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# THE IMPACT OF LONG-TERM ELEVATED ATMOSPHERIC CARBON DIOXIDE ON BELOWGROUND MICROBIAL COMMUNITY AT CONTRAST NITROGEN CONDITIONS

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THE IMPACT OF LONG-TERM ELEVATED ATMOSPHERIC CARBON DIOXIDE ON BELOWGROUND MICROBIAL COMMUNITY AT CONTRAST NITROGEN CONDITIONS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

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## Abstract

Global terrestrial ecosystems are subjected to various climate change factors, including the concurrent elevated $CO_2$ ($eCO_2$) and nitrogen deposition (eN). Despite the increasing appreciation that $eCO_2$ and eN can interactively affect aboveground plants, how they will affect soil microbial communities and associated ecoprocesses remain understudied. This dissertation addresses this gap by examining the response of soil microbial communities associated with two plant functional groups (C3 grass and legumes) to $eCO_2$ and eN in a long-term (12-year) field experiment (BioCON).

In the beginning of this study, we investigated soil bacterial and archaeal communities subjected to $CO_2$ (ambient, 368 μmol $mol^{-1}$, versus elevated, 560 μmol $mol^{-1}$) and N (ambient, 0 g $m^{-2}$ $yr^{-1}$, versus elevated, 4 g $m^{-2}$ $yr^{-1}$) treatments using Illumina MiSeq sequencing of 16S rRNA gene amplicons. Over 2.3 million passing sequences were obtained from a total 24 samples, corresponding to 38 known phyla, 96 classes, and 565 genera. Elevated $CO_2$ significantly altered the diversity and structure of microbial communities, but these changes vary greatly depending on soil N conditions and plant functional groups. In C3 grass plots, community diversity increased with $eCO_2$. A positive eN effect on community richness was also observed. These shifts in community structure and composition may be driven by differential responses of microbial taxonomic groups to $eCO_2$ and/or eN. For example, *Actinobacteria* abundance decreased with the main effect of $eCO_2$, accounting for about 20.3% of the total population in the C3 grass. *Chlamydiae* increased with $eCO_2$ but only under eN condition. The abundance of *Woesearchaeota* increased with eN, but no effect of $eCO_2$ on its abundance was observed. Whereas in legume plots, community richness increased with

$eCO_2$. The abundance of *Actinobacteria*, *Chloroflexi*, *Armatimonadetes*, *Saccharibactiera*, and *Euryarchaeota*, accounting for about 21.2% of the total population in legume plots, decreased with $eCO_2$, eN or both. Only *Nitrospirae* and *Latescibacteria* increased with $eCO_2$ in their abundance. Changes in community diversity and composition were significantly related to plant and soil properties including plant biomass, biomass N content and C/N ratio, soil ammonium and nitrate, pH, moisture, temperature, and soil C and N contents by Mantel analysis. In addition, our results suggested that copiotrophic-like bacteria appear to be more abundant in the legume than in the C3 grass plots, whereas oligotrophic-like bacteria appear to be more abundant in the C3 grass than in the legume plots. Collectively, these results revealed different impacts of $eCO_2$ and eN on soil microbial community diversity and composition with few common trends observed across plant functional groups, providing new information for our understanding of the feedback response of soil microbial communities to global change factors.

In the following, we used high-throughput microbial functional gene microarray (GeoChip), stable isotope-based microbial C-sequestration and N-fixation measurements to detect and identify the impacts of $eCO_2$ and eN on soil microbial functional communities. We found that long-term changes in $CO_2$ and N availability dramatically altered the diversity and structure of C3 grass-associated soil microbial functional genes via several mechanisms, such as altering plant fine root production, exudation and soil moisture. There was an antagonistic relationship between $eCO_2$ and eN that affected a large number of microbial functional genes, with $eCO_2$ generally increasing the abundance of these genes at aN, but either decreasing or increasing abundance to a

minimal degree at eN. These results imply that $eCO_2$ may accelerate C and nutrient cycling in the C3 grass system, but the magnitude of effect is strongly dependent on the relative availability of N. Particularly, microbial activities associated with chemically recalcitrant soil organic matter (SOM) turnover significantly increased with $eCO_2$ in low fertility condition (unfertilized C3 grass plots of this study). These changes in C degradation genes suggested enhancement of microbial N mining under long-term $eCO_2$, an effect may limit soil C storage and stability. Meanwhile, $eCO_2$ and eN had surprisingly minor effects in the legume-associated soil microbial functional community with generally lower gene abundances at eCaN condition and higher gene abundances at eCeN condition, suggesting that the impacts of $eCO_2$ and eN are plant-functional-group-specific. This study provides new insights into our understanding of microbial functional processes in response to multiple global change factors.

The potential impact of global change factors (e.g., $eCO_2$, eN) on microbial activities, such as decomposition of various organic compounds, remains largely inferred from metagenomic analysis targeting the 16S, 28S rRNA, ITS or functional genes. However, the actual activity of microorganism can't be directly measured by such DNA-based technologies. Thus, the following study focused on assessing the influence of long-term $eCO_2$ on belowground microbial metabolic potential on different C sources provided on Biolog EcoPlate and determining whether the effect of $eCO_2$ was regulated by soil N conditions in the two plant functional groups (C3 grass and legumes). By cultivating soil samples on Biolog EcoPlate containing 31 low molecular weight C substrates, we constructed sole C source utilization profiles of microbial communities in soil samples mentioned above. We found that community composition of soil microbes based on

metabolic potential (utilization rates of 31 C sources) in the C3 grass plot soils was significantly different from those in the legume plot soils by both DCA and nonparametric dissimilarity tests. Microbial communities in legume plots had significantly higher metabolic potential than in C3 grass plots for decomposing organic substrates. Specifically, compared to the C3 grass plots, microbes in legume plots can use a larger number of C sources provided on EcoPlate and with greater decomposition rates during the measured time. Elevated $CO_2$ and eN didn't significantly alter metabolic potential of microorganisms in the C3 grass plots. In contrast, overall microbial metabolic activities significantly increased with $eCO_2$ by 20.6% in the fertilized legume plots, while there was no evidence for a $CO_2$ effect in the non-fertilized legume soils. Total soil N content, root ingrowth biomass, aboveground biomass, and root biomass N content as environmental attributes were closely correlated with microbial C utilization patterns as suggested by the Mantel test. In addition, PLFA analysis showed both total microbial and bacterial biomass were significantly lower in legume than in C3 grass plots, showing an opposite trend to the microbial metabolic potential in these plots. Collectively, these results demonstrated that $eCO_2$ effects on active microbial metabolic activities are contingent on N conditions, and such effect differs between plant functional groups. Differences in microbial metabolic potential among treatments and between plant functional groups were not attributed to population size (biomass) but likely attributed to changes in community structure and/or enzymatic activities of belowground microbes.

We further analyzed the impacts of long-term $eCO_2$ and eN on AOA communities by using 454 pyrosequencing of archaeal-*amoA* gene amplicons. A total of 87 *amoA* OTUs (95% identity cutoff) were generated from 26,211 qualified reads. The diversity of

AOA communities measured by OTU richness (Chao1), Pielou evenness, Shannon and phylogenetic hill number were significantly reduced by $eCO_2$ but the $CO_2$ effects were confined within ambient N condition. PCoA, βMNTD and non-parametric dissimilarity tests revealed significant $CO_2$ effects on the community structure of AOA regardless of N deposition, but no effect of N was observed. We also detected significant changes of several AOA taxa in their relative abundance, which were significantly correlated with plant root biomass, proportional soil moisture, and pH. In addition, significant positive correlations between AOA taxa and soil nitrification rate were observed, indicating AOA may be actively involved in the nitrification process in grassland soil. Interestingly, $eCO_2$ and eN alone and combined did not significantly alter the abundance of AOA. These results are important in furthering the understanding of the global change impacts on AOA community in the long term.

All studies included in this work provided novel insights into the long-term $eCO_2$ effects on belowground microbial communities. Our results demonstrated that $eCO_2$ effects are contingent on soil N conditions and plant functional groups, underscoring the difficulty toward predictive modeling of soil ecosystem under future climate scenarios and necessitating more detailed studies.

**Keywords**: **Climate change; Elevated $CO_2$; N fertilization; Soil microbial community; 16S rRNA; microbial functional genes; microbial metabolic potential; *amoA*; ammonia oxidizing archaea; AOA; Illumina sequencing; 454 pyrosequencing; GeoChip; Biolog EcoPlate; progressive nitrogen limitation; priming effect; BioCON**

# Chapter 1: Introduction

## 1.1 Atmospheric $CO_2$: the background

Global atmospheric carbon dioxide ($CO_2$) concentration is rising at a record pace. The annual mean global atmospheric $CO_2$ concentration has increased by more than 40% since the start of the Industrial Revolution, reaching 409 ppm in mid-2017 (Stocker 2013). This is an unprecedented level in the human history and likely the highest over the past 2.6 to 5.3 million years. Anthropogenic activities, primarily fossil-fuel combustion, released an enormous amount of ancient carbon (6-8 PgC/year) that locked within the Earth and directly related to the $CO_2$ builds up in the atmosphere (Stocker 2013). Land use change (mainly deforestation and agricultural) also strongly contributed to the $CO_2$ emission. According to the Intergovernmental Panel on Climate Change (IPCC) report, the concentration of atmospheric $CO_2$ is expected to further increase, reaching approximately 985 ppm by the end of this century if fossil fuels maintain their dominant position in the global energy production (Stocker 2013). As one of the most important atmospheric components that interact with Earth's biosphere, such a rapid increase in $CO_2$ concentration would bring dramatic impacts on the Earth's ecosystem. Primary impacts of increasing atmospheric $CO_2$ on ecosystem include the magnitude and rate of climate changes (e.g. global warming, ocean acidification, extreme weather). Secondarily, increasing atmospheric $CO_2$ is expected to change global C and nutrient cycling by altering phenology, physiology, community composition and interaction, and functional activities of macro- and microorganisms (Walther et al. 2002, Drigo et al. 2008, Stocker 2013).

### 1.2 Consequences of elevated atmospheric $CO_2$ for macroecosystems

Elevated $CO_2$ atmospheric concentrations can cause an immediate stimulation of plant photosynthesis and net primary production (NPP), referred to as the $CO_2$ fertilization effect (Amthor 1995). In particular, C3 plants respond markedly to elevated $CO_2$, and in the coming century, a 30-50% increase of C3 plant photosynthetic activity is expected (Kuzyakov and Domanski 2000, Long et al. 2004). The $CO_2$ fertilization effect could increase terrestrial C uptake and is expected to provide negative feedback to $CO_2$ accumulation (IPCC 2007), although it is uncertain how sustainable this effect is and to what extent it will promote C allocation to soil (Oren et al. 2001, Schneider et al. 2004, Reich et al. 2006, Inauen et al. 2012). One major aspect of this uncertainty is that the increase in plant N uptake and immobilization of N at $eCO_2$ could lead to progressive N limitation (PNL), a decrease of soil N availability over time. PNL theory suggests that soil N availability should be gradually reduced over time by elevated $CO_2$ and that the $CO_2$ fertilization effect should occur only in short time in N limited ecosystems (Johnson 2006). However, over the past three decades, empirical evidence across studies in pots, growth chambers, open-top chambers and FACE (Free Air Carbon Dioxide Enrichment) experiments showed inconsistent and controversial results that $CO_2$ fertilization effect either down-regulated over long-term $CO_2$ enrichment (Ainsworth and Long 2005, Norby et al. 2010, Reich and Hobbie 2013), or increased and accompanied by an increase in plant N uptake (Finzi et al. 2007, McCarthy et al. 2010). The mechanism by which plants retain long-term growth response to $eCO_2$ in these studies remains unclear. Soil N levels were extremely limited in some of these studied ecosystems (e.g. BioCON FACE experiment), thus it is unlikely that PNL was simply

delayed. It has been proposed that the priming effect, an opposing mechanism, may play a role simultaneously in these ecosystems in offsetting the PNL effects (Reich and Hobbie 2013). In addition, a stimulation of microbial N fixation by additional plant organic input was documented in some $CO_2$ enrichment studies, which may help in alleviating or preventing the PNL effects (Lee et al. 2003, van Groenigen et al. 2006, He et al. 2010).

Under nutrient limited conditions (particularly low N availability), even if there is no significant $CO_2$ stimulation of total or aboveground plant biomass production (Korner and Arnone 1992, Zak et al. 2000, Oren et al. 2001, Shaw et al. 2002, Hungate et al. 2003, Luo et al. 2004), plants exposed to elevated $CO_2$ can allocate a greater amount of photosynthate to enhance root development and exudation to 'mine' nutrients from soil (Johnson 2006). Such enhancement of belowground C allocation by elevated $CO_2$ was documented in several $CO_2$ enrichment experiments (Korner and Arnone 1992, Diaz et al. 1993, Hungate et al. 1997, Jones et al. 1998, Schlesinger and Lichter 2001, Lukac et al. 2003, Norby et al. 2004, Heath et al. 2005, Korner et al. 2005, Carney et al. 2007, Finzi et al. 2007, Pollierer et al. 2007, Pritchard et al. 2008, Phillips et al. 2009), and to a greater amount with low than high soil N availability (Phillips et al. 2011). Concomitant changes in the chemical composition (e.g. C/N ratio) of litter and exudates were also widely reported in these studies.

### 1.3 Consequences of elevated atmospheric $CO_2$ for microbial communities belowground

The concentration of $CO_2$ in soil pore space is much higher (10 to 15 times) than in the atmosphere, thus the direct influence of rising $CO_2$ atmospheric concentration on soil

microbial community should be negligible compared to the indirect effects, such as stimulating plant organic matter inputs, altering plant physiological characteristics and soil chemistry (Kandeler et al. 1998, Janus et al. 2005).

As soil microorganisms are generally C-limited, the increase in plant-derived carbon (C) inputs could enhance the biomass and activity of belowground microbes. Increased microbial activities, such as decomposition, will enhance organic matter turnover, offsetting the effect of plant C fixation from the air. Meanwhile, the rates of nutrient cycling (e.g. N, S and P) are expected to increase as dynamics of these nutrients are strongly coupled with C turnover. Particularly, under nutrient limited conditions, stimulated microbial population by elevated $CO_2$ can synthesize more extracellular enzymes for depolymerization of N from old soil organic matter (SOM) to meet their increasing nutrient demand (priming effect) (Cheng 2005). This N will eventually be released for plant uptake during microbial biomass turnover, which could support plant growth response under long-term elevated $CO_2$ (Dijkstra et al. 2009). There is a consensus that C cycling mediated by microbes is essential in influencing C storage and soil N availability, thus understanding the response of microbial biomass, activity and the potential shifts in microbial community structure is central to predicting changes in soil C and N cycling under elevated $CO_2$ conditions.

Although common responses of plants (e.g. higher NPP and C allocation to soil, altered nutritional quality) to elevated atmospheric $CO_2$ were documented, a corresponding picture of common patterns in belowground microbial community responses to elevated $CO_2$ have not yet emerged (Jossi et al. 2006, Carney et al. 2007, Jin and Evans 2007, Lesaulnier et al. 2008, Austin et al. 2009, Dunbar et al. 2012, Xu et al.

2013, Berthrong et al. 2014, Yu et al. 2016). Studies examining effects of elevated $CO_2$ concentrations on microbial community biomass and activity are highly variable and ecosystem-specific. Previous studies reported mainly positive effects of elevated $CO_2$ concentration on soil microbial growth and activities (e.g. decomposition and nutrient cycling) as a result of alterations in C inputs (Zak et al. 1993, Cotrufo and Gorissen 1997, Sadowsky and Schortemeyer 1997, van Ginkel et al. 2000, Zak et al. 2000, He et al. 2010, Tu et al. 2015). However, contrasting observations were also found with a decrease (Diaz et al. 1993, Ebersberger et al. 2004) or no change in the growth and/or activities of belowground microbial communities by elevated $CO_2$ (Jones et al. 1998, Kandeler et al. 1998, Hu et al. 1999). Since soil N conditions can drastically regulate plant physiological responses to elevated $CO_2$ and vary among sites and over time, it may cascade to alter the responses of belowground microbial community to elevated $CO_2$ and largely contribute to the mixed results in these studies (Hu et al. 2001).

Community diversity and composition of microorganisms are major factors responsible for ecosystem multifunctioning and stability (McCann 2000, Hector and Hooper 2002, Tilman et al. 2006, Hector and Bagchi 2007, Zavaleta et al. 2010, Wagg et al. 2014). A number of previous studies across FACE experiments detected either no or minor significant changes in microbial community composition by elevated $CO_2$ (Zak et al. 2000, Chung et al. 2006, Austin et al. 2009). In contrast, significant compositional and structural shifts of microbial communities and decreased microbial richness under elevated $CO_2$ were observed as a result of $CO_2$-induced plant and soil properties changes, such as plant biomass, soil moisture, and soil C and N contents (Deng et al. 2012, He et al. 2012). In addition, increased bacterial diversity and/or decreased archaeal diversity as

a result of elevated $CO_2$ were also reported (Janus et al. 2005, Lesaulnier et al. 2008). Disparity of microbial responses to elevated $CO_2$ in these studies could be due to the following major aspects: (1) the extreme diversity of soil microbial communities; (2) ecosystem differences (e.g. ecosystem-specific environmental attributes); (3) differences among the methodologies used, such as restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis(DGGE), enzyme activities, phospholipid fatty acid (PLFA), and 16S rRNA gene-based sequencing, which may resolve differences in the soil community caused by elevated $CO_2$ to differing degrees.

Soil microbial community tends to shift in consistent ways in the taxonomic and functional traits along higher nutrient availability (e.g. N, P), with the faster-growing copiotrophic bacterial taxa being increased in relative abundances and slower-growing oligotrophic bacterial taxa being decreased in relative abundances (Leff et al. 2015). Such changes in microbial community may relate to shifts in general life history strategies with bacterial taxa that are faster growing and more copiotrophic being favored under conditions of elevated nutrient availability (Leff et al. 2015, Roller and Schmidt 2015, Roller et al. 2016).

Methodologically, the fast developing high-throughput technologies (e.g. 454, Illumina sequencing), targeting the 16S rRNA or other functional gene markers (e.g. *amoA*), offer quick screening of community-wide spatial and temporal information on microbial composition and functional potential. More importantly, deep sequencing by these approaches provides more thorough characterization of microbial communities and allows capture of the response of less abundant taxa (Austin et al. 2009, Dunbar et al. 2012). Therefore, advanced sequencing methods may help to detect changes in microbial

community composition at finer, previously unexamined taxonomical levels by relatively coarse methods (e.g. PCR-DGGE, RFLP). However, sequencing-based approaches suffer from high sensitivity to random sampling errors, contaminated non-target DNA and dominant populations (Zhou et al. 2008). Currently, the use of advanced sequencing technologies in characterizing the belowground microbial community is still challenging, especially for the genetic and functional diversity. On the other hand, microarray-based metagenomics technologies (e.g. GeoChip, PhyloChip) do no suffer from these limitations, and provide community-wide information on microbial functional structure and potential activity (Zhou et al. 2008). An integration of high-throughput metagenomic approaches such as 454 and Illumina sequencing and microbial gene microarray such as GeoChip would be ideal to work in a complementary way in characterizing microbial community.

### 1.4 Foci of this study

Despite the wide range of key ecological processes and the mediation of ecosystem responses to global changes that belowground microbial communities support (Chapin et al. 2000, Francis et al. 2007), the response of these communities to elevated atmospheric $CO_2$, particularly possible interactive effects of elevated $CO_2$ concentration with dynamic soil N availability, remains largely unclear (van Groenigen et al. 2006, Craine et al. 2007). Meanwhile, in contrast to the numerous studies on small scale, short-term $CO_2$ enrichment experiments, results of long-term elevated $CO_2$ effects on belowground microbial community biomass, composition, and activities are still rare due partly to the low number of long-term experiments manipulating $CO_2$ concentrations (e.g. FACE

experiments). Short duration of $CO_2$ enrichment experiments often ignored the co-limitation by N supply on $CO_2$ fertilization effect, which can generate inconsistent results and make the extrapolation of these results to long-term ecosystem feedbacks to elevated $CO_2$ problematic (He et al. 2010, Xiong et al. 2015). By integrating traditional microbiological methods (e.g. PLFA, Biolog), high-throughput metagenomic approaches (e.g. 454 and Illumina sequencing, functional gene array), and stable isotope-based microbial C-sequestration and $N_2$-fixation measurements, this study aimed to reveal whether and how soil N conditions affect the ecological effects of long-term elevated $CO_2$ on microbial community and soil ecoprocess in a comprehensive manner. Major results are presented in chapter 2 to chapter 5.

This study was conducted at the BioCON (the Biodiversity, $CO_2$, and Nitrogen Experiment) experiment site, one of the longest $CO_2$ manipulation experiments (established since 1998) in the world by using the free-air $CO_2$ enrichment (FACE) technology (Reich et al. 2001, Reich et al. 2004). The BioCON experiment is located at the Cedar Creek Natural History Area, in central Minnesota, USA with $CO_2$, N deposition and plant diversity treatments arranged in 296 2 × 2 m plots. All the plots were merged into six rings with nearly 20 m diameter each. Half of the rings receive air with increased concentration of $CO_2$ (560 μmol $mol^{-1}$) during the growing seasons (April to October), the other half of the rings receive ambient $CO_2$ (368 μmol $mol^{-1}$). N enrichment was conducted by applying N fertilizer (4 g $m^{-2}$ $yr^{-1}$ of $NH_4NO_3$) to half of these plots three times per growing season (mid-May, mid-June, and mid-July). Plant species numbers were directly manipulated (1, 4, 9, or 16 perennial grassland species randomly chosen from a pool of 16 species, planted as seed in 1997). The BioCON experiment allows us

to examine the effects of elevated $CO_2$ concentration in natural field conditions (e.g., natural amounts of rain, wind, light). With N manipulation, it allows the study of elevated $CO_2$ concentration impacts on plant growth and soil microbial changes as affected by soil N availability.

**Chapter 2** describes the plant-mediated impact of elevated $CO_2$ on belowground microbial communities of two plant functional groups, C3 grass and N-fixing legume. Analyses focused on characterizing the profile of bacterial and archaeal community diversity and structure. The microbial communities were profiled by high-throughput Illumina sequencing, targeting the 16S rRNA gene amplicons. Greater sequencing depth of such technology provides more thorough characterization of microbial communities and allows capture of the responses of previously undetected less abundant taxa. In **chapter 3**, I examined effects of elevated $CO_2$ on microbial functional structure and activities, using respectively microbial functional gene array (GeoChip 3.0), soil respiration measurement and stable isotope-based microbial C-sequestration and N-fixation measurements. Subsequently, multivariate statistical analyses were used to link those community responses with plant and properties, and compare the relative impact of elevated $CO_2$, N addition treatments versus plant and soil effects on these communities. **Chapter 4** aims to assess if soil N status influences the response of microbial metabolic potential to elevated $CO_2$. To gain insight into the actual activity of active microbial populations that can't be directly measured by such DNA-based technologies, community-level physiological profile (CLPP), based on the utilization pattern of various carbon sources was measured using Biolog EcoPlate. **Chapter 5** focuses on a subset of soil microbes: ammonia oxidizing archaea (AOA), a group of phylogenetically distinct

microorganisms that possess the same functional trait — the gene *amoA* encoding ammonia monooxygenase and functioning as nitrification. Using a PCR-based 454-pyrosequencing approach, this chapter examines how AOA community are influenced by $CO_2$ treatment and N addition. The results of the different studies are summarized, discussed and evaluated in **chapter 6**. Overall, this work provided novel insights into the microbial community diversity, composition, functional structure and potential activity changes in response to long-term elevated $CO_2$ under contrast soil N conditions, and be of merit for a mechanistic understanding of the ecosystem responses to elevated atmospheric $CO_2$.

# Chapter 2: Responses of soil microbial communities to elevated carbon dioxide and nitrogen deposition differ by C3 and legume plants

## 2.1 Abstract

Understanding soil microbial responses to global change factors, such as elevated $CO_2$ ($eCO_2$) and N deposition (eN), is crucial for estimating ecosystem feedbacks and predicting future climate change. This study investigated soil bacterial and archaeal communities subjected to $CO_2$ (ambient, 368 µmol $mol^{-1}$, versus elevated, 560 µmol $mol^{-1}$) and N (ambient, 0 g $m^{-2}$ $yr^{-1}$, versus elevated, 4 g $m^{-2}$ $yr^{-1}$) treatments for over 12-years in a grassland (BioCON) using Illumina MiSeq sequencing of 16S rRNA gene amplicons. Over 2.3 million passing sequences were obtained from a total 24 samples, corresponding to 38 known phyla, 96 classes, and 565 genera. Elevated $CO_2$ significantly altered the diversity and composition of microbial communities, but these changes varied greatly depending on soil N conditions and plant functional groups. In C3 grass plots, community diversity and richness increased with $eCO_2$ and eN, respectively. *Actinobacteria* abundance decreased with $eCO_2$, accounting for about 20.3% of the total population in the C3 grass. *Chlamydiae* increased with $eCO_2$ and $eCO_2$+eN, and *Woesearchaeota* increased with eN. In legume plots, community richness measured by Chao1 estimator increased significantly with $eCO_2$ at both N conditions. The abundance of *Actinobacteria*, *Chloroflexi*, *Armatimonadetes*, *Saccharibactiera*, and *Euryarchaeota*, accounting for about 21.2% of the total population in legume plots, decreased with $eCO_2$, eN or both. Only *Nitrospirae* and *Latescibacteria* significantly increased with $eCO_2$ in their relative abundances. Changes in community diversity and composition were significantly correlated to plant and soil properties including plant biomass, biomass N

content and C/N ratio, soil ammonium and nitrate, pH, moisture, temperature, and soil C and N contents by Mantel analysis. Collectively, our results revealed different impacts of $eCO_2$ and eN on soil microbial community diversity and composition with few common trends observed across plant functional groups. This study provides new information of the feedback response of soil microbial communities to global change factors.

