



Soil bacteria and fungi respond differently to plant diversity and plant family composition during the secondary succession of abandoned farmland on the Loess Plateau, China

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Abstract

Aims This study aimed to determine the responses of soil bacteria and fungi to plant species diversity and plant family composition (PFC) following secondary succession on former farmland (FL).

Methods Illumina sequencing of 16S rRNA and ITS genes was used to determine soil microbial communities along a chronosequence of FL left abandoned for 0, 10, 20, 30, 40, and 50 years on the Loess Plateau. Soil properties, plant diversity, and PFC were also investigated.

Results Fungal communities were dominated by *Ascomycota* and *Basidiomycota*. Fungal diversity and *Ascomycota* abundance increased with time, while *Basidiomycota* abundance decreased. The fungal diversity and dominant phyla were related to the increasing levels of

plant species diversity and evenness with succession. Bacterial diversity first increased and then decreased as succession proceeded, peaking at 30 years. Bacterial communities transitioned from *Actinobacteria* to *Proteobacteria* dominance during the first 30 years, after which *Actinobacteria* was dominant. Plant family composition exerted indirect effects on the diversity and dominant phyla of bacterial communities, mainly through direct effects on soil organic carbon and total nitrogen content. Bacterial diversity and *Proteobacteria* abundance were higher at Leguminosae- and Gramineae-dominant succession stages, but lower in Compositae-dominant plots; *Actinobacteria* showed the opposite result.

Conclusions Plant species diversity and evenness might be the key drivers for shaping fungal communities, but bacteria are influenced more by changes in PFC and abiotic soil nutrient levels during succession.

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Keywords Soil bacteria and fungi · Plant-microbial diversity · Plant families · Secondary succession · Soil nutrients

Abbreviations

AL	Abandoned land
Comp	Compositae
FL	Farmland
Gram	Gramineae
Legu	Leguminosae
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen

MBP	Microbial biomass phosphorus
NMDS	Non-metric multidimensional scaling
PFC	Plant family composition
PLS-PM	Partial least squares path models
SOC	Soil organic carbon
TN	Total nitrogen
TP	Total phosphorus.

Introduction

Secondary succession of former arable lands with poor soil fertility and productivity is an effective practice to withstand ecosystem deterioration in ecologically fragile regions (Jiang et al. 2009; Zhang et al. 2016). The total area of succession on abandoned farmlands (FLs) in China was 33,500 km² over the past two decades, and such widespread changes in ecosystem structures have strong potential to alter key ecological processes. Theoretically, shifts in plant communities during succession could regulate belowground communities via altering the heterogeneity of food resources and physical microhabitats (e.g., root morphology, soil pore structure, and aeration) (Burns et al. 2015; Chen et al. 2016; Mahaming et al. 2009). In turn, soil microbes could affect aboveground communities by enhancing the bioaccessibility of nutrients (Prober et al. 2015; Singh et al. 2004; Zhang et al. 2016), and influence plant dominance through symbiotic and pathogenic microorganisms (van der Heijden et al. 2008). Therefore, plant succession is essentially the interaction between aboveground plants and belowground microorganisms (He et al. 2008; Kielak et al. 2008).

Previous studies have examined the positive consequences of plant indexes (i.e., diversity, richness, and evenness) on soil microbial communities by diversifying food resources (soil exudates and litter) (Chen et al. 2016; Cline and Zak 2015; Dassen et al. 2017; Eisenhauer et al. 2011). Moreover, various studies on the impact of plant species on soil microbial communities indicated that changes in plant family composition (PFC) might have a stronger influence than plant diversity per se (Bezemer et al. 2006; Kennedy et al. 2004). For example, certain soil microorganisms form host-specific relationships with a specific plant family, such as Leguminosae and nitrogen-fixing bacteria (Dassen et al. 2017; van der Heijden et al. 2008). Additionally, different plant families that have various plant traits (such as the differences in C:N ratio between

Leguminosae and Compositae) are likely to have differing effects on a variety of soil properties, and thereby affect soil microbial community composition (Marshall et al. 2011; Zhang et al. 2018). In general, for vegetation, succession consists in the substitution of species, whereas in the microbial community, it consists in shifts in the metabolic activity adapted to successively transformed substrates (Mahaming et al. 2009). Therefore, soil bacteria and fungi might respond in different ways to secondary succession owing to their different metabolic pathways. For instance, a shift toward plant communities with greater lignin contents (e.g., C₄ plants) can be expected to alter the fungal:bacterial biomass ratio toward a more fungal-dominated system, since fungi are capable of colonizing a wider range of substrates than are bacteria (Cline and Zak 2015; van der Wal et al. 2006); however, the presence of legumes generally increases the soil bacterial biomass (Dassen et al. 2017). Although such studies have shown that aboveground plant community variations can impact the soil microbial diversity and composition, most have been limited to a subset of the plant (single effect of plant diversity or PFC identity) or soil microbial community (single response of the fungi or bacteria). Therefore, a critical knowledge gap is emerging on whether the plant diversity and family composition bring about similar changes in soil microbial groups (i.e., bacteria and fungi), especially in fragile ecological regions where species diversity and composition change rapidly.

The Loess Plateau of China, which has a total area of 624,000 km², is subjected to severe soil erosion and desertification, and is considered to be one of the most ecologically fragile areas worldwide (Ren et al. 2016; Zhang et al. 2016). Intensive soil erosion has resulted in land productivity decline, environmental degradation, and enormous economic losses (Jiang et al. 2009; Zhong et al. 2018). Abandoning sloped FLs to allow secondary succession is the prevalent practice for improving ecological condition in the Loess Plateau; studying succession is the starting point for developing vegetation recovery and management strategies in this region. The effects of secondary succession on soil properties (Zhang et al. 2018; Zhong et al. 2018), microbial dynamics (Deng et al. 2019; Jiang et al. 2009), and their interrelationships (Zhang et al. 2016) have been reported in recent years. However, information on the responses of soil bacterial and fungal communities to the changes in vegetation characteristics with succession is still limited. Such information is required

for a better understanding of the functional connection between the aboveground and belowground components of terrestrial ecosystems and for the appropriate management and conservation of the ecological environment.

To elucidate the response of soil bacteria and fungi to secondary succession, Illumina sequencing was used to examine the soil microbial diversity and compositions from 15 sites representing a 50-year secondary succession on abandoned FL. In the current study, we hypothesized that soil bacterial and fungal diversity respond differently to secondary succession, and that plant diversity may help to explain these different responses. Additionally, we predicted that variation in specific microbial taxa (bacterial and fungal taxa) might be associated with the changes in PFC and soil nutrient levels during secondary succession (Mahaming et al. 2009; Marshall et al. 2011). The objectives of this study were therefore to (i) evaluate the patterns of change in the plant community characteristics, soil properties, and the diversity and composition of microbial communities with succession; (ii) determine the relationships between plant diversity, evenness, richness, and the diversity and composition of bacterial and fungal communities; and (iii) determine the response patterns of the diversity and composition of bacterial and fungal communities to PFC changes with succession.

Materials and methods

Site description

The study area is located in the Zhifanggou watershed of Ansai County, Shaanxi Province, China (109°19′–109°22′ E, 36°51′–36°52′ N), with an area of 5.88 km² and elevation ranging from 1010 to 1400 m, which is a typical hill-and-gully region on the Loess Plateau. The region is temperate and semi-arid, with a mean annual temperature of 8.8 °C and average annual precipitation, which mainly falls from July to September, of 535 mm. Loessial soil [Calcic Cambisols; FAO 1972] is the main soil type and characterized by its weak cohesion, high infiltrability, low nutrient retention, and proneness to erosion. The original vegetation in this region has been devastated completely over the last two centuries because of wars and calamities (Zhong et al. 2018). Since the 1970s, the area has been used as an experimental site for rebuilding vegetation and controlling soil erosion by

the Institute of Soil and Water Conservation of the Chinese Academy of Science. A series of integrated management processes of vegetative restoration and soil and water conservation were carried out based on the soil quality thereafter. In particular, all FLs with slopes greater than 15° were abandoned or converted to artificial forest when the ‘Grain-to-Green’ Program was implemented by the Chinese government in 1999 (Zhong et al. 2018). This long-term natural restoration along the soil quality gradient is an important driver for the changes in soil and plant characteristics (Deng et al. 2019; Zhang et al. 2016), and therefore provides an ideal chronosequence to study the effects of the secondary succession of abandoned land (AL) on ecosystem processes.

