





## RESEARCH ARTICLE

# Microbially enhanced methane uptake under warming enlarges ecosystem carbon sink in a Tibetan alpine grassland

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## Abstract

The alpine grasslands of the Tibetan Plateau store 23.2 Pg soil organic carbon, which becomes susceptible to microbial degradation with climate warming. However, accurate prediction of how the soil carbon stock changes under future climate warming is hampered by our limited understanding of belowground complex microbial communities. Here, we show that 4 years of warming strongly stimulated methane (CH<sub>4</sub>) uptake by 93.8% and aerobic respiration (CO<sub>2</sub>) by 11.3% in the soils of alpine grassland ecosystem. Due to no significant effects of warming on net ecosystem CO<sub>2</sub> exchange (NEE), the warming-stimulated CH<sub>4</sub> uptake enlarged the carbon sink capacity of whole ecosystem. Furthermore, precipitation alternation did not alter such warming effects, despite the significant effects of precipitation on NEE and soil CH<sub>4</sub> fluxes were observed. Metagenomic sequencing revealed that warming led to significant shifts in the overall microbial community structure and the abundances of functional genes, which contrasted to no detectable changes after 2 years of warming. Carbohydrate utilization genes were significantly increased by warming, corresponding with significant increases in soil aerobic respiration. Increased methanotrophic genes and decreased methanogenic genes were observed under warming, which significantly ( $R^2 = .59, p < .001$ ) correlated with warming-enhanced CH<sub>4</sub> uptakes. Furthermore, 212 metagenome-assembled genomes were recovered, including many populations involved in the degradation of various organic matter and a highly abundant methylo-trophic population of the *Methyloceanibacter* genus. Collectively, our results provide compelling evidence that specific microbial functional traits for CH<sub>4</sub> and CO<sub>2</sub> cycling

processes respond to climate warming with differential effects on soil greenhouse gas emissions. Alpine grasslands may play huge roles in mitigating climate warming through such microbially enhanced CH<sub>4</sub> uptake.

#### KEYWORDS

alpine grassland, climate warming, metagenome, methane uptake, microbial community, soil respiration

## 1 | INTRODUCTION

The Qinghai-Tibetan Plateau is the highest and largest plateau in the world, with an area of more than 2.4 million km<sup>2</sup> mostly lying above 4000m (above sea level), often called “the roof of the world” (Yao et al., 2012). Alpine grasslands cover more than 60% of the plateau, storing 23.2 Pg soil organic carbon (SOC) equivalent to 23.4% of SOC stock in China (Genxu et al., 2002). This large C stock has accumulated for thousands of years, partly due to low temperatures of the plateau, which constrain microbial SOC mineralization (Liu et al., 2012; Yang et al., 2008). Due to the high terrain, the plateau has experienced more substantial climate warming, whose rate is about twice the global average in the past 50 years (Chen et al., 2013; Jia et al., 2017). This rapid warming is expected to stimulate soil microbial processes resulting in more release of greenhouse gases such as CO<sub>2</sub> and CH<sub>4</sub>, which could threaten the stability of SOC stock (Yi et al., 2014; Yuan et al., 2021). Considering the significance of the Qinghai-Tibetan Plateau as Asia's water tower and regulator for climate (Yao et al., 2012; Yuan et al., 2021), such changes pose a major risk to future climate change in regional and even global scale. However, prediction of the magnitude of soil C change is hampered by our knowledge gaps regarding microbial metabolisms of SOC and their controls that operate in these environments.

Soil microbial communities play crucial roles in ecosystem responses to climate warming through their effects on carbon and nutrient cycling (Guo et al., 2019; Gutknecht et al., 2012; Yuan et al., 2021; Zhou et al., 2012). Previous studies of laboratory-incubated soil under increased temperatures indicated that microbial community structure and functions of alpine grasslands on the Qinghai-Tibetan Plateau significantly shifts toward increased carbon respiration and/or more methane release (Johnston et al., 2019; Liu et al., 2017). However, these incubation experiments in the laboratory cannot simulate the complexity of the natural environment closely (Johnston et al., 2019). Furthermore, climate warming not only increases soil temperature, but also affects water availability, redox conditions, nutrient allocation, and aboveground plant composition (Guo et al., 2020; Gutknecht et al., 2012; Johnston et al., 2019; Liu et al., 2018). These soil physicochemical changes and plant community shifts can further influence soil microbial communities and their functions (Guo et al., 2018, 2020; Xue et al., 2016). Consistently, plant-mediated effects of warming on soil microbial community structure and interactions could substantially explain the changes of soil respiration rates (Chen et al., 2021). A long-term warming experiment in the Tibetan alpine grassland showed that

precipitation substantially modulated the warming effects on soil microbial respiration, resulting in the temporal variations of warming response of microbial respiration (Wang et al., 2021). Hence, both microbial and environmental features serving as key candidates to evaluate soil C release at the ecosystem level are vast and interconnected (Johnston et al., 2019). Understanding the microbial clades and functional potentials and their environmental controls operating in situ could help to explain greenhouse gas fluxes and predict ecosystem-scale response to climate warming (Schoor et al., 2009; Woodcroft et al., 2018).

To address these issues, a climate change experiment with warming and altered precipitation as main treatment factors was established in July 2011 at the Haibei Alpine Grassland Ecosystem Research Station (37°36'N, 101°19'E, mean elevation of 3215m) in the northeastern Qinghai-Tibetan Plateau (Jia et al., 2019; Ma et al., 2017). Our previous study revealed rapid responses of soil physicochemical properties and microbial physiological features in the first 2 years of warming at this site (Qi et al., 2021). However, the community-wide changes in microbial structure were less discernible (Qi et al., 2021; Zhang et al., 2016). In this study, a total of 36 soil surface (0–10 cm) samples were collected from all plots and analyzed by integrated metagenomic sequencing to assess the responses of soil microbial communities in situ after 4 years of experiment. Our hypotheses were as follows: (i) microbial communities would be sensitive to warming treatment after 4 years of experiment; (ii) microbial functional shifts induced by warming would result in more releases of CO<sub>2</sub> and/or CH<sub>4</sub>; and (iii) the changes in the relative abundances of microbial functional genes under warming would explain the changes of soil C fluxes. Our results revealed that 4 years of warming significantly shifted soil microbial community structure and functional gene traits, leading to the increase in both microbial CH<sub>4</sub> uptake and aerobic respiration. Due to no significant effects of warming on NEE, the warming-stimulated soil microbial CH<sub>4</sub> uptake enlarged the carbon sink capacity of whole alpine grassland ecosystem.

## 2 | MATERIALS AND METHODS

### 2.1 | Field site description and sampling

This field experiment site was established in July 2011 at the Haibei Alpine Grassland Ecosystem Research Station in the northeastern Qinghai-Tibetan Plateau. Based on the local climatological survey

data, the annual mean temperature at this site is  $-1.5^{\circ}\text{C}$  and the annual accumulated precipitation is 501.3 mm, generally concentrated from May to September (Qi et al., 2021). The alpine grassland is dominated by *Kobresia humilis*, *Helictotrichon tibeticum*, *Carex przewalskii*, and *Stipa aliena* (Jia et al., 2019). In most years, vegetation reaches the maximal plant biomass during late July to early August. The soil at this site is Mat-Gryic Cambisol with a clay loam texture, with a mean pH of 8.0 (Jia et al., 2017).

