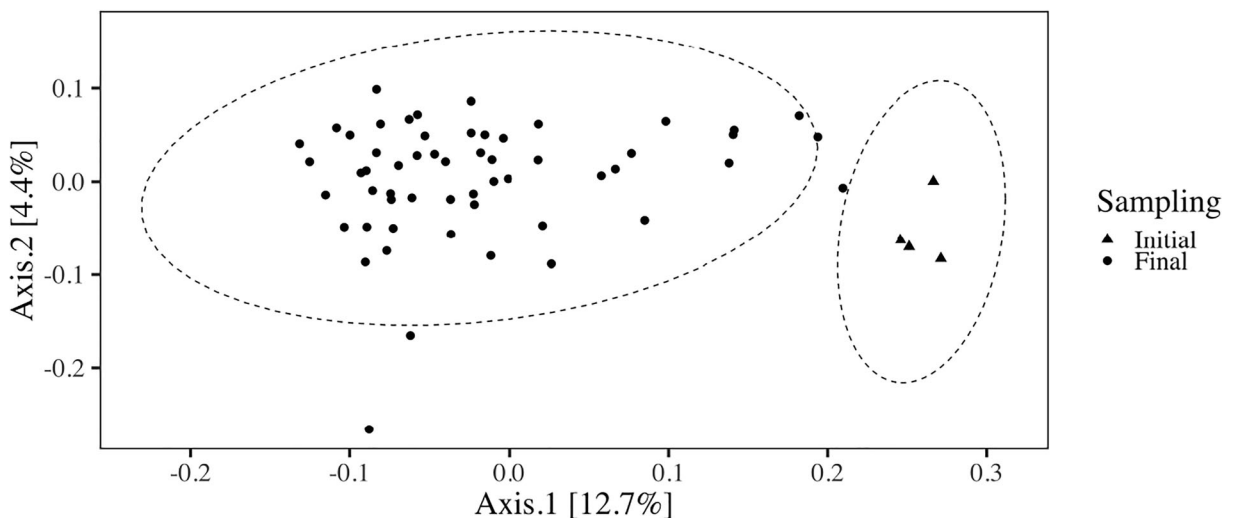

Fig. 5 Bacterial *β*-diversity presented with principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarities. Shown are ellipses on 95% confidence level for separation of bacterial community structure according to the factor sampling with initial (triangles) and final (dots). Axis 1 and 2 account for 12.7 and 4.4% of the variation, respectively
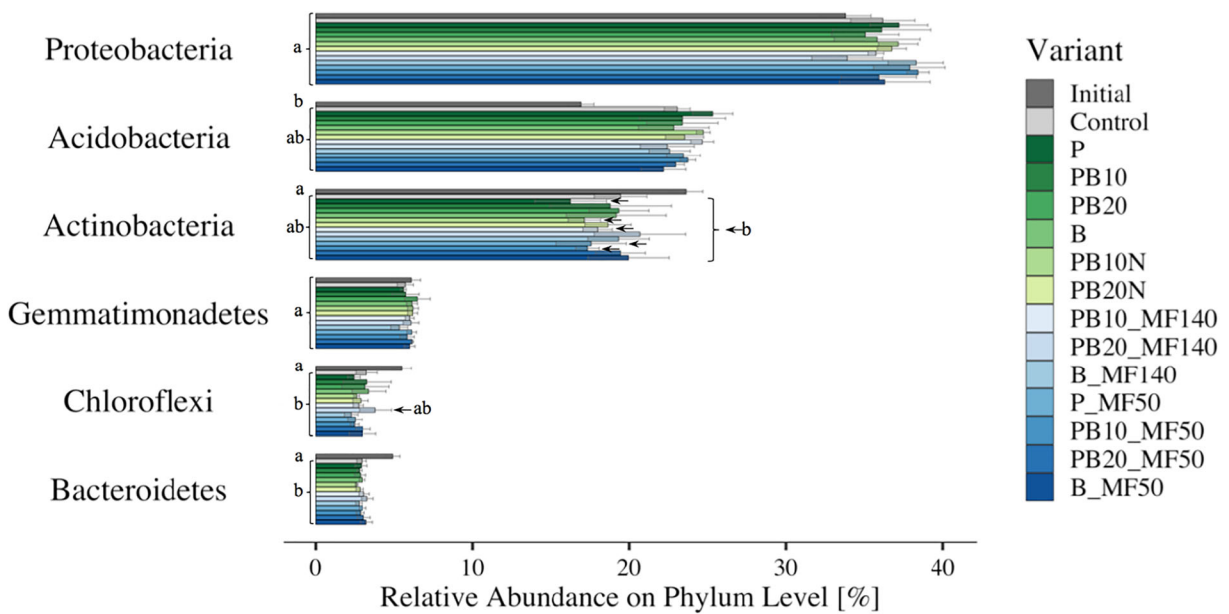
Fig. 6 Mean relative abundances for the six most abundant phyla observed for the 15 variants (*n* = 4) in percent. Different letters (**a** and **b**) indicate significant differences (*p* < 0.05) calculated with multivariate ANOVA (Tukey's post-hoc test)

