Plant diversity and community age shape soil microbial communities

Marc W. Schmid[1,2,6,*], Sofia J. van Moorsel[1,6], Terhi Hahl[1], Enrica De Luca[1], Cameron Wagg[1,4], Pascal A. Niklaus[1] & Bernhard Schmid[1,3,5]

[1]Department of Evolutionary Biology and Environmental Studies, University of Zürich, Winterthurerstrasse 190, CH-8057, Switzerland

[2]MWSchmid GmbH, Möhrlistrasse 25, CH-8006 Zürich

[3]Department of Geography, University of Zürich, Winterthurerstrasse 190, CH-8057, Switzerland

[4]Agriculture and Agri-Food Canada, 850 Lincoln Road, Fredericton, New Brunswick, Canada

[5]Institute of Ecology, College of Urban and Environmental Sciences, Peking University, 100871 Beijing, China

[6]These authors contributed equally

[*]To whom correspondence should be addressed:
contact@mwschmid.ch

ABSTRACT

Plants influence belowground microbial communities, which in turn drive nutrient cycling, plant productivity, and the maintenance of plant diversity. Over time, associations between plant communities and their belowground microbiota may strengthen and become more specific. However, we do not know whether plant diversity affects bulk soil microbial community composition and diversity, and whether this association strengthens as micro-evolutionary processes modify plant communities through time. To address this, we used plant communities and soil from an eight-year-old biodiversity experiment (the Jena Experiment; "old" plant communities and "old" soil), plant communities without such history ("new" plants), and soil from which any soil legacy was removed by sterilization ("new" soil) to set up a field biodiversity experiment in which we factorially crossed plant community age with soil legacy for 52 different plant species compositions. We grew these plant-soil communities for four years in experimental plots with plant species richness ranging from 1, 2, 4, 8 to 60 species. At the end of the experiment, we sequenced bacterial 16S rRNA and fungal ITS fragments to compare soil microbial communities under new vs. old plant communities in new vs. old soils. Soil microbial diversity was positively but weakly associated with plant diversity, with the strongest difference in microbial diversity between plant monocultures and any community of more than one plant species. Particular plant community compositions were associated with particular microbial community compositions. Soil legacy (old soil) increased soil bacterial richness and evenness more strongly than did plant community age. Plant community age (old plant communities) significantly affected the abundance of 10% of fungal OTUs. Some of the effects of soil legacy on bacterial and fungal diversity were indirect via changes in soil abiotic and biotic properties. Our findings suggest that as experimental ecosystems develop over time, plant communities associate with specific microbiomes and plant species associate with specific soil microbial species. With increasing knowledge about the taxonomic identities of the microbial OTUs, our dataset in the future may be used for function-based analyses of the detected associations. This may eventually help to identify microbiomes that improve plant health and consequently plant community productivity.

Keywords: 16S-rRNA gene sequencing; bacteria; biodiversity experiment; community age; fungi; ITS; nitrogen mineralization; plant species richness; soil microbiome

INTRODUCTION

Soil biota are essential for soil health and are critical drivers of soil processes such as nutrient cycling, which in turn provide ecosystem services (van der Heijden et al. 2008, Haines-Young and Potschin 2010, Wagg et al. 2019). Soil microbial communities are in constant interaction with plants that provide habitat and resources for a vast diversity of organisms. Knowing how soil microbial communities co-assemble with plant communities over time and whether this can be manipulated by plant species richness is essential for understanding how above- and belowground biodiversity supports ecosystem processes (Wardle et al. 2004).

The soil microbiome often changes in response to different plant species, which can in turn modify the composition and productivity of plant communities (Klironomos 2002, Bartelt-Ryser et al. 2005, Kardol et al. 2007, Petermann et al. 2008, van der Putten et al. 2013). These plant-soil feedbacks can drive co-adaptation (Lau and Lennon 2011, Wagg et al. 2014, Schweitzer et al. 2014). For example, plant-soil feedbacks may incur selection for plant individuals able to reduce antagonistic and improve beneficial associations with soil organisms (van der Putten et al. 2013, Wagg et al 2014), potentially even leading to positive feedbacks (Zuppinger-Dingley et al. 2016). However, little is known about how plant and soil communities co-assemble and adapt through time and how this affects soil feedback.

Soil properties such as pH, clay content, or soil moisture can also affect specific soil microbiota (Fierer et al. 2007). Fast changes in soil moisture can completely overhaul the active soil community (Barnard et al. 2013), however, microbial community structure can also be very robust to short-term environmental changes (Carey et al. 2015). Soil microbial biomass (Zak et al. 2003b, Weisser et al. 2017, Prommer et al. 2019, Chen et al. 2019) and activity (Weisser et al. 2017, Chen et al. 2019) has been known to increase in diverse plant communities, although interactive effects with environmental factors have been found, such as in a factorial plant-diversity x CO_2 experiment (Grüter et al. 2006, Niklaus et al. 2007). Increased plant species richness may diversify the resource pool to accommodate a larger variety of soil microbes through litterfall and root exudates (Stephan et al. 2000, Milcu et al. 2006, 2013, Sauheitl et al. 2010). Additionally, a higher plant diversity further yields higher community biomass production (Hector et al. 1999), which in turn may increase the biomass of soil fungi and bacteria through the above-belowground transfer of photosynthetically derived carbon (De Deyn et al. 2011).

Although often overlooked, it is important to note that plant-soil interactions are not static and may take time to develop and are ever changing (Lau and Lennon 2011, 2012, terHorst et al. 2014). Microbial communities can theoretically adjust to novel conditions, such as a reduction of plant biodiversity (Schweitzer et al. 2014), by changes in abundance distributions or evolution. Simultaneously the plant community may also change. For example, plant-soil feedbacks have been shown to vary over plant generations (Wagg et al. 2014) and significant evolutionary changes were observed after eight years of community history in a large grassland biodiversity experiment in Jena, Germany (van Moorsel et al. 2018a, 2020). How

such temporal changes in plant communities ("old" vs. "new" plant communities) and associated soil microbial communities ("old" vs. "new" soil) interact remains largely unexplored.

Here we address this interaction in one direction, i.e. how plant community age and soil legacy ("age") affect the diversity and composition of soil microbial communities by sequencing the small subunit ribosomal RNA markers of both bacteria and fungi (16S and ITS fragments, respectively). We then ask whether plant species richness, varying from 1, 2, 4, 8 to 60 plant species, affects the soil microbial communities and modifies the effects of plant community age. For this, we created communities of these species-richness levels using plant or soil material collected from plots with corresponding plant diversity in the Jena Experiment eight years after its start (old plant communities or old soil). At the same time, we created plant communities of the same species compositions from plant material sourced from outside the Jena Experiment (a commercial seed supplier) or from gamma-irradiated soil re-inoculated with soil from a sugar beet field (new plant communities or new soil). We factorially crossed plant community age (new vs. old plant communities) with soil legacy (new vs. old soil) to obtain different types of plant-soil communities for each plant species composition and grew these communities for four years in subplots of the Jena Experiment (see Fig. 1). We use the term "plant community age" when we specifically refer to the history in the Jena Experiment from its establishment in 2002 to 2010 (or the lack thereof). During these eight years, plant species grew and aged in the company of one another, potentially developing stronger interactions or associations with both the plant and soil community partners over time (van Moorsel et al. 2020), which should have created legacy effects in the soil.

We hypothesized (1) that the prolonged history of interactions between plants and soil microbes in old communities leads to more diverse soil microbial communities and (2) that this effect should be strongest in the most plant-diverse communities, due a positive relationship between plant and soil microbial diversity (Hooper et al. 2000). Furthermore (3), we hypothesized that differences between the soil microbiome of new and old plant communities could be affected mainly by plant community age or by soil legacy, and (4), that each plant community composition (even within a species richness level) would sustain its own specific microbial community composition with different fungal and bacterial phyla associating with different plant functional groups and plant species. Finally, we hypothesized (5), that such specific matching between plant and soil microbial communities should be strongest when old plant communities are combined with old soil.

METHODS

Study site and experimental design

The study was conducted at the Jena Experiment field site (Jena, Thuringia, Germany, 51 °N, 11 °E, 135 m a.s.l.) from 2011 to 2015. The Jena Experiment is a long-term biodiversity experiment in which 60 grassland species have been grown in different combinations since 2002 (Roscher et al. 2004, Weisser et al. 2017). The present study was conducted in

experimental plots ("boxes") established within the larger plots of the Jena Experiment and thus represent a biodiversity experiment within a biodiversity experiment (van Moorsel et al. 2018a). We used Illumina sequencing markers of both bacteria and fungi (16S and ITS fragments, respectively) to determine the community structure and diversity of bacteria and fungi in bulk soil (see below). Bacterial and fungal OTU richness, effective species richness, and evenness were examined in response to the treatments and we included co-variates characterizing abiotic and biotic soil properties, such as nitrogen mineralization and soil microbial carbon and nitrogen (see below).

We used a split-split plot design with the three factorially crossed treatments: plant species richness, soil legacy, and plant community age. The latter two represent various aspects of shared community (evolutionary) history (new soil-new plants, new soil-old plants, old soil-new plants, old soil-old plants). Plant species richness was manipulated at plot level and included 12 monocultures, 12 two-species mixtures, 12 four-species mixture, and 12 eight-species mixtures, all distinct species composition. It also included four replicates (i.e. four different plots) of the full 60-species mixture containing all species used in the experiment. Soil legacy was manipulated at the split-plot level and had three levels, two with the old microbial communities taken after eight years from the Jena Experiment and one with new microbial communities taken from outside (see below). Plant community age was manipulated at the split-split-plot level and had two levels: plant communities established with material obtained after eight years form the Jena Experiment and with material taken from outside (see below). Some treatment combinations were removed because the wrong plant species was planted (van Moorsel et al. 2018a) or DNA extraction from soil samples failed. Hence, the final design included 11 to 12 plant communities for each combination of the three treatment factors, except for the 60-species plant mixtures for which we only tested new communities (i.e. the new soil-new plant communities combination; Fig. 1). The 60-species mixtures were only included in some of the analyses.

Details about soil legacy treatments

In 2010, 8-years after the establishment of the Jena Experiment, within the larger experimental plots of the Jena Experiment, we established the boxes surrounded by plastic frames used in this study. Each box, subsequently called plot, contained four 1 m^2 quadrats (split-plots) with different soil treatments in each. To create the soil treatments, within each of these 2 x 2 m plots, we removed the original plant cover in September 2010 and the soil was excavated to a depth of 0.35 m and sieved. To minimize the exchange of soil components between the four 1 m^2 quadrats and with the surrounding soil, the borders of the quadrats were separated by plastic frames. Two 5-cm layers of sand, separated by a 0.5-mm mesh net, were added to the bottom. Using the excavated original soil from each of the plots, four soil treatments were prepared.