Experimental design, vegetation surveys, and soil sampling

Based on the duration of land abandonment, 15 sites were chosen under the guidance of the local forestry bureau in August 2016. The AL chronosequence we selected consisted of five age classes differed by ca. 10 years and were: 11-, 10-, and 9-; 18-, 18-, and 20-; 29-, 28-, and 30-; 40-, 39-, and 38-; 48-, 52-, and 48-year-old, respectively (which will be referred to in this study as 10-, 20-, 30-, 40-, and 50-year-old sites). All of the sites had similar slope gradients, elevations, and previous farming practices. The main crops grown in these FLs before abandonment were maize (*Zea mays*) and foxtail millet (*Setaria italica*) in rotation. When the FLs were abandoned, the plants were spontaneously colonized on these lands, without seeding or human disturbance. Three active sloped FLs growing maize and millet under traditional cultivation were used as references. The crops were manually harvested, and the plots at these sites were manually plowed to a depth of 20 cm each year after harvesting. In the FLs, the farmers regularly removed economically relevant weeds by hand, remaining weeds were scarce and thus not further investigated here. No irrigation (depending on rainfall) was provided during the growth season, and manure was the main fertilizer. Detailed information on these experimental sites are shown in Table 1. Three 25 × 25 m plots (each plot was 50 m apart) were randomly set up at each site for subsequent investigation and sampling.

In order to reveal the linkages of soil microbial and plant compositions and further reflect the synergistic

change between plants and microbes during the secondary succession, in mid-August 2016, ten 1×1 m quadrats were established with an “S” shape in each replicated plot for vegetation investigation, and plant and soil sampling. All plant species were identified to the species level and the total number of each species in the quadrats was recorded. The cover of every species was visually estimated as percent canopy cover (%) by the same pair of observers working together (so that bias, if it exists, was similar at all sites). The scales of species coverage in plant communities were applied according to Braun-Blanquet (1964). The height was measured with a ruler on ramets (culms) that represented the majority of the total ramets of the species. In this study, importance value (*IV*) was used to indicate the importance of species in the community, and the Gleason richness index (*G*), Shannon–Wiener diversity index (*H*), and Pielou evenness index (*E*) were used to describe species diversity; these four values were calculated with the following formulas:

$$IV = \frac{(\text{relative density} + \text{relative frequency} + \text{relative coverage})}{\text{relative height}} / 4 \times 100\% \quad (1)$$

$$G = \frac{S-1}{\ln N} \quad (2)$$

$$H = - \sum_{i=1}^S (P_i \ln P_i) \quad (3)$$

$$E = \frac{H}{\ln S} \quad (4)$$

where *S*, *N*, and *P_i* are the total number of species *i*, the total number of individuals, and the density proportion of *i* species, respectively.

In each quadrat, one sample was collected from the top 10 cm using a soil auger (5 cm inner diameter). A total of 10 soil samples were collected in each plot, and then mixed to provide one sample per site. Thus, a total of 18 soil samples were collected (6 age classes \times 3 replicate sites). Roots, stones, and other visible plant debris were carefully removed by hand and the soil samples were separated into three parts. One part was air-dried and sieved through a 2-mm sieve for C, N, and P analyses. The two other parts were either stored at

–80 °C or 4 °C for the extraction of DNA or microbial biomass analysis, respectively.

Soil chemical analysis

Soil organic carbon (SOC) and total nitrogen (TN) were determined by the $K_2Cr_2O_7$ oxidation method (Nelson and Sommers 1982) and the Kjeldahl procedure (Bremner and Mulvaney 1982), respectively. Total phosphorus (TP) was analyzed colorimetrically after the digestion with H_2SO_4 and $HClO_4$ (Parkinson and Allen 1975). Soil microbial biomass were determined by the chloroform fumigation-direct extraction method (Brookes et al. 1985; Vance et al. 1987). In brief, organic C and total N were extracted with 40 ml of 0.5 M K_2SO_4 by shaking for 1 h from fumigated and non-fumigated soils, and measured in the filtered extract using a TOC-TN analyzer (Shimadzu, Kyoto, Japan). Values for microbial biomass C (MBC) and N (MBN) were reported as the difference between fumigated and non-fumigated samples, with a conversion factor for C (*Ec*) of 2.64 (Vance et al. 1987) and for N (*En*) of 2.22 (Brookes et al. 1985). Microbial biomass P (MBP) was measured using a similar method as for MBC and MBN except that the P was extracted by 0.5 M $NaHCO_3$ (pH 8.5). Phosphorus content in the filtrate was determined colorimetrically, and was calculated using a conversion factor (*K_p*) of 0.4 (Brookes et al. 1984).

Soil DNA extraction, PCR amplification, and Illumina sequencing

DNA was extracted in triplicate from 0.5 g of fresh sample using the FastDNA spin kit for soil (MP Bio-medicals, Cleveland, USA), following the manufacturer’s instructions. The quality and integrity of the DNA extracts were checked by 1.0% agarose gel electrophoresis (Caporaso et al. 2010). The V4 region of bacterial 16S rRNA genes were amplified using the primer combination of 515F (5’-GTGCCAGC MGCCGCGG-3’) and 907R (5’-CCGTC AATTCMTTTRAGTTT-3’) (Biddle et al. 2008). The fungal ITS-1 region was amplified by using fungi-specific primers ITS1F (5’-ACTTGGTCATTTAGAG-GAAGTAA-3’) and ITS2 (5’-BGCTGCGTTCTTCAT CGATGC-3’) (Mukherjee et al. 2014). The primers were tagged with unique barcodes for each sample. The reaction system of bacterial PCR amplification contained 0.4 μ l of each primer, 1.25 μ l of template DNA (10 ng),

Table 1 Geographical features and soil physical properties of the selected studied sites

Abandoned sites ^a	Age (year)	Geographical coordinates	Elevation (m)	Slope gradient (°)	Slope aspect (°)	Clay content (<0.002 mm) (%)	Silt content (0.002–0.05 mm) (%)	Sand content (0.05–2 mm) (%)	Bulk density (g·m ⁻³)	Soil water content (%)
FL	–	36°51'59.3"N; 109°20'43.4"E	1212	7	E39°N	14.59 ± 0.41d	43.74 ± 0.09d	41.67 ± 0.33a	1.26 ± 0.03a	9.05 ± 0.38c
	–	36°51'54.1"N; 109°20'45.1"E	1197	7	E47°N					
	–	36°51'58.3"N; 109°20'48.4"E	1206	5	E39°N					
AL10	9	36°51'54.4"N; 109°20'59.1"E	1242	23	E70°N	18.86 ± 0.98c	50.01 ± 0.84c	31.13 ± 1.81b	1.05 ± 0.02b	9.29 ± 0.49c
	10	36°51'52.6"N; 109°20'57.2"E	1237	29	E74°N					
	11	36°51'51.8"N; 109°20'57.5"E	1241	31	E69°N					
AL20	18	36°51'54.9"N; 109°20'53.2"E	1194	33	W25°N	19.65 ± 0.18abc	56.66 ± 0.33a	23.68 ± 0.43e	1.02 ± 0.01b	10.26 ± 0.57c
	18	36°51'52.2"N; 109°20'53.4"E	1198	33	W21°N					
	20	36°51'55.9"N; 109°20'52.4"E	1196	30	W21°N					
AL30	28	36°51'43.1"N; 109°21'01.5"E	1289	35	E40°N	20.92 ± 0.71ab	52.39 ± 1.92bc	26.69 ± 1.21 cd	1.06 ± 0.04b	14.19 ± 0.31a
	29	36°51'40.2"N; 109°21'04.7"E	1291	33	E38°N					
	30	36°51'38.5"N; 109°20'59.6"E	1297	39	E44°N					
AL40	38	36°51'47.9"N; 109°20'51.8"E	1203	32	E10°N	21.32 ± 0.42a	54.41 ± 0.60ab	24.27 ± 0.27de	1.08 ± 0.06b	12.24 ± 0.52b
	39	36°51'48.2"N; 109°20'52.9"E	1197	35	E14°N					
	40	36°51'49.3"N; 109°20'54.1"E	1208	37	E24°N					
AL50	48	36°51'50.0"N; 109°20'58.2"E	1250	28	E31°N	19.29 ± 0.13bc	51.93 ± 0.07bc	28.79 ± 0.16bc	1.09 ± 0.04b	9.78 ± 1.01c
	48	36°51'49.0"N; 109°20'59.3"E	1252	28	E31°N					
	52	36°51'48.8"N; 109°20'58.5"E	1247	31	E23°N					

