Science of the Total Environment 652 (2019) 1474–1481

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Long-term elevated CO_2 shifts composition of soil microbial communities in a Californian annual grassland, reducing growth and N utilization potentials

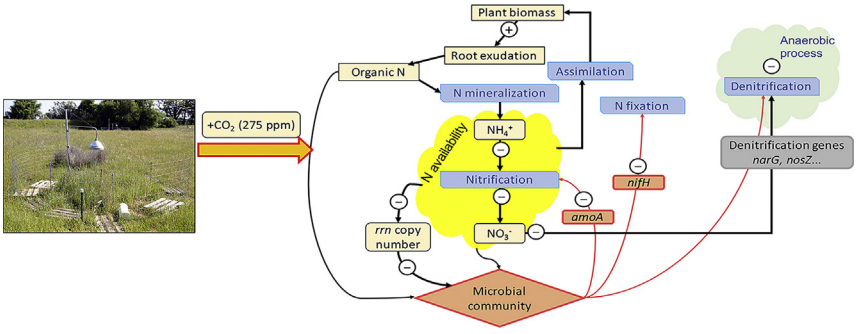
Sihang Yang [a], Qiaoshu Zheng [a], Mengting Yuan [b], Zhou Shi [b], Nona R. Chiariello [c], Kathryn M. Docherty [d], Shikui Dong [e], Christopher B. Field [c], Yunfu Gu [b,f], Jessica Gutknecht [g,h], Bruce A. Hungate [i], Xavier Le Roux [j], Xingyu Ma [a], Audrey Niboyet [k,l], Tong Yuan [b], Jizhong Zhou [a,b,m], Yunfeng Yang [a,*]

[a] State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing 100084, PR China
[b] Institute for Environmental Genomics, Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA
[c] Stanford University, Stanford, CA 94305, USA
[d] Department of Biological Sciences, Western Michigan University, Kalamazoo, MI 49008, USA
[e] State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Beijing normal university, Beijing 100875, PR China
[f] Department of Microbiology, College of Resource Sciences and Technology, Sichuan Agricultural University, Chengdu 611130, PR China
[g] Department of Soil Ecology, Helmholtz Centre for Environmental Research - UFZ, Halle 06120, Germany
[h] Department of Soil, Water, and Climate, University of Minnesota, Twin Cities, Saint Paul, MN 55104, USA
[i] Center for Ecosystem Science and Society, Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA
[j] Microbial ecology Centre, INRA, CNRS, University of Lyon, University Lyon 1, UMR INRA 1418, UMR CNRS 5557, 43 boulevard du 11 novembre 1918, 69622 Villeurbanne, France
[k] Institut d'Ecologie et des Sciences de l'Environnement de Paris (Sorbonne Université, CNRS, INRA, IRD, Université Paris Diderot, UPEC), 4 place Jussieu, 75005 Paris, France
[l] AgroParisTech, 75005 Paris, France
[m] Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

HIGHLIGHTS

- Effects of 14 years of experimentally elevated CO_2 on soil microbes in a semi-arid grassland were examined.
- The abundance-weighted average *rrn* copy number of significantly changed OTUs declined by elevated CO_2.
- The nitrogen fixation gene *nifH* and the ammonium-oxidizing gene *amoA* significantly decreased by elevated CO_2.
- Elevated CO_2 constrained microbial N decomposition, thereby slowing potential maximum growth rate of microbial community.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 August 2018
Received in revised form 22 October 2018
Accepted 26 October 2018
Available online 28 October 2018

Editor: Henner Hollert

ABSTRACT

The continuously increasing concentration of atmospheric CO_2 has considerably altered ecosystem functioning. However, few studies have examined the long-term (i.e. over a decade) effect of elevated CO_2 on soil microbial communities. Using *16S rRNA* gene amplicons and a GeoChip microarray, we investigated soil microbial communities from a Californian annual grassland after 14 years of experimentally elevated CO_2 (275 ppm higher than ambient). Both taxonomic and functional gene compositions of the soil microbial community were modified by elevated CO_2. There was decrease in relative abundance for taxa with higher ribosomal RNA operon (*rrn*) copy number under elevated CO_2, which is a functional trait that responds positively to resource availability in

* Corresponding author.
E-mail address: yangyf@tsinghua.edu.cn (Y. Yang).

https://doi.org/10.1016/j.scitotenv.2018.10.353

Keywords:
Elevated CO_2
Microbial communities
Annual grassland
MiSeq sequencing
GeoChip

culture. In contrast, taxa with lower *rrn* copy number were increased by elevated CO_2. As a consequence, the abundance-weighted average *rrn* copy number of significantly changed OTUs declined from 2.27 at ambient CO_2 to 2.01 at elevated CO_2. The nitrogen (N) fixation gene *nifH* and the ammonium-oxidizing gene *amoA* significantly decreased under elevated CO_2 by 12.6% and 6.1%, respectively. Concomitantly, nitrifying enzyme activity decreased by 48.3% under elevated CO_2, albeit this change was not significant. There was also a substantial but insignificant decrease in available soil N, with both nitrate (NO_3^-) (−27.4%) and ammonium (NH_4^+) (−15.4%) declining. Further, a large number of microbial genes related to carbon (C) degradation were also affected by elevated CO_2, whereas those related to C fixation remained largely unchanged. The overall changes in microbial communities and soil N pools induced by long-term elevated CO_2 suggest constrained microbial N decomposition, thereby slowing the potential maximum growth rate of the microbial community.

1. Introduction

Increasing atmospheric CO_2 concentration, which is largely attributed to anthropogenic activities (IPCC, 2014), is well-documented to cause rising global temperature. Ecosystem responses to these global environmental changes can feed back to affect climate. As the increase in atmospheric CO_2 could continue in the coming decades, it is critical to assess its influence on terrestrial biogeochemical cycling and ecosystem functioning. Elevated CO_2 typically increases plant growth (Ainsworth and Long, 2005) and plant organic matter inputs to the soil via litter or fine root and root exudate production (de Graaff et al., 2006; Hungate et al., 2009; Reich et al., 2001). Elevated CO_2 also increases both soil microbial biomass and respiration (Dijkstra et al., 2006; Reid et al., 2012). However, the influence of elevated CO_2 on plants and microbial communities may be constrained when available nitrogen (N) becomes depleted in the long-term (i.e., progressive N limitation) (Reich et al., 2006). Limited N availability can also affect microbial decomposition of soil organic matter (Hu et al., 2001), stimulating the CO_2-induced priming effect (i.e. the use of more recalcitrant fractions which are sources of additional N from soil) (Fontaine et al., 2011).

Elevated CO_2 could change the soil C pool and increase soil aggregate turnover rates. For example, the quantity of aggregate-occluded light fraction (OLF) of soil organic matter was decreased but the OLF lignin concentration was increased by elevated CO_2 in a grassland ecosystem (Henry et al., 2005). Simultaneously, the occlusion of soil aggregates could decrease the amount of soil organic matter accessible for microbial decomposition (Rovira and Vallejo, 2003). Although additional C fixation feeds rapidly cycling C pools (Hungate et al., 1997), the priming effect theory suggests that an increase in labile C pools under high CO_2 may evoke the breakdown of recalcitrant C (Hoosbeek et al., 2004). Extracellular enzyme activities, often used as indices for assessing changes in the function of soil microbial communities (Henry et al., 2005), can be affected by elevated CO_2. For example, Dhillion et al. (1996) reported that elevated CO_2 increased the activity of cellulose-degrading enzymes, likely as a result of increased turnover of fine roots.

