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Research article

Alteration of microbial carbon and nitrogen metabolism within the soil metagenome with grazing intensity at semiarid steppe

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ABSTRACT

Grazing causes changes in microbiome metabolic pathways affecting plant growth and soil physicochemical properties. However, how grazing intensity affects microbial processes is poorly understood. In semiarid steppe grassland in northern China, shotgun metagenome sequencing was used to investigate variations in soil carbon (C) and nitrogen (N) cycling-related genes after six years of the following grazing intensities: G0, control, no grazing; G1, 170 sheep days ha^{-1} $year^{-1}$; G2, 340 sheep days ha^{-1} $year^{-1}$; and G3, 510 sheep days ha^{-1} $year^{-1}$. Taxa and functions of the soil microbiome associated with the C cycle decreased with increasing grazing intensity. Abundances of genes involved in C fixation and organic matter decomposition were altered in grazed sites, which could effects on vegetation decomposition and soil dissolved organic carbon (DOC) content. Compared with the control, the abundances of nitrification genes were higher in G1, but the abundances of N reduction and denitrification genes were lower, suggesting that light grazing promoted nitrification, inhibited denitrification, and increased soil NO_3^- content. Q-PCR further revealed that the copies of genes responsible for carbon fixation (*cbbL*) and denitrification (*norB*) decreased with increasing grazing intensity. The highest copy numbers of the nitrification genes *AOA* and *AOB* were in G1, whereas copy numbers of the denitrification gene *nirK* were the lowest. A multivariate regression tree indicated that changes in C fixation genes were linked to changes in soil DOC content, whereas soil NO_3^- content was linked with nitrification and denitrification under grazing. Thus, genes associated with C fixation and the N cycle affected how C fixation and N storage influenced soil physicochemical properties under grazing. The findings indicate that grazing intensity affected C and N metabolism. Proper grassland management regimes (e.g., G1) are beneficial to the balances between ecological protection of grasslands and plant production in the semiarid steppe.

1. Introduction

The structure and function of grassland ecosystems strongly depend on management, including grazing intensity (Augustine and Frank, 2001; Altesor et al., 2005). Grazing is the most widespread land use on grasslands; it can greatly affect plant species diversity, community composition and productivity, as well as carbon (C) and nitrogen (N) cycling. Excessive grazing reduces plant species diversity and primary productivity and nutrient availability and soil stability (Bai et al., 2012; Zhang et al., 2021). In contrast, moderate grazing can increase the productivity of plant communities by promoting compensatory plant growth and stimulating nutrient recycling. (McNaughton, 1985; Frank and Evans, 1997). Soil microorganisms are the predominant mediators of C and N cycling in grasslands at soil–plant–animal interface. Previous studies primarily examined the effects of grazing intensity upon soil microbial communities' diversity and its makeup (Yang et al., 2019; Fan et al., 2020). However, cumulative effects of grazing intensity, determined by timing, frequency, and duration of grazing, on functions of soil

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microbial communities remain unclear.

Microbes are vital as primary producers and decomposers, and as drivers of biogeochemical cycles, they are the mediators of organic matter decomposition and nutrient storage and turnover (Bardgett and van der Putten, 2014; Zhang et al., 2020a). With grazing, microbial taxa and functions may be affected by livestock trampling, feces and urine deposition, and vegetation removal (Zhao et al., 2017; Yang et al., 2019). Trampling by the livestock affects functions and compositions of soil microbial communities by compacting the soil and altering soil moisture, aeration, and hydraulic conductivity (Yang et al., 2013). For instance, trampling by animals compacts soil and leads to anaerobic conditions that favor microbial denitrification by communities of denitrifiers dominated by *Proteobacteria* (Hou et al., 2018). Feces and urine deposition accelerate N cycling and availability by affecting soil bacterial community composition (e.g., abundances of *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, and *Alphaproteobacteria*) and functions (e.g., inhibiting N fixation or altering nitrification) (Cusack et al., 2009; Han et al., 2018). Removal of aboveground vegetation with grazing changes partitioning of C and N between above- and belowground portions of the plant community, leading to increases in root exudates in the rhizosphere, which include enzymes and soil extractable C (Guitian and Bardgett, 2000; Mueller et al., 2017). With the increase in root exudates under grazing, soil organic C and N undergo various changes, which alter composition and function of soil microbial communities (Liu et al., 2015; Eldridge et al., 2017). Vegetation removal with grazing also reduces litter input into the soil (Yang et al., 2019; Wang et al., 2020). Therefore, microbial mediated C cycling under grazing decreases because of low abundances of genes associated with C fixation, C degradation, and CH_4 production (Yang et al., 2013; Wang et al., 2016a). Notwithstanding, the soil microbiome roles in driving ecosystem functions under particular disturbance or management regimes need to be explored further.

Grazing practices alter C and N cycling in grasslands (Zhou et al., 2007; Hu et al., 2016; Pan et al., 2018). Quantities of aboveground biomass, litter and belowground biomass are affected by grazing intensity (Bai et al., 2012). However, how grazing intensity interacts with biotic and abiotic factors to effect soil microbial communities remains elusive. Furthermore, it is unclear to what extent alterations in soil microbial communities mediate effects of grazing on C and N cycles. Therefore, shotgun metagenomics was used to evaluate the effect that grazing intensity associated with soil microbiome in a semi-arid steppe in northern China. Plant community productivity, soil physic-chemical properties, together with soil microbial groups and their roles were studied to evaluate plant-soil-microbiome interactions at different grazing intensities.

2. Materials and methods

2.1. Study site

This investigation was performed at Chowk Ula in Xilinhot, Inner Mongolia, northern China (44°15′24.4″-44°15′40.7′′N, 116°32′08.2″-116°32′28.3′′E). The average rainfall is 286 mm annually and the average temperature is 1.0 °C. Soils are classified as calcareous chernozems (IUSS WRB Working Group, 2006) or loamy sands. Dominant grass species were *Stipa grandis* P.A. Smirn., *Leymus chinensis* (Trin.) Tzvelev, and *Cleistogenes polyphylla* Keng.

2.2. Field investigation and sampling

An experiment was conducted in a moderately grazed site in a semiarid steppe using a randomized block design with three replicates. There were four levels of grazing intensity by sheep: G0 (no grazing), G1 (low grazing intensity), G2 (medium grazing intensity), and G3 (high grazing intensity), coded 0, 170, 340, and 510 sheep days ha^{-1} $year^{-1}$, respectively, and initiated in May 2014. Every year, grazing was followed from the middle of June to the middle of September. During that time, sheep were kept in the field for the entire day. Each of the 12 experimental plots was 110 m × 124 m.

2.3. Plant and soil sampling

All experimental data were collected in 2019. Before grazing six mobile cages (1.5 m × 1.5 m), were positioned randomly in each plot. Herbaceous plants were cut near the ground in mid-August. Aboveground plant tissue was separated from the previous year's standing litter and surface litter by species. Harvested aboveground plant tissue samples were oven-dried at 65 °C for 48 h and weighed. Total aboveground biomass of all plant species was used to determine aboveground net primary production (ANPP). 0–20 cm of soil samples were collected in mid-August 2019 using a core drill (diameter = 3.5 cm). Thirty soil cores collected per plot, which were composited and mixed to provide a pooled soil sample from each plot. Samples were transferred to the laboratory in an ice-box. Composite soil samples from each plot were separated into three subsamples. One subsample was air-dried, sieved (2 mm), and used to assess soil physicochemical properties; one subsample was stored at 4 °C (for one week) to determine NH_4^+ and NO_3^- concentrations, and one subsample was immediately stored at −80 °C to evaluate taxa and functions of soil microbial communities.