^a Means that the abandoned lands had been restored for approximately 10 years (AL10), 20 years (AL20), 30 years (AL30), 40 years (AL40), and 50 years (AL50). FL: farmland. Values are means ± standard error (*n* = 3). Lowercase letters indicate significant difference among different successional stages (*P* < 0.05)

0.4 μl of FastPfu polymerase (Beijing TransGen Biotech Co., Ltd., China), 2.5 μl of $10 \times$ PCR buffer, 2.0 μl of dNTPs (2.5 mM), 5.0 μl of $5 \times$ high enhancer, and 13.45 μl of sterile ultrapure water, in a total reaction volume of 25 μl . The PCR amplification of 16S rRNA was performed at an initial denaturation temperature of 95 °C for 3 min, followed by 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. A final elongation step at 72 °C was run for 10 min. The PCR reaction system of ITS gene was also performed in a 25 μl mixture, which contained 0.5 μl of the two primers (30 $\mu\text{mol } \mu\text{l}^{-1}$), 1.5 μl of template DNA (10 ng), and 22.5 μl of Platinum PCR SuperMix (Invitrogen, Shanghai, China). The thermal cycling program consisted of an initial annealing temperature at 95 °C for 2 min, followed by 30 cycles with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. The three replicated amplification products of bacteria and fungi in each soil sample were mixed to provide one final PCR product. Each mixed PCR product was purified using the Qiagen™ PCR purification kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol, and then were eluted in sterile water. Subsequently, 2.0% agarose gel electrophoresis were used to quantified the concentration of each mixed gene (16S rRNA gene and ITS rRNA gene). Finally, the purified amplicons were pooled in equimolar and paired-end sequenced (2×300) on an Illumina MiSeq platform (Personal, Shanghai, China) according to the standard protocols.

Sequence processing

Raw FASTQ files of sequencing were de-multiplexed and quality-filtered using Quantitative Insights Into Microbial Ecology (QIIME, Version 1.9.0) workflow (Caporaso et al. 2010). Briefly, tags sequence, primer sequence, sequences <200 bp, an average quality score of <25 and reads containing ambiguous bases or any unresolved nucleotides were removed to obtain the high-quality sequence for subsequent analysis (Caporaso et al. 2010). Forward and reverse reads were incorporated into full-length sequences with FLASH (Fast Length Adjustment of Short reads). The above processed sequences were clustered into operational taxonomic units (OTUs) defined by 97% similarity using UPARSE (version 7.1) program (Edgar 2013), and chimeric sequences were identified and removed

using UCHIME (Schloss et al. 2009). Subsequently, the largest number of sequences in each OTU were selected as the representative sequence to prepare OTUs table (Zhang et al. 2016). The obtained bacterial and fungal OTUs were performed sequence alignment in Ribosomal Database Project (DPR) and National Center for Biotechnology Information (NCBI), respectively, with the lowest similarity of 0.8 for both bacteria and fungi (Cole et al. 2005). Python Nearest Alignment Space Termination (PyNASt) workflow was applied to align the representative sequence to the reference sequence (Caporaso et al. 2010) and phylogenetic analysis of 16S and ITS sequences were conducted by Fast tree software (Price et al. 2009). To compare the variations in microbial community during secondary succession, all the microbial DNA sequence were further classified according to certain sequence number for subsequent analysis. The complete dataset was sent to the Sequence Read Archive (SRA) database of the NCBI under the accession numbers of SRP223557 for bacteria and SRP223562 for fungi.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the effects of secondary succession on soil properties, plant characteristics (diversity and PFC), and the diversity and composition of soil bacterial and fungal communities. If significant effects were observed by ANOVA, a least significant difference [LSD (0.05)] test was used. We quantified the responses of the contents and stoichiometries of C, N, and P in soil and microbial biomass to secondary succession, with the FL as control. For each variable, the response ratios (RRs) were calculated as below:

$$\text{RRs} = \ln \left\{ \frac{X_t}{X_c} \right\} = \ln(X_t) - \ln(X_c) \quad (5)$$

where X_t and X_c were means of SOC, TN, TP, MBC, MBN, MBP, and their stoichiometric ratios in ALs and in FLs, respectively. Significant responses ($P < 0.05$) were determined if RRs did not overlap with zero. Shannon index was used to reflect bacterial and fungal alpha diversities and calculated using the same equation to plant community did (Eq. 3). The unweighted Bray-Curtis distances between the ALs and FLs were used to represent microbial beta diversity. We therefore generated a non-metrical multidimensional scaling (NMDS)

plot based on Bray-Curtis distance to illustrate the clustering of the different samples and further evaluate microbial beta diversity (NMDS1 and NMDS2). Moreover, the redundancy analysis (RDA) was used to analyze the correlations between the plant diversity, evenness, and richness and the microbial diversity and community composition. Furthermore, model evaluation of Partial Least Squares Path (PLS-SM) is a data analysis approach for studying observed variables that can be summarized by the use of a latent variable and the fact that linear relationships exist between latent variables (Sanchez 2013). Therefore, PLS-SM was applied to explore the relationships among the PFC, soil nutrient content, the dominant microbial phyla, and microbial alpha and beta diversities, which was based on the overall model goodness-of-fit index and on the R^2 coefficient for the three soil stability measures. We select the PLS-PM approach since it is based on a series of ordinary least square regressions, and as a consequence has minimum demands regarding sample size, a characteristic that was required in our study. On the other hand, PLS-PM generally achieves high levels of statistical power and can still be applied when other methods do not converge or provide inadmissible solutions (Ali et al. 2017). All statistical analyses were done using R software package v.3.2.3.

Results

Changes in vegetation characteristics during secondary succession

The crops on the FLs had been harvested, so the plant characteristics of these sites were not analyzed (Table 2). A total of 51 herbaceous species and five shrub species were recorded on all ALs, belonging to 18 plant families (Table S1). The *IV* of total herbs gradually increased during the first 30 years and then decreased at the late successional stages. The dominance of total shrubs was extremely low (average of 0.7%) at the early successional stages, but their *IV* increased dramatically (average of 19.1%) after 30 years of secondary succession.

No obvious trends in species richness were found, which followed the order of AL20 (4.0) > AL50 (3.5) > AL30 (3.1) > AL10 (3.0) > AL40 (2.8) (Table 2). The species diversity (H) and evenness (E) of plant communities generally increased over the entire period of succession, except for at the 20-year site, following the

order of AL50 > AL20 > AL40 > AL30 > AL10 for H and AL50 > AL40 > AL20 > AL30 > AL10 for E .

The plot of NMDS (Fig. 1a) indicated obvious differences in vegetation composition along coordinate 1 among the successional stages. Compositae, Gramineae, and Leguminosae dominated the PFC in our study area, with 14, 13, and 9 herbaceous species, respectively (Table S1). The percentage of plants belonging to the Compositae family first decreased and then increased with successional time, with the maximum proportion at AL50 and minimum at AL30 (Fig. 1b). In contrast, the percentages of Gramineae and Leguminosae increased during the first 30 years and then dropped as succession proceeded, peaking at the AL30 site.