edgeR and DESeq2 did also not reveal any significant differences among treatments (FDR < 0.05), even for rare phyla.

Although *Proteobacteria* did not show any change on phylum level, changes on class level could be observed (Supplementary Fig. S7). *Alphaproteobacteria,* the most abundant class, resulted in increasing and *Gammaproteobacteria,* the second most abundant class, in decreasing abundances at the final sampling. The increase of *Alphaproteobacteria* was mainly influenced by *Xanthobacteraceae,* the most abundant family of the experiment (Supplementary Table S4). The decrease of *Gammaproteobacteria* was mainly caused by a 23.7 ± 12.9% decrease of *Nitrosomonadaceae*, the third most abundant family (Fig. 7).

Besides *Nitrosomonadaceae,* the most pronounced decrease on family level compared to the initial soil was observed for *Chitinophagaceae* (30.6 ± 7.8%), *Nitrospiraceae* (27.0 ± 13.2%), *Xanthomonadaceae* (37.8 ± 14.8%), and *Burkholderiaceae* (57.7 ± 6.6%) (Supplementary Table S5). On the contrary, the most pronounced increase compared to initial was observed for *Xanthobacteraceae* (38.0 ± 15.1%), *Mycobacteriaceae* (57.6 ± 21.1%) and *Pyrinomonadaceae* (98.4 ± 37%). Abundances of *Gemmatimonadaceae* and *Gaiellaceae* remained almost constant within the experiment.