The soil water content (SWC) was evaluated by gravimetric method and oven-dried at 105 °C for 12 h until constant weight. Soil pH was determined (1:1, soil water ratio) using CO_2-free water (Fierer and Jackson, 2006). Soil total N (TN) content was determined after a Kjeldahl digestion (Wang et al., 2016). Dissolved organic carbon (DOC) in soil was measured following the method reported by Jones and Willett (2006). Soil inorganic nitrogen (NH_4^+ and NO_3^-) were determined using 2 M KCl extract (1:5, w: v), and analyzed using a continuous-flow auto-analyzer.

2.4. DNA extraction, library construction, and metagenomic sequencing

The total genomic DNA (1 μg) from the soil samples was extracted in accordance with the manufacturer's instructions via NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA). Through an Illumina HiSeq4000 platform, generated libraries were sequenced, and 150-bp paired-end reads were produced. Illumina HiSeq4000 platform obtained raw data that were preprocessed using Readfq (v8) to gather transparent data for further analysis. Comprehensive datasets concerning metagenomic sequencing is provided within Supplementary Material (Supplement text and Table S1).

2.5. Extraction of total DNA from soils

For quantitative PCR (qPCR) analysis, 50-fold diluted DNA samples were used. Based on similar previously reported methods, qPCR was used to quantify C fixation and the N cycle genes (Supplement text and Table S2).

2.6. Data analyses

Effects of the grazing intensity on vegetation characteristics were analyzed using R v3.0.2R statistical software (R Core Team, 2016). One-way ANOVA was employed to analyze effects of grazing treatments on ANPP, SWC, soil pH, TN, DOC, soil NH_4^+ and NO_3^- content, C fixation genes (*cbbL* and *cbbM*), and N cycling associated genes (*nif*H, AOA, AOB, *nir*K, *nir*S, *nor*B, and *nos*Z). To determine the effects of grazing intensity on soil bacterial community composition, a multivariate PERMANOVA was performed (Zhang et al., 2020a). We used principal coordinate analysis (PCoA) dependent upon Bray–Curtis distances for visualizing the distribution patterns of bacterial communities as a function of grazing intensity (Fan et al., 2021). The Vegan package's multivariate PERMANOVA with 999 permutations (Adonis function) was utilized to

investigate differences in bacterial community composition between pairwise grazing intensities. Linear discriminant analysis of effect sizes (LEfSe) investigated the significant differences between grazing intensities in taxa, functional level-3 KEGG pathways, and functional genes (C and N cycle) (Segata and Huttenhower, 2011). LEfSe analysis via Kruskal-Wallis and Wilcoxon rank frequency tests followed by linear discriminant analysis (LDA) to evaluate the trait effect sizes. Relative abundances of functional categories and taxa were averaged for each treatment, z-score transformed, and visualized as heat maps using the R package 'pheatmap'. A multivariate regression tree (MRT) assessment was done for measuring the key variables affecting C fixation and the N cycle through appling the 'mvpart' package (De'Ath, 2002).

3. Results

3.1. Effects of grazing intensity on plant community biomass and soil properties

Grazing intensity significantly affected ANPP, (SWC, soil pH, and soil TN, DOC, and NO_3^- contents ($P < 0.05$, Table 1). ANPP was significantly lower in G2 and G3 than in G0 and G1 ($P < 0.05$, Table 1). All grazing treatments significantly reduced SWC compared with that in G0 ($P < 0.05$, Table 1). Compared with G0 and G1, soil pH was higher in G2 but lower in G3. The TN content in the soil was highest at moderate grazing (G2) and lowest at heavy grazing (G3). Grazing intensity significantly reduced DOC content in the order heavy and moderate grazing < light grazing < no grazing ($P < 0.05$, Table 1).Grazing did not affect soil NH_4^+ content but altered NO_3^- contents, but light grazing increased, and heavy grazing decreased NO_3^- contents, compared with no grazing ($P < 0.05$, Table 1).

3.2. Effects of grazing intensity on microbial taxa

Principal coordinate analyses showed clear differences in overall microbial community composition among grazing intensities (Adonis test, $P = 0.002$; Fig. 1a). Compared with no grazing, grazing (G1, G2, and G3) significantly reduced relative abundances of *Proteobacteria* and *Thaumarchaeota* but significantly increased those of *Cyanobacteria*, *Firmicutes*, and *Armatimonadetes* ($P < 0.05$, Fig. 1c). Light grazing also increased abundances of *Candidatus Tectomicrobia* (G1), *Planctomycetes* (G1, G2), and *Nitrospirae* (G1) ($P < 0.05$, Fig. 1c), and abundances of *Chloroflexi*, *Planctomycetes*, and *Bacteroidetes* were higher in both G1 and G2 than in G3, and also G0 (except for *Bacteroidetes*) ($P < 0.05$, Fig. 1c).

3.3. Effects of grazing intensity on soil microbial functional characteristics

Composition of functional genes was also markedly variable among grazing intensities (Adonis test, $P < 0.001$; Fig. 1b). Functional genes (>0.3%) were clustered into 21 level-3 categories based on the subsystem KEGG database. Grazing (G1, G2, and G3) had significantly lower relative abundances of genes linked to ABC transporters, purine metabolism, pyrimidine metabolism, carbon fixation pathways in prokaryotes, two-component system, translation, ribosome, methane metabolism, aminoacyl-tRNA biosynthesis, glycine, serine, and threonine metabolism, and amino sugar and nucleotide sugar metabolism than the control ($P < 0.05$, Fig. 1d). In G2, there were greater relative abundances of involved genes in starch and sucrose metabolism than G0 ($P < 0.05$, Fig. 1d).

Table 1
Effects of different grazing intensity treatments on plant community ANPP and soil physicochemical properties in semiarid steppe grassland.

Variable	G0	G1	G2	G3	*F*	*P*
ANPP (g m^{-2})	125.08a	115.32a	91.83b	51.73c	123.04	<0.001
SWC (%)	13.19a	12.33b	12.14b	11.81b	16.81	<0.001
pH	7.27b	7.24b	7.37a	7.16c	17.64	<0.001
DOC (mg kg^{-1})	51.88a	48.04b	41.71c	42.75c	10.84	<0.001
TN (g kg^{-1})	1.47b	1.48 ab	1.56a	1.41b	7.69	<0.001
NH_4^+ (mg kg^{-1})	1.35	1.30	1.32	1.26	0.36	0.783
NO_3^- (mg kg^{-1})	6.69b	7.94a	6.33bc	5.64c	20.54	<0.001

Note: ANPP, plant community aboveground net primary productivity; SWC, soil water content; DOC, soil dissolved soil organic carbon content; TN, soil total nitrogen content.
G0, control; G1, light grazing; G2, moderate grazing; G3, heavy grazing.
Means with different letters are statistically different at $P < 0.05$.

3.4. Changes in carbon metabolism

Relative abundances of 15 KEGG modules associated with C fixation varied among grazing intensities (Fig. 2a and b). Compared with G0, the relative abundances of genes associated with enzymatic functions involved in carbon fixation pathways, including M00170 (C4 dicarboxylic acid cycle), M00171 (reductive pentose phosphate cycle), and M00172 (C4 dicarboxylic acid cycle), and prokaryotic carbon fixation pathways, including M00620 (incomplete reductive citrate cycle), M00374 (dicarboxylate hydroxybutyrate cycle), "M00375 (hydroxypropionate hydroxybutylate cycle), M00376 (3-hydroxypropionate Bi cycle), and M00377 (reductive acetyl-CoA pathway), decreased significantly in G1 and G3 ($P < 0.05$, Fig. 2). Relative abundances of M00168 (Crassulacean acid metabolism: CAM), M00170, and M00377 (reductive acetyl-CoA pathway) were less under G2 than under G0 ($P < 0.05$, Fig. 2).