In addition to C processes and associated microbial groups, elevated CO_2 can also affect N and phosphorus (P) cycling and the corresponding microbial communities. Elevated CO_2 may stimulate N_2O efflux from soil, though the effect can be variable (Brown et al., 2011; Niboyet et al., 2011a). This could be caused by stimulated heterotrophic denitrification as elevated CO_2 enhances root-derived carbon inputs to soil (Regan et al., 2011). It could also be due to the enhanced anaerobic denitrification, in response to the reduced soil oxygen concentration owing to increases in soil moisture (Arnone III and Bohlen, 1998). The effects of elevated CO_2 on nitrification and nitrifier abundance are idiosyncratic (Barnard et al., 2006; Horz et al., 2004; Le Roux et al., 2016; Niboyet et al., 2009; Niboyet et al., 2011b; Regan et al., 2011; Simonin et al., 2015): elevated CO_2 sometimes decreases nitrification and nitrifier abundances due to increased soil moisture and decreased soil oxygen or to decreased availability of N for nitrifiers (Barnard et al., 2004). N mineralization can also increase under high CO_2, possibly caused by higher soil moisture and increased root biomass (Ebersberger et al., 2003). P may function as a secondary limiting resource for primary production in many ecosystems (Vitousek, 2004). Some studies showed that elevated CO_2 did not alter P limitation or demand (Menge and Field, 2007). However, other studies showed that elevated CO_2 could increase the activities of alkaline phosphatase and urease, thus promoting P acquisition (Ebersberger et al., 2003). While the effects of rising CO_2 on plant growth and soil biogeochemical cycling are reasonably well understood, the effects of elevated CO_2 on the soil microbiome are controversial.

Soil microbial diversity has been reported to increase (Lesaulnier et al., 2008), decrease (He et al., 2012), or remain unchanged (Austin et al., 2009) in response to elevated CO_2. This inconsistency could be ascribed to high complexity of soil microbial communities (Gans et al., 2005), differences among ecosystems, techniques used to profile microbial communities, and the time scale of various studies. Previous studies focused mainly on taxonomic composition (He et al., 2012) or the composition of certain functional groups such as nitrifiers (Horz et al., 2004; Le Roux et al., 2016; Simonin et al., 2015); but comprehensive investigation into both taxonomic and functional compositions of the soil microbial community, especially looking at a broad range of functions, is still lacking. Moreover, studies of the effect of elevated CO_2 on the soil microbial community are often conducted using a relatively short-term CO_2 exposure. Only a limited number of studies examine the long-term (e.g. over 10 years) effect of elevated CO_2 on the soil microbial community. Since long-term manipulations are often necessary for detecting changes in soil microbial community dynamics (Gutknecht et al., 2012), this knowledge gap calls for attention, as the need is underscored by observations that microbes mediate important soil biogeochemical processes (Falkowski et al., 2008) and that plants rely on soil microbes for their mineral nutrition (Field et al., 1992). Thus, soil microbial communities must be considered when assessing the long-term environmental impact of elevated CO_2 and elucidating the underlying mechanisms.

Here, we report a study in a California annual grassland subjected to 14 years of elevated CO_2 by using a free-air CO_2 enrichment. We adopted high-throughput MiSeq sequencing of 16S rRNA gene amplicons to measure fine-resolution composition of microbial communities, and the microarray-based metagenomic tool, GeoChip, to detect functional genes related to C, N and P cycles. Our objectives were to: (i) determine the effect of elevated CO_2 on the overall taxonomic and functional gene composition of soil microbial communities; (ii) examine the effect of elevated CO_2 on microbial functional potentials related to C, N and P cycling; and (iii) link soil geochemistry and plant variables with soil microbial community. We hypothesize that, in response to elevated CO_2, (1) the microbial community would shift towards fast-growing taxa as soil C inputs are expected to increase; (2) the relative abundance of functional genes related to labile C degradation would increase; (3) but those related to recalcitrant C degradation would decrease; and (4) the relative abundance of some functional genes related to N cycling (e.g. nitrification genes) would decrease, due to higher N limitation.

2. Materials and methods

2.1. Site description and sampling

The study was conducted as part of the Jasper Ridge Global Change Experiment (JRGCE) located at Jasper Ridge Biological Preserve (37°40 N, 122°22 W), CA, USA (Menge and Field, 2007). The site is an annual grassland with sandstone-derived soil. The plant community is dominated by grasses, mainly non-native annuals (including *Avena barbata*, *Avena fatua*, *Bromus hordeaceus*, and *Lolium multiflorum*) and native perennials. The remainder of the vegetation is a mix of native and non-native annual and perennial forbs. The Mediterranean-type climate has cool, wet winters and warm, dry summers, with most of the growing season from November to April.

The elevated CO_2 experiment is part of a global change experiment set up in fall 1998 (Shaw et al., 2002) to test grassland responses to elevated CO_2, warming, N deposition, precipitation and all possible combinations between two levels (ambient and elevated) of these four main global change treatments. The experiment comprised 32 circular grassland plots of 2 m-diameter, which were each divided into four quadrants of 0.78 m^2 resulting in a total of 128 subplots. Half of the subplots were intentionally burned in July 2011. Fiberglass barriers were used underground to separate subplots, discouraging roots and resources from escaping. Aboveground mesh was also used to limit plants, seeds, and litter from crossing subplot boundaries.

In the present study, we focus on the control and elevated CO_2 treatments. Therefore, we examined samples from ambient and elevated CO_2 treatments with 4 replicates, which experienced ambient levels of temperature, N deposition, and precipitation and were unburned. CO_2 was elevated using a free-air CO_2 enrichment system, which continuously adjusted the speed and direction of CO_2 fumigation to keep CO_2 concentrations near the target level of +275 ppm compared to ambient subplots. One soil core per subplot of 5 cm diameter × 7 cm depth was collected on April 26 and 27, 2012. The soil was mixed and sieved through 2 mm mesh, and soil samples were then stored at 4 °C for a few days before conducting enzyme activities assays, at −20 °C before soil geochemical measurements, or at −80 °C before DNA extraction.

2.2. Measurements of soil and vegetation variables

Soil temperature data were recorded hourly from thermo-couples at depths of 2 cm and 10 cm. A Carlo Erba Strumentazione Model 1500 Series I analyzer was used to determine the total soil C (TC) and total soil N (TN). Soil Dissolved Organic Carbon (DOC) content was measured on a $CaCl_2$ extract using a Shimadzu TOC-analyzer. Soil ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were measured by filtering 2 M KCl solution extracts from 5 g of soil, with a SEAL Automated Segmented Flow analyzer. Soil pH was measured in water using 5 g of soil, and soil moisture was measured by the difference in weight after drying 10 g of soil at 105 °C for 24 h.

Aboveground plant material in each subplot was harvested the day before soil sampling. Individual plants were separated into functional groups (annual grasses, annual forbs, perennial grasses, and perennial forbs), and the biomass was oven-dried at 70 °C and weighed. The biomass of plant total shallow roots and fine roots was collected and oven-dried at 70 °C and weighed. Litter was also collected, dried at 70 °C and weighed.

Nitrifying and denitrifying enzyme activities, also called potential nitrification and denitrification rates, were measured on fresh soil samples. Nitrifying enzyme activity was measured according to Niboyet et al. (2011b) as nitrite (NO_2^-) plus NO_3^- production rate from 3 g soil samples amended with NH_4^+ (50 μg N-NH_4^+ g^{-1} dry soil) and incubated at 28 °C for 10 h with constant agitation at 180 rpm. NO_2^- and NO_3^- concentrations were measured after 0, 2, 4, 6, 8 and 10 h on an ion chromatograph (DX120, DIONEX, Salt Lake City, USA). Denitrifying enzyme activity was measured as nitrous oxide (N_2O) production rates from 5 g soil samples amended with NO_3^- (50 μg N-NO_3^- g^{-1} dry soil) and labile C (0.5 mg C-glucose g^{-1} dry soil and 0.5 mg C-glutamic acid g^{-1} dry soil) and incubated at 28 °C for 5 h under anaerobic conditions using acetylene to inhibit N_2O reduction (Patra et al., 2005). N_2O concentration was measured after 3 h of incubation every 25 min during 2 h on a gas chromatograph equipped with an electron capture detector (Agilent P200, Santa Clara, CA, USA).