The experimental design of this site has been described previously (Qi et al., 2021). Briefly, a total of 36 plots were set up with a completely random block design to simulate climate warming and altered precipitation. There are six experimental blocks, each containing six  $2.2 \times 1.8$  m plots. The six plots within each block were under one of six randomly distributed treatments (i.e., control, warming, decreased precipitation, increased precipitation, combined warming and decreased precipitation, and combined warming and increased precipitation). Therefore, each treatment has six replicates, which are distributed across the six blocks. Two infrared heaters were suspended at the height of 1.5 m above the ground in each warmed plot to stimulate the whole ecosystem warming of  $+2^{\circ}\text{C}$ , while two dummy heaters were installed for the other plots. Transparent resin polycarbonate channels covering 50% of plot area were used to halve precipitation in the plots with decreased precipitation. The intercepted precipitation by the polycarbonate channels was collected and then added to the plots with increased precipitation, resulting in a 50% increase in precipitation.

Soil surface (0–10 cm) samples were collected in August 2015 from each plot. Each sample was mixed from three soil cores randomly taken from each plot. Therefore, a total of 36 samples were collected in the same day and transported to the laboratory on ice. Then, all soil samples were immediately sieved with a 2 mm mesh to remove stones and visible plant roots. Soil samples were further divided into two parts, with one part for physiochemical measurements stored at  $4^{\circ}\text{C}$  refrigerator and the other part for microbial analyses stored at  $-80^{\circ}\text{C}$  refrigerator.

## 2.2 | Environmental and soil chemical measurements

All the measurements of soil and plant variables were conducted in 2015. Soil temperature and moisture were automatically monitored by 5-TM probe sensors at the soil depth of 5 cm with an EM-to data logger (Meter, Inc.) in each plot. To represent microclimates under different treatments, the seasonal and annual average temperatures and moistures were calculated to further analyze in this study.

Total carbon and total nitrogen (TN) were determined using a 2400 II CHN elemental analyzer (Perkin-Elmer, Inc.). SOC was calculated by subtracting carbon bound to  $\text{CaCO}_3$  from total carbon. Soil nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ) were analyzed with a continuous flow AutoAnalyzer 3 (Seal Analytical, Inc.). Soil dissolved organic carbon (DOC) and dissolved organic nitrogen were measured with a Multi N/C 3000 TOC analyzer (Analytik Jena AG). Soil pH was

determined at a water-to-soil ratio of 5:1 (volume/weight) using a FE20 pH meter (FiveEasy).

## 2.3 | Aboveground and belowground plant communities

Plant survey was carried out at the peak of plant biomass as previously described in 2015 (Qi et al., 2021). Three subplots ( $0.5 \times 0.5$  m) were selected for each plot, and the corresponding plant species and coverage were counted in each subplot. The average coverage in three subplots represented the species coverage of a whole plot, and the total species richness represented the plant richness of a whole plot. A detailed description of aboveground plant biomass and root biomass estimation was described previously (Qi et al., 2021). The estimated aboveground plant biomass was considered as the aboveground net primary production (ANPP), and root biomass was considered as the belowground net primary production (BNPP).

## 2.4 | Soil respiration, soil $\text{CH}_4$ fluxes and ecosystem C fluxes

Soil aerobic respiration (soil  $\text{CO}_2$  flux) was automatically measured once an hour by the LI-8150 Multiplexer composed of LI-8100-104 long-term chambers (Li-Cor Inc.) and a LI-8100 Automated soil  $\text{CO}_2$  flux system (Wang et al., 2014). As for soil  $\text{CH}_4$  fluxes, gas samples were collected from all plots twice or three times per month between 9:00 a.m. and 12:00 p.m. on sunny days (Qi et al., 2021). Specifically, a stainless steel collar was inserted 10 cm into the soil in each plot and a static opaque chamber (40 cm in length  $\times$  40 cm in width  $\times$  40 cm in height) was used to collect gas samples from soil at this site (Yuan et al., 2021). At each measurement, 60 ml gas sample was collected in each plot and analyzed within 24 h using gas chromatography (Agilent 7890A; Agilent Technologies) to present a 1-day average flux. Soil  $\text{CH}_4$  fluxes were calculated using a linear regression between sampling time (0, 10, 20, 30 min) and gas concentration as the following equation:

$$F = \frac{M}{V_0} \frac{P}{P_0} \frac{T_0}{T} \frac{\Delta c}{\Delta t} H,$$

where  $F$  is the  $\text{CH}_4$  flux rate;  $M$  is the molecular mass of  $\text{CH}_4$ ;  $P$  is the air pressure at sampling time;  $T$  is the air temperature;  $V_0$ ,  $T_0$ , and  $P_0$  are the gas mole volume, standard air temperature, and atmospheric pressure, respectively;  $\Delta c/\Delta t$  is the slope of the linear regression of the gas concentration gradient along time; and  $H$  is the height of the chamber (Yuan et al., 2021).

Similar with soil  $\text{CH}_4$  fluxes, ecosystem C fluxes were also measured twice or three times per month between 9:00 a.m. and 12:00 p.m. on sunny days (Qi et al., 2021). We used a LI-6400 infrared gas analyzer (LI-COR, Inc.) with a transparent chamber (0.4 m in length  $\times$  0.4 m in width  $\times$  0.6 m in height) to measure net ecosystem

CO<sub>2</sub> exchange (NEE). Ecosystem respiration (ER) was measured using the similar method with the transparent chamber covered by an opaque cloth. Gross ecosystem production (GEP) was estimated as the difference between NEE and ER (Qi et al., 2021). In this study, the more negative NEE represents more CO<sub>2</sub> sequestration by terrestrial ecosystem. The annual average values of ecosystem C fluxes (GEP, NEE and ER) and soil CO<sub>2</sub> and CH<sub>4</sub> fluxes were calculated by taking the average of all the measurements of an environmental attribute in a year as follows:

$$C\_flux_{avg} = \frac{1}{n} \sum_{i=1}^n C\_flux_i,$$

$n$  is the number of measurements for one specific carbon flux in a year,  $C\_flux_i$  is every measure value of one specific carbon flux. Furthermore, the averages of soil temperature, soil moisture and C fluxes in spring (March–May), summer (June–August), autumn (September–November), and winter (December–February) were calculated to depict the seasonal variation of alpine grassland (Figure S1).

## 2.5 | DNA extraction and sequencing analysis

Soil DNA extraction and amplicon sequencing of bacterial and archaeal 16S rRNA gene and fungal internal transcribed spacer (ITS) region in this study were performed similarly to the method used for samples collected after the first 2 years of experiment (Qi et al., 2021). These sequences for 16S rRNA gene or ITS were processed to generate amplicon sequence variants (ASVs) by DADA2 (Callahan et al., 2016). Subsequently, 34,077 sequences for the 16S rRNA gene and 57,960 sequences for ITS were randomly selected for each sample to normalize samples to the same total read abundance. The sequencing depth of amplicon sequencing was estimated using rarefaction analyses. For 16S rRNA gene, the ASVs were taxonomically annotated by USEARCH 11 with Silva database (Quast et al., 2013) at a confidence cutoff of 50%. The taxonomic classification of the ITS representative sequences was performed by USEARCH 11 with UNITE database (Nilsson et al., 2019) at a confidence cutoff of 50%.

Metagenomic library for shotgun sequencing was prepared using a NEBNext® Ultra™ DNA Library Prep Kit and sequenced at the Magigene Biotechnology Co Ltd using Illumina HiSeq X Ten platform with a 2 × 150bp paired-end kit. A total of 4.01 billion reads were generated from all 36 samples. All sequencing reads were quality-trimmed with fastp (Chen et al., 2018) and Trimmomatic (Bolger et al., 2014). Trimmed sequences were analyzed by Nonpareil version 3.0 to estimate the sequencing coverage of microbial communities using Nonpareil R package (Rodriguez-R et al., 2018).

To determine microbial composition of soil communities, Metaxa 2.2 was applied to read pairs of metagenomic data from each sample to extract 16S rRNA and 18S rRNA gene sequence with default options (Bengtsson-Palme et al., 2015). Read pairs identified as

16S rRNA or 18S rRNA gene were further assigned to their closest matching lineages in Silva database with 99% identity by QIIME1.9.1.

