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Title page

Title: Shrub encroachment is associated with changes in soil bacterial community composition in a temperate grassland ecosystem

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Abstract

Aims: The effects of shrub encroachment on plant and soil properties have been well studied. However, little is known about how shrub encroachment influences soil bacterial communities. We investigated the effects of shrub encroachment on grassland soil bacterial communities along a soil depth gradient in the Inner Mongolian region of China.

Methods: The belowground bacterial communities were examined using high-throughput sequencing of the 16S rRNA gene (V4-V5 region, Illumina MiSeq).

Results: Bacterial alpha-diversity was higher in shrub-encroached soils than in control grassland soils. Bacterial OTU richness was highest at 0-20 cm soil depths, while phylogenetic diversity was greatest at 10-20 cm soil depths. At each soil depth layer, shrub encroachment was associated with a significant shift in bacterial community composition. Change in soil pH was the factor most strongly related to change in bacterial community composition associated with shrub encroachment at all four depth horizons in the soils. Shrub encroachment appears to alter the distribution of bacterial life history strategies in the surface soil (i.e., showing an enrichment in copiotrophs and a depletion in oligotrophs) and shrubs are associated with an increase in nitrification potential in deeper soil horizons.

Conclusions: Our results indicate that the influence of shrub encroachment on bacterial community composition extends deep into the soil. The intensity of shrub encroachment at this study site suggests that this ecosystem is undergoing dramatic succession towards shrub-dominance, which will likely trigger shifts in ecosystem function.

Keywords: shrub encroachment · bacterial community · soil pH · soil depth · grassland ecosystem

Introduction

Shrub encroachment - defined as an increase in cover, abundance and dominance of shrubs (Jackson et al. 2002; Briggs et al. 2005; Kulmatiski et al. 2013) - has been reported extensively in grassland ecosystems, from Australia (Brown and Carter 1998; Costello et al. 2000), Africa (van Vegten 1983; Moleele and Perkins 1998), and Eurasia (Rivest et al. 2011; Peng et al. 2013; Li et al. 2015), to North and South America (Adamoli et al. 1990; Dussart et al. 1998; Houghton et al. 1999). Shrub encroachment appears to depend on multiple drivers, including grazing pressure (Brown and Archer 1999; Coetzee et al. 2008), wildfire frequency (Scholes and Archer 1997; Briggs et al. 2002), changes in climate (Knapp et al. 2008; D'Odorico et al. 2010), elevated atmospheric carbon dioxide (CO₂) concentration (Archer et al. 1995) and nitrogen (N) deposition (Briggs et al. 2005).

Shrub encroachment into grasslands usually creates a mosaic landscape, with shrub patches interspaced with open patches of grass. These shrub and open patches usually have quite distinct sets of plant species present. An increase in shrub cover is often associated with large changes in ecosystem functioning with either decreases (Throop et al. 2008; Gomez-Rey et al. 2013) or increases (Soliveres and Eldridge 2014) in the primary productivity of the landscape. There are often long-term effects on overall biodiversity, primary production, plant allocation, rooting depth, soil erosion, and soil faunal community composition (Grover and Musick 1990; Trumbore et al. 1997; Jobbágy et al. 2000; Hibbard et al. 2001; Briggs et al. 2005; Breshears 2006; Zhang et al. 2006; Knapp et al. 2008). Shrub encroachment influences the cycling of water and energy, and increases oxygenation and nutrient content in surface soils (Kurc and Small 2004; Bragazza et al. 2015). The effects of shrub encroachment are not always consistent. In some cases, encroachment leads to increases in soil C and N contents (Hibbard et al. 2001; Baer et al. 2006; Liao et al. 2006; McClaran et al. 2008; McKinley and Blair 2008; Li et al. 2015) and net C losses (Jackson et al. 2002; Lett and Knapp 2003), while in other cases no significant changes are found in soil C and N (McCarron et al. 2003; Smith and Johnson 2003).

