

# Interactions of soil bacteria and fungi with plants during long-term grazing exclusion in semiarid grasslands

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## ABSTRACT

Microbial succession has been extensively investigated during the restoration of degraded environments, but the interactions of microbes with plants and soils have not been well documented. We examined changes in the plant communities, soil variables, and microbial communities of grasslands after different periods of grazing exclusion (0, 10, 25, and 35 y) on the Loess Plateau in China. The microbial communities were characterized based on their biomass, enzymatic activities, quantity of functional microbes, and composition using high-throughput sequencing. Grazing exclusion increased the plant diversity, above- and belowground biomass, organic carbon content, total nitrogen content, microbial biomass, enzymatic activities, abundance of ammonia-oxidizing microbes, and diversities of the bacterial and fungal communities; however, the highest values of these variables occurred at the 25-y exclusion site and subsequently declined, indicating that long-term exclusion could have a negative effect on this grassland. Decreases in the abundances of *Alphaproteobacteria* and *Leotiomycetes* and increases in *Acidobacteria* and *Sordariomycetes* along the chronosequence indicated different successional patterns in the microbial communities. The patterns of change in the composition and diversity of the plant, bacterial, and fungal communities suggest that plant and bacterial succession occurred in parallel and proceeded faster than fungal succession. Indicators of the bacterial and fungal communities, including their biomass, enzymatic activities, and community composition and diversity, were affected by the plant diversity and organic carbon, total nitrogen, and nitrate nitrogen contents. Fungal succession was also susceptible to changes in the soil moisture content. These results suggest that plant diversity plays an important role in shaping the microbial communities, likely by altering the levels of soil nutrients and moisture.

## 1. Introduction

An estimated 30% of the terrestrial area around the world is arid or semiarid, and these regions offer many ecosystem services (Zhan et al., 2007). Many grasslands in these regions have been degraded and are gradually disappearing due to anthropogenic interference, especially overgrazing (Slimani et al., 2010). Nearly 4 million km<sup>2</sup> of grassland in China covers > 40% of the land area (Ren et al., 2008), and approximately 90% of the grasslands have been degraded by long-term livestock overgrazing and overexploitation, which has become a serious environmental problem for the Chinese government (Cheng et al., 2016). Restoring degraded grasslands has been a serious concern in recent decades (Bai et al., 2004; Simmons et al., 2007; Jing et al., 2014). Microorganisms are important contributors to the structure and function of ecosystems, so addressing microbial successional patterns and their interactions with plants and soils is essential for increasing our understanding of the mechanisms of restoration, improving our

capacity to predict the responses of ecosystems to human disturbance, and optimizing the design of large-scale restoration projects (Kardol et al., 2013).

The exclusion of grazing is an efficient approach for restoring degraded semiarid grasslands in China (Cheng et al., 2016). Grassland restoration is a long-term and complex process (Millard and Singh, 2010; Zeng et al., 2017a; Zhang et al., 2016) and mainly focuses on three essential elements: vegetation structure, soil variables, and microbial communities (Liu et al., 2018; Fry et al., 2016). Plants affect microbial communities during restoration and drive changes in soil physicochemical properties through the decomposition of litter, the turnover of roots, and root exudation (Haichar et al., 2008). Soil microorganisms in turn can either positively or negatively affect plant growth through the maintenance and transformation of soil nutrients, which further influence the plant-community composition (Philippot et al., 2013; van der Putten et al., 2013). Interactions between plants and soil microbes may therefore have important consequences for the

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**Table 1**

Geographical characteristic of study sites. The soil of all the sites is a montane gray-cinnamon soil.

Sites	Latitude (N)	Longitude (E)	Altitude (m)	Slope gradient(°)	Slope aspect	Dominant species
GE0	36°17'06"	106°23'28"	2017	18	E26°N	<i>Potentilla bifurca</i> Linn., <i>Stipa przewalskyi</i> Roshev., <i>Carex tangiana</i> Ohwi.
GE10	36°16'57"	106°23'28"	2034	20	E38°N	<i>Leymus secalinus</i> Tzvel., <i>Carex tangiana</i> Ohwi, <i>Stipa grandis</i> P. Smirn.
GE25	36°16'31"	106°23'27"	2070	18	W21°N	<i>Stipa grandis</i> P. Smirn. <i>Artemisia sacrorum</i> Ledeb, <i>Oxytropis bicolor</i> .
GE35	36°15'05"	106°23'10"	2071	21	E29°N	<i>Stipa przewalskyi</i> Roshev., <i>Carex tangiana</i> Ohwi.

dynamics of plant communities, becoming determining factors of community assemblages and ecosystem functioning (Kardol et al., 2013). Previous studies of the effect of grazing exclusion on degraded grassland, however, have almost exclusively focused on the dynamics of plant communities (coverage, biomass, and diversity) (Jing et al., 2013) and soil variables (nutrient levels, aggregates, and enzymes) (Cheng et al., 2016), and the few studies of soil microorganisms have focused only on microbial activity and community composition (Zeng et al., 2017a; Hu et al., 2017). The relationships between aboveground plant communities and belowground microbial communities are thus poorly understood. Experimental evidence of predictable patterns in microbial community structure during the natural restoration of grassland where grazing has been excluded is lacking, particularly compared to the amount of evidence for plant community patterns.

Bacterial and fungal communities are important functional groups in soils that play different roles in regulating ecosystem function and soil biogeochemistry, and these communities are differentially affected by environmental factors (Sun et al., 2017; Geisseler and Scow, 2014; Rinnan and Bååth, 2009). Bacteria are regarded as important mediators of the rapid pathways of carbon cycling in soil, and their growth tends to be approximately 10-fold greater than that of fungi (Rousk and Bååth, 2007). Fungi generally have more symbiotic relationships with plants, and their dispersal may be more limited because of their larger size (Schmidt et al., 2014). Given the differences in phenotype, phylogeny, and life history between bacteria and fungi, a comparison of their patterns of succession and their responses to plants and soils should enhance our understanding of the responses of various components of soil microbial communities to ecosystem restoration and help our assessment of the environmental impacts of land-use changes.

In the present study, four grasslands that had experienced different periods of grazing exclusion (0, 10, 25, and 35 years) in a typical semiarid area, the Chinese Loess Plateau, were selected to investigate the effects of long-term exclusion on the above- and belowground communities. We hypothesized that (i) grazing exclusion is beneficial for restoring degraded grassland, including the aboveground productivity (biomass), soil nutrient level, and microbial activity and diversity; (ii) there are different succession patterns between the plant and microbial communities, and bacterial succession likely proceeds more rapidly than fungal succession; and (iii) plant diversity plays a significant role in structuring the bacterial and fungal communities. To test these hypotheses, we evaluated the changes in plant communities, soil variables, and microbial communities (bacteria and fungi) and determined their relationships along a 35-year grazing-exclusion chronosequence. The microbial community compositions were analyzed by sequencing the bacterial 16S ribosomal RNA (rRNA) and a fungal internal transcribed spacer (ITS) gene. The functional microbes involved in nitrification, denitrification, and N<sub>2</sub> fixation were determined using real-time quantitative PCR.

## 2. Materials and methods

### 2.1. Study sites

Our experiment was conducted in the largest grassland on the Loess Plateau, which occurs in the Yunwushan National Natural Grassland Protection Zone (106°21'–106°27'E, 36°10'–36°17'N) in the Ningxia

Autonomous Region of China. This region has been protected since 1982 to monitor the long-term restoration of degraded grassland. The climate in this area is semiarid, with a mean annual precipitation of 425 mm, > 60% of which falls from July to September. The mean annual temperature is 7 °C, with an average minimum of –8.2 °C in January and an average maximum of 25.2 °C in August. The soil is a montane gray-cinnamon soil.

