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Reduction of microbial diversity in grassland soil is driven by long-term climate warming

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Abstract [No more than 150 words please]

Anthropogenic climate change threatens ecosystem functioning. Soil biodiversity is essential for maintaining the health of terrestrial systems, but how climate change affects the richness and abundance of soil microbial communities remains unresolved. We examined the effects of warming, altered precipitation and annual biomass removal on grassland soil bacterial, fungal and protistan communities over 7 years to determine how these representative climate changes impact microbial biodiversity and ecosystem functioning. We show that experimental warming and the concomitant reductions in soil moisture played the predominant role in shaping microbial biodiversity by decreasing the richness of bacteria (9.6%), fungi (14.5%), and protists (7.5%). Our results also show positive associations between microbial biodiversity and ecosystem functional processes such as gross primary productivity and microbial biomass. We conclude that the detrimental effects of biodiversity loss might be more severe in a warmer world.

MAIN

Biodiversity, the variety of genes, species, and ecosystems which constitute life on our planet[1], is dramatically affected by human alterations of global environment[2]. Biodiversity underscores healthy ecosystem functions and assures the production of essential goods, services, and benefits to society, such as climate regulation, landscape stability, fibers, and food production[1]. However, such benefits are threatened by the unprecedented biodiversity loss[3,4] caused by anthropogenic global environmental changes like climate warming, altered precipitation patterns, and land use changes[5]. Studies demonstrate that biodiversity loss impairs the functioning of natural ecosystems

and diminishes the number and quality of services they provide[6]. Thus, it is imperative to understand how global environmental change affects biodiversity and the underlying mechanisms[7].

Anthropogenic climate changes are the greatest threats to biodiversity from local to global scales[5,6]. The effects of climate change on biodiversity include shifts in species' geographical ranges[4], extinction[8-10], changes in abundance within species ranges[11], loss of phylogenetic community diversity[12], and increased genetic mutation and selection[13]. In contrast to plants and animals, our understanding of the climate change effects on microbial biodiversity are poorly understood. Previous studies demonstrated the effects of climate warming on soil microbial communities in terms of respiratory feedback responses[14,15], decomposition[16], microbial biomass[17], community composition[14,15,18,19], community succession[18], temporal scaling[19], and network complexity and stability[20]. However, there is a paucity of information on the effects of warming on below-ground microbial biodiversity (i.e., alpha diversity) due to the lack of well-replicated, long-term time-series observations under realistic field settings that is necessary to discern clear warming impacts. Therefore, despite a longstanding interest in this topic, whether and how climate warming would result in net soil microbial biodiversity gain or loss, and their underlying mechanisms remain unresolved.

Because different species differ greatly in their temperature-dependent metabolic rates, rising temperature would have dramatic effects on resource consumption, growth, reproduction and interactions between species (e.g., competition, predation, parasitism, and symbiosis)[9]. On one hand, certain species with higher fitness at elevated temperature are likely to have a competitive advantage over other species that are less fit[21]. Consequently, warming could trigger extinction

events at local scales and drive biodiversity loss, which may further cause extinction of other species through coextinction cascades[8]. Similarly, warming and associated environmental changes like decreased moisture would act as strong filtering factors against existing microbial species, which could also cause biodiversity loss. On the other hand, in general, warming promotes plant productivity[14,15]. Such potentially higher plant diversity and/or quantity of resources could support more microbial species by providing more niches with more ways that species can coexist[21], and result in biodiversity gain. In addition, the effects of these factors could be intertwined, resulting in no change in biodiversity.

To determine whether and how climate warming affects soil biodiversity, we examined the taxonomic and phylogenetic diversity (PD) of grassland soil bacteria, fungi and protists in a multifactor global change experiment[19] over seven consecutive years from 2009 to 2016, which has warming (+3 ºC), altered precipitation levels, i.e., -50% (half precipitation) and +100% (double precipitation) of ambient precipitation, as primary factors and clipping (annual removal of above-ground biomass to simulate the land-use practice of mowing for hay[22] or bioenergy[23]) as a secondary factor. We address the following major questions: whether and how experimental warming, altered precipitation, and clipping affect soil microbial biodiversity over time; whether such effects vary among different microbial lineages; and what are their underlying mechanisms. We hypothesize that warming would reduce the biodiversity of soil bacteria, fungi, and protists via alternation of both environmental filtering and biotic interactions.

RESULTS

Effects of climate change factors on soil and plant variables

Linear mixed-effects models for determining the sources of variations in hierarchical biological data were first employed to test the effects of treatments and their interactions on soil biogeochemistry and plant communities, in which the regression coefficients represent the directions and magnitudes of the treatment effects, namely effect sizes (β). By comparing the β values, our results revealed that experimental warming, compared to altered precipitation levels and clipping (Fig. 1a, Extended Data Fig. 1), had predominant effects on soil microclimate by increasing temperature, but decreasing moisture (Extended Data Fig. 2a, b; Supplementary note A), and on geochemistry (e.g., decreasing soil pH, increasing NO_3^-) (Extended Data Fig. 2c, d; Supplementary note A). For example, on average, warming decreased soil moisture by 1.5% (absolute) ($\beta = -1.5$, $p < 0.0001$; Extended Data Fig. 2b). In comparison, half precipitation only decreased soil moisture by 0.35% while double precipitation increased soil moisture by 0.7% ($\beta = 0.7$, $p < 0.0001$; Extended Data Fig. 2b). As expected, clipping had significant negative effect on plant biomass, but positive on plant richness (Extended Data Fig. 2f, g; Supplementary note A).

Impacts of warming on microbial biodiversity

It is expected that the alterations in soil microclimate, geochemistry and plant communities would lead to changes in soil microbial biodiversity. Here we define biodiversity[21] as taxonomic (i.e., species richness and their relative abundance), and phylogenetic[19] diversity in a local community. To test this prediction, all samples were analyzed for bacteria (56,182±27,613 reads per sample), fungi (23,569 ± 16,323 reads per sample), and protists (11,146 ± 10,528 reads per sample) (Extended Data Fig. 3 and S4). Linear mixed-effects models revealed that warming had strong negative effects ($\beta = -0.84 \sim -0.11$, $p < 0.007$) on richness and other taxonomic diversity indices,

and Faith's phylogenetic diversity (Fig. 1b-f; Table S1; Supplementary note B1). In general, although precipitation alteration or clipping could exert significant effects on richness (for example, precipitation alteration on bacterial richness; Fig. 1b), the effect sizes of warming on richness were 3 ~ 41 times larger than those of other treatments (Fig. 1b; Table S1; Supplementary note B1). In addition, the effects of treatment interactions were rarely significant except for the positive interactive effects of warming and clipping on fungal and protistan diversity ($\beta = 0.08 \sim 0.91$, $p < 0.05$) (Table S1), indicating that the warming effect was largely independent of altered precipitation and clipping. Collectively, these results suggest that the diversity of soil bacteria, fungi, and protists is predominantly shaped by experimental warming. A possible explanation is that the changes of microbial biodiversity are mainly driven by soil microclimate and geochemistry such as soil temperature, moisture, and pH[24-26]. As shown above, experimental warming had larger effects on these variables as compared to the effects of the half/double precipitation and clipping treatments. Therefore, we will primarily focus warming-induced treatment effects in subsequent sections.