Plants influence bacterial communities by determining the quantity and quality of

aboveground litterfall and by releasing root exudates into the soil. Plant litter and root exudates feed heterotrophic soil microorganisms (Staddon et al. 2003; Wallenstein et al. 2007). Shrub encroachment significantly increased microbial biomass (Yannarell et al. 2014) and altered fungal community composition (Bragazza et al. 2015). Gellie et al. (2017) found that revegetation has a profound effect on belowground bacterial community structure. In addition to vegetation shaping belowground community composition, soil microbes can stimulate plant growth, serving as sources and sinks for key nutrients and as catalysts for nutrient transformations (Hart et al. 2005). Dynamic and complex feedback mechanisms exist between aboveground vegetation and belowground bacterial community (Xiang et al. 2014). Despite a substantial body of work on how shrub encroachment affects plant ecology and soil chemistry, the response of bacterial communities is not well understood. A better understanding of how soil bacterial communities respond to shrub encroachment will help us to predict how encroachment will impact grassland ecosystem processes.

The Inner Mongolian grassland, part of the Eurasian steppe belt, includes approximately 86.6 million ha of grassland ecosystem. Shrub encroachment has impacted over 5.1 million ha of Inner Mongolian grassland (Zhang et al. 2006; Peng et al. 2013; Chen et al. 2015). Shrub encroachment affects plant communities and belowground soil properties (McCarron et al. 2003; Briggs et al. 2005), which might trigger shifts in soil bacterial community composition (Yannarell et al. 2014). In addition, shrub encroachment alters plant rooting depth, which might affect the depth profile of soil bacterial communities by changing the spatial distribution of root exudates. Understanding bacterial distributions along a soil depth profile following shrub encroachment is important for clarifying how shrub encroachment impacts grassland ecosystems. We carried out this study in order to evaluate how soil bacterial communities differ in the presence of shrubs, along a soil depth gradient, in a representative shrub-encroached grassland of Inner Mongolia. Due to the characterized effects of shrub encroachment on surface soil parameters, i.e. oxygenation, nutrients, etc (Kurc and Small 2004; Bragazza et al. 2015), our major hypotheses were: i) that shrub encroachment will lead to an increase in the relative

abundance of copiotrophic taxa that thrive at higher nutrient concentrations, especially in surface soil; and ii) that the effects of shrub encroachment on bacterial community composition would weaken with soil depth.

Materials and Methods

Site selection and soil sampling

The study was conducted in a representative area of shrub-encroached grassland (42°57'N, 112°43'E; 1208 m), located in Xinlinguole, Inner Mongolia. The average annual temperature and precipitation in this region are 5.1 °C and 195 mm (Chen et al. 2015), respectively. The soil type is chestnut loam (Chen et al. 2015). The landscape is dominated by the grasses *Cleistogenes songorica* and *Agropyron mongolicum*, but *Caragana microphylla* (shrub) is encroaching into this area, possibly due to climate change (e.g. less precipitation) and/or overgrazing (Zhang et al. 2006; Chen et al. 2015).

We identified five shrub-encroached sample sites and four control sites (i.e. without shrub encroachment) to include in this study. The control samples were collected several hundred meters away from shrub-encroached areas (Fig. 1). Shrub coverage (SC) showed a slight increase, from 9.2-16.9%, for sites that were further away from the controls (Table S1). Spatial distance was not significantly correlated with bacterial community composition (Mantel $P = 0.104$) or soil properties (Mantel $P = 0.112$). All samples were taken > 10 kilometers away from human settlements to avoid anthropogenic disturbances.