## 2.6 | Functional gene annotation and abundance calculation

Gene calling and annotation of short nucleotide reads derived from metagenomic sequencing, and calculation of relative abundances of functional genes were performed using an internal pipeline Automatic Read-based Metagenomic Analysis Pipeline (ARMAP version 1.6, [http://zhoulab5.rccc.ou.edu/pipelines/ARMAP\\_web/job\\_submission.php](http://zhoulab5.rccc.ou.edu/pipelines/ARMAP_web/job_submission.php)). To obtain high-quality nucleotide reads, CD-HIT was used to remove duplicates in reads with an identity cutoff of 100% (Li & Godzik, 2006). Then, NGS QC Toolkit (version 2.3.3) was used for quality trimming and filtering (Patel & Jain, 2012). Poor-quality bases with quality score < 20 were trimmed from 3' end until the first base with a quality score ≥ 20. The trimmed reads were further filtered with an average score cutoff of 20, a length of > 120 bp, and ≤ 1 ambiguous base to acquire high-quality reads. High-quality reads were then converted to a fasta format and split into multiple partitions prior to the DIAMOND search (BLASTx; Buchfink et al., 2015) against NR database with  $E$  value cutoff of 1e-5, coverage cutoff of 0.5, and maximum target number of 50. The BLASTx outputs were submitted to MEGAN (Ultimate Edition, version 6.6; Huson et al., 2007) for taxonomic classification and function profiling with parameter of top percent of hits 10%, minimum score 50 and minimum support 1. The functional profiles of SEED subsystem and eggNOG were reported for each gene. The functional profiles were consolidated into the corresponding SEED categories or eggNOG terms as described previously (Huerta-Cepas et al., 2016; Overbeek et al., 2014). All of these genes were further mapped to E.C. numbers with the database of all E.C. annotated genes gathered from the Carbohydrate-Active enZymes (CAZy) website (<http://www.cazy.org/>; Lombard et al., 2014). Annotations to the CAZy database were consolidated further into six broad functional modules, including glycoside hydrolases, glycosyl transferases, carbohydrate esterases, polysaccharide lyases, auxiliary activities, and carbohydrate binding (Lombard et al., 2014). For each sample from metagenomic sequencing, the relative abundances of all functional genes were normalized by dividing the read numbers of functional genes with the total number of high-quality reads used as query for functional annotation.

## 2.7 | MAG assembly and genome binning

Metagenomic sequences for each sample were assembled individually using MEGAHIT (options: -meta) with an estimated insert size of ~300bp (Li et al., 2015). The resulting contigs ≥ 1.0 kbp were then binned using MetaBAT 2 (default options), MaxBin 2 (default options), and CONCOCT (default options) to recover microbial population genomes (metagenome-assembled genomes [MAGs]). MAGs were then refined using DASTools (version 1.3.4) with search engine

USEARCH (version 11.0.667). The completeness and contamination of MAGs were determined by CheckM (version 1.1.3) "lineage\_wf" pipeline (default options; Parks et al., 2015). A total of 212 bacterial and archaeal MAGs with more than 50% completeness and less than 10% contamination were retained in this study. For redundant MAGs obtained independently with fastANI value  $\geq 95\%$ , the MAG with the highest quality score or largest overall size was retained by dRep version 2.2.3 (Olm et al., 2017). Protein-encoding genes of all non-redundant MAGs were predicted with Prokka version 1.11.1, and the amino acid sequences were annotated by kofamscan against Kyoto Encyclopedia of Genes and Genomes (KEGG) Kofam database. Projection of the MAGs on the KEGG pathways was performed using iPath version 3 (<https://pathways.embl.de/>). Taxonomic assignments and phylogenetic inferences of the non-redundant MAGs were conducted using GTDB-Tk (version 1.5.0) with the genome taxonomy database (Chaumeil et al., 2020). The non-redundant set of MAGs was selected to visualize in ITOL (<https://itol.embl.de/>) for the overall Bacteria and Archaea trees. For the *Rhizobiales* tree, methylophilic bacterial species including one MAG obtained in this study were incorporated within the *Rhizobiales* based on GTDB-Tk analysis.

To calculate the relative abundances of MAGs, high-quality reads in each sample were mapped to the contigs of all non-redundant MAGs using Bowtie 2 (version 1.2.3), and SAMtools (version 0.1.19-44428cd) were used to sort and convert SAM files to BAM format. BamM "filter" was used to remove low-quality mappings (minimum identity 95%, minimum aligned length 75% for each query read). The coverage of each contig was calculated with BamM "parse" using "tpmean" mode, and then the coverage of each MAG was calculated using the average of contig coverages weighting each contig length.

## 2.8 | Statistical analyses

All statistical analyses were carried out using R software 3.1.1 unless otherwise indicated. Taxonomic alpha diversity indices (i.e., richness, Shannon index, inverse Simpson index, and Pielou's evenness) were calculated based on resampled Metaxa counts of metagenomics shotgun sequencing and ASV tables generated from amplicon sequencing using R vegan package (Dixon, 2003). Given the block design in this study, linear mixed-effects models (LMMs) were used to evaluate the effects of treatments on environmental attributes, ecosystem C fluxes, microbial diversity, and the relative proportions of CAZy modules or eggNOG biological process terms. The R packages lme4 (Bates, 2010) and lmerTest (Kuznetsova et al., 2017) were used to implement LMMs, with warming (0 for ambient temperature and 1 for warming) and precipitation alternation (-0.5 for decreased precipitation, 0 for normal precipitation, and 0.5 for increased precipitation) treatments and their interaction as fixed effects, and the block as a random intercept effect ( $y \sim \text{warming} \times \text{precipitation} + (1|\text{block})$ ). The regression coefficient of LMMs was calculated to quantify the effect size of different