Half of the soil (around 600 kg per plot) was gamma-sterilized with a dose of 50 kGy to kill soil biota (McNamara et al. 2003). Half of the sterilized soil was then inoculated with 4 % (by weight) of sugar beet soil and 4 % of sterilized soil ("new" soil). Sugar beet soil was

added to create a natural soil community and was previously collected in a sugar beet field not associated with the Jena Experiment, but with comparable soil properties. The second half of the sterilized soil was inoculated with 4 % sugar beet soil and 4 % unsterilized soil ("inoculated" soil, first variant of old soil). From the remaining unsterilized soil (also around 600 kg per plot), half was filled back into one quadrat ("native" soil, second variant of old soil). The other half of the unsterilized soil was mixed among all plots and filled into the remaining quadrats, thus equalizing potential differences due to plot history and location. This fourth soil treatment was abandoned after 2 years because the plant community was excavated for another experiment. Hence, soil legacy only comprised three of the four soil treatments: new soil, inoculated old soil and native old soil.

Details about plant species richness and plant community age treatments

We used two plant community age treatments: "old plant communities" (with eight years of co-occurrence history in the Jena Experiment) vs. "new plant communities" (plants without such co-occurrence history). Seeds for the new communities were obtained from the original seed supplier of the Jena Experiment (Rieger Hofmann GmbH, in Blaufelden-Raboldshausen, Germany). To produce seeds for the old communities, cuttings made after eight years (2010) in the Jena Experiment were transferred and planted in the original species combination in plots of an experimental garden in Zurich, Switzerland. Plots were surrounded by nets to reduce pollination between communities and only left open on top to allow pollinator access (Zuppinger-Dingley et al. 2014). Seeds for the old communities were thus offspring of plant populations that had been sown in 2002 and grown until 2010 in plots of the Jena Experiment.

All seeds were germinated in potting soil (BF4, De Baat, Holland) in mid-January 2011 in a glasshouse in Zurich. In March 2011, the seedlings were transferred to the field site of the Jena Experiment and planted within the 2 x 2 m plots described above. Each 1 x 1 m quadrat was further divided into two equally sized halves (split-split-plots). The seedlings of old communities were transplanted into one half and seedlings of new communities into the other half of each quadrat at a density of 210 plants per m^2 (Fig. 1). Species were planted in equal proportions. Species that were no longer present in the original plot of the Jena Experiment were excluded from both planted communities. Five out of 60 plant species were excluded because they had gone extinct in the sampled communities from the Jena Experiment. The plant communities were grown from 2011–2015 and maintained by weeding three times a year and by cutting twice a year in late May and August, which are typical grassland harvest times in central Europe.

Soil sampling and soil microbial biomass and activity

Once the old and new plant communities and their associated soil microbial communities had been allowed to develop for four years, soil samples were collected (early October 2015) in each of the six split-split-plots (two in case of the 60-species mixtures). Three soil cores were collected per split-split-plot and pooled. This yielded in a total of 6 x 48 + 2 x 4 – 4 missing combinations = 292 samples. Soil samples were then sieved to 2 mm and divided into

two sub-samples of which one used for soil chemical analysis and the other one for DNA extraction and subsequent 16S/ITS sequencing. For the DNA extraction, we weighed approximately 0.5 g of fresh soil per sample, added buffer, and froze the samples at –80 °C.

Gravimetric soil water content was determined by oven-drying (105 °C for 24 h). Soil microbial carbon and nitrogen were determined by chloroform-fumigation extraction (Brookes et al. 1985, Vance et al. 1987). In brief, 10 g of soil were extracted with 25 mL 0.5 M K_2SO_4 (45 min, 150 rpm), the suspension filtered (MN 615, Macherey-Nagel AG, Oensingen, Switzerland), and dissolved organic carbon and nitrogen in the filtrate quantified with a TOC analyzer (Dimatoc 2000; Dimatec Analysentechnik GmbH, Germany). A second sample was processed similarly after fumigation with ethanol-free chloroform. Microbial C and N were calculated assuming an extraction efficiency of kEC = 0.45 (Vance et al. 1987) and kEN = 0.54 (Brookes et al. 1985), respectively.

Net nitrogen mineralization was determined in laboratory incubations under anaerobic conditions. Soil samples (20 g fresh weight) were incubated for 7 days at 40 °C in 30 ml centrifuge tubes containing 20 mL of extra water, leaving minimal headspace. After incubation, the tubes were vortexed, samples transferred to 100 ml polypropylene cups and 40 ml of 3M KCl added, yielding a concentration of 2M KCl in the suspension. The suspension was extracted on a table shaker for 30 min. After sedimentation and filtration of the supernatant, the now soil-free extract was stored frozen (–18°C) until determination of NH_4^+ concentrations (Skalar SAN+ segmented flow analyzer, Skalar Analytical B.V., Breda, The Netherlands). Note that no nitrate was formed due to the aerobic nature of the incubation.

Available phosphorus was determined using the method of Olsen (Olsen et al. 1954). 2 g of fresh soil were extracted with 40 ml 0.5 M NaHCO3 (pH 8.5, table shaker, 30 minutes) and the supernatant filtered as described above. Phosphate in the extract was determined colorimetrically using the molybdate blue method (Watanabe and Olsen 1965) on the same segmented flow analyzer.

Bacterial 16S rDNA and fungal ITS sequencing

In June 2016, DNA was isolated from 500 mg of bulk soil using the FastDNA SPIN Kit for Soil (MP Biomedicals, Illkirch-Graffenstaden, France) following the manufacturer's instructions. We used the primer pair ITS1-F_KYO2 (5'-TAGAGGAAGTAAAAGTCGTAA) and ITS2_KYO2 (5'-TTYRCTRCGTTCTTCATC) to amplify the internal transcribed spacer subregion 1 (ITS1, Toju et al. 2012) and the primer pair S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC) to amplify the variable regions V3 and V4 of the prokaryotic ribosomal RNA gene (Herlemann et al. 2011). 16S/ITS specific sequences were fused to generic adapters (forward: 5'-ACACTGACGACATGGTTCTACA, reverse: 5'-TACGGTAGCAGAGACTTGGTCT) for the first round of PCR. The PCR conditions for the amplification of the 16S and ITS regions consisted of an initial denaturation at 94 °C for 15 min, 30 cycles of denaturation at 94 °C for 40 s, an annealing at 58 °C for 40 s, and an elongation at 72 °C for 1 min followed by a final elongation at 72 °C for 10 min. The PCR products were purified according to the manufacturer's instructions (NucleoSpin Gel and

PCR Clean-up) and amplicon concentrations were measured with Qubit (Tecan Spark M10 plate reader with Pico Green). All samples were diluted to a concentration of 0.5 ng/µl and shipped to GenomeQuebec (Montréal, Canada) for library preparation and sequencing. Samples were paired-end sequenced (2 x 250 bp) on the Illumina HiSeq system (GenomeQuebec, Montréal, Canada). Short-reads were deposited at SRA (accession number PRJNA639013).

Identification and annotation of OTUs

Operational taxonomic units (OTUs) were generated with UPARSE (version 10.0.024, Edgar 2013) following the example and the tutorial given for paired-end Illumina data (drive5.com/uparse/). Reads were first quality-checked with FastQC (bioinformatics.babraham.ac.uk/projects/fastqc). Following removal of adapter sequences and low-quality bases with Trimmomatic (version 0.33 with the parameters ILLUMINACLIP:adapterSeqs:2:30:10 SLIDINGWINDOW:5:15 MINLEN:100; Bolger et al. 2014), paired-end reads were merged using usearch (with the parameters -fastq_maxdiffs 25 -fastq_maxdiffpct 10 for merging; Edgar 2013).

OTU sequences were annotated with the taxonomy data (Appendix Table S1) available from the Ribosomal Database Project (bacterial sequences, version 16, Cole et al. 2014) and UNITE (fungal ITS1 sequences, version 7, Nilsson et al. 2019) with usearch (version 10.0.240, with the parameters -sintax -strand both -sintax_cutoff 0.8, Edgar 2016). Fungal ITS-OTUs were further annotated with functional categories using FUNGuild (version 1.1, Nguyen et al. 2016). OTU abundances were finally obtained by counting the number of sequences (merged and filtered) matching to the OTU sequences (version 10.0.024 with the parameters -usearch_global -strand plus -id 0.97, Edgar 2013, Appendix Table S1). OTUs annotated as chloroplast were removed to avoid a potential bias caused by plant DNA (mitochondrial sequences were not present in the databases and, therefore, not removed). To avoid sequencing artifacts, OTU sequences with less than 30 counts in total or with counts in less than four samples were removed from all further analyses (14,469 bacterial and 5,214 fungal OTUs remained after this filter).

Data normalization and identification of differentially abundant OTUs

Normalized OTU counts were calculated with DESeq2 and log2(x+1)-transformed to obtain approximately normally distributed OTU abundances. Sequencing data were not rarefied (McMurdie & Holmes 2014). Variation in relative abundances of individual OTUs was analyzed with the same models used for the biodiversity indices and community compositions described below, i.e., using the regular ANOVA and linear model functions in R. However, we removed the plots with 60 species from the analysis because these did not occur in more than one treatment combination. Following the removal of these plots, the OTUs were re-filtered as described above. For a given term in the model, *P*-values from all OTUs were adjusted for multiple testing (Benjamini and Hochberg 1995), and OTUs with an adjusted P-value (false discovery rate, FDR) below 0.01 and explaining more than 1 % of the variation in relative abundances of individual OTUs were considered to be differentially abundant.

Assessment of microbial community structure

Distances between all samples using all OTUs passing the filter were visualized with a redundancy analysis (RDA). The RDA was conducted in R with the package vegan (version 2.4-4, function rda(); Oksanen et al. 2017). Input data were the log2-transformed and normalized OTU counts as response variables and the treatment factors with all interactions as explanatory terms.

To assess the overall impact of the treatment factors on the dissimilarity between the microbiomes' bacterial community structure, we analyzed the variation in dissimilarities between microbiomes with a multivariate ANOVA in R with the package vegan (version 2.4-4, function adonis(); Oksanen et al. 2017). We used the Manhattan distance as a dissimilarity measure because it performs better than the Euclidean distance for high-dimensional data (Aggarwal et al. 2014). Similarly, to assess the impact of the treatment factors on the phylogenetic composition of the bacterial communities, we performed a multivariate ANOVA with UniFrac distances (Lozupone et al. 2011). These are based on phylogenetic trees whereas the above dissimilarities do not consider the phylogenetic distances between bacterial taxa.