### 2.2. Experimental design and sampling

We studied four grassland sites along a chronosequence of grazing exclusion in August 2017, when the aboveground biomass was the highest. Three of these sites have not been grazed by livestock, allowing natural restoration, since 1982, 1992, and 2007, corresponding to grazing exclusion for 35 year (GE35), 25 y (GE25), and 10 y (GE10), respectively. Meanwhile, the remaining site has been continuously grazed (4 sheep/ha) throughout the year and served as a reference (GE0). Before grazing exclusion, all the investigated sites had been intensively grazed (> 60 sheep/ha) (Jing et al., 2014). The sites have similar soil types, altitudes, slope gradients, slope aspects, and previous management practices (Table 1). Three 50 m × 100 m plots were established in each site and were separated by 80–100 m. Soil samples were collected from the top 20 cm of the soil profile using an auger (5 cm in diameter and 20 cm long) after the litter layer was removed. Ten soil cores were collected from each plot along a sigmoidal transect and then combined to make one sample. Roots, stones, litter, and debris were removed, and each bulked sample was divided into three subsamples. One subsample was immediately stored at –80 °C for DNA extraction, another was stored at 4 °C for the determination of microbial biomass and enzymatic activities, and the third was air dried for physicochemical analysis. Five 1 × 1 m subplots were randomly established within each plot for the measurement of vegetation coverage, above- and belowground biomass, maximum/mean height, and number of species. The Shannon-Wiener index ( $H = -\sum P_i \ln P_i$ ) was used to estimate the diversity of the plant communities, where  $P_i$  is the ratio of the number of each species to the total number of all species. The aboveground biomass was determined by drying the aboveground tissues, including the shoots, leaves, and litter, at 60 °C for 36 h. The belowground biomass was measured by washing the roots in distilled water and then drying them at 60 °C for 36 h.

### 2.3. Analysis of soil physicochemical properties

The amount of soil organic carbon (OC) in the samples was determined using dichromate oxidation. The total nitrogen (TN) content was measured using an automatic Kjeldahl instrument (Kjeltec 8400, FOSS Corporation, Denmark). The available phosphorus (AP) content was determined using the Olsen method. The NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N contents were determined using a continuous-flow auto-analyzer (Alpkem, OI Analytical, USA) after sample extraction with 2 M KCl at a soil: KCl ratio of 1:5. The soil pH was measured using a 1:2.5 soil: water mixture. The soil moisture was measured using the oven-drying method.

## 2.4. Microbial biomass and enzymatic activities

Soil microbial biomass C and N were determined using fumigation extraction (Vance et al., 1987). Briefly, 25 g of the oven-dry equivalent of field-moist soil was fumigated at 25.8 °C for 24 h with CHCl<sub>3</sub>. The soil was added to 100 ml of 0.5 M potassium sulfate by shaking at 200 rpm for 1 h and then filtered after fumigant removal. Another 25 g of non-fumigated soil was simultaneously extracted. The OC and TN contents of the extracts were determined using a Liqui TOCII analyzer (Elementar Analyses system, Hanau, Germany).

The activities of three enzymes involved in C, N, and P cycling, namely, β-1,4-glucosidase (BG), β-1,4-N-acetylglucosaminidase (NAG), and alkaline phosphatase (AKP), were measured using a fluorescence microplate assay with substrates labeled with 4-methylumbelliferone (MUB) (Marx et al., 2001, Table S1). Briefly, 1.0 g of fresh soil and 125 mL of deionized water were mixed for 2 h at 25 °C using an oscillator and served as soil buffer suspensions. One milliliter of each suspension and 250 μL of the substrate solution were then pipetted into a 2-mL centrifuge tube. The centrifuge tube was incubated at 25 °C for 4 h in the dark, the reaction was terminated by adding 50 μL of NaOH, and 250 μL of each sample mixture, a negative control, the reference standard, a blank control, and a quench control were then pipetted into a black 96-well plate. The negative control well received 6 mL of buffer, 150 μL of substrate, and 180 μL of 0.5 M NaOH. The reference standard well received 6 mL of buffer, 150 μL of the reference standard (10 mM MUB), and 180 μL of NaOH. The blank control well received 1 mL of the sample suspension and 250 μL of deionized water. The quench control well received 1 mL of the sample suspension and 250 μL of the standard. Activities were determined fluorometrically using a fluorescence plate reader (SpectraMax M2, Molecular Devices, USA) at 365 nm excitation and 450 nm emission. The activities were expressed as nmol substrate converted per mL of sample, with units of nmol h<sup>-1</sup> g<sup>-1</sup>. Six replicate wells were tested for each sample.

## 2.5. Real-time quantitative PCR (qPCR)

DNA was extracted from 0.25 g of the homogenized soil subsamples using the FastDNA<sup>®</sup> SPIN Kit for Soil (MP Biomedicals, Cleveland, USA) according to the manufacturer's instructions. The concentrations of the DNA extracted from both sample replicates were independently measured using a NanoDrop<sup>®</sup> ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Three replicates of the real-time PCR runs were performed for each concentration using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The following genes from different microbial groups were measured using qPCR: genes encoding the ammonia monooxygenase enzymes of archaea (*amoA* AOA) and bacteria (*amoA* AOB) to represent the nitrifying ammonia-oxidizing community (Regan et al., 2017) and genes encoding *narG*, *nirS*, *nirK*, *norB* and *nosZ*, which represent nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively, among the denitrifying community. The *nifH* gene was also used to evaluate the N<sub>2</sub>-fixing community. The primers of the genes are presented in Table S2. The reactions were set up in microcapillary tubes using the following final concentrations: 0.4 μM each of the sense and antisense primers, 2.5 μM MgCl<sub>2</sub>, 1 × SYBR Green Master Mix and 2 μL of DNA. The cycling conditions were as follows: denaturation (95 °C for 5 min), amplification and quantitation (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) repeated 35 times, a melting curve program (60–95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement) and a cooling step at 40 °C. The unit of abundance of the N cycling genes is copies g<sup>-1</sup> dry soil.

## 2.6. Soil microbial community composition

### 2.6.1. Soil DNA extraction and sequencing

DNA was extracted from 0.5 g of the soil samples using a FastDNA

Spin Kit (MP Biomedicals, Cleveland, USA) following the manufacturer's specifications. The quality and quantity of the DNA was tested using 1% agarose gel electrophoresis and a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, USA), respectively. The V4-V5 hypervariable region of the bacterial 16S rRNA gene was amplified using PCR with the primers 515F (5'-GTGCCAGMCGCCGG TAA-3') and 907R (5'-CCGTCAATTCCTTGTAGT TT-3'). The forward primer was modified to contain a unique 6-nt barcode at the 5' end. The fungal ITS1 region was amplified using the primers ITS5-1737F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2-2043R (5'-GCTGCGT TCTTCATCGATGC-3'). The PCR reactions contained 2 μL of sterile ultrapure water, 15 μL of 2 × Phusion Master Mix, 3 μL of 6 μM primers, and 10 μL of template DNA (5–10 ng). The amplification program was 98 °C for 60 s; 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s; and 72 °C for 5 min. Triplicate PCR amplicons were pooled and then mixed with the same volume of 1 × loading buffer. The amplicons were detected using electrophoresis in a 2% (w/v) agarose gel. The amplicons were mixed at equal density ratios and purified using a Qiagen Gel Extraction Kit (Qiagen Co., Ltd, Germany). The purified amplicons were sequenced using an Illumina MiSeq PE300 platform (Illumina Corporation, San Diego, USA). The raw sequences were deposited in the NCBI Sequence Read Archive (accession number SRP131234).

### 2.6.2. Sequence processing

Paired-end reads were merged using FLASH (<http://ccb.jhu.edu/software/FLASH/>), and the quality of the reads was described by Caporaso et al. (2011). Chimeric sequences were detected and removed using USEARCH based on the UCHIME algorithm (Edgar et al., 2011). Quality-filtered sequences were complete-linkage clustered into operational taxonomic units (OTUs) using UCLUST with a cutoff of 97% similarity (Edgar, 2010). Low-abundance OTUs were eliminated from the OTU table if they did not present a total of at least two counts across all samples in the experiment. The most abundant sequence in each OTU was chosen as the representative sequence and was aligned using PyNAST. The bacteria were identified using the Silva reference database (<http://www.arb-silva.de>) with the RDP classifier, and the fungi were identified using the Unite database (<https://unite.ut.ee/>) with the BLAST tool in QIIME (<http://qiime.org/index.html>). Community diversity indicators, including rarefaction curves, observed species, the Shannon-Wiener index, and the Chao1 estimator, were calculated using 40,800 and 73,250 reads per sample for bacteria and fungi, respectively (the minimum number of sequences required to normalize the differences in sequencing depth), using QIIME.