2.3. DNA extraction, purification and quantification

DNA was extracted from 5 g soil samples using a freeze-grinding mechanical lysis method (Zhou et al., 1996). DNA extraction steps included liquid-N grinding, proteinase K lysis, phenol-chloroform extraction and isopropanol precipitation, followed by ~1% gel purification. The final DNA concentration was quantified with a PicoGreen method (Yang et al., 2014), and DNA quality was assessed by ratios of light absorbance at 260 nm/280 nm and 260 nm/230 nm.

2.4. 16S rRNA amplicon sequencing

PCR amplicons targeting the V4 region of 16S rRNA gene with primers F515 (5′-GTGCCAGCMGCCGCGG-3′) and R806 (3′-TAAT CTWTGGGVHCATCAG-5′) were sequenced with a MiSeq machine (Illumina, San Diego, CA) using a previously described protocol (Caporaso et al., 2012). Paired barcodes were used to identify paired-end raw sequences and then FLASH was used to combine the raw sequences. Btrim was used to trim unqualified sequences, and then joint sequences with multiple Ns were deleted. Then sequences were trimmed to 200–300 bp to remove potential non-16S rRNA gene sequence. Chimeras were removed using Uchime. UCLUST was used to identify operational taxonomic units (OTUs) at the 97% identity level, corresponding to species-like OTUs. Singlet OTUs, which were detected in only one sample, were deleted to improve data quality.

2.5. GeoChip 4.6 hybridization and signal processing

Microbial DNA was labeled with Cy-3 fluorescent dye using a random priming method. DNA was then purified with a DNA purification kit (Qiagen, Valencia, CA, USA) and dried at 45 °C for 45 min with a SpeedVac (ThermoSavant, Milford, MA, USA). Labeled DNA was then hybridized with GeoChip in a MAUI hybridization system (BioMicro, Salt Lake City, UT, USA) at 42 °C for 16 h. After manually washing the slides with three buffers provided by NimbleGen, a MS 200 Microarray Scanner (Roche Diagnostics, Madison, WI, USA) was used to scan the fluorescent intensities of each probe on GeoChip. Then the fluorescent intensities were digitalized using NimbleScan version 2.5 (Roche Diagnostics, Madison, WI, USA).

Raw GeoChip data were processed as previously described (Liu et al., 2015), including the following steps: (i) remove poor-quality spots with a signal-to-noise ratio (SNR) <2.0; (ii) remove genes detected only in one replicate out of four samples from the same treatment; (iii) perform logarithmic transformation of the signal intensity of each gene; and (iv) normalize the data by dividing the transformed signal intensities of each gene by the mean intensity in the sample.

2.6. Statistical analyses

To determine the effects of elevated CO_2 on taxonomic and functional compositions of the soil microbial community (objective 1), analyses were performed with the Vegan package in R v.3.1.0. Detrended correspondence analysis (DCA) and two dissimilarity tests, multiple response permutation procedure (MRPP, based on Bray-Curtis distance) and permutation multivariate analysis of variance (Adonis, based on Bray-Curtis distance), were used to assess the overall difference of microbial communities between the control and elevated CO_2 treatments. Shannon-Weaver and Simpson indices were used to estimate the alpha

diversity. Phylogenetic diversity and species richness were measured using the picante R package. The *rrn* copy number for each OTU on the rrnDB database was calculated as previously described, based on the closest relatives with known *rrn* copy number (Roller et al., 2016). To address objective 2, analysis of variance (ANOVA) was performed to analyze the differences in microbial functional gene abundance between elevated CO_2 and ambient CO_2 samples. The significance of differences was determined by two-tailed Student's *t*-test (two sample equal variance). The *P* value was adjusted for false discovery rate using the Benjamini-Hochberg method to take into account the high number of differences tested. The phylogenetic tree was constructed from OTUs showing the largest fold change in response to elevated CO_2 using MEGA 6 and visualized with the pipeline (http://itol.embl.de/).

To address objective 3, canonical correspondence analysis (CCA) and Mantel tests were used to explore the correlations between soil and vegetation variables and relative abundance of microbial phyla or functional genes. Soil variable measurements include soil temperature, moisture, pH, NH_4^+, NO_3^-, DOC, TC and TN. Vegetation variable measurements include annual/perennial grass and forb biomass, shallow and fine roots, and litter biomass. Before CCA modeling, variance inflation factors (VIFs) were calculated for each plant/soil variable to identify redundant constraints, using VIF values of over 10 as the criterion. Based on VIFs, soil temperature at the depth of 2 cm, soil moisture, soil NO_3^- concentration and annual grass biomass were selected for the CCA model with MiSeq sequencing data. For the CCA model with GeoChip data, the selected variables were soil moisture, soil total N, dissolved organic C, soil NO_3^-, and annual grass biomass.

3. Results

3.1. Bacterial and archaeal taxa

A total of 586,684 sequences were obtained, ranging from 63,037 to 82,066 reads per sample (Fig. S1). After re-sampling with 29,275 sequences per sample, 29,214 OTUs were obtained. We have a total of 128 samples in this JRGCE experiment, the lowest number of sequences in all these samples were 29,275, but this sample was not involved in this study. We stick to 29,275 sequence reads so that there is a potential to compare with other studies. Shannon-Weaver and Simpson indices indicated no significant differences in taxonomic diversity between ambient CO_2 and elevated CO_2 (Table S1). However, the overall taxonomic composition of soil microbial communities tended to differ between elevated CO_2 and ambient CO_2 (DCA, Fig. S2A), which was verified by the nonparametric multivariate statistical tests of dissimilarity ($P = 0.08$ by MRPP and $P = 0.08$ by adonis, Table S2). In addition, the phylogenetic diversity increased ($P = 0.05$) under elevated CO_2 (Table S1).

Significant differences of bacterial and archaeal taxonomic composition at the phylum level between elevated CO_2 and ambient CO_2 were observed (Fig. S3). The relative abundance of *Bacteroidetes* was lower ($P = 0.02$) under elevated as compared with ambient CO_2 (5.7% and 7.6% of all taxa, respectively). At the finer taxonomic level, 27 bacterial genera were altered ($P < 0.05$) in relative abundance (Fig. S4). Microbial taxa with high *rrn* copy numbers decreased ($P < 0.05$) under elevated CO_2. Responsive taxa include the *Bacteroidetes* genera *Ferruginibacter* (estimated *rrn* copy number of 4.50), *Segetibacter* (estimated *rrn* copy number of 4.50), *Ohtaekwangia* (estimated *rrn* copy number of 2.89), *Pseudomonas* belonging to γ-*Proteobacteria* (estimated *rrn* copy number of 5.01) and *Skemanella* belonging to α-*Proteobacteria* (estimated *rrn* copy number of 3.83) (Fig. S4). By contrast, genera with low *rrn* copy number increased ($P < 0.05$) under elevated CO_2. Responsive taxa include *Gp1 and Edaphobacter* belonging to *Acidobateria* (estimated *rrn* copy number of 1.13), *Labrys* (estimated *rrn* copy number of 2.00), and *Pedomicrobium* belonging to α-*Proteobacteria* (estimated *rrn* copy number of 1.67). The abundance-weighted average *rrn* copy number of all the significantly changed OTUs for each sample was decreased from 2.27 ± 0.018 under ambient CO_2 to 2.01 ± 0.008 under elevated CO_2 ($P < 0.01$, Fig. 1).

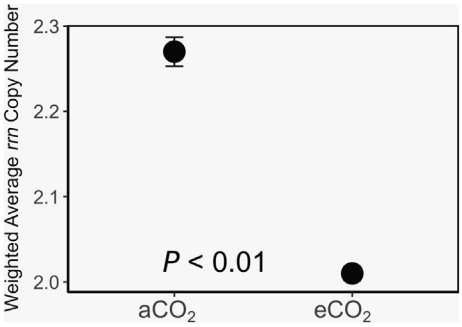


Fig. 1. Estimated weighted average *rrn* copy number of significantly changed OTUs under ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2) samples. Error bars indicate standard errors (n = 4).