**Keywords: 16S rRNA; Illumina sequencing; Climate change; Elevated $CO_2$; N fertilization; Soil microbial community; BioCON**

## 2.2 Introduction

Elevated $CO_2$ ($eCO_2$) fertilization effect is commonly found to stimulate plant growth and enhance carbon (C) allocation toward soils, which may lead to negative feedback to climate change (Zak et al. 2000, Reich et al. 2001, Ainsworth and Long 2005, Drigo et al. 2008, Souza et al. 2010). However, an increase in the soil organic C pool is not always observed (Lichter et al. 2008) under $eCO_2$, despite greater plant primary productivity and root exudation. For example, Jastrow et al (2005) reported a 5.6% increase in soil C over 2-9 years of $eCO_2$ at a median rate of 19 g C $m^{-2}$ $yr^{-1}$ (Jastrow et al. 2005). While other studies revealed barely any change (van Groenigen et al. 2014) through meta-analysis or a slight decrease of soil C pool as a result of enhanced decomposition via long-term (3 years) field experiments (Peralta and Wander 2008). The discrepancy between modeling and empirical evidence suggests our understanding of ecosystem feedback mechanisms to $eCO_2$ is incomplete.

Elevated $CO_2$-enhanced plant primary production doesn't always lead to greater C accumulation in the soil because rates of microbial growth and metabolism can also increase in response to the extra organic matter input (Blagodatskaya et al. 2010) (Field et al. 1995, Reed et al. 2011). These phenomena could be more pronounced under N-limited conditions, releasing larger amount of C than fixed due to enhanced microbial decomposition of both fresh substrates and native soil organic matter, known as 'priming effect' (Kuzyakov et al. 2000, Hoosbeek et al. 2004). N fertilization (eN), another major environmental change factor, could decrease the priming effect by suppressing the oligotrophic microbial taxa (e.g., *Actinobacteria, Verrucomicrobia*) and their N-mining activities on SOM (Fierer and Schimel 2003, van Groenigen et al. 2006, Ramirez et al.

2010, Ramirez et al. 2012, Chen et al. 2014). However, it is also suggested that N fertilization may induce greater decomposition from higher availability of labile litter with high C/N ratio possibly by favoring copiotrophic microbial taxa (e.g., *Firmicutes*) (Wild et al. 2014). Therefore, the consequences of increased plant C allocation toward soil on ecosystem C storage depend on the responses of soil microbial communities of different composition and C decomposition capabilities (Phillips et al. 2011). However, few common responses of microbial community composition and structure, biomass and activity to these environmental change factors were found across sites (Jossi et al. 2006, Carney et al. 2007, Jin and Evans 2007, Lesaulnier et al. 2008, Austin et al. 2009, Dunbar et al. 2012, Xu et al. 2013, Berthrong et al. 2014, Yu et al. 2016), making it hard to predict the changes in soil C storage under $eCO_2$. In addition, although impacts of $eCO_2$ and eN on soil microorganisms have been thoroughly studied individually, their interactive effects on soil microbial community remain notoriously understudied which further hampers our ability to accurately predict soil C dynamics under global climate change.

Our study was conducted at the BioCON experiment site, one of the longest $CO_2$ manipulation experiments in the world by using the free-air $CO_2$ enrichment (FACE) technology (Reich et al. 2001, Reich et al. 2004). It allows us to examine the effects of $eCO_2$ in natural field conditions (i.e. natural amounts of rain, wind, light). Ammonium nitrate was applied to the experimental plots, allowing the study of $eCO_2$ impacts on plant growth and soil microbial changes as affected by soil N availability. A number of previous studies on BioCON and other FACE experiment soils detected either no or minor changes in microbial community composition by $eCO_2$ (Zak et al. 2000, Chung et al. 2006, Austin et al. 2009). However, significant changes in functional gene abundances

and enzymatic activities were noted under conditions of $eCO_2$ (Chung et al. 2006, He et al. 2010), indicating changes in microbial community composition at finer, previously unexamined taxonomical levels due largely to the relatively coarse methods (i.e. PCR-DGGE, clone libraries), or altered metabolic strength. To address this knowledge gap, we conducted in-depth analysis to characterize more complete profile of microbial diversity, compositional and structural responses to $eCO_2$ and eN, and how it is affected by different plant functional groups (C3 grass and legume). We also aimed to link those community responses with plant properties and soil geochemistry. The microbial communities were profiled by high-throughput Illumina sequencing, targeting the 16S rRNA gene amplicons. Greater sequencing depth of such technology would provide more thorough characterization of microbial communities and allows capture of the responses of previously undetected less abundant taxa (Austin et al. 2009, Dunbar et al. 2012). Given that $eCO_2$ and eN commonly change C inputs to soil through greater litter fall and root activity (Lukac et al. 2009, Phillips et al. 2011), and alter nutritional quality of plant litter or root exudates (Parsons et al. 2008, Phillips et al. 2009) and soil chemistry (Field et al. 1995, Saarsalmi et al. 2006), we predicted that increased C input into soil as a result of stimulated plant biomass production under $eCO_2$ would reduce microbial competitions and increase overall microbial diversity, and significantly change the community structure and composition. The response of certain microbial populations to $eCO_2$ may be altered by soil N status. For example, low limiting resource (N) availability should select for efficient growth and have greater oligotrophic membership in response to $eCO_2$, whereas the relative abundance of copiotrophs may promote in response to $eCO_2$-induced additional organic matter input in N enrich soil (Koch 2001, Roller and Schmidt 2015).

We also predicted that microbial communities in the C3 grass and legume plots will respond differently to $eCO_2$ and eN due to varied plant mediated soil C, N inputs and soil chemistry (Lee et al. 2001, Craine et al. 2003, Craine et al. 2003, Lee et al. 2003).

## 2.3 Materials and Methods

*2.3.1 Site description and experimental design:*

The BioCON experiment at Cedar Creek Natural History Area, in central Minnesota (45°24′13.5″N, 93°11′08″W), was established on a glacial outwash comprised of loamy sand soils with poor nitrogen availability (Reich et al. 2001). A total of 296 2 × 2 m plots were merged into six rings with nearly 20 m diameter each. Half of the rings receive air with increased concentration of $CO_2$ (560 μmol $mol^{-1}$) during the growing seasons (April to October), the other half of the rings receive ambient $CO_2$ (368 μmol $mol^{-1}$). The $CO_2$ manipulation was achieved through Free-Air Carbon dioxide Enrichment (FACE) technology. N enrichment was conducted by applying N fertilizer (4 g $m^{-2}$ $yr^{-1}$ of $NH_4NO_3$) to half of these plots 3 times per growing season (mid-May, mid-June, and mid-July). For a more detailed site description refer to Reich, et al. (2001) (Reich et al. 2001).

Soil samples without plant root from a subset of plots of the FACE experiment were collected for analyses, specifically, the 24 4-plant species plots (12 with C3 grass, and 12 with legume) receiving $CO_2$ and/or N treatments, with 3 biological replicates each. C3 grass and N-fixing legume were used to assess soil microbial community responses to the treatment factors ($eCO_2$ and eN) as affected by distinct plant functional groups. Plots were manually weeded on a regular basis to remove unwanted species. Bulk soil samples were taken in June 2009, during the growing season, and each sample was

composited from five randomly collected soil cores at a depth of 0-15 cm. All samples were immediately transported to the laboratory and stored at 4$^{o}$C and -80$^{o}$C for following analyses.

*2.3.2 Plant and soil property measurements*

*Plant biomass*. The aboveground and belowground (0–20 cm) biomass were measured as previously described (Reich et al. 2001, Reich et al. 2006). Briefly, a 10 x 100 cm strip was clipped at just above the soil surface, and all plant material was collected, sorted to live material and senesced litter, dried and weighed. Roots were sampled at 0–20 cm depth using three 5-cm diameter cores in the area used for the aboveground biomass clipping. Roots were washed, sorted into fine (< 1 mm diameter) coarse and crowns classes, dried and weighed.

*Soil physical properties.* Soil pH and volumetric soil moisture were measured at depths of 0-17, 42-59, and 83-100 cm in a KCl slurry and with permanently placed TRIME Time Domain Reflectometry (TDR) probes (Mesa Systems Co., Medfield MA), respectively (Fig. S2.1). Other soil properties including temperature, total soil C and N concentrations, soil $NO_3^+$-N and $NH_4^-$-N were measured on site and described previously (Reich et al. 2006).

*Soil processes*. Net N mineralization rates were measured concurrently in each plot for one-month in situ incubations with a semi-open core at 0-20 cm depth during mid-summer of each year (Reich et al. 2001, Reich et al. 2006). Net N mineralization rates were determined by the difference between the final and initial $NH_4^+$-N + $NO_3^-$-N pool sizes determined with 1 M KCl extractions. Net ammonification was determined by

the difference between the final and initial $NH_4^+$-N pool sizes. Net nitrification was determined by the difference between the final and initial $NO_3^-$-N pool sizes.

*2.3.3 DNA isolation, PCR amplification of the 16S rRNA gene and Illumina sequencing*

Microbial community DNA was extracted from 5 g of soil from each sample using a freeze-grinding method and purified through agarose gel electrophoresis (Zhou et al. 1996). DNA quality was assessed by the ratios of 260/280 nm and 260/230 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and DNA concentration was measured by PicoGreen using a FLUOstar OPTIMA fluorescence plate reader (BMG LABTECH, Jena, Germany). The V4 region of the 16S rRNA genes was amplified using a two-step PCR amplification method with the primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') combined with Illumina adapter sequences, a pad and a linker of two bases, as well as barcodes on the reverse primers (Wu et al. 2015). Sample libraries were generated from purified PCR products. The Miseq 300 cycles kit was used for 2 × 150 bp paired-ends sequencing on Miseq machine.

*2.3.4 Sequence data preprocessing and statistical analysis*

Illumina sequencing of the 16S gene was performed on a Miseq (Illumina, San Diego, CA, USA), producing 250 bp reads per end, according to manufacturer's instructions. Raw sequences were processed through Galaxy pipeline developed by Yujia Qin. Briefly, raw sequences with 100% matches to barcodes were split to sample libraries and were trimmed using BTRIM (Kong 2011) with threshold of QC higher than 20 over 5 bp window size and the minimum length of 100 bp. Forward and reverse reads with at least 10 bp overlap and lower than 5% mismatches were joined using FLASH (Magoc and

Salzberg 2011). After trimming of ambiguous bases (i.e. N), joined sequences with lengths between 240 and 260 bp were subjected to chimera removal by U-Chime (Edgar et al. 2011). Operational taxonomic unit (OTU) clustering was performed through UCLUST (Edgar 2010) at 97% similarity level, and taxonomic assignment was through SILVA public 16S rRNA database (Quast et al. 2013). Singletons were removed from each treatment for downstream analyses. Samples were rarefied at 50,000 sequences per sample. Subsequent analyses were performed in R , version 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria). Dissimilarity tests were based on Jaccard and Bray-Curtis dissimilarity indices using Adonis under the package of VEGAN (Dixon 2003). To make a principal coordinate analysis (PCoA) of UniFrac distances, OTU representative sequences were aligned against GreenGenes 16S rRNA database using PYNAST, followed by tree computation with FASTTREE. The PCoA analysis was based on weighted and unweighted UniFrac distance matrix using the classical multidimensional scaling method. Differences in abundance across treatments were determined by ANOVA analysis followed by Least Significance Difference (LSD) test.

## 2.4 Results

### *2.4.1 Structure of soil bacterial and archaeal communities*

Over 2.3 million qualified sequences were obtained from the total 24 samples. After OTU clustering at 97% sequence identity, removal of singletons and rarefaction at 50,000 sequences per sample, 62,248 OTUs remained (with 42,446 in C3 grass plots and 39,841 in legume plots), out of which 25,653 OTUs were affiliated to 38 known phyla, 96 classes, and 565 genera. Archaea accounted for 0.44% (113 OTUs) of the total population, of which 91% were *Thaumarchaeota* and 8% were *Euryarchaeota*. Within the bacterial

domain, *Proteobacteria* were the most abundant phylum in both C3 grass (29.8% in relative abundance) and legume plots (34.7%), followed by *Actinobacteria* (20.3% in C3 grass and 16.1% in legume), *Acidobacteria* (18% in C3 grass and 16% in legume) and *Verrucomicrobia* (14.6% in C3 grass and 10.4% in legume) (Fig. 2.1). Meanwhile, 0.23% OTUs in the C3 grass and 0.25% OTUs in legume plots were not assigned to any known phylum (Fig. 2.1).

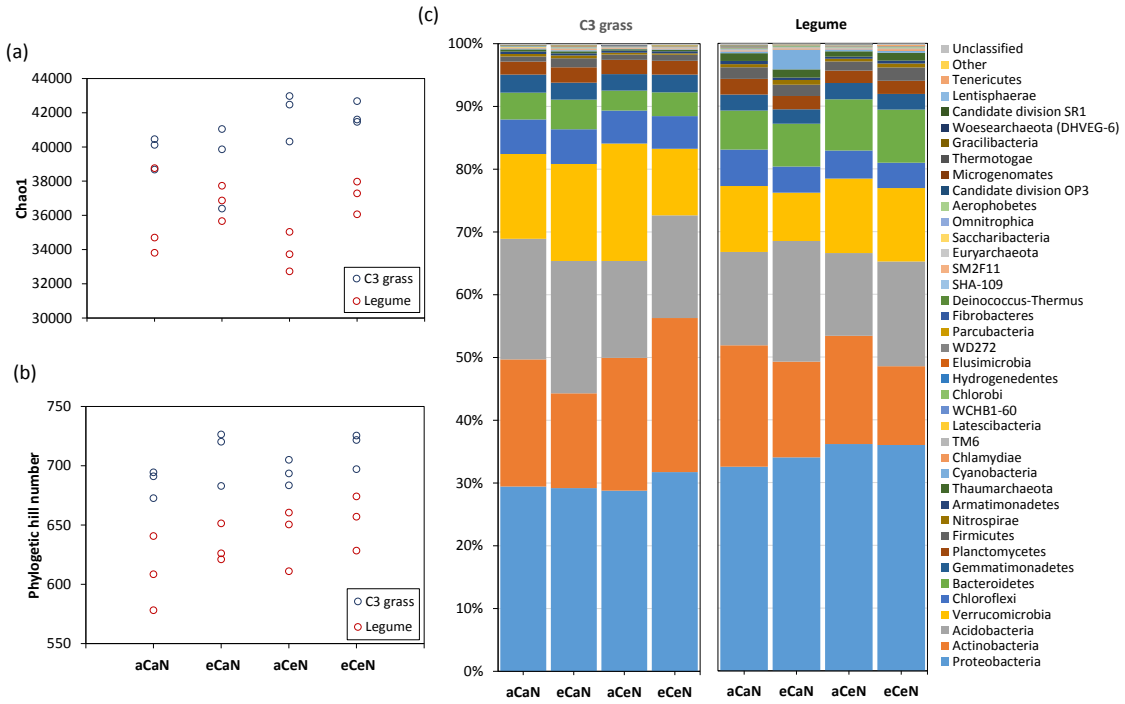



**Fig. 2.1** Effects of $eCO_2$ and eN on microbial richness measured as Chao1 estimator (a) and diversity measured as phylogenetic hill number (b). Community composition of C3 grass and legume plots under different $CO_2$ and N regimes at the phylum level (c).

At the genus level, OTUs belonging to the *Chthoniobacterales* DA101 soil group of *Verrucomicrobia* was the most dominant group in both C3 grass (13.0%) and legume plots (8.8%), along with Subgroup 4 of *Acidobacteria* (7.9%), *Afipia* (7%), Subgroup 6 of *Acidobacteria* (3.8%), *Sphingomonas* (3.1%), *Massilia* (2.7%), *Gaiellales* of *Actinobacteria* (2.1%), *Gemmatimonadales* of *Gemmatimonadetes* (2.0%),

*Solirubrobacter* (1.2%), *Chitinophagaceae*, *Acidobacteriaceae* (Subgroup 1) (1.2%), *Pseudonocardiaceae* (1.1%), *Micrococcaceae* (1.1%), *Nitrosomonadaceae* (1.0%), and *Mycobacteriaceae* (1.0%), accounting for 50.5% of total abundance in C3 grass; and along with Subgroup 4 of *Acidobacteria* (6.9%), *Sphingomonas* (4.2%), Subgroup 6 of *Acidobacteria* (3.0%), *Sphingobacteriales* of *Bacteroidetes* (2.4%), *Afipia* (2.3%), *Variibacter* (2.0%), *Massilia* (2.0%), *Gemmatimonadales* of *Gemmatimonadetes* (1.8%), *Acidobacteriales* of *Acidobacteria* (1.7%), *Gaiellales* of *Actinobacteria* (1.7%), *Arthrobacter* (1.4%), and *Nitrosomonadales* of *Proteobacteria* (1.1%), accounting for 39.5% of total abundance in legume plots.

*2.4.2 Effect of $eCO_2$ and eN on microbial community diversity and structure*

Elevated $CO_2$ and eN differentially altered the α-diversity of microbial communities of C3 grass and legume plots. Phylogenetic diversity as measured by phylogenic hill number (q=0, a species richness estimator) (Chao et al. 2010) in C3 grass plots and OTU richness as measured by Chao1 diversity estimator (Chao 1984, Colwell and Coddington 1994) in legume plots increased with $eCO_2$ ($P < 0.05$) (Fig. 2.1). OTU richness also increased in C3 grass plots with eN ($P < 0.05$) (Fig. 2.1).

The principle coordinates analysis (PCoA) based on both weighted and unweighted UniFrac distances showed a combined effect of $eCO_2$, eN and soil physio-chemical properties on the β-diversity of the bacterial communities in C3 grass and legume plots (Fig. S2.2). Based on the weighted PCoA models, the first three dimensions together explained 69.94% and 65.32% of the observed variations in C3 grass and legume plots, respectively. Community compositions under four $CO_2$ and N levels were

significantly different from each other based on Jaccard distance method regardless of plant functional groups (Table 2.1), which was also reflected on the ordination plot.

**Table 2.1** Effects of $eCO_2$ and eN on additional plant and soil attributes.

| | Groups | adonis.F (Jaccard) | adonis.p (Jaccard) | adonis.F (Bray-Curtis) | adonis.p (Bray-Curtis) |
|---|---|---|---|---|---|
| C3 grass | aCaN vs eCaN | 1.374 | **0.001** | 0.866 | 0.600 |
| | aCaN vs aCeN | 1.373 | **0.001** | 1.036 | 0.506 |
| | aCaN vs eCeN | 1.345 | **0.017** | 0.984 | 0.609 |
| Legume | aCaN vs eCaN | 1.399 | **0.001** | 1.049 | 0.379 |
| | aCaN vs aCeN | 1.355 | **0.001** | 0.772 | 0.756 |
| | aCaN vs eCeN | 1.374 | **0.001** | 0.976 | 0.670 |

*2.4.3 Changes in community composition along $eCO_2$ and eN*

Treatment effects of $eCO_2$ and eN, individually or together, were inconsistent on the abundance of microbial groups at the phylum level between C3 grass and legume plots. Within C3 grass plots, the abundances of three phyla were significantly altered by treatments as compared to the control receiving no additional $CO_2$ and N, including *Actinobacteria*, *Chlamydiae* and *Woesearchaeota*, accounting 20.4% of the total population (Fig. 2.2, $P < 0.05$). Among these phyla, the abundance of *Actinobacteria* (20.3% in relative abundance) decreased with $eCO_2$ but only in unfertilized soil (aN). *Chlamydiae* increased with $eCO_2$ in both fertilized and unfertilized soil. Within legume plots, abundances of seven phyla were altered by treatments, accounting 21.8% of the total population within legume plots (Fig. 2.2, $P < 0.05$). Most of these phyla decreased with $eCO_2$ in the unfertilized soil, including *Chloroflexi*, *Armatimonadetes*, *Saccharibactiera*, and *Euryarchaeota* (5.1% in relative abundance). *Actinobacteria*, accounting 16.1% of the total population in legume plots, decreased with $eCO_2$ regardless

of N fertilization. Whereas, *Nitrospirae* and *Latescibacteria* increased with $eCO_2$ in unfertilized and fertilized soil, respectively.

| | a | ab | b | bc | c |
|---|---|---|---|---|---|

| | Phylum | aCaN mean | eCaN mean | aCeN mean | eCeN mean | Abundance (%) | Treatment effects vs. aCaN |
|---|---|---|---|---|---|---|---|
| C3 | *Actinobacteria* | 10116 | 7538 | 10573 | 12259 | 20.262% | Decreased with eCaN |
| | *Chlamydiae* | 41 | 77 | 46.7 | 60 | 0.116% | Increased with eCaN & eCeN |
| | *Woesearchaeota* | 0 | 0 | 4 | 0 | 0.001% | Increased with aCeN |
| | *Saccharibacteria* | 2.3 | 0.3 | 0.7 | 4 | 0.004% | None |
| Le | *Chloroflexi* | 2902.3 | 2079 | 2226.3 | 1966.7 | 4.624% | Decreased with eCaN |
| | *Armatimonadetes* | 253.7 | 188.7 | 169.7 | 225.7 | 0.422% | |
| | *Saccharibacteria* | 7 | 3 | 5 | 3.7 | 0.009% | |
| | *Euryarchaeota* | 7 | 0 | 3.3 | 0.7 | 0.006% | |
| | *Actinobacteria* | 9661 | 7618 | 8597.7 | 6102.7 | 16.099% | Decreased with eCaN & eCeN |
| | *Latescibacteria* | 10 | 30 | 13.3 | 21.3 | 0.038% | Increased with eCaN |
| | *Nitrospirae* | 251.7 | 378.7 | 219.3 | 303.3 | 0.582% | Increased with eCaN & eCeN |
| | *Chlamydiae* | 73.3 | 93.7 | 52.3 | 115.7 | 0.170% | None |
| | *Acidobacteria* | 7423 | 9603 | 6578.3 | 8116.3 | 15.996% | None |

**Fig. 2.2** Phyla with different abundance levels among treatments. The color panel under 'aCaN', 'eCaN', 'aCeN' and 'eCeN' reflects both the absolute abundance (mean of sequence count) of the corresponding phylum and the relative abundance level at each treatment by LSD test. Red toned cells, (a); Blush toned cells, (ab); White toned cells, (b); Sapphire toned cells, (bc); and Blue toned cells, (c).

Further examination revealed significant changes occurred in several microbial populations at finer taxonomic levels, many of which were associated with those unchanged phyla under treatments at the phylum level. At the class level, the abundance of 13 and 22 classes were significantly altered in response to treatments in the C3 grass and legume plots, respectively (Fig. S2.3, $P < 0.05$). Of these groups, four classes decreased in response to $eCO_2$ within C3 grass plots (20.0% in relative abundance), with *Actinobacteria* and *Thermoleophilia* in the unfertilized soil, and *Acidimicrobiia* and *Planctomycetes* BD7-11 in fertilized soil. On the contrary, abundance of four classes (1.8% in relative abundance), including *Gammaproteobacteria*, *Chlamydiae*, *Verrucomicrobia* UA11, and *Bellilinea*, increased with $eCO_2$ in unfertilized soil. Within legume plots, nine classes decreased in abundance in response to $eCO_2$ (9.5% in relative abundance), of which three decreased in unfertilized soil (*Deltaproteobacteria*,

*Phycisphaerae*, and *Chloroflexi* JG30-KF-CM66), three decreased in fertilized soil (*Chloroflexi* TK10, *Chloroflexi* Gitt-GS-136, and *Actinobacteria* TakashiAC-B11), and another three decreased regardless of soil N levels (*Chloroflexi* JG37-AG-4, *Thermoleophilia*, *and Thermoplasmata*). Contrarily, five classes were increased in response to $eCO_2$ (2.6% in relative abundance), among which *Acidobacteria* Subgroup 22 and 26 increased in the unfertilized soil, *Gammaproteobacteria* and *Bacteroidetes* VC2.1 Bac22 increased in fertilized soil, and *Latescibacteria* increased in both soil N levels. At the genus level, the abundance of 20 genera in C3 grass plots were decreased with $eCO_2$, either in unfertilized soil (*Patulibacter* and *Virgisporangium* associated with *Actinobacteria*), or in fertilized soil (10 genera, i.e. *Acidobacteria* Subgroup 5 and Subgroup 17, *Frankiales*), or in both (eight genera, i.e. *Sphingobacteriales*, *Acidimicrobiales*, *Herpetosiphon*) (Fig. S2.4, $P < 0.05$). Whereas abundance increased with $eCO_2$ in another 84 genera, of which 46 increased in unfertilized soil (i.e. *Acidobacteria* Subgroup 4, *Hyphomicrobium*, *Paenibacillus*), 30 increased in fertilized soil (i.e. *Mycobacterium*, *Solirubrobacterales*, *Micromonosporaceae*), and eight increased in both soil N levels (i.e. *Kribbella*, *Gammaproteobacteria* NKB5, *Legionella*). In legume plots, 34 genera decreased in abundance, with 11 decreased in unfertilized soil (i.e. *Solirubrobacterales*, *Haliangium*, *Sorangium*), nine decreased in fertilized soil (i.e. *Acidimicrobiales*, *Singulisphaera*, *Actinobacteria* TakashiAC-B11) and 14 decreased in both N levels (i.e. *Chloroflexi*, *Phycisphaerae* WD2101 soil group, *Jatrophihabitans*) (Fig. S2.5, $P < 0.05$). There were 54 genera increased with $eCO_2$ in legume plots. Of these groups, 25 increased in unfertilized soil (i.e. *Rhodoplanes*, *Betaproteobacteria*, *Adhaeribacter*), 19 increased in fertilized soil (i.e.

