

Warming and altered precipitation independently and interactively suppress alpine soil microbial growth in a decadal-long experiment

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Abstract

Warming and precipitation anomalies affect terrestrial carbon balance partly through altering microbial growth and death in soil. However, little is known about how such eco-physiological traits responds to simultaneous regime shifts in temperature and precipitation. We used the ^{18}O -water quantitative stable isotope probing approach to estimate bacterial growth in alpine meadow soils of the Tibetan Plateau after a decade of warming and altered precipitation manipulation *in situ*. Our results showed that the growth of major taxa was suppressed by either individual or interactive global change factors, eliciting 40-90% of growth reduction of whole community. The antagonistic interactions of warming and altered precipitation on population growth were common (~70% taxa), represented by the weak antagonistic interactions of warming and drought, and the neutralizing effects of warming and wet. These results are important to understand and predict the soil microbial dynamics in alpine meadow ecosystems suffering from multiple climate change factors.

eLife assessment

This **valuable** study addresses the long-term effect of warming and altered precipitation on microbial growth, as a proxy for understanding the impact of global warming. While the methods are **compelling** and the evidence supporting the claims is **solid**, additional analysis of the data would strengthen the study, which should be of broad interest to microbial ecologists and microbiologists.

Global climate change is threatening multi-dimensional ecosystem services, such as terrestrial primary productivity, soil carbon storage, and microbial diversity (Jansson & Hofmockel, 2020 [DOI](#); Walker *et al.*, 2022 [DOI](#); Zhou *et al.*, 2022 [DOI](#)), especially in high-elevation ecosystems (Ma *et al.*, 2017 [DOI](#); Liu *et al.*, 2018 [DOI](#)). Of these, the effects of global climate change on processes related to soil nutrient cycling and carbon storage should receive more extensive attention, because carbon loss will have positive feedbacks on climate system, and further reinforce/diminish the net impact on ecosystem functioning (Jansson & Hofmockel, 2020 [DOI](#)). Microbial growth, the critical population dynamics parameter, serves as the major engine of soil organic carbon (SOC) turnover and further dominates the feedback on climate (Hicks *et al.*, 2022 [DOI](#); Sokol *et al.*, 2022 [DOI](#)). However, the response of decomposer growth rates to climate regime shifts may be strongly idiosyncratic, varying among taxa, thus making predictions at the ecosystem level difficult. Quantitative estimates of trait-based responses of microbes to multiple climate factors is critical for improved biogeochemical models and predicting the feedback effects to global change.

Climate warming and precipitation regime shift (reduced precipitation, hereafter ‘drought’, or enhanced precipitation, hereafter ‘wet’) can influence soil microbial physiological activities directly or indirectly (Schimel, 2018 [DOI](#); Jansson & Hofmockel, 2020 [DOI](#); Purcell *et al.*, 2022 [DOI](#); Sokol *et al.*, 2022 [DOI](#)). The Tibetan Plateau is considered among the most sensitive ecosystems to climate change (Liu *et al.*, 2018 [DOI](#)). In alpine regions, warming can alleviate low temperature limitations to enzymatic activity, stimulating SOC mineralization and microbial respiration (Dieleman *et al.*, 2012 [DOI](#); Streit *et al.*, 2014 [DOI](#)). The evidence is overwhelming that long-term warming reduces soil organic carbon pools and exacerbates carbon limitation of soil microbes, causing a negative effect on microbial growth and eco-physiological functions (Jansson & Hofmockel, 2020 [DOI](#); Melillo *et al.*, 2017 [DOI](#); Purcell *et al.*, 2022 [DOI](#); Streit *et al.*, 2014 [DOI](#)). Altered precipitation, particularly drought or heavy precipitation events, also tends to negatively influence soil processes and biodiversity (Oppenheimer-Shaanan *et al.*, 2022 [DOI](#); Yuan *et al.*, 2017 [DOI](#)). Precipitation fluctuation is expected to be the major consequence of future climate change in mesic grassland ecosystems, constraining microbial physiological performance and functions (Cook Benjamin *et al.*, 2015; McHugh *et al.*, 2017 [DOI](#); Schimel, 2018 [DOI](#)). Increased frequency and magnitude of precipitation events could cause microbial species loss by ‘filtering out’ the taxa with low tolerance to increased soil moisture and drying-rewetting (Evans & Wallenstein, 2014 [DOI](#)). In addition, higher mean annual precipitation (MAP) triggers an increase in SOC decomposition (Zhou *et al.*, 2022 [DOI](#)), which could cause a negative effect on microbial growth in long term. As a result of these negative consequences on the microbe-associated processes in terrestrial ecosystems, we propose the hypothesis that microbial taxa in soil exhibit reduced growth rates after the long-term warming and altered precipitation regimes (i.e., drought or wet) in alpine meadow soils.

As temperature and precipitation are of particular relevance, the interactive effects of warming and altered precipitation remain largely illusive, especially on the population dynamics of soil microbes (Zhu *et al.*, 2016 [DOI](#); Song *et al.*, 2019 [DOI](#)). Drought limits the resistance of the entire system to warming (Hoepfner & Dukes, 2012 [DOI](#)). Higher evapotranspiration in a warmer world will result in chronically lower average soil moisture, further reducing the eco-physiological performance of soil microbes (Reich *et al.*, 2018 [DOI](#)). Increasing drought also reduces the transfer of recently fixed plant carbon to soil bacteria and shifts the bacterial community towards slow growth and drought adaptation (Fuchslueger *et al.*, 2014 [DOI](#)). In contrast, enhanced precipitation relieved overall water limitations caused by warming and improved primary productivity and soil respiration (20-56%) (Fay *et al.*, 2008 [DOI](#)), suggesting positive warming effects under wet climate conditions (Song *et al.*, 2019 [DOI](#)). The responses of microbial population dynamics to multiple climate factors could be complex because i) the changed climate conditions can directly affect the eco-physiological characteristics of soil microbes and ii) indirectly affect microbial functioning by altering soil physicochemical properties (e.g., soil temperature, water availability, redox conditions, nutrient allocation) and aboveground plant composition (Qi *et al.*, 2022 [DOI](#); Yang *et al.*, 2021 [DOI](#)). Previous

studies have demonstrated that additive effects were rare and the net effects of biological responses (both aboveground and belowground) declined with increasing numbers of manipulated factors, suggesting antagonistic interactions between concurrently multiple global change drivers (Dieleman *et al.*, 2012 [↗](#); Leuzinger *et al.*, 2011 [↗](#); Sheik *et al.*, 2011 [↗](#)). As the complexity of the interaction between climate factors, we further pose the hypothesis that the interactive effects between warming and altered precipitation on microbial population growth rates are not simply additive.

Here, we performed the ^{18}O -quantitative stable isotope probing (^{18}O -qSIP) to estimate soil bacterial growth response to a decade of warming and altered precipitation manipulation on the alpine grassland of the Tibetan Plateau (**Fig. 1A** [↗](#)). We focused on the single-factor and interactive effects of warming and precipitation regimes on the population-specific growth of soil bacteria. We classified the potential interaction types, i.e., additive, synergistic and antagonistic (weak antagonistic, strong antagonistic and neutralizing) interactions between two climate factors (**Fig. 1B** [↗](#)) by using the effect sizes and Hedges' *d* (an estimate of the standardized mean difference) (Côté *et al.*, 2016 [↗](#); Harpole *et al.*, 2011 [↗](#); Ma *et al.*, 2019 [↗](#); Yue *et al.*, 2017 [↗](#)). We also aim to determine the difference in the interaction types under various temperature and precipitation scenarios, as well as the potential key microbial taxa. These results are critical to understand and predict the eco-physiological responses of soil microorganisms to climate change in the sensitive ecosystems like the Tibetan Plateau.

Materials and methods

Study design and soil sampling

The warming-by-precipitation experiment was established in 2011 at the Haibei National Field Research Station of Alpine Grassland Ecosystem (37°37'N, 101°33'E, with elevation 3215 m), which is located on the northeastern Tibetan Plateau in Qinghai Province, China. The climate type is a continental monsoon with mean annual precipitation of 485 mm and the annual average temperature approximately -1.7°C . The high rainfall and temperature mainly occur in the peak-growing season (from July to August (Liu *et al.*, 2018 [↗](#))). The soils are Mat-Gryic Cambisols, with the average pH value of surface soil (0-10 cm) being 6.4 (Ma *et al.*, 2017 [↗](#)).