## 2.7. Statistical analysis

One-way analysis of variation (ANOVA) was used to evaluate the effect of the length of exclusion on plant characteristics (coverage, above- and belowground biomass, and diversity), soil properties, microbial biomass, enzymatic activities, and microbial diversity, followed by *post hoc* comparisons using Turkey's HSD test. Significance was established at  $P < 0.05$ . The one-way ANOVAs and multiple comparisons were conducted using SPSS 18.0 (SPSS Incorporation, Chicago, USA). A principal coordinate analysis (PCoA) was used to assess the differences in the structures of the plant and microbial communities among the exclusion sites based on Bray-Curtis distances. We also tested the significance of the differences using analysis of similarities (ANOSIM). Specific microbial groups with different abundances among the exclusion sites that could be used as potential microbial biomarkers were detected using linear discriminant analysis (LDA) effect size (LEfSe) (<http://huttenhower.sph.harvard.edu/lefse/>) based on a relative abundance matrix. LEfSe uses the non-parametric factorial Kruskal-Wallis test to identify features that significantly differ among samples (Segata et al., 2011). An LDA threshold of 3.0 and an  $\alpha$  of 0.05 were used. Mantel tests of the Bray-Curtis distance similarities calculated

**Table 2**

**Plant characteristics along the grazing-exclusion time.** Results is reported as the mean  $\pm$  SE (n = 3). Values of *F* and *P* are the significance by the one-way ANOVA. Different letters in a row indicate significant differences between the sites by the Tukey HSD test (*P* < 0.05).

Parameters	GE 0	GE 10	GE25	GE35	<i>F</i>	<i>P</i>
Cover (%)	86.7 $\pm$ 1.9 b	94.7 $\pm$ 2.6 a	95.0 $\pm$ 0.6 a	92.3 $\pm$ 1.5 a	4.69	0.036
Aboveground biomass (g m <sup>2</sup> )	160.0 $\pm$ 17.4 c	440.4 $\pm$ 60.8 b	1298.2 $\pm$ 150.3 a	1019.0 $\pm$ 131.1 a	24.82	< 0.001
Belowground biomass (g m <sup>-2</sup> )	77.9 $\pm$ 5.6 b	134.7 $\pm$ 22.2 a	75.9 $\pm$ 9.5 b	38.6 $\pm$ 3.9 c	9.99	0.004
Shannon diversity	0.43 $\pm$ 0.04 d	1.63 $\pm$ 0.03 c	3.53 $\pm$ 0.05 a	3.01 $\pm$ 0.03 b	1384.56	< 0.001
Richness	18 $\pm$ 2 a	12 $\pm$ 1 b	9 $\pm$ 2 b	12 $\pm$ 2 b	8.392	0.007

based on the number of OTUs were used to identify the plant and soil factors correlating with the microbial community composition. A canonical correspondence analysis (CCA) and a Pearson correlation analysis were also performed to identify the environmental factors accounting for the patterns of microbial community structure and enzymatic activities. The PCoA, ANOSIM, Mantel tests, Pearson correlation analysis, and CCA were conducted using the vegan package in R-3.4.3, and the LEfSe analysis was conducted using the MASS package.

### 3. Results

#### 3.1. Plant communities

The plant community composition significantly differed among the grazing-excluded sites (Table 2). Grazing exclusion generally significantly increased the coverage, diversity, and aboveground and root biomass values (*P* < 0.05). Plant coverage was much higher at GE10 than GE0 but did not differ significantly among GE10, GE25, and GE35. The aboveground biomass and Shannon diversity behaved similarly, increasing during the first 25 y and peaking at GE25. The belowground biomass was highest at GE10 and then decreased. Species richness was highest at GE0.

#### 3.2. Soil nutrients and microbial activity

The effects of grazing exclusion on the status of soil nutrients, microbial biomass, and enzymatic activities are presented in Table 3. The soil nutrient contents significantly differed among the four grasslands in which grazing had been excluded, except for the NH<sub>4</sub><sup>+</sup>-N content and the C:N ratio. Grazing exclusion significantly increased the OC content, which peaked at GE25. The variation in the TN and OC contents behaved similarly. In contrast, the NO<sub>3</sub><sup>-</sup>-N content generally decreased when grazing was excluded from a grassland and was lowest at GE25. Grazing exclusion significantly decreased the AP content in the first 10 y relative to GE0, but then it increased over time. The pH ranged from 8.31 to 8.53 and was highest at GE10. The soil moisture content

**Table 3**

**Soil properties along the grazing-exclusion time.** Results is reported as the mean  $\pm$  SE (n = 3). Values of *F* and *P* are the significance by the one-way ANOVA. Different letters in a row indicate significant differences between the sites by the Tukey HSD test (*P* < 0.05).

Parameters	GE 0	GE 10	GE25	GE35	<i>F</i>	<i>P</i>
Organic C (g kg <sup>-1</sup> )	20.85 $\pm$ 0.92 c	28.54 $\pm$ 0.88 b	38.11 $\pm$ 1.13 a	28.67 $\pm$ 0.46 b	64.19	< 0.001
Total N (g kg <sup>-1</sup> )	2.73 $\pm$ 0.15 c	3.67 $\pm$ 0.13 b	4.16 $\pm$ 0.13 a	3.38 $\pm$ 0.19 b	15.35	0.001
Available P (mg kg <sup>-1</sup> )	6.80 $\pm$ 0.39 ab	2.90 $\pm$ 0.10 c	5.41 $\pm$ 1.08 b	7.64 $\pm$ 0.49 a	11.04	0.003
NO <sub>3</sub> <sup>-</sup> -N (mg kg <sup>-1</sup> )	14.07 $\pm$ 0.63 a	13.17 $\pm$ 0.96 ab	10.18 $\pm$ 0.23 b	11.84 $\pm$ 0.59 ab	6.62	0.015
NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )	3.64 $\pm$ 0.23 a	3.79 $\pm$ 0.11 a	3.74 $\pm$ 0.37 a	4.26 $\pm$ 0.77 a	0.38	0.771
pH	8.31 $\pm$ 0.03 b	8.53 $\pm$ 0.03 a	8.36 $\pm$ 0.04 b	8.32 $\pm$ 0.02 b	11.17	0.003
C:N	7.73 $\pm$ 0.75 a	7.81 $\pm$ 0.37 a	9.17 $\pm$ 0.29 a	8.53 $\pm$ 0.46 a	1.84	0.218
NO <sub>3</sub> <sup>-</sup> /NH <sub>4</sub> <sup>+</sup>	3.89 $\pm$ 0.19 a	3.47 $\pm$ 0.17 a	2.76 $\pm$ 0.02 b	2.95 $\pm$ 0.47 b	4.19	0.048
Soil moisture (%)	17.8 $\pm$ 0.01 c	21.4 $\pm$ 0.01 b	24.9 $\pm$ 0.01 a	27.1 $\pm$ 0.00 a	28.16	< 0.000
Microbial biomass C (mg kg <sup>-1</sup> )	522.8 $\pm$ 45.2 b	629.9 $\pm$ 5.9 ab	761.6 $\pm$ 84.2 a	601.3 $\pm$ 18.2 b	4.16	0.047
Microbial biomass N (mg kg <sup>-1</sup> )	78.6 $\pm$ 9.9 b	98.0 $\pm$ 0.6 ab	126.1 $\pm$ 13.7 a	110.8 $\pm$ 6.4 a	4.97	0.031
β-1,4-glucosidase	123.6 $\pm$ 14.1 c	215.4 $\pm$ 6.5 ab	244.2 $\pm$ 7.1 a	210.7 $\pm$ 17.7 b	17.94	0.001
β-1,4-N-acetylglucosaminidase	14.4 $\pm$ 1.6 c	26.4 $\pm$ 2.2 b	36.8 $\pm$ 0.6 a	19.7 $\pm$ 1.6 bc	35.79	< 0.001
Alkaline phosphatase	320.5 $\pm$ 4.8 c	498.0 $\pm$ 4.5 a	451.9 $\pm$ 25.5 ab	435.1 $\pm$ 6.7 b	30.95	< 0.001

The unit of β-1,4-glucosidase, β-1,4-N-acetylglucosaminidase, and alkaline phosphatase is nmol g<sup>-1</sup> h<sup>-1</sup>. The unit of polyphenol oxidase is mg g<sup>-1</sup> h<sup>-1</sup>.