We selected the 137 sequences that exhibited the largest relative changes (positive or negative) in response to elevated CO_2 to construct a phylogenetic tree of CO_2-responsive microorganisms. Among them, three OTUs with lower estimated *rrn* copy number from *Acidobacteria*, OTU-1182 (*Gp1* genus), OTU-42814 (*Gp6* genus) and OTU-20644 (*Gp1* genus), were more abundant ($P < 0.05$) under elevated CO_2 (Fig. S5). In contrast, OTUs with higher estimated *rrn* copy number, such as OTU-18988 (*Cellvibrio* genus of *Proteobacteria*), OTU-64624 (*Klebsiella* genus of *Proteobacteria*), OTU-26206 (*Flavobacterium* genus of *Bacteroidetes*) and OTU-101605 (*Adhaeribacter* genus of *Bacteroidetes*) showed lower ($P < 0.05$) abundance under elevated CO_2 (Fig. S5).

3.2. Functional genes

A total of 63,755 functional genes were detected by GeoChip 4.6. Microbial functional gene composition was affected by elevated CO_2 (DCA, Fig. S2B), which was verified by nonparametric multivariate statistical tests of dissimilarity ($P = 0.05$ based on MRPP and $P = 0.02$ based on Adonis, Table S2). In contrast, microbial functional diversity and evenness remained unchanged under elevated CO_2 (Table S1). The relative abundance of genes related to C cycling, metal homeostasis, and stress decreased (Fig. S6, $P < 0.05$) under elevated CO_2 whereas those of P cycling increased with elevated CO_2 (Fig. S6, $P < 0.05$). The relative abundance of genes related to N cycling remained similar between elevated CO_2 and ambient CO_2.

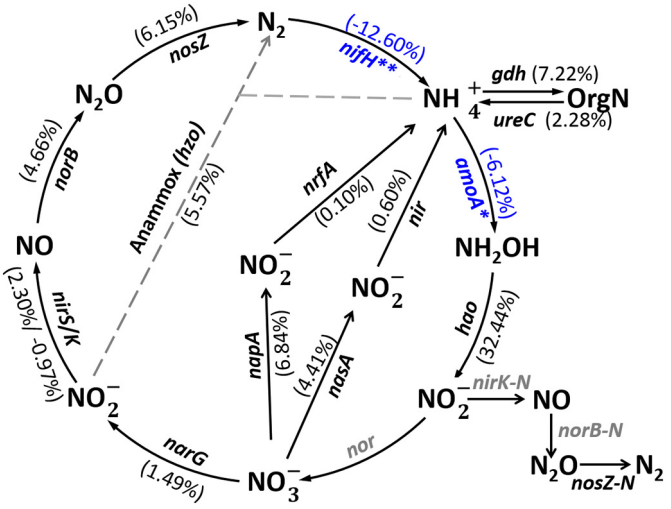


Fig. 2. Percent changes in abundances of N cycling genes between elevated CO_2 and ambient CO_2 treatments. When the CO_2 treatment had a significant effect, the significance of the difference is indicated as ** when $P < 0.01$ and * when $P < 0.05$.

3.2.1. N cycle genes

The relative abundances of the *nifH* gene encoding nitrogenase and the gene *amoA* encoding the ammonia monooxygenase decreased under elevated CO_2 by 12.6% and 6.1%, respectively (Fig. 2, $P < 0.01$). Other N cycling genes remained unchanged. Concomitantly, nitrifying (−48.3%) and denitrifying (−25.5%) enzyme activities, indicators of soil potential nitrification and denitrification rates, were lower under elevated CO_2 as compared to ambient CO_2 though those changes were not significant ($P > 0.05$; Table S3).

3.2.2. C cycle genes

We did not observe a significant effect of CO_2 treatment on the relative abundances of any genes associated with C fixation. The relative abundances of genes associated with starch degradation decreased ($P < 0.01$), whereas genes associated with pectin, hemicellulose, cellulose, chitin and lignin degradation remained unchanged (Fig. S7). More precisely, the relative abundances of *amyA* (−13.3%, $P < 0.01$) encoding alpha-amylase and *cda* (−11.3%, $P < 0.01$) encoding cytidine deaminase decreased, whereas that of *glucoamylase* increased (+9.5%, $P < 0.01$) under elevated CO_2 (Fig. 3). Similarly, *exoglucanase* (+13.4%, $P < 0.01$) and *cellobiase* (+9.3%, $P < 0.01$) increased under elevated CO_2, whereas CDH encoding CDP-diacylglycerol phosphotidylhydrolase decreased (−4.8%, $P < 0.01$). Among chitin degradation genes, *chitinase* (+3.5%, $P < 0.01$) and *endochitinase* (+1.2%, $P < 0.01$) increased but *N-acetyglucosaminidase* (−2.0%, $P < 0.01$) decreased. In addition, some of the pectin degradation genes (*pel*, *pectate lyase* and *rgl*) decreased whereas others (*exopolygalacturonase of fungi*, *pec* and *rgh*) increased.

3.2.3. P cycle genes

The relative abundances of the two P cycle genes targeted by GeoChip 4.6 were significantly increased under elevated CO_2, i.e. the genes encoding exopolyphosphatase (*ppx*) for inorganic polyphosphate degradation, and polyphosphate kinase (*ppk*) for polyphosphate biosynthesis (*ppx*: +8.8%, $P < 0.01$; and *ppk*: +4.9%, $P = 0.05$) (Fig. S8).

3.3. Relationships among microbial communities and soil and plant characteristics

To identify possible environmental drivers for changes in the soil microbial community, we examined soil and plant variables under ambient and elevated CO_2 (Table S3). In 2012, elevated CO_2 significantly increased the biomass of fine root (+70.6%, $P < 0.05$). Despite suggestions, effects on biomass of annual grasses (+29.3%), perennial forbs (+77.6%), perennial grass (+123.5%) and total shallow roots (+60.5%) (Table S3) were not significant. In April 2012, there were also no significant effects of elevated CO_2 on soil N pools and microbial N activities, despite suggestions of decreased soil NO_3^- (−27.4%), soil NH_4^+ (−15.4%), nitrifying enzyme activity (−48.3%) and denitrifying enzyme activity (−25.6%) under elevated CO_2. Other environmental variables, such as soil pH, TC, TN and litter biomass, were similar between ambient and elevated CO_2.

We performed CCA to explore soil and plant variables for their linkages with microbial communities. Four variables (soil temperature at 2 cm, soil NO_3^-, soil moisture, and annual grass biomass in April 2012) were selected to perform CCA on microbial taxonomic composition (see Materials and Methods for details). The resulting a model explained 58.9% of the total variance ($P = 0.05$, Fig. 4A). Soil temperature, soil NO_3^-, and soil moisture appeared to be the important environmental variables linking to changes in microbial taxonomic composition.

Five variables (soil moisture, dissolved organic C, soil NO_3^-, total N and annual grass biomass) were selected for the CCA model on microbial functional composition, which explained 62.5% of the total variance ($P = 0.05$, Fig. 4B). Among them, soil NO_3^-, dissolved organic carbon and annual grass biomass were major variables related to microbial functional composition.