ANOVA models

The structure of all ANOVA models followed general design principles that have been applied in other biodiversity experiments (Schmid et al. 2002, 2017). For all models, factors were fitted sequentially (type I sum of squares) as indicated in Table 1 and 2. Significance tests were based on *F* tests using appropriate error terms and denominator degrees of freedom (Schmid et al. 2017). The fixed terms of the models were spatial variation across the field site (composed of five directional contrasts, see Le Roux et al. (2013), plant species richness and composition contrasts comparing monocultures with mixtures, assessing log-linearized species richness (logSR) and comparing plots with legumes/grasses/herbs with others, soil legacy (composed of two contrasts, new vs. old followed by native vs. inoculated), plant community age (PA) and two- and three-way interactions between treatment terms. Note that the spatial variation was chosen as an alternative to using the blocks in the field because this model can better account for differences in soil chemistry (Le Roux et al. 2013). The random terms were plot and its two-way interaction with soil legacy (split-plots) and the three-way interaction with soil legacy and plant community age (split-split-plots).

Structural Equation Modeling

To test whether the effect of the soil legacy and plant species richness treatments were direct or indirect due to differences in soil chemistry, we used the package lavaan (Rosseel 2012). To account for spatial variation of the soil chemistry in the field (Le Roux et al. 2013), we first fitted a model with the plot coordinates and used the residuals for further analyses. We formulated the model in such a way that it tests for a direct effect of soil legacy or plant species richness and for indirect effects of these via each soil chemistry in parallel; plant community age was not included in these models because it only had weak effects in the ANOVA models.

Enrichment and depletion of bacterial taxa

To test for enrichment/depletion of bacterial taxa occurrences in each set of OTUs (e.g., OTUs with significant difference in abundance between treatment levels), we constructed for each taxon a contingency table with the within/outside phyla counts for the given set of OTUs and all OTUs passing the filter. We then tested for significance with Fisher's exact test. *P*-values were adjusted for multiple testing (Benjamini and Hochberg 1995), and taxa with an adjusted *P*-value (false discovery rate, FDR) below 0.05 were considered to be significantly enriched/depleted (Appendix Table S3).

RESULTS

Characterization of soil microbial OTUs according to soil legacy, plant species richness, and plant community age

Of 14,469 16S-OTUs, 11,664 were classified as Bacteria, 8 were classified as Archaea and 2,797 remained unclassified or unknown. Within the bacterial domain, the ten most frequent phyla accounted for 96.3 % of all 16S-OTUs (Appendix Table S1 and Table S2). Whenever we mention bacterial OTU's, we include Archaea as well, but because they represent so few OTU's and for brevity, we believe it is reasonable to only refer to Bacteria. Of the 5,214 ITS-OTUs, 2,258 were classified as Fungi and 2,956 remained unclassified or unknown. The ten most frequent phyla accounted for 99.8 % of all ITS-OTUs (Appendix Table S1 and Table S2).

To evaluate the overall differences in normalized 16S-/ITS-OTU abundances between microbiomes of the different treatment combinations we conducted an RDA with the treatment factors and their interactions as explanatory terms (Fig. 2). The first two RDA axes explained 13.1 % and 6.0 % of the total variation in 16S- and ITS-OTUs, respectively. These RDA axes clearly separated the three soil legacies and to some extent also ordered the samples according to the different levels of plant species richness. No separation was apparent between old and new plant communities.

Diversity and composition of soil microbial communities

To characterize the overall impact of the treatment factors on bacterial and fungal diversity, we analyzed the variation in 16S-/ITS-richness, effective richness (exponent of the Shannon index; Magurran 2004) and evenness (Pielou 1975) (Table 1, Fig. 3). For the 16S-OTUs, richness was significantly different between the plots representing different plant species compositions and soil legacies, including both contrasts, the first comparing the native old soil to the others, and the second comparing the inoculated old to the new soil. Bacterial richness was highest in the native soil and lowest in the new soil. The soil legacy was also significant in the analysis for effective richness and evenness, both being highest in the native soil and lowest in the new soil (Table 1, Fig. 3). In other words, soil legacy had a clear positive effect on bacterial diversity. Tested against the significant variation among species compositions, plant species richness contrasts did not have a significant effect on bacterial

diversity. Bacterial OTU richness was higher, and evenness was lower when plant species compositions contained grasses than when they did not. Significant interactions between plot and plant community age for bacterial richness and effective richness (Table 1) indicated that, depending on the species composition of the plant community, old communities differentially affected bacterial diversity. Effective bacterial richness also varied spatially across the Jena field site.

The results for the ITS-OTUs, mainly representing fungal diversity, were quite different from those for bacterial diversity (Table 1, Fig. 3). Soil legacy and its contrasts were only significant for evenness. However, the effect was opposite to that on bacterial evenness, with fungal evenness being highest in the new and lowest in the native old soil. Plant monocultures had significantly lower fungal species richness and effective species richness than plant mixtures (Table 1) and a trend of higher (effective) fungal species richness with higher plant species richness was apparent in the corresponding graphical display (Fig. 3). The presence of legumes in plant communities significantly decreased effective fungal species richness and evenness (Appendix Table S4 for an ANOVA including legumes instead of grasses). In contrast, the presence of grasses significantly increased all three indices of fungal diversity. Fungal species richness and effective species richness, like bacterial OTU richness, also varied spatially significantly across the Jena field site.

We obtained comparable results with multivariate ANOVAs testing for the impact of the treatment factors on the bacterial and fungal community compositions (Table 2) and the phylogenetic community composition (Appendix Table S5). Plot (i.e. plant species composition), soil legacy, including both contrasts, and the presence of grasses had significant effects on bacterial and fungal community composition. Soil legacy explained more variation in the bacterial data (14.2 % sum of squares) than in the fungal data (6.6 %). This may explain the differences between the two data sets observed in the analysis of diversity indices. For the ITS-OTUs, the presence of legumes was also significant (Appendix Table S6). The effects of treatment factors on phylogenetic community composition were similar with the same explanatory terms being significant. Spatial variation had a significant effect on 16S-OTUs but not on ITS-OTUs.

In summary, the overall bacterial community structure was primarily determined by spatial variation across the field site, plot (i.e., the effect of a particular plant community composition), soil legacy and to a lesser extent by the presence of grasses. The fungal community structure was additionally influenced by the presence of legumes and differed between plant monocultures and mixtures, reflecting a tendency for fungal diversity to increase with plant species richness (Fig. 3). Interestingly, we could not detect any main effects of plant community age (old vs. new plant communities) on soil microbial communities.

Relative abundance of different soil microbial OTUs in the treatment combinations

To identify the OTUs that contributed to the differences between microbial communities from different treatment combinations, we tested each 16S/ITS-OTU for differential abundance with the models used for the biodiversity indices and community composition

(Table 3). Of the 11,883 and 4,219 16S- and ITS-OTUs tested, 7,804 (65.6 %) and 2,489 (59.0 %) showed one or several significant model terms. In both 16S- and ITS-OTUs, soil-legacy treatments, plots (i.e. different plant species compositions) and their interactions were often significant (16S-OTUs: plot: 2,032, soil legacy: 4,964, interaction: 640; ITS-OTUs: plot: 1,173, soil-legacy: 614, interaction: 673). Considering that we corrected for the overall spatial variation, the effect of the plot is likely an effect of the specific species composition (the summarized factor "spatial variation" was frequently significant as well with 1,321 significant 16S-OTUs and 156 significant ITS-OTUs). In terms of species composition contrasts, we frequently observed significant effects of the presence of grasses (741 16S-OTUs and 296 ITS-OTUs) or legumes (46 16S-OTUs and 109 ITS-OTUs). However, the presence of the two other plant functional groups, small or tall herbs, which were taxonomically more heterogeneous than grasses and legumes, had no significant effect. Plant species richness had no significant effects as well.

When we tested for the effect of biomass proportions of each plant species (Table 4) we found that many plant species significantly affected the relative abundance of multiple 16S- and ITS-OTUs (on average 13.7 or 0.115 % 16S- and 30.7 or 0.728 % ITS-OTUs). The 16S-OTUs were clearly less responsive than the ITS-OTUs to the biomass proportions of individual plant species. The number of significantly affected OTUs per plant species further increased when the interactions of their biomass proportions with soil-legacy treatments were considered (on average 26.9 16S-OTUs and 52.0 ITS-OTUs; Table 4). Significant interactions between biomass proportions of individual plant species and plant community age were less frequent (on average 8.0 16S-OTUs and 21.1 ITS-OTUs). The three-way interaction was less often significant for 16S-than for ITS-OTUs (on average 9.7 16S-OTUs and 37.5 ITS-OTUs). It is important to note that the biomass proportion of specific plant species cannot be entirely separated from plant composition effects because plant species composition in the Jena Experiment is partially confounded with the presence of a particular plant species (see e.g. Le Roux et al. 2013).

Taxonomy of differentially abundant soil-microbial OTUs

We assessed the taxonomy (phyla) of the 16S- and ITS-OTUs significantly affected by soil-legacy treatments, plots (plant community composition), their interaction, and the plot x plant community age interaction. The plot x plant community age interaction only mattered for the ITS-OTUs because there were too few significant 16S-OTUs. For the ITS-OTUs, we also assessed the "trophic mode" annotations from FUNGuild (Nguyen et al. 2016), Appendix Table S3). With enrichment/depletion of a certain taxum we refer to a significantly more/less frequent occurrence of the taxum in a set of OTUs compared to if the taxa in the set of OTUs were randomly sampled (i.e., expected number of OTUs of taxum A = number of OTUs in a set * frequency of taxum A in all identified OTUs).

For the 16S-OTUs significantly affected by soil legacy we observed an enrichment of *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Latescibacteria*, *Planctomycetes* and unknown bacteria and a depletion of *Bacteroidetes*, *Candidatus Saccharibacteria*, *Parcubacteria* and *Proteobacteria*. 16S-OTUs changing their abundance in dependence of plot were enriched for *Bacteroidetes*, *candidate division WPS-1*, *Cyanobacteria*, *Candidatus Saccharibacteria*,

Gemmatimonadetes, *Proteobacteria*., and *Verrucomicrobia* and depleted of *Actinobacteria*, *Latescibacteria*, *Planctomycetes* and unknown bacteria. Finally, for the 16S-OTUs significantly affected by plot x soil legacy interactions we observed an enrichment *Acidobacteria*, *Latescibacteria*, *candidate division WPS-1* and unknown bacteria and a depletion of *Actinobacteria*, *Bacteroidetes*, *Planctomycetes*, and *Proteobacteria*

ITS-OTUs that were significantly affected by the legacy were enriched for *Zoopagomycota* and unknown fungi and depleted of *Basidiomycota*. In contrast, ITS-OTUs changing their abundance in dependence of plot most were enriched for *Ascomycota* and *Basidiomycota* and depleted of unknown fungi. Likewise, the ITS-OTUs with significant plot x soil legacy interactions were enriched for *Basidiomycota* and depleted of unknown fungi.