Overall, warming significantly reduced bacterial richness by 9.6% ($\beta = -0.83$, $p < 0.0001$), fungal richness by 14.5% ($\beta = -0.84$, $p < 0.0001$) and protistan richness by 7.5% ($\beta = -0.99$, $p < 0.0001$). Such negative effects varied yearly with significant impacts on bacteria ($\beta = -1.72 \sim -0.68$, $p < 0.05$) after 2011, and fungi ($\beta = -2.15 \sim -0.36$, $p < 0.05$) after 2013, and protists in 2011, 2013 and 2014 ($\beta = -1.44 \sim -0.60$, $p < 0.05$) (Extended Data Fig. 5, Supplementary note B2). Rarefaction analyses indicated that the observed richness for bacteria, fungi, and protists were always lower under warming than non-warming control except warming & double precipitation & clipping (WDC) versus double precipitation & clipping (DC) for fungi and warming & clipping (WC)

versus clipping (C) for protists (Paired *t* test, p <0.0001, Extended Data Fig. 4). Warming also significantly decreased the phylogenetic diversity of bacteria by 7.2% (β = -0.49, p < 0.0001), fungi by 9.3% (β = -0.47, p = 0.002), and protists by 4.5% (β = -0.80, p = 0.003) based on Faith's PD, the phylogenetic analogue of taxon richness (Fig. 1f, Table S1). In addition, consistent to warming-induced biodiversity decrease, warming significantly reduced microbial biomass as determined by phospholipid fatty acid analysis (PLFA) (β = -0.83, p = 0.046), and DNA yields (β = - 0.72, p = 0.002; Fig. 1g and Table S2; Supplementary note B1). Collectively, all of these results indicate that experimental warming significantly reduced microbial biodiversity.

The negative warming effects on microbial biodiversity varied considerably among different microbial lineages. Warming significantly decreased the richness of most microbial phyla (Fig. 2a), as well as their phylogenetic diversity (Extended Data Fig. 6a; Supplementary note C1). Warming had the largest negative effects on the richness of Acidobacteria, Verrucomicrobia, and Planctomycetes (β = -1.21 to -1.19, p < 0. 01), but had a significant positive effect on the richness of Firmicutes (β = 1.52, p < 0. 01; Fig. 2a). Similar to species richness, warming significantly decreased the relative abundance of Acidobacteria, Verrucomicrobia and Planctomycetes (β = -0.88 ~ -0.84, p < 0. 01). In contrast, warming increased the relative abundance of Actinobacteria, Firmicutes and Gemmatimonadetes (β = 0.52 ~ 1.05, p < 0.05; Extended Data Fig. 6b), which could be due to their preference for drier soils[27-29]. Notably, the increase of Firmicutes and Actinobacteria may be in part due to their spore-forming ability[30], which makes them resistant to desiccation stress. In support of this, we examined the characteristics of spore-forming bacteria in more detail. Almost all the families of Firmicutes and Actinobacteria that were increased under warming are known spore-formers[30] (Extended Data Fig. 7a, b). In addition, the relative

abundances of the major sporulation genes in Firmicutes (*spo0A*) and Actinobacteria (*bldD*), as identified from shotgun sequencing metagenome data, also significantly or marginally significantly increased under warming ($p < 0.0001$ for *bldD;* $p = 0.08$ for *spo0A*; Extended Data Fig. 7c). Similar patterns were also observed at individual ASV (amplicon sequence variant) level (Fig. 2b). For instance, most taxa in Verrucomicrobia (78.5% of ASVs of Verrucomicrobia, 91.8% of relative abundance) decreased under warming, while most taxa in Firmicutes (88.0% of ASVs, 98.9% of relative abundance) increased (Fig. 2b; Supplementary note C2). In addition, warming effects varied among different fungal guilds as classified by FUNGuild[31]. Warming reduced the richness, phylogenetic diversity, and abundance of arbuscular mycorrhiza fungi (AMF) ($\beta = -1.05 \sim -0.42$, $p < 0.01$; Fig. 2a; Extended Data Fig. 6a, b; Supplementary note D1), which are beneficial microorganisms capable of forming mutualistic symbiosis with plants. The negative warming effect on AMF abundance was also supported by AMF biomass decreases as determined by PLFA ($\beta = -0.54$, $p = 0.013$; Fig. 1g). Interestingly, although warming decreased the richness of putative plant pathogenic fungi (Fig. 2a), it marginally increased their relative abundance ($\beta = 0.43$, $p = 0.055$; Extended Data Fig. 6b), which could have negative effects on plant growth. Moreover, warming significantly reduced the richness, phylogenetic diversity and abundance of Cerozoa and Ochrophyta ($\beta = -1.07 \sim -0.20$, $p < 0.002$) but increased the richness and phylogenetic diversity of Conosa ($\beta = 0.05 \sim 0.12$, $p < 0.02$) (Fig. 2a; Extended Data Fig. 6a, b; Supplementary note C1). Similarly, warming significantly decreased the richness and phylogenetic diversity of various functional groups of protists (i.e., consumers, phototrophs, and parasites) ($\beta = -0.98 \sim -0.39$, $p < 0.04$). Warming also reduced the relative abundance of phototrophic protists ($\beta = -0.17$, $p = 0.01$) (Fig. 2a; Extended Data Fig. 6a, b). These results suggest that warming has differential impacts on various microbial lineages and/or functional guilds, which are consistent with our previous

observations that warming effects vary greatly among different microbial functional groups[15]. Warming-induced diversity decrease on most microbial categories could have significant impacts on ecosystem functioning, as suggested by previous reports in macroecology[3,4,6] and microbial ecology[32]. Particularly, since warming decreased beneficial taxa such as AMF, the aboveground plant community could be negatively impacted.

Mechanisms underlying reduced microbial biodiversity

As we posited earlier, warming-induced biodiversity decrease could be due to changes in biotic interactions and abiotic environmental conditions caused by warming. Under warmer conditions, many microbes with adaptive traits (e.g., Firmicutes and Actinobacteria with spore-forming ability) would survive and outcompete other microbes (e.g., Acidobacteria, Verrucomicrobia, and Planctomycetes) (Fig. 2a, b). Consequently, species coexistence patterns would be substantially altered, as revealed by a network analysis showing that the occurrence network was more complex under warming than non-warming control[20]. The increased positive connections may indicate more microbial cooperations[33], which could be important for their survival under warming. Also there were more negative connections under warming than control[20], suggesting there might be more intense competition under warming. Eventually, the warming-induced changes in microbial activities and interactions could trigger various extinction events and ultimate biodiversity decrease due to cascading effects[8]. Alternatively, warming could just act as a deterministic filtering factor to impose significant positive selection on spore-forming microorganisms (e.g., Bacillaceae_2) and/or negative selection on nonspore-forming microorganisms (e.g. Acidothermaceae), which is consistent with the observation that warming enhanced homogeneous selection on Bacillales in Firmicutes[34]. All of these results suggest that both biotic interactions and

environmental filtering could play important roles in mediating warming-induced biodiversity decrease.

It is anticipated that soil environmental conditions should also play important roles in driving microbial biodiversity decrease. As shown in Fig. 3a, bacterial, fungal, and protistan richness were highly correlated with soil moisture, temperature and NO_3^--N content (LMM's r = -0.25 ~ 0.24, p < 0.01). Bacterial richness also showed significant correlations with plant richness and biomass (LMM's r = 0.11 ~ 0.19, p < 0.05; Supplementary note D1). However, obvious collinearity among these variables also occurred (Fig. 3a) (Supplementary note D1). Thus, to further disentangle the direct and indirect effects of the environmental drivers on microbial biodiversity, structural equation modeling (SEM) analyses were performed with the presumed relationships (Extended Data Fig. 8) among the selected subsets of plant and soil variables which were least-correlated (see Methods for details of model selection). Soil moisture, which was negatively affected by warming (standardized path coefficient, b = - 0.69) and half precipitation (b = -0.16), but positively by double precipitation (b = 0.45), played the strongest role in shaping bacterial richness directly (b = 0.43, p = 0.001; Fig. 3b; Supplementary note D2). Soil pH, plant richness and the biomass of C_3 plants were also significantly and positively (b = 0.23-0.31, p < 0.02) correlated to bacterial richness. Furthermore, bacterial richness directly and positively affected protistan richness (b = 0.69, p < 0.001). In comparison, among the variables which directly contribute to fungal richness, only paths of soil moisture (b = 0.44, p = 0.001) and plant richness (b = 0.26, p = 0.015) were significant (Extended Data Fig. 9), suggesting that the environmental drivers appear different between bacteria and fungi. Overall, those variables can explain 61%, 51%, and 50% of the variations in bacterial, fungal and protistan richness (Fig. 3b, Extended Data Fig. 9), respectively.