Differences in relative abundance between treatments

Although the most pronounced difference in bacterial abundance was observed between initial and final soils, also minor changes were seen between treatments at the final sampling. Especially treatment B_MF140 was striking, exhibiting highest relative abundances for *Xanthobacteraceae* (12.4 ± 1.0%) and *Mycobacteriaceae* (2.6 ± 0.3%) and lowest for *Haliangiaceae* (1.0 ± 0.1%) at the final sampling (Supplementary Table S4). On the contrary, treatment PB20_MF140 outlined lowest relative abundances for *Xanthobacteraceae* (9.6 ± 0.9%) and *Mycobacteriaceae* (1.9 ± 0.2%) across all treatments. Similar trends were shown for the ratio of the respective treatments compared to initial as visualized in Fig. 7. No significant differences across different fertilizer combinations (treatments) could be observed for *Gemmatimonadaceae, Nitrosomonadaceae, Solirubrobacterales (67–14), Gaiellaceae, Chitinophagaceae, Nitrospiraceae, Pyrinomonadaceae,* and *Burkholderiaceae* at the final sampling (Supplementary Table S5). Additional in-depth analyses among treatments using edgeR including all families, revealed significant differentiation (FDR < 0.05) for ten families with only three of them being members of the 12 most abundant ones
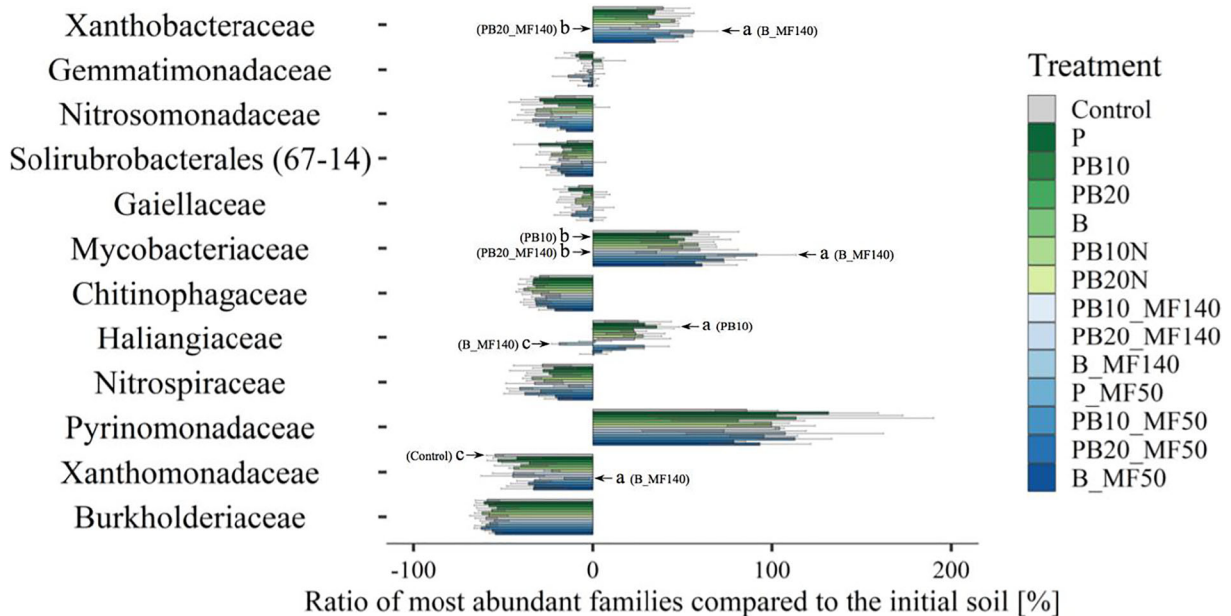
Fig. 7 Relative abundance ratio of the 12 most abundant families compared to the mean of the initial soil for the 14 different treatments ($n = 4$). Arrows and letters (**a**, **b** and **c**) indicate significant differences among the treatments ($p < 0.05$) calculated with multivariate ANOVA (Tukey's post-hoc test) for the highest and lowest abundances. Statistical data not included in the plot is given in Supplementary Table S5

(Supplementary Table S6). Similar to the pattern observed for relative abundance analysis and ratio plot (Fig. 7) the majority of families (7 out of 10) revealed the most pronounced difference for treatment B_MF140 compared to the other treatments.

Relationships of soil and plant parameters to the most abundant families at the final sampling

Highly significant associations ($p < 0.01$) of eight soil and plant parameters with the 12 most abundant families at the final sampling were observed (Fig. 8). The majority of families exhibited an associative relationship to microbial carbon (MBC) and nitrogen (MBN) at the final sampling time. Only *Gaiellaeceae*, *Haliangiaceae*, and *Xanthomonadaceae* did not show any association to MBC and MBN. Nevertheless, *Gaiellaeceae* showed a relationship to TDN and *Xanthomonadaceae* to nitrate-N. The family *Haliangiaceae* was associated not only to TDN and nitrate-N but also to the plant parameters TPB and LAI. Another association to those plant parameters was only found for unclassified bacteria *67–14* of the order *Solirubrobacterales*. Even though the maximum potential activity of β-glucosidase (MUG) and acid phosphatase (MUP) was weakest at final sampling

(Fig. 3), an association to the families *Pyrinomonadaceae*, *Burkholderiaceae* and *Nitrosomonadaceae* (for MUP only) was found.

Discussion

Stable soil quality and intensified plant performance

Effects of organic amendments and mineral fertilizer on soil-rhizosphere microbiota are known to directly influence soil quality and plant health. Since soil quality cannot be measured directly, different fast responding chemical and biological indicators had to be assessed within this study. Soil total carbon and soil structure analyses were not performed within this pot study due to the short-term experimental design.