**Table 4**

**Abundance of N cycling gene copy numbers along the grazing-exclusion time.** Results is reported as the mean  $\pm$  SE (n = 3). Values of *F* and *P* are the significance by the one-way ANOVA. Different letters in a row indicate significant differences between the sites by the Tukey HSD test (*P* < 0.05).

Parameters (copies g <sup>-1</sup> soil)	GE 0	GE 10	GE25	GE35	<i>F</i>	<i>P</i>
<i>amoA</i> AOA ( $\times 10^6$ )	4.8 $\pm$ 0.4 d	13.8 $\pm$ 0.5 c	85.9 $\pm$ 2.3 a	56.6 $\pm$ 3.28 b	351.62	< 0.001
<i>amoA</i> AOB ( $\times 10^7$ )	2.4 $\pm$ 0.15 c	6.3 $\pm$ 0.28 c	17.8 $\pm$ 2.0 a	9.2 $\pm$ 0.5 b	43.37	< 0.001
<i>narG</i> ( $\times 10^5$ )	3.1 $\pm$ 0.3 a	3.2 $\pm$ 0.3 a	1.5 $\pm$ 0.09 b	2.7 $\pm$ 0.07 a	14.16	0.001
<i>nirS</i> ( $\times 10^8$ )	2.2 $\pm$ 0.16 a	1.9 $\pm$ 0.19 a	0.4 $\pm$ 0.04 c	0.8 $\pm$ 0.03 b	48.58	< 0.001
<i>nirK</i> ( $\times 10^9$ )	3.7 $\pm$ 0.3 a	3.1 $\pm$ 0.2 a	0.3 $\pm$ 0.02 b	0.3 $\pm$ 0.02 b	86.89	< 0.001
<i>norB</i> ( $\times 10^5$ )	1.9 $\pm$ 1.7 a	5.1 $\pm$ 0.5 b	8.6 $\pm$ 0.4 b	15.4 $\pm$ 1.4 a	11.174	0.003
<i>nosZ</i> ( $\times 10^5$ )	2.2 $\pm$ 0.3 a	2.2 $\pm$ 0.2 a	1.7 $\pm$ 0.09 a	1.7 $\pm$ 0.3 a	1.95	0.20
<i>nifH</i> ( $\times 10^6$ )	0.7 $\pm$ 0.04 b	1.1 $\pm$ 0.08 b	2.8 $\pm$ 0.4 a	3.7 $\pm$ 0.3 a	30.65	< 0.001

was higher and the *Alphaproteobacteria* and *Gammaproteobacteria* abundances were lower at GE25 and GE35 than at GE0 and GE10. The PCoA based on the Bray-Curtis distances (number of OTUs) clearly identified a large change in bacterial structure along the exclusion chronosequence (Fig. 2); this finding was supported by the ANOSIM test, indicating that the OTU-based taxonomic bacterial community composition significantly differed among the four grasslands (Table S3). *Sordariomycetes* was the dominant fungal class (26.6%), followed by *Dothideomycetes* (23.4%) and *Leotiomycetes* (16.2%) (Fig. 1b). The abundances of these three classes significantly differed (*P* < 0.05) among the sites. Interestingly, the *Sordariomycetes* abundance increased and the *Leotiomycetes* abundance decreased with the length of exclusion. The fungal community composition significantly differed among GE10, GE25, and GE35 but did not significantly differ between GE0 and GE10 (Table S3).

The abundances of a total of 20 bacterial orders and 17 fungal families significantly differed among the sites, and these taxa were chosen as specific microbial biomarkers (Fig. S1, LDA values are shown in Fig. S2). These microbes were mainly from *Actinobacteria*, *Proteobacteria*, *Acidobacteria*, and *Chloroflexi*. The specific fungal groups were from *Ascomycota* and *Basidiomycota*. *Thermomicrobia* and *Ktedonobacteria* were the main bacterial biomarkers at GE0, and Teratosphaeriaceae and Ceratostomataceae were the main fungal biomarkers. *Xanthomonadales* and *Sphingomonadales* were the main bacterial biomarkers at GE10, and *Montagnulaceae*, *Didymosphaeriaceae*, and *Pezizaceae* were the main fungal biomarkers. *Desulfurellales*, *Rhodobacterales*, and *Halosphaeriaceae* were the main biomarkers at GE25, and *Gaiellales*, *Blastocatellales*, *Gemmatimonadales*, *Hygrophoraceae*, and *Schizoporaceae* were the main biomarkers at GE35.

### 3.4. Comparison of the variation in the plant and microbial communities

The diversity and composition ordinations of the plant and microbial communities were further compared to determine whether the patterns of change were similar between the plant and microbial communities. The diversities of the plant and bacterial fungal communities behaved similarly and increased along the chronosequence in the initial 25 years (Table 2, Table 5) but then drastically decreased. The diversity of the fungal community, however, did not significantly differ between the GE0 and GE10 sites, although the highest value occurred at GE25 (Table 5). The plant and bacterial communities

clearly differed among the exclusion sites and showed the same pattern of distribution (Fig. 2), unlike the fungal communities, which were closely clustered for GE0 and GE10. There was a strong correlation between the first coordinates of the plant and bacterial communities (*r* = 0.79; *P* < 0.001), indicating that these two community types changed in parallel over time.

### 3.5. Soil and plant properties associated with the microbial community structure

pH was not taken into account in the evaluation of the relationships among the soil and the plant and microbial communities because of the low variation among sites. The Mantel test indicated that the above-ground biomass, plant diversity (Shannon index), OC content, and TN content were significantly correlated with the overall compositions of the bacterial and fungal communities (*P* < 0.05; Table 6), whereas the soil NH<sub>4</sub><sup>+</sup>-N content had no obvious effect on the community composition (*P* > 0.05). The vectors in the CCA plot (Fig. 3) indicated that the plant diversity, OC, and NO<sub>3</sub><sup>-</sup>-N contents were the most important factors affecting the changes in specific bacterial groups, ammonia-oxidizing microbes and denitrifying microbes and that plant diversity, NO<sub>3</sub><sup>-</sup>-N content, AP, and soil moisture content were the most important factors affecting the specific fungal groups. We also found a close association among the plants, soils, and microbial communities (Tables S4 and S5). For example, the plant diversity and OC and TN contents were positively correlated with the microbial biomass, the bacterial and fungal diversity, the activities of BG, NAG, and AKP, and the abundances of *Acidobacteria*, *Betaproteobacteria*, and *Sordariomycetes*. The soil moisture content was positively correlated with the fungal diversity and *Sordariomycetes* abundance and negatively correlated with the *Leotiomycetes* abundance. The AP content was positively correlated with the *Acidobacteria* abundance and negatively correlated with the AKP activity and *Alphaproteobacteria* and *Gammaproteobacteria* abundances.

## 4. Discussion

### 4.1. Effects of grazing exclusion on plant and soil properties

Coverage, biomass, and diversity greatly increased in the plant communities in the grazing-excluded grasslands compared to the

**Table 5**

**Soil microbial diversities along the grazing-exclusion time.** Results is reported as the mean  $\pm$  SE (n = 3). Values of *F* and *P* are the significance by the one-way ANOVA. Different letters in a row indicate significant differences between the sites by the Tukey HSD test (*P* < 0.05).

