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Recovery through proper grazing exclusion promotes the carbon cycle and increases carbon sequestration in semiarid steppe

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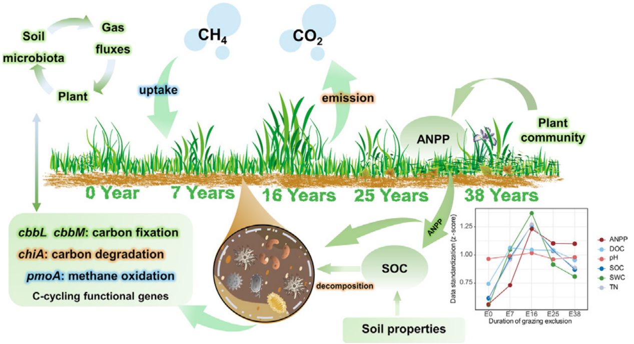
HIGHLIGHTS

- Exclusion duration affected soil carbon cycling in semiarid steppe.
- Grazing exclusion, especially when lasting 16 years, is beneficial for restoring degraded grassland.
- Aboveground net primary productivity (ANPP) drove C-cycle functional genes and microbial communities.
- ANPP, soil organic carbon, and *pmoA* modulated CO_2 emission and CH_4 uptake rates.

GRAPHICAL ABSTRACT



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ABSTRACT

Grazing exclusion changes soil physical-chemical characteristics, rapidly affects microbial community composition and function, and alters biogeochemical processes, e.g., carbon (C) cycle, over time. However, the temporal dynamics of CO_2 emission and CH_4 uptake during grassland restoration chronosequences remain poorly understood. We investigated soil CO_2 emission and CH_4 uptake, the genes related to CO_2 and CH_4 production and reduction (*cbbL*, *cbbM*, *chiA*, and *pmoA*), and associated microbial communities under different durations of grazing exclusion (0, 7, 16, 25, and 38 years) to reveal the mechanisms and potential of soil CO_2 emission and CH_4 uptake in a semi-arid steppe. The results showed that a proper exclusion period could significantly improve soil physical-chemical conditions, vegetation community, and soil C-cycling. The abundance of C-cycling functional genes (*cbbL*, *cbbM*, *chiA* and *pmoA*), CH_4 uptake and CO_2 emission rates showed a single-peak pattern with increasing duration of grazing exclusion, peaking at 16 years and then decreasing in the period between 25 and 38 years, indicating that the effect of exclusion weakened when the exclusion period was too long. The changes in C-cycling functional genes and microbial communities are primarily influenced by aboveground net primary productivity (ANPP), and are associated with CO_2, CH_4, soil water content (SWC), and soil organic carbon (SOC). Structural equation modeling showed that increases in SOC content and *pmoA* abundance caused by an increase in ANPP accelerated CO_2 emission and CH_4 uptake rates, respectively. Our results provide valuable insights into the critical role of grazing exclusion in promoting grassland restoration and carbon sequestration, and have potential implications for sustainable land management practices.

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1. Introduction

Improper grassland management practices, especially overgrazing, have had serious negative impacts on the structure and function of grassland ecosystems (Bai et al., 2012). About 50 % of the world's grasslands have experienced degradation (Ebrahimi et al., 2016). It has been proven that grassland degradation and land use change (Zhou et al., 2008) due to overgrazing has changed the composition of plant community (Jing et al., 2014), reduced species diversity and primary productivity (He et al., 2022), and decreased soil organic carbon and nutrient availability (Wang et al., 2022a). These effects may reduce greenhouse gas fluxes, thus diminishing the function of grasslands as sinks of CH_4 and sources of CO_2 emissions (Ma et al., 2018). Thus, it is imperative to address the issue of grassland management practices and implement measures to restore degraded grassland ecosystems. Since 2003, China has implemented a program to recover degraded grasslands by excluding grazing through fencing (Jiang et al., 2006). Grazing exclusion has achieved remarkable results by triggering self-healing capacities (Listopad et al., 2018), which not only revitalizes the vegetation but also affects subterranean microbiota (Wang et al., 2022b). In this process, soil microorganisms play an crucial role in storing carbon (C) in the soil, especially those with functional genes related to carbon cycling (Liu et al., 2016).

Soil and vegetation biomass are the major C pools in the ecosystem. Plants are responsible for fixing carbon from the atmosphere, but soil organisms determine the turnover of the soil organic matter pool (Crowther et al., 2019). Soil microbes decompose plant substrates through “ex vivo modification” and accelerate carbon accumulation from plant sources and “in vivo turnover” of small molecular plant substrates, thus contributing microbial carbon sources to the soil (Liang and Zhu, 2021). Soil microbiota are generally regarded as a means to support soil biological functions, regulate vegetation succession, and accelerate vegetation restoration (Cheng et al., 2016). Subterranean microorganisms are involved in a variety of C-cycling processes, such as carbon fixation, methane metabolism and carbon degradation (Deng et al., 2014), and play an important role in steppe by limiting CO_2 emissions to the atmosphere. For instance, the Calvin cycle is the main pathway of carbon sequestration and the enzyme that plays a major role is ribulose bisphosphate carboxylase (Rubisco) (Berg, 2011), which is encoded by functional genes *cbbL* and *cbbM* (Liu et al., 2016). Chitin represents a common organic polymer in the soil and is degraded by chitin degrading enzymes (Turner et al., 2019). The *chiA* chitin hydrolase gene is related to the mineralization process in the soil, and the mineralization of precipitated organic material is the main driver of the initial soil C and nitrogen (N) turnover (Brankatschk et al., 2011). Functional genes involved in the Calvin cycle for CO_2 fixation (*cbbL* and *cbbM*) (Liu et al., 2016), and the decomposition of organic matter using chitinases (*chiA*) (de Menezes et al., 2015), are present in bacterial phyla *Actinobacteria* and *Proteobacteria*. Microorganisms respond to soil carbon dynamics by changing their community composition, regulating the abundance of microbial functional populations, and strengthening nutrient cycling (Zhou et al., 2011). Elevated CO_2 is thought to be associated with abundance of photosynthetic bacteria and functional genes involved in CO_2 assimilation (Shi et al., 2020). The increased abundance of functional genes reflects the decomposition and transformation of nutrients by microorganisms, and the soil nutrient content increases, which means the increase of resources available to microorganisms (He et al., 2020). Specifically, 58 % of the CO_2 released by soil respiration comes from soil microbial action, with the remainder coming from plants and their litter (Bowden et al., 1993). CH_4 consumption is divided into aerobic oxidation of methane and anaerobic oxidation. *PmoA* functional gene is mainly responsible for encoding the β subunit of pMMO protein in methane monooxygenase (Mmo), which participates in the CH_4 oxidation process and has been widely used to detect methane oxidizing flora in the environment (Li et al., 2020). The activity of transcribed genes is related to changes in the abundance and composition of active methanotrophs and CO_2 production (Liu et al., 2022). The rate of CH_4 uptake is largely determined by the physical-chemical properties of the soil (Chen et al., 2019) and soil cluster (USCα and γ) proportions and abundance of *pmoA* transcripts (Täumer et al., 2022). During grassland enclosure, soil aeration improves due to reduced trampling by livestock. Aerobic ventilation is the key to improve the activity of methanotrophs and the rate of CH_4 uptake (Abichou et al., 2015). Jang et al. (2006) studied the effect of soil nitrogen on the rate of CH_4 uptake and concluded that inorganic nitrogen, especially ammonium nitrogen, could inhibit the oxidation rate of methanotrophic bacteria. With the prolongation of the enclosure, the soil moisture content increases, thereby affecting the diffusion of CH_4 into the soil, leading to a decrease in CH_4 uptake (Chen et al., 2011). Furthermore, atmospheric CH_4 is mainly oxidized by methanotrophs containing *pmoA* functional genes under aerobic conditions and this serves as the sole biological sink for CH_4 uptake (Shrestha et al., 2012). In general, environmental variables alter CH_4 oxidative uptake rates either through their effects on methanotrophic communities or by affecting soil properties in grassland ecosystems (Kou et al., 2017).