4. Discussion

4.1. Long-term elevated CO_2 treatment induces a shift in bacterial taxonomic composition

The microbial community was shifted towards taxa with lower *rrn* copy number under elevated CO_2 (Fig. 1). The *rrn* copy number is

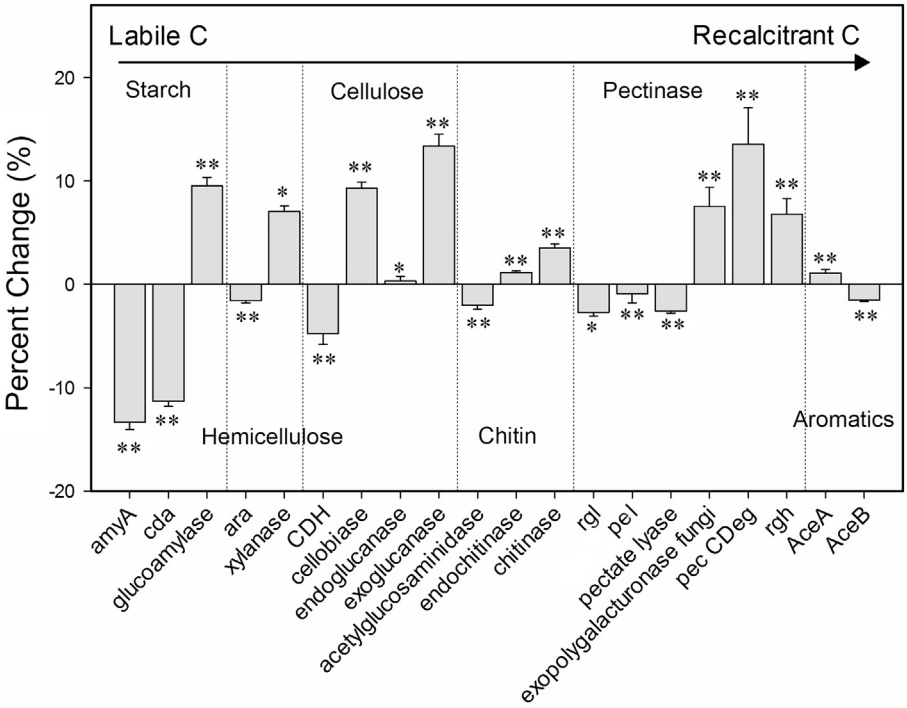


Fig. 3. Percent changes in abundances of C degradation genes between elevated CO_2 and ambient CO_2 (C sources ranked from labile on the left to recalcitrant on the right). Abundances of all C degradation genes that were significantly affected by the CO_2 treatment are indicated. Error bars indicate standard errors (n = 4). **, $P < 0.01$ and *, $P < 0.05$.

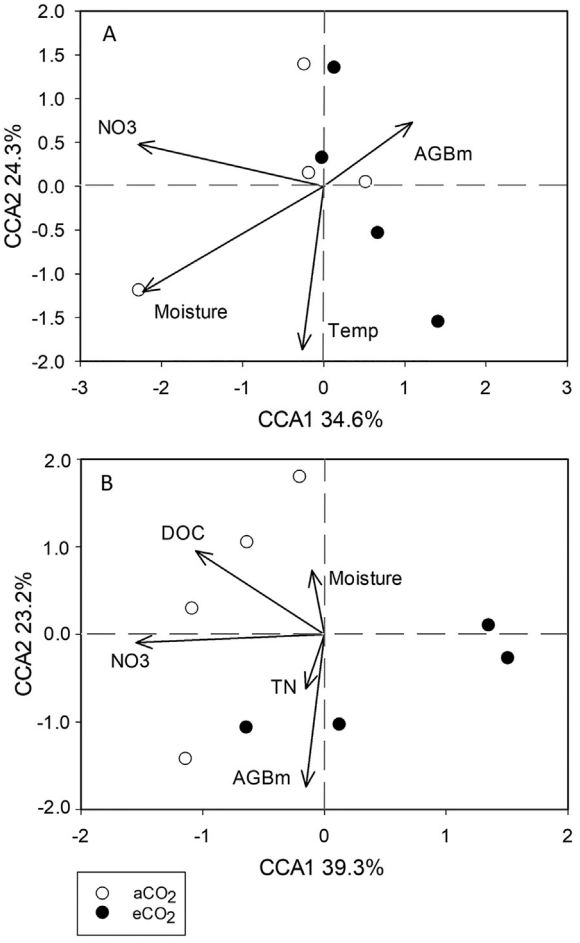


Fig. 4. Canonical correspondence analysis models of (A) the taxonomic composition of the bacterial community, accounting for all OTUs at the significance $P = 0.05$; and (B) the functional composition of the microbial community, accounting for all probes at the significance $P = 0.05$. The value associated to each axis is the proportion of total variation attributed to the corresponding axis. Temp, average soil temperature at 2 cm in April 2012; DOC, soil dissolved organic C; AGBm, aboveground grass biomass; TN, total soil N concentration; Moisture, soil moisture; NO_3, soil nitrate concentration.

thought to reflect ecological strategies, with high *rrn* copy number indicating rapid responses to resource input (i.e. r strategists) and low *rrn* copy number reflecting efficient allocation of resources under constant, slow-growth environments (i.e. K strategists) (Klappenbach et al., 2000; Lee et al., 2009). The *rrn* copy number is indicative of the maximum growth rate of bacteria in culture, whereas the carbon use efficiency is inversely related to maximal growth rate and *rrn* copy number (Roller et al., 2016). Our result was consistent with a previous study showing that average *rrn* copy number decreased with successional time in salt marsh sediment, deglaciated Alaskan soil and a burned soil from Colorado (Nemergut et al., 2016).

Bacteroidetes include many fast-growing members with strong cellulolytic and chitinolytic capacity (Lladó et al., 2015) and the potential to metabolize both plant and fungal cells (Lesaulnier et al., 2008; Lladó et al., 2015). We found that *Bacteroidetes*, especially the genera *Segetibacter*, *Ferruginibacter* and *Ohtekwangia*, characterized by high *rrn* copy number, decreased under elevated CO_2 (Fig. S4). *Opitutae* genus of *Verrucomicrobia* also decreased under elevated CO_2 (Fig. S4), consistent with the observation of decreased *Verrucomicrobia* in an elevated CO_2 experiment in trembling aspen (Lesaulnier et al., 2008). In contrast, there was a significant increase in the *group 1* (*Gp1*) genus of *Acidobacteria* (Fig. S4), which was reported to be abundant among very slow-growing and mini-colony-forming soil bacteria (Davis et al., 2011), and whose growth is suppressed by the addition of organic matter (Stevenson et al., 2004). These results contrast with those from other CO_2 experiments. In plantations and in scrub and tidal marsh exposed to 10 years of elevated CO_2, results from Sanger sequencing showed that *Acidobacteria Gp1* declined (Dunbar et al., 2012). In grassland mesocosms exposed to short-term elevated CO_2, PLFA analysis showed no responses of the fungal/bacterial ratio and Gram positive/Gram negative bacteria ratios used as proxies of the prevalence of K-strategists in the microbial community (Simonin et al., 2017). These contrasting responses to elevated CO_2 might be caused by differences in nutrient and other resource limitations across ecosystems (Shaw et al., 2002) or in the technologies used to characterize microbial communities (Hayden et al., 2012). In the grassland experiment we assessed, the shift towards microorganisms with a lower *rrn* copy number under elevated CO_2 may indicate a shift in the microbial community towards slow-growing taxa, possibly in response to increased competition for nutrients under elevated CO_2.

4.2. Long-term effects of elevated CO_2 on microbial functions related to soil biogeochemical cycling

The decreases of *nifH* and *amoA* genes in relative abundances were concurrent with substantial but insignificant decreases of soil nitrifying and denitrifying enzyme activities (Table S3), and were in agreement with previous studies conducted at the JRGCE where elevated CO_2 was reported to decrease the total abundance of bacterial ammonia-oxidizers (Horz et al., 2004) and potential nitrification rates (Barnard et al., 2006), but contrasted with a previous study that reported no effect of elevated CO_2 on potential nitrification and denitrification rates after 7 and 8 years of CO_2 exposure (Niboyet et al., 2011b). The CO_2 effect on nitrification and nitrifiers could vary according to N availability (Niboyet et al., 2009).