Parameters		GE 0	GE 10	GE25	GE35	<i>F</i>	<i>P</i>
Observed OTUs	Bacteria	1859 $\pm$ 41.3 a	1837 $\pm$ 20.2 a	1885 $\pm$ 23.3 a	1734 $\pm$ 20.6 b	5.670	0.022
	Fungi	1244 $\pm$ 24.2 a	1070.7 $\pm$ 101.7 ab	1367.3 $\pm$ 41.3 a	855.7 $\pm$ 73.6 b	10.937	0.003
Chao1 estimator	Bacteria	2435.4 $\pm$ 55.7 a	2424.9 $\pm$ 26.5 a	2424.7 $\pm$ 55.3 a	2169.5 $\pm$ 78.2 b	5.171	0.028
	Fungi	1622.3 $\pm$ 36.0 a	1388.0 $\pm$ 116.5 b	1676.9 $\pm$ 55.1 a	1085.7 $\pm$ 52.9 c	14.008	0.002
Shannon index	Bacteria	6.14 $\pm$ 0.01 c	6.29 $\pm$ 0.03 ab	6.38 $\pm$ 0.01 a	6.25 $\pm$ 0.01 b	41.086	< 0.001
	Fungi	3.92 $\pm$ 0.13 b	4.03 $\pm$ 0.19 ab	4.84 $\pm$ 0.12 a	3.26 $\pm$ 0.18 c	22.596	< 0.001

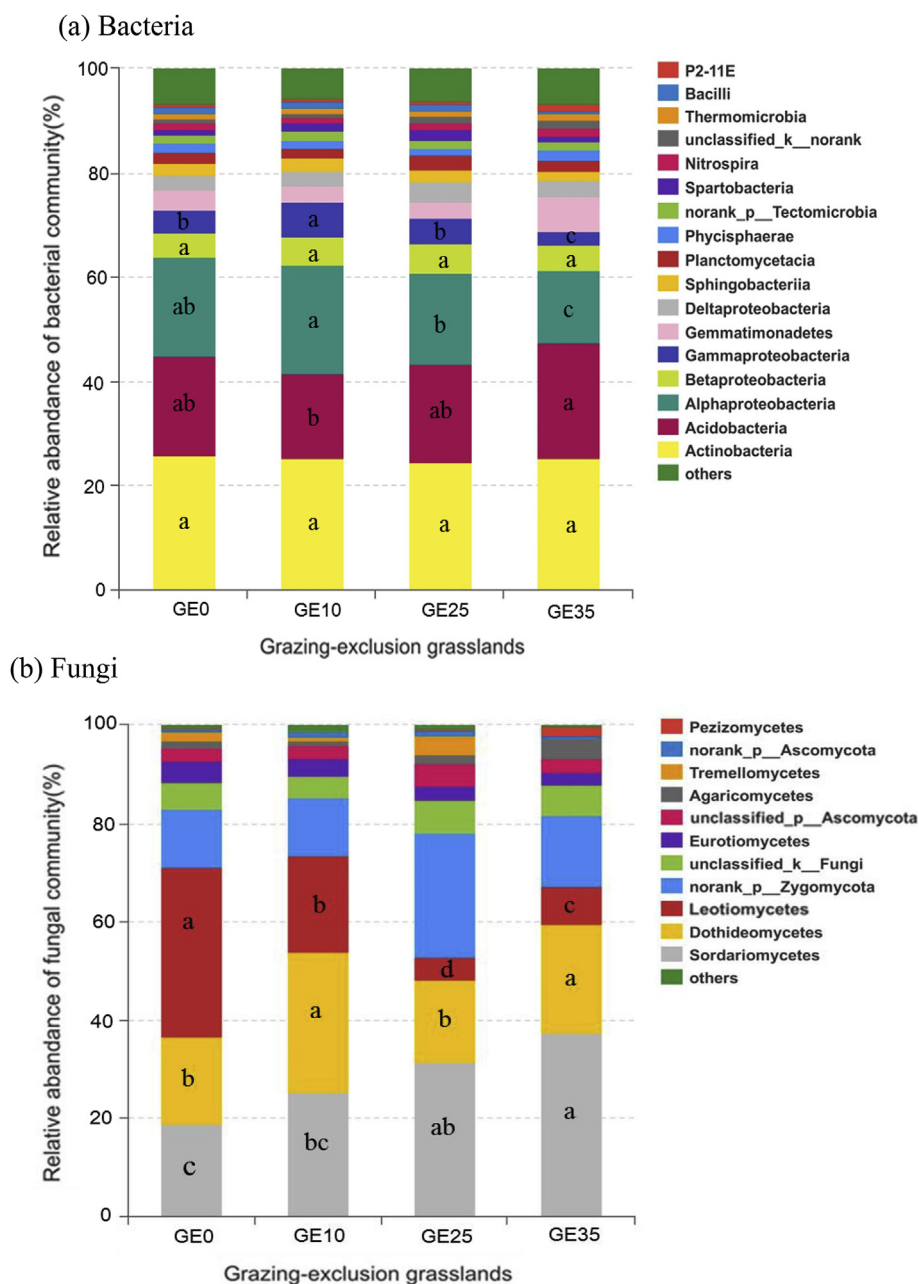


Fig. 1. Changes in the microbial communities at the class level. a: bacteria; b: fungi.

grazed grassland, indicating that grazing exclusion had a positive effect on the aboveground productivity. Plant biomass and diversity, however, peaked at GE25 and then drastically decreased, in agreement with a previous finding that diversity is usually highest during the middle-late stage of restoration (Odum, 1969; Wardle and Peltzer, 2007). The initial increase and later decrease in species diversity, biomass, and coverage at our field sites may indicate that long-term (> 25 y) exclusion is not beneficial for the restoration of degraded grasslands, even though the aboveground diversity and biomass were significantly higher at GE35 than at GE0. Community richness was higher at GE0 than at the grazing-excluded sites, indicating that grazing disturbance favors plant richness.

Grazing exclusion greatly improved the soil nutrient levels compared to those in the continuous grazing sites due to the increased aboveground biomass. The lower nutrient contents at GE0 may have been due to the faster loss of leaves and shoots through ingestion by domestic animals (primarily sheep), which greatly decreases the

accumulation of aboveground biomass (Cheng et al., 2016). The higher aboveground biomass along the chronosequence would lead to the accumulation of organic matter in the soil from the decomposition of the litter. The sharply decreased OC, TN, and moisture contents after 25 years indicated that organic matter contents can decrease when exclusion exceeds 25 y, despite the initial positive effect in the former 25 years. By contrast, the C:N ratio in the soils remained remarkably stable along the chronosequence, which is consistent with previous studies reporting a constant C:N ratio in grassland soils (Peng and Wang, 2016; Zhang et al., 2016; Baddeley et al., 2017; Zeng et al., 2017b). In contrast, other studies have reported a variable C:N ratio in soils, such as those in forestland and shrubland, as some individual studies observed a higher C:N ratio in coniferous forests than in broadleaf forests (Xu et al., 2016; Yang and Luo, 2011). Compared with the abundant nutrients provided to soils from the decomposition of aboveground plant parts in forestland, grasslands, with relatively lower aboveground biomass, add less C and N to soils. Given the resources of aboveground