Previous studies have mainly focused on short-term exclosure, and long-term time series investigations are limited. By conducting long-term grassland restoration monitoring of vegetation and soil under similar soil and climatic conditions, we studied the effects of exclosure recovery in a typical grassland in Inner Mongolia on soil, C-cycling functional gene microbial communities, CH_4 uptake, CO_2 emissions, and the interaction mechanisms among them. We hypothesized that 1) Soil C storage can recover following removal of grazing pressure by improving soil nutrient status and promoting rapid recovery of the vegetation; 2) Grazing exclusion affects the expression of C-cycling functional genes by changes the composition of C-cycling microbial communities; 3) Plant characteristics and soil properties are the main controlling factors of CO_2 emission and CH_4 uptake by regulating the soil microbial community associated with C-cycling functional genes.

2. Material and methods

2.1. Description of the study area

The study area was located at a long-term grassland ecological research station (E 116°40′-116°44′, N 43°33′-43°35′) (Table S1) in Inner Mongolia, Northern China. The climate is typical of a continental, semiarid climate. The mean annual temperature is 0.9 °C, and the average annual precipitation is 344 mm (2021). The maximum monthly mean temperature in August is 19 °C. The soil type of the study site was chestnut soil, and the soil texture was sandy soil. The total carbon content of the soil ranged from 8.92 to 11.43 g kg^{-1} (Zhong, Li et al. 2018) and microbial biomass carbon content was between 118 and 317 mg kg^{-1} (He, Qi et al. 2013).

2.2. Experimental design and sampling

Four grasslands with different durations of grazing exclusion were selected on August 2021. Grazing has been prohibited at these sites since 1983, 1996, 2005, and 2014, corresponding to grazing prohibition for 38 (E38), 25 (E25), 16 (E16), and 7 years (E7), respectively. A long-term grazing site (2.25 sheep ha^{-1}) served as the reference (E0). Six plots of 20 m × 20 m were established in each of these five grazing and exclosure treatment areas for replicated assessment of vegetation and soil characteristics. Three replicated soil subplots (1 × 1 m) were randomly selected for each plot. Three soil samples (0–10 cm) were collected at each subplot with an 8-cm diameter soil drill, and then mixed into a composite sample; sampling was at least 1 m away from the edge of the subplot to avoid edge effects. These composite soil samples were sieved through a 2-mm mesh and separated into two parts. Both samples were initially stored in a cooler containing dry ice, and promptly transported to the laboratory. The samples intended for soil microbial analysis were preserved under −80 °C conditions, while those intended for analysis of soil physicochemical properties were preserved at 4 °C. Three 1 × 1 m subplots were randomly established within each plot, with a total of 6 × 5 = 30 plots, for the measurement of vegetation height, coverage and the number of species of each plant in each quadrat. Then the aboveground parts of each plant were collected according to the plant species. All samples were collected within one day. In short, we had 30 composite soil samples and 30 plant samples obtained from five plots in each of five grasslands with different durations of grazing exclusion, with six replicates for soil samples and plant samples. The collected fresh plant samples were dried in an oven for 48 h (at 65°) and then weighed. Aboveground net primary productivity ANPP is the total aboveground biomass of all plant species in the subplot.

To distinguish the dominance degree of plant species in the community, the relative importance value was used as a comprehensive measurement index of species (Xu et al., 2021), with the relative importance value calculated as (relative coverage + relative height + relative abundance + relative biomass) / 4.

During the entire growing season from May to October, we employed static chamber-based gas chromatography (GC) to collect CH_4 and CO_2 fluxes from six replicate sampling plots across five enclosed grasslands. The sampling chamber was made of colorless organic glass plate with a length and width of 0.65 m and a height of 0.90 m. On site, a 100-ml gas syringe was used to extract gas for 3 times from a closed sampling box embedded in the soil of the sampling site every 10–15 min. The extracted gas sample was transferred to an air bag and taken back to the laboratory for gas chromatography analysis (Yue et al., 2022). Sampling occurred during the growing season before soil sampling. CH_4 uptake rate and CO_2 emission rate were calculated by the following equation (Dong, Zhang et al. 2000):

$$F = \rho \times \frac{V}{A} \times \frac{\Delta C}{\Delta T}$$

where F is the gas flux (mg CO_2 m^{-2} h^{-1}; mg CH_4 m^{-2} h^{-1}); ρ represents gas density (mg m^{-3}); V and A represent volume (m^3) and cover area (m^2) respectively; ΔC is the rate of change of gas concentration per unit time; ΔT represents the time interval. A positive CO_2 value represents emissions from the soil, and a negative CH_4 value represents uptake from the atmosphere into the soil.

2.3. Analysis of soil physicochemical properties

Soil water content (SWC) of each soil sample plot was measured by a portable time-domain-reflectometry (TDR) probe (HH2, Delta-T Devices, Cambridge, UK). Soil pH was determined by a pH meter (Mettler Toledo, Switzerland) measuring 1:2.5 (weight/volume) soil suspensions that were produced by mixing the soil with deionized water. The potassium dichromate oxidation spectrophotometric method was used to measure soil organic C content. The soil total nitrogen (TN) content was determined using the VARIO EL III analysis system (Elementar, Germany). The soil was mixed with distilled water and shaken well for 1 h. The supernatant was poured into a 0.45-micron filter for vacuum filtration, and then the DOC was measured by a TOC analyzer (Liu, Yang et al. 2021).

2.4. Quantitative PCR

The number of bacterial functional genes was quantitatively assessed by real-time fluorescent quantitative PCR (ABI7900, Applied Biosystems Inc., USA). PCR reaction system (10 μL) contained 5.0 μL Power qPCR PreMix (Genecopoeia), upstream and downstream primers 0.25 μL each, DNA template 1 to 10 ng and 4 μL Milli-Q water. Then the standard curve (Tang, Yu et al. 2018) and the number of soil carbon sequestration functional genes were determined. Specific primers for target genes are shown in Table S2. Amplification efficiencies were 92 % for *cbbL*, 97 % for *cbbM*, 98 % for *chiA* and 99 % for *pmoA*, respectively.

2.5. Soil DNA extraction and MiSeq sequencing

DNA was extracted from 2-g soil samples and genomic DNA was isolated using the FastDNA®SPIN kit (MP Biomedicals, Santa Ana, CA, USA). DNA concentration and purity were determined at a wavelength of 260 nm by Thermo Scientific, Wilmington, USA, and DNA integrity was checked by agarose gel electrophoresis. PCR amplification was performed using specific primers (Table S2), and the products were purified, quantified, and homogenized to form sequencing libraries. The DNA fragments were processed on the Illumina HiSeq4000 platform and sequenced to obtain raw Sequenced Reads. Vsearch software was used to merge the raw sequence at paired-end reads, based on the following sequence of actions: reserve the sequence length by product size (bp) (Table S2), remove the primer sequence, and then perform fastq-filter to remove the low-quality sequence. Through the cluster-size module, the deduplicated sequences were clustered at the 98 % similarity level, while chimeras were removed using the uchime-denovo module. Subsequently, high-quality sequences were obtained by filtering the chimeras in the sequence set up after quality control (Haas et al., 2011). The resulting final valid sequences were clustered by 97 % similarity level using the cluster-size module with the OTU table as output. An abundance table of OTUs was generated. Some subsequent analysis steps required each sample to be performed at the same sequencing depth level, so some transformation processing of this abundance table was required. The rarefaction method can be used to predict the observed OTUs and relative abundance of each sample at this sequencing depth by randomly extracting a certain number of sequences from each sample to reach a uniform depth (Edgar et al., 2011). The original sequence data were deposited in the SRA database of the National Center for Biotechnology Information (accession number: PRJNA945012).