The most variable soil quality indicators were nitrate-N, TDN and DON. Decreases of nitrate-N in most of the treatments compared to the initial soil indicated nitrate uptake by the plant, which was also proven by the negative correlation of total plant biomass (TPB) to nitrate-N. Even though nitrate-N was reduced in soil by plant uptake, TDN and DON remained almost constant during the experiment, indicating a stable pool of

Fig. 8 Heatmap showing associations between the 12 most abundant families (decreasing order) to the 60 different soil and plant parameters taken at the final sampling. Associative relationships (darkred) with $p < 0.01$ (univariate ANOVA) are considered significant and those with $p > 0.01$ are considered insignificant (white). Shown are only those soil and plant parameters sharing at least two significant p-values with the most abundant families
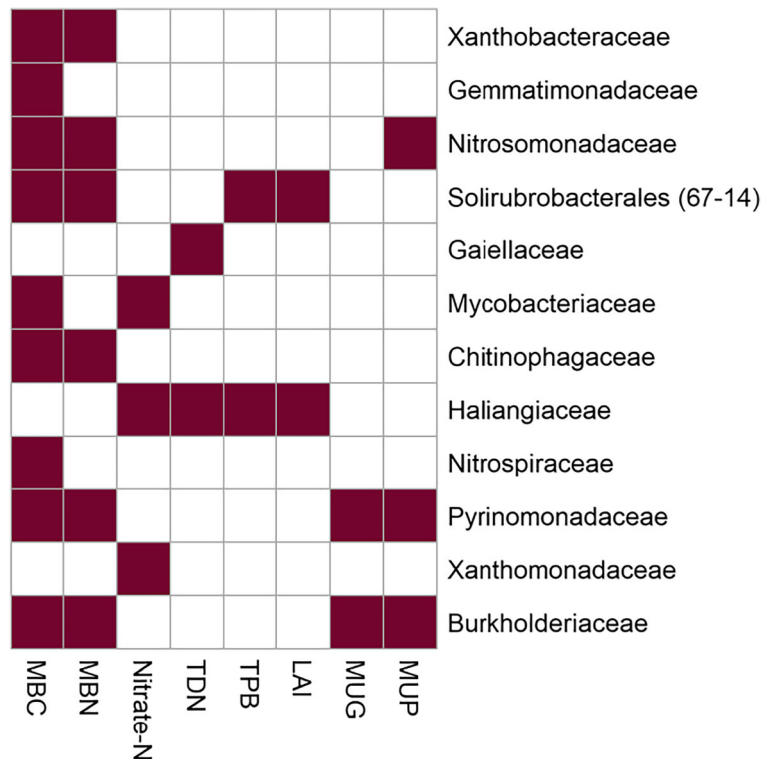
organic nitrogen. The unchanged DOC content within the microcosms also reflected this stability of the organic pool. In addition, the unaffected content of MBN in the soil-rhizosphere environment indicated that the C/N ratio of the OAs did not induce microbial N immobilization.

The significant increase of nitrogen observed for treatment B_MF140 is likely due to application of mineral fertilizer, which was almost 3-times as high as for all other MF treatments (Supplementary Table S1). Nonetheless, a trend evolved towards higher nitrate-N contents remaining in soils amended with higher amounts of biochar and MF compared to treatments containing no or only low amounts of biochar and MF (Supplementary Fig. S2). This trend might be explained by the N_{min} retention capacity of biochar, described by Prendergast-Miller et al. (2014) who also observed higher nitrate-N after cultivating *H. vulgare* plants in biochar amended soils. However, the effects observed in this short-term experiment are only weak, since the initial soil (see controls) contained sufficient nitrate-N for plant growth.

Best plant performance was observed in treatments P and P_MF50 and was therefore independent of mineral

fertilizer application. Nutrients provided by the pellets alone were sufficient to intensify plant performance and maintain good soil quality. No beneficial effects of combining organic amendment with mineral fertilizer on plant performance, different from Zhao et al. (2016), were observed within this study. However, a slight trend towards decreasing TPB alongside with an increase of biochar could be observed (Supplementary Fig. S2). This is in accordance with Liu et al. (2018) who outlined the importance of the correct dosage of biochar for positive effects on plant performance.