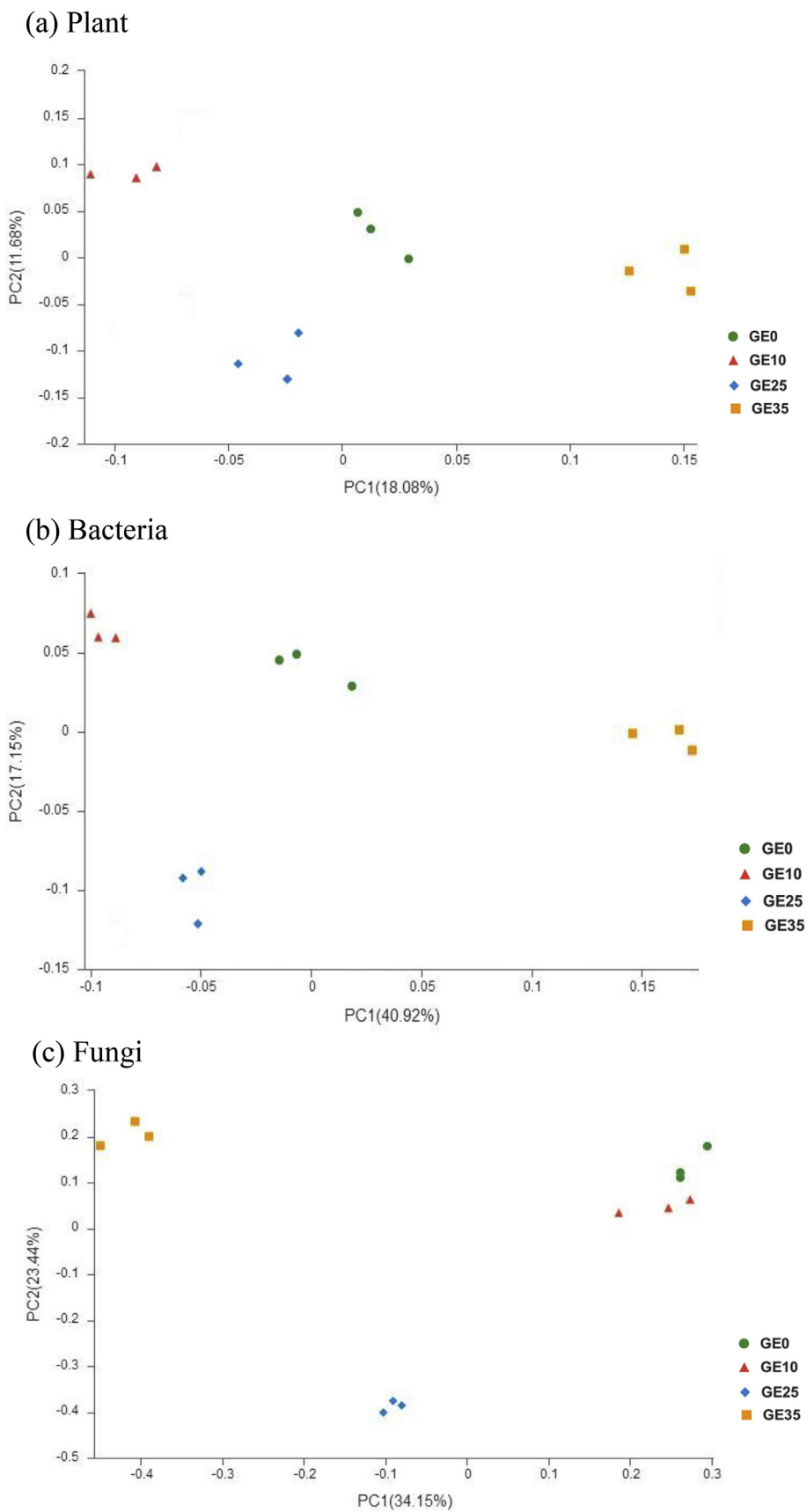


Fig. 2. Ordination of the plant (cover of different species) and microbial communities (OTU abundance) along an exclusion chronosequence using PCoA with the Bray-Curtis similarity index. a: plants; b: bacteria; c: fungi.

**Table 6**

Mantel test results for the correlation between community composition and environmental variables for bacteria and fungi based on OTU along the exclusion time.

Community	Parameters	<i>r</i>	<i>p</i>
Bacteria	Cover	0.357	0.531
	AB	<b>0.475</b>	<b>0.042</b>
	BB	0.408	0.061
	P-Shannon	<b>0.628</b>	<b>0.002</b>
	OC	<b>0.579</b>	<b>0.009</b>
	TN	<b>0.554</b>	<b>0.021</b>
	C: N	0.118	0.192
	NO <sub>3</sub> <sup>-</sup> -N	<b>0.498</b>	<b>0.037</b>
	NH <sub>4</sub> <sup>+</sup> -N	0.220	0.089
	NO <sub>3</sub> <sup>-</sup> /NH <sub>4</sub> <sup>+</sup>	0.018	0.410
	AP	0.181	0.137
	Moisture	0.049	0.205
	Fungi	cover	0.036
AB		0.183	0.194
BB		0.125	0.303
P-Shannon		<b>0.677</b>	<b>0.001</b>
OC		<b>0.608</b>	<b>0.001</b>
TN		<b>0.575</b>	<b>0.005</b>
C: N		0.157	0.325
NO <sub>3</sub> <sup>-</sup> -N		0.414	0.030
NH <sub>4</sub> <sup>+</sup> -N		0.019	0.472
NO <sub>3</sub> <sup>-</sup> /NH <sub>4</sub> <sup>+</sup>		0.1954	0.106
AP		0.116	0.307
Moisture		<b>0.665</b>	<b>0.001</b>

AB: aboveground biomass, BB: aboveground biomass,  $H_{\text{plant}}$ : plant Shannon diversity, OC: organic C, TN: total N, AP: available P. Values in bold indicate significant correlation ( $P < 0.05$ ).

litter and belowground roots in terms of soil nutrients, it can be assumed that the stable C:N ratio in the soil along the chronosequence could be driven by the relatively constant C:N ratio in litter and roots (Yang and Luo, 2011; Alberti et al., 2008). Although we did not attempt to quantify the contributions of litter and roots to the soil nutrient contents, an investigation by Zeng et al. (2017b), who conducted a grassland experiment along a similar chronosequence in the Yunwushan grassland reserve, supported the assumption that no significant changes in litter C: N and root C: N occurs during 30 years of grazing exclusion. The relatively constant C: N ratio in the litter and roots may be ascribed to the close C–N coupling in their components over successional time. It is known that soil C and N are mainly derived from the stable organic matter formed after the decomposition of dead organisms (Cleveland and Liptzin, 2007). Organisms tend to have a stable C: N ratio, and the close C–N coupling exists even after plant detritus enters the soil as litter (McGroddy et al., 2004). Consequently, the close C–N coupling in litter and roots could lead to the tight association between C and N in soils. The stable C:N ratio suggests that grazing exclusion had little effect on the balance of the soil C and N levels in degraded grassland in this semiarid area. The similar trends in the contents of OC, TN, and microbial biomass C and N and the activities of BG, NAG, and AKP were consistent with a previous report that soil microbial biomass and enzymatic activities are closely associated with nutrient contents (Sinsabaugh et al., 2009).

#### 4.2. Effects of grazing exclusion on the microbial communities

Plant succession is usually characterized by an initial increase in species diversity and accumulation of aboveground biomass, followed by a decrease in diversity caused by the strong competition dominating the middle-late stage (Zhang et al., 2016; Jing et al., 2014). Our results indicate that this pattern also applied to the diversities of the bacterial and fungal communities, which were much higher at GE25 than at the other sites, suggesting the negative effect of long-term exclusion on the microbial community. This was further confirmed by the changes in the