2.6. Statistical analysis

Statistical analyses were all performed in R (version R-4.2.0; https://www.r-project.org). Shapiro-Wilk tests showed that plant and soil properties data fitted the normal distribution model. We then used the Bartlett test to assess homogeneity of variance. One-way ANOVA and Least—Significant Difference method (LSD test) were used to show differences among treatments. The RandomForest package was used to estimate the importance of some environmental factors in the responses to the duration of the exclosure (Wang et al., 2022c). Meanwhile, the importance score of variables was predicted using the rfPermute package and the significance information was provided based on the permutation test (Jiao et al., 2018). Variance test and multiple comparison (LSD test) were performed for CH_4 uptake rate and CO_2 emission rate to distinguish the differences between different periods of exclusion. The correlations between CH_4 uptake rate, CO_2 emission rate and environmental factors were analyzed by least square linear regression and a linear regression model was established. Relationships between environmental factors and C-cycle functional gene microbial communities were analyzed based on CCA (Canonical Correspondence Analysis) using the vegan package in R (Sun et al., 2021). The correlation between each environmental factor and community structure was calculated using the envfit function of the vegan package, so as to determine the important environmental factors that can explain the distribution of microbial community of C-cycling functional genes (Lavergne et al., 2020). After the C-cycle gene copy number data was standardized, linkET and the vegan package were used to analyze the gene correlation of C-cycle functional genes and the relative importance and variance explained by the random forest is shown. This was done to determine the primary environmental factors that contribute to microbial communities and the abundance of C-cycle functional genes. A Pearson correlation heat map was established to show the relationships between the relative abundance of microbial community composition of C-cycle functional genes and environmental variables. The piecewiseSEM package was used to construct structural equation models (SEM) to determine the direct and indirect effects of C-cycle functional genes on greenhouse gases.

3. Results

3.1. Plant and soil properties under different periods of grazing exclusion

Grazing exclusion had a positive effect on the plant community. With the extension of the exclosure period, ANPP increased significantly and peaked at E16, but with a small but stable decline thereafter (Fig. 1a). ANPP, soil organic carbon content (SOC), soil water content (SWC) and total nitrogen (TN) significantly increased in the short- and medium-term, increasing by 54.4 %, 51.4 %, 59.6 % and 51.8 % from E0 to E16, respectively, and peaked in year 16, while the values were 116 g m^{-2}, 18.0 g kg^{-1}, 18.6 % and 1.78 g kg^{-1}, respectively. ANPP, pH and TN had a significant downward trend after 16 years of enclosure, and reached a plateau between 25 and 38 years (Fig. 1a).

Vegetation coverage showed a consistent pattern of change with ANPP and soil properties, and grazing exclusion had a positive effect on plant community, with vegetation coverage in the enclosed plots being significantly higher than that of E0 ($P < 0.05$, Fig. 2b). The dominant species of grassland community during grazing exclusion were *Leymus chinensis*, *Stipa grandis*, *Caldesia korshinskii* and *Cleistogenes squarrosa*, accounting for 47.8 % (E0), 95.6 % (E7), 76.2 % (E16), 95.9 % (E25), and 43.6 % (E38) of the importance value in the different enclosure durations. With the extension of grazing exclusion, the importance value of *L. chinensis* and *S. grandis* increased significantly, but the important value of *C. squarrosa* significantly decreased. The importance values of L. *chinensis* and *S. grandis* in E7, E16 and E25 were significantly higher than those in E0

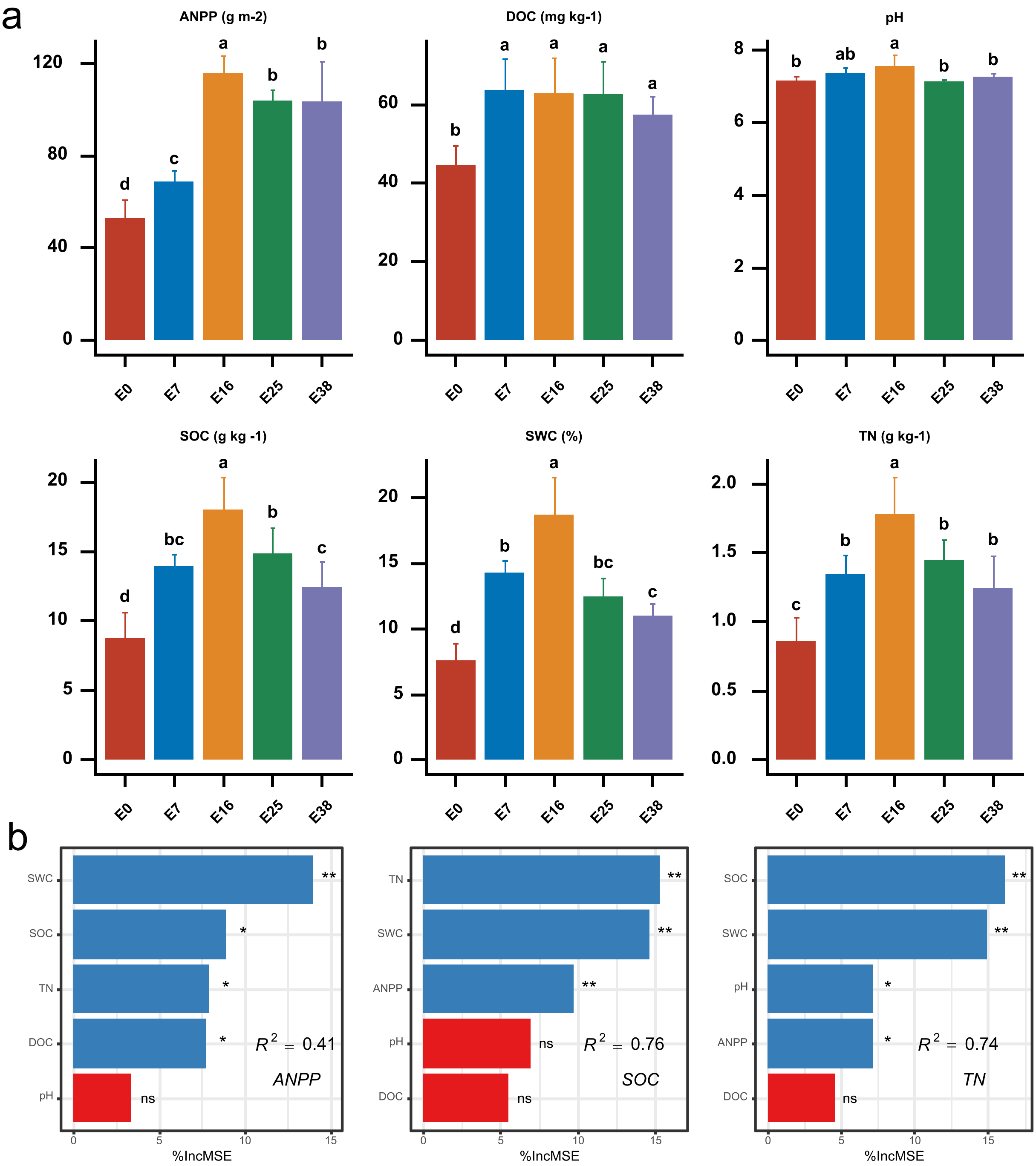

Fig. 1. (a) Effect of different grazing exclusion durations on plant and soil properties. *ANPP* aboveground net primary productivity, *PH* soil pH value, *TN* soil total nitrogen content, *SWC* soil water content, *SOC* soil organic carbon, *DOC* soil dissolved organic carbon. Results are reported as the mean ± sd (n = 6). Different letters indicate significant differences ($P < 0.05$), based on LSD tests. (b) The importance of plant and of soil properties in explaining the variations of *ANPP*, *SOC*, *TN*. Random forest (RF) mean predictor importance (percentage of increase of mean square error, %IncMSE) was used to determine the variable importance, and higher MSE% values imply more important predictors. R^2 represents the total variance that could be explained by selected environmental factors. Significance levels are as follows: $*P < 0.05$ and $**P < 0.01$.