In addition, SEM analysis revealed that warming played predominant roles in shaping microbial diversity (standardized total coefficient = -0.61 for bacteria, -0.56 for fungi and -0.51 for protists, Fig. 3c) as compared to precipitation or clipping treatments (standardized total coefficients = -0.05 ~ 0.31, Fig. 3c), which was consistent with linear mixed-effects model analysis (Fig. 1a). These results indicate that soil and plant variables, particularly soil moisture, are also important in mediating warming-induced soil microbial diversity decrease directly and indirectly. Since the SEMs could explain over half of the variations in microbial diversity, the environmental filtering effects, especially the induced desiccation stress, could be the main driver for microbial diversity decrease via affecting microbial activities and interactions.

Links between microbial biodiversity and ecosystem functions

A following important question is whether the warming-induced changes in microbial diversity affects ecosystem functional processes. Consistent with the reduced microbial biodiversity, warming also decreased the ecosystem functions of total microbial biomass, bacterial biomass, GPP, and ER (β = -0.17 ~ -0.84, Extended Data Fig. 10). In agreement with various reports in macroecology[6], the overall bacterial richness had significant positive correlations with total microbial biomass, bacterial biomass, gross primary productivity (GPP), and ecosystem respiration (ER) (r = 0.14 ~ 0.22, $p < 0.002$) (Fig. 3d; Supplementary note E). Similar positive correlation patterns were also observed for most bacterial groups (e.g., Proteobacteria, Bacteroidetes, Planctomycetes) (Fig. 3d; Supplementary note E) except for Firmicutes, which showed significant negative correlations with total microbial biomass, bacterial and fungal biomass, and ER (r = -0.26 ~ -0.10, $p < 0.04$). In addition, the overall richness of fungi and most fungal phyla/guilds showed significant positive correlations with GPP and ER (Fig. 3d;

Supplementary note E). The overall protistan richness, major protistan lineages and functional groups also had significant positive correlations with total microbial biomass, bacterial biomass, GPP and ER ($r = 0.08 \sim 0.22$, $p < 0.04$) (Fig. 3d; Supplementary note E). All of these results indicate that there are significant positive linkages between microbial community diversity and relevant ecosystem functional processes.

DISCUSSION

Understanding how climate change affects microbes and the underlying mechanisms is a critical issue in climate change and microbiology research[35]. By examining the dynamic changes of soil microbial biodiversity in a well replicated long-term climate change experiment, this study provides explicit evidence that climate warming consistently reduced the taxonomic and phylogenetic diversity of soil bacteria, fungi, and protists across different years. In addition, by examining the interactive effects of warming, precipitation level, and clipping on microbial diversity, this is also the first study to demonstrate that warming plays a predominant role in driving soil biodiversity decrease via altering biotic interactions and soil biogeochemical conditions, particularly soil moisture, which is in agreement with the fact that warming had prevalent effects on accelerating the temporal scaling rates of soil microbial biodiversity[19]. Finally, warming-induced diversity decrease could have significant impacts on ecosystem functioning, which augments previous reports in macroecology[3,4,6].

Our findings have important implications for predicting ecological consequences of climate change and for ecosystem management. Because warming as a deterministic filtering factor drives

292 microbial biodiversity decrease[18], the ecosystems under future climate change scenarios would be
293 less diverse. Along with faster biodiversity turnover rates as previously demonstrated[19], it is
294 expected that the linked ecosystem functions and services could become more vulnerable in a
295 warmer world[6]. Particularly, since warming has differential effects on different microbial lineages,
296 such as decrease of beneficial taxa (e.g., AMF), the detrimental effects of biodiversity loss on
297 future ecosystem functioning could be more severe. In addition, since warming-effects on
298 biodiversity is primarily via reduced moisture, it is expected that warming-induced biodiversity
299 decrease could be more severe in drylands (i.e., arid, semi-arid, and dry-subhumid ecosystems),
300 covering 41% of Earth's land[36], as compared to wet regions. The future warming-induced
301 precipitation changes could also be important in mediating warming-induced biodiversity decrease.
302 However, further research is necessary to determine whether the warming-induced biodiversity
303 decrease and associated mechanisms are applicable to other ecosystems.
304
305 **Methods**
306 **Study site and sampling**
307 We conducted the warming experiment at the Kessler Atmospheric and Ecological Field Station
308 (KAEFS) in the US Great Plains in McClain County, Oklahoma (34° 59′ N, 97° 31′ W)[18-20,22]. The
309 acting director of the site is Meghan Bomgraars (mbomgaars@ou.edu). Detailed site description
310 can be found in the Supplementary note F. In brief, KAEFS is an old-field tall-grass prairie with
311 dominant plants of C_3 forbs (*Ambrosia trifida*, *Solanum carolinense* and *Euphorbia dentate*) and
312 C_4 grasses (*Sorghum halepense* and *Tridens flavus*)[22]. Based on Oklahoma Climatological Survey
313 data from 1948 to 1999, the air temperature ranges from 3.3 °C in January to 28.1 °C in July with
314 mean annual temperature 16.3 °C, and the precipitation ranges from 82 mm in January and

February to 240 mm in May and June with mean annual precipitation 914 mm[15]. The soil type of this site is Port–Pulaski–Keokuk complex, and soil texture class is loam with 51% of sand, 35% of silt and 13% of clay[37].

The field site experiment was established on July in 2009 with a blocked split-plot design (Extended Data Fig. 1), in which warming (continuous heating at a target of +3 °C above ambient temperature) and precipitation alteration (targets of −50% and +100% ambient precipitation) were primary factors nested with clipping (annual removal of aboveground biomass in peak growth season) as the secondary factor[18-20]. The clipping treatment was used to mimic the land-use practice of hay harvest, which is widely practiced in the southern Great Plains of the US[22], and biomass harvest for bioenergy[23]. In brief, the site has four experimental blocks, each including six plots. Each plot has the size of 2.5 × 3.5 m^2, which was further divided into one 2.5 × 1.75 m^2 clipped subplot and one 2.5 × 1.75 m^2 unclipped subplot, resulting in a total of 48 subplots (Extended Data Fig. 1).

From 2009 to 2016, surface (0–15 cm) soil samples were collected annually from subplots one day before annual clipping. Each sample was mixed from three soil cores (2.5 cm diameter × 15 cm depth) from a soil sampler tube. In the first year (2009), we collected 24 pre-warmed soil samples from the southern subplots. As for the following years, a total of 48 annual soil samples were collected from all subplots in each year. A total of 360 annual soil samples from 2009 to 2016 were collected in this study and stored in a freezer at −80 °C.