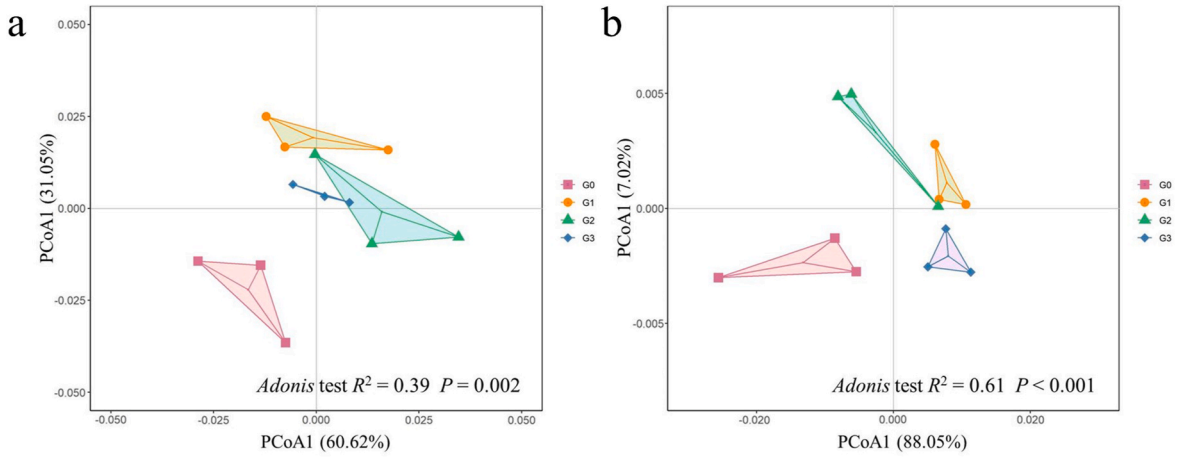
Microbial genera involved in C fixation included *Bradyrhizobium*, *Mycobacterium*, *Hydrogenophaga*, *Rhodopseudomonas*, *Stappia*, *Verminephrobacter*, *Burkholderia*, and *Magnetospirillum* (Fig. 3a). Relative abundances of some of those genera (e.g., *Bradyrhizobium* and *Magnetospirillum*) were lower under grazing than under G0 (Fig. 3a). The highest relative abundance of *Mycobacterium* was in G2 (Fig. 3a). Grazing (G1, G2, and G3) showed lower relative abundances of the functional genes *rbcL*, *rbcS*, *accB*, *accC*, and *pccA* associated with C fixation than the G0 ($P < 0.05$, Fig. 3b). Relative abundance of *oorA* increased by 96.3% in G1, 98.3% in G2, and 120.0% in G3 compared with G0. Relative abundances of *acsA* and *pccB* were lower in G1 and G3 than in G2 ($P < 0.05$, Fig. 3b).

Genera associated with C decomposition were primarily *Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Steroidobacter*, *Novosphingobium*, *Comamonas*, *Sphingobium*, *Gemmobacter*, and *Limnobacter* (Fig. 4a). Relative abundance of *Limnobacter* was lower in G2, that of *Sphingobium* was lower in G1, and those of *Flavobacterium* and *Sphingobium* were lower in G3 than in G0 (Fig. 4a). Additionally, functional genes linked to C degradation were significantly impacted by grazing intensity. (Fig. 4b). Compared with G0, G2 had higher relative gene abundance related to cellobiose, lignin, and chitin degradation, whereas G1 had relative gene abundance was greater for chitin degradation (Fig. 4b).

3.5. Changes in the nitrogen cycle

Of the KEGG modules involved in the N cycle, relative abundances of genes associated with dissimilatory nitrate reduction (M00530) and denitrification (M00529) were lower under grazing than in G0 ($P < 0.05$, Fig. 2c). In contrast, grazing increased relative genes abundances related to complete nitrification (M00804) and nitrification (M00528), with the greatest increases in G1 ($P < 0.05$, Fig. 2c).

Microbial genera involved in the N cycle were primarily *Nitrosopumilus*, *Nitrosomonas*, *Nitrosospira*, *Nitrosococcus*, *Nitrospira*, *Rhizobium*, *Bacillus*, *Rhodobacter*, *Sinorhizobium*, *Alcaligenes*, *Agrobacterium*, *Brucella*, *Pseudomonas*, *Campylobacter*, and *Geobacillus* (Fig. 5a). Relative abundances of *Nitrosomonas*, *Nitrosococcus*, and *Nitrospira* were higher in G1, whereas those of *Nitrosopumilus*, *Alcaligenes*, and *Brucella* were lower than in G0 ($P < 0.05$, Fig. 5a). Relative abundances of *Geobacillus* were significantly higher in G2, whereas those of *Rhizobium* were significantly lower than in G0 ($P < 0.05$, Fig. 5a). Relative *Nitrosopumilus* and



c

	G0	G1	G2	G3	*F*	*P*
Actinobacteria	50.73	49.81	52.39	51.06	1.49	0.247
Proteobacteria	15.34a	12.87b	12.38b	13.58b	5.58	0.006
Chloroflexi	3.69b	4.06a	4.00a	3.76b	6.68	0.003
Acidobacteria	3.26	3.07	3	2.56	3.02	0.054
Verrucomicrobia	1.59	1.95	1.74	1.85	0.83	0.490
Candidatus Rokubacteria	1.31	1.73	1.35	1.61	4.11	0.020
Thaumarchaeota	1.60a	1.03b	0.92b	1.03b	22.04	<0.001
Candidatus Tectomicrobia	0.92b	1.23a	1.03b	1.05b	7.63	0.001
Cyanobacteria	0.96b	1.18a	1.12a	1.14a	7.80	0.001
Gemmatimonadetes	0.92	0.87	0.9	0.85	0.60	0.625
Firmicutes	0.61c	0.69ab	0.70a	0.66b	13.86	<0.001
Planctomycetes	0.44b	0.47a	0.47a	0.42b	9.24	<0.001
Nitrospirae	0.32b	0.38a	0.29b	0.31b	5.37	0.007
Bacteroidetes	0.26ab	0.28a	0.27a	0.25b	6.41	0.003
Armatimonadetes	0.20b	0.24a	0.23a	0.24a	17.33	<0.001