*Bradyrhizobiaceae*, *Thaumarchaeota* Soil Crenarchaeotic Group, *Myxococcales*) and 10 increased in both N levels (i.e. *Acidobacteria* Subgroup 4, *Holophagae*, *Phyllobacteriaceae*).

Collectively, the results show that significant changes in microbial community diversity, richness, and community composition under $eCO_2$ were plant functional group-specific (C3 grass and legume), and such changes were largely affected by soil N status.

*2.4.4 Distribution of copiotrophic-, oligotrophic-like microorganisms along treatments in C3 grass and legume plots*

Previous studies demonstrated that oligotrophic bacteria tend to encode fewer ribosomal RNA operons (*rrn*) copies in their genomes than copiotrophs (Lauro et al. 2009, Eichorst et al. 2011), thus *rrn* copy numbers can be used to infer bacteria of different life strategies: copiotrophic or oligotrophic. Here, we further examined the distribution of copiotrophic- and oligotrophic-like microorganisms among the 24 soil samples in this study by examining the number of *rrn* in detected bacteria genomes. The *rrn* copy numbers of detected bacteria using 16S rRNA sequencing were determined using the Gene Copy Number Adjustment function of RDP Classifier (https://rdp.cme.msu.edu/classifier/class_help.jsp#copynumber). Copy numbers of *rrn* of 168 genera were identified across the 24 samples, ranging from 1 to 15 copies per genome. Cluster analysis showed that samples in C3 grass plots were clustered together and well separated from samples in legume plots (Fig. 2.3). Bacteria encoding a larger number of *rrn* copies were more abundant in legume plots (e.g., Group 2), whereas smaller copy number of *rrn* encoding bacteria were more abundant in C3 grass plots (e.g.,

Group 5). However, there was no clear clustering pattern for samples under treatments of $eCO_2$ and eN within each plant functional groups. These results suggest that N richer legume plots may harbor more copiotrophic-like, but less oligotrophic-like microorganisms compare to the C3 grass plots. And long-term of $eCO_2$ and eN didn't alter the distribution of copiotrophic- and oligotrophic-like microorganism within neither C3 grass nor legume plot soils based on this test.

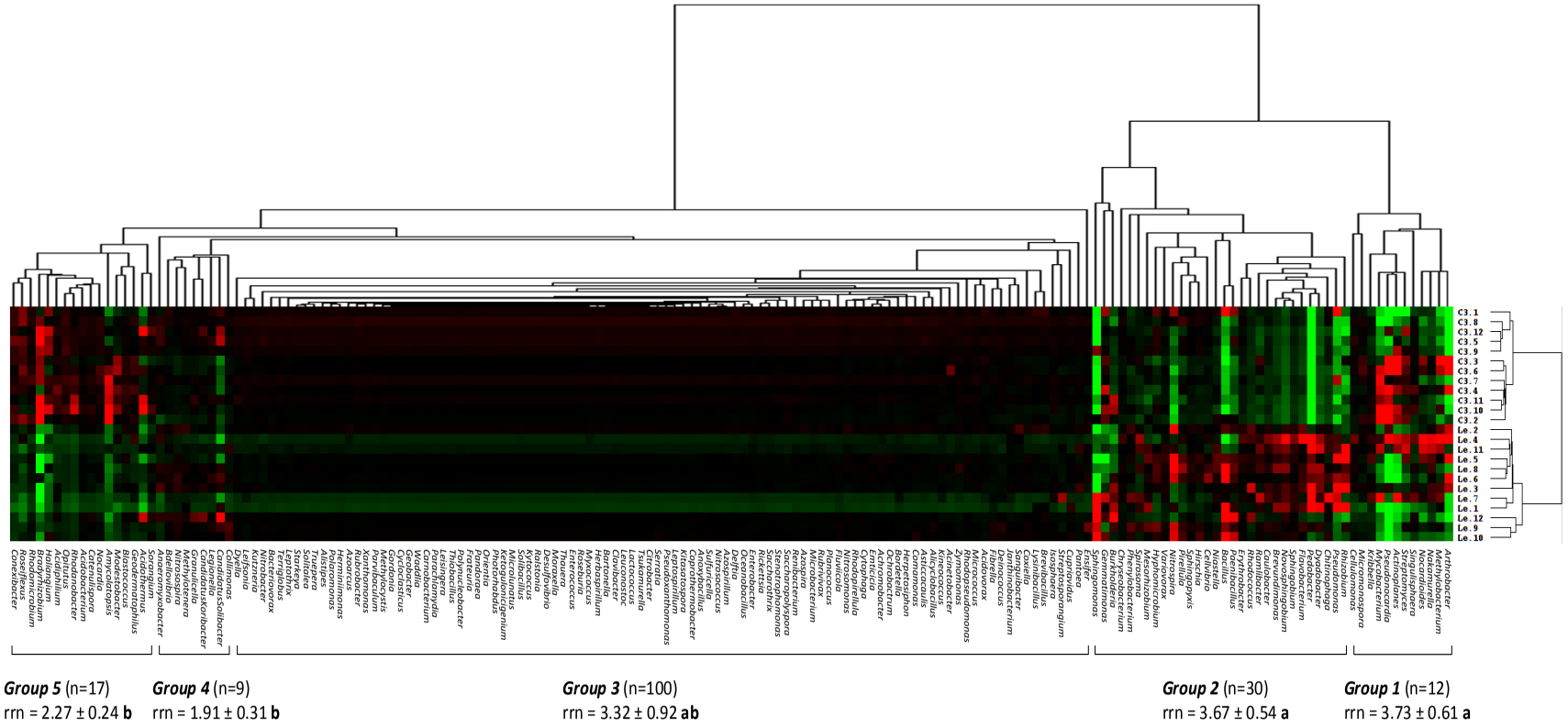



**Fig. 2.3** Hierarchical cluster analysis of relative abundances of 168 genera among the 24 samples from C3 grass and legume plots. *rrn*: 16S rRNA copy number. Data are presented with mean ± se. Values labeled with different letters are significantly different ($P < 0.05$) according to ANOVA with pairwise comparison. Results were generated in CLUSTER and visualized using TREEVIEW. Green indicates signal intensities below background, whereas red indicates signal intensities above background. Brighter red color indicates higher signal intensities. Sample in C3 grass plots clustered together and were well separated from samples in legume plots. No clear clustering pattern for samples under treatments of $CO_2$ and N within each plant functional groups.

*2.4.5 Linkages between microbial composition and selected environmental properties*

We performed partial Mantel tests to link the observed taxonomic structure of microbial communities with selected plant properties (Clarke and Ainsworth 1993), including aboveground biomass, root biomass (0-20 cm, fine roots), aboveground biomass N and C/N, root biomass N and C/N, root ingrowth biomass (RIB), and soil geochemistry, including pH, moisture, temperature (Tm), total C (TC) and N (TN) content, nitrate, and ammonium. The results showed that microbial community was significantly correlated with selected plant properties on the whole ($r = 0.457$, $P = 0.001$), but not with soil properties ($r = 0.157$, $P = 0.058$).

Variation partition analysis (VPA) (Ramette and Tiedje 2007) was performed to further assess the contributions of $CO_2$, N, plant and soil properties to the taxonomic structure of microbial communities (Fig. S2.8). Individual variables $CO_2$ and N were found to contribute similarly to the observed variations in microbial taxonomic structure (4.8% and 4.5%). Seven plant properties and seven soil properties could independently explain 28.6% and 17.6% of the variation, respectively. The interactive effects between plant and soil, plant and $CO_2$, plant and N, Soil and N, and $CO_2$ and N were 6.8%, 4.8%, 3.4%, 3.2%, and 1.2%. Furthermore, the interactions among plant, soil, $CO_2$, and N was 5.2%, and other interactive effects were less than 1.0%. 27.5% of the variation in microbial community composition remains unexplained by the environmental variables measured. Plant properties play the most important role in shaping microbial communities in this study.

Individual taxa at different taxonomic categories (phylum, class, and genus) were further screened for potentially significant correlations with measured

environmental properties using Spearman's rank correlation coefficient. Changes in taxa were found to be significantly related to several of the selected environment properties, with more significant correlations found with plant properties ($P < 0.05$, Fig. 2.4, S2.6, and S2.7). At the phylum level, 10 phyla were significantly correlated with the plant and/or soil properties (Fig. 2.4, $P < 0.05$). Of these phyla, similar patterns were observed for *Acidobacteria, Bacteroidetes, Chlamydiae, Nitrospirae,* and *Verrucomicrobia* which generally positively correlated with aboveground biomass, aboveground and root biomass N content, and negatively correlated with root biomass, aboveground and root biomass C/N ratio, and root ingrowth biomass (Fig. 2.4). Contrarily, *Chloroflexi* and *Cyanobacteria* were negatively correlated with aboveground biomass, aboveground and root biomass N content, and soil $NO_3^+$, and positively correlated with aboveground and root biomass C/N ratio. Positive correlations were observed for *Actinobacteria* with soil temperature, $NH_4^-$, root biomass and ingrowth root biomass. In addition, negative correlations were observed for *Thaumarchaeota* with soil temperature, $NH_4^-$, root biomass, aboveground and root biomass C/N ration, and ingrowth root biomass. Similarly, significant correlations between microbial taxa and the selected plant and/or soil properties were detected in 21 classes and 90 genera (Fig. S2.6 and S2.7, $P < 0.05$). In addition, many unclassified phylotypes were significantly correlated with the selected plant and/or soil properties, suggesting that plant and soil factors may also greatly shape taxonomically uncharacterized microorganisms ($P < 0.05$).

| | pH | Moisture | Tm (°C) | TC (%) | TN (%) | $NO_3^+$ (mg $kg^{-1}$) | $NH_4^-$ (mg $kg^{-1}$) | AB (g $m^{-2}$) | RB (g $m^{-2}$) | AB-N (%) | AB-C/N | RB-N (%) | RB-C/N | RIB (g $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acidobacteria* | | | -0.52** | | | 0.42* | -0.51* | 0.67*** | -0.75*** | 0.62** | -0.65*** | 0.66*** | -0.68*** | -0.75*** |
| *Actinobacteria* | | | 0.59** | | | | 0.52** | | 0.55** | | | | | 0.50* |
| *Armatimonadetes* | | | | | | | | | | | | | | |
| *Bacteroidetes* | | | -0.44* | | | | | | -0.54** | 0.41* | -0.47* | 0.46* | -0.48* | -0.51* |
| *Chlamydiae* | | | | | | 0.52* | | 0.49* | | 0.48* | -0.51* | 0.46* | -0.45* | |
| *Chloroflexi* | | | 0.54** | | | -0.54** | 0.41* | -0.70*** | 0.75*** | -0.68*** | 0.71*** | -0.82*** | 0.82*** | 0.77*** |
| *Cyanobacteria* | | 0.50* | | | | -0.60** | | -0.69*** | | -0.59** | 0.58** | -0.58** | 0.55** | |
| *Firmicutes* | | | -0.43* | | 0.44* | | -0.47* | | -0.49* | | | | | -0.47* |
| *Gemmatimonadetes* | | | | | | | | | | | | | | |
| *Nitrospirae* | | | -0.55** | | | | -0.55** | 0.51* | -0.65*** | 0.49* | -0.52* | 0.44* | -0.48* | -0.66*** |
| *Planctomycetes* | | | | | | | | | | | | | | |
| *Proteobacteria* | | | | | | | | | | | | | | |
| *Thaumarchaeota* | | | -0.57** | | | | -0.48* | | -0.52** | | -0.42* | | -0.43* | -0.55** |
| *Verrucomicrobia* | | | | 0.43* | 0.43* | 0.59** | | 0.51* | -0.45* | 0.45* | -0.50* | 0.46* | -0.48* | |

**Fig. 2.4** Spearman's rank correlation coefficient rho of selected phyla (with different abundance levels among treatments) with environmental attributes. Coral toned cells represent significant positive correlations; Cyan toned cells represent significant negative correlations. Only phyla with different abundance levels among treatments were subjected to this test. P values have been adjusted for multiple comparisons by FDR. The first seven columns correspond to correlations of each phylum with soil attributes including pH, moisture, temperature (Tm), total C (TC) and N (TN) content, nitrate, and ammonium. The next seven columns correspond to correlations of each phylum with plant attributes including aboveground biomass (AB), root biomass (RB) ( 0-20 cm, fine roots), aboveground biomass N (AB-N) and C/N (AB-C/N), root biomass N (RB-N) and C/N (RB-C/N), and root ingrowth biomass (RIB).

## 2.5 Discussion

Predicting long-term climate change impacts to ecosystem carbon and nitrogen dynamics require detailed knowledge of biodiversity response patterns and the linkages between such responses to environmental factors. By using 16S rRNA Illumina deep sequencing to characterize the profile of soil microbial communities, we showed that the microbial community diversity and structure was significantly altered under $eCO_2$ conditions. Particularly, the responses of several microbial populations to $eCO_2$ differ at contrasting N levels. With $eCO_2$, several environment properties significantly correlated with the changes in microbial populations at varied soil N levels, with plant properties, such as biomass production and C/N ratio, playing a more important role in shaping microbial taxonomic structure.

Current efforts to predict whether terrestrial ecosystems will serve as a carbon sink or source under future climate scenarios are hampered by the difficulty of characterizing processes beneath the earth's surface (e.g. soil C dynamics). A major approach to understanding the soil processes under climate changes is to survey the responses of soil microbial communities, which mediate important biogeochemical cycles, such as C, N, P and S. However, existing observations on microbial communities regarding the responses of their biomass, community composition and structure, and activities to climate change factors are highly variable and site-specific (Zak et al. 2000, Lipson et al. 2005, Jossi et al. 2006, Carney et al. 2007, Drissner et al. 2007, Drigo et al. 2009, Deng et al. 2012, Dunbar et al. 2012). Site-specific microbial behavior under climate change yields conflicting observations on soil C and nutrient dynamics, which further limit robust ecological modeling. Our results showed variability in community composition and the response to $eCO_2$ between C3 grass and legume plots, providing limited predictive value to the response of microbial communities across a wide range of ecosystems.

The experimental plots in this study have been exposed to contrasting $CO_2$ and N levels for over 12 years. Thus, the observed changes in microbial community composition and structure are expected to represent permanent, rather than transient responses of microbial communities to $eCO_2$ and/or eN. We detected only three and seven significantly changed phyla in response to treatments in the C3 grass and legume plots, respectively, among a total of 38 phyla detected, suggesting that microbial communities were generally resilient to $eCO_2$ at coarse taxonomic levels. Similar results were also reported in previous studies (Chung et al. 2006). For example, a study

on soil samples at the Rhinelander WI FACE experiment using PLFA and PCR-DGGE methods showed that microbial community composition did not significantly change between each of the triplicate FACE plots for either ambient or elevated $CO_2$ (Chung et al. 2006). At the same site, a follow-up experiment by Lesaulnier et al. (2008) examined detailed and deeper branching profile of population responses to $eCO_2$ by analyzing the 16S and 18S rRNA clone libraries and qPCR quantification, which revealed no changes in trembling aspen associated bacterial community structure at the phylum level (Lesaulnier et al. 2008).

Furthermore, it was proposed that numerically dominant microbial taxa may be less responsive to $eCO_2$ effects compared with less abundant taxa (Dunbar et al. 2012). A study assessing soil microbial community responses across six filed FACE experiments showed that only a small fraction (about 2%) of identified bacterial taxa were potentially responsive to $eCO_2$ (Dunbar et al. 2012). Our 16S Illumina deep sequencing results were generally in agreement with these results. We evaluated overall changes in soil microbial communities using both the quantitative dissimilarity index (Bray-Curtis), which gives abundant OTUs the most weight, and a non-quantitative index (Jaccard), which is based on presence or absence and OTUs with lower abundance exert a larger effect. Both C3 grass and legume ecosystems showed significant treatment effects on Jaccard index but not on Bray-Curtis index, indicating that overall community shifts were largely due to changes in abundances of those less dominant or rare microbial taxa. Such changes may have implications for ecosystem function, especially via effects on the cycling of essential elements.

In both C3 grass and legume soils of this study, the soil microbial communities were primarily dominated by members of the phylum *Proteobacteria*, followed by the *Actinobacteria*, *Acidobacteria*, and *Verrucomicrobia*, as is typical for soils (Janssen 2006). This result agrees well with our former synthesis of soil microbial community composition studies at BioCON in the 16-plant species plots during 2007 that showed *Proteobacteria* (23.2%), *Actinobacteria* (17.0%), *Bacteroidetes* (12.8%), and *Acidobacteria* (10.5%) dominated microbial community regarding their abundance (Deng et al. 2012). Similar dominant groups were also detected in soils of other FACE study (e.g., Oak Ridge National Laboratory FACE site) (Austin et al. 2009). Together, these results indicate a general consistency of dominant groups of microbial communities across BioCON plots of different plant functional groups and richness.

*Actinobacteria* are microorganisms that tend to prefer oligotrophic habitats (Fierer et al. 2003), and most of them seem to be out competed in soils of $eCO_2$ plots in this study likely as a result of increased flux of C. It is noteworthy that the not all *Actinobacteria* subgroups showed consistent trend to decrease in relative abundance at $eCO_2$. For example, several genera of *Actinobacteria* (e.g., *Microbispora, Smaragdicoccus, Streptacidiphilus, Hamadaea, Streptomonospora, Tsukamurella, Microlunatus, and Sinosporangium*) were selectively increased in response to $eCO_2$ in unfertilized soil. *Actinobacteria* play important role in organic matter turnover (e.g., cellulose and chitin decomposition) (He et al. 2010), and abundance changes in these microorganisms under $eCO_2$ may alter soil C cycling in this grassland ecosystem. *Chloroflexi*, another abundant phylum detected in this study, decreased in abundance at $eCO_2$ in the legume plots. Such change was also observed previously at this site (He et

al. 2012), supporting assumptions of adaptation of *Chloroflexi* to nutrient-limited habitats. However, a closer look at populations at the genus level showed there was a pretty diverse response to $eCO_2$ for taxa that closely affiliated with *Chloroflexi*. For example, the abundance of *Herpetosiphon*, *Ktedonobacteria*, and an unclassified *Chloroflexi*-affiliated genera significantly decreased at $eCO_2$ in the unfertilized soil, whereas *Ktedonobacterales, Bellilinea,* and *Litorilinea* significantly increased at $eCO_2$. These results reflect the various metabolic feature of *Chloroflexi* that capable of utilizing recalcitrant organic compounds and other more labile sources (Cho and Azam 1988, Jorgensen and Boetius 2007). It could also be related to soil heterogeneity or to the persistence of micro-niches of nutrient poor environments.

Although a phylum level significant change was lacking, a large number of *Proteobacteria* at lower taxonomic levels across α-, β-, γ-, and δ-subgroups were predominantly stimulated by $eCO_2$, either in unfertilized or in N enriched soils (e.g. *Hirschia, Holosporaceae, Desulfuromonas, Nitrosococcus, Rudaea, Coxiella*), which are generally consistent with our previous observation at BioCON and from other FACE sites (Feng et al. 2009, He et al. 2012). Enhanced plant growth under $eCO_2$ require higher rates of inorganic nitrogen uptake and assimilation (Reich et al. 2006, Reich and Hobbie 2013), microbial populations which are able to produce $NH_4^+$ from various sources were proposed to be favored under such conditions (Zehr et al. 2003, Craine et al. 2007), thus we would expect the trend towards an increase in these populations. Indeed, populations such as *Rhodobacter, Azospirillum, Rhizobium, Mesorhizobium,* and *Rhizobiales* that capable of fixing $N_2$ were generally promoted at $eCO_2$ in this study. Also, a recent study at the same site showed that the abundance of key genes involved in

microbial N fixation was significantly increased at $eCO_2$. These changes may modify soil N availability and cycling at this site.

Other than the indirect effects mediated by plants, $eCO_2$ may have direct impacts on the soil microbial community and their processes. A former study at this site measuring microbial functional genes showed that microbial genes involved in C fixation were significantly increased at $eCO_2$ (He et al. 2010). Similarly, using C table isotope analysis, our following experiment showed that $eCO_2$ significantly increased the C sequestration potential as measured by the changes in $^{13}C$ in soils. These changes in soil microbial functional potential are evidence for the direct impacts of $eCO_2$ on soil microorganisms. However, given that the concentration of $CO_2$ in soil pore space is 10 to 15 times higher than in the atmosphere, it is expected that such direct effects of $eCO_2$ on soil microbial community should be negligible compared to the indirect effects, such as altering plant organic matter inputs and soil chemistry (Kandeler et al. 1998, Janus et al. 2005). The results in the current study support this hypothesis and showed that direct effects of $eCO_2$ contribute to nearly 5% of the observed community variations, which is much smaller than measured plant properties (28.6%) and soil variables (17.6%) in shaping microbial community structure.

Previous studies demonstrated that *rrn* copy number is a reliable and generalizable proxy for determining bacteria of different life strategies and their adaptation to resource availability (Koch 2001, Lauro et al. 2009, Eichorst et al. 2011, Roller and Schmidt 2015). Consistent with the observations by Roller and colleagues showing that higher nutrients (e.g., N, P) availability tend to favor copiotrophs and reduce the abundance of oligotrophs (Koch 2001, Roller and Schmidt 2015), our results showed that copiotrophic bacteria

appear to be more abundant in the legume than the C3 grass plots. This was likely due to the significantly higher N content in legume plots. Furthermore, copiotrophs are expected to be more capable of decomposing labile C substrates with low C use efficiency (CUE), while oligotrophs are specialized in degrading recalcitrant SOM for N (Koch 2001). The differential distribution of copiotrophic- and oligotrophic-like bacteria in C3 grass and legume plots may lead to selective decomposition of organic substrates in these plot soils.

In conclusion, this study examined the profile of soil microbial communities in response to 12 years of $eCO_2$ at the BioCON site and demonstrated dramatic changes in community diversity, composition and structures and their relationship to the direct effects of $CO_2$ and indirect effects through plant and soil properties. We also demonstrated that the responses of several microbial populations to $eCO_2$ significantly depend on soil N levels. The long-term experiment enabled us to avoid transient responses, as such providing prolonged responses to assess the influence of climate change factors on the community dynamics of soil microorganisms. These findings provide fundamental, predictive understanding of ecosystem responses to global climate change and their feedbacks.

# Chapter 3: Contingency in belowground microbial functional responses to rising atmospheric $CO_2$ at contrast nitrogen conditions

## 3.1 Abstract

Soil N availability alters plant photosynthate partitioning under elevated $CO_2$ ($eCO_2$), with effects that cascade to belowground microbial communities and associated ecological processes. Plants exposed to rising $CO_2$ increase root development and exudation, and such effect by $eCO_2$ is commonly greater at low than high nutrient conditions. These interactive effects between $CO_2$ and N can also change plant tissue chemistry. For example, previous meta-analysis of litter exposed to elevated $CO_2$ found a 7.1% decline in N concentration and a 6.5% increase in lignin concentration across species. The C/N ratio of both leaf and root has also been shown to change with $CO_2$ enrichment. These chemistry changes in plant tissue under $eCO_2$, however, were generally weaker when additional N is applied. Plant organic matter input through litter, root biomass turnover and root-exudation dramatically affects belowground microbial community composition and functional activities.How quantitative and qualitative changes in plant organic matter input in response to the interactive effects of $CO_2$ and N will affect soil microbial communities and associated ecoprocesses remain understudied.

Here, we used high-throughput microbial functional gene microarray (GeoChip), stable isotope-based microbial C-sequestration and N-fixation measurements to detect and identify the impacts of $eCO_2$ and eN on soil microbial functional communities. We found that long-term changes in $CO_2$ and N availability dramatically altered the diversity and structure of C3 grass-associated soil microbial functional genes via several mechanisms, such as altering plant fine root production, exudation and soil moisture.