The experimental design has been described in previously (Ma *et al.*, 2017 [↗](#)). Briefly, experimental plots were established in an area of 50 m $\times$ 110 m in 2011, using a randomized block design with warming and altered precipitation treatments. Each block contained six plots (each plot was 1.8 m $\times$ 2.2 m), crossing two levels of temperature [ambient temperature (T^0), elevated temperature of top 5 cm layer of the soil by 2°C (T^+)], and three levels of precipitation [natural precipitation (nP, represents ambient condition), 50% reduced precipitation (-P, represents 'drought' condition) and 50% enhanced precipitation (+P, represents 'wet' condition)]. In the warming treatments, two infrared heaters (1,000 mm length, 22 mm width) were suspended in parallel at 150 cm above the ground within each plot. Rain shelters were used to control the received precipitation in the experimental plots. Four 'V'-shaped transparent polycarbonate resin channels (Teijin Chemical, Japan) were fixed at a 15° angle, above the infrared heaters, to intercept 50% of incoming precipitation throughout the year. The collected rainfall from the drought plots was supplied to the wet plots manually after each precipitation event by sprinklers, increasing precipitation by 50%. To control for the effects of shading caused by infrared heaters, two 'dummy' infrared heaters and four 'dummy' transparent polycarbonate resin channels were installed in the control plots. Each treatment had six replicates, resulting in thirty-six plots.

Soil samples were collected in August 2020. For each treatment, we randomly selected three out of the six plots, serving as three replicates, to sample the topsoil for qSIP incubation experiment. In each plot, three soil cores were randomly collected and combined as a composite sample. The

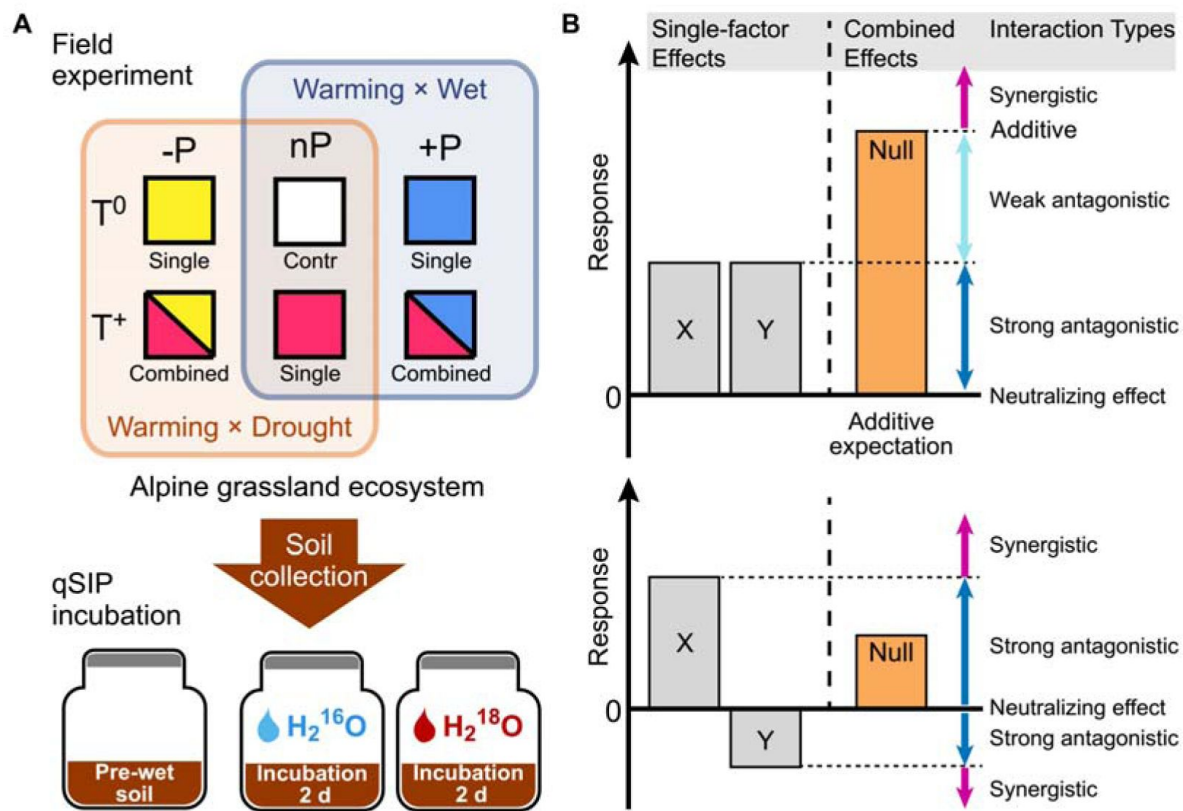


Fig. 1

Field experiment design for simulated warming and altered precipitation, qSIP incubation, and the growth responses of soil bacteria to changing climate regimes.

A To examine the effects of warming and altered precipitation on an alpine grassland ecosystem, two levels of temperature (T^0 , T^+), and three levels of precipitation (-P, nP, +P) were established in 2011. The soil samples were collected in 2020 and used for ^{18}O -qSIP incubation. **B** Potential interaction types between multiple climate factors. The diagram shows that two factors (X and Y) of warming and altered precipitation impact a biological response in the same direction (upper) or have opposing effects on when acting separately. Their combined effect could be additive, i.e. the sum of the two factor effects. Alternatively, the interaction types can be antagonistic or synergistic. Null model (we use the additive expectation as the null model here) provides the threshold for distinguishing between these interactions.

fresh soil samples were shipped to the laboratory and sieved (2-mm).

18O-qSIP incubation

We estimated the population-specific growth rates of active microbes by conducting a ^{18}O -water incubation experiment combined with DNA quantitative stable isotope probing (Fig. 1A). The incubations were similar to those reported in a previous study (Ruan *et al.*, 2023). Soil samples of ambient temperature treatments (including $\text{T}^0\text{-P}$, T^0nP , and $\text{T}^0\text{+P}$) were air-dried for 48 h at 16 °C (average temperature across the growth season), while the soil samples of warming treatments (including $\text{T}^+\text{-P}$, T^+nP , and $\text{T}^+\text{+P}$) were air-dried at 18 °C (increased temperature of 2 °C). A portion of the air-dried soil samples was taken as the pre-wet treatment. We incubated the air-dried soils (2.00 g) with 400 μl of 98 atom% H_2^{18}O (^{18}O treatment) or natural abundance water (^{16}O treatment) in the dark for 48 h by using sterile glass aerobic culture bottles (Diameter: 29 mm; Height: 54 mm). After incubation, soils were destructively sampled and stored at -80°C immediately. A total of 54 soil samples, including 18 pre-wet soil samples (6 treatments $\times$ 3 replicates) and 36 soil samples for 48h incubation (6 treatments $\times$ 3 replicates $\times$ 2 types of H_2O addition), were collected.

DNA extraction and isopycnic centrifugation

Total DNA from all the collected soil samples was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's instructions. The concentration of extracted DNA was determined fluorometrically using Qubit® DNA HS (High Sensitivity) Assay Kits (Thermo Scientific™, Waltham, MA, USA) on a Qubit® 4 fluorometer (Thermo Scientific™, Waltham, MA, USA). The DNA samples of 48 h incubation were used for isopycnic centrifugation, and the detail pipeline was described previously (Ruan *et al.*, 2023). Briefly, 3 μg DNA were added into 1.85 g ml^{-1} CsCl gradient buffer (0.1 M Tris-HCl, 0.1 M KCl, 1 mM EDTA, pH = 8.0) with a final buoyant density of 1.718 g ml^{-1} . Approximately 5.1 ml of the solution was transferred to an ultracentrifuge tube (Beckman Coulter QuickSeal, 13 mm $\times$ 51 mm) and heat-sealed. All tubes were spun in an Optima XPN-100 ultracentrifuge (Beckman Coulter) using a VTi 65.2 rotor at 177,000 g at 18 °C for 72 h with minimum acceleration and braking.

Immediately after centrifugation, the contents of each ultracentrifuge tube were separated into 20 fractions (~250 μl each fraction) by displacing the gradient medium with sterile water at the top of the tube using a syringe pump (Longer Pump, LSP01-2A, China). The buoyant density of each fraction was measured using a digital hand-held refractometer (Reichert, Inc., Buffalo, NY, USA) from 10 μl volumes. Fractionated DNA was precipitated from CsCl by adding 500 μl 30% polyethylene glycol (PEG) 6000 and 1.6 M NaCl solution, incubated at 37 °C for 1 h and then washed twice with 70% ethanol. The DNA of each fraction was then dissolved in 30 μl of Tris-EDTA buffer.