d

	G0	G1	G2	G3	*F*	*P*
ABC transporters	1.40E+00a	1.36E+00b	1.36E+00b	1.34E+00b	4.74	0.012
Purine metabolism	1.25E+00a	1.17E+00c	1.21E+00b	1.16E+00c	10.07	<0.001
Quorum sensing	1.14E+00	1.12E+00	1.10E+00	1.10E+00	1.99	0.102
Oxidative phosphorylation	9.23E-01a	9.06E-01ab	9.29E-01a	8.77E-01b	5.01	0.009
Pyrimidine metabolism	8.70E-01a	7.95E-01c	8.30E-01b	7.85E-01c	11.12	<0.001
Glyoxylate and dicarboxylate metabolism	8.80E-01	8.14E-01	8.57E-01	8.15E-01	9.57	<0.001
Pyruvate metabolism	8.23E-01a	7.90E-01b	8.27E-01a	7.79E-01b	6.70	0.003
Carbon fixation pathways in prokaryotes	7.55E-01a	7.18E-01b	7.08E-01b	7.01E-01b	7.86	0.001
Two-component system	7.86E-01a	7.49E-01b	7.51E-01b	7.45E-01b	8.80	<0.001
Glycolysis / Gluconeogenesis	6.92E-01a	6.80E-01ab	6.97E-01a	6.69E-01b	4.59	0.013
Alanine, aspartate and glutamate metabolism	6.38E-01a	5.80E-01c	6.07E-01a	5.80E-01b	12.55	<0.001
Citrate cycle (TCA cycle)	6.41E-01a	6.13E-01a	6.41E-01a	5.96E-01b	5.87	0.005
Translation; Ribosome	6.42E-01a	5.92E-01bc	6.10E-01b	5.82E-01c	10.02	<0.001
Methane metabolism	6.18E-01a	5.92E-01b	6.12E-01a	5.91E-01b	6.17	0.004
Aminoacyl-tRNA biosynthesis	6.08E-01a	5.72E-01c	5.92E-01b	5.72E-01c	12.04	<0.001
Glycine, serine and threonine metabolism	5.82E-01a	5.22E-01c	5.44E-01b	5.40E-01b	23.86	<0.001
Propanoate metabolism	5.77E-01a	5.45E-01b	5.73E-01a	5.38E-01b	8.39	<0.001
Butanoate metabolism	5.75E-01a	5.44E-01b	5.64E-01a	5.41E-01b	8.53	<0.001
Starch and sucrose metabolism	5.05E-01b	5.06E-01b	5.23E-01a	5.06E-01b	3.78	0.027
Amino sugar and nucleotide sugar metabolism	4.95E-01a	4.69E-01c	4.80E-01b	4.80E-01b	9.61	<0.001
Nitrogen metabolism	3.22E-01a	2.99E-01bc	3.10E-01b	2.96E-01c	9.08	<0.001
Carbon fixation in photosynthetic organisms	3.11E-01a	3.05E-01b	3.03E-01b	3.01E-01b	4.84	0.011

Fig. 1. Effects of grazing intensity (G0: no grazing; G1: 170 sheep days ha^{-1} $year^{-1}$; G2: 340 sheep days ha^{-1} $year^{-1}$; and G3: 510 sheep days ha^{-1} $year^{-1}$) on microbial community structure according to principal coordinate analysis (PCoA) principled on (a) Phyla and (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) (level-3 paths). ANOVA tables showing the effects of grazing on (c) major phyla and (d) level-3 categories undergrazing intensity treatments.

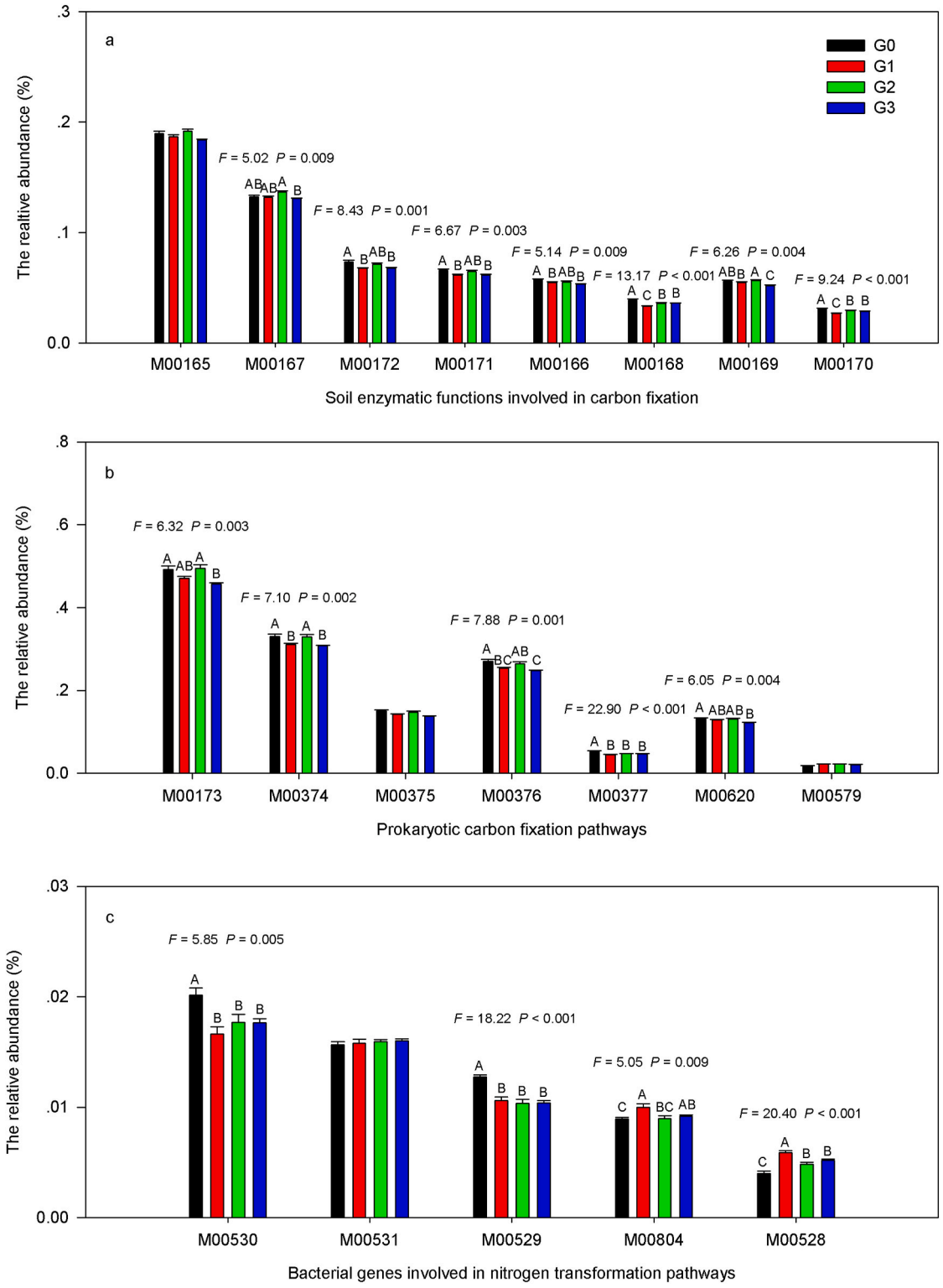
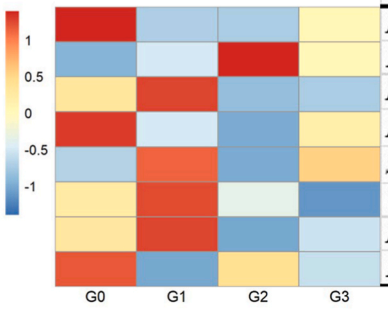