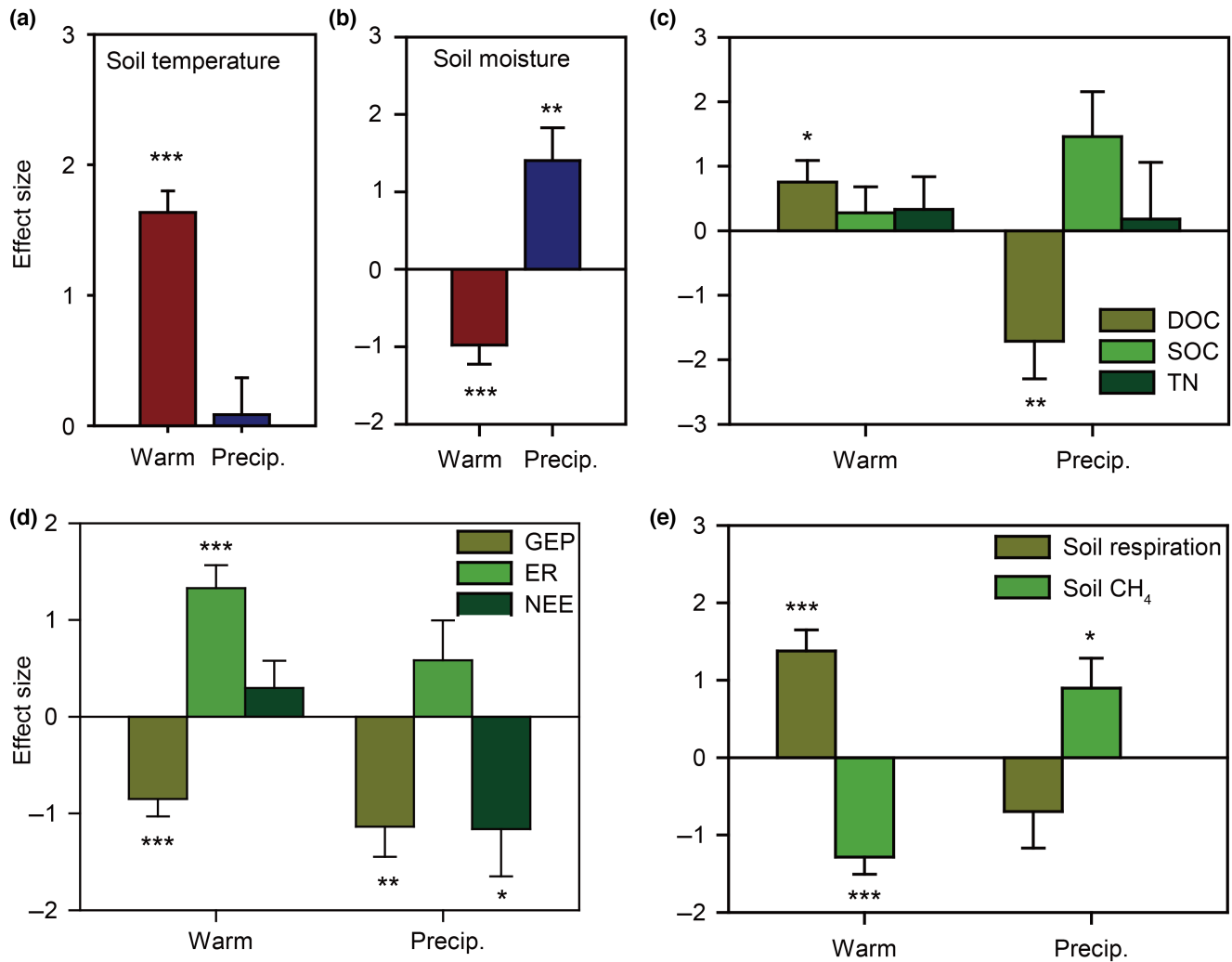
treatments. A Tukey's honest significant difference (HSD) test was used as a post hoc analysis to evaluate the statistical significance of differences among six treatments using *TukeyHSD* in R stats package (Johnston et al., 2019). Permutational multivariate analysis of variance (Adonis) was used to test the significance of treatment effects on phylogenetic and functional structure of soil microbial communities based on Bray-Curtis dissimilarity metrics using R vegan package. The dissimilarity of soil microbial communities under different treatments was determined by non-metric multidimensional scaling (NMDS) ordination analysis based on Bray-Curtis dissimilarity metrics. Canonical correspondence analysis (CCA) was used to determine the linkage between environmental attributes and microbial community structures, and the significance of CCA models was tested using *anova.cca* function in vegan package. Pearson correlations between environmental attributes and certain community measures (i.e., lineages or metagenome annotations) were conducted with the *cor.test* function in R stats package. Then, *P* values were adjusted using the Bonferroni algorithm in the stats package. All figures in this study were generated by using SigmaPlot 11.0.

## 3 | RESULTS

### 3.1 | Changes of environmental attributes, soil C fluxes, and ecosystem C fluxes under warming

After 4 years of experiment, soil temperature, soil moisture, soil aerobic respiration, soil CH<sub>4</sub> fluxes, and ecosystem C fluxes exhibited strong seasonal fluctuations under all treatment conditions, mostly reaching a peak in summer (Figure S1). Overall, warming more significantly altered environmental attributes than the treatment of altered precipitation did, as indicated by the LMMs in conjunction with Tukey's HSD test (Figure 1; Tables S1 and S2). On average, experimental warming significantly increased soil temperature (0–10 cm) by 2.0°C across the entire year (coefficient = 1.64,  $p < .001$ ; Figure 1a). Soil moisture was significantly decreased by warming (coefficient = -0.98,  $p < .001$ ; Figure 1b) but increased by precipitation alternation (coefficient = 1.40,  $p = .005$ ; Figure 1b). Consistent with our observation in the first 2 years of experiment (Qi et al., 2021), warming significantly (coefficient = -1.10,  $p < .001$ ) decreased plant richness, whereas plant coverage, ANPP, and BNPP were not affected by warming (Table S2). While ANPP was significantly increased by precipitation alternation (coefficient = 1.29,  $p = .004$ ). In addition, the concentration of soil DOC was significantly increased by warming (coefficient = 0.76,  $p = .036$ ) but decreased by precipitation alternation (coefficient = -1.71,  $p = .008$ ; Figure 1c).

Warming altered ecosystem C fluxes and soil C fluxes (Figure 1; Tables S1 and S2). Specifically, warming significantly stimulated GEP (coefficient = 0.85,  $p < .001$ ) and ER (coefficient = 1.33,  $p < .001$ ). Consequently, NEE was not significantly affected by warming (coefficient = -0.30,  $p = .315$ ; Figure 1d). Interestingly, significant but



**FIGURE 1** Warming effects on environmental attributes and ecosystem C fluxes (a–e). The effect sizes of warming (warm) and altered precipitation (Precip.) on soil surface temperature (a), soil moisture (b), soil carbon and nitrogen contents (c), in situ annual ecosystem C fluxes (d), and soil respiration and soil CH<sub>4</sub> fluxes (e). Soil carbon and nitrogen contents were showed as soil dissolved organic carbon (DOC), soil organic carbon (SOC), and total nitrogen (TN). Ecosystem C fluxes were estimated on the basis of the C amount from CO<sub>2</sub> emissions of whole ecosystem. ER, ecosystem respiration; GEP, gross ecosystem productivity; NEE, net ecosystem C exchange. Soil respiration was estimated on the basis of the C amount from soil CO<sub>2</sub> emissions. Soil CH<sub>4</sub> fluxes represented the C amount from soil CH<sub>4</sub> emissions. The estimated effect sizes are regression coefficients based on rescaled response attributes in the linear mixed-effects model. Significances are denoted by asterisks: \*\*\**p* < .001, \*\**p* < .01, \**p* < .05. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

opposite effects of warming were observed in the aerobic and anaerobic C processing, that is, soil CO<sub>2</sub> fluxes produced by soil aerobic respiration increased by 11.3% (coefficient = 1.38, *p* < .001), but soil CH<sub>4</sub> fluxes decreased by 93.8% under warming (coefficient = -1.28, *p* < .001; Figure 1e; Table S2). Actually, the methane oxidation is much larger than methanogenesis at this site, resulting in a more obvious net methane uptake under warming (Tables S1 and S2). Furthermore, there were no significant interaction effects of warming and precipitation alternation on NEE (coefficient = -1.08, *p* = .146) and soil CH<sub>4</sub> fluxes (coefficient = 0.93, *p* = .114; Table S2), despite significant precipitation effects were observed on them (coefficient = -1.16 to 0.90, *p* < .035; Figure 1d,e). These results indicated that precipitation alternation did not alter such warming effects on NEE and soil CH<sub>4</sub> fluxes.

### 3.2 | Sequencing information and soil microbial diversity assessment under warming

To reveal the shifts of soil microbial communities under warming and altered precipitation, amplicon sequencing of the 16S rRNA gene for bacteria and archaea, and the ITS for fungi, and metagenomic shotgun sequencing were used to analyze all 36 soil samples (Table S3). In the amplicon sequencing, an average of 91,000 ± 30,000 and 88,000 ± 15,000 sequence reads per sample were obtained for 16S rRNA gene and ITS region, respectively. All samples approached saturation in rarefaction curves analysis, indicating that the sequencing effort was sufficient to estimate the responses of these soil microbial communities to warming and altered precipitation (Figure S2). In the metagenomic shotgun sequencing,

a total of 1.2 Tb sequence data were generated from 36 samples (30.1–36.3 Gbp per sample, Table S3). The average coverage of the microbial communities estimated by Nonpareil 3 was 0.47–0.52 (Figure S3), suggesting that, beyond the sequencing effort achieved, there is 47%–52% likelihood that the additional sequences would be redundant with those already observed.