Quantitative PCR and Sequencing

Total 16S rRNA gene copies for DNA samples of all the fractions and the day-0 soils were quantified using the primers for V4-V5 regions: 515F (5'-GTG CCA GCM GCC GCG G-3') and 907R (5'-CCG TCA ATT CMT TTR AGT TT-3') (Guo *et al.*, 2018). Plasmid standards were prepared by inserting a copy of purified PCR product from soil DNA into *Escherichia coli*. The *E. coli* was then cultured, followed by plasmid extraction and purification. The concentration of plasmid was measured using Qubit DNA HS Assay Kits. Standard curves were generated using 10-fold serial dilutions of the plasmid. Each reaction was performed in a 25- μl volume containing 12.5 μl SYBR Premix Ex Taq (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.5 μl of forward and reverse primers (10 μM), 0.5 μl of ROX Reference Dye II (50 $\times$), 1 μl of template DNA and 10 μl of sterile water. A two-step thermocycling procedure was performed, which consisted of 30 s at 95°C, followed by 40 cycles of 5 s at 95°C, 34 s at 60°C (at which time the fluorescence signal was collected). Following

qPCR cycling, melting curves were conducted from 55 to 95°C with an increase of 0.5°C every 5 s to ensure that results were representative of the target gene. Average PCR efficiency was 97% and the average slope was -3.38, with all standard curves having $R^2 \geq 0.99$.

The DNA of pre-wet soil samples (unfractionated) and the fractionated DNA of the fractions with buoyant density between 1.703 and 1.727 g ml⁻¹ (7 fractions) were selected for 16S rRNA amplicon sequencing by using the same primers of qPCR (515F/907R). The fractions with density between 1.703 and 1.727 g ml⁻¹ were selected because they contained more than 99% gene copy numbers of each sample. A total of 270 DNA samples [18 total DNA samples of prewet soil + 252 fractionated DNA samples (6 treatments × 3 replicates × 2 types of water addition × 7 fractions)] were sequenced using the NovaSeq6000 platform (Genesky Biotechnologies, Shanghai, China).

The sequences were quality-filtered using the USEARCH v.11.0 (Edgar, 2010). In brief, sequences <370 bp and total expected errors> 0.5 were removed. Chimeras were identified and removed. Subsequently, high-quality sequences were clustered into operational taxonomic units (OTUs) using the UPARSE algorithm at a 97% identity threshold, and the most abundant sequence from each OTU was selected as a representative sequence. The taxonomic affiliation of the representative sequence was determined using the RDP classifier (Wang *et al.*, 2007). In total, 19,184,889 reads of the bacterial 16S rRNA gene and 6,938 OTUs were obtained. The sequences were uploaded to the National Genomics Data Center (NGDC) Genome Sequence Archive (GSA) with accession numbers CRA007230.

Quantitative stable isotope probing calculations

As ¹⁸O labeling occurs during cell growth via DNA replication, the amount of ¹⁸O incorporated into DNA was used to estimate the growth rates of active taxa. The density shifts of OTUs between ¹⁶O and ¹⁸O treatments were calculated following the qSIP procedures (Hungate *et al.*, 2015; Koch *et al.*, 2018). Briefly, the number of 16S rRNA gene copies per OTU in each density fraction was calculated by multiplying the OTU's relative abundance (acquisition by sequencing) by the total number of 16S rRNA gene copies (acquisition by qPCR). Then, the GC content and molecular weight of a particular taxon were calculated. Further, the change in ¹⁸O isotopic composition of 16S rRNA genes for each taxon was estimated. We assumed an exponential growth model over the course of the incubations, and average population growth rates within 48 h incubation were estimated. The absolute rate of growth is a function of the rate of appearance of ¹⁸O-labeled 16S rRNA genes. Therefore, the growth rate of taxon *i* was calculated as:

$$g_i = \ln \left(\frac{N_{TOTALit}}{N_{LIGHTit}} \right) \times \frac{1}{t} \quad (1)$$

where $N_{TOTALit}$ is the number of total gene copies for taxon *i* and $N_{LIGHTit}$ represents the unlabeled 16S rRNA gene abundances of taxon *i* at the end of the incubation period (time *t*). $N_{LIGHTit}$ is calculated by a function with four variables: $N_{TOTALit}$, molecular weights of DNA (taxon *i*) in the ¹⁶O treatment (M_{LIGHTi}) and in the ¹⁸O treatment (M_{LABi}), and the maximum molecular weight of DNA that could result from assimilation of H ¹⁸O (*M*) (Koch *et al.*, 2018). We further calculated the average growth rates (represented by the production of new 16S rRNA gene copies of each taxon per g dry soil per day) over each of the three time intervals of the incubation: 0-1, 0-3, and 0-6 d, using the following equation (Stone *et al.*, 2021):

$$\frac{dN_i}{dt} = N_{TOTALit} (1 - e^{-g_i t}) \times \frac{1}{t} \quad (2)$$

where *t* is the incubation time (day). All data calculations were performed using the qSIP package (<https://github.com/bramstone/qsip>) in R (Version 3.6.2) (Team 2006).

Single and combined effects of climate change factors

To address the effects of warming and altered precipitation on microbial growth rates, three single-factor effects (warming, 50% reduced precipitation only, and 50% enhanced precipitation only) and two combined effects (combined warming and reduced precipitation manipulation and combined warming and enhanced precipitation manipulation) were calculated by the natural logarithm of response ratio (lnRR), representing the response of microbial growth rates in the climate change treatment compared with that in the ambient treatment (Yue *et al.*, 2017). The lnRR for growth rates was calculated as:

$$\ln RR = \ln \left(\frac{X_t}{X_c} \right) \quad (3)$$

where X_t is the observed growth rates in climate treatment and X_c is that in control. Three lnRR values (i.e., three replicates) were calculated, and 95% confidence interval (CI) was estimated using a bootstrapping procedure with 1000 iterations (Ruan *et al.*, 2023). If the 95% CI did not overlap with zero, the effect of treatment on microbial growth is significant.

The interaction between warming and altered precipitation

Hedges' d , an estimate of the standardized mean difference, was used to assess the interaction effects of warming $\times$ drought (i.e. reduced precipitation) and warming $\times$ wet (i.e. enhanced precipitation), respectively (Yue *et al.*, 2017). Briefly, all six climate treatments were divided into two groups, warming combined with reduced precipitation scenario (Warming $\times$ Drought), and warming combined with enhanced precipitation scenario (Warming $\times$ Wet), by using the ambient temperature and precipitation treatment (T^0NP) as control (Fig. 1A). The interaction effect size (d_I) of warming $\times$ drought or warming $\times$ wet was calculated as:

$$d_I = \frac{(X_{AB} - X_A) - (X_B - X_C)}{2s} J(m) \quad (4)$$

where X_C , X_A , X_B and X_{AB} are one of three duplicate values of growth rates in the control, treatment groups of factor A, B, and their combination (AB), respectively. Total three d_I values were calculated in each comparison, and 95% CI was estimated using a bootstrapping procedure with 1,000 iterations. The s and $J(m)$ are the pooled standard deviation and correction term for small sample bias, respectively, which were calculated by the following equations:

$$s = \sqrt{\frac{(n_C - 1)s_C^2 + (n_A - 1)s_A^2 + (n_B - 1)s_B^2 + (n_{AB} - 1)s_{AB}^2}{n_C + n_A + n_B + n_{AB} - 4}} \quad (5)$$

$$J(m) = 1 - \frac{3}{4(n_C + n_A + n_B + n_{AB} - 4) - 1} \quad (6)$$

where n_C , n_A , n_B and n_{AB} are the sample sizes, and s_C , s_A , s_B and s_{AB} are the standard deviations in the control, experimental groups of A, B, and their combination (AB), respectively.

The interaction types between warming and altered precipitation were mainly classified into three types, i.e. Additive, Synergistic and Antagonistic, according to the single-factor effects and 95% CI of d_I . If the 95% CI of d_I overlapped with zero, the interactive effect of warming and altered precipitation was additive. The synergistic interaction included two cases: 1) the upper limit of 95% CI of $d_I < 0$ and the single-factor effects were either both negative or have opposite directions; 2) the lower limit of 95% CI of $d_I > 0$ and both single-factor effects were positive. The antagonistic interaction also included two cases: 1) the upper limit of 95% CI of $d_I < 0$ and both single-factor effects were positive; 2) the lower limit of 95% CI of $d_I > 0$ and the single-factor effects were either both negative or have opposite directions (Yue *et al.*, 2017). We further divided antagonistic interaction into three sub-categories: weak antagonistic interaction, strong antagonistic interaction, and neutralizing effect, by comparing the single-factor and combined effect sizes (Fig.