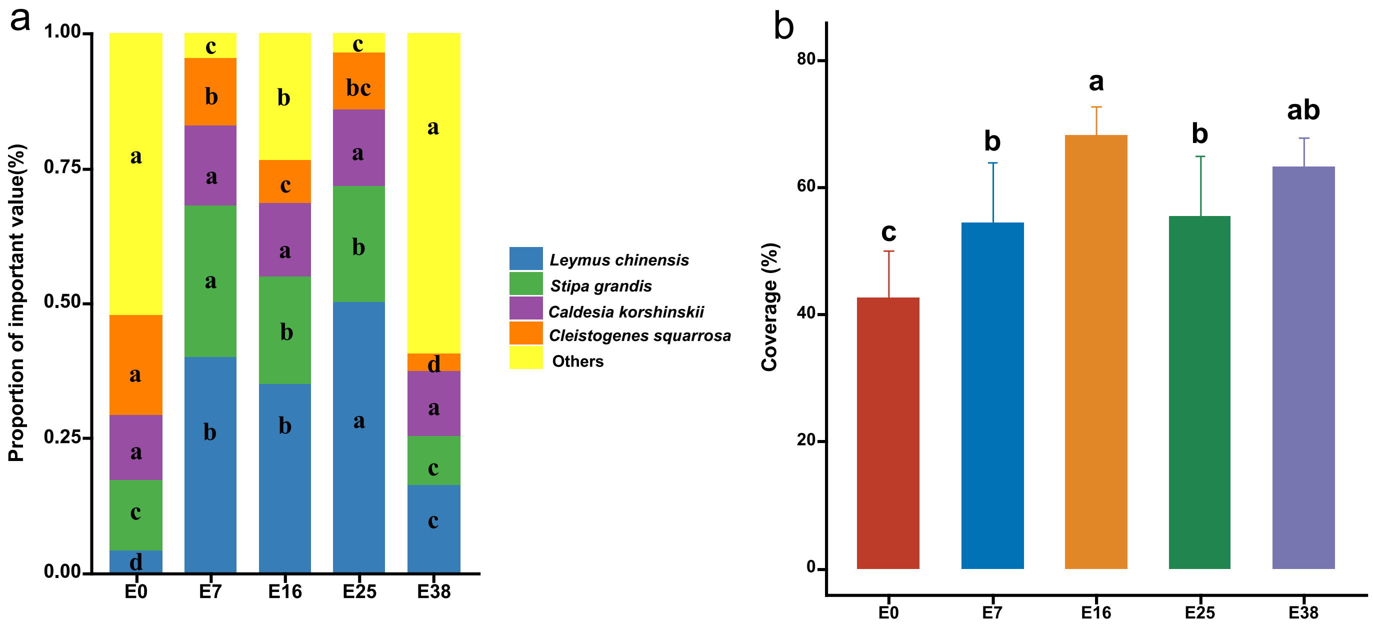

Fig. 2. (a) The proportion of importance value for dominant plant species and other plant species during different periods of exclosure. (b) Effect of different periods of exclosure on plant coverage. Results are reported as the mean ± sd (n = 6). Different letters indicate significant differences ($P < 0.05$), based on LSD tests.

and E38 ($P < 0.05$, Fig. 2a), while the importance values of *C. squarrosa* gradually decreased with the extension of the exclosure period ($P < 0.05$, Fig. 2a).

To disentangle the potential main drivers of soil nutrient cycling during the enclosure period, we conducted a random forest analysis to assess important environmental factors affecting ANPP, SOC, and TN (Fig. 1b). SWC was the most important environmental factor for ANPP, followed by SOC, TN and DOC. TN became the most important environmental factor influencing SOC, whereas SOC was the most significant environmental factor having an impact on TN. SWC had significant effects on ANPP, SOC and TN, indicating that SWC plays an important role in the grassland ecosystem. Although soil pH was not a significant variable accounting for variation in ANPP and SOC, it significantly contributed to explain variation in TN (Fig. 1b).

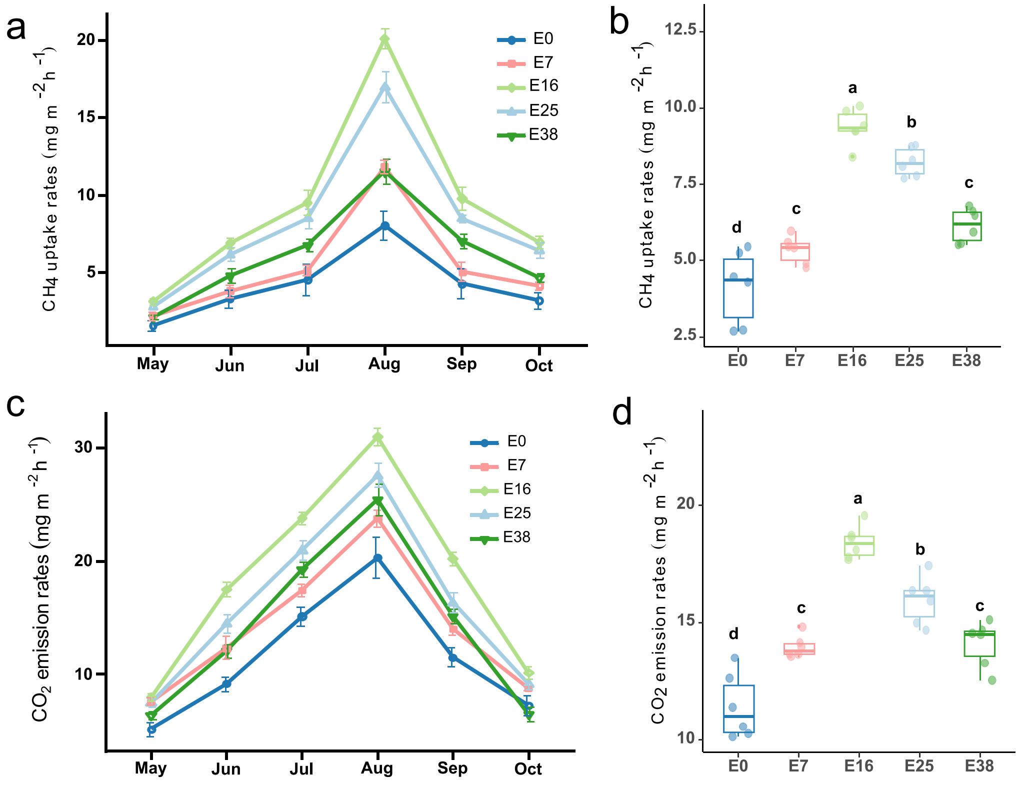

Fig. 3. (a) Changes in CH_4 uptake rates in different exclusion sites from May to October and (b) Average rates under different exclusion sites. (c) Changes in CO_2 emission rates in different exclusion sites from May to October and (d) Average rates under different exclusion sites. Results reported as the mean ± sd (n = 6). Different letters indicate significant differences ($P < 0.05$), based on LSD tests.