In soybean agro-ecosystems, the relative abundances of genes associated with N fixation and denitrification were significantly increased by elevated CO_2, possibly due to the increased labile C inputs into soil via litter and root exudation (He et al., 2014). In the BioCON grassland, elevated CO_2 stimulated the relative abundances of genes related to N fixation (He et al., 2010) and the growth and N_2-fixation rates of legume species in monoculture (Lee et al., 2003). The presence of N_2-fixing legume species also stimulated net photosynthesis and leaf N concentration of co-occurring non-fixing species, though legumes did not enhance the overall plant biomass response (Lee et al., 2003). In our study, the decreases in the abundances of N cycling genes and trends towards decreasing N enzyme activities may reflect increased N limitation to microbes under elevated CO_2.

Alpha amylase (*amyA*), which hydrolyses the α (1–4) glycosidic bond of starch, decreased under elevated CO_2, but glucoamylase, which hydrolyses α (1–4) and α (1–6) glycosidic bonds of starch, increased under elevated CO_2 (Fig. 2). Since the changes of labile C degradation genes were inconsistent, and labile C is of a small portion of the C pool (Carol Adair et al., 2011; Reid et al., 2012), changes in labile C caused by elevated CO_2 might make a negligible contribution to long-term storage (Gill et al., 2002). In contrast to our findings, a study conducted in the BioCON grassland reported significant increases in the relative abundances of carbon degradation genes, including *amyA*, *pullulanase*, *arabinofuranosidase*, and *vanilate demethylase*, after 8–10 years of elevated CO_2 (He et al., 2010). This inconsistency might be caused by different soil N availability. Specifically, in their study, elevated CO_2 increased gene abundances for symbiotic N_2 fixation and labile C degradation, and it stimulated soil microbial biomass, together indicating increased resource availability that stimulated fast-growing microorganisms, which were previously at a low abundance but grow quickly (He et al., 2010).

C stocks generally increase under elevated CO_2 due to the continuous input of C belowground by plants, though a few studies also observed unchanged or decreased soil C stocks (Qiao et al., 2014). In this study, we did not find any evidence of a change in soil microbial

community or soil characteristics corresponding to a changed soil C storage under elevated CO_2. More precisely, no significant changes were observed in soil TC (Table S3).

We observed a significant increase of the relative abundance of inorganic polyphosphate degradation gene ppx under elevated CO_2 (Fig. S8), possibly suggesting an increase in the degradation of polyphosphates, as well as the availability of inorganic P under elevated CO_2 (He et al., 2010). This result implies that long-term elevated CO_2 in the JRGCE might lead to increased utilization of soil P. However, a four-year elevated CO_2 study in this same grassland showed that neither phosphatase activity nor P demand was altered (Menge and Field, 2007).

The effects of elevated CO_2 on plant C inputs to soil, soil moisture, or nutrient availabilities are likely the key drivers of any shifts in the soil microbial community (Carol Adair et al., 2011). The shift towards taxa with low *rrn* copy numbers (putative slow-growing taxa) may suggest that elevated CO_2 shifts the interaction between plants and microbes towards the priority of plant utilization of nitrogen, since plants outcompete microbes for N when N is limiting (Hu et al., 2001). Plants can aggravate N limitation for microbes by decreasing the allocation of N-rich metabolites to root exudates released to soil under elevated CO_2 (Tarnawski and Aragno, 2006). When N limits the growth of microbes (Henry et al., 2006), the "priming effect" theory suggests that soil microbes could utilize recalcitrant C to gain N when the labile C input is increased under elevated CO_2 (Gill et al., 2002). Whether this effect would affect soil C stocks depends on the availability of substrates in the soil (Qiao et al., 2014).

When N availability is high, increased C input caused by elevated CO_2 could stimulate microbial activity and N utilization (Diaz et al., 1993; Zak et al., 1993). The main effects of elevated CO_2 on soil microbiota occur via plant metabolism and root secretion, especially in C3 plants (Drigo et al., 2008). Under elevated CO_2, C3 plants increased root growth without increasing soil N input (i.e., total efflux rate of amino acids), but C4 plants increased soil N input (total efflux rate of amino acids) without promoting root or shoot growth (Drigo et al., 2008). Elevated CO_2 accelerated the successional replacement of C4 grasses by C3 forbs (Polley et al., 2003), which possibly decreased soil N input. Therefore, elevated CO_2 could reduce microbially available soil N, exacerbate N limitation to microbes, and reduce microbial respiration per unit biomass (Hu et al., 2001). In ecosystems where N strongly limits plant growth, both fine root production and mycorrhizal infection of plants may increase, reducing the path length for soil N in solution to diffuse to roots (Rillig et al., 1998). As a result, microbially available N could be outcompeted by plant N uptake (Hu et al., 2001). We observed a significant linkage between plant aboveground biomass and microbial taxonomic communities (Fig. 4). Such a response of the soil-plant system to elevated CO_2 was also observed in nutrient-poor grassland on a silty clay loam where N stocks in living plants and surface litter increased under elevated CO_2, but N in soil organic matter and microbes remained unaltered (Niklaus et al., 2003). However, plant biomass production in the JRGCE study is generally unresponsive (Dukes et al., 2005; Zavaleta et al., 2003) or suppressed by elevated CO_2 (Henry et al., 2006; Shaw et al., 2002). Over the entire experiment, elevated CO_2 had no significant effect on above or below-ground biomass (Zhu et al., 2016).

5. Concluding remarks

This study examines soil microbial communities in an annual grassland in California, USA, after 14-years of elevated CO_2 exposure. Our hypothesis that elevated CO_2 shifts microbial community towards fast-growing taxa was refuted, which might be attributed to lack of fundamental understanding in ecological consequences of long-term elevated CO_2 treatment. In particular, this may suggest that other resources like nutrients become increasingly limiting under high CO_2, thus counteracting any selection of fast-growing taxa by the enhanced labile C availability. The decreased *rrn* copy number of significantly changed OTUs suggested a shift of microbial community towards slow-growing taxa, possibly indicative of increased competition for nutrients. Consistently, the relative abundance of the *amoA* gene decreased under elevated CO_2, which might also reflect higher N (NH_4^+) limitation to soil microorganisms. The relative abundance of the *nifH* gene also decreased under elevated CO_2, suggesting that the decreased N availability in soil induced by higher plant N uptake was likely not compensated by increased N_2 fixation. Long-term elevated CO_2 exposure may shift N reallocation from soil to plant biomass, resulting in decreased available N in soil. Overall, those results suggest that the response of soil microorganisms to long-term CO_2 elevation is not simply driven by the treatment-induced increase in labile C sources, which should favor fast-growing taxa. In contrast, the changes in the soil microbial community seem to be driven by cascading treatment effects on the availability of other resources (decreased soil nutrient availability), which steers the soil microbial community towards slow-growing taxa without stimulation of microbial functional groups involved in N dynamics.

Acknowledgements

The authors wish to thank staffs of the Jasper Ridge Global Change Experiment for site preserving and sampling assistance. This study was funded by grants from the U.S. Department of Energy, the US NSF (DEB-0092642/0445324), the National Natural Science Foundation of China (41471202/41430856), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15010102), the David and Lucile Packard Foundation, the Morgan Family Foundation, the INSU, CNRS - EC2CO Program (project INTERACT) and State Key Joint Laboratory of Environmental Simulation and Pollution Control.

Data availability

GeoChip data are available online (www.ncbi.nlm.nih.gov/geo/) with the accession number GSE107168. MiSeq data are available in NCBI SRA database with the accession number SRP126539.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.10.353.

References

Ainsworth, E.A., Long, S.P., 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytol. 165, 351–372.

Arnone III, J.A., Bohlen, P.J., 1998. Stimulated N_2O flux from intact grassland monoliths after two growing seasons under elevated atmospheric CO_2. Oecologia 116, 331–335.