1B [↗](#)). The weak antagonistic interaction determined if the combined effect size was larger than the single-factor effect sizes, but smaller-than-additive. The strong antagonistic interaction determined if the combined effect size was smaller than the single-factor effect sizes but not equal to zero. The neutralizing effect represented the combined effect size is equal to zero, and at least one single-factor effect size is not equal to zero.

Statistical analyses

Uncertainty of growth rates (95% CI) was estimated using a bootstrapping procedure with 1,000 iterations (Ruan *et al.*, 2023 [↗](#)). The cumulative growth rates at the phylum-level were estimated as the sum of taxon-specific growth rates of those OTUs affiliated to the same phylum. Significant differences of bacterial growth rates for each group between climate treatments were assessed by two-way ANOVA in R (version 3.6.2). Phylogenetic trees were constructed in Galaxy /DengLab (<http://mem.rcees.ac.cn:8080> [↗](#)) with PyNAST Alignment and FastTree functions (Caporaso *et al.*, 2009 [↗](#); Price *et al.*, 2009 [↗](#)). The trees were visualized and edited using iTOL (Letunic & Bork, 2016 [↗](#)). To estimate the phylogenetic patterns of different interaction types, the nearest taxon index (NTI) was calculated by the “picante” package in R (version 3.6.2) (Webb *et al.*, 2002 [↗](#)). NTI with values larger than 0 and their *P* values less than 0.05 represent phylogenetic clustering. The *P* values of NTI between 0.05 and 0.95 represent random phylogenetic distributions. KO gene annotation of taxa was performed by PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), which predicted functional abundances based on marker gene sequences (Douglas *et al.*, 2020 [↗](#)). The marker genes related to carbon (C), nitrogen (N), sulfur (S), and phosphorus (P) cycling were selected according to the conclusions reported in previous documents (Dai *et al.*, 2019 [↗](#); Llorens-Marès *et al.*, 2015 [↗](#); Nelson Albright *et al.*, 2015 [↗](#)).

Results

Overall growth response of soil bacteria to warming and altered precipitation

Excess atom fraction ^{18}O value (**Fig. 2** [↗](#)) and the population growth rate of each OTU were calculated using the qSIP pipeline. Collectively, 1373 OTUs were identified as “ ^{18}O incorporators” (i.e. OTUs with growth rates significantly greater than zero) and used for subsequent data analyses. The maximum cumulative growth rates of the whole communities occurred in the ambient temperature and ambient precipitation condition ($T^0\text{nP}$), and all climate manipulations had negative effects on soil bacterial growth (**Fig. 3A** [↗](#)). Three single-factor manipulations, warming ($T^+\text{nP}$), 50% reduced (i.e. drought, $T^0\text{-P}$) and 50% enhanced precipitation (i.e. wet, $T^0\text{+P}$), exerted larger negative effects on the overall bacterial growth rates than the two combined-factor manipulations (i.e. $T^+\text{-P}$ and $T^+\text{+P}$), indicating the antagonistic interaction between warming and altered precipitation. We further determined the interaction types of warming $\times$ drought and warming $\times$ wet, according to the framework presented in **Fig. 1** [↗](#). Both drought and wet treatment showed strong antagonistic interactions with warming (**Fig. 3B** [↗](#)). Moreover, the combined effect size of wet and warming was larger than that of drought and warming, indicating a higher degree of antagonism of wet and warming than that of drought and warming.

Similar with the result of whole growth of bacterial community, the growth responses of the major bacterial phyla were also negatively influenced by single climate factors (**Fig. 3C** [↗](#) and **3D** [↗](#)). The antagonistic interactions between warming and altered precipitation were prevalent among the major phyla (except Bacteroidetes showed the additive interaction between drought and warming). We also found the significant larger combined effect sizes of warming $\times$ wet in the major phyla compared with that of warming $\times$ drought ($P < 0.05$), such as Actinobacteria,

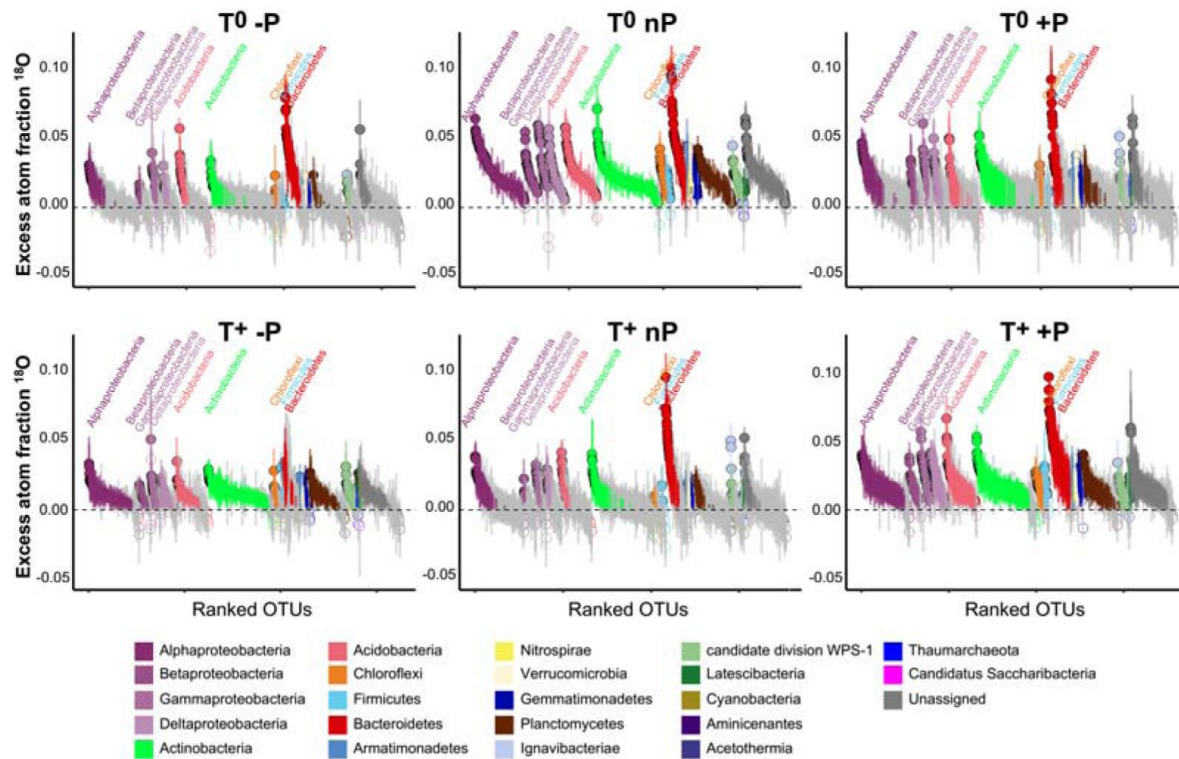


Fig. 2

Species-specific shifts of ^{18}O excess atom fraction (EAF- ^{18}O).

Bars represent 95% confidence intervals (CIs) of OTUs. Each circle represents an OTU and color indicates phylum. The open circles with gray bars represent OTUs with 95% CI intersected with zero (indicating no significant ^{18}O enrichment); Closed circles represent the OTUs enriched ^{18}O significantly, whose 95% CIs were away from zero (i.e., the OTUs had detectable growth).