There was an antagonistic relationship between $eCO_2$ and eN that affected a large number of microbial functional genes, with $eCO_2$ generally increasing the abundance of these genes at aN, but either decreasing or increasing abundance to a minimal degree at eN. These results imply that $eCO_2$ may accelerate nutrient cycling in the C3 grass system, but the magnitude of effect is strongly dependent on the relative availability of N. Elevated $CO_2$ and eN exerted surprisingly minor effects in the legume-associated soil microbial functional community, with $eCO_2$ generally decreasing gene abundance at aN, but increasing it at eN, suggesting that the impacts of $eCO_2$ and eN are ecosystem-specific. This study provides new insights into our understanding of microbial functional processes in response to multiple global change factors.

**Keywords: elevated $CO_2$; nitrogen fertilization; microbial community; GeoChip; functional genes; progressive nitrogen limitation; priming effect**

## 3.2 Introduction

Current efforts to accurately predict ecosystem feedbacks to the rising atmospheric $CO_2$ are hampered by uncertainties of the microbial responses beneath the Earth's surface which mediate key biogeochemical cycles (Luo et al. 2006, Reich et al. 2006, van Groenigen et al. 2006, Carney et al. 2007, He et al. 2010). Numerous studies have reported that rising atmospheric $CO_2$ stimulates belowground microbial growth and activities (He et al. 2010, Xu et al. 2013, Xiong et al. 2015, Butterly et al. 2016), and alters microbial community composition and structure (Austin et al. 2009, Berthrong et al. 2014) through increasing primary production in plants and C inputs into soil (Rich et al. Norby et al. 2005; Finzi et al. 2007). However, the commonly found nutrient limitations, particularly nitrogen (N) limitations in terrestrial ecosystems, restrict the magnitude of $CO_2$-induced plant growth enhancement (Zak et al. 2000, Oren et al. 2001, Shaw et al. 2002, Hungate et al. 2003, Luo et al. 2004) and alters plant physiological activities in response to rising $CO_2$ (e.g. photosynthate partitioning) (Gleeson and Tilman 1992, Reich and Hobbie 2013) thus, potentially altering belowground microbial processes accordingly.

Conceptual models of nutrient limitation suggest that plants exposed to rising $CO_2$ should allocate a greater amount of photosynthate to enhance root development to 'mine' nutrients from soil (Johnson 2006), and such belowground C allocation by elevated $CO_2$ should be greater at low than high N conditions (Reich and Hobbie 2013). In support of this, empirical evidence showed increased belowground C allocation and fine root production in low N availability soils in several $CO_2$ enrichment experiments (Lukac et al. 2003, Norby et al. 2004, Pritchard et al. 2008). It is also widely observed

that root exudation, low molecular weight organic substrates (e.g. sugars, amino acids and low molecular weight organic acids), increases in response to $eCO_2$ (Korner and Arnone 1992, Diaz et al. 1993, Hungate et al. 1997, Jones et al. 1998, Schlesinger and Lichter 2001, Heath et al. 2005, Korner et al. 2005, Carney et al. 2007, Finzi et al. 2007, Pollierer et al. 2007, Phillips et al. 2009), and to a greater amount with low than high soil N availability (Phillips et al. 2011). These changes in plant organic matter input in response to the interactive effects of $CO_2$ and N may further affect soil microbial communities and associated ecoprocesses but yet remain less studied.Fine root turnover, root cell sloughing, and root exudation deliver chemically labile organic substrates directly to soil (Meier et al. 2008). Compare to other plant-derived C input (e.g. leaf litter), they are preferred and readily consumed by soil microbes, providing essential C and energy sources (Drigo et al. 2008). Enhanced root growth and exudation under $eCO_2$ may therefore greatly impact ecosystem C and nutrient dynamics by altering belowground microbial community and activities (Korner and Arnone 1992, Diaz et al. 1993, Hungate et al. 1997, Jones et al. 1998, Schlesinger and Lichter 2001, Heath et al. 2005, Korner et al. 2005, Carney et al. 2007, Finzi et al. 2007, Pollierer et al. 2007)**.** For example, in responses to this extra C input under $eCO_2$, stimulated microbial population may synthesize more extracellular enzymes for depolymerization of N from soil organic matter (SOM) to meet their increasing nutrient demand, an effect known as 'priming effect' (Cheng 2005). Consequently, greater microbial immobilization of N will eventually be released for plant uptake during microbial biomass turnover, which may help sustaining plant N requirements under long-term $eCO_2$ (Dijkstra et al. 2009). Previous study showed that the strength of priming effect largely depends on the amount

of soil available N as it is more energetically efficient for microbes to assimilate mineral N from soil than mining it from SOM (Cheng 2005).

Meanwhile, a number of studies have suggested that $eCO_2$-induced increase in leaf litter production can stimulate SOM decomposition through priming effects and drive soil C loss (Carney et al. 2007, Talhelm et al. 2009). Similarly, recent work also showed that enhanced root growth and exudation in response to $eCO_2$, particularly in low fertility soils, stimulated SOM decomposition which may prevent soil C accumulation in forest ecosystems (Phillips et al. 2011). However, no studies have yet comprehensively measured how N availability affects the responses of belowground microbial functional processes under $eCO_2$. Moreover, most $CO_2$ enrichment experiments have been relatively short in duration (3 years or less), thereby reducing their utility for understanding long-term interactive effects between $eCO_2$ and N availability on belowground microbial processes.

In this study, we examined belowground microbial communities associated with C3 grass and legume plant functional groups in a 12-year grassland free-air $CO_2$ enrichment (FACE) experiment. We predicted that the abundance of microbial functional genes related to C and N cycling, and associated activities under $eCO_2$ will be higher than under ambient $CO_2$ ($aCO_2$) as a result of the greater primary production of plants. We predicted that the strength of these $CO_2$ effects would be reduced for plots fertilized with inorganic N, particularly for microbial functional groups related to chemically recalcitrant C decomposition and N-fixation, due to a lesser amount of root production and exudation.

## 3.3 Materials and Methods

### *3.3.1 Site description and sampling*

The BioCON experiment is located at Cedar Creek Natural History Area, in central Minnesota, USA (Inouye and Tilman 1988, Reich et al. 2001) with $CO_2$, N deposition and plant diversity treatments arranged into 296 2 × 2 m plots. A free-air-$CO_2$ enrichment (FACE) technology was employed to maintain elevated $CO_2$ concentration at 560 ppm versus the ambient $CO_2$ concentration at 368 ppm in 6 rings (20 m diameter). Half of the plots receive an N addition of 4 $g \cdot m^{-2} \cdot y^{-1}$ ($NH_4NO_3$) in three applications (mid-May, mid-June, and mid-July), which doubles the rate of N deposition at the site. There are 4 levels of plant diversity with 1, 4, 9 and 16 species, respectively. A detailed description of the site can be found in Reich *et al*. (2001).

In this study, soil samples were collected in July (the growing season) 2009, 12 years after establishment of the BioCON experiment, from all 24 plots with 4 plant species associated with two functional groups: C3 grass (*Agropyron Repens, Bromus Inermis, Koeleria Cristata, Poa Pratensis*) and N-fixing legume (*Amorpha Canescens, Lespedeza Capitata, Lupinus Perennis, Petalostemum Villosum*). All plant species in this study were native or naturalized to the Cedar Creek Ecosystem Science Reserve. Within each plant group, there were two treatments: $CO_2$ and N with 3 biological replicates.

Five soil cores were collected at a depth of 0-20 cm from each plot and mixed as one composite sample and sealed in a plastic sampling bag, then placed on ice until transported to the laboratory. All samples were then passed through a 2 mm sieve to remove roots. A subsample was kept at 4$^{o}$C for soil characterization, C sequestration and N deposition measurements and the remainder was stored at -80$^{o}$C for DNA extraction.

Plant and soil properties were measured routinely on site (described elsewhere, (Reich et al. 2001, Reich et al. 2006)).

*3.3.2 Soil DNA extraction, amplification, and labeling*

Microbial community DNA was extracted from 5 g of soil from each sample using a freeze-grinding method (Zhou et al. 1996) and purified by agarose gel electrophoresis. DNA quality was assessed by the ratios of 260⁄280 nm and 260⁄230 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and final DNA concentrations were quantified with PicoGreen (Ahn et al. 1996) using a FLUOstar Optima (BMG Labtech, Jena, Germany). DNA (100 ng) was then amplified through a rolling circle amplification using the Templiphi Kit (GE Healthcare, Piscataway, NJ, USA). Single-strand binding protein (200 ng $ul^{-1}$) and spermidine (0.04 mM) were applied to the reaction buffer to facilitate amplification (Wu et al. 2006). The reactions were incubated at 30ºC for 3 h. All the amplified DNA were then labeled with cyanine-5 dye, purified and dried at 45ºC for 45 min (SpeedVac, ThermoSavant, Waltham, MA, USA) (Wu et al. 2006).

*3.3.3 Microarray hybridization*

Hybridizations were performed using GeoChip 3.0 on a Maui Hybridization Station (BioMicro, Salt Lake City, UT, USA). Before hybridization, dried samples were resuspended in 50 ul hybridization solution (45% formamide, 5 × SSC, 0.1% SDS and 0.1 mg/ml Salmon sperm DNA) and 2 ul of universal standard DNA labeled with cyanine-3 dye (Liang et al. 2010), incubated at 98ºC for 3 min and then kept at 65ºC until hybridization.

GeoChip slides were placed in a prehybridization buffer (74% DI water, 5 × SSC, 0.1% SDS and 1 mg/ml BSA) at 42°C for 45 min. The slides were then washed twice using wash buffer III (0.1 × SSC) for 5 min each and dried using the Maui Wash Station (BioMicro, Salt Lake City, UT, USA). Maui Mixer AO (BioMicro) was adhered to the dried slides to form hybridization chambers. Hybridization solution with dissolved DNA (50 ul) was then injected into the chamber and hybridization was performed at 42°C for 12 h on a Maui Hybridization station (BioMicro). After hybridization, the Mixer was removed, and the slide was washed and dried using Maui Wash Station at 42°C with 40 s agitation using buffer I (1 × SSC, 0.1% SDS), 30 s agitation at RT using buffer II (0.1 × SSC, 0.1% SDS), 50 s agitation at RT using buffer III and for 3 min drying.

*3.3.4 Microarray scanning and data pre-processing*

Microarrays were scanned using a ScanArray® 5000 (PerkinElmer, Boston, MA, USA), and digitally analyzed using ImaGene™ v6.0 to quantify the pixel density (intensity). A signal-to-noise ratio [SNR, (signal mean-background mean)/background standard deviation] of ≥ 2 was considered as a positive signal (He and Zhou 2008). Then, the signal intensity was normalized using a two-step normalization method as described previously (Xue et al. 2013). Sample intensity was first normalized by using the intensity of universal standards within each sub-grid. The normalized sample intensity was then further normalized by the mean universal standard intensity across all slides. For data reliability, a gene was considered positive only if it was detected at least twice among the three replicates (He et al. 2010, Xiong et al. 2010). Then, relative abundance of each sample was calculated by dividing the individual signal intensity of each probe by the total signal intensity for all detected probes in that sample. The relative abundance was then multiplied

by the mean value for the sum of signal intensity of all samples. A natural logarithm transformation was performed afterward for the amplified relative abundance plus 1.

*3.3.5 Ecosystem C flux measurement*

Soil heterotrophic respiration measurement: soil samples (50 g, dry weight equivalent) were put into Duran bottles and incubated for 24 days. The concentration of $CO_2$ was measured with a Micro-Oxymax Respirometer (Columbus Instrument, Columbus, OH) every 12 hours. Soil C flux was measured routinely on site (described elsewhere, (Reich et al. 2006)).

*3.3.6 Stable isotope analysis of soil C sequestration and N fixation*

Microbial incorporation of $^{13}CO_2$ into soil in relation to controls were measured through lab incubation. Soil samples (50 g, dry weight equivalent) were incubated in containers at 25ºC, 40% water holding capacity, with air supplemented with either 368 or 568 ppm of 99% atom $^{13}CO_2$ (same as the BioCON site). A halogen lamp (400 – 700 nm, visible spectrum) with an infrared filter was used to provide incident irradiance from 8 am to 6 pm during the experiment, and samples were incubated in the dark for the rest of time. Gas was sampled every 5 days to measure $^{13}CO_2$ concentration and then renewed. After 30 days, 75 mg subsamples of the soil were collected and the $^{13}C$ content was measured by a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) in the Stable Isotope Facility at the University of California, Davis (Davis, CA).

Microbial incorporation of $^{15}N_2$ into soil in relation to controls were measured through lab incubation. Since $^{15}NH_4{}^{15}NO_3$ was used as N fertilizer in all N-enriched plots at the BioCON experiment, the lab incubation for measuring $^{15}N_2$ fixation was carried out

only using samples from the aN plots. Soil samples (50 g, dry weight equivalent) were placed into 25 ml Balch tubes (Bellco Glass, Vineland, NJ, USA). The headspace of the tubes was evacuated and replaced with synthetic air (20% $O_2$ and 80% $^{15}N_2$; 98 atom % $^{15}N$, Isotec, Miamisburg, OH, USA). Controls were set up using unlabeled $N_2$. Tubes were incubated horizontally at 25ºC in the dark for 30 days. After incubation, 75 mg subsamples were weighted out and sent to the Stable Isotope Facility at the University of California, Davis (Davis, CA) to determine the $^{15}N$ content by a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The net potential N-fixation rate was then calculated.

*3.3.7 Statistical analysis*

Diversity indices were calculated as described previously (He et al. 2010). Detrended correspondence analysis (DCA) was performed to determine the distances between microbial functional composition and structure among treatments. Permutational multivariate analysis of variance (Adonis) was applied to test the significant differences in overall functional structure among treatments. Multivariate regression tree (MRT) analysis was used to partition the relative effects of plants, $CO_2$ and N amendment on soil microbial functional composition and structure. Canonical correlation analysis (CCA) was used to evaluate possible linkages between overall microbial functional structure and environmental attributes. A forward selection procedure and variance inflation factors values (999 Monte Carlo permutations) were used to identify common sets of plant and soil attributes important in shaping the microbial community and these attributes were used in the CCA model. Mantel tests were used to elucidate correlations between soil

processes (e.g., soil C flux, heterotrophic respiration, C sequestration potential, N fixation potential, ammonification rate, nitrification rate) and detected key functional gene categories. The significant differences were defined as $P < 0.05$, or with listed $P$ values. All analyses were performed by using R, v.2.9.1 (R foundation for Statistical Computing, Vienna, Austria). Effects of $CO_2$ and N on plant, soil and microbial functional genes were tested by a two-way analysis of variance (ANOVA), using the agricolae-package in R. In all ANOVA models, $CO_2$ and N were considered fixed effects and the block effect was random. The *post hoc* Fisher's least-significant-difference (LSD) test with Holm-Bonferroni adjustment was used to establish the significance of the differences among means.

## 3.4 Results

### *3.4.1 Effects of $eCO_2$ and eN on plant and soil attributes*

Elevated $CO_2$ and eN had greater influence on C3 grass than legume in terms of total biomass, aboveground biomass, different types of roots and C/N ratio. For example, total $C_3$ grass biomass significantly increased with $eCO_2$ compared to $aCO_2$ ($P = 0.006$) and to a greater extent at eN (+60.9%) than aN condition (+6.1%) ($CO_2 \times N$ interaction $P = 0.012$). Aboveground biomass and coarse root of C3 grass significantly increased with $eCO_2$ at eN condition only (+28.1% and +492.5%, respectively. Fig 3.1a). In contrast, fine root of C3 grass significantly increased by 355.7% with $eCO_2$ at aN, but was unaffected by $eCO_2$ at eN condition (Fig 3.1a). No changes were observed for legume growth, except a significant decrease in aboveground biomass by 44.4% with eCO2 at aN condition (Fig 3.1a).

Neither $eCO_2$ nor eN had a strong influence on measured soil attributes including pH, temperature, and total soil C and N. However, the moisture of C3 grass plot soils significantly increased (+70.4%) with eCO2 at aN, while there was no $CO_2$ effect at eN (Table S3.1).

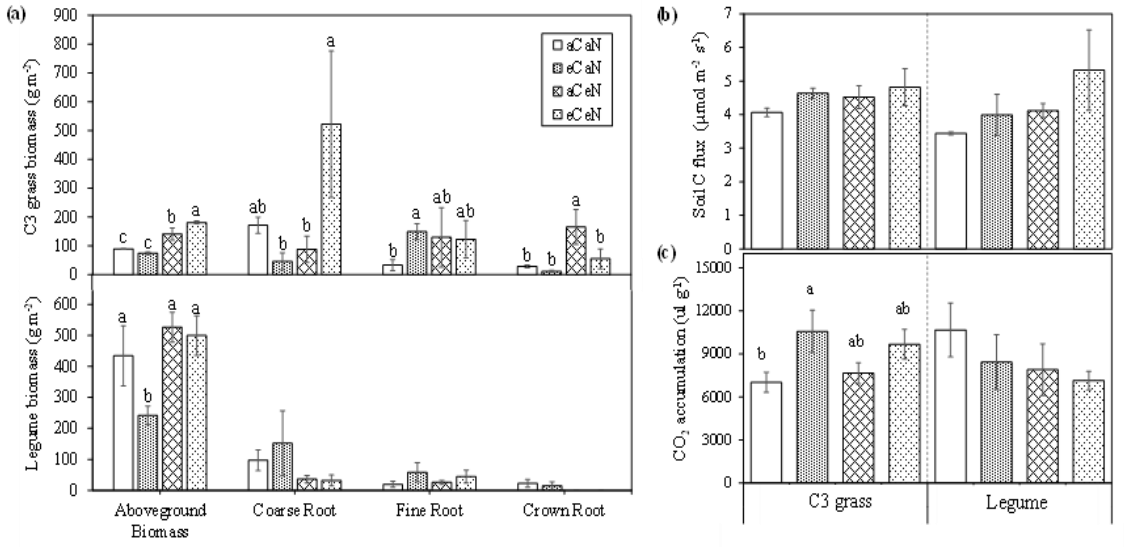



**Fig. 3.1** Effects of $eCO_2$ and eN on plant biomass and ecosystem C fluxes. a. Aboveground and root biomass (0-20 cm, coarse, fine, and crown root) of C3 grass and legume in June 2009, the sampling year. b. Soil C flux, calculated as the average of all five measurements from June to August during the sampling year to minimize variation caused by time the reading was taken during the day. c. Heterotrophic respiration rate. All data are presented with mean ± SE (error bars). Values labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

*3.4.2 Overall responses of soil microbial functional genes to $eCO_2$ and eN*

Nearly 1.7 times as many probes/genes (13956 *vs.* 8433) were detected by GeoChip in C3 grass than in legume plots ($P < 0.05$), indicating higher microbial functional potential in C3 plots. Within each plant group, only around half of the detected functional genes at $eCO_2$ and eN alone or together overlapped with those receiving no treatments (Tables

S3.2). In C3 plots, $eCO_2$ plots under aN harbored the largest number of unique genes (25.19%), coinciding with a significantly ($P < 0.05$) higher overall microbial functional richness and diversity (Tables S3.3). Ambient $CO_2$ plots under eN harbored the least number of unique genes (8.97%). In legume plots, $eCO_2$ plots under aN had the least number of unique genes (9.26%) (Tables S3.2). No significant treatment effects were observed on overall microbial functional richness and diversity (Tables S3.3).

Overall microbial community structures of both plant groups were significantly altered by $eCO_2$ and eN as indicated by three non-parametric multivariate statistical tests (ANOISM, Adonis, and MRPP) (Table 3.1).

**Table 3.1** Significance tests of the effects of $eCO_2$ and eN on the overall microbial functional gene structure

| | | | $CO_2$ | N | $CO_2 \times N$ |
|---|---|---|---|---|---|
| C3 grass | ANOSIM[1] | *R* | 0.250 | 0.5222 | NA |
| | | *P* | **0.035** | **0.005** | NA |
| | Adonis[2] | *F* | 0.1982 | 0.2696 | 0.1903 |
| | | *P* | **0.025** | **0.002** | **0.001** |
| | MRPP[3] | $\delta$ | 404.4 | 404.4 | NA |
| | | *P* | **0.009** | **0.003** | NA |
| Legume | ANOSIM | *R* | 0.4037 | 0.237 | NA |
| | | *P* | **0.003** | **0.025** | NA |
| | Adonis | *F* | 0.2155 | 0.2044 | 0.214 |
| | | *P* | **0.001** | **0.007** | **0.001** |
| | MRPP | $\delta$ | 330.5 | 330.5 | NA |
| | | *P* | **0.003** | **0.009** | NA |

Based on 999 permutations.
1. Analysis of similarities.
2. Non-parametric multivariate analysis of variance (MANOVA) with the Adonis function.
3. A Nonparametric approach depends on the internal variability of the data.

DCA-based ordination of overall detected microbial functional genes revealed a clear separation of samples between the two plant groups, and with more distinct treatment effects on assembling the C3 associated samples (Fig. 3.2). Moreover, multiple

regression tree was first split by plant groups. Of the C3 grass branch, it was further split by "N" and then "$CO_2$". Whereas, within the legume branch, it has first split by "$CO_2$" and then "N" (Fig. S3.1).

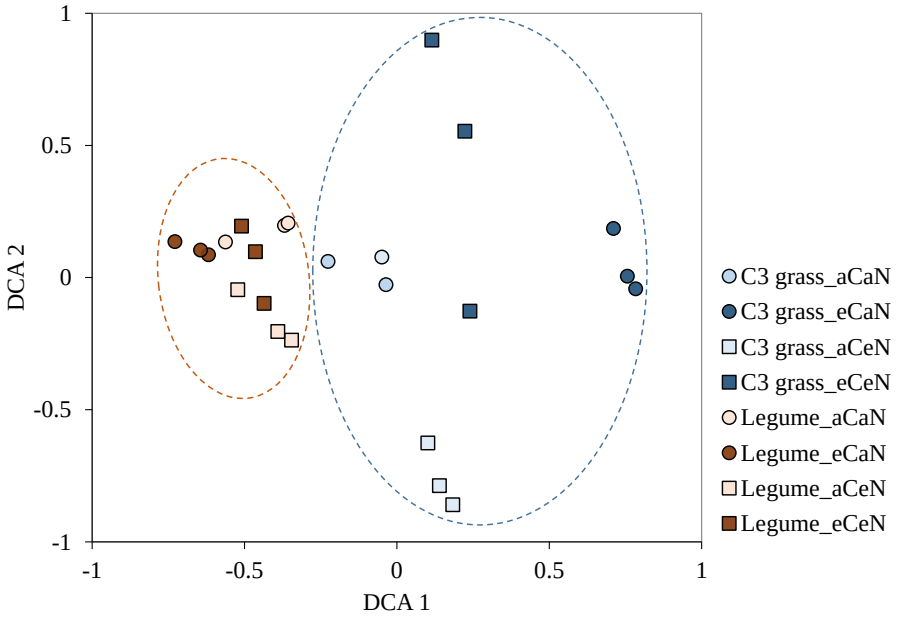



**Fig. 3.2** Detrended correspondence analysis (DCA) showing that $eCO_2$ and eN had substantial influences on microbial functional gene composition and structure

*3.4.3 Effects of $eCO_2$ and eN on key functional genes*

Key function genes involved in important biogeochemical processes such as C, N, P and S cycling were further examined.

(i) C decomposition genes. A total of 26 gene families involved in C degradation were detected by GeoChip. These ranged from genes involved in the degradation of simple sugars (e.g., starch, hemicellulose, cellulose) to recalcitrant polycyclic C structures (e.g., chitin, aromatics and lignin). Among these genes, $eCO_2$ and eN significantly altered the abundance of several of them ($P < 0.05$), but to a greater extent in the C3 grass (Fig. 3.3) than in legume plots (Fig. 3.4).

In C3 grass plots, there were significant $CO_2$ × N interactions on 12 C degradation gene families ($P < 0.05$, Fig. 3.3). Compared to $aCO_2$, $eCO_2$ consistently increased the abundance of these genes (+24% to +151%) at aN, but either increased to a lesser extent (+0.1% to +5%) or decreased them (-3% to -41%) at eN. Notably, the interactive effects were predominantly affected genes involved in recalcitrant C decomposition (e.g., acetylglucosaminidase, endochitinase, isocitrate lyase, malate synthase, vanillate monooxygenase, vanillin dehydrogenase, manganese peroxidase, phenol_oxidase). As a main effect, $eCO_2$ significantly altered the abundance of 13 C degradation genes (12 increase and 1 decrease) across N levels ($P < 0.05$, Fig. 3.3). N also had a highly significant effect for 19 C degradation genes ($P < 0.05$, Fig. 3.3). Relative to aN, eN significantly ($P < 0.05$) decreased the abundance of all these genes.

In legume plots, $CO_2$ × N interactions significantly affected eight C degradation genes, wherein $eCO_2$ predominantly decreased the abundance of these genes (-4% to -56%) at aN, but increased them (+0.3% to +95%) at eN ($P < 0.05$, Fig. 3.4). Very few significant changes by the main effects of $eCO_2$ were observed for C degradation genes in legume plots (Fig. 3.4). Interestingly, the main effects of eN significantly increased the abundance of five genes involved in degrading labile C substrates (e.g. alpha, amylase, pullulanase, xylanase, and endoglucanase) ($P < 0.05$, Fig. 3.4).