Soil samples were taken on the 9th of August, 2015. At each sample site (20 m x 20 m plots), soils were collected in four layers: 0-10 cm, 10-20 cm, 20-30 cm, and 30-50 cm. For each layer, soil was collected from five points (four vertices and the center of plots) and then mixed as one sample for control soils. The shrub-encroached soils were collected under five shrub canopies (close to roots), which were nearest to the four vertices and the center of a plot and then mixed as one sample. A total of 36 samples, 16 (4 sites x 4 layers) from control grassland and 20 (5 sites x 4 layers) from shrub-encroached soils, were analyzed in this study. The soils were kept in a cooler

and transported refrigerated to the lab as quickly as possible. The samples were homogenized within each bag and sieved to remove stones and roots, and then divided into three parts: one part was stored at 4 °C for biogeochemical analysis; the second part was stored at -20 °C for DNA extraction and the third part was placed in long-term storage at -40 °C.

Soil properties

We measured soil pH, soil moisture (SM), total carbon (TC) and nitrogen (TN), total phosphorus (TP), dissolved organic C (DOC) and N (DON), ammonium (NH_4^+), nitrate (NO_3^-), soil inorganic (SIC) and organic (SOC) carbon contents, as described in the Supporting Information.

Soil DNA extraction and purification

DNA extractions were carried out on 0.5 g fresh soil according to the manufacturer's instructions (FastDNA® SPIN Kit for soil, MP Biomedicals, Santa Ana, CA). DNA was purified using a PowerClean® DNA Clean-Up Kit (MO BIO Laboratories, Carlsbad, CA, USA) to remove PCR inhibitors. The extracted DNA was dissolved in 50 µl of elution buffer, quantified by NanoDrop ND-1000 (Thermo Scientific, USA), and stored at -20 °C.

PCR and amplicon library preparation

An aliquot (50 ng) of purified DNA from each sample was used as template for amplification. Primer sets F515/R907 equipped with sequencing adapters and unique identifier tags were used to amplify the V4-V5 hypervariable regions of the bacterial 16S rRNA genes fragments (Biddle et al. 2008) for the Illumina MiSeq platform (PE 300) at Majorbio (Shanghai, China). PCR was carried out in 50 µl reaction mixtures containing each deoxynucleoside triphosphate at a concentration of 1.25 mM, 1 µl of forward and reverse primers (20 mM), 2 U of Taq DNA polymerase (TaKaRa, Japan), 2 mM of MgCl_2 , and 50 ng of DNA. The following cycling parameters were used: 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at

72 °C for 45 s; with a final extension at 72 °C for 10 min. To check for contamination, PCR negative controls were performed without added DNA template. Negative PCR controls did not contain detectable PCR product, and were not processed for sequencing. Triplicate reaction mixtures per sample were pooled together and purified using an agarose gel DNA purification kit (TaKaRa). The PCR products were pooled in equimolar amounts (10 pg for each sample) before sequencing.

Processing of sequence data

Sequences were merged by FLASH (Magoc and Salzberg 2011) and then processed using Quantitative Insights Into Microbial Ecology (QIIME v.1.9.0; <http://www.qiime.org/>) (Caporaso et al. 2010). Poor-quality sequences (below an average quality score of 25) and short sequences (< 200 bp) were removed. Sequences were clustered into Operational Taxonomic Units (OTUs) using a 97% identity threshold (default QIIME settings) by UCLUST (Edgar 2010) and all singleton OTUs were deleted. Chimera filtering was also performed to remove sequencing errors with USEARCH tool in QIIME. The most abundant sequence within each cluster was selected as the representative sequence for that OTU. Representative sequences were aligned using PyNAST (Caporaso et al. 2010). The taxonomic identity of each OTU was determined using the ribosomal database project (RDP) Classifier (Wang et al. 2007). To have similar sequencing effort and homogenize among samples, we used a randomly selected subset of 15,000 reads per sample to compare bacterial community composition and diversity among samples. A read depth of 15,000 was chosen because this was the lowest sequence read depth among our samples.