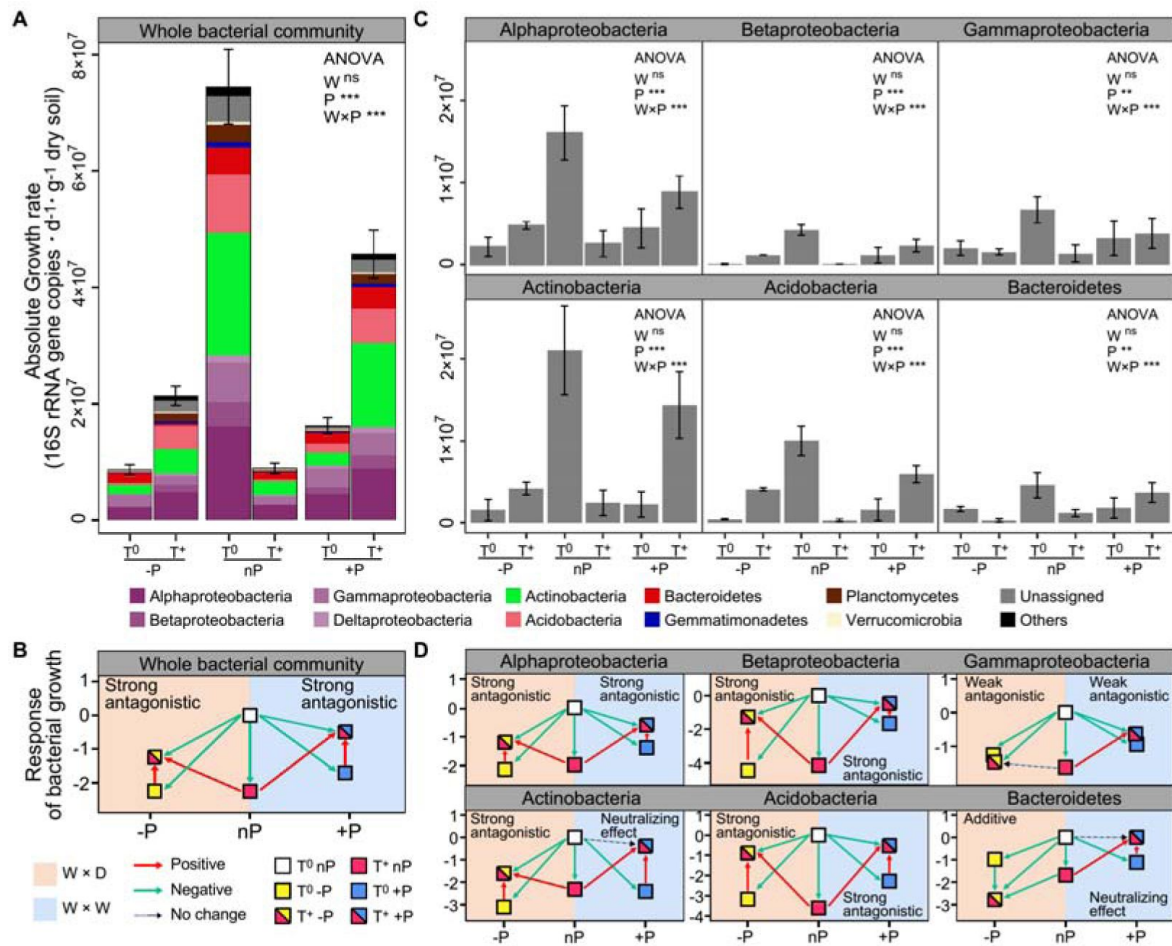


Fig. 3

Bacterial growth responses to climate change and the interaction types between warming and altered precipitation.

A The growth rates, and **B** responses of soil bacteria to warming and altered precipitation at the whole community level. **C** The growth rates, and **D** responses of the dominant bacterial phyla had similar trends with that of the whole community.

Bacteroidetes and Betaproteobacteria, indicating higher degree of antagonism. In particular, the effects of wet and warming neutralized each other, resulting the net effects became zero on the growth rates of the phyla Actinobacteria and Bacteroidetes.

The phylogeny for the species with different interaction types

We constructed a phylogenetic tree including all ^{18}O incorporators in all six climate treatments (Fig. 4A). The single-factor effects on the growth of incorporators tended to be negative (Fig. 4B): The individual warming treatment (T^+nP) reduced the growth rates of 75% incorporators, followed by the individual drought treatment (74% of incorporators) and individual wet treatment (67% of incorporators). The combined manipulations of warming and altered precipitation lowered the percentages of incorporators with negative responses compared with single factor manipulation, especially warming and enhanced precipitation manipulation (T^0+P , 43% of incorporators showed negative growth responses). Further, the interaction type of both warming $\times$ drought and warming $\times$ wet on the growth of most incorporators was antagonistic (70% and 69% of incorporators, respectively), while the proportion of incorporators subjected to weak antagonistic interaction and neutralizing effect varied between different warming and rainfall regimes (Fig. 4C). The weak antagonistic interaction on bacterial growth was dominant under the warming $\times$ drought conditions (41% of incorporators), while more incorporators (34%) whose growth subjected to neutralizing effect was found under the warming $\times$ wet conditions.

The phylogenetic distribution patterns of the incorporators subjected to different interaction types varied between different warming and rainfall regimes (Table S1). Overall, the incorporators whose growth was influenced by the antagonistic interaction of both warming $\times$ drought and warming $\times$ wet clustered at the phylogenetic branches ($NTI > 0$, $P < 0.05$), except the incorporators subjected to the weak antagonistic interaction of warming $\times$ wet. The incorporators subjected to the additive interaction of warming $\times$ drought clustered near the tips of the phylogenetic tree ($P < 0.05$), but randomly distributed under warming $\times$ wet scenario ($P = 0.116$). In addition, incorporators subjected to the synergistic interaction were phylogenetically random distribution under both warming $\times$ drought and warming $\times$ wet scenarios (both $P > 0.05$).

Higher degree of antagonism in the warming and wet scenario

We further assigned the antagonistic intensity to the five interaction types on a 5-point scale, from -1 to 3 for synergistic, additive, weak antagonistic, strong antagonistic and neutralizing effect, respectively (Fig. S1A), where the larger values represent higher degree of antagonism. Then, the overall antagonistic intensities of all incorporators under warming $\times$ drought and warming $\times$ wet scenarios were estimated by weighting the relative proportions of incorporators subjected to different interaction types (Fig. S1B). We found higher overall antagonistic intensity of warming $\times$ wet than that of warming $\times$ drought, contributing by a higher proportion of incorporators whose growth subjected to neutralizing effect (Fig. 4C and Fig. S1B).

Of the total 1373 incorporators, 1218 were shared in both warming $\times$ drought and warming $\times$ wet scenarios (Fig. 5A). That is, the difference in interactive effects between warming $\times$ drought and warming $\times$ wet we observed was due to a within-species change in growth response, rather than changes in species composition. Of these species identified in both warming $\times$ drought and warming $\times$ wet scenarios, 453 were assigned a higher degree of antagonistic interaction of warming $\times$ wet than that of warming $\times$ drought. Further, the growth of 215 incorporators were influenced by the weak antagonistic interaction of warming $\times$ drought, and neutralizing effect of warming $\times$ wet. The growth response of these 215 species could contribute mainly to the overall growth patterns observed in grassland bacterial community under warming and altered precipitation scenarios, because of the prevalence of weak antagonistic interaction of warming $\times$ drought and neutralizing effect of warming $\times$ wet (Fig. 4C).