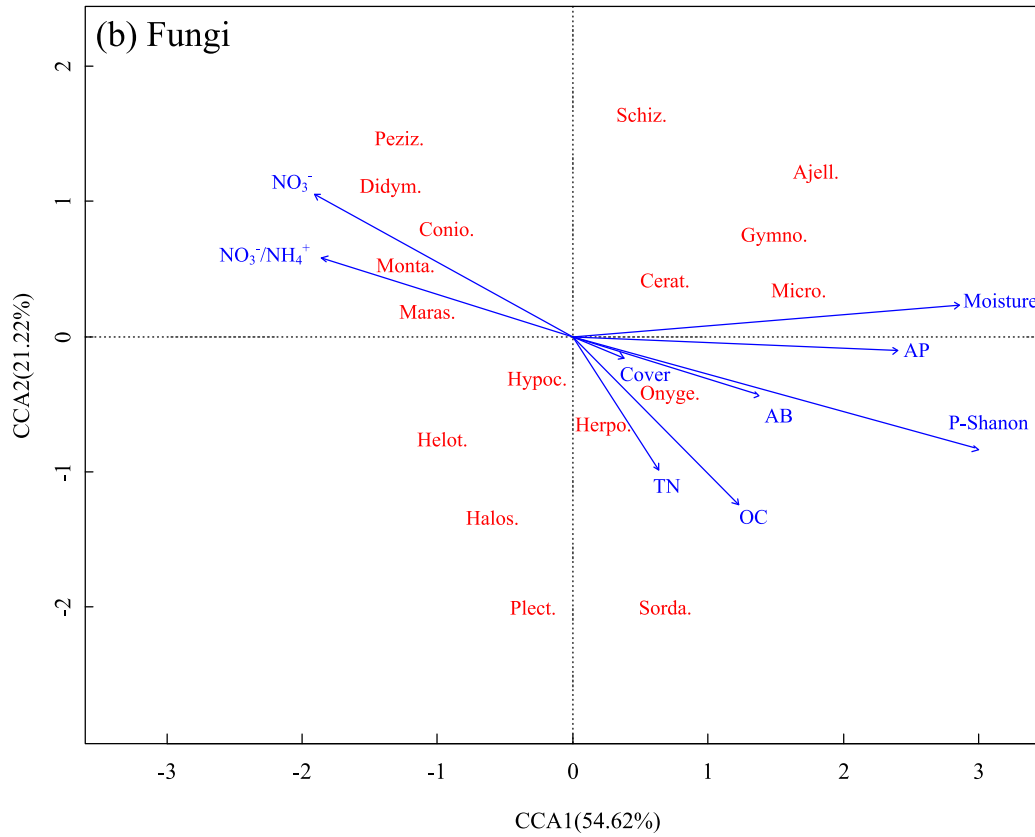
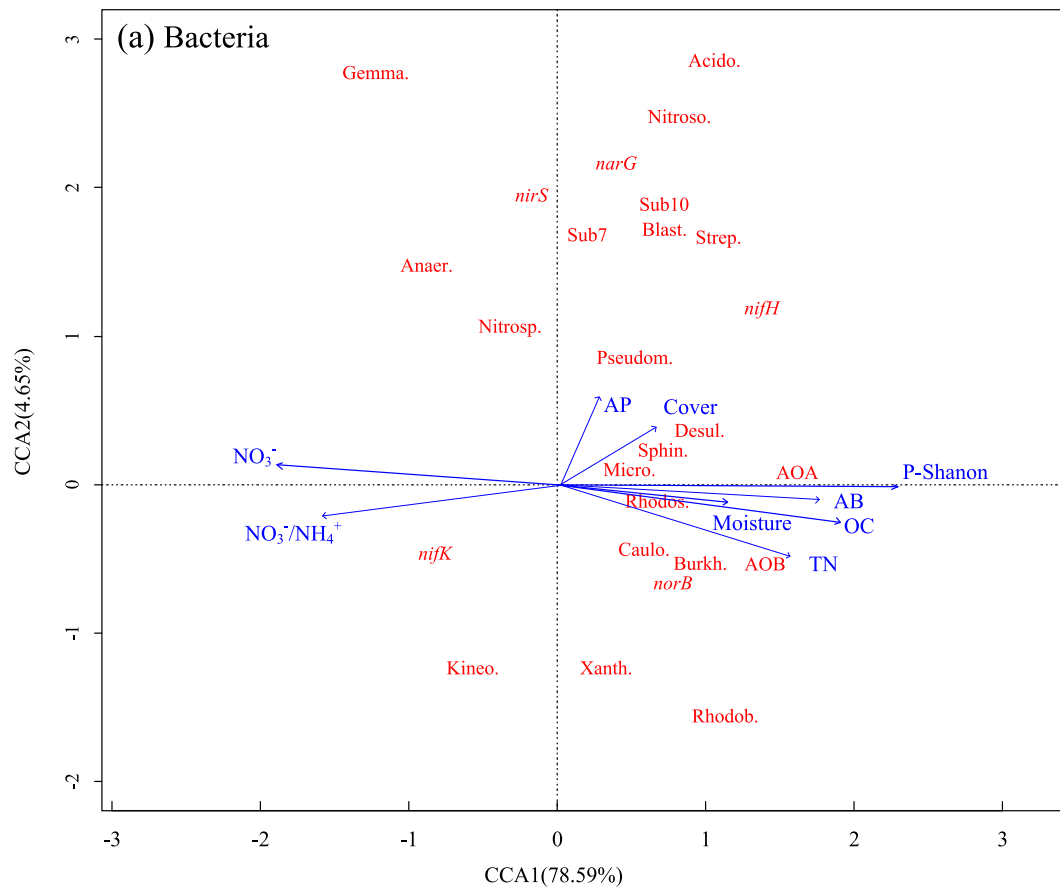
abundance of nitrifying ammonia-oxidizing (AOA, AOB) microbes and denitrifying microbes (*narG*, *nirS*). Our results agree with the findings of Cheng et al. (2016), who reported that 33 years of grazing exclusion had a negative effect on the diversity and abundance of soil bacteria. The trends in the bacterial community diversity contrasted with the findings of Lozano et al. (2014), showing that bacterial diversity increased linearly along a successional chronosequence of restored farmland. This discrepancy may be associated with the substrates that provided nutrients to the microorganisms, which will be discussed in the next section.

The successional pattern of microbial communities has been a subject of debate. We found a clear pattern of succession in the bacterial community composition in the grazing-excluded grasslands, in agreement with the results of Zeng et al. (2017a), who found distinctly different bacterial communities along a grazing-exclusion chronosequence. Kuramae et al. (2011), however, reported high overlap in microbial communities, with no clear differences among successional stages along a chronosequence in chalk grasslands. Interestingly, we found that the fungal community compositions did not significantly differ between GE0 and GE10 (Fig. 2c) but that the compositions at these sites significantly differed from those at GE25 and GE35, suggesting that 10 y of exclusion would not cause changes in the fungal communities, which was supported by the lack of a significant difference in fungal diversity in the first 10 y (Table 5). Our data also identified changes over time in the relative abundances of several microbial groups. The bacterial communities shifted from fast-growing oligotrophic groups to slow-growing copiotrophic groups (Zhou et al., 2017), characterized by decreases in *Alphaproteobacteria* and *Gammaproteobacteria* abundances and increases in *Acidobacteria* abundance along the chronosequence (Banning et al., 2011). This shift may have been due to the increase in C and N substrates in the soils, consistent with the higher OC and TN contents at the middle-late stage compared to the early stage. Microbial communities containing r strategists, such as some members of *Proteobacteria*, usually dominate communities at the early stages of succession, suggesting less crowded conditions with higher resource availability (Fierer et al., 2007). Communities containing K strategists, such as *Acidobacteria*, can dominate a community as the ecosystem matures (Banning et al., 2011).

The consistent patterns of composition and diversity in the plant and bacterial communities indicated that their succession proceeded in parallel. This result differed from the finding by Lozano et al. (2014), in which plant and bacterial succession were incongruous in an arid grassland that had developed on abandoned cropland, with plant succession proceeding faster than bacterial succession. The different starting points of community succession between the two grassland ecosystems could be responsible for this discrepancy. The plant and microbial succession in our study began from established grassland with high aboveground coverage and biomass, but the grassland in the study by Lozano et al. (2014) began from abandoned cropland with scarce plant coverage, which could cause differences in nutrient supply and consequently lead to different impacts of plants on the microbial communities. This inference is also supported by the OC content data, which significantly differed between GE0 and GE10 in our study but did not significantly differ between the first two successional stages in the study by Lozano et al. (2014). The difference in bacterial succession between the two studies could thus be directly due to the different substrates used by microbes, which were influenced by the decomposition of the aboveground biomass.

The majority of the fungi collected in our study were saprophytic. Only < 1% were mycorrhizal fungi, including arbuscular mycorrhizal fungi, such as *Glomeraceae* (Welch et al., 2014), and ectomycorrhizal fungi, such as *Lactarius* (Zhang et al., 2017). Mycorrhizal fungi are generally abundant in forest communities such as pine forests and scarce in grassland, especially in arid areas. The fungal communities showed distinctive succession along the chronosequence, as expected. *Sordariomycetes*, the second largest class of Ascomycota, increased in





(caption on next page)

**Fig. 3.** Canonical correspondence analysis (CCA) identified the effects of plants and soils on specific microbial groups. a: bacteria; b: fungi. Acido.: Acidobacteriales; Anaer.: Anaerolineales; Blast.: Blastocatellales; Burkh.: Burkholderiales; Caulo.: Caulobacteriales; Desul.: Desulfurculales; Gemma.: Gemmatimonadales; Kineo.: Kineosporiales; Micro.: Micromonosporales; Nitroso.: Nitrosomonadales; Nitrosp.: Nitrospira; Pseudon.: Pseudonocardiales; Pseudom.: Pseudomonadales; Rhodob.: Rhodobacteriales; Rhodos.: Rhodospirillales; Sphin.: Sphingomonadales; Strep.: Streptomycetales; Xanth.: Xanthomonadales; AOA, AOB: ammonia-oxidizing microbes; Hypoc.: Hypocreaceae; Schiz.: Schizoporaceae; Micro.: Microasceae; Ajell.: Ajellomycetaceae; Gymno.: Gymnoasceae; Halos.: Halosphaeriaceae; Sorda.: Sordariaceae; Herpo.: Herpotrichiellaceae; Conio.: Coniophoraceae; Onyge.: Onygenaceae; Plect.: Plectosphaerellaceae; Monta.: Montagnulaceae; Helot.: Helotiaceae; Didym.: Didymosphaeriaceae; Peziz.: Pezizaceae; Cerat.: Ceratobasidiaceae; Maras.: Marasmiaceae.