In terms of their trophic guilds, ITS-OTUs significant for the plot x soil legacy interaction were enriched for "pathotroph-symbiotroph" fungi and depleted of saprotroph and "pathotroph-saprotroph-symbiotroph" fungi. ITS-OTUs affected by plot x plant community age interactions were enriched for the guild "endophytes". This guild was also most more responsive to plot (i.e., plant species composition) but not to soil legacy. ITS-OTUs significantly affected by plot vs. soil-legacy treatment were enriched for plant pathogens and leaf saprotrophs vs. animal pathogens, lichenized fungi, and different saprotrophs (soil and wood saprotrophs).

Indirect effects of plant community age, soil legacy, and plant species richness via changes in soil microbial biomass and activity

Lastly, we assessed whether plant community age, soil legacy, and plant species richness affected soil microbial communities directly or indirectly through changes in soil microbial biomass and activity. To test whether soil microbial biomass and activity were at all different between treatments, we applied the same models used for the biodiversity indices above (Appendix Table S7). All soil property variables were significantly affected by the location of the plots and the plots (plant species composition). Soil legacy, its contrasts, and some of the interactions with plot were significant for microbial C and N, Olsen's P, and mineral N, but not microbial C:N ratio or (gravimetric soil water content). The monoculture contrast and its interaction with soil legacy was significant for mineral N and microbial N, respectively. Besides that, plant species richness had no significant effect on any measured soil property. Plant community age effects were insignificant. Hence, indirect effects of treatment factors on biodiversity variables via soil biotic and abiotic properties might have occurred in cases of plant species richness (monoculture contrast), soil legacy, and plot (plant species composition). We then tested whether effects were direct or indirect with a path analysis (Fig. 4).

Plant community age effects were insignificant in the path analysis. The soil-legacy treatment had multiple significant indirect effects on both, bacterial and fungal, diversity indices. These were limited to the four soil property variables that were significant in the ANOVA, but not all indirect paths were significant for each biodiversity index. For example, the path via Olsens's P was significant for bacterial OTU richness and effective species richness, but insignificant for bacterial evenness and the fungal biodiversity indices (Fig. 4).

In contrast to the effects of soil-legacy treatments, the effects of plant species richness on soil microbial communities was at most marginally explained through indirect effects operating via changed soil abiotic and biotic properties, instead, these effects were direct or potentially mediated by other, unmeasured covariates.

DISCUSSION

We set out to study whether a prolonged history of interactions between plants and soil microbes in old communities would increase soil microbial diversity, in particular in more diverse plant communities (hypotheses 1–3). We further wanted to test whether a specific plant community composition would "select" its own specific microbial community composition (hypothesis 4), and consequently expected such a specific matching between plant and soil microbial communities to get stronger as plant communities age (hypothesis 5). Our results support the overall hypothesis that prolonged interactions among plants and their soil communities influence the composition of the soil microbiome, that plant diversity had an effect (albeit a weak one beyond the differences between monocultures and mixtures) and that, indeed, particular plant species can "select" for a particular microbial community. These results are in accordance with new findings (Chen et al. 2020) that plant communities may actively try to prevent dysbiosis of their associated microbiomes.

Plot effects: spatial variation, community composition, and specific species influence soil community composition

A previous study in the Jena Experiment, which showed positive relationships between plant diversity and bacterial and fungal diversity (Lange et al. 2015), led us to examine this hypothesis in more detail. We expected diverse old communities to have the highest belowground diversity (hypotheses 1 and 2). We found that the overall effect of plant species richness on bacterial and fungal diversity was only weakly positive, but that this overall relationship was complicated by influences of spatial variation and particular plant community species compositions, including the presence of particular functional groups such as grasses or legumes. In contrast to hypothesis 1, plant species richness did not have a significant positive effect on bacterial OTU richness and evenness. For fungal species richness, the positive effect of plant diversity was mainly due to a positive difference between plant mixtures and monocultures; whereas among the mixtures increasing diversity only had a marginally significant positive effect on fungal species and effective richness. This is in line with a previous study conducted on bulk soil from the Jena Experiment (Dassen *et al*. 2017) that reported a marginally positive effect of plant species richness on fungal diversity in the large main plots at the Jena Experiment. In contrast, a plant–soil feedback study using some of the same plant species did not confirm that plant species richness increases fungal diversity (Mommer et al. 2018). Different sampling procedures may explain some of these differences. While we and Dassen *et al.* (2017) characterized microbial communities within bulk soils, Mommer and colleagues studied root-associated fungi. As Dassen *et al.* (2017) noted, plant species richness may not affect fungal diversity *per se* but through changes in soil properties,

for example increased litter availability. If true, this would unlikely affect root-associated fungi but rather saprotroph fungi in the soil matrix.

In terms of bacterial and fungal community composition our samples tended to cluster along a plant species richness gradient from monocultures to 60 species-mixtures (see Fig.2), suggesting that, despite relatively weak effects of plant diversity on the diversity of soil microbial communities, plant diversity did lead to "directed" microbial species turnovers. Similar results have been found for example by Grüter et al. (2006) but other studies on the influence of plant diversity on bacterial and fungal community composition have produced variable results. Two studies using 454 pyrosequencing found that plant diversity did influence fungal (LeBlanc et al. 2015) or bacterial community composition (Schlatter et al. 2015). A more recent study conducted in a grassland ecosystem in Texas using Illumina sequencing, however, found that fungal community composition was not influenced by plant diversity *per se* but rather by the addition of a single exotic plant species (Checinska Sielaff et al. 2018). More research applying novel methods offering a higher resolution in terms of community composition is needed to further investigate how strongly plant species richness *per se* can influence bacterial and fungal community composition.

Plots with different plant species compositions considerably varied in overall bacterial and fungal community diversity and structure. In part this was due to the spatial position of the plots within the field site, which meant slightly different soil conditions (i.e. sand, silt and clay content) depending on the plots' positions relative to the nearby river Saale (Le Roux et al. 2013, Weisser et al. 2017). But large variation among plots remained after accounting for spatial variation. We interpret this as a sign of the influence of particular plant community compositions, which were different for each plot (except the four replicate 60-species plots), thus confirming our fourth hypothesis. Whereas for bacteria this variation among plots was partly due to the presence vs. absence of grasses, for fungi variation among plots was more strongly due to the presence vs. absence of grasses or legumes. The presence of legumes tended to increase fungal effective species richness and evenness. Legumes can modify arbuscular mycorrhizal fungi (AMF) community composition and increase AMF diversity (König et al. 2010). Dassen et al. (2017) also found that legumes influenced the bulk soil community structure and increased diversity. However, they reported stronger effects on rhizobia (bacteria) and archaea than on fungi. We found that the presence of grasses increased all fungal diversity indices and bacterial richness but decreased bacterial evenness, which could mean that grasses promote the abundance of some microbes over others.

To assess whether each plant community would sustain its own specific microbial community composition with different fungal and bacterial phyla (hypothesis 4), we identified the OTUs that contributed to the differences in the microbial communities. Testing each 16S/ITS-OTU for differential abundance revealed a significant effect of community composition, i.e. the identity of a plot. Especially grasses and legumes seem to have "selected" for different and specific sets of OTUs, whereas the taxonomically more diverse small or tall herb plant functional groups had no consistent effects on soil microbial communities. The latter is not surprising, because it is difficult to envision a mechanism that would link the abundance of an individual microbe to the presence or absence of a diverse set

of plant species. In other words, our results rather suggest that specific microbial OTUs are commonly associated with specific plant species, as we indeed found in the corresponding analyses presented in Table 4 and an earlier study including a subset of species in a controlled glasshouse inoculation experiment (Schmid et al. 2019).

Soil effects: The effects of soil sterilization are long-lasting

We hypothesized that interactions between plants and soil microbes would over time lead to particularly diverse soil microbial communities (hypothesis 1). As expected, native old soil, i.e., non-sterilized soil including co-evolved microbial communities, had significantly higher bacterial richness, evenness, and fungal richness than sterilized soil inoculated with native microbes. It also had richer microbial communities than sterilized soil inoculated with microbes from an external sugar beet field. Furthermore, soil legacy affected the bacterial phyla differentially. The strong differences between soil treatments were observed four years after the establishment of the plots, suggesting that the potential exchange of microbes between adjacent treatments could not remove initial differences. Furthermore, the differences between soil treatments were even maintained through a severe natural flood at the field site in spring 2013 (Blöschl et al. 2013, Wright et al. 2015, van Moorsel et al. 2020) during which the plots were completely submerged by standing water.

The two soil treatments which underwent the sterilization process were more similar than the native soil, as they cluster closer together in the RDA (Fig. 2). This was to be expected, given that a large fraction of soil microbes is oligotrophic and grows very slowly (Lennon and Jones 2011). The inoculum likely contained eutrophic microbes only, or at least these were the ones that could multiply and dominate. Nevertheless, the distinct soil microbial assemblages in all three soils support in part our first hypothesis that microbial community composition changes over time in response to the associated host community.

Effects of plant community age are weak

The Jena Experiment has yielded evidence for significant effects of plant community age on plant community productivity (van Moorsel et al. 2018a, Vogel et al. 2019) and stability (van Moorsel et al. 2020). We, therefore, expected plant community age to have equally significant effects on soil microbial communities. We hypothesized that species-rich plant communities with a history of co-occurrence would yield the highest soil diversity (hypothesis 2). We were not able to confirm this hypothesis, as there was no significant influence of plant community age on soil bacterial or fungal diversity. We also hypothesized that the matching between specific plant species and bacterial or fungal phyla would be stronger in old communities than in new communities (hypothesis 5). However, plant community age only affected fungal communities, i.e., about 10% of the fungal OTU's were influenced by whether the plant community hosting them was old or new. Interestingly, we also could detect some OTUs with a significant effect of the plot x plant community age interaction (6 16S-OTUs and 62 ITS-OTUs). This suggests a potential co-adaptation of microbes and plants in communities with specific plant species compositions. For the ITS-OTUs we observed a particular sensitivity of *Ascomycota* and *Basidiomycota* to community age among different plant community compositions which may be caused by species-specific

interactions between particular plants and particular mycorrhizal fungi belonging to these phyla (Crowther et al. 2019).