Statistical analysis

The relative abundances of dominant phyla and community alpha-diversity followed a normal distribution across samples (Kolmogorov-Smirnov test; $P > 0.05$ in all cases), and significant differences in relative abundances of dominant phyla and alpha-diversity measures among treatments were determined by one-way analysis of variance (ANOVA; SPSS 20.0 for Windows). Identification of bacterial taxa that

differed significantly among sampling groups was performed using linear discriminant analysis (LDA) effect size (LEfSe) (<http://huttenhower.sph.harvard.edu/lefse/>; Segata et al. 2011). LEfSe uses a non-parametric Kruskal-Wallis rank sum test to detect significant features and performs LDA to estimate the effect size for each feature. Vegetation type and depth were input as 'class' and 'sub-class', respectively, to identify bacterial biomarkers for these metadata categories. An alpha value of 0.05 and an effect size threshold of 2 were used to identify biomarkers in this analysis (Segata et al. 2011). The differences of bacterial community composition among treatments were analyzed by Non-metric multidimensional scaling (NMDS), with corresponding soil properties using envfit, and by permutational multivariate analysis of variance (PERMANOVA; permutations = 999) using the vegan package (Version 2.0-2; Oksanen et al. 2011) in R v.2.8.1 (R Development Core Team, 2006). Multicollinearity of soil metadata variables was tested using the variance inflation factor (VIF). Variables with VIF values less than 3 were selected (Zuur et al. 2010; Xiang et al. 2015) for variance partitioning analysis (VPA) and Mantel tests (permutations = 999), both of which were used to show the effect of soil properties on bacterial community composition.

Data availability

Raw sequence data were submitted to the Sequence Read Archive (SRA) of NCBI under the accession number SRP092383.

Results

Soil chemistry

Soil pH and total phosphorus (TP) were significantly lower following shrub encroachment at all four depth horizons relative to the controls (Table S2). Soil NH_4^+ and dissolved organic nitrogen (DON) were significantly higher in the 0-10 cm soil of shrub sites, compared to control locations. Shrub encroachment was associated with higher content of NO_3^- in 0-30 cm soils and lower content of total carbon (TC) in 10-50 cm soils. Total nitrogen (TN) and soil organic carbon (SOC) levels were

significantly lower in the 10-20 cm and 30-50 cm horizons of shrub-encroached sites, relative to control sites. Dissolved organic carbon (DOC) was higher in 0-10 cm soils and lower in 30-50 cm soils for shrub encroached sites, relative to controls (Table S2). Soil moisture (SM) increased with soil depth in both grassland and shrub-encroached soils. TC and TP showed increases in control soils and decreases in shrub-encroached soils along with soil depth. Soil NH_4^+ , TN, DOC, DON and SOC concentrations declined along with soil depth in shrub-encroached soils (Table S2).

Bacterial alpha-diversity

Soil bacterial alpha-diversity (i.e. OTU richness and phylogenetic diversity) was calculated at a depth of 15,000 randomly selected sequences per sample. Generally, alpha-diversity was higher in shrub-encroached soil than in control soils at all four soil depth horizons. Bacterial OTU richness was significantly higher in shrub-encroached sites at 0-20 cm soil depths and phylogenetic diversity was significantly greater at 10-20 cm soil depths, relative to controls (Fig. 2). OTU richness was negatively correlated with pH at all four depth horizons and phylogenetic diversity showed a negative correlation with pH in 10-30 cm soil horizons (Table S3). In control soils, OTU richness was significantly negatively correlated with SM, TC, TP, SIC, and positively correlated with DON; phylogenetic diversity showed significant negative correlations with SM, TP, SIC, and positive correlation with DON (Table S3). In shrub-encroached soils, both OTU richness and phylogenetic diversity were significantly negatively correlated with SM, and positively correlated with NH_4^+ , TC, TN, TP, DOC, DON, and SOC (Table S3).

Bacterial community composition

Across all soil samples, we obtained a total of 1,041,706 high quality bacterial sequences with 15,476-56,886 sequences per sample. Soil bacterial community composition was analyzed at a depth of 15,000 randomly selected sequences per sample. The dominant phyla across soils were Actinobacteria (32.2%), Acidobacteria (24.3%), Proteobacteria (19.0%), Chloroflexi (5.7%), Gemmatimonadetes (4.5%),

Planctomycetes (4.1%), Nitrospirae (3.7%), Bacteroidetes (2.3%) and Firmicutes (1.0%).