Responses of the contents and stoichiometries of C, N, and P in soil and microbial biomass to secondary succession.

The Shapiro–Wilk test was used to check the distribution of all data, and all variables followed a normal distribution. Further variance analysis showed that the C and N contents in the soil and microbial biomass significantly increased after land was abandoned in general; however, they responded differently to successional time (Fig. 2 and Table S2; $P < 0.05$). Specifically, SOC, TN, and MBN increased during the first 30 years and then decreased gradually, with a peak at the AL30 site, while MBC and MBP showed increasing trends with succession, despite the fact that the increases in MBC at AL40 and AL50 were not significant ($P > 0.05$). No significant difference in TP concentration was found among sites. Because of the disproportionate increases in C, N, and P during succession, the C:N:P stoichiometry in the soil and microbial biomass also showed a different response to secondary succession. The C:N ratio in the soil decreased with time; however, this ratio in the microbial biomass increased, while the C:P and N:P ratios increased until 30 years and then decreased.

Variations in microbial diversity and composition during secondary succession

The OTUs were clustered at a similarity above 97% to calculate the diversity indices. The soil microbial alpha diversity values in all abandoned sites, except for the bacterial alpha diversity at AL50 and fungal alpha diversity at AL10, were higher than that in the FL (Fig. 3). In addition, the alpha diversity values of bacteria and fungi took on different trends with successional time.

Table 2 Characteristics of the vegetation during secondary succession

Abandoned sites ^a	FL	AL10	AL20	AL30	AL40	AL50
Plant coverage (%)	^b	35.25 ± 2.40d	46.40 ± 0.70c	68.27 ± 0.27a	58.32 ± 3.95b	47.53 ± 2.22c
Aboveground biomass (g·m ⁻²)	–	162.38 ± 6.57d	257.54 ± 14.25c	324.89 ± 27.34a	301.68 ± 20.56b	289.94 ± 23.06bc
Gleason richness index	NA	3.00 ± 0.19a	4.01 ± 0.25b	3.12 ± 0.06b	2.84 ± 0.25b	3.45 ± 0.20ab
Shannon–Wiener diversity index	NA	2.65 ± 0.08c	3.47 ± 0.03ab	3.06 ± 0.22bc	3.27 ± 0.19ab	3.72 ± 0.02a
Pielou evenness index	NA	0.92 ± 0.04c	1.09 ± 0.03ab	1.04 ± 0.08bc	1.15 ± 0.03ab	1.23 ± 0.02a
Dominant plant species	<i>Setaria italic</i> (Gramineae) <i>Glycine max</i> (Leguminosae)	<i>Artemisia gmelinii</i> (Compositae) <i>Stipa grandis</i> (Gramineae)	<i>Setaria viridis</i> (Gramineae) <i>melilotoides</i> (Leguminosae)	<i>Cleistogenes squarrosa floribunda</i> (Gramineae) <i>Lespedeza</i> (Leguminosae)	<i>Artemisia gmelinii floribunda</i> (Compositae) <i>Lespedeza</i> (Leguminosae)	<i>Artemisia gmelinii</i> (Compositae) <i>Digitaria sanguinalis</i> (Gramineae)
Minor species	–	<i>Dendranthema indicum</i> (Compositae) <i>Lespedeza floribunda</i> (Leguminosae) <i>Artemisia sieversiana</i> (Compositae)	<i>Artemisia capillaris</i> (Compositae) <i>Setaria faberi</i> (Gramineae) <i>Heteropappus altaicus</i> (Compositae)	<i>Phragmites australis</i> (Gramineae) <i>Setaria viridis</i> (Gramineae) <i>Glycyrrhiza uralensis</i> (Leguminosae)	<i>Stipa grandis</i> (Gramineae) <i>Cleistogenes squarrosa</i> (Gramineae) <i>Astragalus melilotoides</i> (Leguminosae)	<i>Lespedeza floribunda</i> (Leguminosae) <i>Heteropappus altaicus</i> (Compositae) <i>Ixeridium sonchifolium</i> (Compositae)

^a Means that the abandoned lands had been restored for approximately 10 years (AL10), 20 years (AL20), 30 years (AL30), 40 years (AL40), and 50 years (AL50). FL: farmland

^b Plant coverage and aboveground biomass were not taken into account because the farmland was harvested. Values are means ± standard error (n = 3). Lowercase letters indicate significant difference among different successional stages (P < 0.05)

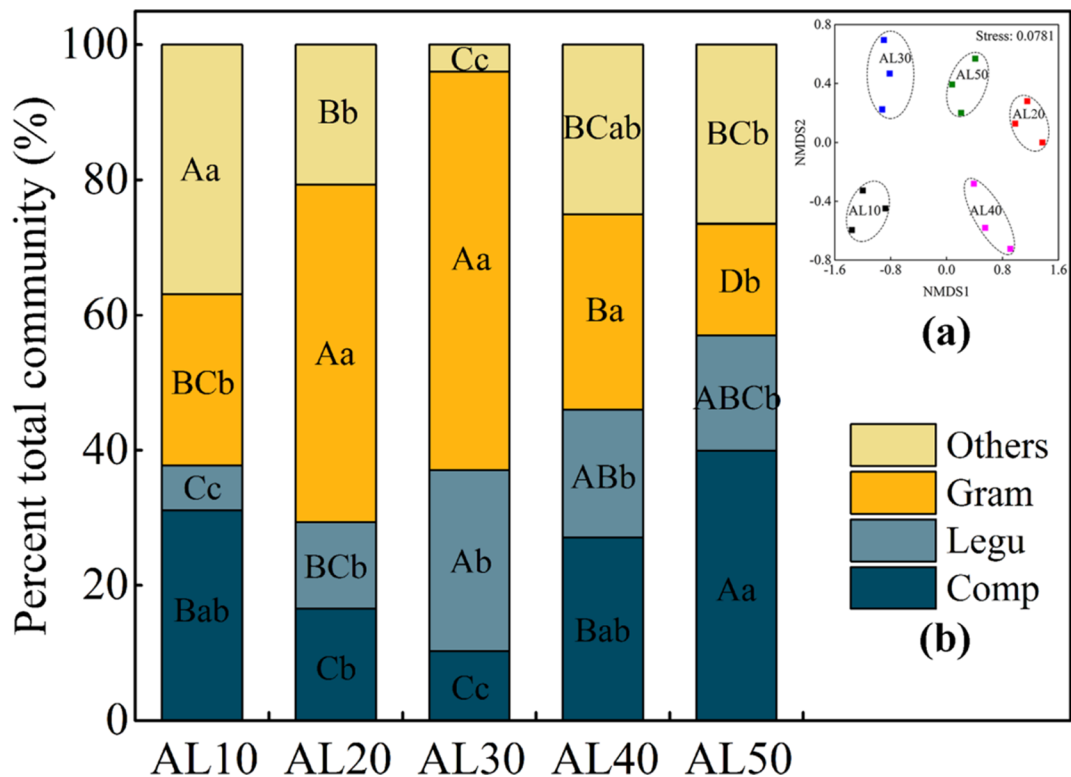


Fig. 1 Non-metric multidimensional scaling analysis (NMDS) of plant composition based on Bray-Curtis distances among different successional stages (a) and Composition of the plant communities at the family level (b). Legu: Leguminosae; Gram: Gramineae; Comp: Compositae; Others: Other families except

Leguminosae, Gramineae and Compositae. Different capital letters within the same plant family indicate significant difference at 0.05 ($P < 0.05$) levels among different successional stages. Different lowercases at the same successional stage indicate significant difference at 0.05 ($P < 0.05$) levels among different plant families

Specifically, the bacterial alpha diversity significantly increased during secondary succession, and tended to be the highest under AL30; thereafter, it decreased dramatically with time, but the fungal alpha diversity showed a monotonic increasing trend with abandonment time. The plot of NMDS (Fig. 4) clearly suggests variations in microbial beta diversity among the sites, with the response of soil bacterial beta diversity to successional time being larger than that of fungal beta diversity, despite the significant variation in both groups with restoration time. The profiles of the bacterial beta diversity at the FL, AL10, and AL50 sites tended to group together and were clearly separated from those at the AL20, AL30, and AL40 sites (Fig. 4a). For fungi, the profiles of fungal beta diversity at the AL10 and AL20 sites tended to group together but were clearly separated from those at the other three successional stages. The profile of fungal beta diversity at the FL site was also clearly separated from the others (Fig. 4b).