Dynamics of microbial activity

The influence of treatments/amendments on soil quality was further assessed by measuring soil enzyme activities, since higher activities often seem to be linked to healthier soils and accelerated nutrient transformation (Caldwell 2005). Many studies report on increasing enzyme activities after organic amendment application, with organic matter input as driver of microbial activity (Li et al. 2018; Zhao et al. 2016; Zhou et al. 2019). This increase was not observed in the present study, where overall microbial activity (FDA), metabolic diversity of

the microbial community (BIOLOG) and the three potential EEAs did not differ between treatments and controls, regardless of mineral fertilizer application. Hence, no increase of enzyme activity was observed in soils amended with a combination of OA and MF, which is different from observations of Zhao et al. (2016). However, these results must be interpreted carefully, since only the potential activity is measured and microbial processes are the result of multiple enzymatic reactions (Nannipieri et al. 2012). Furthermore, due to functional redundancy (Louca et al. 2018) it is possible, that the microbial activity still remained stable, despite the changes observed in bacterial community structure on family level. Whether also rare species play a role for functional redundancy is an interesting aspect for further studies and needs to be investigated in detail.

Changes in average maximum potential activity of β-glucosidase, acid phosphatase and β-N-acetylhexosaminidase with sampling time can be linked to plant growth stages and rhizodeposition. Plants secrete root exudates and thereby influence the microbial community and activity in the rhizosphere (Nannipieri et al. 2008). Furthermore, Philippot et al. (2013) showed that rhizodeposition changes throughout the plant life cycle and alongside with changes in microbial activity. Although the activity of β-glucosidase and acid phosphatase was weakest at the final sampling point (Fig. 3) an association to the bacterial families *Pyrinomonadaceae*, *Burkholderiaceae* and *Nitrosomonadaceae* (for MUP only) was found (Fig. 8). The decrease of β-glucosidase in line with a decrease of *Burkholderiaceae* might be explained by findings of Kim et al. (2006), who described members of this family as β-glucosidase producer.

Changes in bacterial abundance as response to plant growth

Within this study, the most pronounced effect on soil-rhizosphere microbiota was observed via molecular barcoding. While α-diversity (Shannon) of the soil bacterial community remained constant, different indices including species evenness, β-diversity, as well as comparative abundance analysis revealed strong changes. These were strongest between the initial and the final sampling (across all treatments). Since experimental conditions were controlled, and the initial soil represents only bulk soil while the final samples are a soil-rhizosphere mixture, the most pronounced difference

seems to be plant development. It may therefore be hypothesized that the major changes in bacterial abundance are caused by plant growth and rhizodeposition, reflecting also the dynamics seen for the average maximum potential EEAs. Similarly, Philippot et al. (2013) reported strong influence of plants on rhizosphere microbiota in natural and agricultural ecosystems. However, also minor effects on family level arose among treatments including controls, which can be related to OA and MF application. Soil pH as important driver of bacterial community structure as shown by Lauber et al. (2009) is not as pronounced in this study because its values remained almost unchanged and no relationship could be detected with the most abundant families (Fig. 8).

Phylogenetic lineage analyses revealed highest abundances of phyla to be common in the soil environment similar to observations made by Fierer (2017), Lauber et al. (2009) and Obermeier et al. (2020). In addition, Buée et al. (2009) have shown that *Proteobacteria*, *Actinobacteria* and *Acidobacteria* are highly abundant in the rhizosphere with strong variations between treatments and studies. However, our study did not reveal any significant change for the phyla between treatments except when initial bulk soil was compared with the final soil-rhizosphere mixtures. This was most pronounced for the strong increase of the highly abundant phyla *Acidobacteria* in line with the decrease of *Actinobacteria*, *Chloroflexi*, and *Bacteroidetes* and most likely caused by plant growth. A strong decrease of *Chloroflexi* in the rhizosphere of different barley varieties compared to bulk soil has already been proven (Bulgarelli et al. 2015). Furthermore, the strong increase of *Acidobacteria* observed in the final soil mixtures can be explained by the input of inorganic and organic nutrients since its abundance is correlated to organic carbon availability (Kielak et al. 2016). The high root density in final soils is likely to promote this high carbon content by rhizodeposition (Philippot et al. 2013). In addition, Buée et al. (2009) showed that *Acidobacteria* are highly dominant in rhizospheres of different plant species which also may explain the observed increase. On class level the strong increase of *Alphaproteobacteria* observed in the final soil samples can also be related to carbon availability (Zhou et al. 2016). However, Bulgarelli et al. (2015) and Yang et al. (2017) showed that depending on the barley variety rhizosphere microbiota may strongly differ.