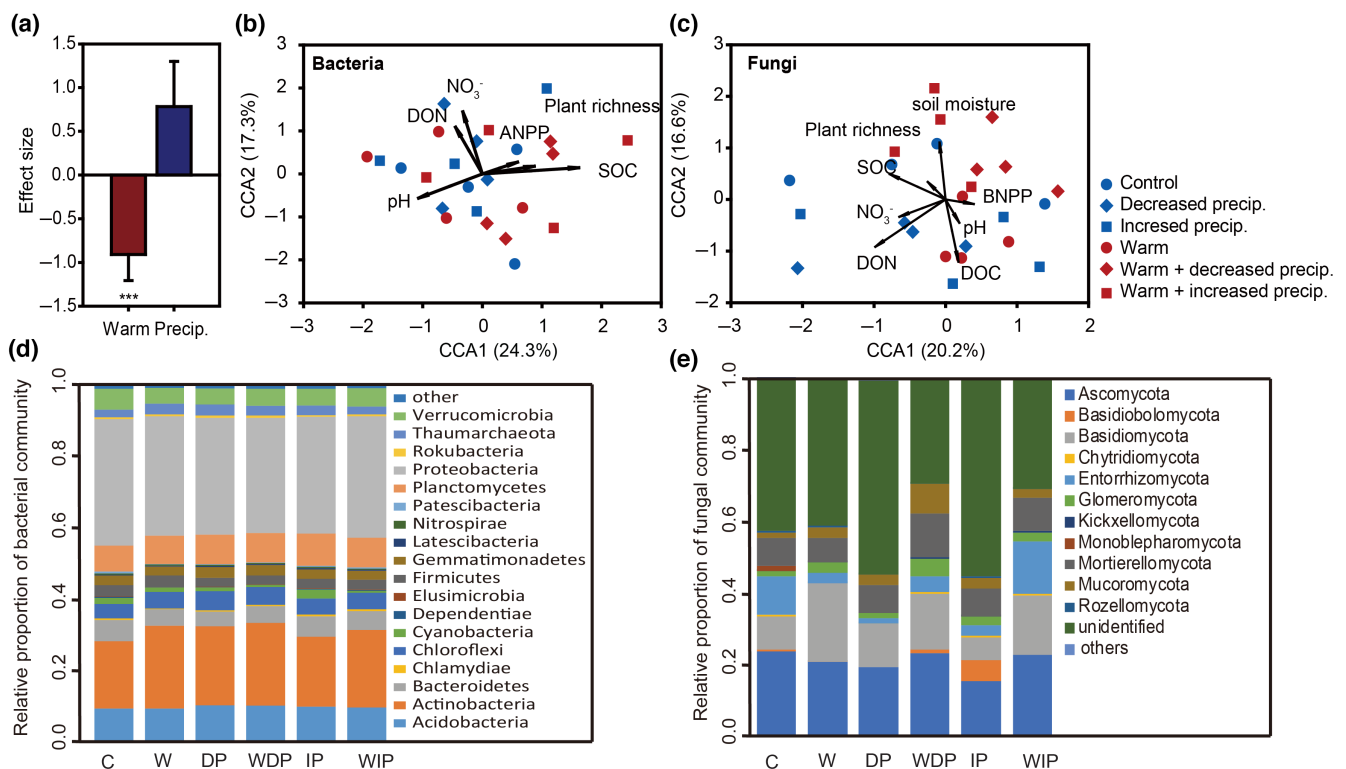
Soil microbial communities were also estimated by Metaxa2 with metagenomic shotgun sequencing, indicating that 2242 bacteria, 104 archaea, 420 fungi, and 134 protists survived in the soil of this site. Warming substantially reduced richness, Shannon–Weaver information, and inverse Simpson of soil microbial communities (coefficient =  $-0.91$  to  $-0.61$ ,  $p < .066$ ; Figure 2a; Table S4). However, no significant precipitation alternation or treatment interactive effects were observed on soil microbial diversities at this site (Table S4).

### 3.3 | Soil microbial community structures and their responses to warming

Contrast with no warming effects in the first 2 years of experiment (Qi et al., 2021), significant community-wide shifts were observed in both bacterial ( $p = .036$ ) and fungal ( $p = .027$ ) taxonomic structures

based on amplicon sequencing datasets after 4 years of warming (Table 1). Moreover, the phylum-level community structures estimated by Metaxa2 with metagenomic shotgun sequencing were also significantly altered by warming ( $p = .002$ ; Table 1). Precipitation alternation also exhibited significant effects on bacterial and fungal communities ( $p < .026$ ). However, there were no significant ( $p > .155$ ) interactions of warming and precipitation alternation on bacterial and fungal communities (Table 1). Although no obvious treatment-induced differences of the bacterial and fungal community structures were observed in the NMDS analysis (Figure S4), CCA indicated that the shifts of soil bacterial and fungal taxonomic structures were significantly ( $F = 1.087$ – $1.130$ ,  $p < .010$ ) shaped by several plant and soil attributes, including plant richness, ANPP, BNPP and soil pH,  $\text{NO}_3^-$ , SOC and soil moisture (Figure 2b,c). These results indicated that 4 years of warming led to significant community-wide shifts in taxonomic structures of soil microbial communities, and these shifts are strongly controlled by plant and soil environmental conditions.

Furthermore, warming differentially changed the relative abundances of microbial phyla (Figure 2d,e). In bacterial and archaeal community, the dominant phyla were *Proteobacteria* (32.7%–35.5%), *Actinobacteria* (18.9%–23.2%), *Acidobacteria* (9.4%–10.4%), and *Planctomycetes* (7.2%–9.1%) across all treatments



**FIGURE 2** Warming effects on bacterial and fungal diversity and community structure (a–e). (a) The effect sizes of warming (warm) and altered precipitation (Precip.) on soil microbial richness based on rescaled response attributes in the linear mixed-effects models. Significances are denoted by asterisks: \*\*\* $p < .001$ . (b, c) Canonical correspondence analyses (CCA) of bacterial (b) and fungal (c) communities with environmental attributes. Bacterial and fungal structures of microbial communities were significantly shaped by soil organic carbon (SOC), soil dissolved organic carbon (DOC) and organic nitrogen (DON), soil nitrate ( $\text{NO}_3^-$ ), soil pH, soil moisture, plant richness, and aboveground and belowground net primary production (ANPP and BNPP). (d, e) Taxonomic composition of bacterial (d) and fungal (e) communities at phylum level under different treatments. Phyla with  $<0.1\%$  abundances were summarized in the group of other. Statistical test of treatment effects for each phylum was listed in Table S5. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

**TABLE 1** Significance tests of the effects of warming and altered precipitation on microbial community structures with permutational multivariate analysis of variance

	Warm		Precipitation		Warm × precipitation	
	R <sup>2</sup>	p	R <sup>2</sup>	p	R <sup>2</sup>	p
16S rRNA gene amplicon	.034	<b>.036</b>	.076	<b>.006</b>	.056	.163
ITS amplicon	.040	<b>.027</b>	.071	<b>.026</b>	.060	.155
Plyla composition by Metaxa	.057	<b>.006</b>	.071	.077	.055	.392
SEED categories	.094	<b>.026</b>	.062	.217	.083	.152
NOG terms	.078	<b>.001</b>	.138	<b>.001</b>	.140	<b>.001</b>
CAZy families	.100	<b>.016</b>	.057	.267	.089	.119

Note: Permutational multivariate analysis of variance (Adonis) was used to test the effects of warming and altered precipitation based on Bray–Curtis dissimilarity metrics. Numerical values represent probability scores (i.e., R<sup>2</sup> values and p values) from each test. Significant effects (p ≤ .05) were highlighted in bold text.