Compared to control soils in the 0-10 cm horizon, the relative abundance of Actinobacteria was significantly higher, and the relative abundance of Acidobacteria was significantly lower in shrub encroached sites. Gemmatimonadetes was significantly less abundant in the 30-50 cm horizon in the shrub encroached sites (Fig. 3). Shrub encroachment was associated with lower relative abundance of Planctomycetes at all four depth horizons. The relative abundances of Nitrospirae and Firmicutes were higher in the 20-50 cm shrub-encroached horizons, relative to controls (Fig. 3). LEfSe analysis further identified specific bacterial taxa that were differentially abundant across the two vegetation types. The results showed that bacteria in three phyla (Cyanobacteria, Planctomycetes and Thermi) and 14 classes were significantly more abundant in grassland soil; bacteria in one phyla (WPS_2) and six class were significantly more abundant in shrub-encroached soil (Fig. 4a). Soil bacterial biomarkers associated with soil depth were also identified in this study. Most group-specific biomarkers were identified in the 0-10 cm soil horizon, while 10-20 cm soils had the lowest number of biomarkers that distinguished native grasslands from shrub-encroached ecosystems (Fig. 4b).

Compared to control grassland soils, shrub encroachment resulted in significant shifts of bacterial community compositions at all four depth horizons in soils (Fig. 5; Table S4). The bacterial community composition was significantly different along soil depth horizons in both control and shrub-encroached soils (Fig. 5; Table S4). Variation in bacterial community composition was largely associated with soil pH ($r^2 = 0.799$), SM ($r^2 = 0.698$), DOC ($r^2 = 0.524$), NO_3^- ($r^2 = 0.512$), TN ($r^2 = 0.450$) and NH_4^+ ($r^2 = 0.392$) (Fig. 5). At each soil depth, bacterial community composition was highly correlated with soil pH (Table 1). Community composition significantly correlated with SM, NO_3^- and TP in control grassland soils, and SM and NH_4^+ in shrub-encroached soils along depth profiles (Table 1). In addition, variance partitioning analysis (VPA) was used to investigate the effects of soil properties on bacterial community composition. The VPA showed that soil pH explained the largest

proportion of bacterial community variation at all four soil depths (Table 2). SM explained much of the variance in bacterial community composition along soil depth profiles for both control and shrub-encroached soils (Table 2).

Discussion

Our first hypothesis was that shrub encroachment would be associated with enrichment in copiotrophic taxa, especially in surface soil. In this study, we found that shrub encroachment was associated with higher relative abundance of Actinobacteria and lower relative abundance of Acidobacteria in the 0-10 cm soil horizons relative to controls. Actinobacteria are generally considered to be more copiotrophic, while Acidobacteria are considered soil oligotrophs (Leff et al. 2015). Prior work has also shown that shrub encroachment increases oxygenation and nutrient content in surface soil (Schlesinger et al. 1990; Archer et al. 1995; Schlesinger and Pilmanis 1998; Reynolds et al. 1999; Kurc and Small 2004; López-Pintor et al. 2006; Coetsee et al. 2012; Bragazza et al. 2015), suggesting that shrub encroachment might influence the distribution of soil bacterial life history strategies (i.e., showing an enrichment in copiotrophs and a depletion in oligotrophs).