The lack of strong effects of plant community age, i.e. plant community selection (van Moorsel et al. 2018a), in this study is in line with earlier findings showing significant effects of plant species identity but not plant community age on associated rhizosphere communities (Schmid et al. 2019). Together, these results suggest that plant community age, which can change plant traits, plant community productivity (Zuppinger-Dingley et al. 2014, van Moorsel et al. 2018b, 2019), and plant–soil feedbacks (Zuppinger-Dingley et al. 2016), did at most only have weak effects on associated microbial communities. This may also explain why the soil treatments used here could not modify the positive effects of community age on plant diversity–productivity (van Moorsel et al. 2018) and plant diversity–stability (van Moorsel et al. 2020) relationships. Overall, the present study supports the previous studies in that plant community age may exert a stronger influence on plant–plant interactions than on soil microbial communities; and the lack of associated changes in microbial communities may explain why effects of plant community age are not modified by associated soil treatments.

Soil legacy and plant diversity act also indirectly via changes in soil abiotic and biotic properties

Lastly, we assessed whether the effects of soil legacy and plant species richness on soil bacterial and fungal diversity were direct or indirect via one or more soil abiotic and biotic variables. Soil legacy had indirect and direct effects. Indirect effects would be expected, as the sterilization process released nutrients in the soil (Gebremikael et al. 2015), with significant consequences for soil abiotic and biotic properties in these sterilized soils. The pathways via microbial C and N were significant for indirect effects of soil legacy on almost all diversity indices. However, it is conceivable that the pathway should be reversed here, i.e. microbial C and N may be a consequence rather than a cause of microbial richness and diversity. It is impossible to disentangle whether one very dominant species contributed to most of the microbial mass or whether a more diverse microbial community led to higher biomass (Thakur et al. 2015), analogue to the positive biodiversity–productivity relationship known for plant communities (Hooper et al. 2005).

Using the path analysis, we could also demonstrate direct effects of soil legacy on soil diversity, which indicates that not only abiotic and biotic soil properties mattered, but that the soil communities themselves played a role in becoming more (or less) diverse over time. This direct effect of soil legacy, thus the "true" soil *community* age effect, increased bacterial richness, i.e. old communities are more diverse, confirming our first hypothesis. In contrast, old communities tend to have slightly lower fungal diversity (in terms of evenness), thus the fungal community may have been selected for a specialized subset of fungal species particularly suited for this type of soil (Sosa-Hernández et al. 2018). Alternatively, the sterilization process, by removing differential abundances of fungal species, may have created an even playing field for all new fungal colonizers in the soil.

It is known that plant community composition can have strong effects on belowground processes (Niklaus et al. 2006), such as legumes driving N mineralization in soils (Lama et al.

2020), which then might influence soil bacterial and/or fungal diversity. We found that the effects of plant diversity on soil microbial communities were largely direct. However, mixtures (2-, 4- and 8-species communities), indirectly via N mineralization, sustained overall a higher bacterial effective richness than monocultures. This is in line with previous studies showing a positive effect plant species richness in N mineralization (Zak et al. 2003a, Rosenkranz et al. 2012) and separate studies showing increased bacterial richness in response to increasing plant species richness (Bartelt-Ryser et al. 2005). Our results suggest that these two findings are linked and that further research is needed to unravel these indirect pathways between plant community properties and belowground diversity via soil chemical processes.

CONCLUSIONS

Soil microbial diversity and community composition were significantly influenced by plant diversity and soil legacy. Overall, fungal communities were more responsive than bacterial communities to our plant and soil treatments. Plant community age had no consistent effects on the diversity or the structure of soil microbial communities but did significantly affect particular OTU abundances in plant communities with particular species composition (10 % of all fungal OTUs), suggesting co-adaptation between plant and soil communities. We further demonstrate that effects of soil legacy and plant diversity are both direct and indirect via soil abiotic and biotic properties. Finally, we present a large data set about soil microbial communities present in more than 200 experimentally manipulated plant communities. Here we could only hint at many specific associations between plant and microbial species and specific combinations of plant and microbial species. Future research should aim at further revealing these interactions, mirroring advances in the field of gut microbiomes (Tso et al. 2018) or phyllosphere microbiome dysbiosis (Chen et al. 2020). Our data might become more valuable in the future when technological advances will make it possible for more OTUs to be assigned to specific microbial species and their functions.

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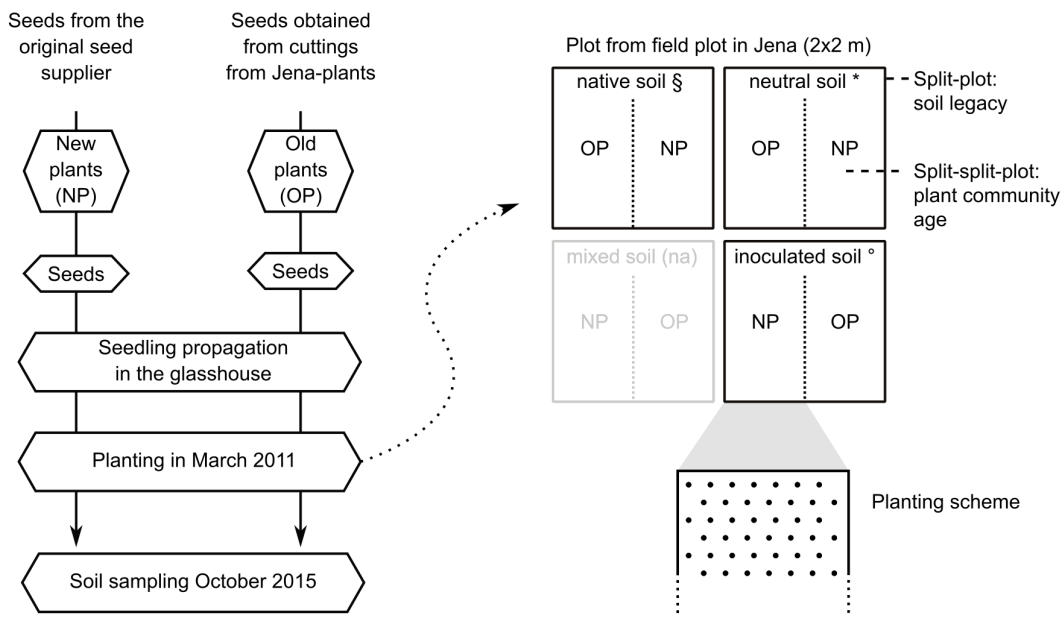
Data accessibility

Data is publicly available on Zenodo (DOI: 10.5281/zenodo.3894768) and SRA (accession PRJNA639013).

Authors' contributions

S.J.V.M, T.H. and B.S. planned and designed the study. T.H. and S.J.V.M. carried out the field experiment. T.H. and S.J.V.M. performed the DNA extraction and sequencing preparation. E.D.L. collected soil samples and conducted soil analyses. C.W. and P. A. N. conducted soil analyses. P.A. N. processed the soil data. M.W.S. processed the sequencing data. M.W.S. performed all data analyses. The paper was written by S.J.V.M., M.W.S. and B.S. with all authors contributing to the final version.

A



Soil legacy (SL)		new soil *		inoculated old soil °		native old soil §	
Community age (PA)		old plants	new plants	old plants	new plants	old plants	new plants
Plant species richness (SR)	1	11/10	11	11	11	11	12
	2	12	11	12	12	12	12/10
	4	11/10	12	12	12	11	12
	8	12	12	12	11	12	12
	60	0	0	0	0	0	4

(*) new soil: sterilized soil + 4% sugar beet soil
(°) inoculated old soil: sterilized soil + 4% sugar beet soil + 4% native soil
(§) native old soil: untreated soil

NP: No co-selection, planted in identical composition
OP: Co-selected plants, grown in Jena Field Experiment since 2002

B

Figure 1. (A) Schematic of the experimental design (see Methods section for details). Plants with a history of growing at the Jena Experiment for 8-years in their respective communities (old plant communities) were planted in communities adjacent to identical communities consisting of plants without such a history (new plant communities). This set up was replicated three times on three different soil treatments. In total, there were 52 of these smaller experimental plots within the large plots across the Jena field site. After planting, the communities were monitored for four years after which the soil samples for the present study were collected. Numbers in the table are replicate communities. (**B**) Photos showing the experimental plots before (left) and after adding the soil and planting the plants (right).