Field measurements and soil chemical analyses

Soil temperature was measured every 15 min at depth of 7.5, 20, 45 and 75 cm in the center of each plot using constantan-copper thermocouples wired to a Campbell Scientific CR10x data logger (Campbell Scientific)[18-20]. Annual average values of temperature at depth 7.5 cm were used to represent soil temperature across experimental years. Volumetric soil water content (%V) was measured using a portable time domain reflectometer (Soil Moisture Equipment Corp.) once or twice a month, and annual average values were used to represent soil moisture[18-20]. Ecosystem carbon (C) fluxes, including ecosystem respiration (ER), net ecosystem exchange (NEE), soil total respiration (R_s) and heterotrophic respiration (R_h) were measured once or twice a month between 10:00 and 15:00 (local time)[18-20]. The gross primary productivity (GPP) was then estimated as the difference between net ecosystem exchange and ecosystem respiration.

Above-ground plant community surveys were conducted at peak biomass (usually September) each year. All species within each plot were identified to estimate species richness. From 2009 to 2015, the above-ground plant biomass, separated into C_3 and C_4 species, were estimated by a modified pin-touch method, as described by Sherry et al[38]. Since 2016, thorough plant survey was conducted, and linear regression models were used to estimate above-ground biomass based on plant height and abundance[39]. Every individual plant was surveyed in the whole plot, with their species identity and height recorded. For each species, we also measured the height across different individuals off-plot, after which we harvested, dried, and weighed the vegetation for which we had recorded. We then constructed regression relationships between plant height and dry weight biomass for each species. The regression model for one species, *Pseudognaphalium obtusifolium* was insignificant (R^2=0.07, p = 0.48), and we used the mean biomass across individuals collected off-plot as the biomass estimate for its individual in the plot. The average adjusted R^2 for all other

regression models was 0.76 and the average *p* value was 0.0056. The total above-ground biomass was then calculated as the sum of estimated biomass for every individual.

Visible stones and plant roots were removed from the soil by metal forceps before chemical and microbial analyses. The chemical properties of all soil samples were analyzed in the Soil, Water, and Forage Analytical Laboratory at Oklahoma State University (Stillwater, OK, USA). Briefly, the total C and total nitrogen (N) contents were determined using a dry combustion C and N analyzer (LECO). Soil nitrate (NO_3^-) and ammonia (NH_4^+) were analyzed using a Lachat 8000 flow-injection analyzer (Lachat). Soil pH was determined using a pH meter with a calibrated combined glass electrode at a water-to-soil mass ratio of 2.5:1[40].

Soil phospholipid fatty acids (PLFA)

Lipids were extracted from the soil samples based on the modified Bligh-Dyer method as described by Buyer and Sasser[41]. In brief, soil samples were freeze-dried and sifted to remove any rocks or large debris. 2 g of each freeze-dried soil sample was then incubated in a 2:1:0.8 solution of methanol, chloroform, and K_2HPO_4 buffer. The chloroform phases were collected and the phospholipids were separated from neutral lipids and glycolipids through silicic acid chromatography, subsequently saponified and methylated to fatty-acid methyl esters. The resulting fatty acid methyl esters were separated and identified using gas chromatography (Agilent 6890N, Wilmington, DE). The peak responses were translated into molar responses using an internal standard, and were fitted with a MIDI Sherlock microbial identification system (Version 4.5, MIDI, Newark, NJ). Further, the peak responses were assigned to microbial groups including gram-negative bacteria, gram-positive bacteria, Actinobacteria, anaerobic bacteria, common fungi, and

384 arbuscular mycorrhizal fungi (AMF) using the Agilent Chemstation software (Agilent
385 Technologies). The total bacterial biomass of the soils was calculated as the total PLFA of all
386 bacterial groups, i.e., the sum of the biomass of gram-negative bacteria, gram-positive bacteria,
387 actinobacteria, and anaerobic bacteria. The total fungal biomass was calculated as the sum of the
388 biomass of common fungi and AMF.
389
390 **DNA extraction**
391 It is well known that sequence quality is subjected to big variations from DNA extraction, PCR
392 amplification and sequencing. It is critical to control each step to generate the high quality of
393 experimental data. Thus, great cautions were taken in this study to ensure the highest quality of
394 sequencing data with more tedious and laboratory experimental protocols. For DNA extraction,
395 the experimental method with grinding, freeze-thawing and sodium dodecyl sulfate (SDS)-based
396 cell lysis[42] was used. This method has been most widely used in microbial molecular ecology. In
397 brief, for each soil sample, microbial DNA was extracted from 1.5 g soil using this grinding,
398 freeze-thawing method[42], and purified with a PowerSoil® DNA isolation kit (MoBio Laboratories)
399 following the manufacturer's protocol. DNA quality was evaluated based on the 260/280 nm and
400 260/230 nm absorbance ratios using a NanoDrop ND-1000 Spectrophotometer (NanoDrop
401 Technologies). All samples had the 260/230 ratios larger than 1.7 and 260/280 ratios larger than
402 1.8. DNA concentration was measured by PicoGreen using a FLUOstar Optima fluorescence plant
403 reader (BMG Labtech). DNA samples were stored at −80 °C until use.
404
405 **Amplicon sequencing and data preprocessing**

406 We used a two-step PCR amplification method for library preparation of 16S rRNA gene (V4
407 region), the intergenic region (ITS) between the 5.8S and 28S rRNA genes and 18S rRNA gene
408 (V9 region) to improve sequence representation and quantification[43,44]. During the first
409 amplification step, 10 ng DNA from each sample was PCR-amplified for 10 cycles in triplicate in
410 25 µl reaction with the primers without adaptors. The obtained PCR products were purified and
411 dissolved in 50 µl D.I. water. This initial amplification step avoided potential amplification bias
412 caused by long tail of adaptors and other added components. During the second amplification step,
413 15 µl of the PCR products from each sample were amplified using the primers with all adaptor,
414 barcode, and spacers in triplicate for an additional 15 cycles. The low total cycle numbers (25-30
415 cycles) ensure that the PCR amplification is not saturated and limits amplification artifacts. Finally,
416 the triplicate amplified products were combined, purified, and quantified for subsequent
417 sequencing using the same MiSeq instrument with 2 × 250 base pair kits at the Institute for
418 Environmental Genomics, University of Oklahoma. The two-step PCR amplification method with
419 phasing primers in triplicate can help reduce sequencing errors, minimize amplification bias, and
420 preserve semi-quantitative information of PCR amplification[43], which is critical for subsequent
421 data analysis, data interpretation, and biological inference[44].

422

423 The primer sequences were trimmed from the paired-end sequences, which were then merged
424 using FLASH[45]. Any merged sequences with an ambiguous base or a length of < 245 bp for the
425 16S rRNA gene, < 220 bp for the ITS, or < 330 bp for 18S rRNA gene were further discarded. An
426 average of 56,182±27,613, 23,569 ± 16,323, and 56,874 ± 55,642 sequence reads were obtained
427 for 16S rRNA gene, and ITS and 18S rRNA gene, respectively (Extended Data Fig. 3a, b). These

high-quality 16S rRNA gene, ITS or 18S rRNA gene sequences were processed to generate amplicon sequence variants (ASVs; also known as unique sequence variants and zero-radius operational taxonomic units) by UNOISE3[46]. Rarefaction analyses (Extended Data Fig. 4) indicated that the sequencing depth was sufficient for assessing the effects of various climate change factors on the diversity of these soil microbial communities.