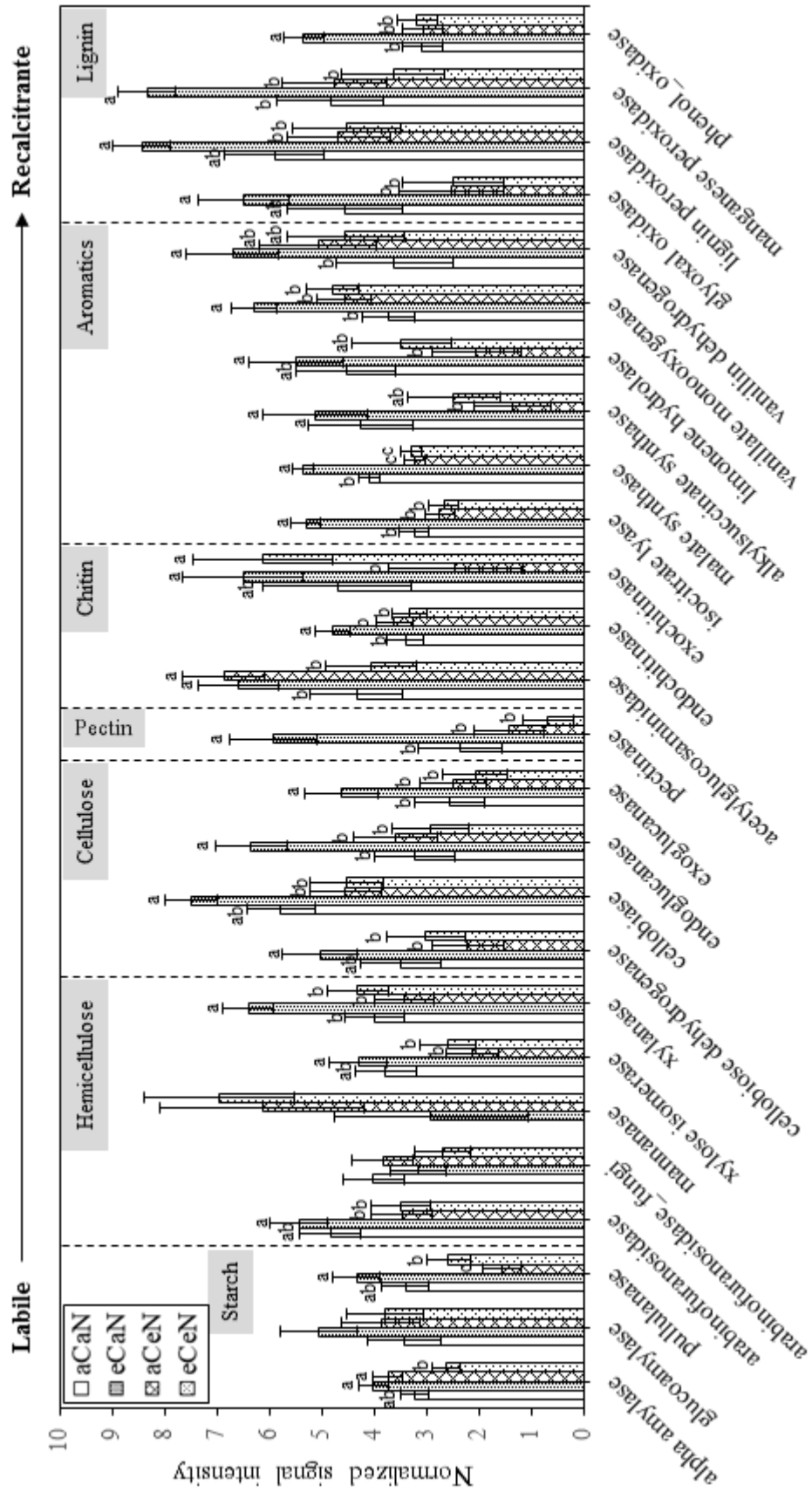



**Fig. 3.3** Effects of $eCO_2$ and eN on C degradation genes detected in C3 grass plots. Bars labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment

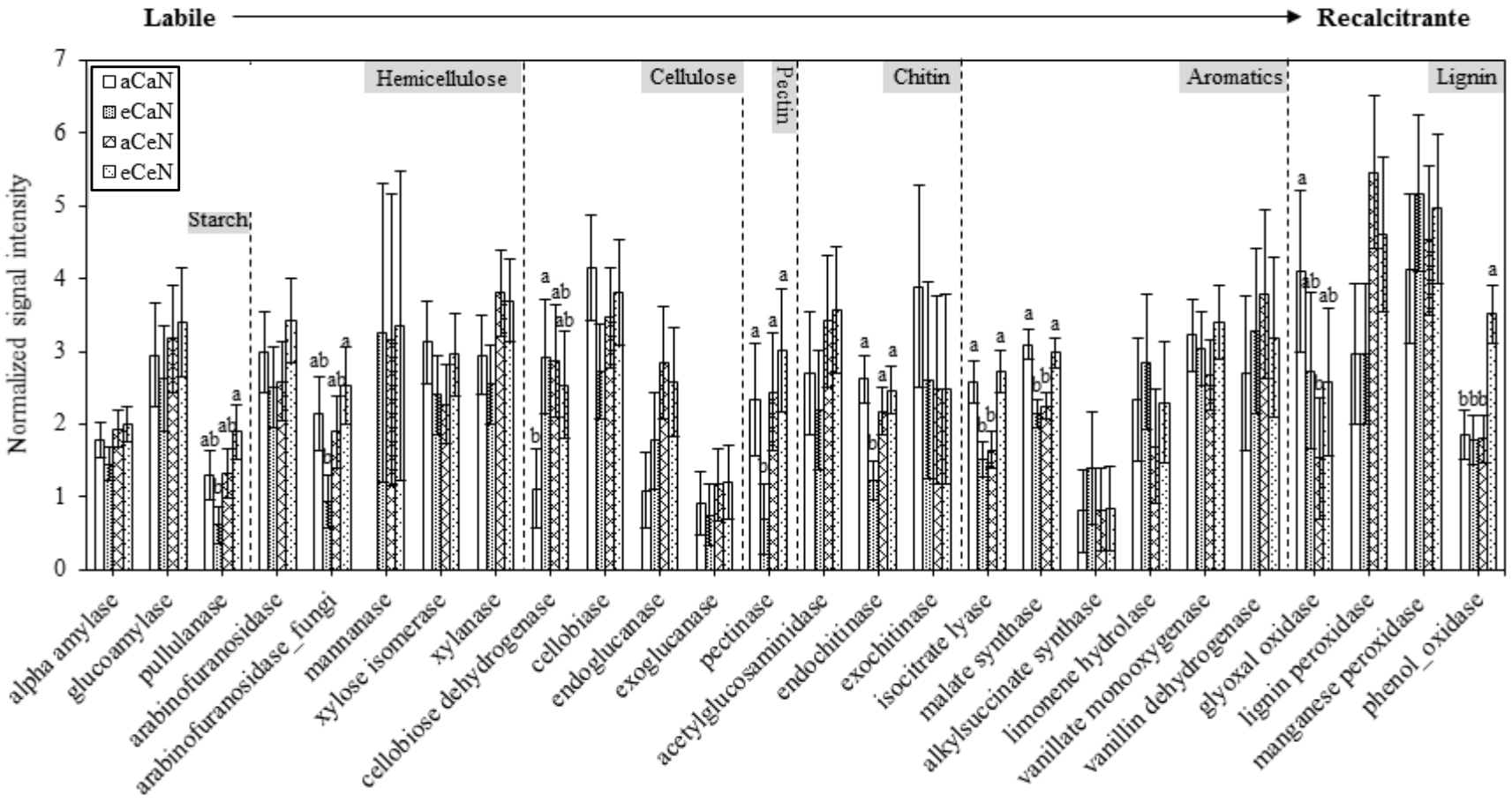



**Fig. 3.4** Effects of $eCO_2$ and eN on C degradation genes detected in legume plots. Bars labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment

(ii) C fixation genes. $CO_2$ and N exhibited significant main and interactive effects on all the detected genes involved in microbial C fixation ($P < 0.05$), including *pcc* encoding propionyl-CoA carboxylase, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisco) genes, and C monoxide dehydrogenase (CODH) genes, but with different patterns between C3 grass and legume plots. In C3 grass plots, relative to $aCO_2$, $eCO_2$ stimulated the abundance of C fixation genes to a greater extent at aN (+52% to +101%) than at eN (+8% to +9%) (Fig. S3.2). Across two N levels, $eCO_2$ significantly increased the abundance of all the three C fixation genes ($P < 0.05$) (Fig. S3.2). Whereas, across two $CO_2$ conditions, eN significantly reduced the abundance of these genes ($P < 0.05$) (Fig. S3.2). In legume plots, $CO_2$ × N interactions significantly altered the abundance of two C fixation genes (CODH and Rubisco), wherein $eCO_2$ decreased the abundance of these genes at aN but increased them at eN ($P < 0.05$, Fig. S3.2).

(iii) N cycling genes. A total of 15 genes related to N fixation, ammonification, nitrification, denitrification, dissimilatory N reduction, assimilatory N reduction and anammox were detected. Among these genes, six were significantly affected by the $CO_2$ and N interactive effects in C3 grass plots including *nifH* for N fixation, *ureC* for ammonification, *nirK* and *nirS* for denitrification, *nasA* for assimilatory N reduction, and *hzo* for anammox ($P < 0.05$, Fig. S3.2). Relative to $aCO_2$, $eCO_2$ increased the abundance of these genes (+36% to +192%) at aN, but increased to a smaller extent (*nirK* and *nasA*, +28% to +35%) or even decreased them (*nifH*, *ureC*, *nirS*, and *hzo*, -2% to -67%) at eN. As a main effect, $eCO_2$ consistently increased the abundance of *nifH*, *ureC*, *narG*, *nirK*, *nirS*, *nosZ*, *nasA*, and *nirA*; whereas eN significantly reduced the abundances of these genes except for *nirA* ($P < 0.05$, Fig. S3.2). In legume plots, $CO_2$ × N interactions had

significant impacts on *nifH*, *ureC*, *narG*, *nirS* and *hzo* ($P < 0.05$, Fig. S3.3). Relative to $aCO_2$, $eCO_2$ reduced the abundance of those genes under aN but increased them under eN. Furthermore, only a few genes were significantly affected by the main effects of $CO_2$ and N. Significant positive correlation was detected between soil ammonification rate and the abundance of *ureC* gene (Mantel test $P < 0.05$, Fig. S3.4d). Significant positive correlation was also found between soil nitrification rate and the abundance of bacterial-amoA but not archaeal-amoA gene abundances (Mantel test $P < 0.05$, Fig. S3.4d), indicating that ammonia oxidizing bacteria (AOB) were more important than ammonia oxidizing archaea (AOA) in mediating soil N cycling at this site.

(iv) P/S cycling genes. In C3 grass plots, interactive effects between $CO_2$ and N significantly affected all the three detected genes involved in P cycling, including *ppk* for polyphosphate biosynthesis, phytase for organic phytate hydrolysis, and *ppx* for inorganic polyphosphate hydrolysis ($P < 0.05$, Fig. S3.2). Relative to $aCO_2$, $eCO_2$ increased the abundances of *ppk* by 31% and *ppx* by 27% at aN, but decreased *ppk* by 21% and increased *ppx* by only 2% at eN. And phytase was only detected in $eCO_2$ plots. In legume plots, only *ppk* was significantly reduced by $eCO_2$ at aN ($P < 0.05$, Fig. S3.2). Meanwhile, four genes involved in S cycling were detected including *aprA* encoding adenylylsulfate reductase, *dsrA* and *dsrB* encoding sulfite reductase, and *sox* for sulfur oxidation. In C3 grass plots, half of these genes were significantly altered by $CO_2 \times N$ interactions ($P < 0.05$, Fig. S3.2). Specifically, the abundance of *dsrA* and *sox* were increased by $eCO_2$ at aN but decreased at eN, as compared to those from $aCO_2$. $CO_2$ main effect significantly increased the abundance of these genes except for *dsrB* ($P < 0.05$, Fig. S3.2). Whereas, all the four genes were significantly reduced by eN across two $CO_2$ levels. By contrast,

in legume plots, three genes (*aprA*, *dsrA*, and *sox*) were reduced by $eCO_2$ at aN but increased by $eCO_2$ at eN compare to those from $aCO_2$ ($P < 0.05$, Fig. S3.3). Only main effect of N was observed that significantly stimulated the relative abundance of *dsrA* and *dsrB* ($P < 0.05$, Fig. S3.3).

*3.4.4 Soil and microbial heterotrophic respiration*

In C3 grass plots, a significant $CO_2$ main effect on belowground microbial heterotrophic respiration (HR) was detected, which was increased by 38% at $eCO_2$ than $aCO_2$ ($P < 0.05$, Fig. 1c). However, no treatment effects were detected on the ecosystem respiration (SCF, represented by the soil C flux measured on site, Fig. 1b). Furthermore, strong positive correlations were observed between both SCF and HR, and the functional gene groups involved in degrading various C compounds targeted by GeoChip, including starch, cellulose, pectinase, chitin, aromatics and lignin (Fig. S3.4a). Thus, changes in abundance of these genes appeared to contribute to the alteration of SCF and HR in C3 grass plots. By contrast, no significant changes in HR or SCF were observed in response to treatments in legume group (Fig. 1b and 1c).

*3.4.5 Stable isotope analysis of soil C sequestration and N fixation*

In C3 grass plots, $eCO_2$ significantly increased the C sequestration potential across two N levels as measured by the changes in $^{13}C$ in soils ($P < 0.05$, Fig. 3.5). Mantel tests showed that the changes in C sequestration potential were significantly correlated with the C fixation genes as a whole ($P < 0.05$, Fig. S3.4c). For individual genes involved in C fixation, significant (CODH, $P < 0.05$) or marginally significant (*pcc* and Rubisco, $P < 0.1$) correlations with the C sequestration potential were also detected, indicating that CODH had a greater contribution to the microbial assimilation of $CO_2$ in C3 grass plots

of this study. In addition, there were no significant treatment effects on C sequestration potential in legume plots were detected (Fig. 3.5).

No significant changes in N fixation potential were detected either in the C3 grass or in legume plots in response to $eCO_2$, possibly due to a lack of statistical power (Fig. 3.5). However, a marginally significant correlation between soil $N_2$-fixation potential and *nifH* gene was detected ($P < 0.1$, Fig. S3.4c).

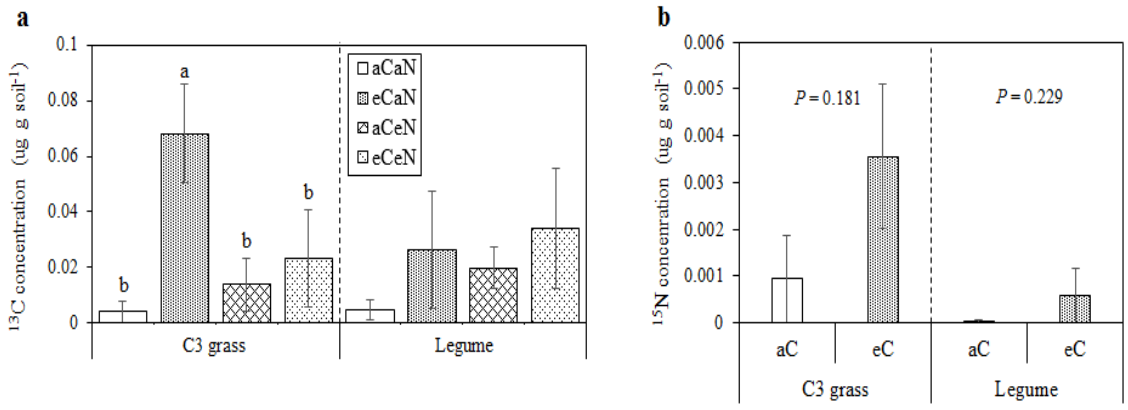



**Fig. 3.5** Quantitative $^{13}C$ and $^{15}N$ enrichment in soils from C3 grass and legume plots at $CO_2$ and N treatments. $^{13}C$ (a) and $^{15}N$ (**b**) concentrations (mean, error bars indicate SEs, n=3) were calculated from the $^{13}C$ and $^{15}N$ excess values (obtained by IRMS) in the soils over the incubation time. *P*-values shown in (a) are based on two-way ANOVA and *P*-values in (b) are based on student's t-test.

*3.4.6 Linkages between microbial functional structure and selected environmental attributes*

We performed canonical correspondence analysis (CCA) to link microbial functional structure and attributes of plant and soil. A forward selection procedure and VIF (variance inflation factors with 999 Monte Carlo permutations) assessment were used to identify common sets of plant and soil attributes important in shaping the microbial community. CCA revealed that community functional structure within the C3 grass plots was significantly shaped by plant (e.g. aboveground biomass and C/N ratio; root biomass

including coarse, crown and fine roots, and root C/N ratio) and soil attributes (e.g. moisture) (Fig. 3.6a, $P$ = 0.005). Samples under different treatments were clustered closely with the first two CCA constrained axes explaining 51.9% of the total variance. As indicated by the projections of environmental attributes, fine root biomass, root C/N ratio and soil moisture seem to play important roles in shaping community structure under $eCO_2$ at aN condition; while aboveground and coarse root biomass, and crown root biomass seem to predominantly influence the structure of communities under $aCO_2$ and $eCO_2$, respectively, at eN condition. In contrast, only three plant attributes were selected (e.g. aboveground and crown root biomass, and root C/N ratio) for CCA model of the legume plots which could explain 29.5% of the total variance for the first two CCA constrained axis (Fig. 3.6b, $P$ = 0.06). CCA axis 1 and 2 appear to reflect the effects of eN and eCO2, respectively. Noticeably, aboveground biomass had a negative relationship to communities under eCO2 at aN condition, whereas crown root biomass may positively affect communities at the aN condition.

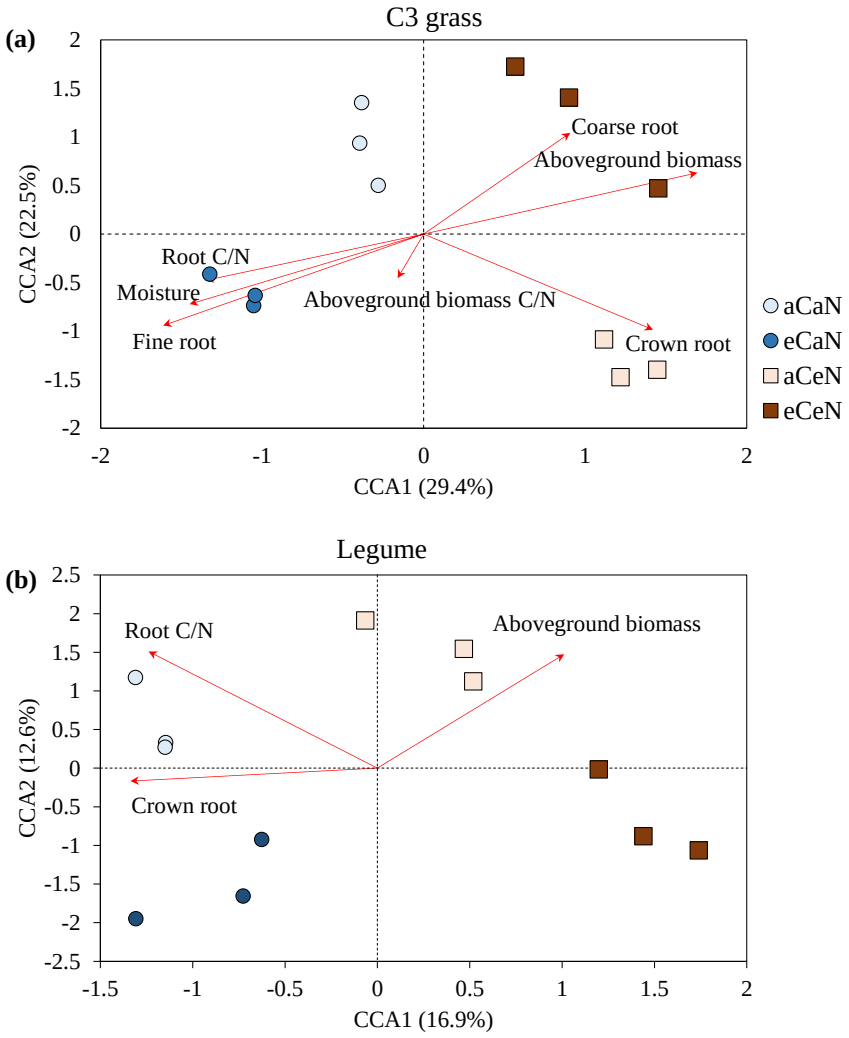



**Fig. 3.6** Canonical correspondence analysis (CCA) of microbial community composition based on detected functional genes with selected environmental variables. a. C3 grass plots. b. Legume plots

Variation partition analysis (VPA) (Ramette and Tiedje 2007) was performed to further assess the contributions of plant and soil attributes to the functional structure of microbial communities (Fig. 3.7). Selected belowground and aboveground plant properties, and soil property could explain 36.3%, 15.3%, and 9.3% of the total variation of community structure in $C_3$ grass plots, respectively. Interactions of belowground × aboveground C3 grass properties, belowground C3 grass properties × soil moisture, and

all three groups could explain 6.0%, 1.0% and 5.9% of the total variation, respectively. In legume plots, belowground and aboveground legume properties, and their interactions could explain 22.3%, 10.9% and 3.1% of the total variation, respectively.

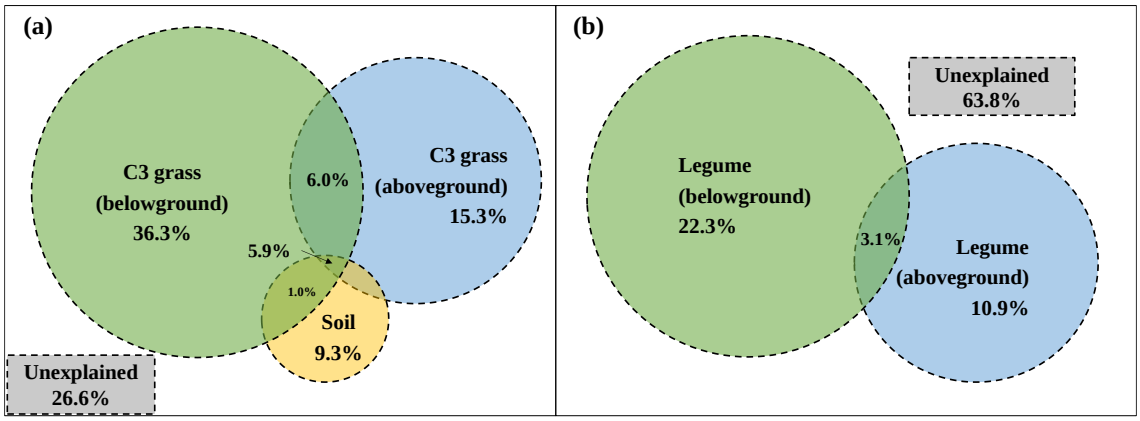



**Fig. 3.7** Variation partitioning analysis (VPA) of microbial functional gene structures explained by plant and soil variables in C3 grass plots (a) and legume plots (b). Each circle shows the percentage of variation explained by a single factor alone. The overlapped area represents the percentage of variation explained by interactions between two or three of the factors. Same plant and soil variables screened for the CCA model were used. Only contribution of variation larger than 1% was shown.

### 3.5 Discussion

Grassland ecosystems cover about one fourth of Earth's surface and provide various ecosystem services (e.g. C storage, provision of food and water, biodiversity preservation) (IPCC 2007, Joyce et al. 2016). Understanding the mechanisms by which belowground microbial communities mediate C and nutrient cycling under elevated $CO_2$ is critical for predicting grassland ecosystem feedbacks to climate change. Here, we show that 12-year elevated $CO_2$ increased belowground microbial functional potential and activities, but the magnitude of such effect depends on soil fertility.

Although previous studies have widely reported elevated $CO_2$-induced stimulation of soil microbial growth and activities, these studies were largely conducted in short-term $CO_2$ enrichment experiments and ignored the co-limitation by N supply

on $CO_2$ fertilization effect (He et al. 2010, Xiong et al. 2015), making the extrapolation of these results to long-term ecosystem feedbacks to elevated $CO_2$ problematic. Our results indicate that belowground microbial functional composition and structure are strongly influenced by elevated $CO_2$, and microbial functional potential related to C, N, S and P cycling in low fertility soils were stimulated more by elevated $CO_2$ than fertilized ones. At the BioCON site, ambient N supply was reported to limit potential biomass accumulation in response to elevated $CO_2$ (Reich and Hobbie 2013), an effect that was observed in the C3 grass response to elevated $CO_2$ of this study. However, fine root production of C3 grass significantly increased in response to elevated $CO_2$ in non-fertilized soils only (Fig. 3.1a). Similar results were observed in the Duke Forest FACE site that $CO_2$ enrichment increases C allocation to fine root production (Pritchard et al. 2008), with greater total C allocation to soil occurring in low fertility soils (Palmroth et al. 2006). Another study at the Duck FACE site also documented higher exudation rates with 13 years of $CO_2$ enrichment during the primary growing season only when N was not added (Phillips et al. 2011). Thus, much of the additional plant growth under elevated $CO_2$ in low nutrient soils is allocated to soil in the form of labile organic matter which can be readily consumed by microorganisms, and likely contributed to the greater microbial functional responses to elevated $CO_2$ in N-poor than N-rich soils of this study.