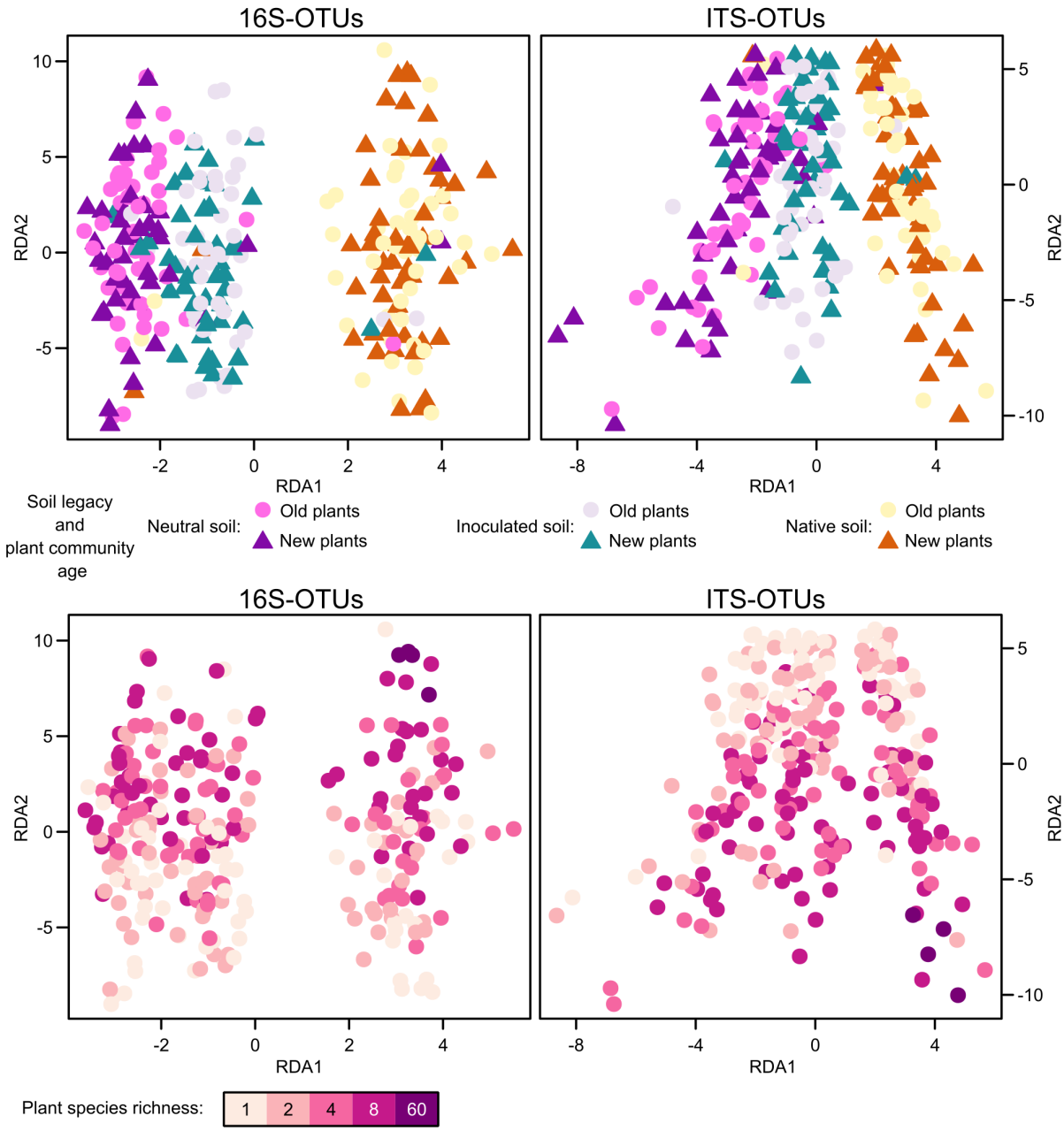


Figure 2. Redundancy analysis (RDA) using the normalized operational taxonomic unit (OTU) abundances of all samples analyzed. For the 16S-OTUs (left), the first two RDA axes explained 19.4 % of the variance (all RDA axes together: 24.7 %). For the ITS-OTUs (right), the first two axes explained 6.4 % of the variance (all RDA axes together: 13.1 %).

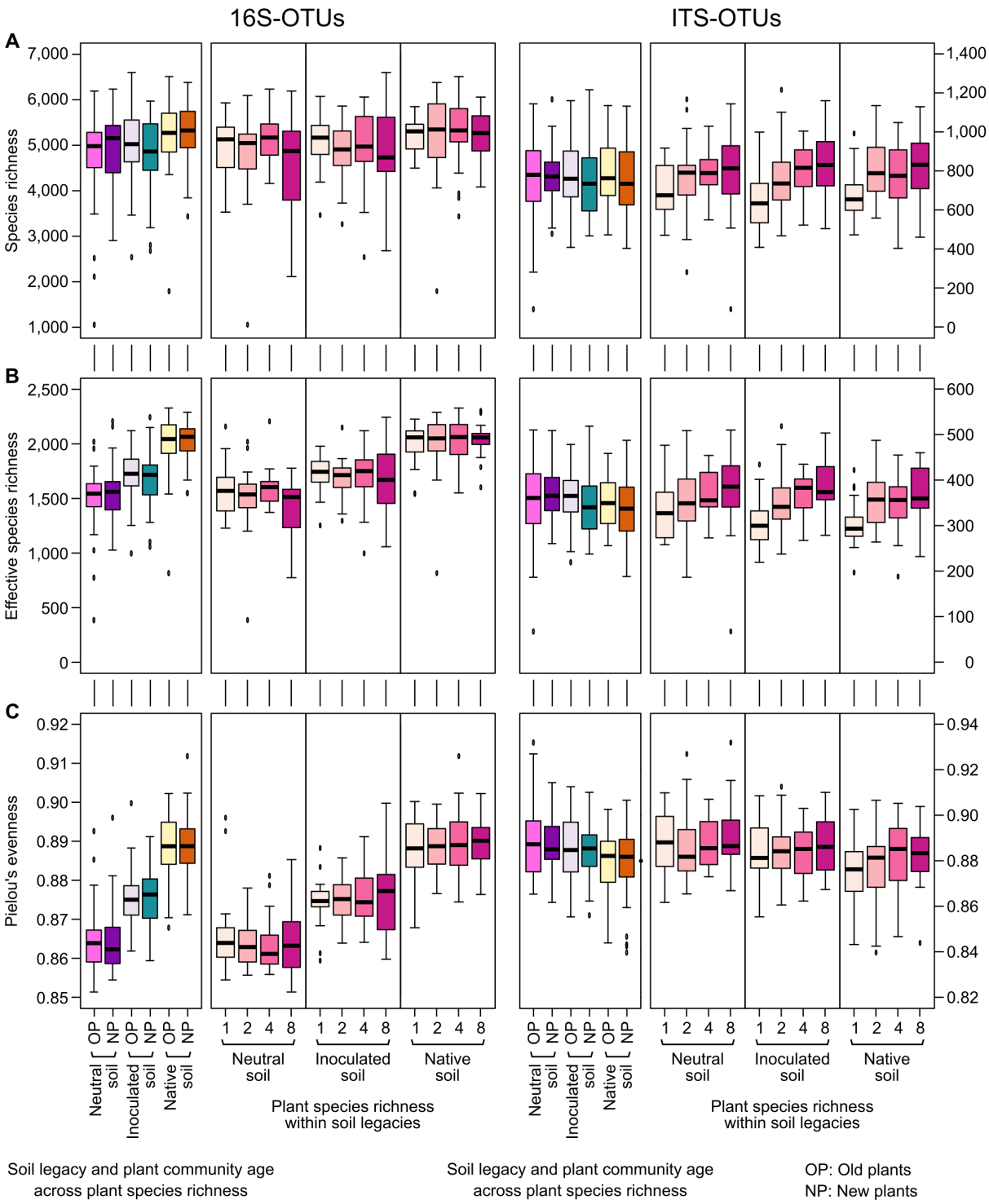


Figure 3. Left half: 16S-OTUs, right half: ITS-OTUs. A) Microbial richness (number of OTUs with more than 0 reads), B) microbial effective OTU richness and C) Pielou's evenness. In a series of four panels, the left most shows the biodiversity indices within each combination of plant community age and soil legacy (all levels of plant species richness are collapsed). The three other panels show the indices within each combination of soil legacy and plant species richness (both levels of plant community age are collapsed). The ANOVA results are given in Table 1. Boxplots: the bottom and top of the boxes correspond to the lower and upper quartiles and the center line marks the median. Whiskers extend to the lowest/highest values unless these values are lower/higher than the first/third quartile

minus/plus 1.5 times the inner quartile range (IQR), which equals the third minus the first quartile.

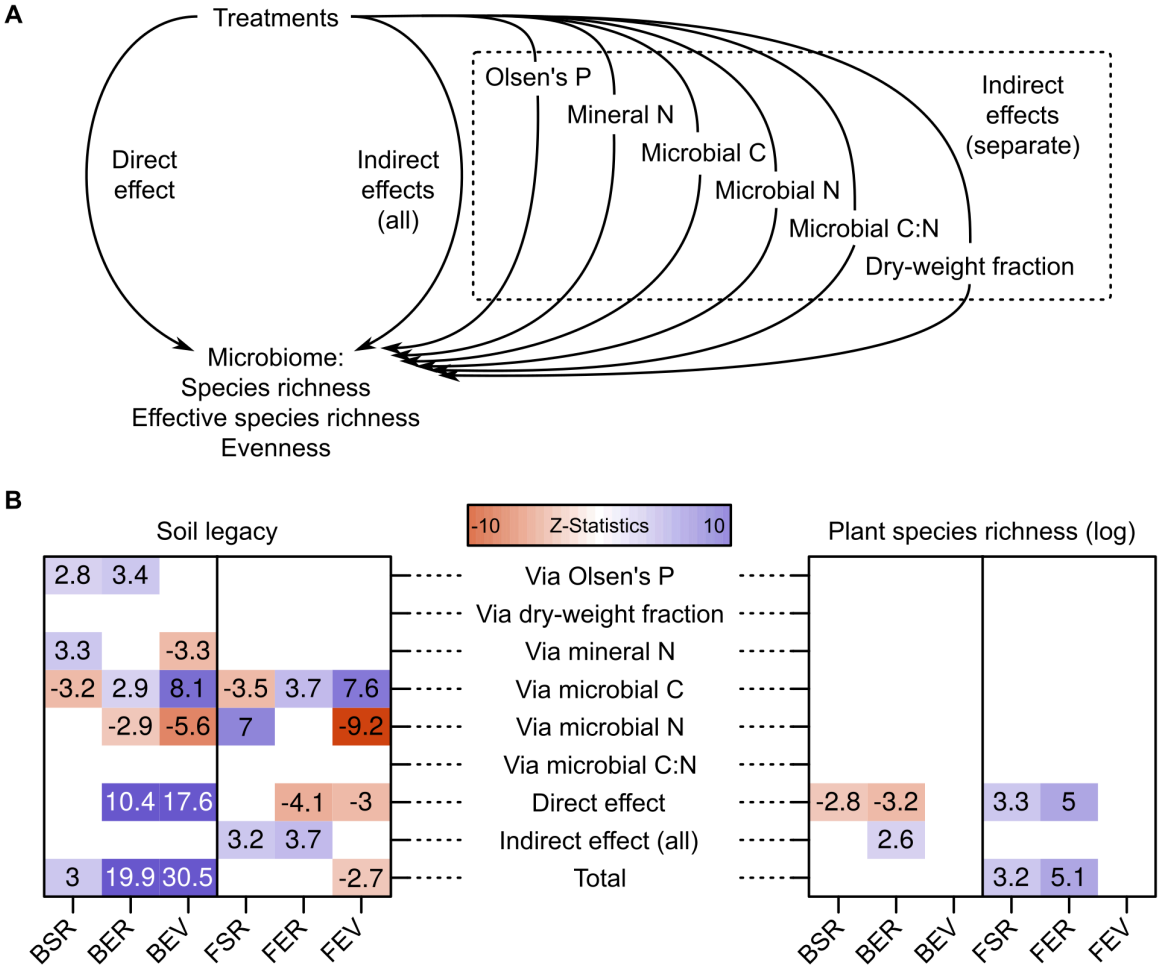


Figure 4. Path analysis to test whether the effects of the soil legacy and plant species richness were direct or indirect through one or more soil variables. A): Schematic representation of the path model. B) Numbers are path coefficients and indicate by how many standard deviations the response would change if the explanatory would be changed by one standard deviation. Insignificant paths are masked with white. Plant community age had no significant term in any of the models and is thus not shown. BSR/FSR: bacterial/fungal richness, BER/FER: bacterial/fungal effective richness, BEV/FEV: bacterial/fungal evenness.

Table 1. Analysis of variance of microbial richness (number of OTUs), effective richness (exp(Shannon Index)) and Pielou's evenness. Contrasts among soil-legacy treatments and their interactions are indented. Significant *P*-values are highlighted in bold. isMonoculture: a contrast to compare plant monocultures to plant species mixtures, logSR: log2 of plant species richness, hasGrasses: contrast for presence/absence of grasses, SL: soil legacy, AGE: new soil compared to the two other soils, INO: inoculated compared to native soil, PA: plant community age. Top: 16S-OTUs, bottom: ITS-OTUs.