We also hypothesized that the effects of shrub encroachment on bacterial community composition would decrease with soil depth. Contrary to our expectation, shrub encroachment was associated with significant changes in bacterial community composition at all four soil depth horizons, relative to control soils. Empirical work has demonstrated that vegetation type can affect soil microbial community composition (Staddon et al. 2003; Hart et al. 2005; Wallenstein et al. 2007; Xiang et al. 2015). A recent study showed substantial differences in the near-surface soil (0-5 cm) bacterial communities at a landscape scale among four vegetation types (Shi et al. 2015), suggesting that soil bacterial communities are sensitive indicators of vegetation types (Kaye and Hart 1997; Schweitzer et al. 2004; Hart et al. 2005). Similarly, we found that shrub encroachment was associated with significant differences in bacterial community composition at soil depths of up to 50 cm, relative to control soils, suggesting that the putative effects of shrub encroachment on bacterial community

composition extended deep into the soil. Furthermore, shrub encroachment might affect deep soil bacterial function. Shrub encroachment may enhance deep soil nitrification by increasing the relative abundance of Nitrospirae, which can perform all the steps in the nitrification pathway and plays an important role in soil nitrogen cycling (Daims et al. 2015).

Several studies have demonstrated that soil pH is the primary driver shaping soil bacterial community structure (Fierer and Jackson 2006; Baker et al. 2009; Xiang et al. 2014). In this study, pH explained most of the variation in soil bacterial community composition at all four depth horizons between the grassland and shrub-encroached soils. In addition, we found that shrub encroachment was associated with a significant drop in pH, which might be driven by the root exudates and ‘fertile island’ effect (i.e. accumulation of nutrients in the surface soil surrounding shrubs) (Archer et al. 1995; Eldridge et al. 2011). Thus, changes in bacterial community composition at all four depth horizons are likely due to shrub-induced shifts in soil pH.

Soil physicochemical properties change predictably with depth, which in turn was associated with shifts in soil microbial community structure (Chu et al. 2016). In this study, soil bacterial community composition showed significant turnover across depth profiles in both control and shrub-encroached soils. Soil moisture (SM) showed the strongest correlation with bacterial community composition along the soil depth gradient within both control and shrub-encroached sites, confirming that SM may be the dominant factor driving shifts in bacterial community composition along soil depth profiles (Angel et al. 2010; Yuan et al. 2014; Zhang et al. 2014).

This work helps to build a more complete picture of how shrub encroachment affects belowground bacterial communities in grassland ecosystems. However, there were certain limitations in this study. We did not explore how grass-associated bacterial communities within the shrub-encroached sites differed from grass-associated bacterial communities within control sites. Furthermore, our study site did not allow us to look at how belowground communities responded to continuous gradients of shrub density. Lastly, the effect of shrub encroachment on bacterial communities was studied within one shrub-encroached region rather than

across multiple regions, so our different sampling sites should be considered geographic pseudo-replicates. These limitations should be addressed in future studies.

In conclusion, we found that shrub encroachment was associated with significant shifts in soil bacterial community composition at four different soil depths, which appear to be largely mediated by shrub-induced changes in soil pH. Shrub encroachment might be associated with shifts of bacterial life history strategies in the surface soil (i.e., enriching copiotrophic phyla and depleting oligotrophic phyla) and increases in nitrification potential in deeper soil horizons. Overall, grasslands are experiencing greater intensities and frequencies of shrub encroachment worldwide, which will trigger large-scale shifts in aboveground vegetation and belowground ecosystem services across the region.

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Table 1. Correlation coefficients (r) and significance (P) were determined by Mantel tests: comparing differences between samples in bacterial community composition to differences between samples in soil properties. SM: soil moisture; TN: total nitrogen; TP: total phosphorus; DOC: dissolved organic carbon; DON: dissolved organic nitrogen; SIC: soil inorganic carbon; SOC: soil organic carbon. Significant correlations are shown in bold ($P < 0.05$). -: variables which have been excluded due to multicollinearity.