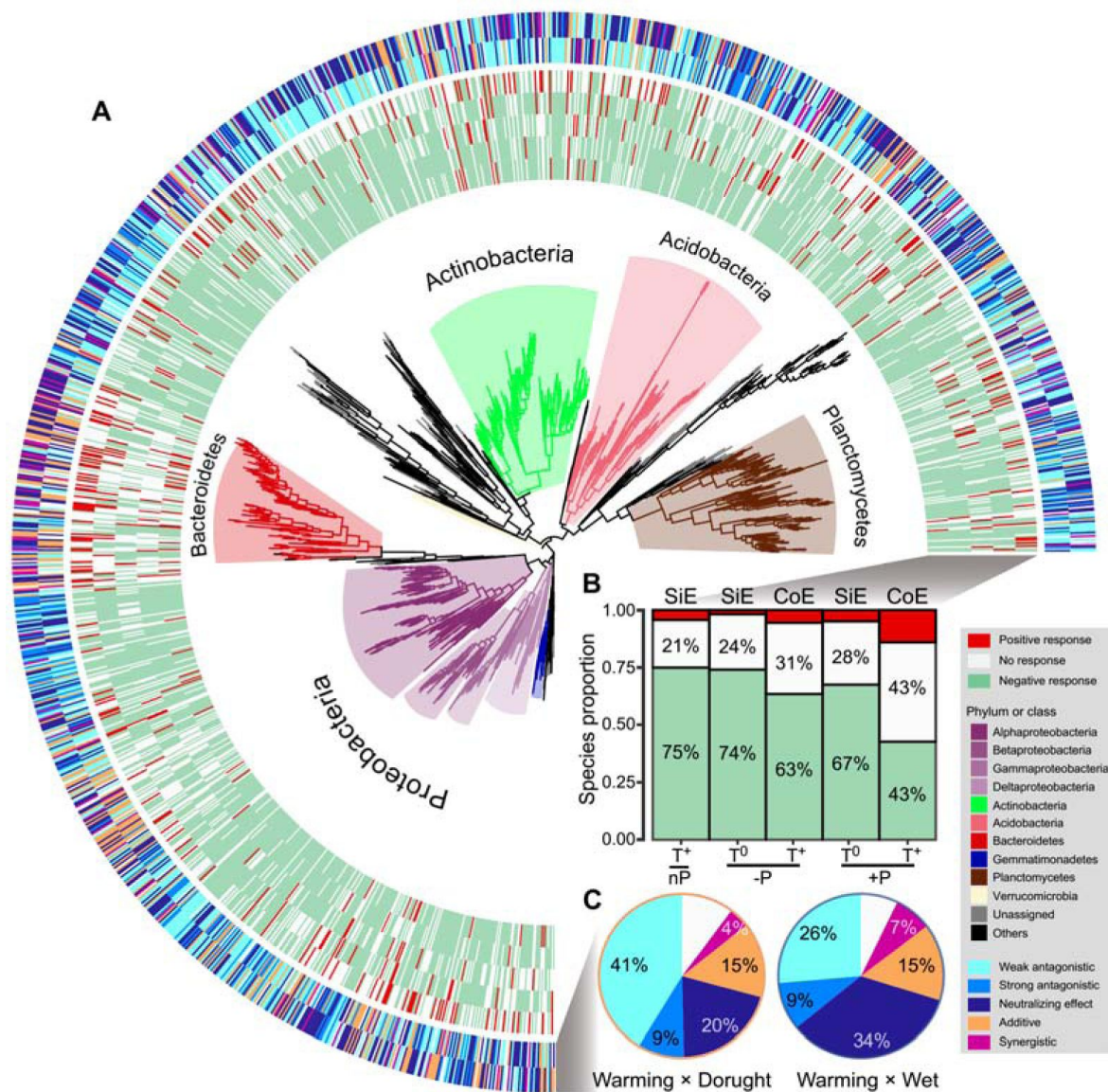


Fig. 4

The growth responses and phylogenetic relationship of incorporators subjected to different interaction types under two climate scenarios.

A The phylogenetic tree of all incorporators observed in the grassland soils. The inner heatmap represents the single and combined factor effects of climate factors on species growth, by comparing with the growth rates in T^0nP . SiE: single-factor effects; CoE: combined effects. The outer heatmap represents the interaction types between warming and altered precipitation under two climate change scenarios. **B** The proportions of positive or negative responses in species growth to single and combined manipulation of climate factors. **C** The proportions of species growth influenced by different interaction types under two climate change scenarios.

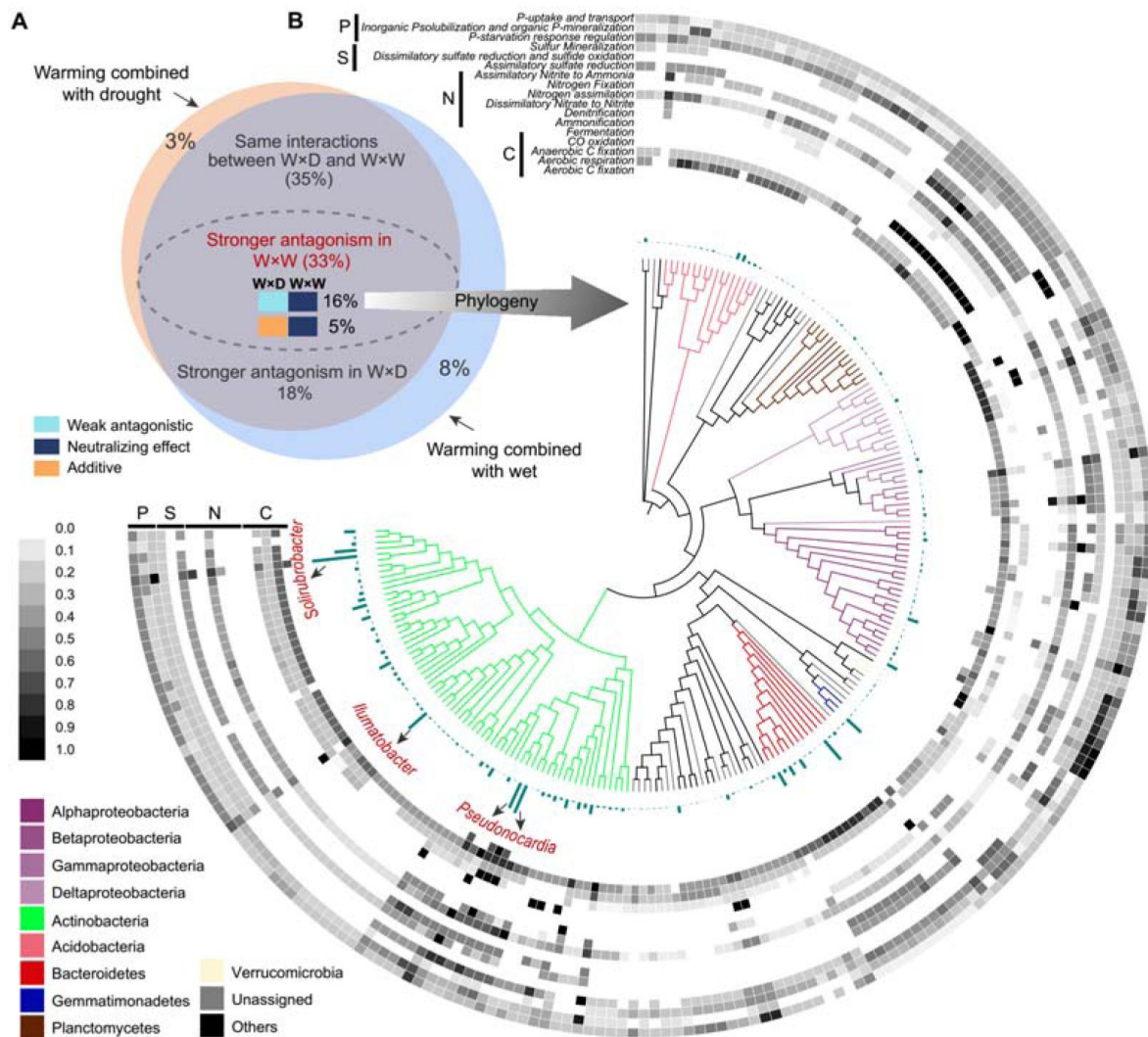


Fig. 5

Within-species shift in interaction types contributed to the variance of the whole community growth response under two climate scenarios.

A Venn plots showing the overlaps of incorporators, and their interaction types between two climate scenarios. **B** The phylogenetic relationship of the 215 incorporators whose growth dynamics were influenced by the weak antagonistic interaction of warming × drought and by the neutralizing effect of warming × wet. The blue-green bars represent the average growth rates of incorporators across different climate treatments. The heatmap displayed the potential functions associated with carbon and nutrient cycles predicted by Picrust2. The values of function potential were standardized (range: 0-1). The “anta” represents the antagonistic interaction; “W×D” represents warming × drought and “W×W” represents warming × wet.

We further assessed the potential functional traits of these 215 incorporators subjected to the dominant interaction types by PICRUST2 software (**Fig. 5B**). The top three OTUs with the highest growth rates possessed extremely high species abundance (Supplementary Data 1). The three taxa also possessed a higher functional potential: the member affiliated to *Solirubrobacter* (OTU 14), has the high functional potential for aerobic C fixation and CO oxidation, nitrogen assimilation and assimilatory nitrite to ammonia, and phosphatase synthesis and phosphate transport transport-related functions. The members affiliated to the genus *Pseudonocardia* (OTU 5 and OTU 31), harbor a higher functional potential for aerobic C fixation, aerobic respiration, and CO oxidation, dissimilatory nitrate to nitrite and nitrogen assimilation, and sulfur mineralization functions. Furthermore, we annotated the genomic characteristics by aligning species sequences to the GTDB database (Genome Taxonomy Database), and we found that OTU 14 (*Solirubrobacter*) was predicted to have larger genomes and proteomes (Supplementary Data 1). All these results suggested that these three species could play essential roles at the species and functional levels of ecosystems.

Discussion

Individual microbial populations might respond differently to environmental changes, resulting in differential contributions of active populations to ensuing biogeochemical fluxes (Blazewicz *et al.*, 2020). Controlled experiments simulating climate change have investigated changes in microbial community composition as measured by shifts in the relative abundances (Evans & Wallenstein, 2014; Barnard *et al.*, 2015). However, changes in relative abundances may be poor indicators of population responses to environmental change in some cases (Blazewicz *et al.*, 2020). Another challenge is the presence of a large number of inactive microbial cells in the soil, which hinders the direct, quantitative measure of the ecological drivers in population dynamics (Fierer, 2017; Lennon & Jones, 2011). Here, we estimated microbial growth responses by using the qSIP technique to decadal-long warming and altered precipitation regimes in the alpine grassland ecosystem on the Tibetan Plateau, which is considered highly susceptible and vulnerable to climate change (Ma *et al.*, 2017). To the best of our knowledge, we are the first to quantitatively describe the growth responses of soil microbes, the proxy for species fitness and the important soil C cycling variable, under warming and altered precipitation scenarios.