3.2. CH_4 uptake rate and CO_2 emission rate

The CH_4 uptake and CO_2 emission rates at the E0-E38 sites showed an increasing trend from May to August, but a decreasing trend from September to October. During May to October, CH_4 uptake rate ranged between 1.57 ± 0.56 mg m^{-2} h^{-1} (E0-May) and 20.06 ± 1.08 mg m^{-2} h^{-1} (E16-August), while CO_2 emission rate fluctuated between 5.15 ± 1.02 mg m^{-2} h^{-1} (E0-May) and 30.69 ± 1.30 mg m^{-2} h^{-1} (E16-August) (Fig. 3a, c). Using the average greenhouse gas flux data throughout the entire growing season from May to October, we compared CH_4 uptake and CO_2 emission rates under different exclusion durations. During the entire growing season, there were significant differences in the average CH_4 uptake and CO_2 emission rates under different exclusion durations. The CH_4 uptake rate of E16 (9.38 ± 0.59 mg m^{-2} h^{-1}) was significantly higher than that of E25 (8.22 ± 0.47 mg m^{-2} h^{-1}), E38 (6.14 ± 0.55 mg m^{-2-} h^{-1}), E7 (5.35 ± 0.45 mg m^{-2} h^{-1}), and E0 (4.14 ± 1.20 mg m^{-2} h^{-1}), while there was no significant difference between E7 and E38 (Fig. 3b). The CO_2 emission rate of E16 (18.42 ± 0.71 mg m^{-2} h^{-1}) was significantly higher than that of E25 (15.96 ± 1.01 mg m^{-2} h^{-1}), E38 (14.09 ± 0.99 mg m^{-2} h^{-1}), E7 (13.96 ± 0.48 mg m^{-2} h^{-1}), and E0 (11.40 ± 1.38 mg m^{-2} h^{-1}) (Fig. 3d).

The constructed linear regression equation between CO_2 emission rate and CH_4 uptake rate shows that there was a strong linear, positive correlation between CO_2 emission and CH_4 uptake ($R^2 = 0.73$, $P < 0.001$) (Fig. S1a). There was a significant, positive correlation between ANPP and CH_4 uptake rate ($R^2 = 0.65$, $P < 0.001$) and CO_2 emission rate ($R^2 = 0.64$, $P < 0.001$). SOC was positively correlated with CH_4 uptake rate ($R^2 = 0.62$, $P < 0.001$) and CO_2 emission rate ($R^2 = 0.57$, $P < 0.001$); SWC was positively correlated with CH_4 uptake rate ($R^2 = 0.53$, $P < 0.001$) and CO_2 emission rate ($R^2 = 0.57$, $P < 0.001$) (Fig. S1).

3.3. Abundance of C-cycle functional genes and microbial community composition in chronosequence of grazing exclusion

The abundance of soil C-cycling functional genes was significantly different in grasslands with different duration of grazing exclusion. The gene copy number of *pmoA* had the largest variation with exclusion (10.20×10^4–44.20×10^5 copies g^{-1} dry soil). *cbbL* (18.37×10^7 copies g^{-1} dry soil), *cbbM* (79.25×10^4 copies g^{-1} dry soil), *chiA* (10.43×10^8 copies g^{-1} dry soil), *pmoA* (35.45×10^5 copies g^{-1} dry soil) gene copy number showed the highest relative abundance. There was no significant difference in gene copy number between *cbbL* and *cbbM* in grasslands 16 or 25 years of exclosure (Fig. 4a).

Using random forest analysis to identify the main drivers of C-cycling functional gene abundance, we found that CO_2, CH_4, ANPP were the main factors leading to functional gene abundance changes, and ANPP was highly correlated with *cbbL* and *pmoA*, with R^2 values of 0.84 and 0.89, respectively. The explanation rate of environmental factors in *pmoA* was as high as 91.5 %, indicating that the model fit was good, which may be related to the high correlation between *pmoA* abundance and most environmental factors. It is worth noting that SWC also had a high importance value for *pmoA* and *chiA*, which were 9.05 and 10.74 respectively (Fig. 4b).

After quality control, OTUs were clustered and identified to the phylum level based on 97 % similarity threshold. At phylum level, C-cycling functional genes communities were primarily comprised of *Actinobacteria* and *Proteobacteria*. *Proteobacteria* are the common dominant bacteria of *cbbL*, *cbbM* and *pmoA*, accounting for 44.1 %, 60.8 % and 99.9 % of their relative abundances, respectively. The functional genes of *pmoA* are almost completely composed of *Proteobacteria*. *Actinobacteria* is another dominant phylum, occupying 48.8 % of *cbbL* and 92.7 % of *chiA* (Fig. S2). At genus level, the *cbbL* functional gene microbial community mainly consisted of *Pseudonocardia*, *Bradyrhizobium* and *Mesorhizobium* (Fig. S3a). The *cbbM* functional genes community was mainly composed of *Acidithiobacillus*, *Bradyrhizobium* and *Rhodopseudomonas* (Fig. S3b). The *chiA* functional genes community was mainly composed of *Actinoplanes*, *Streptomyces* and *Amycolatopsis* (Fig. S3c).

Shannon indexes of *cbbL* and *pmoA* were significantly higher for E7 than for E0 ($P < 0.05$, Fig.S5a, d). Chao1 and Observed_species index of *cbbL* and *chiA* showed similar changes ($P < 0.05$, Fig. S5a, c). Goods_coverage was significantly lower for E7 than for E0 for all functional genes ($P < 0.05$, Fig. S5).

3.4. Environmental factors that drive the abundance of carbon cycle functional genes and microbial communities

The main environmental factors affecting the microbial community of C-cycling genes were analyzed by CCA, and the environmental factors significantly related to the microbial community were evaluated by the envfit

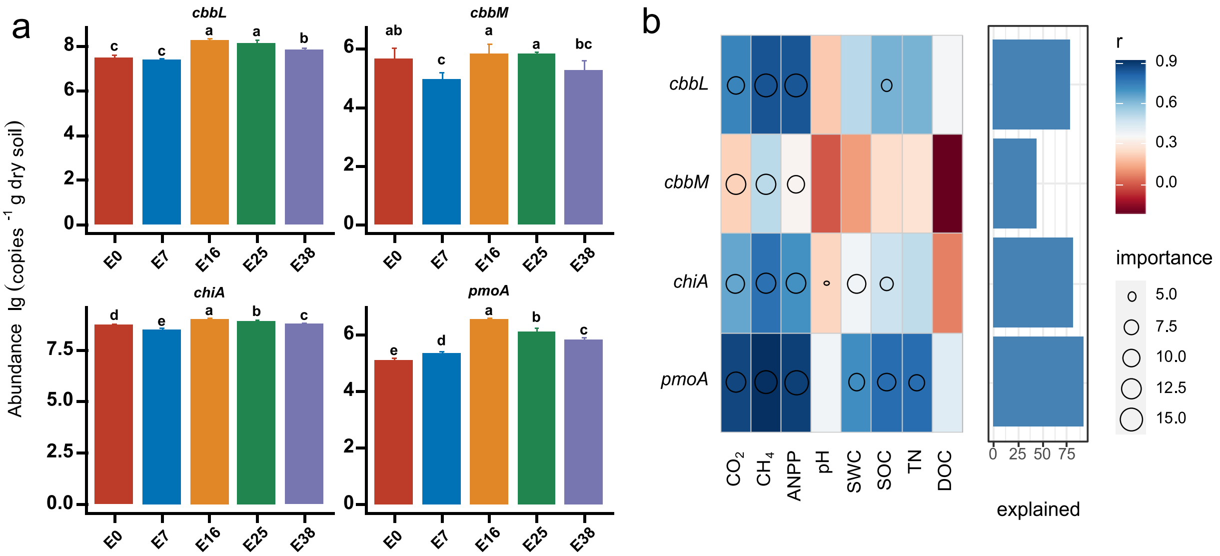

Fig. 4. (a) The abundances of soil C-cycling functional genes during the grazing exclusion. Results are reported as the mean ± sd (n = 6). Different letters indicate significant differences ($P < 0.05$), based on LSD tests. (b) Relationship between environmental factors and C-cycling genes during grazing exclusion. Contribution of environmental factors to abundance of C-cycling functional genes based on correlation and random forest models; the importance of variables is represented by the size of the circle (proportion of explained variation was calculated using multiple regression modeling and variance decomposition analysis). The color represents the Pearson correlation.