The representative 16S rRNA or 18 rRNA gene sequences were aligned using Clustal Omega v1.2.2[47] for constructing the phylogenetic tree by FastTree2 v2.1.10[48]. The FastTree topology search was constrained with the relatively reliable 16S-based bacterial tree in Silva Living Tree Project[49] release 132. The fungal phylogenetic tree was constructed using 'constrained topology search' in FastTree v2.1.11[50]. A guide tree was built from the full-length SSU sequences of 511 representative species, one species from each fungal family. Then, the full tree was built from the observed ITS sequences with the constraint alignment converted from the guide tree. The SSU sequences were retrieved from Silva 138.1 Ref NR database. For 16S rRNA gene, the ASVs or OTUs were taxonomically annotated with RDP Classifier using 16S rRNA gene training set 16 with a confidence cutoff of 50%[51], and Chloroplast and Mitochondria were further removed from the bacterial profiles. For ITS, the ASVs or OTUs were taxonomically annotated with RDP Classifier using UNITE Fungal ITS training set (version of August 2018)[52]; ITS sequences were further assigned into three functional groups—plant pathogens, AMF, and saprotrophs using FUNGuild[31]. Sequences that had multiple function assignments or in FUNGuild were termed as unassigned guild in this analysis. The sequence number in each sample was rarefied to the same depth for 16S rRNA gene (22,599) or ITS sequences (7,761) in subsequent comparative analyses. For 18S rRNA gene, the ASVs were taxonomically annotated with PR2 databases[53]. We also

assigned the major protistan lineages to their dominant mode of energy acquisition (i.e., trophic functional groups)—either phototrophic, parasitic, or as consumers, following the classifications in Oliverio et al[54]. Sequences annotated as protists were further rarefied to 1,100 for subsequent comparative analyses.

Sporulation gene profiling from metagenomic dataset

The soil samples under single treatment of warming (warming and normal precipitation and unclipped) and control (ambient temperature and normal precipitation and unclipped) were selected for metagenomic sequencing. That is, 8 subplots × 8 years = 64 metagenomic samples. Libraries were constructed using genomic DNA with KAPA Hyper Prep Kit (KR0961) based on the manufacturer's instruction, and DNA was sequenced using an Illumina HiSeq 2500 platform. A total of 1100.14 gigabases (Gb) were generated, with an average of 17.19 ± 2.68 Gb per sample. The quality of the metagenomic data was evaluated using FastQC v0.11.6[55]. CD-HIT[56] was used to remove duplicates with an identity cutoff of 100%. NGS QC Toolkit (version 2.3.3)[57] was used for quality filtering, where poor-quality bases with quality score <20 were trimmed from the 3 end until the first base had a quality score ≥ 20. Trimmed reads with length of > 120 and the average quality score ≥ 20 were kept. In addition, reads with more than one ambiguous base were removed. High-quality reads were then converted to fasta format, split into multiple partitions, and searched against NR database (BLASTx) using DIAMOND[58] with E value cutoff of 1×10^{-5}, coverage cutoff of 0.5, and maximum target number of 50. The outputs were submitted to MEGAN6 (Ultimate Edition, version 6.6)[59] for function profiling with parameter of top percent of hits 10%, minimum score 50 and minimum support 1. The annotated functional profiles of SEED Subsystem (3 levels) were exported, and two major sporulation genes annotated at level 3, i.e., *spo0A* gene of

Firmicutes and *bldD* gene of Actinobacteria were selected. Their relative abundances were then determined by dividing the annotated sequence counts by the total number of high-quality sequences of the corresponding metagenomes.

Statistical analyses

This study is based on a well-designed long-term climate change experiment with a blocked split-plot design, with 12 treatment combinations of warming, precipitation levels, and clipping. Each combination has 4 replicated plots (Extended Data Fig. 1). Also, the same plots were repeatedly sampled over 8 years and high quality of experimental data were generated, which greatly increased the power for various robust data analyses to ensure the liability of the statistical inference.

Diversity analyses

Richness was used to measure taxonomic α-diversity, using the *Picante* R package[60,61]. Other taxonomic α-diversity indices, including Shannon index, inverse Simpson index and Pielou's evenness were also calculated using the *vegan* R package[62]. Faith's index, which is the sum of the total phylogenetic branch length based on the phylogenetic tree constructed, was used to measure phylogenetic α-diversity using the *Picante* R package[60].

Treatment effects by linear mixed-effects models

Due to block design and repeated measurements, the experimental data are not completely independent. Therefore, linear mixed-effects models (LMMs) were used to assess the effects of

497 experimental treatments on environmental variables, microbial diversity or the relative abundance
498 of microbial groups. The lme4 R package was used to implement LMMs[63]. In the linear mixed-
499 effects models, warming (0 for ambient temperature and 1 for warming), precipitation level (0.5
500 for half, 1 for normal and 2 for double precipitation level) and clipping (0 for unclipped and 1 for
501 clipped) treatments and their interactions were considered as fixed effects, while the sampling time
502 (year) and the block were termed as random intercept effects (y ~ warming × precipitation level ×
503 clipping + (1|Block) + (1|year)). That is, three variables (warming, precipitation level, and clipping)
504 were created to denote the experimental treatments for soil samples. The reason that the
505 precipitation treatments were not treated as categorical variables is because a categorical variable
506 can not capture the gradient of precipitation levels, which is assumed to linearly correlate with soil
507 moisture and other variables.
508
509 We also tested alternative models in which the effects of sampling year and its interaction with
510 experimental treatments were considered as fixed effects (y ~ warming × precipitation level ×
511 clipping × year + (1|Block); or y ~ warming × precipitation level × clipping + year (1|Block)), yet
512 the corresponding model with sampling year as random intercept effect was better, based on lower
513 AIC values. Thus, we decided to use the model with experimental treatments as fixed effects with
514 year and block as random intercept effects. Effect sizes of treatments or treatment interactions
515 were represented by the regression coefficients in the LMMs. Wald type II χ^2 tests were used to
516 calculate the *p* values from the LMMs using the *car* R package[64]. Since the precipitation level is
517 considered as a continuous variable in the LMM (0.5 for half precipitation, 1 for normal and 2 for
518 double precipitation), only one regression coefficient of precipitation treatment would be derived
519 by the LMM. The effect size of half precipitation (as compared to ambient precipitation) can be

derived by multiplying the regression coefficient by - 0.5, while the effect size of double precipitation (as compared to ambient precipitation) can be derived by multiplying the regression coefficient by 1. For instance, as shown in Extended Data Fig. 2b, precipitation level was positively correlated to soil moisture with $\beta = 0.7$ per fold change (+100%) of precipitation ($p < 0.0001$; Extended Data Fig. 2b). That is, the double precipitation treatment only caused a $0.7\% \times 1 = 0.7\%$ (absolute) increase in soil moisture, while half precipitation changed soil moisture by $0.7\% \times (-0.5) = -0.35\%$, i.e., half precipitation decreased soil moisture by 0.35% on average.

Predicting microbial diversity with environmental variables

To link the environmental variables to microbial diversity, the correlations between the individual environmental variable and bacterial or fungal diversity was tested by the linear mixed-effects model, in which the sampling year and the block were termed as random intercept effects. Since richness is highly correlated with other diversity indexes (Fig.S1), it is used to represent microbial biodiversity. The marginal coefficient of determination (pseudo-R-squared) was calculated using function 'r.squaredGLMM' of the MuMIn R package[65], which represents the variance explained by the fixed effect in the linear mixed-effects model.

Because most soil and plant predictor variables were strongly correlated with each other (Fig. 3a), we further performed a model selection analysis to compile sets of variables that are strong predictors of the data, least-correlated, or potentially biologically informative on the basis of *a priori* assumptions. The following tests were performed. First, the contribution of each variable on predicting bacterial or fungal richness was ranked using the method of bootstrap forest partitioning, conducted by the function of 'Predictor Screening' in JMP 15.0 (SAS Institute) (Table S3 & S4).