A total of 757,856 and 1,509,586 high-quality sequences of bacterial and fungal communities were obtained across the soil samples, respectively. Bacterial sequences ranged from 32,064 to 51,429 per sample (mean = 42,103), whereas fungal sequences ranged from 45,907 to 131,310 per sample (mean = 83,866). For the downstream analysis of microbial sequences, the bacterial and fungal datasets were rarefied to 32,000 sequences. Regarding the bacterial community, the dominant phyla (> 1%) were *Proteobacteria* (the number of classified sequences in this phylum ranged from 20.8% to 34.9% in all samples), *Actinobacteria* (20.5%–46.0%), *Acidobacteria* (12.1%–19.8%), *Chloroflexi* (4.5%–14.3%), *Gemmatimonadetes* (7.6%–11.3%), *Nitrospirae* (1.1%–3.0%), *Bacteroidetes* (1.1%–4.1%), and *Verrucomicrobia* (0.1%–2.8%; Fig. 5). Notably, *Proteobacteria* and *Acidobacteria* significantly increased during the first 30 years (including in AL30) and then decreased as succession proceeded, with a peak

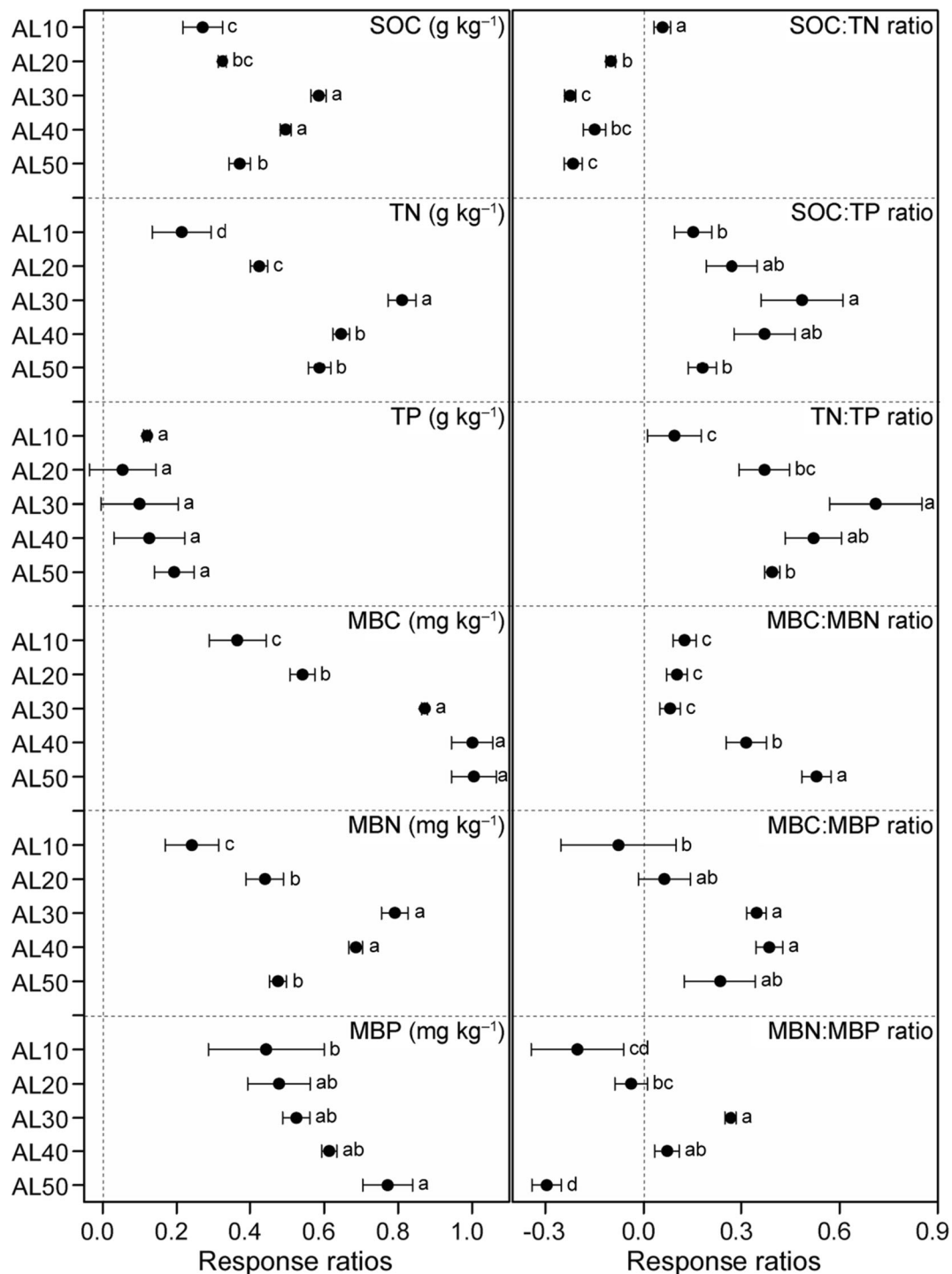
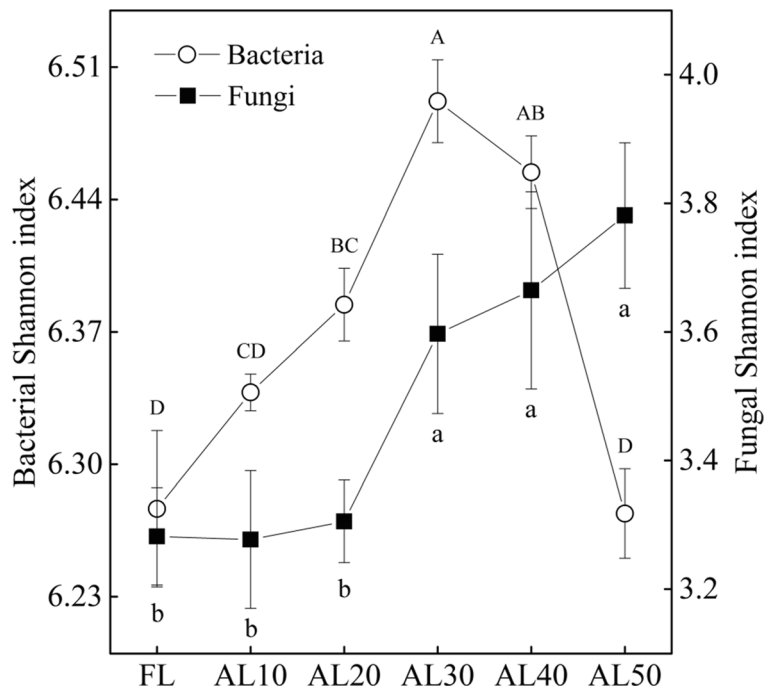


Fig. 2 The effects of secondary succession on the contents and stoichiometry of C, N, and P in soil and microbial biomass. Error bars represent bootstrap 95% confidence intervals (CIs). The effect of secondary succession is considered significant if the CI of the effect size does not overlap with zero. Different letters indicate

significant differences ($P < 0.05$) among the successional stages based on a one-way ANOVA followed by an LSD test. SOC: soil organic carbon, TN: total nitrogen, TP: total phosphorus, MBC: microbial biomass carbon, MBN: microbial biomass nitrogen, MBP: microbial biomass phosphorus

Fig. 3 Comparison of the alpha diversity (Shannon diversity index) between bacterial and fungal communities during succession. The error bars are the standard errors. Different uppercase letters and lowercase letters indicate significant differences ($P < 0.05$) among different successional stages for the bacterial alpha diversity and fungal alpha diversity, respectively



at AL30. *Actinobacteria* showed the opposite trend. Other phyla showed different responses to secondary succession, but these responses were insignificant ($P > 0.05$). In the case of the fungal community, fungi could be classified into three different phyla, predominantly *Ascomycota* (52.6%–83.3%), *Basidiomycota* (5.3%–22.9%), and *Zygomycota* (2.5%–15.8%; Fig. 5). The abundances of *Ascomycota* were significantly higher in the FL and AL50 sites and were ranked as

follows: AL50 > FL > AL40 > AL30 > AL20 > AL10, while *Basidiomycota* showed the opposite trend. No successional regulation of *Zygomycota* was observed.