16S		**BSR**	**BSR**	**BSR**	**BER**	**BER**	**BER**	**BEV**	**BEV**	**BEV**
Source of variation	**df**	***F***	***P***	**%-SS**	***F***	***P***	**%-SS**	***F***	***P***	**%-SS**
SpatialVariation	5	2.13	0.083	5.98	2.92	**0.025**	3.51	1.31	0.279	1.73
isMonoculture	1	1.33	0.256	0.75	2.65	0.111	0.64	0.01	0.909	0
logSR	1	0.06	0.806	0.03	0.04	0.852	0.01	0.02	0.881	0.01
hasGrass	1	6.11	**0.018**	3.44	2.79	0.103	0.67	4.86	**0.033**	1.28
Plot	39	2.03	**0.003**	21.98	0.99	0.507	9.37	1.73	**0.018**	10.27
old vs new (AGE)	1	7.59	**0.009**	2.51	99.11	**<0.001**	26.9	208.59	**<0.001**	43.94
inoculated vs native (INO)	1	11.27	**0.002**	2.54	82.06	**<0.001**	17.75	209.24	**<0.001**	19.62
Soil legacy (SL)	2	9.08	**<0.001**	5.05	91.55	**<0.001**	44.65	208.79	**<0.001**	63.56
isMonoculture:AGE	1	0.01	0.932	0	0.4	0.532	0.11	1.33	0.255	0.28
isMonoculture:INO	1	0.9	0.349	0.2	0.8	0.376	0.17	0.29	0.592	0.03
isMonoculture:SL	2	0.37	0.693	0.2	0.58	0.564	0.28	1.01	0.369	0.31
logSR:AGE	1	0.5	0.482	0.17	0.79	0.378	0.22	0.04	0.837	0.01
logSR:INO	1	0.48	0.493	0.11	0.62	0.434	0.13	0.01	0.924	0
logSR:SL	2	0.49	0.613	0.28	0.72	0.49	0.35	0.03	0.968	0.01
hasGrass:AGE	1	0	0.98	0	0.26	0.613	0.07	0.78	0.382	0.16
hasGrass:INO	1	0.36	0.55	0.08	0.11	0.74	0.02	3.78	0.058	0.35
hasGrass:SL	2	0.15	0.864	0.08	0.19	0.824	0.09	1.71	0.188	0.51
Plot:AGE	43	1.25	0.239	14.23	2.04	**0.012**	11.67	3.14	**<0.001**	9.06
Plot:INO	43	1.17	0.31	9.67	2.37	**0.003**	9.3	1.64	0.057	4.03

Plot:SL	86	1.21	0.188	23.9	2.17	**0**	20.97	2.45	**<0.001**	13.09
PA	1	0.11	0.747	0.04	0.17	0.68	0.03	0.07	0.799	0.01
isMonoculture:PA	1	0.36	0.551	0.14	0.43	0.516	0.09	0.08	0.778	0.01
logSR:PA	1	2.43	0.126	0.95	1.72	0.196	0.35	1.47	0.231	0.12
hasGrass:PA	1	0.22	0.643	0.09	0.66	0.42	0.13	0.39	0.536	0.03
Plot:PA	43	1.71	**0.019**	16.78	1.81	**0.011**	8.72	1.25	0.188	3.36
AGE:PA	1	0.03	0.857	0.01	0.02	0.894	0	0.08	0.784	0.01
INO:PA	1	2.15	0.15	0.41	3.12	0.085	0.28	0	0.954	0
SL:PA	2	0.92	0.401	0.42	1.28	0.284	0.28	0.04	0.958	0.01
isMonoculture:SL:PA	2	1.78	0.175	0.81	1.36	0.263	0.31	2.4	0.097	0.3
logSR:SL:PA	2	0.46	0.635	0.21	0.9	0.41	0.2	0.08	0.923	0.01
hasGrass:SL:PA	2	0.15	0.858	0.07	0.56	0.572	0.12	2.39	0.098	0.3
Plot:SL:PA	82			18.77			9.2			5.1
Total	374									

ITS		***FSR***	***FSR***	***FSR***	***FER***	***FER***	***FER***	***FEV***	***FEV***	***FEV***
Source of variation	**df**	***F***	***P***	**%-SS**	***F***	***P***	**%-SS**	***F***	***P***	**%-SS**
SpatialVariation	5	5.43	**0.001**	8.05	6.12	**<0.001**	12.12	1.57	0.191	4.72
isMonoculture	1	20.71	**<0.001**	6.15	24.55	**<0.001**	9.71	1.94	0.171	1.17
logSR	1	1.08	0.306	0.32	1.96	0.17	0.77	0.77	0.386	0.46
hasGrass	1	7.54	**0.009**	2.24	17.75	**<0.001**	7.02	7.63	**0.009**	4.58
Plot	39	0.95	0.554	11.58	1.64	**0.029**	15.43	1.82	**0.011**	23.41
neutral vs others (AGE)	1	0.08	0.785	0.03	1.86	0.18	0.55	11.9	**0.001**	3.91
inoculated vs native (INO)	1	0.31	0.581	0.07	1.3	0.26	0.24	7.88	**0.007**	2.6
Soil legacy (SL)	2	0.16	0.849	0.1	1.65	0.199	0.79	9.89	**<0.001**	6.51
isMonoculture:AGE	1	0.57	0.454	0.22	1.71	0.198	0.51	0.31	0.579	0.1

isMonoculture:INO	1	0.31	0.579	0.07	0.23	0.632	0.04	0.69	0.412	0.23
isMonoculture:SL	2	0.47	0.626	0.29	1.14	0.325	0.55	0.5	0.608	0.33
logSR:AGE	1	0.05	0.821	0.02	0	0.976	0	0.23	0.631	0.08
logSR:INO	1	1.48	0.23	0.35	1.12	0.296	0.21	0.29	0.59	0.1
logSR:SL	2	0.6	0.553	0.37	0.43	0.651	0.21	0.26	0.769	0.18
hasGrass:AGE	1	0.17	0.681	0.07	0.01	0.909	0	0.15	0.704	0.05
hasGrass:INO	1	1.04	0.314	0.25	1.45	0.235	0.27	0.08	0.779	0.03
hasGrass:SL	2	0.5	0.607	0.32	0.56	0.571	0.27	0.11	0.894	0.08
Plot:AGE	43	1.25	0.245	16.56	1.22	0.267	12.75	1.31	0.197	14.14
Plot:INO	43	0.71	0.864	10.21	0.81	0.747	7.96	1.85	**0.027**	14.2
Plot:SL	86	0.97	0.563	26.77	1.02	0.462	20.71	1.53	**0.028**	28.34
PA	1	0.49	0.487	0.19	0.21	0.646	0.06	0.02	0.879	0.01
isMonoculture:PA	1	0	0.949	0	0.01	0.937	0	0.02	0.876	0.01
logSR:PA	1	1.89	0.176	0.74	1.96	0.168	0.56	0.64	0.427	0.16
hasGrass:PA	1	0.02	0.89	0.01	0.01	0.919	0	0.07	0.791	0.02
Plot:PA	43	1.22	0.222	16.88	1.2	0.236	12.19	1.19	0.245	11.04
AGE:PA	1	0.48	0.492	0.15	2.66	0.111	0.65	0.45	0.504	0.11
INO:PA	1	0.02	0.882	0.01	0	0.986	0	0.01	0.942	0
SL:PA	2	0.24	0.785	0.16	1.37	0.259	0.65	0.27	0.766	0.11
isMonoculture:SL:PA	2	0.62	0.541	0.4	0.29	0.75	0.14	1.98	0.145	0.86
logSR:SL:PA	2	0.24	0.79	0.15	0.13	0.88	0.06	0.89	0.413	0.38
hasGrass:SL:PA	2	0.22	0.807	0.14	0.84	0.436	0.39	2.1	0.13	0.9
Plot:SL:PA	78			25.12			18.37			16.76
Total	370									

Table 2. Multivariate analysis of variance of dissimilarities between microbial communities. Contrasts among soil-legacy treatments and their interactions are indented. Significant *P*-values are highlighted in bold. MS refers to the mean squares of the dissimilarity measure (Manhattan distance between samples calculated with the log2(x+1)-transformed normalized OTU sequence counts). isMonoculture: a contrast to compare plant monocultures to plant species mixtures, logSR: log2 of plant species richness, hasGrass: contrast for presence/absence of grasses, SL: soil legacy, AGE: new soil compared to the two other soils, INO: inoculated compared to native soil, PA: plant community age. Top: 16S-OTUs, bottom: ITS-OTUs.

16S-OTUs Source of variation	**Df**	***F* altern**	***P* altern**	**% SS**
SpatialVariation	5	2.97	0.023	7.18
isMonoculture	1	1.44	0.237	0.7
logSR	1	1.23	0.274	0.59
hasGrass	1	5.53	0.024	2.67
Plot	39	1.76	0.016	18.85
neutral vs others (AGE)	1	26.88	<0.001	7.26
inoculated vs native (INO)	1	24.69	<0.001	6.92
Soil legacy (SL)	2	25.77	<0.001	14.18
isMonoculture:AGE	1	0.96	0.333	0.26
isMonoculture:INO	1	0.93	0.341	0.26
isMonoculture:SL	2	0.94	0.394	0.52
logSR:AGE	1	0.95	0.336	0.26
logSR:INO	1	0.8	0.376	0.22
logSR:SL	2	0.87	0.422	0.48
hasGrass:AGE	1	1.04	0.313	0.28
hasGrass:INO	1	0.86	0.359	0.24
hasGrass:SL	2	0.95	0.391	0.52
Plot:AGE	43	1.28	0.213	11.61
Plot:INO	43	1.22	0.262	12.05
Plot:SL	86	1.25	0.155	23.66
PA	1	1.05	0.312	0.24
isMonoculture:PA	1	1.05	0.311	0.24

logSR:PA	1	1	0.323	0.23
hasGrass:PA	1	0.88	0.353	0.21
Plot:PA	43	1.06	0.407	10
AGE:PA	1	0.85	0.363	0.18
INO:PA	1	1.02	0.318	0.23
SL:PA	2	0.94	0.396	0.41
isMonoculture:SL:PA	2	0.96	0.387	0.43
logSR:SL:PA	2	0.9	0.409	0.4
hasGrass:SL:PA	2	0.98	0.378	0.43
Plot:SL:PA	82			18.04