Abbreviations: CAZy, Carbohydrate-Active enZymes; ITS, internal transcribed spacer.

(Figure 2d). The relative abundance of *Actinobacteria* was significantly increased by warming (coefficient = 0.74, p = .014; Table S5). Whereas warming significantly decreased the relative abundance of *Cyanobacteria* (coefficient = -0.66, p = .032), *Chlamydiae* (coefficient = -0.62, p = .040), *Nanoarchaeaeota* (coefficient = -0.66, p = .018), and *Zixibacteria* (coefficient = -0.89, p = .005). However, compared with warming treatment, fewer abundant bacterial phyla were significantly affected by altered precipitation treatment (Table S5). The dominant fungal phyla across all samples were *Ascomycota* (15.60%–23.79%), *Mortierellomycota* (6.60%–12.49%), and *Basidiomycota* (6.40%–21.68%; Figure 2e). Warming significantly or marginally significantly increased the relative abundance of *Basidiomycota* (coefficient = 0.58, p = .094), *Chytridiomycota* (coefficient = 0.62, p = .065), and *Glomeromycota* (coefficient = 0.59, p = .044; Table S5).

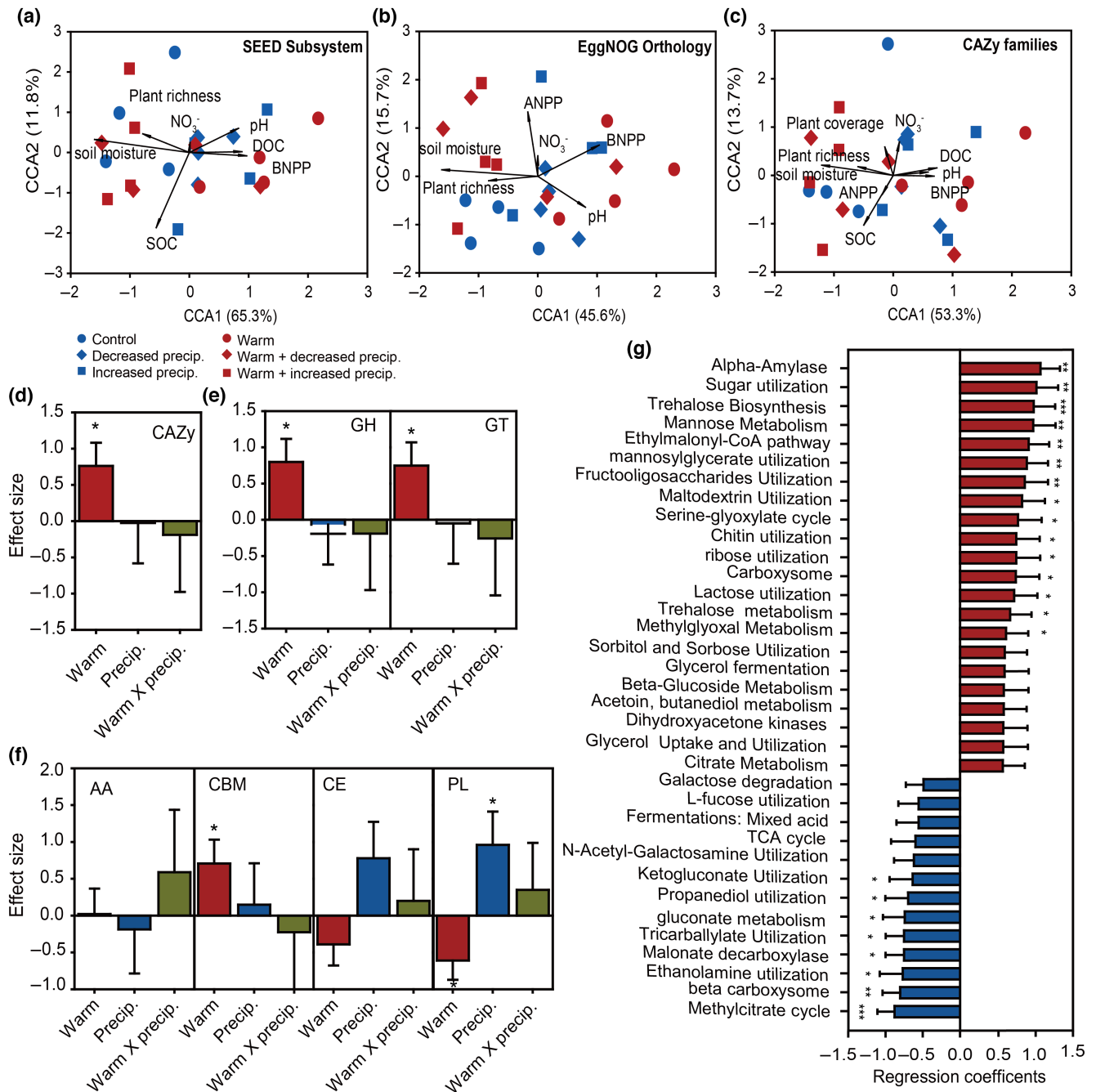
### 3.4 | Shifts of microbial C decomposition and methane metabolism under warming

Experimental warming also significantly shifted functional gene structures of microbial community, annotated as SEED categories (p = .026), EggNOG Orthology (NOG) terms (p = .001), and CAZy families (p = .016; Table 1). Contrast with warming, the effects of altered precipitation on functional gene structures of microbial community were negligible and insignificant (Figure S5), with only significant precipitation effect observed in NOG terms (p = .001; Table 1). Furthermore, the functional shifts of microbial community were tightly linked to plant and soil attributes (i.e., plant richness, BNPP, soil pH, moisture, and NO<sub>3</sub><sup>-</sup>) as revealed by CCA analysis (Figure 3a–c). These results indicated that warming significantly shifted soil microbial metabolic potentials more than altered precipitation.

Consistently, warming significantly increased the relative abundance of functional genes involved in carbohydrate metabolism and binding (p < .001; Table S6), as indicated by CAZy reference

annotation (Figure 3d). Specifically, warming significantly increased the relative abundances of glycoside hydrolase genes and glycosyl-transferase genes compared with corresponding controls (p < .041, Figure 3e). Meanwhile, functional genes encoding for carbohydrate binding were significantly enriched under warming (p < .037, Figure 3f), though negative effects of warming on polysaccharide lyase genes were observed in this study. However, altered precipitation exhibited no significant effects on most of CAZy families, except for polysaccharide lyase genes (Table S6). These results were generally consistent with SEED categories involving in the carbohydrate metabolism. There were 67 of 116 SEED carbohydrate subcategories having higher abundances in the warmed microbial communities than the corresponding controls (Table S7). Among these SEED subcategories, 15 were significantly stimulated by warming (p < .05) but only 8 were significantly (p < .05) suppressed by warming (Figure 3g).