543 This method evaluates the relative contribution of predictors on the response[66], which can identify
544 predictors that might be weak alone, but strong when used in combination with other predictors.
545 Second, the Pearson correlation coefficient between variables were calculated (Table S5) to reveal
546 the colinearty between variables. We then applied different model selection strategies to obtain
547 sets of variables for our linear mixed models, in which the random intercept effects of sampling
548 year and block were included. Three alternative strategies were pursued to select soil and plant
549 variables to be included in candidate models, based on their collinearity and/or informed
550 hypotheses: (i) Model 1 (Table S6 for bacteria and Table S7 for fungi). For highly correlated
551 variables which have similar biological inference, we kept the one with the highest contribution
552 based on Predictor Screening. For example, we kept soil annual mean moisture rather than soil
553 moisture at the sampling month for both LMMs on bacterial and fungal richness. For variables on
554 plant biomass, we kept C_3 plant biomass for the LMM on bacterial richness while we kept C_4 plant
555 biomass for that on fungal richness. Then, we iteratively removed variables with the highest
556 correlation coefficients and kept variables that had high contributions in predictor screening, until
557 the correlation between the remaining variables were less than 0.5. Through this way, each set of
558 six variables were selected for the bacterial and fungal model. (ii). Model 2 (Table S6, S7). We
559 kept six least-correlated variables by removing all variables with a correlation of $r > 0.5$ using R
560 *caret* package[67]. (iii). Model 3 (Table S6, S7). The six variables with largest contributions from
561 predictor screening were kept.
562
563 The random intercept effects of sampling year and block were included in Models 1-3. Models 1-
564 3 were compared based on their AIC values, and the model with the lowest AIC chosen as the
565 preferred model. Soil temperature, moisture, pH, and total plant richness were selected for both

bacteria and fungi although slight differences were observed with respect to nitrogen and plant biomass (Table S6, S7). These variables in the preferred model were used in the downstream structural equation modelling analyses.

Structural equation modeling

To further discern the direct and indirect effects of the environmental drivers on microbial biodiversity, structural equation modelling (SEM) analyses were performed to examine the relationships among experimental treatments, soil and plant variables, and microbial diversity. To correct the potential temporal autocorrelation, we used data at the plot level, by averaging the microbial or environmental data across time points of the same plot. We first considered a hypothesized conceptual model (Extended Data Fig. 8) that included all reasonable pathways. Then, we sequentially eliminated non-significant pathways unless the pathways were biologically informative, or added pathways based on the residual correlations. The procedure was repeated until the model showed sufficient fitting with the p values of χ^2 test larger than 0.05 (i.e., the predicted model and observed data are not significantly different) and the root mean square error of approximation (RMSE) < 0.08. The SEM-related analysis was performed using the lavaan R package[68].

Data availability

The DNA sequences of the 16S rRNA gene, 18S rRNA gene and ITS amplicons were deposited to the National Center for Biotechnology Information (NCBI) under the project accession number PRJNA331185. Raw shotgun metagenomic sequences are deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study no. PRJNA533082. Silva 138.1 Ref NR database

589 is available at https://www.arb-silva.de/documentation/release-138/. Protist Ribosomal Reference
590 database (PR2) databases is available at https://github.com/pr2database/pr2database. The ASV
591 table and ASV representative sequences, soil physical and chemical attributes, and plant biomass
592 and richness are downloadable online at http://www.ou.edu/ieg/publications/datasets.. Source data
593 are provided with this paper.
594
595 **Code availability**
596 R scripts for statistical analyses are available on GitHub at https://github.com/Linwei-
597 Wu/warming_soil_biodiversity.
598
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607
608 **Author contributions**
609 All authors contributed intellectual input and assistance to this study. The original concepts were
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611 M.Y., J.K., Y.F., A.Z., D.N., J.M., S.J., S.H., Z.Y., Y.O. and Liyou W. Sampling collection, soil

chemical and microbial characterization were carried out by M.Y., X.G., Linwei W., J.G., Z.G., and X.Z. Data analysis were done by Linwei W., Y.Z., X.G., and N.X. with the assistance provided by D.N. and J.Z. All data analysis and integration were guided by J.Z. The manuscript was prepared by J.Z., Linwei W., Y.Z., X.G., with significant input from J.M.T., Y.Y., X.L.. Considering their contributions in terms of site management, data collection, analyses and/or integration, Linwei W., Y. Z., and X.G. were listed as co-first authors.

Competing interests

The authors declare no competing interests.

Figure legends

Fig. 1. Effects of experimental warming on soil microbial communities. a, Experimental settings for treatments. **b-d**, The effect sizes of warming, altered precipitation level and clipping on microbial richness (**b**), phylogenetic diversity (**c**) and biomass (**d**). The estimated effect sizes are regression coefficients based on rescaled response variables (with zero mean and unit standard deviation) in the linear mixed-effects models. Statistical significance is based on Wald type II χ^2 tests (n = 360). Bars represent mean effect sizes and error bars represent standard errors. The effects of treatment interactions and exact *p* values are indicated in Table S1 & S2. Significances of χ^2 tests are denoted by asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. PD, Faith's phylogenetic diversity. PLFA, phospholipid fatty acid analysis. AMF, arbuscular mycorrhiza fungi.

Fig. 2. Effects of experimental warming on different microbial taxa. a, Effect sizes of warming on the (rescaled) richness of major microbial groups based on linear mixed-effects models. Data are presented as mean values ± standard errors of the estimated effect sizes. Statistical significance is based on Wald type II χ^2 tests (n= 360), which is denoted by asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Insignificant changes are denoted by grey dots. **b**, The phylogenetic relationship of individual bacterial ASVs (amplicon sequence variants, the first ring inside). Only the ASVs with a significant response (adjusted $p < 0.05$) to warming and their average read numbers $\geqslant 2$ among warmed or unwarmed samples were included in the tree. The outside and inside bars of the second ring represent the positive and negative effect sizes of warming on rescaled taxon relative abundances. Colors of the branches in the first ring and the bars in the second ring correspond to individual phyla or classes, and the grey color in the tree indicates unclassified or other minor phyla. Colors in the third ring represent ASVs with significant increase (yellow) or decrease (blue) under warming. The area sizes of the pies reflect the total relative abundance of bacterial phyla/classes across all samples, where the yellow and blue parts represent the proportions of the total abundance of ASVs which increased and decreased under warming, respectively.

Fig. 3. Environmental drivers of microbial diversity. a, Correlations between environmental variables and microbial diversity. Edge width corresponds to the absolute value of correlation coefficient determined by the linear mixed-effects models. Colors indicate correlation types. Solid and dashed lines denote significant and insignificant correlations, respectively, based on Wald type II χ^2 tests (n= 360 biologically independent soil samples). Pairwise comparisons of environmental factors are shown in the triangle, with a color gradient denoting Pearson's correlation coefficient. **b**, Structural equation models (SEMs) showing the relationships among treatments, soil and plant variables, and bacterial and protistan richness. Blue and red arrows indicate positive and negative relationships, respectively. Solid or dashed lines indicate significant ($p < 0.05$) or nonsignificant

relationships. Numbers near the pathway arrow indicate the standard path coefficients. R^2 represents the proportion of variance explained for every dependent variable. $\chi^2 = 47.69$, df = 34, $p = 0.06$ (large p value indicates the predicted model and observed data are equal, i.e., good model fitting). Comparative fit index (CFI) = 0.955, and n = 48 independent plots. **c**, Standardized total effects (direct plus indirect effects) derived from SEMs. **d**, Correlations between microbial richness and ecosystem functioning. The color denotes the correlation coefficient determined by the linear mixed-effects model. Statistical significance is based on Wald type II χ^2 tests with n =360 independent soil samples. The p values were adjusted by false discovery rate and are denoted by asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. GPP: gross primary productivity; ER: ecosystem respiration; NEE: net ecosystem exchange; R_h: heterotrophic respiration; R_s: soil total respiration.