Austin, E.E., Castro, H.F., Sides, K.E., Schadt, C.W., Classen, A.T., 2009. Assessment of 10 years of CO_2 fumigation on soil microbial communities and function in a sweetgum plantation. Soil Biol. Biochem. 41, 514–520.

Barnard, R., Barthes, L., Le Roux, X., Leadley, P.W., 2004. Dynamics of nitrifying activities, denitrifying activities and nitrogen in grassland mesocosms as altered by elevated CO_2. New Phytol. 162, 365–376.

Barnard, R., Le Roux, X., Hungate, B.A., Cleland, E.E., Blankinship, J.C., Barthes, L., et al., 2006. Several components of global change alter nitrifying and denitrifying activities in an annual grassland. Funct. Ecol. 20, 557–564.

Brown, J.R., Blankinship, J.C., Niboyet, A., van Groenigen, K.J., Dijkstra, P., Le Roux, X., et al., 2011. Effects of multiple global change treatments on soil N_2O fluxes. Biogeochemistry 109, 85–100.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624.

Carol Adair, E., Reich, P.B., Trost, J.J., Hobbie, S.E., 2011. Elevated CO_2 stimulates grassland soil respiration by increasing carbon inputs rather than by enhancing soil moisture. Glob. Chang. Biol. 17, 3546–3563.

Davis, K.E., Sangwan, P., Janssen, P.H., 2011. Acidobacteria, Rubrobacteridae and Chloroflexi are abundant among very slow-growing and mini-colony-forming soil bacteria. Environ. Microbiol. 13, 798–805.

de Graaff, M.-A., van Groenigen, K.-J., Six, J., Hungate, B., van Kessel, C., 2006. Interactions between plant growth and soil nutrient cycling under elevated CO_2: a meta-analysis. Glob. Chang. Biol. 12, 2077–2091.

Dhillion, S., Roy, J., Abrams, M., 1996. Assessing the impact of elevated CO_2 on soil microbial activity in a Mediterranean model system. Plant Soil 187.

Diaz, S., Grime, J., Harris, J., McPherson, E., 1993. Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. Nature 364, 616–617.

Dijkstra, F.A., Hobbie, S.E., Reich, P.B., 2006. Soil processes affected by sixteen grassland species grown under different environmental conditions. Soil Sci. Soc. Am. J. 70, 770.

Drigo, B., Kowalchuk, G.A., van Veen, J.A., 2008. Climate change goes underground: effects of elevated atmospheric CO_2 on microbial community structure and activities in the rhizosphere. Biol. Fertil. Soils 44, 667–679.

Dukes, J.S., Chiariello, N.R., Cleland, E.E., Moore, L.A., Shaw, M.R., Thayer, S., et al., 2005. Responses of grassland production to single and multiple global environmental changes. PLoS Biol. 3, e319.

Dunbar, J., Eichorst, S.A., Gallegos-Graves, L.V., Silva, S., Xie, G., Hengartner, N.W., et al., 2012. Common bacterial responses in six ecosystems exposed to 10 years of elevated atmospheric carbon dioxide. Environ. Microbiol. 14, 1145–1158.

Ebersberger, D., Niklaus, P.A., Kandeler, E., 2003. Long term CO_2 enrichment stimulates N-mineralisation and enzyme activities in calcareous grassland. Soil Biol. Biochem. 35, 965–972.

Falkowski, P.G., Fenchel, T., Delong, E.F., 2008. The microbial engines that drive Earth's biogeochemical cycles. Science 320, 1034–1039.

Field, C.B., Chapin III, F.S., Matson, P.A., Mooney, H.A., 1992. Responses of terrestrial ecosystems to the changing atmosphere: a resource-based approach. Annu. Rev. Ecol. Syst. 201–235.

Fontaine, S., Henault, C., Aamor, A., Bdioui, N., Bloor, J.M.G., Maire, V., et al., 2011. Fungi mediate long term sequestration of carbon and nitrogen in soil through their priming effect. Soil Biol. Biochem. 43, 86–96.

Gans, J., Wolinsky, M., Dunbar, J., 2005. Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309, 1387–1390.

Gill, R.A., Polley, H.W., Johnson, H.B., Anderson, L.J., Maherali, H., Jackson, R.B., 2002. Nonlinear grassland responses to past and future atmospheric CO_2. Nature 417, 279–282.

Gutknecht, J.L.M., Field, C.B., Balser, T.C., 2012. Microbial communities and their responses to simulated global change fluctuate greatly over multiple years. Glob. Chang. Biol. 18, 2256–2269.

Hayden, H.L., Mele, P.M., Bougoure, D.S., Allan, C.Y., Norng, S., Piceno, Y.M., et al., 2012. Changes in the microbial community structure of bacteria, archaea and fungi in response to elevated CO_2 and warming in an Australian native grassland soil. Environ. Microbiol. 14, 3081–3096.

He, Z., Xu, M., Deng, Y., Kang, S., Kellogg, L., Wu, L., et al., 2010. Metagenomic analysis reveals a marked divergence in the structure of belowground microbial communities at elevated CO_2. Ecol. Lett. 13, 564–575.

He, Z., Piceno, Y., Deng, Y., Xu, M., Lu, Z., Desantis, T., et al., 2012. The phylogenetic composition and structure of soil microbial communities shifts in response to elevated carbon dioxide. ISME J. 6, 259–272.

He, Z., Xiong, J., Kent, A.D., Deng, Y., Xue, K., Wang, G., et al., 2014. Distinct responses of soil microbial communities to elevated CO_2 and O_3 in a soybean agro-ecosystem. ISME J. 8, 714–726.

Henry, H.A.L., Juarez, J.D., Field, C.B., Vitousek, P.M., 2005. Interactive effects of elevated CO_2, N deposition and climate change on extracellular enzyme activity and soil density fractionation in a California annual grassland. Glob. Chang. Biol. 11, 1808–1815.

Henry, H.A.L., Chiariello, N.R., Vitousek, P.M., Mooney, H.A., Field, C.B., 2006. Interactive effects of fire, elevated carbon dioxide, nitrogen deposition, and precipitation on a California annual grassland. Ecosystems 9, 1066–1075.

Hoosbeek, M.R., Lukac, M., van Dam, D., Godbold, D.L., Velthorst, E.J., Biondi, F.A., et al., 2004. More new carbon in the mineral soil of a poplar plantation under Free Air Carbon Enrichment (POPFACE): cause of increased priming effect? Glob. Biogeochem. Cycles 18 (n/a-n/a).

Horz, H.P., Barbrook, A., Field, C.B., Bohannan, B.J., 2004. Ammonia-oxidizing bacteria respond to multifactorial global change. Proc. Natl. Acad. Sci. U. S. A. 101, 15136–15141.

Hu, S., Chanpin III, F.S., Firestone, M.K., Field, C.B., Chiariello, N.R., 2001. Nitrogen limitation of microbial decomposition in a grassland under elevated CO_2. Nature 409, 188–191.

Hungate, B.A., Holland, E.A., Jackson, R.B., Chapin, F.S., Mooney, H.A., Field, C.B., 1997. The fate of carbon in grasslands under carbon dioxide enrichment. Nature 388, 576–579.

Hungate, B.A., van Groenigen, K.-J., Six, J., Jastrow, J.D., Luo, Y., de Graaff, M.-A., et al., 2009. Assessing the effect of elevated carbon dioxide on soil carbon: a comparison of four meta-analyses. Glob. Chang. Biol. 15, 2020–2034.

IPCC, 2014. Climate Change 2014: The Physical Science Basis: Fifth Assessment Report of the Intergovernmental Panel on Climate Change. http://www.ipcc.ch.

Klappenbach, J.A., Dunbar, J.M., Schmidt, T.M., 2000. *rRNA* operon copy number reflects ecological strategies of bacteria. Appl. Environ. Microbiol. 66, 1328–1333.