Progressive N limitation (PNL) theory suggests that soil N availability should be gradually reduced over time by elevated $CO_2$, and that the $CO_2$ fertilization effect should occur only in short time in N limited ecosystems (Johnson 2006). However, empirical evidence across FACE experiment sites showed inconsistent and controversial results that $CO_2$ fertilization effect either down-regulated over long-term

$CO_2$ enrichment (Norby et al. 2010, Reich and Hobbie 2013), or increased and accompanied by an increase in plant N uptake (Finzi et al. 2007, McCarthy et al. 2010). Previous studies at BioCON showed, although ambient N constrained the potential $CO_2$ fertilization effect on plant biomass production, it did not preclude stimulation of plant productivity by decade-long elevated $CO_2$ (the stimulation at ambient N was around half of that under enriched N). The mechanism by which plants retain long-term growth response to elevated $CO_2$ at such N-poor grassland ecosystem remains unclear. It is been suggested that the opposing mechanism, Priming, may play a role simultaneously at BioCON in offsetting the PNL effects (Reich and Hobbie 2013).

In this study, microbial genes related to chemically recalcitrant C degradation (e.g. phenol_oxidase, manganese peroxidase, vanillin dehydrogenase, vanillate monooxygenase, malate synthase, isocitrate lyase, endochitinase, acetylglucosaminidase) were selectively increased with elevated $CO_2$ in non-fertilized C3 grass plot soils only, indicating that microbial N-mining through decomposition of soil organic substrates may increase with elevated $CO_2$ in low nutrient conditions at BioCON. This process may be intensified by increased soil moisture in these plots which could stimulate soil microbial biomass and decomposition rate (He et al. 2010). Meanwhile, a positive effect of elevated $CO_2$ on net N mineralization in non-fertilized C3 grass plots, whereas a negative effect in fertilized plots was observed (Fig. S3.5). In support of this, *ureC* abundance, associated with ammonification, was increased with elevated $CO_2$ in non-fertilized C3 grass plot soils only, and a significant correlation between *ureC* and measured rates of ammonification in these plots were found. Collectively, our results suggest that Priming occurs simultaneously with PNL and

being stronger in low than the high nutrient condition of this grassland ecosystems. Such effect may contribute to the long-term stimulation of plant productivity, but persistent turnover of chemically recalcitrant C by microbes also accelerates soil C loss under elevated $CO_2$ particularly when N is limited, offering a partial explanation for the lack of C accumulation in the C3 grass plot soils at this site despite 12 years of $CO_2$-induced increases in NPP (Reich and Hobbie 2013).

Other mechanisms may also contribute to an increase in N cycling under elevated $CO_2$ in non-fertilized C3 grass plots. For example, elevated $CO_2$ can influence N dynamics by stimulating N fixation, an effect commonly observed in several $CO_2$ enrichment experiments including BioCON (Lee et al. 2003, Luo et al. 2006, van Groenigen et al. 2006, He et al. 2010). In this study, the abundance of *nifH* genes increased by 35.5% with elevated $CO_2$ in non-fertilized C3 grass plots, suggesting more N could have been fixed in these plots. Consistently, such change in *nifH* was mirrored by measured N-fixation rate in these plots which showed similar response patterns under elevated $CO_2$ and significantly correlated with the relative abundance of *nifH* genes. Therefore, enhanced N fixation by belowground microbes at elevated $CO_2$ positively affect the soil N budget in this grassland ecosystem, but such effect was confined to low fertility soils. Likely, N-fixing microbes which carry *nifH* genes and respond strongly to elevated $CO_2$ in C3 grass plots were actively using energy derived from decaying fine roots and exudates to fix $N_2$.

Nitrogen addition can decreases SOM decomposition rates, by reducing N mining of microbial decomposers (Craine et al. 2007). Such decline in microbial decomposition of complex C compounds appears to be common in studies with increases

in N (Sinsabaugh et al. 2005, Waldrop and Zak 2006, Ramirez et al. 2012). We found a significant negative effect of N fertilization on genes involved in recalcitrant C decomposition (e.g. endochitinase, isocitrate lyase, malate synthase, alkylsuccinate synthase, limonene epoxide hydrolase, glyoxal oxidase, lignin peroxidase or ligninase, manganese peroxidase, and phenol_oxidase) in the C3 grass plots, suggesting a decrease in SOM decomposition in these plots. However, several labile C decomposition genes (e.g. alpha amylase, pullulanase, arabinofuranosidase, mannanase, xylanase) were also decreased in relative abundance in response to N fertilization. Together, these effects may contribute to C accumulation but reduce N-gain through microbial N-mining in fertilized C3 grass plot soils in the long-term. Furthermore, N fertilization can reduce the competitive advantages of other $NH_4^+$-producing microorganisms (e.g. N-fixers) over non-$NH_4^+$-producing microorganisms, because N uptake from soil is less costly than driving from $N_2$-fixation or mineralization (Lee et al. 2003, Reich et al. 2006). Accordingly, a significantly lower abundance of *nifH* genes was observed in fertilized than non-fertilized C3 grass plots, suggesting fertilization may suppress the N gain via decreased $N_2$-fixation and ammonification in these plots.

### 3.6 Conclusions

Our results demonstrated the contingency in belowground microbial functional responses to long-term elevated atmospheric $CO_2$ at contrast N conditions, and highlight the importance of understanding the role of soil N availability in mediating plant and belowground microbial community responses to elevated atmospheric $CO_2$. Elevated $CO_2$ induces major shifts in overall microbial functional composition and structure; and,

to a greater extent, stimulates microbial functional genes that associated with C, N, S and P cycling in low fertility than fertilized conditions. These responses of belowground microbial community were closely related to the enhancement of fine root production and C flux from roots to soil under elevated $CO_2$. Particularly, microbial activities associated with chemically recalcitrant SOM turnover increases with elevated $CO_2$ in low fertility condition which may limit soil C storage and stability. To what extent, these changes in microbial functional potential will alter total C and N budgets in this grassland ecosystem requires further study.

# Chapter 4: Impact of Elevated $CO_2$ and N Addition on Metabolic Diversity of C3 grass- and Legume-associated Microbial Communities

## 4.1 Abstract

The impact of elevated atmospheric $CO_2$ on qualitative and quantitative changes in plant-derived C inputs belowground will have important consequences of C storage and nutrient cycling in soil. The aim of this work was to assess whether and how soil N conditions affect the influence of long-term $eCO_2$ on belowground microbial metabolic potential and diversity; and whether such effect varies between microbial communities associated with distinct plant functional groups (C3 grass and legumes). We investigated soil microbial communities in a grassland ecosystem exposed to two levels of atmospheric $CO_2$: ambient ($aCO_2$, 368 ppm) vs. elevated ($eCO_2$, 560 ppm); and two levels of N deposition: ambient (aN, 0 $g \cdot m^{-2} \cdot yr^{-1}$) vs. enriched (eN, 4 $g \cdot m^{-2} \cdot yr^{-1}$) for 12 years, growing either C3 grass or N-fixing legume forb. Biolog EcoPlate containing 31 low molecular weight C substrates, was used to construct sole C source utilization profiles of these communities. We found that community composition of soil microbes based on metabolic potential (C utilization rate) in the C3 grass plot soils was significantly different from those in the legume plot soils by both DCA and nonparametric dissimilarity tests. Microbial communities in legume plots had significantly higher metabolic potential than in C3 grass plots for decomposing organic substrates. Specifically, compared to the C3 grass plots, microbes in legume plots can use a larger number of C sources provided on EcoPlate and with greater decomposition rates within the measured time. We also found that $eCO_2$ and eN didn't significantly alter metabolic potential of microorganisms in the C3 grass plots. But overall microbial metabolic activities significantly increased with

$eCO_2$ by 20.6% in the fertilized legume plots, while there was no evidence for a $CO_2$ effect in the non-fertilized legume soils. Mantel test unveiled total soil N content, root ingrowth biomass, aboveground biomass, and root biomass N content as environmental attributes were closely correlated with microbial C utilization patterns. In addition, PLFA analysis showed both total microbial and bacterial biomass were significantly lower in legume than in C3 grass plots, showing an opposite trend to the microbial metabolic potential in these plots. Collectively, these results demonstrated that $eCO_2$ effects on active microbial metabolic activities are contingent on N conditions, and such effect differs between plant functional groups. Differences in microbial metabolic potential among treatments and between plant functional groups were not due to population size (biomass) but likely attributed to changes in community structure and/or enzymatic activities of belowground microbes.

**Keywords: Biolog EcoPlate; microbial metabolic potential; elevated $CO_2$; N fertilization**

## 4.2 Introduction

Grasslands cover about 40% of the global land surface and store approximately 34% of the global terrestrial stock of organic C pool, mostly in grassland soil (Scurlock and Hall 1998, Lal 2004). Microbial communities in grassland soil play a pivotal role in organic matter decomposition and thus essential to the global ecosystem C and nutrient cycling. The composition and activities of soil microbial communities could be significantly altered under global change factors such as elevated $CO_2$ ($eCO_2$) and N deposition (eN) (Field et al. 1995, Fierer and Schimel 2003, van Groenigen et al. 2006, Blagodatskaya et al. 2010, He et al. 2010, Ramirez et al. 2010, Adair et al. 2011, Reed et al. 2011, Deng et al. 2012, He et al. 2012, Ramirez et al. 2012, Chen et al. 2014), thus potentially affect the ecosystem function of grasslands of being sink or source for atmospheric $CO_2$ under future climate scenarios. Yet, the capability of decomposing various organic compounds by soil microbiota as affected by $eCO_2$ and/or eN remain largely inferred from metagenomic analysis targeting the 16S, 28S rRNA, ITS or functional genes (He et al. 2010, Langille et al. 2013, Tu et al. 2015). However, the actual activity of microorganims can't be directly measured by such DNA-based technologies (McGrath et al. 2010, Simon and Daniel 2011).

Community-level physiological profiling (CLPP), based on the utilization pattern of various carbon sources, has been widely employed with the aim to characterize and classify the metabolic versatility of heterotrophic microbial communities in many different environments such as soils, wetlands, marine, and air environments (Grayston et al. 1998, Collins et al. 2004, Lyons et al. 2010, Wu et al. 2013, Mastrogianni et al. 2014). Biolog EcoPlate (BIOLOG Inc, Hayward, CA, USA) represents one of the most

widely used tools for evaluating CLPP of communities as well as microbial isolates. Biolog EcoPlates contain replicated wells, each with a single low molecular weight C source, a nutrient medium, and a tetrazolium dye that becomes blue in proportion to the amount of C used and can be used to identify patterns in heterotrophic microbial metabolic capabilities (Garland and Mills 1991). A total of 31 different C sources along with a blank well used as a negative control are merged on EcoPlate (Table 4.1). EcoPlate has been shown to be useful as an assay to detect and measure environmental change effects on microbial communities (Preston-Mafham et al. 2002, Gryta et al. 2014, Xiong et al. 2015). Particularly, EcoPlate has been proved to be effective in revealing fast growing, copiotrophic bacteria present in environments such as soil and lake (Kenarova et al. 2013, Lima-Bittencourt et al. 2014, Llado et al. 2016).

In this context, this work aims to assess global change factors including $eCO_2$ and eN on the C utilization capabilities of soil microbial communities by using the Biolog EcoPlate approach. Our study was conducted at the BioCON experiment site, one of the longest $CO_2$ manipulation experiments in the world by using the free-air $CO_2$ enrichment (FACE) technology (Reich et al. 2001, Reich et al. 2004).

## 4.3 Materials and Methods

### *4.3.1 Site description and sample collection*

The same samples collected from the BioCON experimental site were used. Please refer to Chapter 2 for more details.

*4.3.2 Sample preparation*

For microbial community recovery, 5 g of soil samples representing each treatment was diluted using 50 mL of sterile deionized water in a 50 ml conical centrifuge tube and agitated for 45 min at 200 rpm. Followed by 30 min settlement at 4°C. Then, the bacterial fraction in the supernatant was serially diluted ($10^{-1}$, $10^{-2}$, and $10^{-3}$) using deionized water, and the $10^{-3}$ dilution was used to inoculate the Biolog EcoPlate.

*4.3.3 EcoPlate assays*

A volume of 100 uL of each $10^{-3}$ soil dilution was loaded into each well of the EcoPlate. The plates were then incubated in a Biolog Omni Log PM System at 25°C for 156 h. The color development of each well was scanned at a wavelength of 595 nm every 15 min by a moving camera located on top of the incubation chamber. Average well color development of each plate ($AWCD_{all}$) and individual C sources on each plate ($AWCD_{sub}$) were calculated by averaging the mean difference between the optical density of all the C-source-containing wells and the control wells.

$$\mathrm{AWCD}_{all/sub} = \frac{\sum(O.D.well - O.D.neg)}{n}, \text{ where}$$

(*O.D. well – O.D. neg*) is the O.D. of C-source-containing well minus the O.D. of the negative control well, and n is the number of C sources (n = 93 in $AWCD_{all}$ and n = 3 in $AWCD_{sub}$). The area under the curves (AUC) based on AWCD values were calculated to better estimate the microbial C utilization among samples (Guckert et al. 1996). Richness (the number of oxidized C substrates), Shannon's diversity (*H'*) and Pielou's evenness (*J'*) indices were calculated using an O.D. of 0.25 as the threshold for a positive response (Garland 1996). Shannon's diversity index was calculated as: $H' = -\sum pi(\ln pi)$ where $p_i$ is the ratio of the activity on each substrate ($O.D._i$) to the sum of activities on all

substrates $\sum(O.D._i)$. Pielou's evenness was calculated as: $J' = H' / H'_{max}$ where $H'$ is the number derived from the Shannon diversity index and $H'_{max}$ is the maximum possible value of $H'$.

*4.3.4 Microbial biomass analysis by Phospholipid Fatty Acid Analysis (PLFA)*

The same soil samples used in the metabolic profiling were used to estimate microbial biomass by PLFA (Chung et al. 2007). Briefly, microbial lipids were extracted from 2g of vacuum-dried soil with a solvent system that included methanol, chloroform, and a phosphate buffer. Extracts were purified on a SPE 96-well plate containing 50 mg of silica per well (Phenomenex, Torrance, CA, USA) and dried in clean vials (60 $^{o}$C for 45 min). Each sample was dissolved with transesterification reagent (0.2 ml) and incubated at 37 ◦C for 15 min. Acetic acid (0.075 M) and chloroform (0.4 ml each) were then added. After shaking for 10 s the phases were allowed to separate. The bottom layer was removed into a clean vial, tried (60 $^{o}$C for 20 min) and dissolved in 100 µl hexane. Fatty acid methyl esters (FAMEs) were identified by an Agilent (Agilent Technologies, Wilmington, DE, USA) 6890 gas chromatograph (GC) based on their retention times and in combination with the Sherlock™ Fatty Acid ID System (MIDI, Inc., Las Vegas, USA).

*4.3.5 Statistical analysis*

Significance of differences in microbial oxidation rates among treatments was examined by two-way analysis of variance (ANOVA), with a probability defined at $P < 0.05$ or with list $P$ values. And the procedure of LSD test with a false discovery rate (fdr) adjustment was used as a post-hoc test. The structure of microbial metabolic potential was ordinated using detrended correspondence analysis (DCA). Three nonparametric tests (multiple-response permutation procedure [MRPP], permutational multivariate analysis of variance

[Adonis], and analysis of similarity [ANOSIM]) were used to evaluate dissimilarities between treatments and control samples. Mantel tests were used to examine the correlation between the microbial community structure and environmental factors (plant and soil properties). All of the above analyses were performed with R, version 2.9.1 (R Foundation for Statistical Computing, Vienna, Austria).

**Table 4.1** Biolog EcoPlate C source guild groupings by chemical structures (Weber and Legge 2009). Each of the carbon sources is replicated 3 times on the 96 well EcoPlate.

| Well number | Carbon source | Compound group |
|---|---|---|
| A1 | Water | – |
| B1 | Pyruvic acid methyl ester | Carbohydrates |
| C1 | Tween 40 | Polymers |
| D1 | Tween 80 | Polymers |
| E1 | α-Cyclodextrin | Polymers |
| F1 | Glycogen | Polymers |
| G1 | D-Cellobiose | Carbohydrates |
| H1 | α-D-Lactose | Carbohydrates |
| A2 | β-Methyl-D-glucoside | Carbohydrates |
| B2 | D-Xylose | Carbohydrates |
| C2 | i-Erythritol | Carbohydrates |
| D2 | D-Mannitol | Carbohydrates |
| E2 | *N*-Acetyl-D-glucosamine | Carbohydrates |
| F2 | D-Glucosaminic acid | Carboxylic and ketonic acids |
| G2 | Glucose-1-phosphate | Carbohydrates |
| H2 | D,L-α-Glycerol phosphate | Carbohydrates |
| A3 | D-Galactonic acid-γ-lactone | Carboxylic and ketonic acids |
| B3 | D-Galacturonic acid | Carboxylic and ketonic acids |
| C3 | 2-Hydroxybenzoic acid | Carboxylic and ketonic acids |
| D3 | 4-Hydroxybenzoic acid | Carboxylic and ketonic acids |
| E3 | γ-Hydroxybutyric acid | Carboxylic and ketonic acids |
| F3 | Itaconic acid | Carboxylic and ketonic acids |
| G3 | α-Ketobutyric acid | Carboxylic and ketonic acids |
| H3 | D-Malic acid | Carboxylic and ketonic acids |
| A4 | L-Arginine | Amino acids |
| B4 | L-Asparagine | Amino acids |
| C4 | L-Phenylalanine | Amino acids |
| D4 | L-Serine | Amino acids |
| E4 | L-Threonine | Amino acids |
| F4 | Glycyl-L-glutamic acid | Amino acids |
| G4 | Phenylethylamine | Amines/amides |
| H4 | Putrescine | Amines/amides |

## 4.4 Results

### *4.4.1 Plant and soil properties*

Hierarchical cluster analysis based on the biogeochemical properties showed that the analyzed samples were well separated into two groups owing to the distinct environmental conditions (Fig. 4.1). Specifically, Group 1 were associated with C3 grass plots and containing significantly lower total soil C (TC) and N (TN) content, aboveground biomass (AB), N content of aboveground biomass (AB-N) and root (RB-N); but higher soil temperature (Tm), plant root ingrowth biomass (RIB), root biomass (RB), C/N of aboveground biomass (AB-C/N) and root biomass (RB-C/N) comparing with Group 2 which were associated with legume plots (*t*-test, $P < 0.05$).

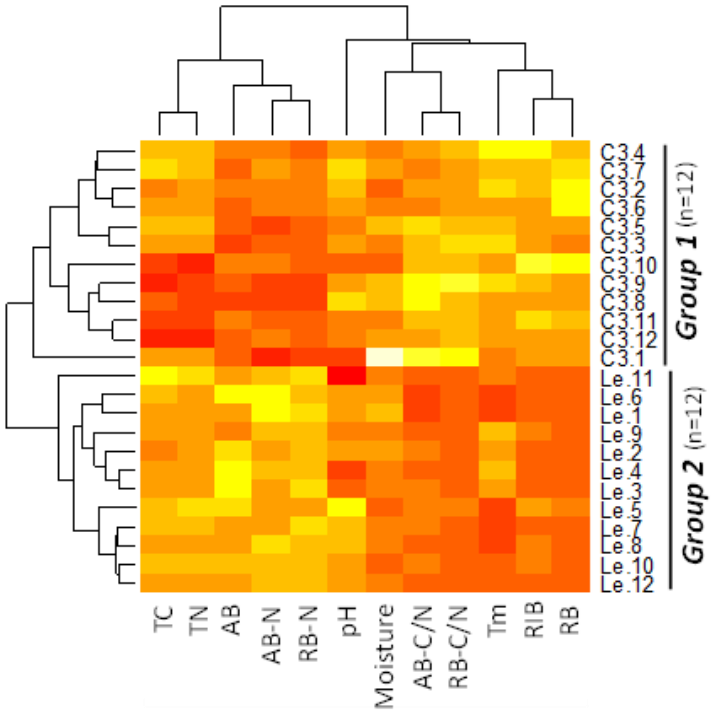



**Fig. 4.1** Hierarchical cluster analysis of environmental properties of 24 samples in both C3 grass and legume plots. The first seven columns correspond to correlations of each phylum with soil attributes including pH, moisture, temperature (Tm), total C (TC) and N (TN) content, nitrate, and ammonium. The next seven columns correspond to correlations of each phylum with plant attributes including aboveground biomass (AB), root biomass (RB) (0-20 cm, fine roots), aboveground biomass N (AB-N) and C/N (AB-C/N), root biomass N (RB-N) and C/N (RB-C/N), and root ingrowth biomass (RIB).

*4.4.2 Community C Substrate Utilization Profile*

In this study, AWCD and AUC were used to determine the utilization of C substrates by microbes, and to estimate the activity and physiological function of microbial communities (Guckert et al. 1996, Zhang et al. 2017). Oxidation of all 31 C substrates on EcoPlates after 156 h of incubation was first measured based on $AWCD_{all}$ which showed that microbial communities in legume plots had significantly higher $AWCD_{all}$ values than in C3 grass plots (Fig. 4.2A, ANOVA, $P < 0.05$). Consistently, greater AUC was observed for samples in legume than in C3 grass plots across the entire incubation period (Fig. 4.2B, ANOVA, $P = 0.017$). Similarly, for individual C substrates, significantly higher oxidation of 11 C substrates were observed by communities in legume plots compare to those in C3 grass plots, including Pyruvic acid methyl ester (Methyl pyruvate), i-Erythritol, L-Asparagine, a-Cyclodextrin, L-Threonine, Glycogen, Itaconic acid, Glycyl-L-glutamic acid, N-Acetyl-D-glucosamine, D-Cellobiose, and a-D-Lactose (ANOVA, $P < 0.001$). Together, these results indicate a greater microbial metabolic capability in the legume than C3 grass plot soils on low-molecular-weight organic substrates provided on EcoPlate.

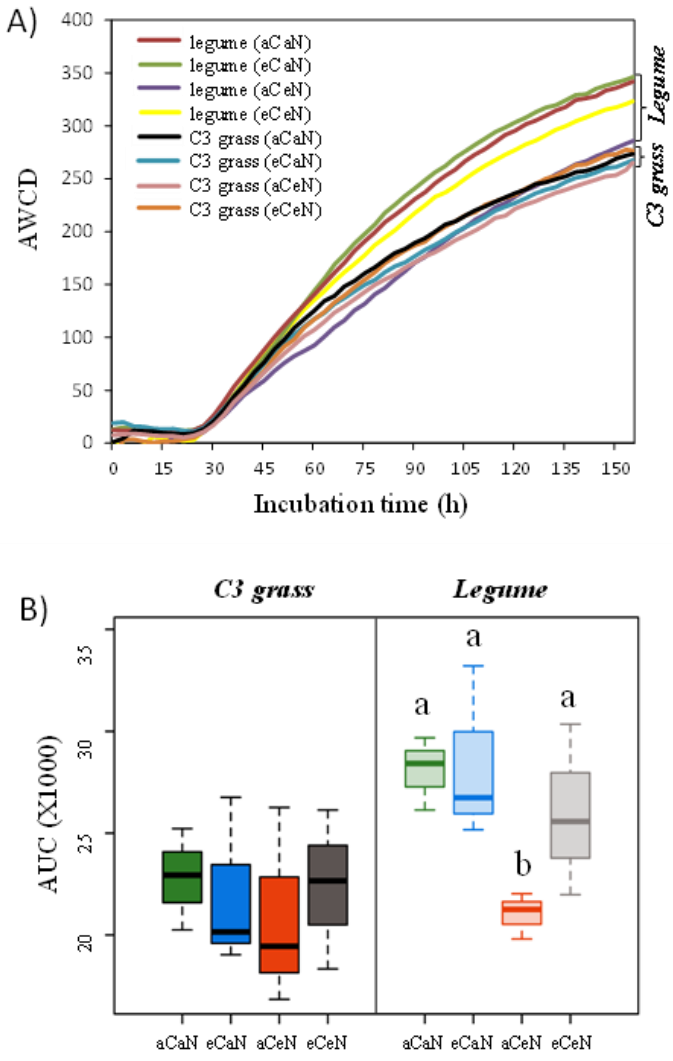
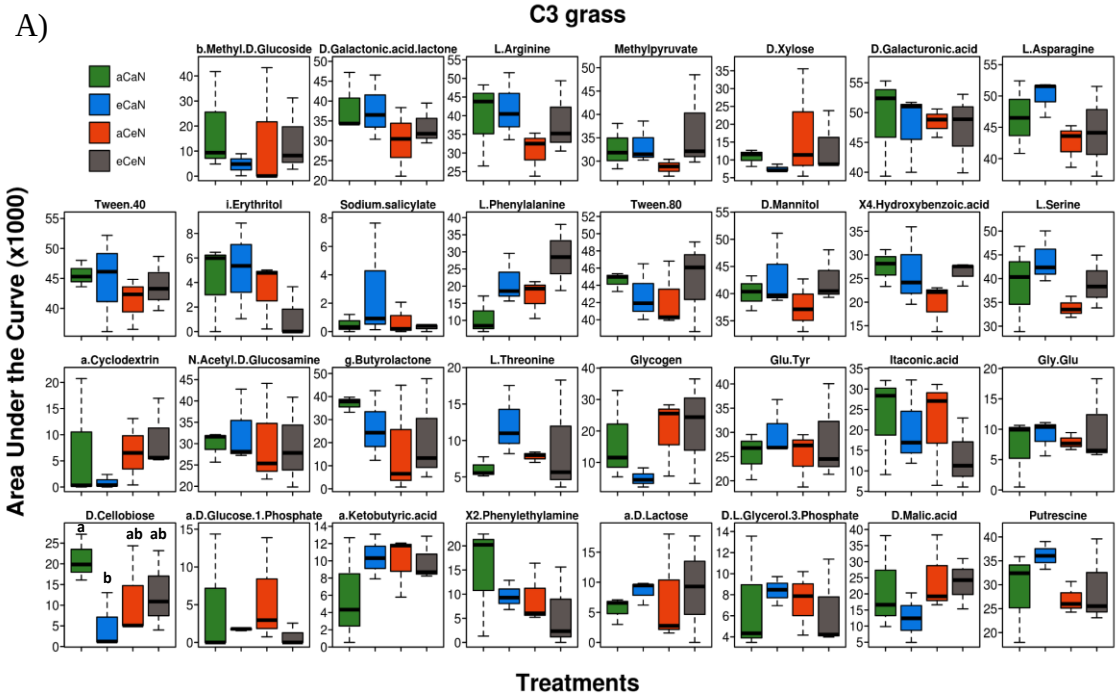



**Fig. 4.2** Microbial C substrate utilization patterns based on 156 h incubation. AWCD (A) and AUC (B) of metabolized substrates of all 31 C substrates in Biolog EcoPlates. Boxes labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

Overall microbial metabolic activities on 31 C substrates responded to experimental treatments ($eCO_2$ and eN) in the legume but not in the C3 grass plot soils. Within the legume plots, overall microbial metabolic activities significantly increased by 20.6% with $eCO_2$ in the fertilized soils, while there was no evidence for a $CO_2$ effect in

non-fertilized soils (Fig. 4.2B). In contrast, none of these effects were significant within the C3 grass plots (Fig. 4.2B). Similar to the pattern of overall C decomposition, $CO_2$ effects on individual C substrate decomposition activities occurred primarily within the legume plots, with significantly increased metabolic activities in fertilized soils but unchanged activities in unfertilized soils (e.g. L. Arginine, L. Asparagine, L. Phenylalanine, and Tween 80) (Fig. 4.3).