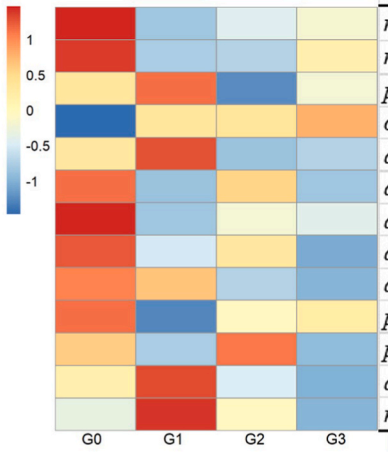
Fig. 2. Relative abundances of Kyoto Encyclopedia of Genes and Genomes (KEGG) modules of genes associated with (a) soil enzymatic functions involved in carbon fixation, "(b) prokaryotic carbon fixation pathways, and (c) bacterial nitrogen transformation pathways in different treatments of grazing intensity. Values are the mean ± SE (n = 3). Soil enzymatic activities involved in carbon fixation: M00165, reductive pentose phosphate cycle (Calvin cycle); M00167, reductive pentose phosphate cycle, glyceraldehyde-3P => ribulose-5P; M00171, reductive pentose phosphate cycle; M00172, C4-dicarboxylic acid cycle, and prokaryotic carbon fixation pathways; M00166, reductive pentose phosphate cycle, ribulose-5P => glyceraldehyde-3P; M00169, Crassulacean acid metabolism (CAM) light; M00168, CAM, dark; and M00170, C4-dicarboxylic acid cycle, phosphoenolpyruvate carboxykinase type. Prokaryotic carbon fixation pathways: M00173, reductive citrate cycle (Arnon-Buchanan cycle); M00374, dicarboxylate-hydroxybutyrate cycle; M00375, hydroxypropionate-hydroxybutylate cycle; M00376, 3-hydroxypropionate bi-cycle; M00377, reductive acetyl-CoA pathway (Wood-Ljungdahl pathway); M00620, incomplete reductive citrate cycle, acetyl-CoA => oxoglutarate; and M00579, phosphate acetyltransferase-acetate kinase pathway, acetyl-CoA => acetate. Bacterial nitrogen transformation pathways: M00530, dissimilatory nitrate reduction: nitrate => ammonia; M00531, assimilatory nitrate reduction: nitrate => ammonia; M00529, denitrification: nitrate => nitrogen; M00804, complete nitrification: ammonia => nitrite => nitrate; M00528, nitrification: ammonia => nitrite; and M00175, nitrogen fixation: nitrogen => ammonia."

a



CO_2 assimilation	G0	G1	G2	G3	*F*	*P*
Bradyrhizobium	3.65E+00a	2.65E+00b	2.64E+00b	3.01E+00b	5.92	0.005
Mycobacterium	1.80E+00b	1.88E+00b	2.20E+00a	1.97E+00b	6.44	0.003
Hydrogenophaga	4.11E-03ab	4.42E-03a	3.72E-03b	3.76E-03b	4.35	0.016
Rhodopseudomonas	2.28E-02a	1.75E-02b	1.60E-02b	1.95E-02ab	6.31	0.003
Stappia	2.34E-03	2.59E-03	2.30E-03	2.51E-03	1.01	0.409
Verminephrobacter	5.11E-04	5.59E-04	4.83E-04	4.46E-04	1.49	0.248
Burkholderia	3.13E-02ab	3.29E-02a	2.91E-02b	2.99E-02b	3.98	0.023
Magnetospirillum	1.76E-02a	1.42E-02b	1.64E-02ab	1.49E-02b	4.21	0.018

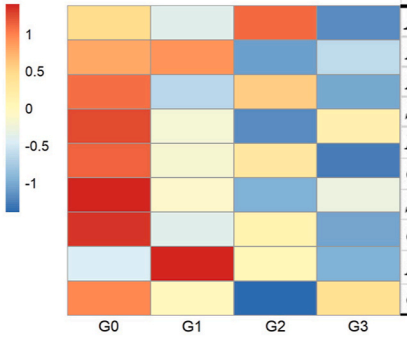
b



C fixation	G0	G1	G2	G3	*F*	*P*
rbcL	4.27E-03a	3.19E-03b	3.39E-03b	3.50E-03b	15.94	< 0.001
rbcS	1.72E-03a	1.36E-03b	1.37E-03b	1.52E-03ab	7.95	0.001
porA	1.72E-03ab	1.86E-03a	1.45E-03ab	1.63E-03b	3.19	0.046
oorA	5.62E-04b	1.10E-03a	1.12E-03a	1.24E-03a	10.67	<0.001
aclB	6.42E-05ab	8.58E-05a	3.74E-05b	4.05E-05b	5.94	0.005
acsA	2.21E-02a	2.00E-02b	2.15E-02a	2.00E-02b	3.68	0.029
accB	6.66E-03a	5.06E-03b	5.51E-03b	5.38E-03b	24.14	<0.001
accC	1.96E-02a	1.74E-02b	1.84E-02ab	1.67E-02b	9.10	<0.001
accD	7.26E-03	7.15E-03	6.73E-03	6.65E-03	3.07	0.051
pccA	5.05E-03a	3.83E-03c	4.45E-03ab	4.58E-03ab	7.53	0.001
pccB	1.86E-02ab	1.65E-02b	1.93E-02a	1.63E-02c	7.91	0.001
accA	1.08E-02	1.09E-02	1.08E-02	1.07E-02	0.19	0.092
nifJ	1.80E-03ab	2.15E-03a	1.86E-03ab	1.67E-03b	3.63	0.031

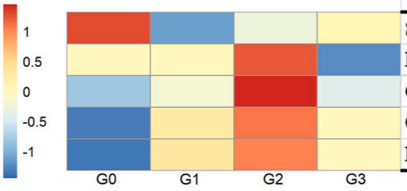
Fig. 3. Relative abundances of (a) microbial genera associated with carbon dioxide assimilation and (b) functional genes involved in carbon fixation in different treatments of grazing intensity. Values are the mean ± SE (n = 3). Heat maps compare relative abundances in different grazing intensities. The key shows z-scores of relative abundances. Analysis of variance (ANOVA) tables indicate significant differences among grazing treatments.

a



C decomposition	G0	G1	G2	G3	*F*	*P*
Acinetobacter	2.63E-03	2.37E-03	2.83E-03	2.12E-03	1.68	0.203
Pseudomonas	4.50E-02	4.51E-02	4.33E-02	4.37E-02	2.73	0.071
Flavobacterium	5.78E-03a	5.09E-03bc	5.58E-03ab	4.94E-03c	4.08	0.021
Steroidobacter	6.73E-02	5.18E-02	4.12E-02	5.56E-02	2.04	0.140
Novosphingobium	8.97E-03	8.86E-03	8.90E-03	8.77E-03	0.14	0.932
Comamonas	1.22E-03	1.13E-03	1.08E-03	1.12E-03	1.18	0.344
Sphingobium	1.01E-02a	8.99E-03ab	9.32E-03ab	8.55E-03b	5.67	0.005
Gemmobacter	1.75E-04	2.01E-04	1.82E-04	1.68E-04	0.65	0.592
Limnobacter	4.64E-04a	3.93E-04a	2.97E-04b	4.24E-04a	6.46	0.003
Gemmobacter	1.75E-04	2.01E-04	1.82E-04	1.68E-04	0.65	0.592

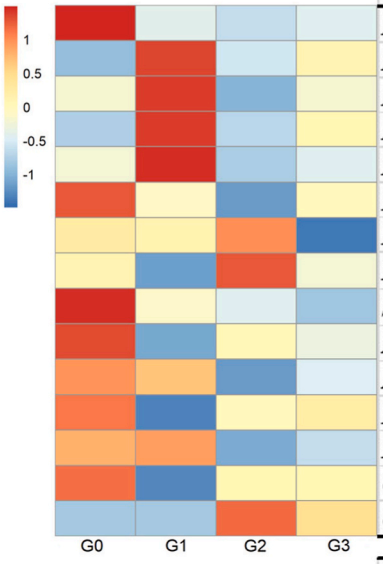
b



C degradation	G0	G1	G2	G3	*F*	*P*
Starch	1.32E-02	1.23E-02	1.26E-02	1.28E-02	2.64	0.078
Hemi-cellulose Degradation	1.74E-02ab	1.75E-02ab	1.90E-02a	1.59E-02b	9.02	0.001
Cellobiose	1.29E-01b	1.32E-01ab	1.38E-01a	1.31E-01b	3.42	0.037
Chitin Degradation	3.35E-03b	3.91E-03a	4.20E-03a	3.81E-03ab	6.64	0.003
Lignin	6.14E-02b	6.56E-02ab	6.74E-02a	6.48E-02ab	7.09	0.002