After a decade of temperature and precipitation regime shift, the pervasive negative impacts of a single climate factors on soil bacterial growth in alpine grassland ecosystem were observed (**Fig. 3**), which supports our first hypothesis that long-term warming and altered rainfall events consistently reduce microbial growth. A previous synthesis suggested that multiple global change factors had negative effects on soil microbial diversity (Yang *et al.*, 2021). Consistent with our findings, a recent experimental study demonstrated that the 15 years of warming generally reduced the growth rate of soil bacteria in a montane meadow in northern Arizona (Purcell *et al.*, 2022). These negative effects of climate factors on microbial growth and diversity are likely due to the variation related to availability of soil moisture and organic carbon (Dieleman *et al.*, 2012; Wu *et al.*, 2011). Previous evidences suggest that warming has a negative impact on soil carbon pools (Jansson & Hofmockel, 2020; Purcell *et al.*, 2022). During the first phase of soil warming (~ 10 years), microbial activity increased, resulting in rapid soil carbon mineralization and respiration (Melillo *et al.*, 2017). Carbon is the critical element in cell synthesis, the reduction of microbially accessible carbon pools may explain the diminished microbial growth after long-term warming. In addition, long-term warming can induce soil water deficiency (Dieleman *et al.*, 2012; Jansson & Hofmockel, 2020), thereby slowing microbial growth.

Altered rainfall patterns that result in increased aridity or wetter conditions, mediate ecosystem cycling by affecting above- and below-ground biological processes (Song *et al.*, 2019). As soil water availability is reduced, changes in osmotic pressure cause microbial death or dormancy, while others can accumulate solutes to survive under lower water potentials (Schimel, 2018).

However, such accumulation of osmolytes could depend on highly energetic expenses (Boot *et al.*, 2013; Jansson & Hofmockel, 2020; Schimel *et al.*, 2007), resulting in less energetic allocation to growth (trade-offs between microbial growth and maintenance). On the other hand, intensified rainfall patterns alter the composition and life strategies of soil bacteria, enriching the taxa with higher tolerance to frequent drying-rewetting cycles (Evans & Wallenstein, 2014). Such taxa may possess physiological acclimation traits, such as synthesizing chaperones to stabilize proteins and thicker cell wall to withstand osmotic pressure (Schimel *et al.*, 2007). However, these adaptation and acclimation strategies also create physiological costs and altered population processes (Schimel *et al.*, 2007). For example, reduced growth contributed by increased allocation of carbon to maintenance instead of new biomass (Lipson, 2015). In addition, climate change factors can influence microbial growth by affecting plant carbon input. A 17-year study of California grassland provided evidence that terrestrial net primary production (NPP) to precipitation gradient are hump-shaped, peaking when precipitation is near the multi-year mean growing season level (Zhu *et al.*, 2016). Reduced NPP could affect plant carbon inputs to the soil, ultimately having a negative effect on microbial growth. As microbial growth is related to microbial biomass carbon (MBC) formation and MBC is a stable form of soil carbon (Wang *et al.*, 2021; Hicks *et al.*, 2022), these results indicate the deleterious consequences of long-term climate change for soil carbon sequestration due to decreased microbial growth.

Characterizing the interactive effects of multiple global change drivers on microbial physiological traits is important for predicting ecosystem responses to changes in rainfall regime or types of environmental change, and soil biogeochemical processes (Song *et al.*, 2019; Zhu *et al.*, 2016). We found that the interaction types between warming and altered precipitation tended to be antagonistic from community-to population-levels, rather than synergistic or additive (Fig. 3 and 4). This result supports our second hypothesis that the interactive effects between warming and altered precipitation on soil microbial growth are not simply additive. A meta-analysis of experimental manipulation revealed that the combined effects of different climate factors were usually less than expected additive effects, revealing antagonistic interactions on soil C storage and nutrient cycling processes (Dieleman *et al.*, 2012; Wu *et al.*, 2011). Moreover, two experimental studies on N cycling and net primary productivity demonstrated that the majority of interactions among multiple factors are antagonistic rather than additive or synergistic, thereby dampening the net effects (Larsen *et al.*, 2011; Shaw *et al.*, 2002). Reduced precipitation can mute organic carbon mineralization by inhibiting soil respiration, which could maintain a relatively adequate soil carbon content and explain the diminished negative effects on microbial growth by the combined manipulation of warming and drought (Jansson & Hofmockel, 2020; Wu *et al.*, 2011). Conversely, enhanced precipitation could stimulate SOM decomposition, causing further loss of soil carbon under warming conditions (Zhou *et al.*, 2022). However, increased rainfall can also alleviate the moisture limitation on plant growth induced by warming, increasing plant-derived carbon inputs (Jansson & Hofmockel, 2020; Wu *et al.*, 2011). The increased carbon inputs may alleviate microbial carbon limitation in soil, which partly explains the higher microbial growth rates under the combined treatment of warming and enhanced precipitation relative to single climate factor manipulation.

About one-third of bacterial species had growth with higher levels of antagonistic interaction of warming $\times$ wet than that of warming $\times$ drought. The growth of these species is mostly subject to weak antagonistic interactions of warming $\times$ drought and neutralizing effect of warming $\times$ wet (Fig. 5A). By annotating the genomic information, we further found that the species with the high growth rate (OTU 14) has a relatively larger genome size and protein coding density (Supplementary Data 1), indicating larger gene and function repertoires. Similar to our finding, a previous study showed that the genus *Solirubrobacter* detected in the Thar desert of India is involved in multiple biochemical processes, such as N and S cycling (Kunjukrishnan Kamalakshi *et al.*, 2018). Members in the genus *Solirubrobacter* are also considered to contribute positively to plant growth (Liu *et al.*, 2020), and can be used to predict the degradation level of grasslands, indicating the critical roles on maintaining ecosystem services (Yan *et al.*, 2022). On the contrary,

we also found that some species with low growth rates and small genome size, tend to perform specific functions (**Fig. 5B** [↗](#) and Supplementary Data 1). For instance, the member in *Bosea* (OTU 190) had the highest potential for organic phosphorus mineralization and transport (P associated Group 2 and Group 3), while the potential for other functions was low. Similarly, the members in *Rathayibacter* (OTU 3021), had higher functional potential for sulfur mineralization. Species with high population density and relative large genome size in a community are generally considered to be microbial generalists, playing keystone roles in multiple, fundamental ecosystem services; Conversely, microbial specialists usually have relatively small genome, which could perform specific functions in environment (Xu *et al.*, 2021 [↗](#)). These results indicate that these two sorts of species jointly contribute to the diverse functional services in the alpine grassland ecosystem.

The evaluation of ecosystem models based on results obtained from single-factor experiments usually overestimate or underestimate the impact of global change on ecosystems, because the interactions between climate factors may not be simply additive (Dieleman *et al.*, 2012 [↗](#); Wu *et al.*, 2011 [↗](#); Zhou *et al.*, 2022 [↗](#)). Our results demonstrate that both warming and altered precipitation have negative effects on grassland bacteria growth, while the interaction between these two factors are usually antagonistic. Further, higher level of antagonistic interaction was found between warming and wet compared to that between warming and drought, which is probably determined by the secondary effects of changing climate regimes on the grassland ecosystem (e.g. changes in resource availability). This also emphasizes the importance of multifactor manipulation experiments in precise prediction and estimation of future ecosystem services and feedbacks. Such work will likely lead to improved, more precise prediction for the carbon dynamics driven by soil microbes in high-elevation ecosystems, e.g., the Tibetan Plateau, suffering from future climate changes.