Plant diversity, evenness, richness, and PFC effects on microbial diversity and community composition

The RDA showed that plant diversity and evenness were the most influential factors driving the

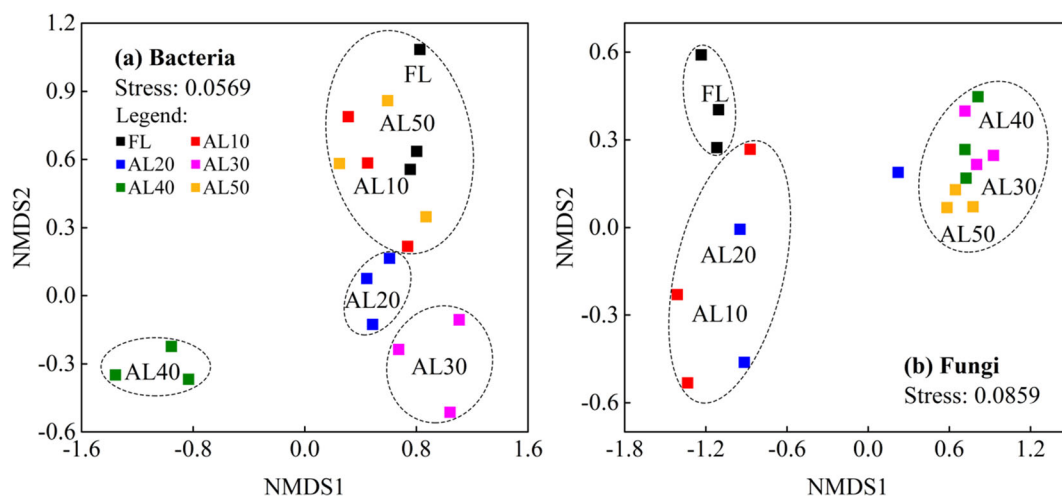
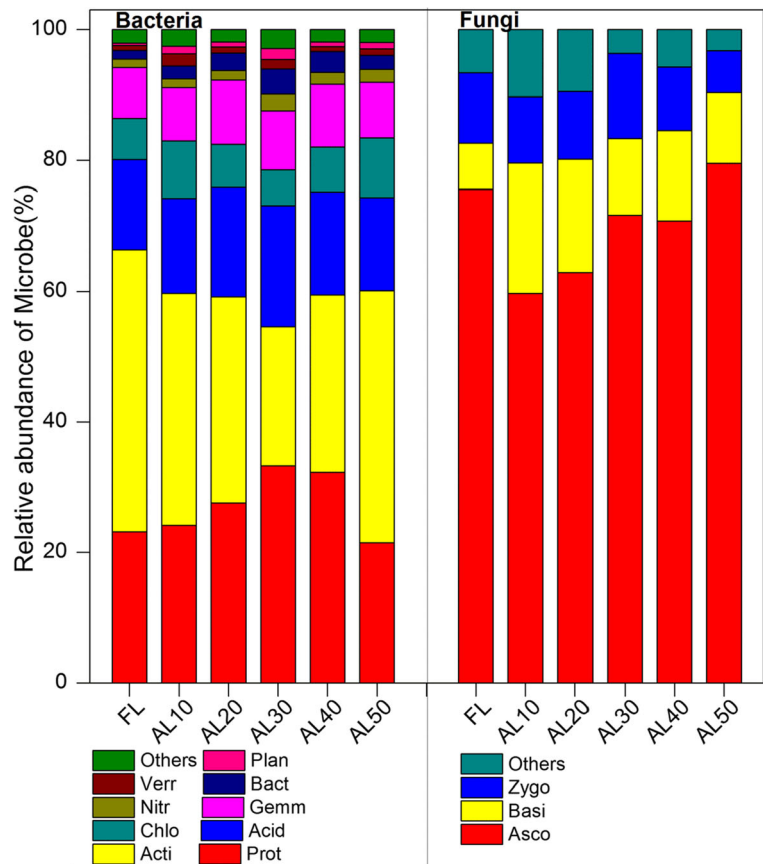


Fig. 4 Non-metric multidimensional scaling analysis (NMDS) of soil bacterial (a) and fungal (b) beta diversities based on Bray-Curtis distances among different successional stages

Fig. 5 Relative abundance of the soil bacterial (*left*) and fungal (*right*) communities at the phylum level. The data for the average relative abundances from three replicates were calculated as the ratio between the abundance of the sequence type and the total number of sequences. All calculations used normalized data. Prot: Proteobacteria; Acti: Actinobacteria; Acid: Acidobacteria; Chlo: Chloroflexi; Gemm: Gemmatimonadetes; Nitr: Nitrospirae; Bact: Bacteroidetes; Verr: Verrucomicrobia; Plan: Planctomycetes; Asco: Ascomycota; Basi: Basidiomycota; Zygo: Zygomycota



changes in the composition and diversity (alpha and beta diversity) of fungal communities (Fig. 6b), but bacterial communities were less influenced by plant diversity, evenness, and richness (Fig. 6a). According to the PLS-PM, secondary succession had both direct and indirect significant associations with the microbial community composition and diversity via all hypothesized pathways in the study, involving soil nutrients, PFC, and a direct path (Fig. 7). Secondary succession was positively and negatively associated with microbial diversity and dominant phyla, respectively, which was consistent with its association with the PFC. Interestingly, the PFC did not exert significant direct effects on microbial communities, but did exert indirect effects on them, mainly through direct effects on the soil nutrient contents. In particular, the bacterial alpha and beta diversities were significantly positively correlated to the SOC and TN contents, but Leguminosae and Gramineae determined the effects of SOC and TN contents on bacterial diversity in a positive

way, while Compositae were a negative determinant of the effects of soil C and N levels on bacterial diversity (Fig. 7). In addition, the PFC not only had direct negative effects on dominant microbial phyla, but also indirectly affected them via its positive effect on the soil nutrient contents, which are negatively associated with dominant microbial phyla (Fig. 7). Gramineae and Leguminosae were significantly and positively associated with the abundance of *Proteobacteria* and *Ascomycota* by positively associating with the soil C and N content, but were negatively associated with the abundance of *Actinobacteria* and *Basidiomycota*. A completely opposite interrelationship was determined among Compositae, soil nutrient levels, and the dominant phyla of the microbial community. Furthermore, the PFC had major effects on the soil bacterial diversity and dominant phyla abundance, either directly or indirectly, but minor effects on the fungal community.