A)



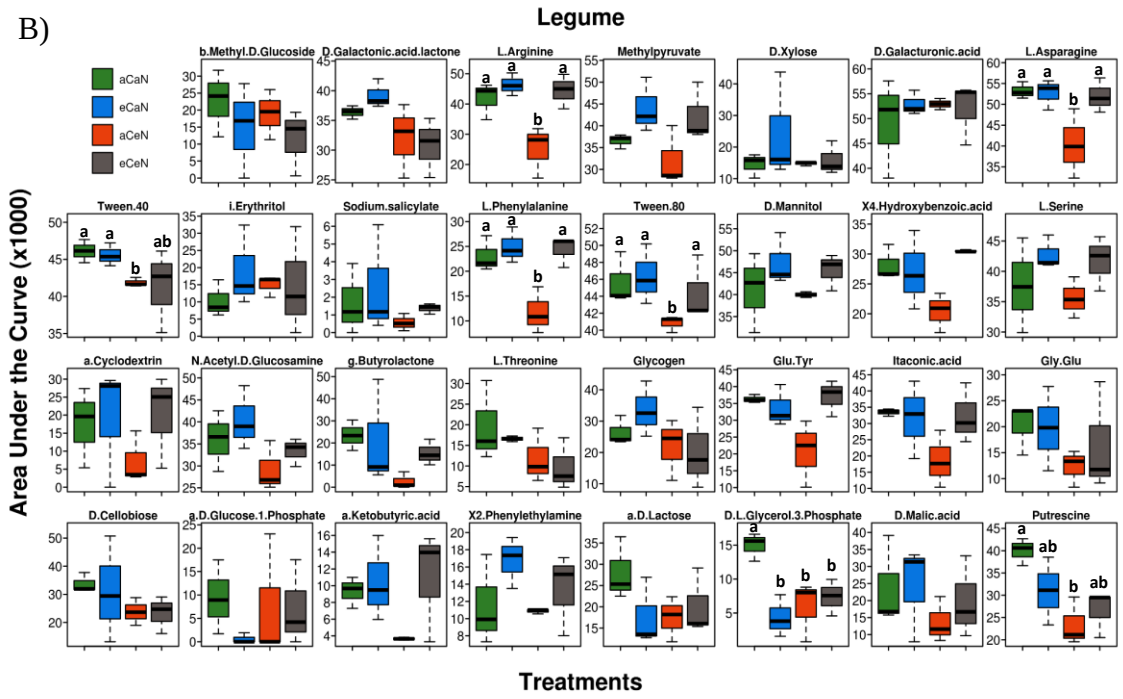



**Fig. 4.3** Oxidation of individual C substrates in the EcoPlate measured by AUC. (A) C3 grass plots. (B) Legume plots. Boxes labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

*4.4.3 Microbial catabolic diversity*

Shannon, richness and evenness indices were used to reflect the functional diversity of microbial metabolisms (Zhang et al. 2017). A significantly higher number of substrates on EcoPlate (Richness) were able to be used by communities in legume than in C3 grass plots (27 vs. 23 on average), showing a metabolic diversification in the C source oxidation between communities in two plant functional groups. Furthermore, the number of usable C substrates under $eCO_2$ was significantly lower than under $aCO_2$ (22 vs. 24 on average) in non-fertilized C3 grass plots, but no significant $CO_2$ effects were detected in fertilized C3 grass or all legume plot samples (Fig. S4.1). In addition, Shannon and Evenness indices significantly increased with $eCO_2$ in non-fertilized legume plots (Fig. S4.1).

*4.4.4 Shifts in microbial communities functional structure under treatments*

Three nonparametric tests (MRPP, Adonis, and ANOSIM) were performed by utilizing the $AWCD_{all}$ value after 156h incubation and consistently showed that the communities in C3 grass were significantly different from those in legume plot soils, while communities within each plant functional groups generally did not differ among different levels of $eCO_2$ and eN (Table 4.2). Consistently, the DCA profile illustrated that C3 grass samples were separated clearly from those of legume along DCA1, while samples of different $CO_2$ or N regimes were less distinctly separated along DCA2 (Fig. S4.2).

**Table 4.2** Significance tests of the effects of $eCO_2$ and eN on microbial metabolic diversity using three different statistical approaches*

| | | MRPP[1] | | anosim[2] | | adonis[3] | |
|---|---|---|---|---|---|---|---|
| | | *Delta* | *P* | *R* | *P* | $R^2$ | *P* |
| C3 grass *vs.* Legume | | 0.209 | < **0.001** | 0.437 | **0.002** | 0.167 | **0.001** |
| C3 grass | aCaN *vs.* eCaN | 0.215 | 0.47 | 0.037 | 0.517 | 0.207 | 0.307 |
| | aCaN *vs.* aCeN | 0.227 | 0.594 | 0.185 | 0.816 | 0.185 | 0.552 |
| | aCaN *vs.* eCeN | 0.228 | 0.802 | 0.111 | 0.892 | 0.183 | 0.607 |
| Legume | aCaN *vs.* eCaN | 0.201 | 0.282 | 0.222 | 0.406 | 0.259 | 0.097 |
| | aCaN *vs.* aCeN | 0.195 | 0.0989 | 0.852 | 0.091 | 0.348 | **0.001** |
| | aCaN *vs.* eCeN | 0.196 | 0.196 | 0.333 | 0.201 | 0.294 | 0.055 |

* Distance method: Bray-Curtis.
1. A Nonparametric approach depends on the internal variability of the data.
2. Analysis of similarities.
3. Non-parametric multivariate analysis of variance (MANOVA) with the Adonis function.

*4.4.5 Linkage between microbial metabolic potential and environmental properties*

Mantel tests were performed based on Bray–Curtis and Euclidean indices to determine if the microbial metabolic potential was associated with measured plant and soil properties (Table 4.3). In this analysis, five soil attributes including moisture, pH, total C and N content, and temperature; and seven plant attributes including root ingrowth biomass, aboveground biomass, root biomass ( 0-20 cm, fine roots), aboveground biomass N

content and C/N ratio, and root biomass N content and C/N ratio were included. Mantel test of microbial metabolic potential patterns and each plant and soil property suggested that soil total N content, aboveground and root biomass, and root biomass N content to be the major factors responsible for differences in microbial metabolic potential (Table 4.3).

**Table 4.3** The relationships of microbial metabolic potential to plant and soil properties by Mantel test

| | | *Statistic r* | *P-value* |
|---|---|---|---|
| Soil | Moisture | -0.07472 | 0.708 |
| | pH | 0.0344 | 0.308 |
| | Total C (%) | 0.1188 | 0.134 |
| | Total N (%) | 0.1768 | **0.045** |
| | Temperature ($^{o}$C) | -0.05238 | 0.727 |
| Plant | Root ingrowth biomass (g $m^{-2}$) | 0.1987 | **0.046** |
| | Aboveground biomass (g $m^{-2}$) | 0.172 | **0.027** |
| | Root biomass (g $m^{-2}$) | 0.07145 | 0.18 |
| | Root ingrowth biomass N content (%) | -0.00191 | 0.486 |
| | Aboveground biomass C/N | 0.03266 | 0.318 |
| | Root biomass N content (%) | 0.1082 | **0.049** |
| | Root biomass C/N | 0.07007 | 0.199 |

*4.4.6 Microbial biomass*

Both total microbial and bacterial biomass were significantly higher in C3 grass plots than in legume plots, whereas fungal biomass showed no difference between plant functional groups (Fig. 4.4 A). Within C3 grass plots, eN significantly increased total microbial biomass and bacterial biomass by 20.4% and 21.3%, respectively (Fig. 4.4 B). Meanwhile, total microbial and bacterial biomass under $eCO_2$ were 7.8% and 9.3% higher than under $aCO_2$, respectively, although these difference were not statistically significant ($P$ = 0.07). Within legume plots, no significant difference was observed for microbial biomass under different treatment conditions (Fig. 4.4 C). It appears that microbial

biomass was not connected to the differences in microbial metabolic potential. Instead, possible changes in community structure and/or enzymatic activities of belowground microbes may be responsible for the difference in microbial metabolic potential between plant functional groups and among treatments.

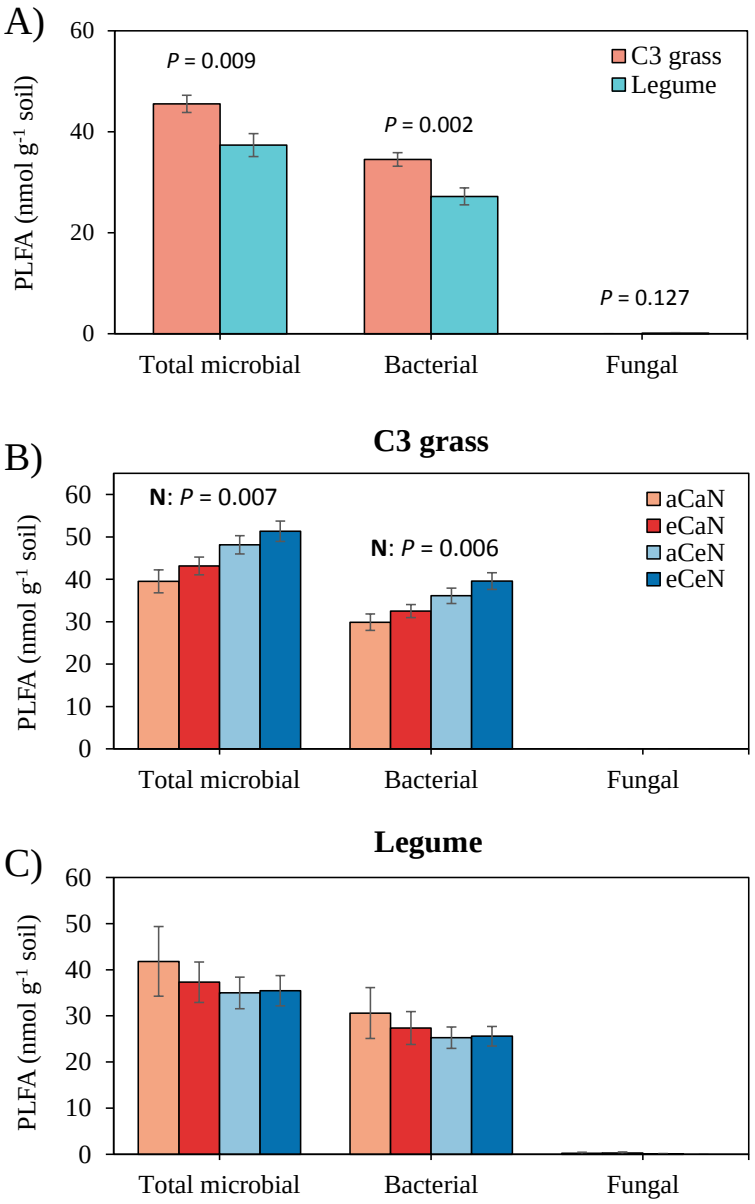



**Fig. 4.4** Microbial biomass in the C3 grass and legume plot soils (A), and the effects of $eCO_2$ and eN on microbial biomass in the C3 grass (B) and legume plots (C).Total microbial, bacterial or fungal biomass was the sum of the signature phospholipid fatty acid (PLFA). All data are presented with mean ± SE (error bars), and the significance of $eCO_2$ or eN on microbial biomass is shown by *P* values.

## 4.5 Discussion

Understanding the mechanism of environmental changes on the soil microorganisms mediated C cycling is essential for estimating the alteration of soil C pools. Analyzing the differences in microbial C utilization abilities were useful to understand functional shifts in soil microbial community (Zhang et al. 2017). In this study, AWCD and AUC of Biolog data were used to represent the utilization of different C substrates by microbes, and to estimate the activity and physiological function of microbial communities (Guckert et al. 1996), while Shannon, Richness and Evenness indices could reflect the functional diversity of microbial metabolisms (Zhang et al. 2017). Our studies indicated that between plant functional groups, microbial communities in legume plots had greater metabolic potential than in C3 grass plots for decomposing organic substrates. Specifically, compared to the C3 grass plots, microbes in legume plots can use a larger number of C sources provided on EcoPlate and with greater decomposition rates within the measured time. We also found that, within each plant functional group, $eCO_2$ and eN had moderate effects on microbial metabolic potential with a significant increase in metabolic potential with $eCO_2$ in fertilized legume plots.

Our results indicate that the community composition of soil microbes based on metabolic potential measured by EcoPlate in the C3 grass plot soils was significantly different from those in the legume plot soils by both DCA and nonparametric dissimilarity tests. The impacts of different plant functional group, plant species on soil microbial community have been widely observed (Bremer et al. 2007, Mendes et al. 2014, Ridl et al. 2016). Physiological traits differ among plant species which affect plant C uptake, release (exudation) and repartition, therefore the structure and functional potential of

microbial communities associated with different plant species tend to vary significantly (Dudley 1996, Pei et al. 2016). Owing to the symbiotic $N_2$-fixation via root symbioses, legume species can abate soil N limitation and often exhibited greater and continuous yield responses compared to non-legumes to $eCO_2$ over long period of time (Hebeisen et al. 1997, Lee et al. 2003, Rogers et al. 2006, van Groenigen et al. 2006). Relative to non-legume species (e.g. C3 grass in this study), this implies that legume-associate microbial communities may drive more organic matter (OM) input and higher N availability. Indeed, overall microbial metabolic patterns in this study were closely linked to soil total N content, aboveground and root biomass, and root biomass N content as suggested by the Mantel test.

Soil N status plays an important role in shaping microbial community composition and structure. A recent large-scale survey examined 25 grassland sites across the globe and showed that soil microbial community composition across these sites shifted in consistent ways with higher nutrients (N, P) (Leff et al. 2015). The high N condition tends to favor copiotrophic bacteria taxa and reduce the abundance of oligotrophic taxa (Leff et al. 2015). Similar results were also observed by Ramirez and colleagues who studied 28 soils from a broad range of ecosystems in North America using high-throughput pyrosequencing and showed that N addition consistently altered bacterial community composition, increasing the relative abundance of *Actinobacteria* and *Firmicutes* which are generally considered to be more copiotrophic, and decreasing relative abundance of the largely oligotrophic *Acidobacteria* phylum (Ramirez et al. 2012). In this study, total soil N content was approximately 1.5 time higher in the legume than in the C3 grass plots ($P < 0.05$), thus there might be more copiotrophic microorganisms in the legume than in

the C3 grass plots. As copiotrophic microorganism are more capable of utilizing labile organic substrates (Koch 2001, Ho et al. 2017), it may explain the overall significantly higher richness and metabolic potential of microbial communities in legume than C3 grass plots on the C sources provided on EcoPlate, all of which are labile, low molecular weight compounds (Garland 1997, Preston-Mafham et al. 2002). Knowing which types of bacteria are specialized in metabolize the labile C may be useful in the assessment of factors that control soil C balance, as microbial decomposition offsets input of plant-derived labile C (*i.e.* litter, decaying roots and root exudates). In addition, population size (biomass) was not likely contributed to the differences in metabolic potential between plant functional groups as greater total microbial and bacterial biomass were detected in the C3 grass than legume plot soils, showing an opposite trend to the microbial metabolic potential in these plots.

It has been reported that over-fertilization disrupts plant-microbe mutualisms, reduce microbial biomass and respiration (Leff et al. 2015, Klinger et al. 2016) which, therefore, may partially explain the significantly lower microbial metabolic potential in N-added legume plots than the control plots. Meanwhile, the microbial metabolic potential was significantly higher under $eCO_2$ than under $aCO_2$ in fertilized legume plots, therefore $eCO_2$ appears to counterbalance the N effects possibly through increasing plant-derived substrate availability and microorganisms capable of readily utilizing these C compounds. Our results showed minor to moderate changes in metabolic profiles of microbial communities in the C3 grass and legume plots in response to $eCO_2$ and eN, suggesting that community composition and structures may have moderately changed in response to these factors. However, it should be noted that EcoPlate contains a relatively

simple combination of C sources which has limited ability to represent and monitor the much more complexed C pool in the rhizosphere and bulk soils, and corresponding microbial metabolic potential. Such limitation may cause underestimation of possible changes in microbial community in response to $eCO_2$ and eN. Also, the Biolog technique is culture-dependent with biases towards fast-growing, easily culturable species (Smalla et al. 1998). Thus, Biolog method should not be seen as a stand-alone approach, but complemented with other approaches (e.g. classical and molecular) in the analysis of microbial communities.

# Chapter 5: Pyrosequencing analysis of *amoA* genes for soil ammonia-oxidizing archaeal communities under elevated $CO_2$ and nitrogen deposition

## 5.1 Abstract

The recent discovery of archaeal *amoA* encoding the α-subunit of ammonia monooxygenase and its widespread distribution of ammonia oxidizing archaea (AOA) in marine, terrestrial and other environments (e.g. hot spring) indicate AOA may play an important role in the global N cycle. Yet, little is known regarding the effect of elevated $CO_2$ on community diversity, composition, and structure of AOA. Furthermore, the interactive effects of multiple factors (i.e., elevated $CO_2$, soil N level, temperature) on the abundance and community structure of AOA remain to be demonstrated. In this study, the AOA community structure and diversity were investigated in a grassland ecosystem subjected to ambient CO2 ($aCO_2$, 368 ppm), elevated $CO_2$ ($eCO_2$, 560 ppm), ambient nitrogen deposition (aN) or elevated nitrogen deposition (eN) treatments for 12-year, using barcoded 454 pyrosequencing coupled with GeoChip functional gene array targeting the archaeal *amoA* gene. Elevated $CO_2$ and N deposition alone and combined did not significantly alter the abundance of AOA. A total of 87 *amoA* OTUs (95% identity cutoff) were generated from 26,211 qualified reads. The diversity of AOA communities measured by OTU richness (Chao1), Pielou evenness, Shannon and phylogenetic hill number was significantly reduced by long-term elevated $CO_2$ but the $CO_2$ effects were confined within ambient N condition. PCoA, βMNTD and non-parametric dissimilarity tests revealed significant $CO_2$ effects on the community structure of AOA regardless of N deposition, but no effect of N deposition was observed. The relative abundance of

several AOA taxa were significantly correlated with plant root biomass, proportional soil moisture, and pH. In addition, significant positive correlations between several AOA taxa and soil nitrification rate were observed, indicating soil AOA may play a role in the nitrification process in grassland soil. These results are important in furthering our understanding of the global change impacts on AOA community structure in the long term.

**Keywords: *amoA*; ammonia oxidizing archaea; AOA; community structure; elevated $CO_2$, N deposition**

## 5.2 Introduction

Ammonia oxidation is the first as well as rate-limiting step in nitrification, converting ammonia to nitrite and then nitrate. It is of great importance to the global nitrogen cycle, economically of paramount importance to agricultural production, and contributes to ground water pollution and greenhouse gas emission ($N_2O$) (Stephen et al. 1998). Catalyzed by the enzyme ammonia monooxygenase, the ability to oxidize ammonia was originally found in a few groups bacteria phylogenetically associated to the β- and γ-*Proteobacteria*. For the past decade, metagenomic approaches and following lab incubation efforts (Treusch et al. 2004, Schleper et al. 2005, Leininger et al. 2006) have revealed the existence of unique ammonia monooxygenase α-subunit (*amoA*) genes derived from archaea (Treusch et al. 2004, Venter et al. 2004, Francis et al. 2005). *amoA-based* phylogenetic analyses have demonstrated that AOA are widely distributed in various environments and often outnumber their bacterial counterparts by orders of magnitudes (Beman and Francis 2006, Leininger et al. 2006, Di et al. 2009). Also, community composition and abundance of AOA have been shown to strongly correlate to soil nitrification potential (Gubry-Rangin et al. 2010, Radax et al. 2012, Xue et al. 2016). These results indicate that that AOA community may be actively involved in and play a more important role than these ammonia oxidizing bacteria (AOB) in nitrogen cycling.

AOA are phylogenetically associated to a newly defined archaeal phylum *Thaumarchaeota* and can be further classified into sub-clusters including *Nitrososphaera, Nitrosopumilus, Nitrosotalea, Nitrosocaldus,* and *Nitrososphaera* sister clusters (Spang et al. 2010, Pester et al. 2011, Pester et al. 2012). Insights into the diversity, structure, and

distribution of AOA communities have been gained by analyzing the archaeal-*amoA* gene family in various environments. *Nitrososphaera* cluster (also called group I.1b) often dominant the AOA community in soils, with the exception of *Nitrosotalea* cluster dominating low pH soils. AOA related to the *Nitrosopumilus* cluster (also called group I.1a) has been shown to be abundant in marine, freshwater, and sediments but absent or represent minor populations in soils (Pester et al. 2012). In addition, *Nitrosocaldus* cluster mainly consists of sequences from hot spring (Pester et al. 2012). Multiple environmental factors such as ammonium concentration, organic C levels, pH, moisture, temperature, and salinity have been documented to influence the community diversity and structure of AOA (Erguder et al. 2009, Biller et al. 2012, Hatzenpichler 2012, Cao et al. 2013). For example, *amoA*-based sequencing analyses targeting agriculture soil showed that pH was a major factor shaping AOA community composition and structure, and different AOA phylotypes are selected in soils of different pH with dominant populations *Nitrosopumilus* and *Nitrososphaera* cluster being negative and positive correlations with soil pH, respectively (Shen et al. 2012). In pristine forest soil, *amoA*-based analysis revealed a decreasing abundance of AOA with increasing soil moisture, suggesting that AOA may sensitive to anaerobic conditions (Szukics et al. 2010).

Elevated $CO_2$ stimulates plant net primary productivity and increases C/N ratio of plant litter (Cotrufo et al. 1998, Norby et al. 2001, He et al. 2010) which could result in slower carbon degradation and N mineralization. Also, soil pH and moisture have been shown to increase in response to elevated $CO_2$ (Reich et al. 2006). As a result, the subsequent AOA community and nitrification process may be strongly affected by elevated atmospheric $CO_2$. Yet, the response of AOA diversity and structure to elevated

$CO_2$ are poorly assessed, particularly for AOA communities in grassland, one of Earth's largest ecosystems. A previous GeoChip survey showed increased *amoA* abundance in response to elevated $CO_2$ in the BioCON grassland experiment, however, the phylogenetic association (either AOA or AOB) of these *amoA* probes was not clear (He et al. 2010). Also, limited by the relatively small *amoA* probe number designed on GeoChip, in-depth analyses of diversity and structure changes of AOA community in response to elevated $CO_2$ were not performed.