Soil properties	Soil layers								Vegetation types			
	0-10 cm		10-20 cm		20-30 cm		30-50 cm		Grass		Shrub	
	r	P	r	P	r	P	r	P	r	P	r	P
pH	0.72	0.003	0.68	0.004	0.90	0.007	0.91	0.003	0.12	0.201	-0.03	0.587
SM	-0.28	0.999	-0.05	0.585	0.13	0.149	-0.15	0.902	0.61	0.001	0.72	0.001
NH ₄ ⁺	0.44	0.015	0.18	0.163	-	-	0.11	0.167	-0.14	0.839	0.39	0.003
NO ₃ ⁻	-	-	-	-	-	-	-	-	0.23	0.028	0.11	0.122
TN	-0.09	0.660	-	-	-	-	-	-	-	-	-	-
TP	-	-	-	-	-	-	-	-	0.43	0.005	-	-
DOC	-	-	0.28	0.056	0.17	0.085	-	-	-0.02	0.528	-	-
DON	-	-	-	-	0.15	0.063	0.17	0.063	-	-	-	-
SIC	-	-	0.10	0.277	-	-	-	-	-	-	0.03	0.324
SOC	-	-	-	-	-	-	-	-	-0.06	0.653	-	-

Table 2: Variance partitioning analysis of the effects of variables on the soil bacterial community structure performed in the vegan package of R project. SM: soil moisture; TN: total nitrogen; TP: total phosphorus; DOC: dissolved organic carbon; DON: dissolved organic nitrogen; SIC: soil inorganic carbon; SOC: soil organic carbon. -: variables which have been excluded due to multicollinearity.

Soil properties	Explained (%)					
	Soil layer				Vegetation type	
	0-10 cm	10-20 cm	20-30 cm	30-40 cm	Grass	Shrub
pH	20.42	21.33	23.85	31.02	7.82	5.43
SM	8.75	8.31	8.62	8.50	24.55	13.92
NH ₄ ⁺	12.21	9.71	-	8.27	5.30	5.94
NO ₃ ⁻	-	-	-	-	10.82	5.97
TN	10.01	-	-	-	-	-
TP	-	-	-	-	5.62	-
DOC	-	12.82	10.18	-	4.55	-
DON	-	-	9.39	12.67	-	-
SIC	-	6.03	-	-	-	4.76
SOC	-	-	-	-	5.43	-

Figure legends

Figure 1. A schematic of sampling locations.

Figure 2. Bacterial alpha-diversity calculated at a rarefaction depth of 15,000 randomly selected sequences per sample. Bars represent mean; error bars denote standard deviation; asterisk above bars represents significant differences from one-way ANOVA with Tukey's HSD comparisons. *: $P < 0.05$; **: $P < 0.01$. GS: control grassland soil; SS: shrub-encroached soil.

Figure 3. Relative abundances of the dominant bacterial phyla in control and shrub-encroached soils. Bars represent mean; error bars denote standard deviation; asterisks above bars represent significant differences from one-way ANOVA with Tukey's HSD comparisons. *: $P < 0.05$; **: $P < 0.01$. GS: control grassland soil; SS: shrub-encroached soil.

Figure 4. LEfSe analysis of soil bacterial biomarkers associated with vegetation type (a) and soil depth (b). Identified phylotype biomarkers ranked by effect size and the alpha value was < 0.05 . Each filled circle represents one biomarker. a: Cladogram representing the taxonomic hierarchical structure of the phylotype biomarkers identified between two vegetation types; Red, phylotypes statistically overrepresented in grassland soil; green, phylotypes overrepresented in shrub-encroached soil; yellow, phylotypes for which relative abundance is not significantly different between the two vegetation types. b: Cladogram representing the taxonomic hierarchical structure of the phylotype biomarkers identified among four depth gradient; Red, phylotypes statistically overrepresented in 0-10 cm soil; Blue, phylotypes statistically overrepresented in 20-30 cm soil; green, phylotypes overrepresented in 30-50 cm soil; yellow, phylotypes for which relative abundance is not significantly different among soil depth.

Figure 5. Non-metric multidimensional scaling (NMDS) plot showing bacterial community composition in control grassland and shrub-encroached soils of Inner Mongolia, with soil property vectors generated using envfit. GS: control grassland soil; SS: shrub-encroached soil.