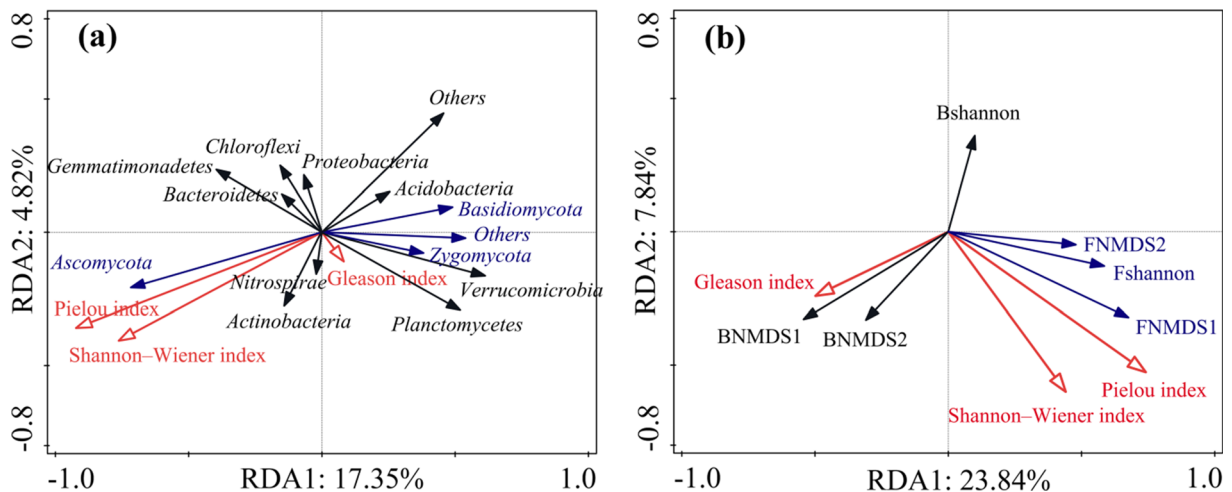


Fig. 6 Ordination plots of the redundancy analysis (RDA) to identify the relationships between the properties of bacteria (Black arrows), fungi (Blue arrows) and plant diversity indexes (Red arrows). **a.** The relationship between the abundance of microbial taxa and plant diversity. **b.** The relationship between the diversities of microbial taxa and plant diversity. Bshannon and

Fshannon represent the alpha diversity (Shannon index) of bacterial and fungal communities, respectively. BNMSD1 and FNMSD1 represent the beta diversity (NMDS1) of bacterial and fungal communities, respectively. Test of significance of all canonical axes please refer to the Table S3

Discussion

Plant diversity and evenness drive changes in soil fungal diversity and community composition

The essence of plant succession is the interaction between aboveground plants and belowground microorganisms (Dassen et al. 2017; Singh et al. 2004; He et al. 2008). In this study, bacterial and fungal diversity responded differently following secondary succession (Figs. 3 and 4). In general, fungi exhibit slower growth and turnover rates than bacteria during the early succession stages (Maharning et al. 2009; Zechmeister-Boltenstern et al. 2015), and thus might remain stable for the first 20 years (Figs. 3 and 4b). As succession proceeds, increased plant diversity and evenness can lead to greater resource availability for microbial growth (Millard and Singh 2010; Zhang et al. 2016), thereby enhancing fungal diversity. The decreasing soil C:N ratio with succession, which was significantly correlated with a higher fungal diversity in our study, supports this idea (Fig. S1). With regard to bacteria, however, we did not find that plant diversity or evenness had a large impact on the bacterial diversity (Fig. 6). The soil bacterial alpha diversity increased until it peaked at AL30 and then decreased, whereas the diversity and evenness of plants were increased monotonically (Table 2 and Fig. 3). A possible mechanism behind this finding could

be the deficiency of a combination of shared environmental drivers and direct functional associations between the plants and soil bacteria in our research area (Chen et al. 2016; Prober et al. 2015). Alternatively, plants can influence multiple soil properties, and thereby indirectly affect the soil bacterial diversity (He et al. 2008; Zhang et al. 2016). Indeed, we found a tight association between the bacterial diversity and the C:P and N:P ratios in the soil and microbial biomass during succession (Fig. S1). Furthermore, the soil fungal diversity corresponded to increased plant diversity and evenness during succession (Table 2 and Figs. 3 and 4b). Similar results for fungi but not for bacteria were also observed by Sugiyama et al. (2008). The positive plant–fungal diversity interaction might be owing to the increased host diversity of functional fungal groups and the increased number of niches that could be filled by a greater array of heterotrophic fungi (Millard and Singh 2010). It might also be the case that bacteria experience more stringent top-down control than fungi, making plant diversity or evenness a less important driver of bacterial diversity (Wardle 2006).

The fungal community composition was significantly correlated with both plant diversity and evenness (Fig. 6a) and a similar pattern was observed in the relationship between the diversities of fungi and plants; our study therefore supports the important influence of plant diversity on soil fungal communities as previously

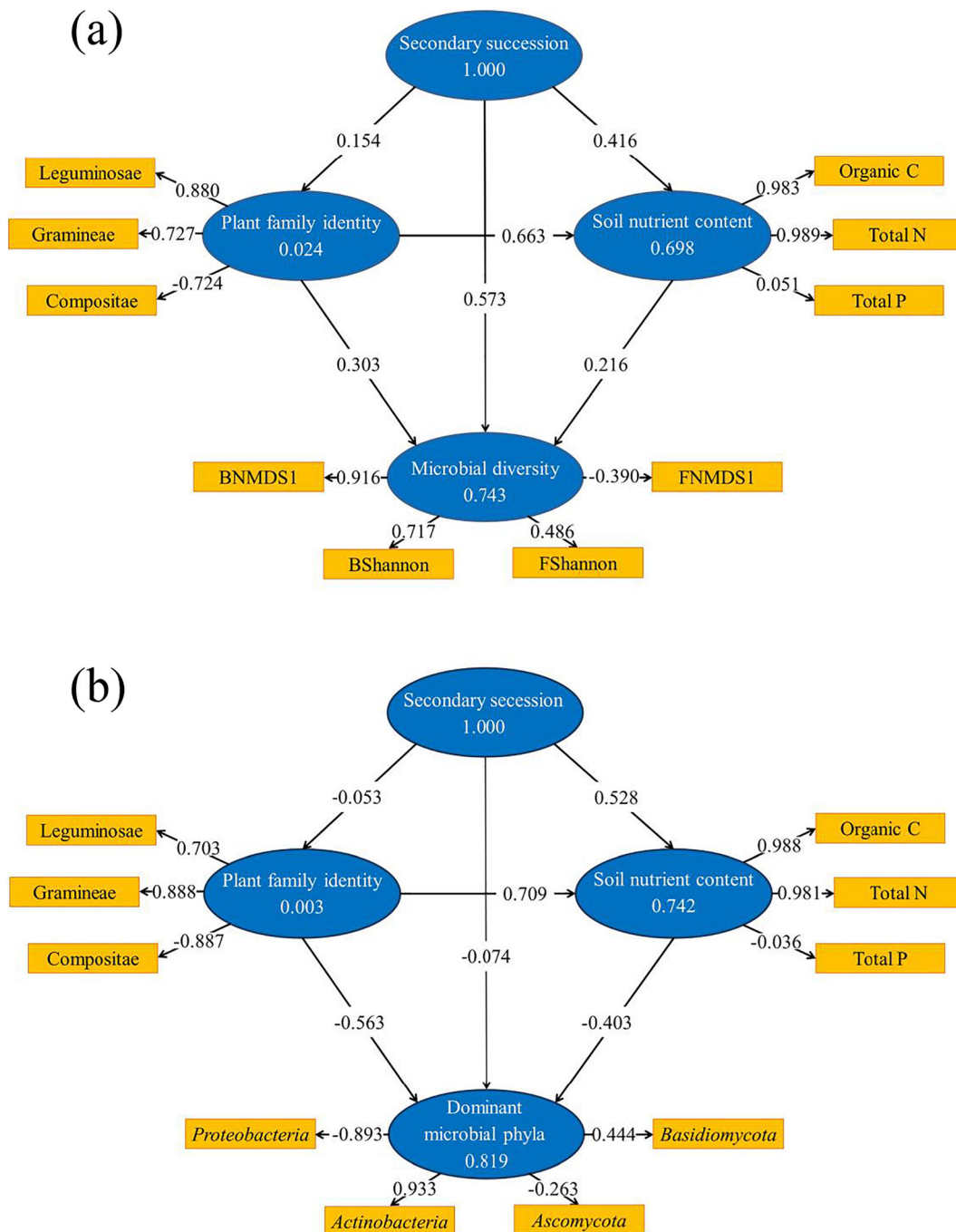


Fig. 7 Partial least squares path models (PLS-PM) of the drivers of soil microbial characteristics. Path analysis results for direct and indirect effects of secondary succession, plant family composition, soil nutrient content on microbial diversity (a) and dominant phyla abundance (b). Numbers on arrows are path coefficients indicating a positive (positive number) or negative