abundance over time and dominated at GE25 and GE35, replacing *Leotiomyces*, which dominated the communities at GE0 and GE10 (Fig. 1). This finding was in accordance with the results reported by Banning et al. (2011), who found an obvious change in the patterns of fungal groups during the succession from grassland to forest, but disagreed with the findings by Davey et al. (2015), who found no successional pattern during natural restoration at Arctic glacial sites. The different types of succession observed in these studies may have been responsible for this discrepancy. The succession in our study and in the study by Banning et al. (2011) was secondary succession in grasslands with sufficient soil nutrients for the establishment of pioneer plants and microorganisms, whereas the succession investigated by Davey et al. (2015) was on glacial land with no original soil or seed pool and so was primary succession. We also found that specific microbial groups were enriched at particular stages of exclusion, indicating succession in soil microbial composition. Overall, these compositional changes in the bacterial and fungal communities suggest distinct successional patterns in the microbial communities during the long-term exclusion of grazing in grassland in this semiarid area.

Unlike the bacterial community structure and diversity, which distinctly differed between the first two stages, the fungal community structure did not significantly differ between GE10 and GE0 (Fig. 2), suggesting that the successional patterns differed between the two communities, with the bacterial community likely developing faster than the fungal community. These results agree with those by Brown and Jumpponen (2015), who reported that fungal communities were largely unresponsive to successional age, while bacterial communities responded strongly, based on an analysis of phylogenetic diversity. Bacterial and fungal communities may have different early successional trajectories because bacteria have a broader range of physiologies than do fungi and are thus more likely to successfully colonize oligotrophic soils at an early successional stage. Fungi are thus more dependent than bacteria on sources of C and N and may not have as many available niches before sufficient organic matter accumulates during succession. This speculation is also supported by Prewitt et al. (2014), who found that bacteria are the initial colonizers during the decomposition of wood, followed by fungal groups. Furthermore, the finding of no significant change in the fungal community composition during the first 10 y suggests that the fungal community develops more slowly than the plant community and that its succession might lag behind plant succession. The plant and bacterial succession in our study generally occurred in parallel and proceeded faster than fungal succession during the long-term exclusion of grazing in grassland.

Plants affect soil microbial communities through the release of C and nutrients derived from the aboveground litter and belowground root system (Cline et al., 2018). These two organic matter sinks are characterized by different chemical compositions (e.g., sugar, carboxylic acids, phenolics) and are utilized by microbes at different rates (Leloup et al., 2018). The composition and quantity of organic matter usually differ among plant species (Eisenhauer et al., 2010), and plant diversity is therefore assumed to be a major factor affecting microbial community structure, abundance and activity (Lange et al., 2015). Our results confirmed this assumption, and the association between plant diversity and the microbial community is further discussed in the following section.

#### 4.3. Soil properties associated with plant and microbial succession

Many studies of grassland succession have documented large differences in bacterial and fungal community taxonomic composition among stages (Fry et al., 2016; LeBlanc et al., 2015). Zeng et al. (2017a) observed that the structure of microbial communities significantly differed among grazing exclusion stages in grasslands on the Loess Plateau. These results suggest that plant succession could be a good predictor of variation in microbial community composition. Plant succession, however, is a complex process, with changes in coverage, biomass, and diversity and the subsequent variation in soil variables. Plant coverage was recently assumed to be an important driver of the horizontal distribution of soil bacteria (Lozano et al., 2014), but we found that plant coverage did not consistently have a significant effect on the microbial community composition or on the relationships of coverage with microbial biomass and enzymatic activity. We instead found that plant diversity had a large effect on the bacterial community diversity and composition (Table 6, Fig. 3). This finding disagreed with that reported by Millard and Singh (2010), who concluded that bacterial diversity is not directly correlated with plant diversity but is more influenced by the quality or composition of the soil organic matter. Soil organic matter (consisting mainly of C and N) in natural ecosystems is mainly derived from the release of root exudates, cell death in roots, and the decomposition of litter biomass, and the quantity and quality of these soil components from plants are determined by the characteristics of the plant community, such as its biomass, composition, and diversity. Changes to microbial communities are thus caused by changes in plant communities that ultimately determine the amount of organic matter. Previous studies have reported strong and consistent associations between the alpha and beta diversity of plants and soil fungi in grassland ecosystems at different scales (Chen et al., 2017; Yang et al., 2017). Our study supported these observations and further found that plant diversity is associated with microbial biomass and enzymatic activity (Tables S4 and S5). It is accepted that a more diverse plant community results in a more diverse composition of litter and root exudates and consequently higher soil microbial diversity (Thakur et al., 2015). An increase in plant diversity is always accompanied by an increase in plant biomass, which increases the accretion of organic materials, such as litter and roots, and consequently favors the accumulation of soil OC and N (Lange et al., 2015). OC reduces the amplitude of fluctuations in soil temperature and increases its water-holding capacity and can thus increase the moisture content (Filep et al., 2015). Plant diversity, because of its effects on OC content and microclimatic conditions, can promote microbial growth and hence microbial biomass (Steinauer et al., 2015). Soil OC and N are mainly derived from root exudates and the decomposition of litter biomass, and we thus inferred that plant diversity likely affected the microbial community diversity by increasing the diversity of root exudates and litter decomposition (Mellado-Vazquez et al., 2016), but this inference requires further investigation.

N content was the main factor driving the bacterial and fungal communities in our study, and similar results were reported by Zhang et al. (2015) in a long-term N addition experiment and by Pommier et al. (2018) in grasslands across Europe. The TN content was strongly associated with biomass, diversity, and the abundance of dominant taxa in the bacterial and fungal communities, but the  $\text{NO}_3^-$ -N content only affected specific groups, and the  $\text{NH}_4^+$ -N content had little influence.

These results indicate that only some forms of N contributed to the succession of the microbial communities.  $\text{NO}_3^-$ -N likely affects the structuring of specific microbial communities in grassland on the Loess Plateau where grazing has been excluded. Our results also indicate that the composition of the fungal communities and the relative abundance of the dominant phyla and specific groups were closely correlated with the soil moisture content, suggesting that soil moisture drove the observed fungal succession. This result is consistent with that reported by Schmidt et al. (2018), who found that a reduction in the soil moisture content affected the composition of grassland fungal communities. The soil moisture content in our study, however, had surprisingly little effect on the bacterial communities, suggesting that the moisture content had a larger impact on the fungal than the bacterial community. The available P content was strongly associated with specific fungal communities, suggesting that P is important in structuring the fungal communities, consistent with the significant negative correlation between the available P content and AKP activity. The increase in AKP activity over time can likely be attributed to the need of the microbes to obtain the scarce P from organic sources.

## 5. Conclusions

Our study suggests that the exclusion of grazing in the semiarid grasslands led to both below- and aboveground succession characterized by changes in soil properties and enzymatic activities, plant biomass and diversity, and microbial biomass, diversity, and community composition. Grazing exclusion could be beneficial for the restoration of degraded grassland, but long-term exclusion could have a negative effect. Therefore, the reasonable utilization of grassland is recommended in the semiarid grasslands of the Loess Plateau, such as proper grazing or rational mowing, when community productivity reaches a peak during the process of grazing exclusion. Plant and bacterial succession occurred in parallel and proceeded faster than fungal succession. Bacterial and fungal succession was affected by plant diversity and the soil OC, TN, and nitrate-N contents. Changes in the fungal community were also susceptible to the variation in soil moisture content. Plant diversity played an important role in shaping the soil microbial communities, likely by altering the nutrient levels and moisture content. Additionally, seasonality is an important factor in shaping our findings because sampling occurred in August, when the aboveground biomass reached the peak. Our results will contribute to the understanding of the mechanisms of ecosystem function and offer a new perspective for the management of semiarid grasslands.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2018.05.026>.

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