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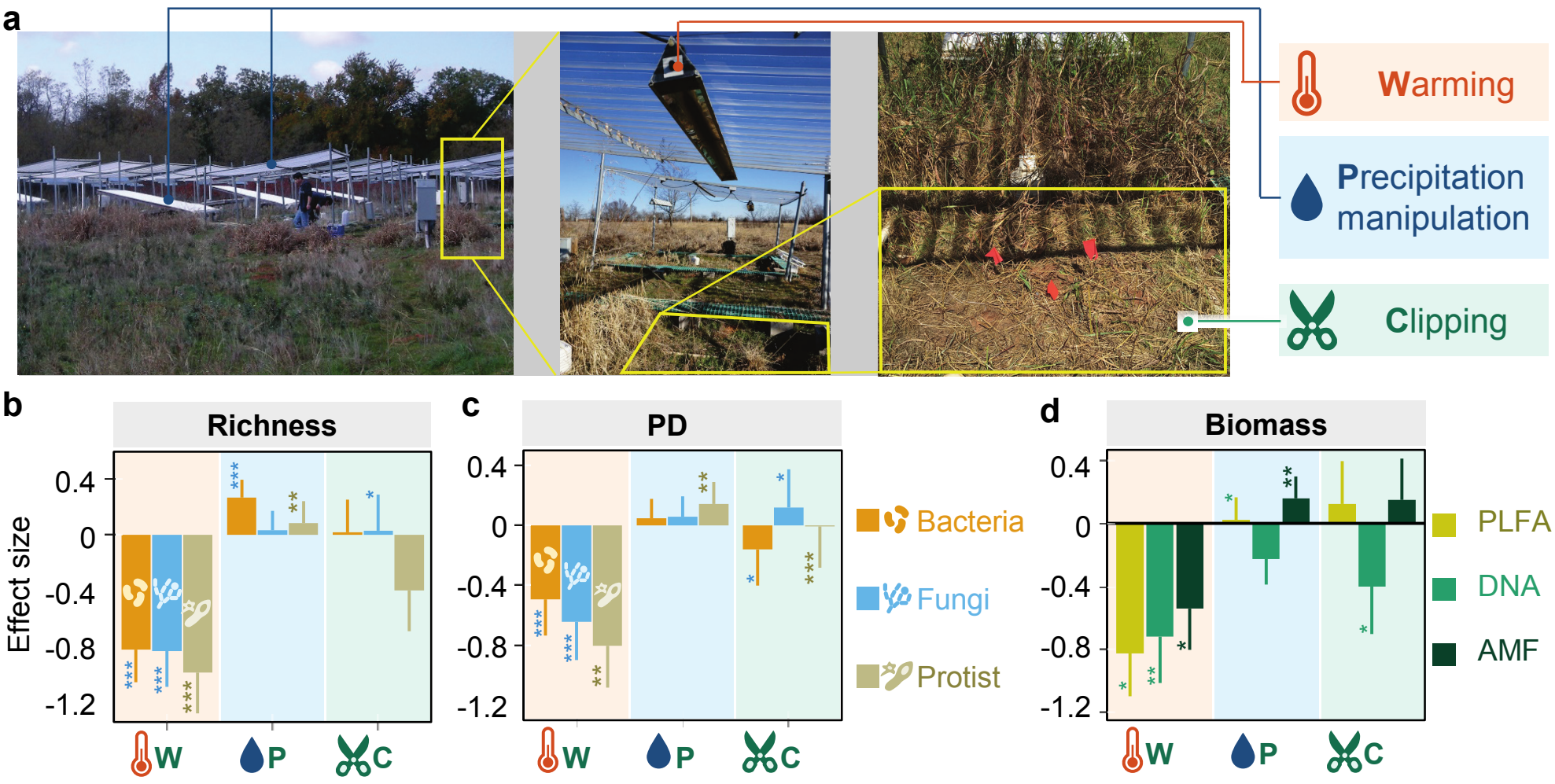
Fig 1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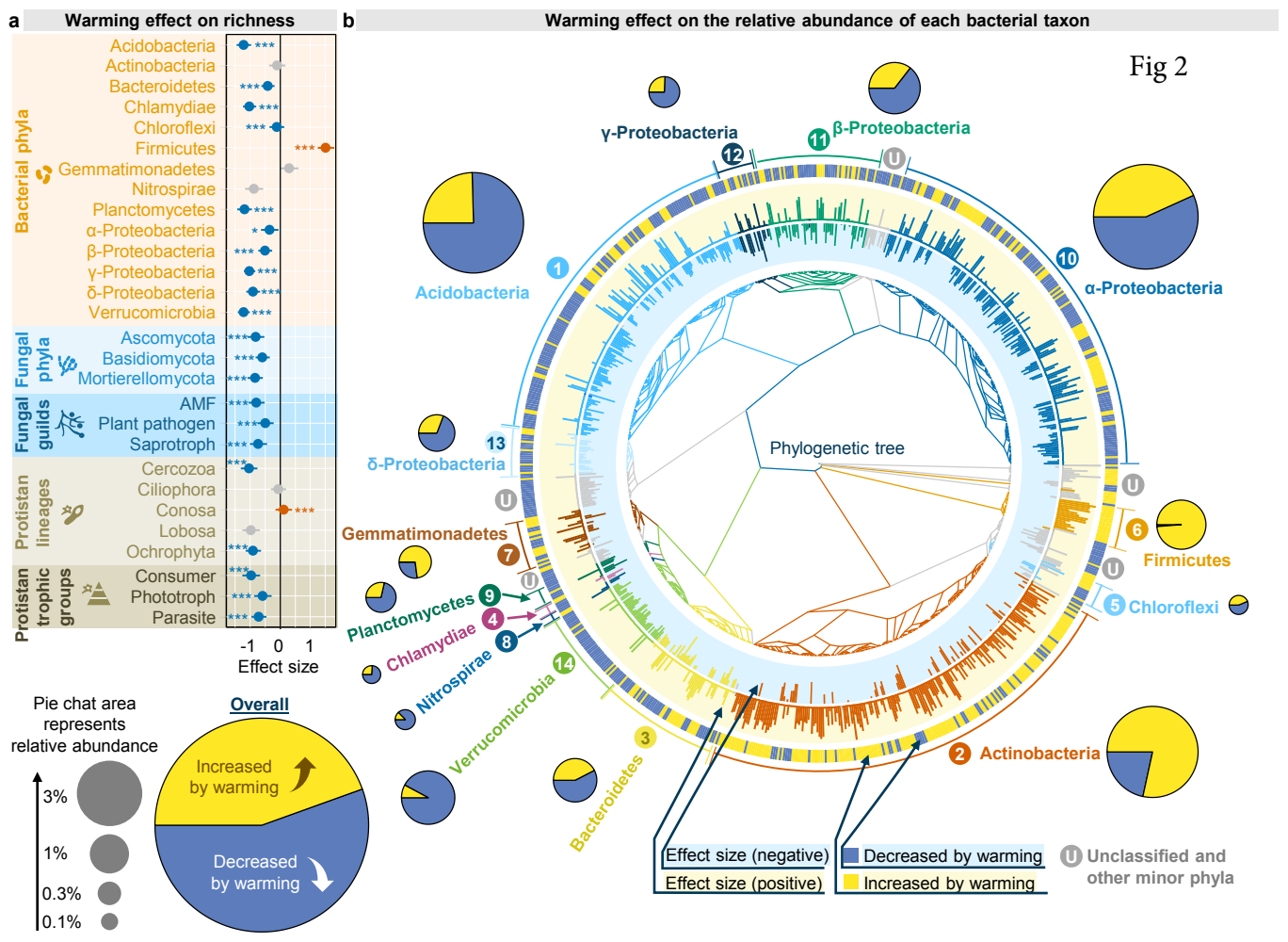
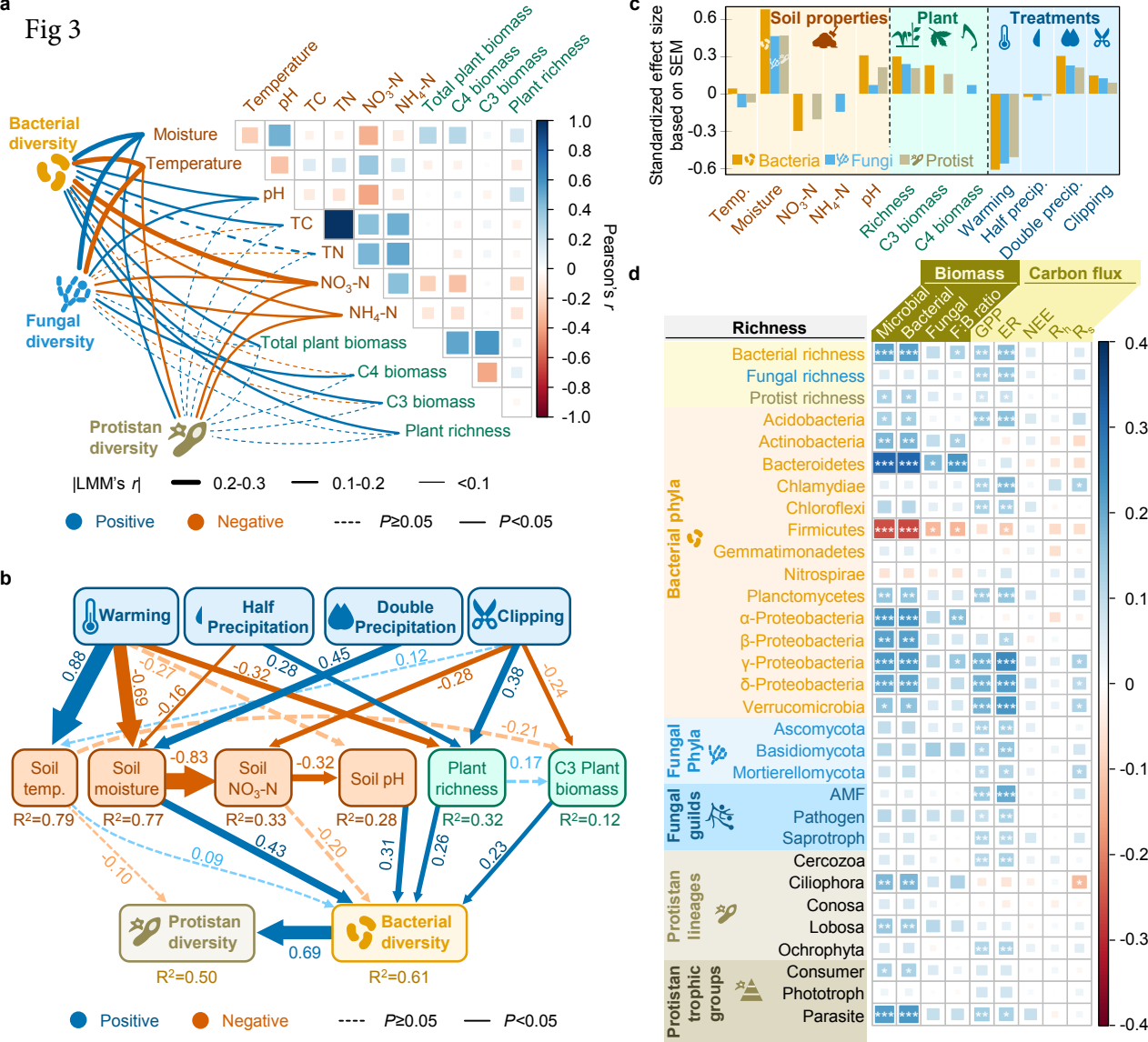