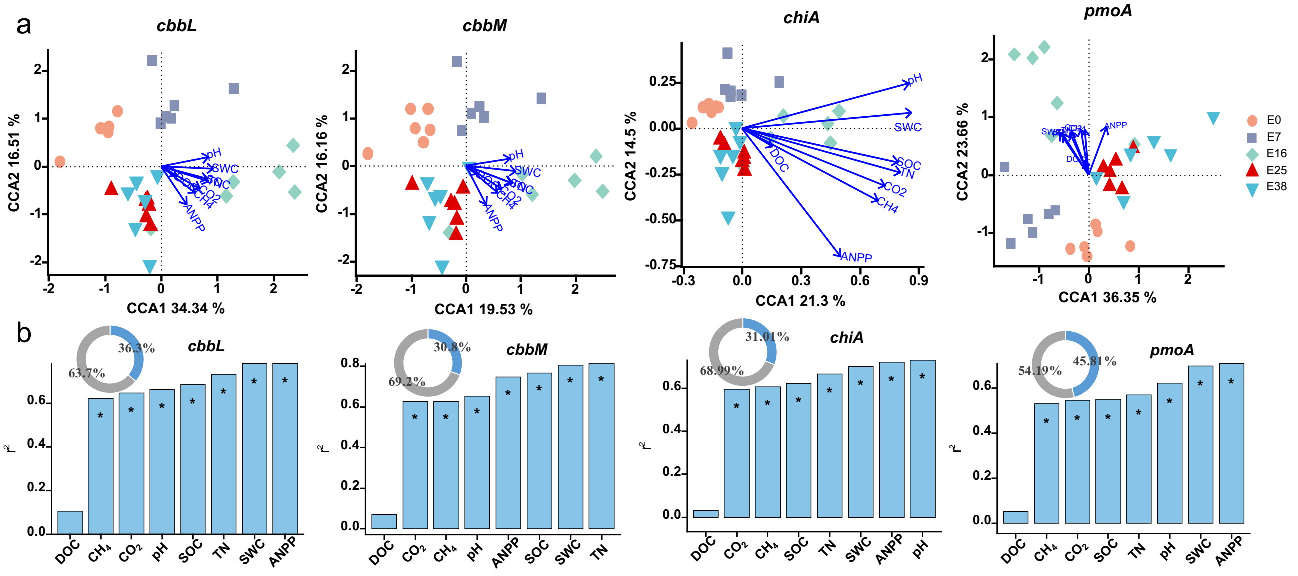

Fig. 5. (a) Canonical Correspondence Analysis (CCA) of soil C-cycling functional genes *cbbL*, *cbbM*, *chiA*, *pmoA* and environmental factors. (b) The Envfit function was used to identify important environmental factors associated with CCA order. The blue part of the circle diagram represents the degree of explanation of community structure differences by environmental factors, while the grey part represents the part of community structure differences that are unexplained by environmental factors. Significance level is as follows: $^*P < 0.05$.

function. The results showed that 36.3 %, 30.8 %, 31.0 %, and 45.8 % of the differences in microbial communities of *cbbL*, *cbbM*, *chiA*, and *pmoA* were explained by environmental factors under different periods of exclosure, respectively (Fig. 5b). ANPP, SWC, TN, SOC, pH, CO_2 and CH_4 significantly affected the functional gene communities of C-cycling ($P < 0.05$). Among them, ANPP was the primary environmental factor affecting the microbial communities of functional genes *cbbL* and *pmoA*. TN was the primary factor affecting the microbial community of functional gene *cbbM*. For the *chiA* microbial community, pH was one of the reasons for the differences in community composition. Meanwhile, CCA analysis showed that the angle between SWC and *cbbL*, *chiA* and *cbbM* was the smallest, indicating that SWC was closely related to microbial community changes, and its environmental factor scores were 0.93, 0.92 and 0.86, respectively (Fig. 5a). According to the PCoA ordination, the composition of the microbial community associated with C-cycling genes varied significantly during grazing exclusion (Adonis, $P < 0.05$) (Fig. S4).

Results of the Pearson correlation analysis showed that *Pseudonocardia* was negatively correlated with ANPP ($P < 0.05$), while *Variovorax* was positively correlated with CO_2 and CH_4 ($P < 0.05$), and significantly positively correlated with ANPP ($P < 0.001$) (Fig. 6a). CO_2 was negatively correlated with *Acidithiobacillus* ($P < 0.01$), and positively correlated with *Bradyrhizobium* ($P < 0.05$) and *Rhodopseudomonas* ($P < 0.01$) (Fig. 6b). *Streptomyces* was positively correlated with CO_2 ($P < 0.01$), CH_4 ($P < 0.05$) and ANPP ($P < 0.05$) (Fig. 6c). Environmental factors (except pH and DOC) were positively correlated with *Methylococcaceae* (Fig. 6d).

3.5. Response of C-cycling to grazing exclusion

Since structural equation model (SEM) analysis is based on a prior conceptual model, we first used the random forest model to determine the important factors in the environment, and then gradually excluded the insignificant environmental factors to simplify the path of the structural equation model.

SEM analysis showed that ANPP had significant, positive effects on DOC and SOC. It is noteworthy that ANPP had no direct significant, positive effect on CO_2 emission ($P = 0.07$), but first affected SOC content and then CO_2. Similarly, ANPP indirectly changed CH_4 uptake rates by directly affecting DOC ($P = 0.0013$) and *pmoA* ($P < 0.001$). *cbbL* and *pmoA*, as functional genes of C-cycling, were positively affected by ANPP. There was no significant effect of pH on CH_4 (Fig. 7).

4. Discussion

4.1. Effects of grazing exclusion on plant community succession and soil carbon sequestration

In this study, grazing exclusion was found to promote vegetation recovery and improve nutrient status, with dominant species playing a crucial role in increasing soil carbon sequestration through biomass accumulation and enhanced ANPP driven by sufficient water and nutrient conditions. ANPP and soil nutrient content reached the peak in the 16th year of exclosure, and then SOC, SWC and TN showed a stepwise decline from 25 to 38 years of exclosure (Fig. 1a). This finding supports previous research showing the effectiveness of grazing exclusion in restoring degraded grasslands, improving carbon sequestration (Hu et al., 2016), altering vegetation community characteristics (Potthoff et al., 2005), and enhancing soil nutrient status by minimizing livestock grazing and underground soil disturbance (Li et al., 2013; Wu et al., 2008). In the absence of livestock interference, dominant plant species are able to acquire more growth space and nutrient resources and occupy significant ecological niches, promoting the rapid recovery of the vegetation, while other species complement each other's ecological niche spatially (Wang et al., 2018). As herbaceous plants are the main source of input for SOC, changes in plant community composition can also affect the accumulation of carbon elements in the soil. This study found that the proportion of dominant plant species in the community of E7 to E25 was higher than that of other enclosure periods (Fig. 2a). However, long-term grazing exclusion led to considerable litter accumulation, decreasing the density of dominant species and encouraging weed growth, which significantly increased the number of other auxiliary species communities after 38 years of exclusion. Compared with E16, the importance values of L. *chinensis* and *S. grandis* were significantly reduced in E38 (Fig. 2a), partly explaining why soil nutrient levels in E16 were significantly higher than in other enclosure periods. Generally, a higher proportion of dominant species from the Poaceae family leads to better nutritional conditions since they possess better decomposition abilities, higher nutritional levels and faster growth rates, thereby increasing ANPP (Craig et al., 2022). Random forest and structural equation models validated that ANPP, SWC and TN are important driving factors affecting SOC (Figs. 1b, 6). This is because sufficient water and nutrient conditions can support richer plant growth and higher ANPP levels, which have a positive feedback effect on plant community structure and productivity (Deng