ITS-OTUs Source of variation	**Df**	***F* altern**	***P* altern**	**% SS**
SpatialVariation	5	2.21	0.073	7.21
isMonoculture	1	1.55	0.221	1.01
logSR	1	1.58	0.216	1.03
hasGrass	1	9.44	0.004	6.16
Plot	39	2.34	<0.001	25.46
neutral vs others (AGE)	1	13.55	<0.001	4.2
inoculated vs native (INO)	1	9.83	0.003	2.43
Soil legacy (SL)	2	11.9	<0.001	6.63
isMonoculture:AGE	1	1.11	0.299	0.34
isMonoculture:INO	1	0.83	0.368	0.21
isMonoculture:SL	2	0.98	0.378	0.55
logSR:AGE	1	1.03	0.315	0.32
logSR:INO	1	1.06	0.308	0.26
logSR:SL	2	1.05	0.356	0.58
hasGrass:AGE	1	2.01	0.163	0.62
hasGrass:INO	1	1.14	0.291	0.28
hasGrass:SL	2	1.63	0.202	0.9
Plot:AGE	43	1.38	0.155	13.34

Plot:INO	43	1.41	0.141	10.64
Plot:SL	86	1.39	0.069	23.98
PA	1	1.08	0.304	0.21
isMonoculture:PA	1	0.92	0.342	0.18
logSR:PA	1	1.19	0.281	0.23
hasGrass:PA	1	1.13	0.293	0.22
Plot:PA	43	0.98	0.525	8.41
AGE:PA	1	0.95	0.336	0.21
INO:PA	1	0.95	0.336	0.17
SL:PA	2	0.95	0.392	0.38
isMonoculture:SL:PA	2	0.86	0.425	0.35
logSR:SL:PA	2	1.08	0.346	0.43
hasGrass:SL:PA	2	1.09	0.341	0.43
Plot:SL:PA	78			15.63

Table 3. The number of 16S-OTUs (top) and ITS-OTUs (bottom) exhibiting significant differential abundance (FDR < 0.01 and %-SS explained > 1 %) and the average percentage sum of squares of either all OTUs or only the OTUs significant for this term. The term "hasFunctionalGroup" corresponds to a factor testing for either presence of grasses, legumes, small herbs, and tall herbs. For example, given the 16S-OTUs, 741 were significantly influenced by the presence of grasses and 46 were significantly influenced by the presence of legumes.

16S-OTUs, Source of variation	No. significant OTUs	Average %-SS (all OTUs)	Average %-SS (significant OTUs)
Spatial variation	1321	5.53	14.8625
isMonoculture	0	0.60	-
logSR	0	0.53	-
hasFunctionalGroup	741 / 46 / 0 / 0	1.82 / 1.20 / 0.67 / 0.60	12.23 / 13.7 / - / -
Plot	2,032	18.66	29.98
Soil legacy (SL)	4,964	7.79	16.93
>> new vs old (OLD)	4,035	3.91	10.02
>> inoculated vs native (INO)	3,362	3.89	11.83
isMonoculture:SL	0	0.6	-
>> isMonoculture:OLD	0	0.3	-
>> isMonoculture:INO	0	0.3	-
logSR:SL	0	0.61	-
>> logSR:OLD	0	0.32	-
>> logSR:INO	0	0.3	-
hasFunctionalGroup:SL	7 / 0 / 0 / 0	0.71 / 0.75 / 0.67 / 0.61	6.54 / - / -/ -
>> hasFunctionalGroup:OLD	0 / 0 / 0 / 0	0.37 / 0.35 / 0.36 / 0.29	- / - / - / -
>> hasFunctionalGroup:INO	0 / 0 / 0 / 0	0.34 / 0.40 / 0.31 / 0.32	- / - / - / -
Plot:SL	640	27.22	29.18
>> Plot:OLD	238	13.50	17.00
>> Plot:INO	118	13.72	18.56
PA	0	0.29	-
isMonoculture:PA	0	0.28	-
logSR:PA	0	0.28	-
hasFunctionalGroup:PA	0 / 0 / 0 / 0	0.26 / 0.26 / 0.27 / 0.28	- / - / - / -
Plot:PA	6	11.72	18.04
SL:PA	0	0.52	-
>> OLD:PA	0	0.24	-
>> SJ:PA	0	0.28	-
isMonoculture:SL:PA	0	0.5	-
logSR:SL:PA	0	0.52	-
hasFunctionalGroup:SL:PA	0 / 0 / 0 / 0	0.53 / 0.54 / 0.52 / 0.54	- / - / - / -
Total	7,804		

ITS-OTUs, Source of variation	No. significant OTUs	Average %-SS (all OTUs)	Average %-SS (significant OTUs)
Spatial variation	156	4.73	18.56
isMonoculture	0	0.69	-
logSR	0	0.70	-
hasFunctionalGroup	296 / 109 / 0 / 0	2.50 / 1.69 / 0.77 / 0.68	14.58 / 13.28 / - / -
Plot	1,173	22.60	35.79
Soil legacy (SL)	614	2.81	11.71
>> new vs others (OLD)	389	1.69	9.74
>> inoculated vs native (INO)	224	1.12	8.81
isMonoculture:SL	0	0.66	-
>> isMonoculture:OLD	0	0.41	-
>> isMonoculture:INO	5	0.26	0
logSR:SL	0	0.71	-
>> logSR:OLD	0	0.37	-
>> logSR:INO	6	0.35	0
hasFunctionalGroup:SL	8 / 4 / 0 / 0	0.90 / 0.77 / 0.68 / 0.66	9.40 / 9.93 / - / -
>> hasFunctionalGroup:OLD	2 / 4 / 0 / 0	0.53 / 0.42 / 0.36 / 0.34	11.37 / 6.13 / - / -
>> hasFunctionalGroup:INO	6 / 6 / 5 / 6	0.37 / 0.35 / 0.32 / 0.32	0 / 1.18 / 0 / 0
Plot:SL	673	28.90	35.81
>> Plot:OLD	408	15.46	20.92
>> Plot:INO	471	13.44	20.11
PA	0	0.27	-
isMonoculture:PA	0	0.25	-
logSR:PA	0	0.28	-
hasFunctionalGroup:PA	0 / 0 / 0 / 0	0.30 / 0.26 / 0.27 / 0.25	- / - / - / -
Plot:PA	62	11.65	21.39
SL:PA	0	0.55	-
>> OLD:PA	0	0.30	-
>> SJ:PA	10	0.25	0.01
isMonoculture:SL:PA	0	0.51	-
logSR:SL:PA	1	0.58	9.06
hasFunctionalGroup:SL:PA	0 / 0 / 0 / 0	0.59 / 0.52 / 0.55 / 0.54	- / - / - / -
Total	2,490		

Table 4. The number of 16S-OTUs (top) and ITS-OTUs (bottom) exhibiting significant differential abundance (FDR < 0.01 and %-SS explained > 1 %) in response to the biomass proportions of specific plant species. The models were similar to the models shown in Table 1, 2, and 3 but replacing the terms associated with functional groups with the terms testing for the biomass proportion.

16S-OTUs, species tested	**species**	**species:SL**	**species:PA**	**species:SL:PA**
A. millefolium	3	3	0	0
A. reptans	7	6	40	0
A. pratensis	4	19	2	35
A. odoratum	7	52	7	0
A. elatius	27	19	0	0
A. pubescens	43	0	0	0
B. erectus	5	4	0	7
B. hordeaceus	1	0	10	0
C. patula	2	0	0	0
C. biennis	4	4	2	4
C. cristatus	5	29	0	0
D. glomerata	4	13	20	0
D. carota	31	18	0	0
F. pratensis	31	2	52	9
F. rubra	143	61	2	24
G. mollugo	11	9	8	34
G. pratense	49	17	2	29
G. hederacea	1	5	2	0
H. sphondylium	8	21	0	0
H. lanatus	2	5	7	0
K. arvensis	43	43	13	21
L. pratensis	0	634	1	15
L. autumnalis	0	7	0	0
L. hispidus	1	0	1	5
L. vulgare	1	1	31	16
L. corniculatus	4	44	3	2
L. campestris	0	10	13	5
M. lupulina	8	14	2	8
M. varia	10	48	115	159
P. pratense	4	6	1	20
P. lanceolata	10	4	1	0
P. media	0	3	6	13
P. pratensis	2	5	0	9
P. trivialis	127	6	2	13
P. veris	0	6	7	5
P. vulgaris	6	32	13	17
R. acris	2	3	0	5
R. repens	4	21	0	0
S. officinalis	12	7	0	2
T. officinale	5	1	0	2
T. campestre	1	3	13	0
T. dubium	0	6	0	0
T. flavescens	1	17	1	5
T. fragiferum	1	43	0	0
T. hybridum	1	10	0	0

T. pratense	20	15	0	0
T. repens	2	7	4	8
V. chamaedrys	17	29	10	3
V. cracca	2	4	0	0

ITS-OTUs, species tested	**species**	**species:SH**	**species:PA**	**species:SH:PA**
A. millefolium	22	95	0	0
A. reptans	7	14	12	0
A. pratensis	50	116	34	88
A. odoratum	44	43	12	0
A. elatius	64	113	80	0
A. pubescens	20	0	0	0
B. erectus	37	85	10	100
B. hordeaceus	13	0	25	0
C. patula	59	0	0	0
C. biennis	29	33	59	80
C. cristatus	21	65	0	0
D. glomerata	229	231	50	0
D. carota	25	113	0	0
F. pratensis	46	52	48	137
F. rubra	138	184	60	211
G. mollugo	16	27	16	35
G. pratense	48	85	43	103
G. hederacea	24	46	24	0
H. sphondylium	17	87	0	0
H. lanatus	16	20	48	0
K. arvensis	31	49	5	69
L. pratensis	10	36	13	39
L. autumnalis	8	47	0	0
L. hispidus	17	17	20	40
L. vulgare	7	28	7	79
L. corniculatus	13	18	5	26
L. campestris	23	13	71	65
M. lupulina	16	36	28	44
M. varia	34	83	28	80
P. pratense	19	56	34	138
P. lanceolata	7	21	0	17
P. media	22	38	40	44
P. pratensis	17	45	33	61
P. trivialis	4	10	34	37
P. veris	9	23	9	0
P. vulgaris	19	64	57	39
R. acris	19	21	15	32
R. repens	38	25	0	0
S. officinalis	37	71	22	87
T. officinale	34	32	5	43
T. campestre	24	12	34	0
T. dubium	12	36	0	0
T. flavescens	15	129	13	43
T. fragiferum	41	70	0	0
T. hybridum	25	23	0	0
T. pratense	26	42	0	0
T. repens	14	28	16	28

V. chamaedrys	21	41	12	74
V. cracca	17	23	12	0