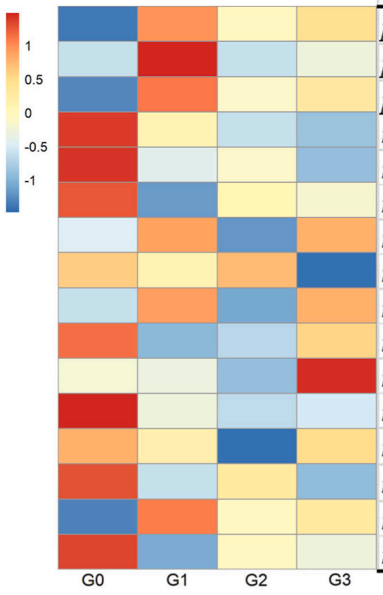
Fig. 4. Relative abundances of (a) microbial genera associated with carbon decomposition and (b) functional genes involved with carbon degradation indifferent grazing intensity treatments. Values are the mean ± SE (n = 3). Heat maps compare relative abundances in different grazing intensity treatments. The key shows z-scores of relative abundances. Analysis of variance (ANOVA) tables indicate significant differences among grazing treatments.

a



N cycle	G0	G1	G2	G3	*F*	*P*
Nitrosopumilus	3.96E-03a	2.51E-03b	2.33E-03b	2.49E-03b	23.34	<0.001
Nitrosomonas	2.08E-02c	2.44E-02a	2.14E-02bc	2.24E-02b	8.64	0.001
Nitrosospira	1.40E-02	1.50E-02	1.35E-02	1.40E-02	2.41	0.097
Nitrosococcus	3.83E-02b	4.61E-02a	3.86E-02b	4.14E-02b	7.02	0.002
Nitrospira	1.06E-01b	1.24E-01a	1.00E-01b	1.04E-01b	5.89	0.005
Rhizobium	6.78E-02a	6.23E-02ab	5.79E-02b	6.26E-02ab	4.33	0.017
Bacillus	5.00E-02ab	4.98E-02ab	5.11E-02a	4.76E-02b	3.18	0.046
Rhodobacter	3.44E-03ab	3.14E-03b	3.73E-03a	3.36E-03ab	3.49	0.035
Sinorhizobium	2.21E-02	2.10E-02	2.08E-02	2.05E-02	2.27	0.112
Alcaligenes	3.03E-04a	1.39E-04c	2.16E-04ab	1.92E-04bc	8.62	0.001
Agrobacterium	4.17E-03	4.10E-03	3.66E-03	3.83E-03	2.74	0.071
Brucella	9.50E-04a	6.52E-04b	8.12E-04a	8.41E-04a	4.08	0.021
Pseudomonas	4.50E-02	4.51E-02	4.33E-02	4.37E-02	2.73	0.071
Campylobacter	1.76E-04	1.30E-04	1.56E-04	1.56E-04	1.09	0.378
Geobacillus	2.59E-03b	2.59E-03b	2.95E-03a	2.82E-03ab	4.25	0.018

b



Genes	G0	G1	G2	G3	*F*	*P*
pmoA-amoA	1.82E-03c	3.00E-03a	2.49E-03bc	2.75E-03ab	26.00	<0.001
pmoB-amoB	1.50E-03b	1.87E-03a	1.50E-03b	1.56E-03ab	4.72	0.012
pmoC-amoC	1.06E-03c	1.48E-03a	1.26E-03bc	1.34E-03ab	10.01	<0.001
narG	3.40E-03a	3.07E-03b	2.88E-03b	2.81E-03b	4.83	0.011
narH	1.97E-03a	1.62E-03b	1.67E-03b	1.52E-03b	5.90	0.005
nirB	1.10E-02	8.61E-03	9.82E-03	9.57E-03	6.68	0.003
nirD	2.39E-03	2.52E-03	2.32E-03	2.51E-03	0.40	0.755
nrfA	7.07E-04	6.78E-04	7.13E-04	5.89E-04	2.14	0.127
nrfH	3.36E-04	3.72E-04	3.25E-04	3.69E-04	0.48	0.702
nasB	1.59E-04a	7.40E-05b	8.53E-05b	1.35E-04ab	4.14	0.02
narB	3.83E-04b	3.79E-04b	3.51E-04b	4.64E-04a	3.10	0.05
napA	1.08E-03a	8.25E-04b	7.66E-04b	7.89E-04b	7.98	0.001
nirK	3.07E-03a	2.87E-03a	2.35E-03b	2.97E-03a	12.50	<0.001
norB	3.15E-03a	2.12E-03c	2.60E-03b	1.94E-03c	14.91	<0.001
norC	1.14E-04b	2.48E-04a	1.84E-04ab	2.03E-04a	5.09	0.009
nosZ	4.00E-05	1.68E-05	2.65E-05	2.43E-05	2.34	0.104

Fig. 5. Grazing intensity influences upon presence of N cycle related the abundance of (a) microbial genera, and (b) functional genes in different grazing intensity treatments. Values are the mean ± SE (n = 3). Heat maps compare relative abundances in different grazing intensity treatments. The key shows z-scores of relative abundances. ANOVA tables indicate significant differences among grazing treatments.

Alcaligenes abundances were greater in G3 than in G0 ($P < 0.05$, Fig. 5a). Relative abundances of *Rhodobacter*, *Brucella*, and *Geobacillus* were greater in G2 than in G1 ($P < 0.05$, Fig. 5a).

Changes in grazing intensity affected the number of genes that were associated with the N cycle (Fig. 5b). The relative abundances of the nitrifying enzymes *amoA*, *amoB*, and *amoC* were greater in G1 in comparison with G0 ($P < 0.05$, Fig. 5b). The abundances of the dissimilatory nitrate reduction genes *narG*, *narH*, and *napA* were significantly lower in the three grazing treatments than in G0 in comparison with the other treatments ($P < 0.05$, Fig. 5b). The relative abundance of *nasB*, which is involved in assimilatory nitrate reduction, was lower in G1 and G2 than in G0 ($P < 0.05$, Fig. 5b), whereas *narB* was more abundant in G3. Compared with G0, the relative abundances of *nirK* and *norB* were significantly lower in G2, whereas the relative abundance of *norC* was significantly greater in both G1 and G3 ($P < 0.05$, Fig. 5b).

3.6. Grazing intensity influences functional genes

According to qPCR results, the *cbbL* gene copy number was significantly lower with grazing (G1, G2, and G3) than without grazing (G0) ($P < 0.05$, Fig. 6a). In comparison with G0, the copy numbers of the *AOA* and *AOB* genes were higher in G1 (Fig. 6d and e), whereas the *nirK* gene copy number was lower in G1 ($P < 0.05$; Fig. 6f). In comparison with G0, the *AOA* and *AOB* genes copy numbers were greatly higher in G3 ($P < 0.05$, Fig. 6d and e), whereas copy numbers of the *norB* gene (Fig. 6h) were significantly lower in the three grazing treatments ($P < 0.05$).

3.7. Variables correlated with soil properties and composition of functional genes

Soil DOC was the primary factor accounting for variance in C fixation genes by multivariate regression tree analysis (Fig. 7a). Soil NO_3^- was a robust indicator of variations in N cycling genes (Fig. 7b).