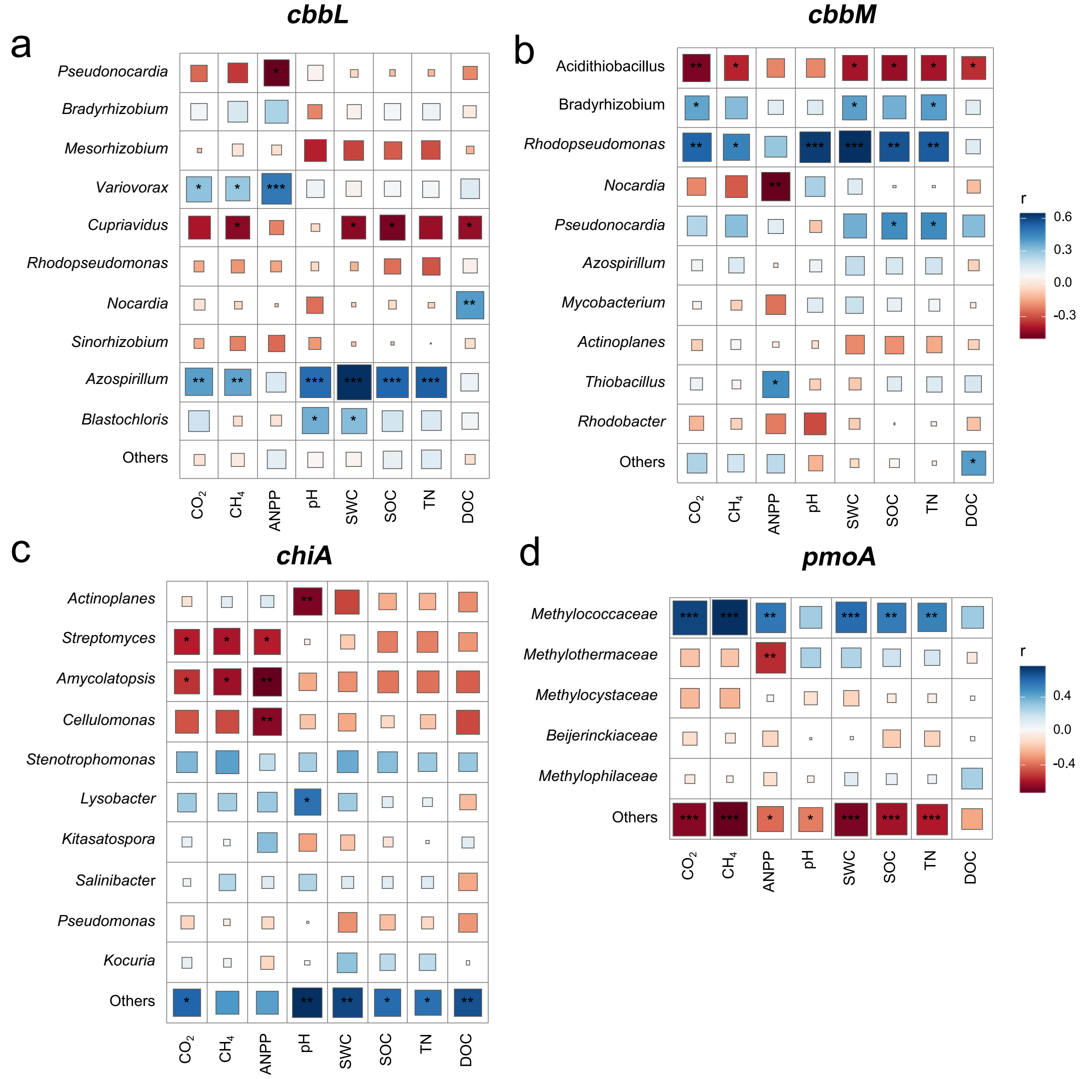

Fig. 6. Pearson's correlation analysis of C-cycling functional genes with environmental factors at genus level (a) *cbbL*, (b) *cbbM*, (c) *chiA* and family level (d) *pmoA*. Significance level are as follows: *** $P < 0.001$, **$P < 0.01$, *$P < 0.05$.

et al., 2017). In particular, higher nutrient conditions favor the competitiveness of Poaceae plants (Wu et al., 2009). These results collectively increase the source of soil carbon inputs (Li et al., 2013), and affect the conversion and storage of SOC (Schulte-Uebbing and de Vries, 2018; Stavi et al., 2008).

4.2. Changes of C-cycle functional genes during grazing exclusion

The functional genes of C-cycling are closely related to the carbon turnover potential (Liu et al., 2016). Through our study, we found that the appropriate exclosure period of a typical Inner Mongolia grassland improved the potential of soil C-cycling (Fig. 4a). Changes in the abundance of functional genes and microbial communities associated with functional genes may be related to soil nutrient status and abundance of dominant bacteria during grazing exclusion.

Our study revealed that the functional gene *cbbL* was substantially more abundant than *cbbM*, and exhibited a stronger positive correlation with environmental factors, explaining 78.9 % of the variation (Fig. 4b). This finding is consistent with prior research indicating that *cbbL* may have a greater impact than *cbbM* genes (Xiao et al., 2014). Notably, *Variovorax* emerged as a dominant bacterial genus within the *cbbL* microbial community, and demonstrated a significant positive correlation with ANPP (Fig. 6a), with evidence indicating its influence on plant root growth via regulation of plant auxin and ethylene levels (Finkel et al., 2020). The relative abundance of *Variovorax* in E16 was significantly higher than that in E0 (Fig. S6a), suggesting its potential role in facilitating grassland restoration during the enclosure process.

The *chiA* functional genes, responsible for mineralization and decomposition of organic matter (Brankatschk et al., 2011), were affected by the increase in SOC during the exclosure process, which provided ample energy sources and raw materials for microorganisms and therefore emerged as an important determinant of changes in *chiA* gene abundance (Fig. 4b). The microbiome associated with the *chiA* genes was primarily dominated by *Actinobacteria* (Fig. S2), with *Streptomyces* as a major bacterial genus playing a pivotal role in organic matter decomposition. *Streptomyces* demonstrated a significant negative correlation with ANPP (Fig. 6c), indicating its association within oligotrophic groups that are better adapted to nutrient-poor environments compared to copiotrophic groups that can reproduce rapidly in high-nutrient environments (Liao et al., 2023). This suggests that *Streptomyces* may be more suited for poor, low-nutrient soil environments (Zeng et al., 2017). Additionally, research has indicated that *Streptomyces* exhibits greater richness in extremely nutrient-limited conditions (Arocha-Garza et al., 2017).

Random forest models showed that the explanatory rate of *pmoA* functional gene abundance and environmental factors was up to 91.5 % (Fig. 4b), and the dominant bacterial family *Methylococcaceae* was positively correlated with environmental factors, indicating that the *pmoA*