Le Roux, X., Bouskill, N.J., Niboyet, A., Barthes, L., Dijkstra, P., Field, C.B., et al., 2016. Predicting the responses of soil nitrite-oxidizers to multi-factorial global change: a trait-based approach. Front. Microbiol. 7, 628.

Lee, T.D., Reich, P.B., Tjoelker, M.G., 2003. Legume presence increases photosynthesis and N concentrations of co-occurring non-fixers but does not modulate their responsiveness to carbon dioxide enrichment. Oecologia 137, 22–31.

Lee, Z.M., Bussema 3rd, C., Schmidt, T.M., 2009. rrnDB: documenting the number of *rRNA* and *tRNA* genes in bacteria and archaea. Nucleic Acids Res. 37, D489–D493.

Lesaulnier, C., Papamichail, D., McCorkle, S., Ollivier, B., Skiena, S., Taghavi, S., et al., 2008. Elevated atmospheric CO_2 affects soil microbial diversity associated with trembling aspen. Environ. Microbiol. 10, 926–941.

Liu, S., Wang, F., Xue, K., Sun, B., Zhang, Y., He, Z., et al., 2015. The interactive effects of soil transplant into colder regions and cropping on soil microbiology and biogeochemistry. Environ. Microbiol. 17, 566–576.

Lladó, S., Žifčáková, L., Větrovský, T., Eichlerová, I., Baldrian, P., 2015. Functional screening of abundant bacteria from acidic forest soil indicates the metabolic potential of Acidobacteria subdivision 1 for polysaccharide decomposition. Biol. Fertil. Soils 52, 251–260.

Menge, D.N.L., Field, C.B., 2007. Simulated global changes alter phosphorus demand in annual grassland. Glob. Chang. Biol. 13, 2582–2591.

Nemergut, D.R., Knelman, J.E., Ferrenberg, S., Bilinski, T., Melbourne, B., Jiang, L., et al., 2016. Decreases in average bacterial community *rRNA* operon copy number during succession. ISME J. 10, 1147–1156.

Niboyet, A., Barthes, L., Hungate, B.A., Le Roux, X., Bloor, J.M.G., Ambroise, A., et al., 2009. Responses of soil nitrogen cycling to the interactive effects of elevated CO_2 and inorganic N supply. Plant Soil 327, 35–47.

Niboyet, A., Brown, J.R., Dijkstra, P., Blankinship, J.C., Leadley, P.W., Le Roux, X., et al., 2011a. Global change could amplify fire effects on soil greenhouse gas emissions. PLoS One 6, e20105.

Niboyet, A., Le Roux, X., Dijkstra, P., Hungate, B.A., Barthes, L., Blankinship, J.C., et al., 2011b. Testing interactive effects of global environmental changes on soil nitrogen cycling. Ecosphere 2, art56.

Niklaus, P.A., Alphei, J., Ebersberger, D., Kampichler, C., Kandeler, E., Tscherko, D., 2003. Six years of in situ CO_2 enrichment evoke changes in soil structure and soil biota of nutrient-poor grassland. Glob. Chang. Biol. 9, 585–600.

Patra, A., Abbadie, L., Clays-Josserand, A., Degrange, V., Grayston, S., Loiseau, P., et al., 2005. Effects of grazing on microbial functional groups involved in soil N dynamics. Ecol. Monogr. 75, 65–80.

Polley, H.W., Johnson, H.B., Derner, J.D., 2003. Increasing CO_2 from subambient to superambient concentrations alters species composition and increases aboveground biomass in a C3/C4 grassland. New Phytol. 160, 319–327.

Qiao, N., Schaefer, D., Blagodatskaya, E., Zou, X., Xu, X., Kuzyakov, Y., 2014. Labile carbon retention compensates for CO_2 released by priming in forest soils. Glob. Chang. Biol. 20, 1943–1954.

Regan, K., Kammann, C., Hartung, K., Lenhart, K., Müller, C., Philippot, L., et al., 2011. Can differences in microbial abundances help explain enhanced N_2O emissions in a permanent grassland under elevated atmospheric CO_2? Glob. Chang. Biol. 17, 3176–3186.

Reich, P.B., Knops, J., Tilman, D., Craine, J., Ellsworth, D., Tjoelker, M., et al., 2001. Plant diversity enhances ecosystem responses to elevated CO_2 and nitrogen deposition. Nature 410, 809–812.

Reich, P.B., Hobbie, S.E., Lee, T., Ellsworth, D.S., West, J.B., Tilman, D., et al., 2006. Nitrogen limitation constrains sustainability of ecosystem response to CO_2. Nature 440, 922–925.

Reid, J.P., Adair, E.C., Hobbie, S.E., Reich, P.B., 2012. Biodiversity, nitrogen deposition, and CO_2 affect grassland soil carbon cycling but not storage. Ecosystems 15, 580–590.

Rillig, M.C., Allen, M.F., Klironomos, J.N., Chiariello, N.R., Field, C.B., 1998. Plant species-specific changes in root-inhabiting fungi in a California annual grassland: responses to elevated CO_2 and nutrients. Oecologia 113, 252–259.

Roller, B.R., Stoddard, S.F., Schmidt, T.M., 2016. Exploiting *rRNA* operon copy number to investigate bacterial reproductive strategies. Nat. Microbiol. 1, 16160.

Rovira, P., Vallejo, V.R., 2003. Physical protection and biochemical quality of organic matter in Mediterranean calcareous forest soils: a density fractionation approach. Soil Biol. Biochem. 35, 245–261.

Shaw, M.R., Zavaleta, E.S., Chiariello, N.R., Cleland, E.E., Mooney, H.A., Field, C.B., 2002. Grassland responses to global environmental changes suppressed by elevated CO_2. Science 298, 1987–1990.

Simonin, M., Le Roux, X., Poly, F., Lerondelle, C., Hungate, B.A., Nunan, N., et al., 2015. Coupling between and among ammonia oxidizers and nitrite oxidizers in grassland mesocosms submitted to elevated CO_2 and nitrogen supply. Microb. Ecol. 70, 809–818.

Simonin, M., Nunan, N., Bloor, J.M.G., Pouteau, V., Niboyet, A., 2017. Short-term responses and resistance of soil microbial community structure to elevated CO_2 and N addition in grassland mesocosms. FEMS Microbiol. Lett. 364.

Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., Breznak, J.A., 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. 70, 4748–4755.

Tarnawski, S., Aragno, M., 2006. The influence of elevated [CO_2] on diversity, activity and biogeochemical functions of rhizosphere and soil bacterial communities. Managed Ecosystems and CO_2. Springer Berlin Heidelberg, pp. 393–412.

Vitousek, 2004. Nutrient Cycling and Limitation: Hawaii as a Model System. Princeton University Press, Princeton, NJ.

Yang, Y., Gao, Y., Wang, S., Xu, D., Yu, H., Wu, L., et al., 2014. The microbial gene diversity along an elevation gradient of the Tibetan grassland. ISME J. 8, 430–440.

Zak, D.R., Pregitzer, K.S., Curtis, P.S., Teeri, J.A., Fogel, R., Randlett, D.L., 1993. Elevated atmospheric CO_2 and feedback between carbon and nitrogen cycles. Plant Soil 151, 105–117.

Zavaleta, E.S., Shaw, M.R., Chiariello, N.R., Thomas, B.D., Cleland, E.E., Field, C.B., et al., 2003. Grassland responses to three years of elevated temperature, CO_2, precipitation, and N deposition. Ecol. Monogr. 73, 585–604.

Zhou, J., Bruns, M.A., Tiedje, J.M., 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62, 316–322.

Zhu, K., Chiariello, N.R., Tobeck, T., Fukami, T., Field, C.B., 2016. Nonlinear, interacting responses to climate limit grassland production under global change. Proc. Natl. Acad. Sci. 113, 10589–10594.