a
Warming effect on richness
Bacterial phyla
Acidobacteria
Actinobacteria
Bacteroidetes
Chlamydiae
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
α-Proteobacteria
β-Proteobacteria
γ-Proteobacteria
δ-Proteobacteria
Verrucomicrobia
Fungal phyla
Ascomycota
Basidiomycota
Mortierellomycota
Fungal guilds
AMF
Plant pathogen
Saprotroph
Protistan lineages
Cercozoa
Ciliophora
Conosa
Lobosa
Ochrophyta
Protistan trophic groups
Consumer
Phototroph
Parasite
-1
0
1
Effect size
b
Warming effect on the relative abundance of each bacterial taxon
Fig 2
γ-Proteobacteria
β-Proteobacteria
α-Proteobacteria
Acidobacteria
δ-Proteobacteria
Gemmatimonadetes
Planctomycetes
Chlamydiae
Nitrospirae
Verrucomicrobia
Bacteroidetes
Actinobacteria
Chloroflexi
Firmicutes
Phylogenetic tree
1
2
3
4
5
6
7
8
9
10
11
12
13
14
U
Pie chat area represents relative abundance
3%
1%
0.3%
0.1%
Overall
Increased by warming
Decreased by warming
Effect size (negative)
Effect size (positive)
Decreased by warming
Increased by warming
Unclassified and other minor phyla

Fig 3


a
Temperature
pH
TC
TN
NO3-N
NH4-N
Total plant biomass
C4 biomass
C3 biomass
Plant richness
Bacterial diversity
Moisture
Temperature
pH
TC
TN
NO3-N
NH4-N
Total plant biomass
C4 biomass
C3 biomass
Plant richness
Fungal diversity
Protistan diversity
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
Pearson's r
|LMM's r|
0.2-0.3
0.1-0.2
<0.1
Positive
Negative
P≥0.05
P<0.05
b
Warming
Half Precipitation
Double Precipitation
Clipping
0.88
-0.69
-0.27
-0.16
-0.32
0.28
0.45
0.12
-0.28
0.38
-0.24
-0.21
Soil temp.
Soil moisture
Soil NO3-N
Soil pH
Plant richness
C3 Plant biomass
R2=0.79
R2=0.77
R2=0.33
R2=0.28
R2=0.32
R2=0.12
-0.83
-0.32
0.17
-0.10
0.09
0.43
-0.20
0.31
0.26
0.23
Protistan diversity
Bacterial diversity
0.69
R2=0.50
R2=0.61
Positive
Negative
P≥0.05
P<0.05
c
Standardized effect size based on SEM
0.6
0.3
0
-0.3
-0.6
Soil properties
Plant
Treatments
Bacteria
Fungi
Protist
Temp.
Moisture
NO3-N
NH4-N
pH
Richness
C3 biomass
C4 biomass
Warming
Half precip.
Double precip.
Clipping
d
Biomass
Carbon flux
Microbial
Bacterial
Fungal
F:B ratio
GPP
ER
NEE
Rh
Rs
Richness
Bacterial phyla
Bacterial richness
Fungal richness
Protist richness
Acidobacteria
Actinobacteria
Bacteroidetes
Chlamydiae
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospirae
Planctomycetes
α-Proteobacteria
β-Proteobacteria
γ-Proteobacteria
δ-Proteobacteria
Verrucomicrobia
Fungal Phyla
Ascomycota
Basidiomycota
Mortierellomycota
Fungal guilds
AMF
Pathogen
Saprotroph
Protistan lineages
Cercozoa
Ciliophora
Conosa
Lobosa
Ochrophyta
Protistan trophic groups
Consumer
Phototroph
Parasite
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4