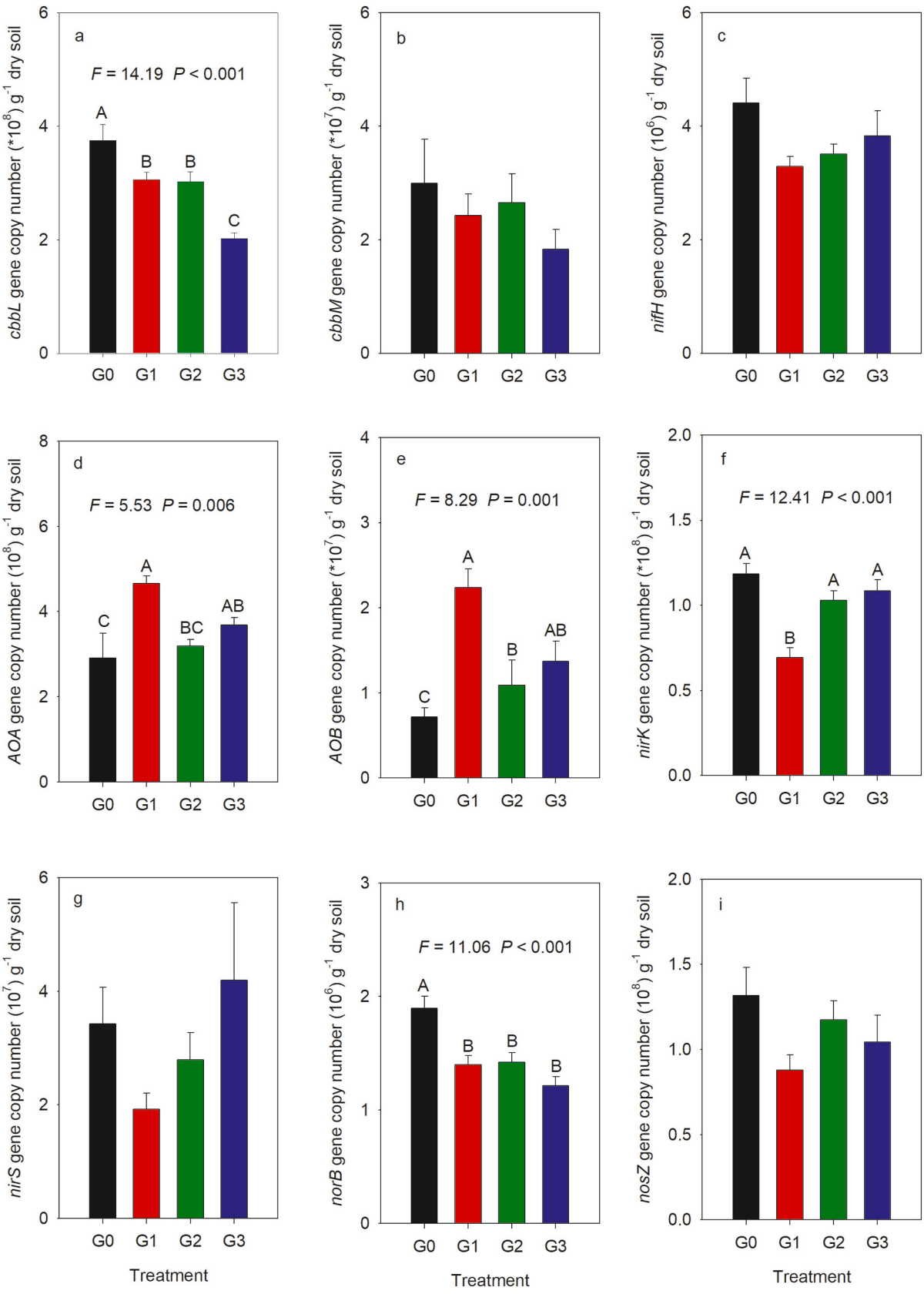


Fig. 6. Effects of grazing intensity on copy number (mean ± SE, n = 3) of the carbon fixation genes (a) *cbbL* and (b) *cbbM*, and the N cycle genes (c) *nifH*, (d) AOA-*amoA*, (e) AOB-*amoA*, (f) *nirK*, (g) *nirS*, (h) *norB*, and (i) *nosZ*.

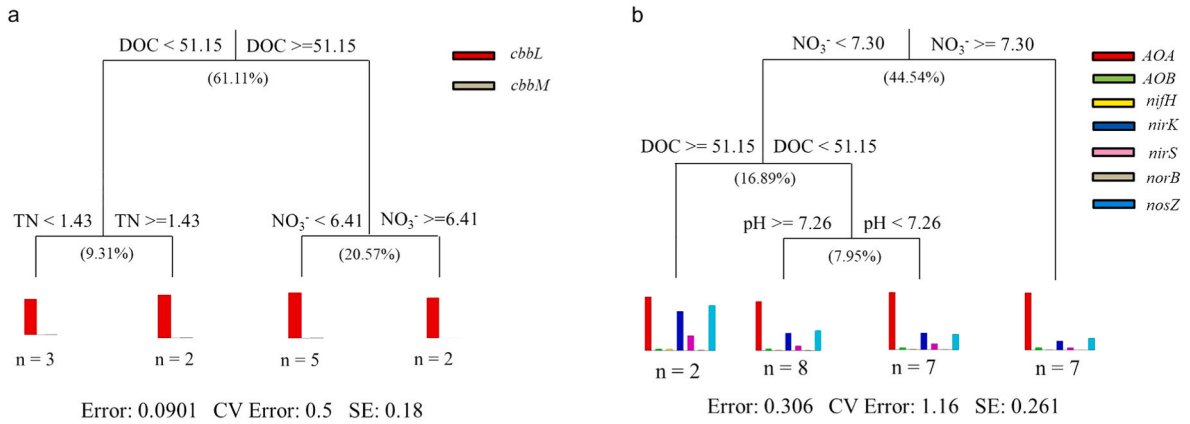


Fig. 7. Multivariate regression tree analysis of (a) carbon fixation genes (*cbbL* and *cbbM*) and soil physicochemical variables and (b) N cycle genes (*nifH*, AOA-*amoA*, AOB-*amoA*, *nirK*, *nirS*, *norB*, and *nosZ*) and soil physicochemical variables. The number of soil samples selected for evaluation is indicated below the bar graphs. The number of soil samples selected for evaluation is indicated below the bar graphs. Soil properties include pH, soil dissolved organic carbon content (DOC), soil total nitrogen content (TN), soil NH_4^+ content (NH_4^+), and soil NO_3^- content (NO_3^-).

4. Discussion

4.1. Effects of grazing on structure of soil microbial communities

Several complementary concrete evidences suggest that grazing intensity significantly affects soil microbial community composition and function in semiarid steppe. First, microbial communities differed in composition at different grazing intensities. Although soil microbial community composition is correlated with soil fertility, the strength of the relationship decreases with increasing grazing intensity (Chen et al., 2020; Zhang et al., 2020b). While grazing intensity increases, trampling by livestock and the removal of vegetation increase, reducing plant cover, plant litter, and soil aggregate structure, thus inhibiting soil microbial activity and growth. Second, several important microbial taxa were found to be sensitive to grazing. In the present study, *Candidatus Tectomicrobia*, *Planctomycetes*, and *Nitrospirae* were more abundant under light grazing, whereas *Cyanobacteria*, *Firmicutes*, and *Armatimonadetes* were more abundant under moderate and heavy grazing than in the non-grazed control. Such taxa-specific responses to different grazing intensities determine the alterations in soil microbiome structure under grazing, with consequent effects on microbial functions and metabolic pathways. In addition, gene associated with C metabolism, including those affecting C fixation pathways in prokaryotes, methane metabolism, and C fixation in photosynthetic organisms, were more abundant with grazing, whereas those associated with N metabolism were significantly less abundant (Fig. 2). The positive relationships between metagenomic metabolic pathways and soil properties are consistent with those reported in a previous study of cross-biome metagenomic analyses (Fierer et al., 2012), in which relative genes abundances related to nutrient cycling and the degradation of plant-derived organic compounds were highest in communities with high fertility. In the present study, plant aboveground biomasses decreased with increasing grazing intensity, indicating a decrease in nutrient recycling and return to the soil via plant litter on the long-term grazing. Moreover, MRT analysis showed that DOC and nitrate levels caused alterations in microbial functional genes implicated within soil C and N cycling. Thus, grazing intensity, particularly moderate and heavy grazing, primarily affected soil microbial community structure and metabolic pathways because of effects on plant biomass, soil DOC, and soil nitrate contents.