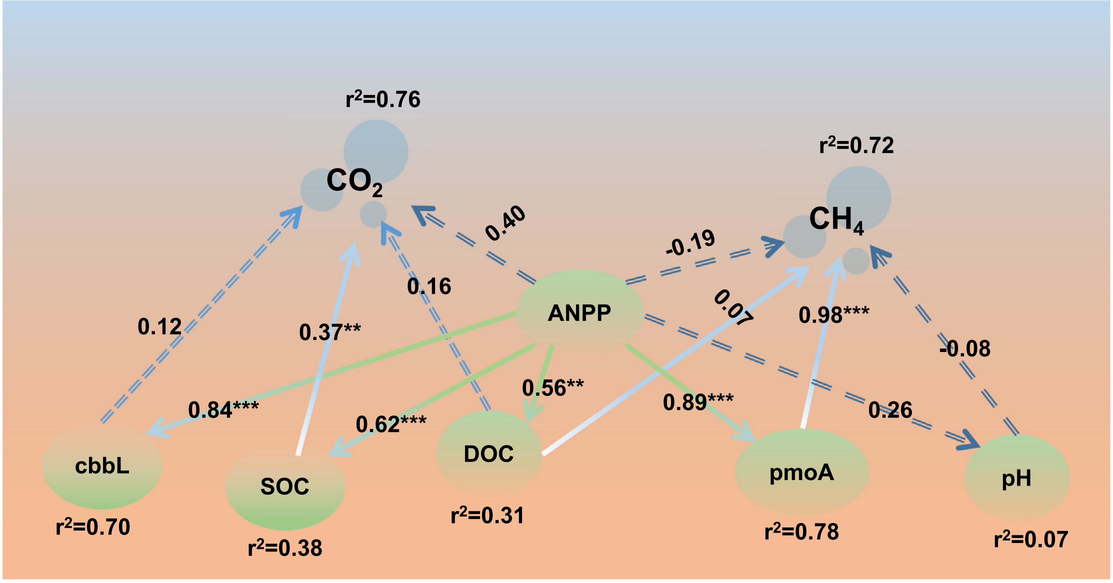

Fig. 7. Structural equation model of the effects of ANPP on soil, C-cycle functional genes and greenhouse gases during grazing exclusion. Solid arrows represent significant positive or negative effects (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$), dotted arrows have no significant effects ($P \geq 0.05$). Normalized path coefficients are marked at the arrow. The R^2 value marked at the response variable represents the proportion of variation explained of the other variables. Fisher's C = 8.069, P = 0.233, DF = 6 (CH_4); Fisher's C = 8.378, P = 0.212, DF = 6 (CO_2).

functional gene was closely related to environmental factors. Additionally, water availability was found to significantly affect the abundance of the *pmoA* functional gene, which can promote the growth and activity of microorganisms, especially those sensitive to water (Wang et al., 2020).

4.3. Effects of exclosure on CO_2 emissions and CH_4 uptake

Grazing exclusion altered CO_2 emission rate by affecting vegetation community, soil properties (e.g., SWC and SOC), and functional genes with the C-cycling. In our study, appropriate exclosure promoted the CO_2 emission rate, which showed a single-peak pattern from the grazing site to 38 years of exclosure site (Fig. 3a), with the minimum and maximum values at E0 and E16, respectively. Exclosure first affected the above-ground canopy; the duration of enclosure had a temporal scale effect on ANPP (Fig. 1a). CO_2 emission rate increased with an increase in ANPP (Fig. 3c), which may indicate that an exclosure increases CO_2 emission rate by improving above-ground vegetation production. This may be because plants are the main pathway of CO_2 emission (Box, 1978), and increased ANPP stimulates the decomposition of organic matter in the form of litter, thereby accelerating the rate of soil respiration and thus also increasing CO_2 emission (Bowden et al., 1993). In addition, both SWC and SOC were positively correlated with CO_2 emission (Fig. S1), mainly because water alters the activity of microorganisms in the soil (Curiel Yuste et al., 2007), controlling the rate of nutrient decomposition and mineralization and thus improves the flux of substrates to the cell surface (Cong et al., 2015). SOC increased nutrient resources for soil microorganisms. Moreover, the microorganisms responsible for C-cycling are mainly present in *Proteobacteria* and *Actinobacteria* (Fig. S2), which have been shown to be closely related to soil organic carbon (SOC) (Hu et al., 2022). An increase in the relative abundance of *Actinobacteria* has been demonstrated to accelerate SOC decomposition, which may lead to an increase in soil CO_2 emissions (Gao et al., 2020). Thus, with a further prolongation of the appropriate enclosure period, the further increase in ANPP content resulted in an increase in soil carbon storage and in the abundance of C-cycle functional genes, which together accelerated the CO_2 emission rate.

Grazing exclusion increases the uptake rate of CH_4, especially at proper exclusion (E16), and the reasons may be as follows. Soil moisture is an important factor affecting CH_4 uptake rate. There is a positive correlation between SWC and *pmoA* abundance and CH_4 uptake rate (Figs. S1d, 4b), which is consistent with the research results of CH_4 uptake rate in desert steppe (Yue et al., 2022). Enclosure measures from 0 to 16 years improved soil moisture status and soil compactness, thereby promoting CH_4 uptake through water-filled pore space (Yue et al., 2019). Interestingly, the opposite results were observed in temperate forests (Dijkstra et al., 2013) and meadows (Shrestha et al., 2012) with high moisture content. Considering the effect of soil moisture content on the methane oxidizing bacteria community, although increasing soil moisture can improve plant diversity, yield (Zavaleta et al., 2003) and the number of methanogens, excessive moisture hinders the supply of CH_4 to methanotrophs in the atmosphere (Phillips et al., 2001). The optimal soil moisture content for CH_4 uptake is 20 %–35 %, while the activity of methanotrophic microbiota is completely inhibited when soil water content is below 5 % (Van den Pol-van Dasselaar et al., 1998). In this study, the SWC was between 5.66 and 20.45 % (Fig. 1a), so there was no excessive moisture and no soil pores being filled with water to inhibit the diffusion of CH_4 to methanotrophic microbiota (Wei et al., 2012), which also explained the opposite results of temperate forest and meadow. The SEM showed a significant correlation between the abundance of *pmoA* functional genes and the CH_4 uptake rate (Fig. 7). The high correlation is because the *pmoA* gene encodes the beta subunit of particulate methane monooxygenase (pMMO) (Holmes et al., 1999), which may be associated with an increase in methanotrophy (Ma et al., 2016). The data indicated that E16 had a higher CH_4 uptake rate compared with other years of grazing exclusion, accompanied by an increased relative abundance of *Methylococcaceae* of *Methanotrophs* and changes in the abundance of *pmoA* gene. Moreover, we also found that most environmental factors had important effects on the abundance of *pmoA* and the microbial community associated with the *pmoA* gene (Fig. 4b, 5b). From 25 to 38 years of exclosure, the CH_4 uptake rate decreased by 14.3 % and 28.4 %, respectively, relative to E16, which may be attributed to the decrease in soil nutrients inhibiting the activity of methanotrophs. The above results further suggest that the effect of exclusion on CH_4 uptake rate is achieved by influencing environmental factors and the abundance and community of methanotrophs.

5. Conclusion

Soil nutrient status and grassland C-cycling were significantly improved by enclosure during the first 16 years, peaked after 16 years and began to decline during in the following years. Compared with grazing land, an appropriate exclosure period could increase ANPP, DOC, SOC, SWC, TN, CO_2 emission rate, CH_4 uptake rate, and the abundance of C-cycling functional genes *cbbL*, *cbbM*, *chiaA*, and *pmoA*. Differences in the abundance of C-cycling functional genes and microbial community composition are primarily influenced by ANPP. The increases in SOC and *pmoA* abundance caused by elevated ANPP accelerated CO_2 emission and CH_4 uptake rates, respectively. Our results showed that an appropriate duration of exclosure improved the C-cycling potential of steppe. In general, ANPP had a significant effect on C-cycling in typical steppe during grazing exclusion and were the main factors affecting the abundance of functional genes of C-cycling and microbial community. However, different C-cycling genes have different responses to specific environmental factors. Our results highlight the important role of C-cycle functional genes in modulating the carbon sequestration and soil fertility during grazing-to-exclusion conversion, and have significant implications for the implementation of future grassland management policies.

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CRediT authorship contribution statement

Zhen Wang conceived and designed the study. Wenbo Zhang, Jing Li, Shengyi Jiang, Yong Zhang, Yuanheng Li and Xiaojiang Yang collected and analyzed the data. Wenbo Zhang drafted the manuscript, and Zhen Wang, Paul C. Struik and Baoming Ji critically revised it for important intellectual content. Ke Jin and Zhen Wang supervised the project. All authors read and approved the final manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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