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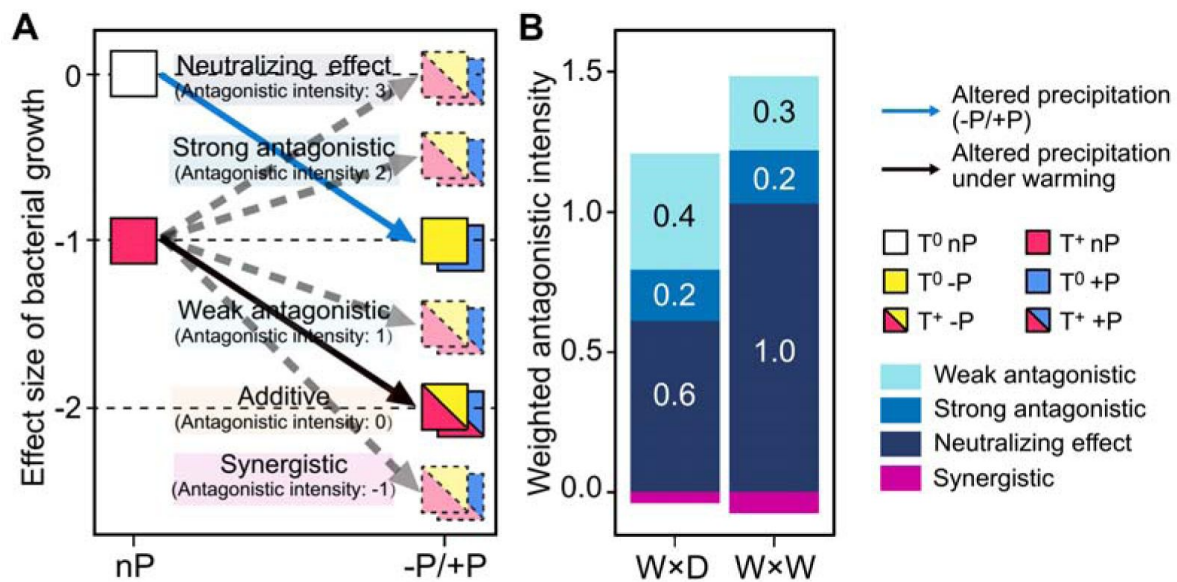


Fig S1.

The higher level of antagonism of wet × warming than that of drought × warming.

A The antagonistic intensities were assigned to the five interaction types on a 5-point scale, from -1 to 3 for synergistic, additive, weak antagonistic, strong antagonistic and nullifying effect, respectively. **B** The overall antagonistic intensities of all incorporators under warming × drought and warming × wet scenarios were estimated by weighting the relative proportions of incorporators subjected to different interaction types.

Climate change scenarios	Interaction types	NTI value	<i>P</i> value
Warming × Drought	Weak antagonistic	1.75	0.036
	Strong antagonistic	3.06	0.003
	Neutralizing effect	2.56	0.005
	Additive	2.33	0.01
	Synergistic	-0.88	0.809
Warming × Wet	Weak antagonistic	1.49	0.063
	Strong antagonistic	2.08	0.021
	Neutralizing effect	1.85	0.036
	Additive	1.16	0.116
	Synergistic	-0.67	0.761

P-value based on comparison of phylogenetic distance observed and that based on 1,000 permutations of a null model. Values in bold: $P < 0.05$. Low *P*-values (and positive indices) indicate that species are more phylogenetically related than expected by chance (phylogenetically clustering).

Table S1

The nearest taxon index (NTI) for incorporators subjected to different interaction types under two climate change scenarios.

P-value based on comparison of phylogenetic distance observed and that based on 1,000 permutations of a null model. Values in bold: $P < 0.05$. Low *P*-values (and positive indices) indicate that species are more phylogenetically related than expected by chance (phylogenetically clustering).

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Reviewer #1 (Public Review):

Warming and precipitation regime change significantly influences both above-ground and below-ground processes across Earth's ecosystems. Soil microbial communities, which underpin the biogeochemical processes that often shape ecosystem function, are no exception to this, and although research shows they can adapt to this warming, population dynamics and ecophysiological responses to these disturbances are not currently known. The Qinghai-Tibet Plateau, the Third Pole of the Earth, is considered among the most sensitive ecosystems to climate change. The manuscript described an integrated, trait-based understanding of these dynamics with the qSIP data. The experimental design and methods appear to be of sufficient quality. The data and analyses are of great value to the larger microbial ecological community and may help advance our understanding of how microbial systems will respond to global change. There are very few studies in which the growth rates of bacterial populations from multifactorial manipulation experiments on the Qinghai-Tibet Plateau have been investigated via qSIP, and the large quantity of data that comprises the study described in this manuscript, will substantially advance our knowledge of bacterial responses to warming and precipitation manipulations.

Reviewer #2 (Public Review):

Summary: The authors aimed to describe the effect of different temperature and precipitation regimes on microbial growth responses in an alpine grassland ecosystem using quantitative ¹⁸O stable isotope probing. It was found that all climate manipulations had

negative effects on microbial growth, and that single-factor manipulations exerted larger negative effects as compared to combined-factor manipulations. The degree of antagonism between factors was analyzed in detail, as well as the differential effect of these divergent antagonistic responses on microbial taxa that incorporated the isotope. Finally, a hypothetical functional profiling was performed based on taxonomic affiliations. This work gives additional evidence that altered warming and precipitation regimes negatively impact microbial growth.

Strengths: A long term experiment with a thorough experimental design in apparently field conditions is a plus for this work, making the results potentially generalisable to the alpine grassland ecosystem. Also, the implementation of a qSIP approach to determine microbial growth ensures that only active members of the community are assessed. Finally, particular attention was given to the interaction between factors and a robust approach was implemented to quantify the weight of the combined-factor manipulations on microbial growth.

Weaknesses: The methodology does not mention whether the samples taken for the incubations were rhizosphere soil, bulk soil or a mix between both type of soils. If the samples were taken from rhizosphere soil, I wonder how the plants were affected by the infrared heaters and if the resulting shadow (also in the controls with dummy heaters) had an effect on the plants and the root exudates of the parcels as compared to plants outside the blocks? If the samples were bulk soil, are the results generalisable for a grassland ecosystem? In my opinion, it is needed to add more info on the origin of the soil samples and how these were taken.

The qSIP calculations reported in the methodology for this work are rather superficial and the reader must be experienced in this technique to understand how the incorporators were identified and their growth quantified. For instance, the GC content of taxa was calculated for reads clustered in OTUs, and it is not discussed in the text the validity of such approach working at genus level.

The selection of V4-V5 region over V3-V4 region to quantify the number of copies of the 16S rRNA gene should be substantiated in the text. Classic works determined one decade ago that primer pairs that amplify V3-V4 are most suitable to assess soil bacterial communities. Hungate et al. (2015), worked with the V3-V4 region when establishing the qSIP method. Maybe the number of unassigned OTUs is related with the selection of this region.

Report of preprocessing and processing of the sequences does not comply state of the art standards. More info on how the sequences were handled is needed, taking into account that a significant part of the manuscript relies on taxonomic classification of such sequences. Also, an OTU approach for an almost species-dependent analysis (GC contents) should be replaced or complemented with an ASV or subOTUs approach, using denoisers such as DADA2 or deblur. Usage of functional prediction tools underestimates gene frequencies, including those related with biogeochemical significance for soil-carbon and nitrogen cycling.

Reviewer #3 (Public Review):

Summary: In this paper, Ruan et al. studied the long-term impact of warming and altered precipitations on the composition and growth of the soil microbial community. The researchers adopted an experimental approach to assess the impact of climate change on microbial diversity and functionality. This study was carried out within a controlled environment, wherein two primary factors were assessed: temperature (in two distinct levels) and humidity (across three different levels). These factors were manipulated in a full factorial design, resulting in a total of six treatments. This experimental setup was maintained for ten years. To analyze the active microbial community, the researchers employed a technique involving the incorporation of radiolabeled water into biomolecules

(particularly DNA) through quantitative stable isotope probing. This allowed for the tracking of the active fraction of microbes, accomplished via isopycnic centrifugation, followed by Illumina sequencing of the denser fraction. This study was followed by a series of statistical analysis to identify the impact of these two variables on the whole community and specific taxonomic groups. The full factorial design arrangement enabled the researchers to discern both individual contributions as well as potential interactions among the variables

Strengths: This work presents a timely study that assesses in a controlled fashion the potential impact of global warming and altered precipitations on microbial populations. The experimental setup, experimental approach and data analysis seem to be overall solid. I consider the paper of high interest for the whole community as it provides a baseline to the assessment of global warming on microbial diversity.

Weaknesses: While taxonomic information is interesting, it would have been highly valuable to include transcriptomics data as well. This would allow us to understand what active pathways become enriched under warming and altered precipitations. Non-metabolic OTUs hold significance as well. The authors could have potentially described these non-incorporators and derived hypotheses from the gathered information. The work would have benefited from using more biological replicates of each treatment.