effect (negative number). Bshannon and Fshannon represent the alpha diversity (Shannon index) of bacterial and fungal communities, respectively. BNMDS1 and FNMDS1 represent the beta diversity (NMDS1) of bacterial and fungal communities, respectively

revealed on a global scale (Prober et al. 2015). Changes in a host plant's community usually shape a specific

fungal community structure (Eisenhauer et al. 2011; Millard and Singh 2010; Prober et al. 2015). In addition,

plant diversity and evenness can affect fungal composition through specific root exudates, as *Ascomycota* and *Basidiomycota* are involved in metabolizing the organic substrates of rhizodeposition (Clemmensen et al. 2013; Hannula et al. 2012). However, it was observed that the diversity or evenness of vegetation and the composition of bacterial community did not show patterns of co-occurrence (Table 2 and Fig. 5). Indeed, our results indicated that plant diversity and evenness were not influential in structuring bacterial communities (Fig. 6). For example, in the few cases where some overlap in vegetation prevailed, such as the occurrence of *Artemisia gmelinii* during early and late succession, the bacterial communities were very similar. These results suggest that broad variations in soil bacterial community composition are not strongly driven by the plant diversity and evenness found in an ecosystem (Chen et al. 2016; Kielak et al. 2008; Zhang et al. 2016), but might be affected by the PFC (Bezemer et al. 2006; Dassen et al. 2017). Overall, the results of this study highlight that plant species diversity and evenness have a larger impact on soil fungal diversity and community composition than bacteria do.

Plant family composition mainly regulates soil bacterial diversity and community composition

Plant families have been shown to differ in their capacity to modify microbial diversity and community composition through specific plant traits and functioning (He et al. 2008; Kielak et al. 2008; Loranger-Merciris et al. 2006). Our results showed that the successional tendency of soil bacterial diversity was consistent with that of the PFC (Figs. 1, 3, and 4a), indicating that bacterial diversity could better reflect the variations in plant families. However, these results contrasted with the findings of a removal experiment by Marshall et al. (2011), which reported that soil microbial communities are resistant to changes in PFC. This inconsistency is most likely because of the low importance of PFC in determining soil nutrient levels over the short term. Interestingly, we found that SOC, TN, and C:P, N:P ratios showed a pattern of co-occurrence with changes in PFC and bacterial diversity, confirming that variation in the PFC is closely associated with changes in bacterial diversity through altering soil C and N contents (He et al. 2008; Wardle 2006). As succession proceeds, increased leguminous plants leads to an elevation in the soil N content because of their N-fixing capability (Venterink 2011). Additionally, gramineous plants are

mostly of the C₄ type, which fix atmospheric C and return it to the soil in the form of litter (Řezáčová et al. 2018). Consequently, the sufficient substrate supply meets the requirements of microbial growth and enhances bacterial diversity (Cline and Zak 2015; Loranger-Merciris et al. 2006). Subsequently, Compositae re-dominates the PFC as in the early succession, benefiting from their higher tolerance of low soil moisture and nutrients (Tables 1 and S2) (Bazzaz 1996). Increased Compositae inhibit the growth of leguminous and gramineous plants by allelopathy for nutrient competition, further exacerbating the adverse environmental conditions for microbial reproduction (Chon and Nelson 2010). Likewise, our results showed that Compositae have a negative impact on the soil nutrient contents and bacterial diversity (Fig. 7). Carvalhais et al. (2019) further revealed that the strigolactones secreted by Compositae roots have a more pronounced effect on promoting fungal diversity than bacterial diversity. Furthermore, we found that PFC had a major effect on bacterial diversity but a minor effect on fungal diversity with secondary succession (Fig. 7). One possible explanation for this discrepancy is that Leguminosae (average 16.4%) is not the preponderant family in our study. This may weaken the positive selective effects between legume–fungal interactions (decreasing mycorrhizal colonization on plant roots) (Dassen et al. 2017) and the selection of saprotrophs by specific litter traits (decreasing the availability of lignocellulose-rich plant residue, which is important for saprotrophic basidiomycete growth) (Maharning et al. 2009). Alternatively, fungi that fulfill more general ecological roles in soil (Clemmensen et al. 2013), such as the decomposition of soil organic matter, are probably less sensitive to changes in the PFC during succession (Marshall et al. 2011).

Given the relatively low proportion of the most dominant bacterial phyla, we opted to focus on two phyla that appeared to be significantly influenced by the PFC, namely *Proteobacteria* and *Actinobacteria* (Fig. 5). In this study, *Proteobacteria* tended to significantly increase during the first 30 years with the increase in leguminous and gramineous plants and then decrease with the increase of Compositae, perhaps owing to the changes in C and N supplies (Burns et al. 2015; Chen et al. 2016). Leguminosae and Gramineae are C- and N-rich plants, the growth of which leads to enriched soil (Zhang et al. 2018), and ecological theory suggests that copiotrophic or r-selected populations should be well adapted to such nutrient-rich conditions (Ren et al. 2016; Zhang

et al. 2016). However, the *Actinobacteria* in this study behaved in a complete oppositely manner to the *Proteobacteria*, with higher abundances in the primary and later stages of succession when Compositae dominated. Aggressive Compositae have strong resource competitiveness and lower C and N contents (Chon and Nelson 2010; Zhang et al. 2018), and oligotrophic *Actinobacteria* might prefer such nutrient-poor environments (Cassman et al. 2016; Siles and Margesin 2016). As a whole, the bacterial community transformed from being oligotrophic *Acidobacteria*-dominant to copiotrophic *Proteobacteria*-dominant, and was finally dominated by *Acidobacteria* as the succession continued, suggesting the occurrence of degraded succession in our research area (Li and Huang 2008; Wei et al. 2013). This may be due to encroachment by small bushes and shrubs (Table S1), which decreased the coverage and aboveground net primary production (Table 2). Shrub encroachment is known to deteriorate the grassland by increasing the consumption of soil nutrients and the spatial heterogeneity of resources (D'Odorico et al. 2012; Wei et al. 2013). This phenomenon could result in an increase in oligotrophs and a decrease in copiotrophs, and could also diversify the fungal communities, which have wider niches than bacteria (Hollister et al. 2010; Kotzé et al. 2017). Although many fungi can affect C and N availability and cycling by adjusting their community composition in response to changes in plant species (Clemmensen et al. 2013; Dassen et al. 2017), no strong correlation was observed between the PFC and fungal community composition. Our examination of fungal community structure using general ITS primers could have missed the potential relationships between plant families and specific fungal functional groups. Analysis of arbuscular mycorrhizal fungi or saprotrophs, for example, could reveal plant effects on that functional group, because we expect some plants to have coevolved relationships with these functional fungi (Burns et al. 2015). Thus, the weaker effects of PFC on fungal community composition could be due to our focus on the overall fungal community structure rather than the functional group distribution, which could yield different results. Altogether, these findings demonstrate more rapid responses of the diversity and composition of bacterial communities than those of fungal communities to changes in PFC, and further shed light on

the role of the PFC in regulating microbial species in restored ecosystems.

Conclusion

The secondary succession of abandoned FL on the Loess Plateau increased the plant species diversity and evenness, which were accompanied by higher fungal diversity and a significant change in the fungal community composition. Plant diversity and evenness had a marginal impact on the bacterial community, but the PFC did significantly affect the diversity and composition of the bacterial community through direct effects on the soil nutrient content, with a positive role for Leguminosae and Gramineae, but a negative one for Compositae. Furthermore, the composition and diversity of the bacterial community is a sensitivity index of the response to potential grassland degradation caused by the PFC transition from Leguminosae + Gramineae-dominant to Compositae-dominant during the 50 years of succession. Therefore, renewing ALs by artificially introducing Leguminosae and Gramineae has important implications for the sustainable development of grassland on the Loess Plateau.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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