4.2. Grazing on affects the carbon cycle

Grazing decreased soil microbial C metabolism in this study, as indicated by decreases in gene abundance and KEGG modules of C fixation (C fixation pathways in prokaryotes and C fixation in photosynthetic organisms). The results support the findings of Liu et al. (2018). Changes in the quantity and quality of plant carbon inputs are likely the cause of such outcomes (Becker et al., 2017). Plant aboveground biomass decreased with increasing in grazing intensity, which likely led to lower nutrient flux from plant to soil, further decreasing C cycling in soil.

In this study, changes in the composition of certain microbial groups were probably crucial for changes in biogeochemical processes. Several microbial taxa related to C metabolism were sensitive to grazing. Of microbes associated with C fixation, *Rhodopseudomonas* and *Bradyrhizobium* were low under grazing but were not sensitive to differences in grazing intensity, whereas *Hydrogenophaga* and *Burkholderia* were low under moderate and heavy grazing. Similarly, microbes related to C decomposition also showed taxa-specific responses to grazing intensities. *Flavobacterium* and *Sphingobium* were low under light or heavy grazing, whereas *Limnobacter* was only low under moderate grazing. Changes in soil microbiome functions (CO_2 assimilation and C decomposition) can result in changes in soil C storage (Tian et al., 2019). Thus, soil microbial taxa sensitive to differences in grazing intensity should be examined because of potentially crucial roles in determining grazing effects on soil microbial C metabolism.

Differences in grazing intensity cause alterations in productivity of plant community and soil fertility that lead to changes in C cycling (Yang et al., 2013; Qu et al., 2016). Functional genes alterations indicate that resource availability and microbial interactions also affect the soil C cycle (Dong et al., 2020). Here, relative genes abundances involved in C fixation (*rbcL*, *rbcS*, *acsA*, *accB*, *accC*, and *pccA*) decreased with increasing grazing intensity. In contrast, relative abundances of genes associated with C decomposition were highest under moderate grazing, including those involved in hemicellulose degradation, cellobiose transport, cellulose degradation, chitin degradation, and lignin degradation. The decrease in C fixation and increase in C decomposition affected the C cycle, and as a consequence, C storage was low under high grazing intensity. Multivariate regression tree analysis also revealed that DOC, NO_3^-, and TN were the key variables involved in changes in C fixation genes. Variations in C fixation genes (*cbbL* and *cbbM*) might be because of the variations in DOC, NO_3^-, and TN in the context of grazing. Therefore, the results of this study suggested that nutrient cycling and organic plant matter degradation are associated with lower relative abundances of genes in a low fertility environment under intensive grazing.

4.3. Effects of grazing on grazing intensity the nitrogen cycle

Changes in soil N content with increasing grazing intensity were

associated with changes in N metabolism. At the KEGG module level, G1 had the highest relative gene abundances linked with complete nitrification (M00804) and nitrification (M00528), whereas the least relative genes abundances were associated with denitrification (M00529) and nitrate reduction (assimilatory, M00530), resulting in the highest soil NO_3^- content in G1. In G1, the relative genes abundances implicated within denitrification (*narG*, *narH*, *nasB*, *narB*, *napA*, *nirK*, and *norB*) decreased, whereas the relative abundances of genes implicated within nitrification (pmoA-*amoA*, pmoB-*amoB*, and pmoC-*amoC*) increased. Those responses likely resulted from the increase in soil NO_3^- content under light grazing. The qPCR results also indicated that light grazing (G1) significantly increased genes associated with nitrification (*AOA* and *AOB*), which is a result consistent with that of previous reports (Tracy and Frank, 1998; Xie et al., 2014) and indicates an increase in animal excretions (Yang et al., 2013). Relative abundances of *AOA* and *AOB* were higher under G1 than under G0, suggesting a shift toward increased nitrification under light grazing. In addition, abundances of several bacterial genera were correlated with decreases in abundances of *AOA* and *AOB* genes, including *Nitrosomonas*, *Nitrosococcus*, *Nitrososphaera*, and *Nitrospira*. Those genera can proliferate with disturbance and might be important in affecting changes in nitrification under light grazing. In contrast, a decrease in denitrification in G1 was indicated by decreases in abundances of *nirK* and *norB*. Lower soil NO_3^- contents under G2 and G3 than in G1 were also attributed to decreases in abundances of genes associated with nitrification (*AOA* and *AOB*) and increases in abundance of a gene linked with denitrification (*nirK*) in both G2 and G3. Alternatively, changes in the soil physicochemical properties of NO_3^-, DOC, and pH could mediate shifts within abundances of *AOA*, *AOB*, *nirK*, and *nosZ* genes (Zhang et al., 2012; Song et al., 2019). Low abundance of *nirK* was linked to decreases within abundances of *Rhodobacter*, *Alcaligenes*, and *Brucella*, and *norB* was correlated with *Nitrosomonas* and *Nitrosococcus*, which are likely candidates for denitrifiers in the system.

4.4. Management implications

Grazing management has the potential to affect microbial carbon and nitrogen metabolism because of changes in plant productivity and soil properties with different levels of grazing intensity. Intensive grazing (G3) reduced the plant community productivity, which resulted in reduced nutrient flux from plants to soil, thereby reducing C and N cycling. An appropriate level grazing, such as in G1, could be sustainable, because plant community productivity and soil nutrient levels were maintained while microbial activity increased. The different responses of microbial carbon and nitrogen metabolism after six years indicated that light grazing (G1) may be might be a suitable management system for long-term sustainable development of semiarid steppe.

5. Conclusions

Changes in plant community aboveground net primary productivity and soil properties under grazing can alter the metabolic potential of microorganisms in biogeochemical cycling, with direct implications for soil C and N cycling and storage. Increases in genes associated with C fixation and decreases in those associated with C decomposition suggest that grazing intensity changed the C cycle. G1 showed increases in genes associated with nitrification (*AOA* and *AOB*) but decreases in those associated with denitrification (*nirK* and *norB*), suggesting that N cycling processes were affected under grazing. Differences in functional genes can modulate grassland ecosystem functions, such as C sequestration and soil fertility, under different grazing intensities.

The study had some limitations. First, six years might not be a sufficient period to study microbial C and N metabolism under grazing. In addition, individual ecosystem functions were not examined further to determine the appropriate level of grazing management. Future research is needed to determine the extent to which changes in soil microbial communities mediate effects of different grazing intensities on ecosystem functions.

CRediT authorship contribution statement

Zhen Wang: and, and, and. **Kai Tang:** and, drafted the manuscript, and. **Paul C. Struik:** and. **Muhammad Nadeem Ashraf:** critically revised it for important intellectual content. **Tongrui Zhang:** and. **Yanning Zhao:** and. **Riliga Wu:** collected and analyzed the data. **Ke Jin:** conceived and designed the study, and, supervised the project. All authors read and approved the final manuscript. **Yuanheng Li:** and, and, and.

Declaration of competing interest

The authors declare that they have no competing interest.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.119078.

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