Similar to the observations made for the different phyla, the dynamics of bacterial families exhibited most pronounced changes when comparing the initial with the final sampling. A strong increase was observed for families *Xanthobacteraceae*, *Mycobacteriaceae*, and *Pyrinomonadaceae*. On the contrary, *Nitrosomonadaceae*, *Chitinophagaceae*, *Xanthomonadaceae*, and *Burkholderiaceae* were subject to the most pronounced decrease. Decreasing abundances of the ammonia-oxidizing family *Nitrosomonadaceae* during cultivation of some cover crops and after the application of organic fertilizer have been reported previously (Fernandez et al. 2016). In accordance with our findings, these authors also described the influence of the rhizosphere as being more pronounced than treatment with organic amendments on shaping the bacterial composition. Although the effects of the different treatments on bacterial community structure at the final sampling time were not as pronounced, interesting effects were observed for treatment B_MF140. Already species evenness (Fig. 4) and pH were lowest for this treatment, which exhibited highest relative abundances of the families *Xanthobacteraceae* and *Mycobacteriaceae* together with lowest abundance of *Haliangiaceae*. An association of the latter two families to nitrate-N has been shown (Fig. 8) and can be explained with the highest nitrate-N ratio remaining at the end of the experiment due to the highest amounts of mineral fertilizer application together with high amounts of biochar. A high relative abundance of *Mycobacteriaceae* (specifically *Mycobacterium*) in biochar amended soils has also been found by Anderson et al. 2011, who highlight the role of several *Mycobacterium* species as nitrate reducers. This indicates that following the development of the crop an application of mineral fertilizer together with higher amounts of biochar seem to strongly shape the bacterial community structure in this short-term experiment.

In conclusion, working hypothesis (1) could be confirmed showing maintained soil quality and improved plant performance after the application of organic amendments. Furthermore, hypothesis (2) needs to be differentiated since differences in the input of organic amendments alone or in combination with mineral fertilizer on microbial activity did not follow a clear pattern. However, the influence of higher doses of mineral fertilizer and biochar on shaping bacterial community structure could be proven (B_MF140). Hypothesis (3) needs to be revised since the potential extracellular enzyme activities did not depend on treatment (OA vs. MF) but were shown to be triggered by plant growth. Finally, hypothesis (4) must be denied since C/N ratios of the treatments did not influence microbial N immobilization.

Conclusion and outlook

The comprehensive approach of the present greenhouse study revealed strong changes of soil-rhizosphere microbiota dependent on plant growth and organic amendment application. Interestingly, plant performance was improved by all treatments but no difference between organic amendment application alone or with mineral fertilizer was observed. The majority of soil parameters remained stable throughout the study and across different fertilizer applications, indicating maintained soil quality. However, the strong shift of the bacterial composition between initial and final soils can be linked to plant growth and emphasizes the importance of considering plant species and taking its specific belowground parameters (e.g. root exudates) into account when analyzing or predicting effects of organic amendments on the soil-rhizosphere microbiota. Differences among treatments on family level were less pronounced and most likely triggered by the higher amounts of mineral fertilizer application in combination with biochar. The unaffected microbial activity in line with the changes seen for the bacterial families indicate microbial functional redundancy which is likely to promote and maintain soil quality. It further highlights the advantage of molecular barcoding approaches for elucidating changes in the soil environment.

The present study provides valuable insights in the response of the soil-rhizosphere microbiota upon fertilization and fosters our understanding of the complexity of plant-soil-microbe interaction. Long-term experiments have to scrutinize these findings under field conditions to find optimal fertilizer combinations for different agroecosystems.

Compliance with ethical standards

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