There are 25 genes involved in methanogenesis and methane oxidation identified in the SEED category annotation (Table S8). The subcategory of soluble methane monooxygenase (sMMO) for methane oxidation were significantly enhanced by warming (Figure 4a), whereas particulate methane monooxygenase and methanogenesis from CO<sub>2</sub>, acetate, methanol, and methylated compounds remained unchanged by warming (Figure 4a,b). Consistently, *smoA* gene encoding for methane monooxygenase component A was significantly (coefficient = 0.96, p = .002) stimulated by warming (Figure 4c; Table S8). For 21 methanogenesis genes, 15 had lower gene abundances under warming (Table S8). Among them, *fmdA* gene encoding for formylmethanofuran dehydrogenase subunit A was significantly (coefficient = 0.99, p = .001) decreased by warming (Figure 4c). Furthermore, warming-induced shifts of sMMO genes significantly correlated with soil temperature and moisture (R<sup>2</sup> = .24–.55, p < .049, Figure 4d,e), suggesting that warming-induced decline of soil moisture and increase of soil temperature greatly contributed to the shifts of methane oxidation genes. Consistently, significant correlations of soil CH<sub>4</sub> fluxes with soil temperature (r = -.61, p < .001) and moisture (r = .90, p < .001; Figure S6) were observed in this

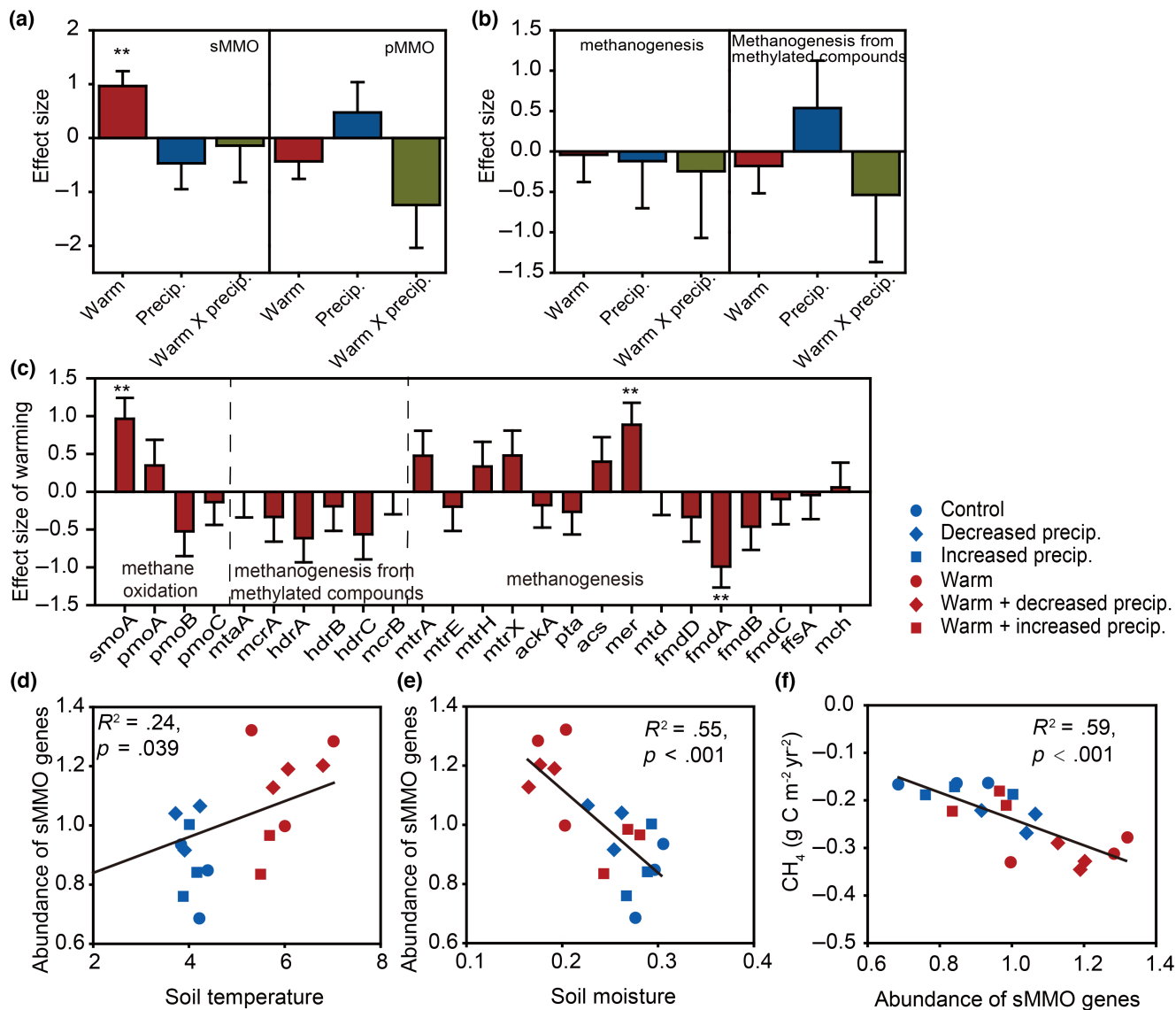


**FIGURE 3** Functional shifts of soil community structure and carbohydrate metabolism genes under warming and altered precipitation treatments. (a–c) Canonical correspondence analyses (CCA) of SEED category (a), EggNOG Orthology (b), and carbohydrate-active enzymes (CAZy) family (c) annotations with environmental attributes. The abbreviations of environmental attribute are the same as Figures 1 and 2. (d–f) the effect sizes of warming (warm), altered precipitation (Precip.) and their interaction on all CAZy annotations (d), and CAZy modules (e, f). AA, auxiliary activities; CBM, carbohydrate binding; CE, carbohydrate esterases; GH, glycoside hydrolases; GT, glycosyl transferases; PL, polysaccharide lyases. (g) The effect sizes of warming on SEED subcategories of carbohydrate metabolism. The estimated effect sizes are regression coefficients based on rescaled response attributes in the linear mixed-effects models. Significances are denoted by asterisks: \*\*\* $p < .001$ , \*\* $.001 \leq p < .01$ , \* $.01 \leq p < .05$ . More information about SEED subcategories of carbohydrate metabolism were summarized in Table S7. [Colour figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

study. Meanwhile, strong correlations between the abundance of *sMMO* genes and soil CH<sub>4</sub> fluxes were observed ( $R^2 = .59$ ,  $p < .001$ , Figure 4d,e). These results suggested that warming enhanced soil methane uptake by simultaneously stimulating methane oxidation and weakening methanogenesis.

### 3.5 | Soil MAGs and their metabolic potentials in alpine grassland

To further identify abundant microbial lineages involving in the degradation of organic matter and methane uptake in response



**FIGURE 4** Shifts of microbial methane oxidation and methanogenesis under warming and altered precipitation treatments. (a, b) The effect sizes of warming (warm), altered precipitation (Precip.) and their interaction on SEED subcategories for methane monooxygenase (a) and methanogenesis (b). Methane monooxygenase included soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO). (c) The effect sizes of warming on functional genes for methane monooxygenase and methanogenesis in the SEED category annotations. The estimated effect sizes are regression coefficients based on rescaled response attributes in the linear mixed-effects models. Significances are denoted by asterisks: \*\*.001 ≤ p < .01, \*.01 ≤ p < .05. The full names of the genes in this plot are listed in Table S8. (d–f) The relative abundance of soluble methane monooxygenase (sMMO) gene correlated with soil temperature (d), moisture (e) and soil CH<sub>4</sub> fluxes (f). Pearson correlation coefficients (R<sup>2</sup>) and p values from linear regressions are shown in each plots. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

to warming, MAGs were recovered from metagenomic shotgun sequencing data in this study. Metagenome assembly and differential coverage binning yielded 212 MAGs with >50% completeness and <10% contamination (Tables S9 and S10). After deduplication of highly similar MAGs, 96 non-redundant MAGs were obtained, with 70 bacterial and 26 archaeal genomes (Table S11). Phylogenetic trees inferred from concatenated sets of single-copy marker genes (120 bacterial and 122 archaeal genes) indicated that the recovered MAGs spanned 10 bacterial and archaeal phyla (Figure 5a). Among bacterial MAGs, there were 28 *Actinobacteria* MAGs, 19 *Acidobacteria*

MAGs, and 17 *Proteobacteria* MAGs. All 26 archaeal MAGs belonged to the phylum *Thermoproteota* (Figure 5a). These MAGs possessed genes involved in the catabolism of various organic matter substrates, such as starch, glycosaminoglycan, and cutin (Figure 5b; Table S12). Interestingly, one bacterial MAG (WP3210.05) matched closely to the methylotrophic genus *Methyloceanibacter* (80.8% average nucleotide identity [ANI]) with GTDB-Tk (Figure 5c), which was first reported to oxidize methane in ocean sediments (Vekeman et al., 2016). Similarly with *Methyloceanibacter*, methane oxidation genes (i.e., methanol dehydrogenase) were identified in this methylotrophic MAG



soil C fluxes. Altogether, these results suggest that specific microbial populations and functional traits for different carbon processes could respond to climate warming with differential influences on soil greenhouse gas emissions (Lee et al., 2012; Woodcroft et al., 2018).

Soil temperature increases and moisture decreases as a result of experimental warming lasted all seasons and were moderately uniform among all precipitation levels in this study. Such soil microclimate changes will undoubtedly change numerous aspects of alpine grassland ecosystem, such as plant richness, which could result in indirect effects of warming on soil microbial community functional traits (Johnston et al., 2019; Zhang et al., 2015, 2016). Thus, the magnitude and direction of soil microbial responses to warming could vary considerably along time (Elberling et al., 2013; Guo et al., 2018). Indeed, more significant community-wide shifts were observed in both bacterial and fungal community structures after 4 years of warming than after the first 2 years (Qi et al., 2021). Such larger microbial responses to warming with time were due to a lag between physiological responses and corresponding community changes at the DNA level, which was also observed in the soils of tundra and temperate grassland (Guo et al., 2018; Johnston et al., 2019).

Significant changes in community functional structure were also observed after 4 years of warming. This included the increased proportion of community functional genes involved in carbohydrate metabolism under warming, including glycoside hydrolase, glycosyltransferase, and carbohydrate binding. Meanwhile, there are more SEED carbohydrate subcategories significantly stimulated than suppressed by warming. The similar changes of carbohydrate metabolism were obtained in the 2 years of experiment, which were related to the increased soil aerobic respiration (Qi et al., 2021). These results indicated that the warming-stimulated microbial functional gene traits for C decomposition and soil respiration are consistent with earlier observations, but also imply an ongoing change a long time. MAGs recovered from metagenomes of Tibetan alpine grassland is barely reported. Recently, one study reported only 20 MAGs from soil samples (>4500 a.s.l.) in the Tibetan Alpine Grassland (Wei et al., 2022). In our study, 96 non-redundant MAGs were obtained from all samples. Catabolism pathways of various organic matter substrates were identified in these MAGs, which reflected the diversity of soil organic matter degradation in the ecosystem of alpine grassland in spite of no significant treatment effects on these MAGs observed (Johnston et al., 2019; Louca et al., 2016; Woodcroft et al., 2018).

The increase in soil aerobic respiration under warming did not necessarily mean a net ecosystem carbon loss, since warming-stimulated plant carbon fixation may offset the carbon loss by soil aerobic respiration (Heimann & Reichstein, 2008; Xu et al., 2016). In our study, both GEP and ER were significantly stimulated by warming, resulting in no changes in NEE. Thus, the C storage of the whole alpine grassland ecosystem at this site maintains unaffected by warming-induced CO<sub>2</sub> flux changes. However, in addition to affecting processes of soil CO<sub>2</sub> production, warming may influence ecosystem C storage by changing soil CH<sub>4</sub> metabolism (Li et al., 2020; Schuur & Mack, 2018). Soil CH<sub>4</sub> fluxes in various grasslands on the

Tibetan Plateau can be decreased, increased or showed no significant change under warming (Yang et al., 2014; Zhao et al., 2017), but a recent meta-analysis indicated that the entire alpine grasslands can act as a CH<sub>4</sub> sink under climate warming scenarios (Li et al., 2020). Consistently, our study indicated that experimental warming significantly stimulated CH<sub>4</sub> uptake by 93.8%. Therefore, warming-enhanced soil CH<sub>4</sub> uptake could enlarge the carbon sink capacity of the alpine grassland ecosystem. Given that a molar basis CH<sub>4</sub> is about 20 times more effective as a greenhouse gas than CO<sub>2</sub> (Beerling et al., 2009), alpine grasslands may play huge roles in mitigating climate warming.

The observed increase in soil CH<sub>4</sub> uptake under warming could be due to the decline of soil moisture more suitable for methane oxidation, rather than for methanogenesis (Lee et al., 2012; Yang et al., 2018). It is well known that soil CH<sub>4</sub> fluxes are determined by the balance between CH<sub>4</sub> production from methanogens (Christiansen et al., 2015) and CH<sub>4</sub> oxidation from methanotrophs (Prabhu Nath, 2013). The lower soil moisture under warming could lead to higher potential redox conditions, in which microbes degraded SOC by gradually using electron acceptors with higher redox potentials, leading to conditions less suitable for methanogenesis (Johnston et al., 2019; Tano et al., 2020). Meanwhile, soil oxygen content could increase with the decline of soil moisture, resulting in more favorable conditions for methane oxidation (Li et al., 2020). In the alpine wetland near our study site, typical methanotrophic genera *Methylobacter* and *Methylosarcina* were significantly stimulated by warming (Zhang et al., 2020). One bacterial MAG matched closely to the marine methylotrophic genus *Methyloceanibacter* was identified in our study. Other study showed the *Methyloceanibacter* was also found in the other terrestrial ecosystems (Yasuda et al., 2020), implying that *Methyloceanibacter* may play key roles in soil CH<sub>4</sub> oxidation of Tibetan alpine grassland. Consistently, the abundance of methanogenic genes (i.e., *fmdA*) was significantly decreased by warming, whereas methanotrophic genes (i.e., *smoA*) were significantly increased by warming at this site. The abundance of *sMMO* genes was highly related to soil moisture and temperature. Furthermore, the increase in the relative abundance of these methane oxidation genes corresponded to increased CH<sub>4</sub> uptake at this site due to our experimental warming. The similar responses of methane oxidation genes to warming were also observed in a recent study of Tibetan alpine grassland (Li et al., 2020). These results indicated that the changes of microbial populations and functional gene traits in response to warming could explain the changes of soil C fluxes. Thus, it is possible to improve our predictive ability for projecting future SOC changes via a better understanding of microbial mechanisms in response to climate changes (Cavichchioli et al., 2019; Guo et al., 2020).

#### AUTHOR CONTRIBUTIONS

All authors contributed intellectual input and assistance to this study. Research questions and experimental strategy were developed by Jin-Sheng He, Yunfeng Yang, and Xue Guo. Field management was carried out by Hao Wang. Sample collection, DNA preparation, and sequencing analysis were carried out by Qi Qi, Jianshu Zhao,

Renmao Tian, Changyi Xie, Qun Gao, and Tianjiao Dai. Soil chemical analyses were carried out by Yufei Zeng and Tianjiao Dai. Various statistical analyses were carried out by Qi Qi, Jianshu Zhao, and Xue Guo. Assistance in data interpretation was provided by Konstantinos T. Konstantinidis, Yunfeng Yang, and Jizhong Zhou. The paper was written by Xue Guo and Qi Qi with the help from Jianshu Zhao and Renmao Tian.

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## CONFLICT OF INTEREST

The authors declare no competing interests.

## DATA AVAILABILITY STATEMENT

DNA sequences of 16S rRNA gene and ITS amplicons and raw shotgun metagenomics sequences were available in NCBI Sequence Read Archive under project no. PRJNA797887. All other relevant data are available in Supplementary Information. The data that support the findings of this study are openly available in "Metadata of the Haibei Station" at <https://doi.org/10.5061/dryad.9s4mw6mks>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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