Natural ecosystems under long-term elevated $CO_2$ concentration are subjected to progressive N limitation due to the stimulated plant growth and immobilization of N compounds in biomass (Hu et al. 2001, Luo et al. 2004, Finzi et al. 2006, Tilman et al. 2006). Inorganic N fertilization could support long-term ecosystem responses to elevated $CO_2$, however, the utilization efficiency of inorganic nitrogen fertilizer largely depends on AOA (Stark et al. 2007). Previous study in an alkaline sandy loam (pH 8.3-8.7) using DGGE revealed no significant change of AOA community after 17 years of fertilization practices using a combination of fertilizer N, P and K (Shen et al. 2008). In contrast, it has been suggested that AOA are favored in ammonia-limited oligotrophic environments, indicating that N fertilization may inhibit their abundance (Erguder et al. 2009, Jia and Conrad 2009, Di et al. 2010). Consistently, long-term fertilization-induced decrease in pH and increase in nutrient supply (e.g. total N and SOM) have been shown to significantly shift AOA community and enzyme activities (Xue et al. 2016). It is of crucial interest to understand how the belowground AOA communities respond to the interactive effects of elevated $CO_2$ and N deposition.

The objective of the present study was to quantitatively assess the response of soil AOA to elevated $CO_2$ and/or N deposition using 454 pyrosequencing of archaeal-*amoA* gene amplicons coupled with GeoChip functional gene array data, and to determine the diversity and structure of soil AOA in the BioCON experimental site after 12-years of treatments (Reich et al. 2001). The following hypotheses were tested: (1) Higher soil moisture at elevated $CO_2$ would reduce *amoA* gene relative abundance as previously reported;(2) Greater plant-derived organic matter input under elevated $CO_2$ and/or N addition may stimulate soil microorganism growth and intensify competition for limiting nutrients, thus decrease AOA diversity. This study provides valuable insights into our understanding of microbial ecology of AOA in soil.

## 5.3 Materials and Methods

### *5.3.1 Site description and sample collection*

A total of 20 bulk soil samples were taken in June 2009 from the BioCON 4 plant species plots under ambient and elevated $CO_2$ conditions for microbial community analysis. Same samples collecting procedure was used as described in Chapter 2.

In this study, the 4 plant species in each plot were randomly chosen from a pool of 16 species including, *Agropyron repens, Bromus inermis, Koeleria cristata, Poa pratensis, Andropogon gerardii, Bouteloua gracilis, Schizachyrium scoparium, Sorghastrum nutans, Amorpha canescens, Lespedeza capitata, Lupinus perennis, Petalostemum villosum, Achillea millefolium, Anemone cylindrica, Asclepias tuberosa,* and *Solidago rigida*. All of these plants are native or naturalized to the Cedar Creek

Ecosystem Science Reserve, and there was no specific functional classification of these plants in this study.

*5.3.2 DNA extraction, purification, and quantification*

Soil DNA was extracted by freeze-grinding mechanical lysis as described previously (Zhou et al. 1996) and was purified using a low melting agarose gel followed by phenol extraction for all 20 soil samples collected. DNA quality was assessed by the ratios of 260/280 nm, and 260/230 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and final soil DNA concentrations were quantified with PicoGreen (Ahn et al. 1996) using a FLUOstar Optima (BMG Labtech, Jena, Germany).

*5.3.3 PCR amplification and 454 pyrosequencing*

Amplification was performed using the Arch-amoAF and Arch-amoAR (Arch-amoAF: STAATGGTCTGGCTTAGACG, and Arch-amoAR: GCGGCCATCCATCTGTATGT) with 8 replicates each, whose products are expected to be approximately 635-bp (Francis et al. 2005). A unique 8-mer barcode was added to each sample at the 5'-end of the forward primer. The barcode-primers were synthesized by Invitrogen (Carlsbad, CA) and used for the generation of PCR amplicons. Octuplicate 25 µl PCR reactions were performed as follows: 2.5 µl Promega GoTaq buffer, 0.25 µl GoTaq DNA polymerase, 2.5 µl Roche 25 mM $MgCl_2$, 2 µl Invitrogen 10 mM dNTP mix, 0.5 µl of each primer (10 pmol $\mu l^{-1}$), 0.25 µl New England BioLabs 10 mg $ml^{-1}$BSA, 1 µl of 25 ng $\mu l^{-1}$ template, and 15.5 µl $H_2O$. Cycling conditions were an initial denaturation at 95°C for 4 min, 30 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 45 s, and a final extension at 72°C for 15 min. Replicated PCR products were pooled andthen subjected to gel purification using

the Qiagen Gel Purification Kit. After adapter ligation, amplicons were sequenced on an FLX 454 system (454 Life Sciences, Branford, CT) by Macrogen (Seoul, South Korea) using Lib-L kits and processed using the shotgun protocol.

*5.3.4 Data analysis*

Raw pyrosequencing reads were extracted from the *sff* file using the *sffinfo* tool from Roche 454. Two files, a *fasta* file containing the sequence and a *qual* file containing the quality information, were generated and then converted into a *fastq* file using the python script "faqual2fastq2.py" that comes with the UPARSE pipeline (Edgar 2013). The quality filtering, chimera removal, and OTU clustering were carried out using the UPARSE pipeline (Edgar 2013). Only the reads with perfectly matched barcodes and a maximum of 2 primer mismatches were kept for further analysis. Barcodes and primers were deleted from reads. The remaining reads were then truncated to 450 bp, and reads with expected errors > 0.5 were discarded. The program FrameBot (Wang et al. 2013) was used to correct potential frame shifts caused by sequencing errors and only reads whose translated proteins got mapped to reference archaeal-*amoA* protein sequences with > 30% identity were kept. The reads were then dereplicated, sorted, and clustered into candidate OTUs with an identity cutoff of 0.95, which is near the average nucleotide identity that approximately corresponds to the species cutoff of 16S rRNA genes (Konstantinidis and Tiedje 2005). Chimeric OTUs were then identified and removed by searching against the *amoA* reference sequences maintained by IEG galaxy pipeline (http://www.drive5.com/uchime/). Finally, qualified reads were mapped to OTU reference sequences for relative abundance calculation.

Taxonomic assignment for archaeal-*amoA* OTUs was carried out by searching OTU representative sequences against reference archaeal-*amoA* sequences with known taxonomic information. A minimum recalculated global identity cutoff of 80% was used to filter BLAST results. A lowest common ancestor algorithm was applied for taxonomic assignment by using CREST v1.0 software (Lanzen et al. 2012) with default parameters (minimum bit-score = 155, LCA range = 2%). Taxonomic information at genus level or higher was assigned. For phylogenetic analysis, representative OTU sequences were aligned by the MUSCLE program (Edgar 2004), and a phylogenetic tree was built by FASTTREE (Price et al. 2009). Significance tests for different taxonomic groups and OTUs were performed by response ratio analysis (Hedges et al. 1999) at 95% confidence interval level. Species richness, evenness, and diversity indices were calculated by the Mothur package (Schloss et al. 2009).

## 5.4 Results

### *5.4.1 Effects of $eCO_2$ and eN on plant biomass, soil N, and amoA gene abundance*

The plant biomass (aboveground and root) and soil nitrogen levels ($NO_3^-$ and $NH_4^+$) were collected and analyzed. Nitrogen addition significantly increased root biomass production under both $aCO_2$ and $eCO_2$ (+66.7%, $P = 0.01$), but no effect of elevated $CO_2$ on plant biomass was observed (Fig. 5.1A). Elevated $CO_2$ significantly increased deep-layer soil moisture content (42-59 cm and 83-100 cm) in unfertilized plots ($P < 0.001$, Fig. 5.1C) and increased soil pH in fertilized plots ($P = 0.02$, Fig. 5.1D). Soil nitrification was calculated based on changes in $NO_3^-$ and $NH_4^+$ concentrations measured through one

month *in situ* incubation, which showed no significant response to elevated $CO_2$ or N addition (Fig. 5.1E).

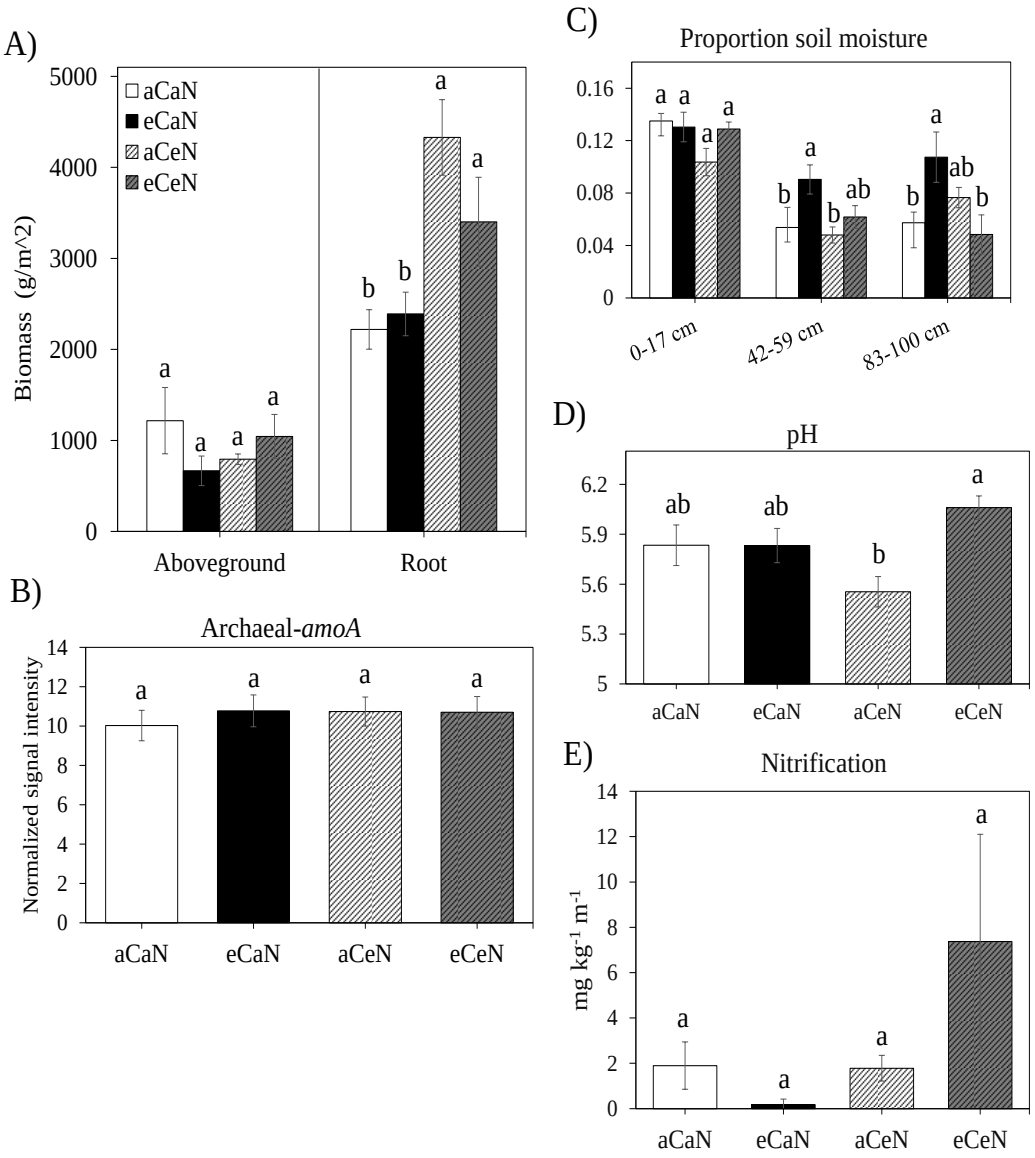



**Fig. 5.1** Effects of $eCO_2$ and eN on plant biomass (A), *amoA* gene abundance (B), proportional soil moisture (C), soil pH (D), and soil nitrification rate (E). Both aboveground and root biomass were averaged from 3 years at the time of sampling, i.e. 2007-2009. Soil nitrification was calculated by $NO_3^-$ and $NH_4^+$ concentrations measured using a semi-open core, one-month *in situ* incubation approach. The abundance of *amoA* genes was obtained from Geochip datasets by extracting probes mapped to archaeal-*amoA* genes. Statistical testing was performed by ANOVA followed by LSD with holm adjustment. aCaN: ambient $CO_2$, no N addition; eCaN: elevated $CO_2$, no N addition; aCeN: ambient $CO_2$, N added; eCeN: elevated $CO_2$, N added.

The abundance of *amoA* genes was assessed by extracting archaeal-*amoA* probes from GeoChip microarray profiling datasets, which the signal intensity of probes are proportional to the corresponding gene abundances (Wu et al. 2001). A total of 43 probes associated to archaeal-*amoA* on GeoChip were detected, and there was no significant difference for the abundance of *amoA* genes under different $CO_2$ and N levels (Fig. 5.1B). This suggested that neither $eCO_2$ nor eN had a significant influence on the abundance of archaeal-*amoA* genes in soils of this grassland ecosystem.

*5.4.2 Sequencing data summary*

Using 454 pyrosequencing, a total of 47,659 raw reverse reads targeting archaeal-*amoA* gene amplicons were obtained for the 20 samples, among which 28,585 were assigned to corresponding barcodes. After quality trimming, frameshift correction and chimera removal, 26,211 reads were clustered into 87 *amoA* OTUs at 95% identity cutoff, of which 64 (a total of 6,153 reads with 1,683; 1,781; 1,037 and 1,652 from aCaN, eCaN, aCeN and eCeN, respectively) were non-singleton OTUs. The number of sequences in each sample ranged from 115 to 546 (308 on average), resulting in 10 to 33 OTUs per sample. A random re-sampling effort of 115 reads per sample was made for further statistical analysis.

*5.4.3 Significant $eCO_2$ and eN effects on overall AOA diversity and structure*

To analyze the Archaeal-*amoA* community diversity and their responses to elevated $CO_2$ at different soil N condition, the OTU richness (Chao1), evenness, taxonomic and phylogenetic diversity indices were calculated (Table 5.1). A total of 40, 28, 39, and 30 OTUs were identified for samples under aCaN, eCaN, aCeN and eCeN, respectively, with the current sequencing effort. Elevated $CO_2$ significantly reduced the evenness and

taxonomic diversity of overall *amoA* community in unfertilized plots ($P < 0.05$), except OTU richness which showed no effect of $CO_2$ or N. Consistently, the phylogenetic diversity of *amoA* gene, which also considers the phylogenetic relationship among OTUs was significantly lower at elevated than ambient CO2 condition in unfertilized plot soils ($P < 0.05$, Table 5.1). No significant differences between fertilized and unfertilized samples were observed for OTU richness, evenness, and diversity at both taxonomic and phylogenetic levels were observed (Table 5.1). All these results suggested that the diversity of *amoA*-community was significantly altered by long-term elevated $CO_2$ but the $CO_2$ effects were confined within ambient N condition in this grassland ecosystem.

**Table 5.1** The diversity of *amoA* genes in the grassland ecosystem under different $CO_2$ and N conditions. Shown are mean values (n=5) ± standard errors. Values labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment.

| Treatment | Chao1 richness | Shannon | Pielou evenness | Phylogenetic diversity (hill_0) |
|---|---|---|---|---|
| aCaN | 20.80 ± 3.22 a | 2.14 ± 0.24 a | 0.76 ± 0.06 a | 17.00 ± 2.30 a |
| eCaN | 16.92 ± 5.94 a | 1.26 ± 0.12 b | 0.55 ± 0.04 b | 9.80 ± 0.86 b |
| aCeN | 20.89 ± 5.72 a | 1.66 ± 0.29 ab | 0.62 ± 0.06 ab | 14.60 ± 2.80 ab |
| eCeN | 17.47 ± 1.42 a | 1.57 ± 0.15 ab | 0.61 ± 0.05 ab | 13.20 ± 0.80 ab |

Long-term elevated $CO_2$ fertilization also significantly alter the overall archaeal *amoA*-community structure in the grassland soil ecosystem with greater influence in low than high N levels (Fig. S5.1). The overall community structural differences among all samples were assessed by both weighted (with relative abundance data)/unweighted (with richness data) UniFrac PCoA and βMNTD analyses. A well separation of elevated $CO_2$ samples from ambient $CO_2$ samples in unfertilized plots were observed by all the analyses (Fig. S5.1). A separation trend of elevated $CO_2$ samples in fertilized plots from ambient $CO_2$ samples in unfertilized plots could be observed with some overlaps (Fig. S5.1).

However, there was no clear separation for elevated $CO_2$ samples from ambient $CO_2$ samples in fertilized plots (Fig. S5.1). Further dissimilarity analysis also suggested that the overall community structure between ambient $CO_2$ and elevated $CO_2$ samples in unfertilized plots was significantly different (ADONIS: $F = 4.920$, $P = 0.015$; ANOSIM: $R = 0.472$, $P = 0$ 0.009; MRPP: $\delta = 0.421$, $P = 0.007$). And samples between ambient $CO_2$ and elevated $CO_2$ samples in fertilized plots were significantly different by ANOSIM ($R = 0.472$, $P = 0.009$) and (ADONIS: $F = 3.359$, $P = 0.041$), and marginally significant by MRPP ($\delta = 0.439$, $P = 0.054$).

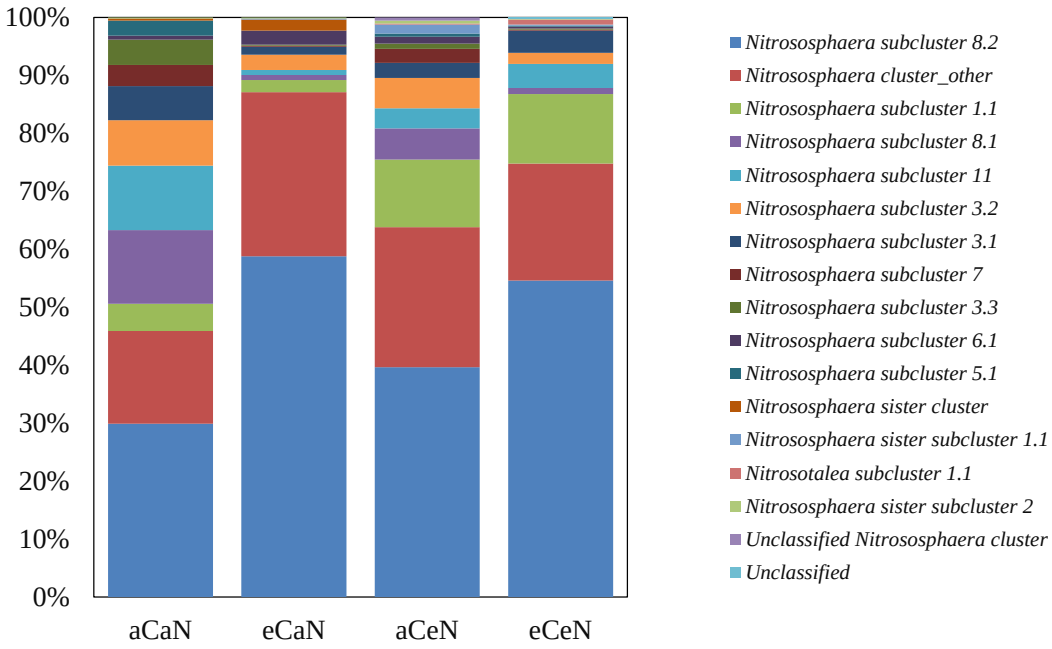
*5.4.4 The taxonomic and phylogenetic composition of archaeal-amoA genes*

Compare to the 16S rRNA and bacterial-*amoA* genes, reference sequences for archaeal-*amoA* genes from cultivated microbial strains/species are still very limited, making it difficult to classify archaeal-*amoA* sequences into their taxonomic groups, especially at the lower taxonomic levels. By assigning taxonomic information to OTUs having a minimum of 95% sequence similarity with references in the *amoA* database, only 63 OTUs could be assigned to known taxonomic groups including *Nitrososphaera* subclusters 1.1, 3.1, 3.2, 3.3, 5.1, 6.1, 7, 8.1, 8.2, and 11; *Nitrososphaera* sister subcluster 1.1 and 2; and *Nitrosotalea* subcluster 1.1 (Fig. 5.2A).

The AOA community was dominated by *Nitrososphaera* cluster (98.4%) across all $CO_2$ and N levels, among which *Nitrososphaera* subcluster 8.2, an unclassified *Nitrososphaera* subcluster, subcluster 1.1 and 8.1 were most dominant, accounting for 45.7%, 22.3%, 7.6%, and 5.0% of the entire AOA community, respectively. Whereas, clusters related to *Nitrososphaera* sister and *Nitrosotalea* only accounted for 1.6% of the entire AOA community in this study. Elevated $CO_2$ significantly reduced the relative

abundances of *Nitrososphaera* subclusters 5.1, 8.1, and 11 in unfertilized plots, but increased *Nitrososphaera* subcluster 8.2 in fertilized plots ($P < 0.05$, Fig. 5.2B); whereas N fertilization significantly increased the relative abundance of *Nitrososphaera* subcluster 1.1 at elevated $CO_2$, but *Nitrososphaera* sister subcluster 1.1 at ambient $CO_2$ ($P < 0.05$, Fig. 5.2B). At OTU level, only 8 OTUs were significantly affected by elevated $CO_2$ and/or N deposition, which however quantitatively accounted for 57.5% of the AOA community ($P < 0.05$). All the 8 OTUs were assigned to *Nitrososphaera* cluster. The most dominant OTU (denovo642), belongs to *Nitrososphaera* subcluster 8.2 and accounted for 47.2% of the archaeal-*amoA* community, was more abundant under elevated than ambient $CO_2$ in unfertilized plots ($P < 0.05$, Fig. S5.2). Less dominant OTUs including denovo301 (0.8%), denovo33 (4.7%) and denovo840 (0.7%) had significant lower relative abundances with elevated $CO_2$ in unfertilized plots ($P < 0.05$, Fig. S5.2). No significant changes of relative abundances for any *amoA* groups at the OTU level were observed between ambient $CO_2$ and elevated $CO_2$ in fertilized plots.

A)




B)

| | $CO_2$ | | N | | $CO_2$*N | | LSD | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *F* | *p* | *F* | *p* | *F* | *p* | aCaN | eCaN | aCeN | eCeN |
| *Nitrososphaera* subcluster 1.1 | 0.12 | 0.74 | 6.58 | **0.02** | 0.20 | 0.66 | ab | b | ab | a |
| *Nitrososphaera* subcluster 5.1 | 6.08 | **0.03** | 2.55 | 0.13 | 3.56 | 0.08 | a | b | ab | ab |
| *Nitrososphaera* subcluster 7 | 5.92 | **0.03** | 0.27 | 0.61 | 0.27 | 0.61 | a | a | a | a |
| *Nitrososphaera* subcluster 8.1 | 17.18 | **0.00** | 3.34 | 0.09 | 3.67 | 0.07 | a | b | ab | b |
| *Nitrososphaera* subcluster 8.2 | 5.96 | **0.03** | 0.10 | 0.76 | 0.60 | 0.45 | b | a | ab | ab |
| *Nitrososphaera* subcluster 11 | 3.74 | 0.07 | 0.77 | 0.39 | 4.90 | **0.04** | a | b | b | ab |
| *Nitrososphaera* sister subcluster 1.1 | 3.16 | 0.09 | 5.23 | **0.04** | 5.23 | **0.04** | b | ab | a | ab |

**Fig. 5.2** Phylogenetic composition of archaeal-*amoA* gene among different $CO_2$ and N conditions (A) and significantly changed AOA groups in response to $CO_2$ and N (B). Variations between different treatments were tested by ANOVA. Different letters denote significant differences among treatments from least-significant-difference (LSD) tests with holm adjustment. Only significant changed AOA groups were listed.

### *5.4.5 Linkage between archaeal amoA gene and environmental factors and soil nitrification rate*

To examine the linkage between detected individual archaeal-amoA taxa and environmental factors, Spearman's rank correlation coefficient was performed. Changes in taxa were found to closely correlate to several of the selected environment properties including aboveground and root plant biomass, soil pH and proportional moisture (Table

S5.1). Root biomass significantly correlated with 7 out of the 16 AOA phylogenetic groups including *Nitrososphaera* subclusters 1.1, 6.1, 8.2, 11 and three *Nitrososphaera* sister clusters (Table S5.1, $P < 0.1$). Soil moisture, particularly at the depth of 42-59 cm, was significantly correlated with 5 AOA groups including *Nitrososphaera* subclusters 1.1, 3.2, 5.1, 6.1 and *Nitrososphaera* sister cluster (Table S5.1, $P < 0.1$).

Linkage between soil nitrification rate and individual archaeal-*amoA* phylogenetic groups were further analyzed based on Spearman's rank correlation coefficient (Table 5.2). A total of 4 AOA taxa including *Nitrososphaera* subclusters 8.2, 6.1, 3.1 and 3.2 were positively correlated with soil nitrification rate ($P < 0.05$), indicating soil ammonia oxidizing archaea were actively involved in the nitrification process in grassland soil.

**5.5 Discussion**

Given the numerically abundant and ubiquitous existence, understanding the diversity, composition, and structure of AOA communities is essential for predicting N dynamics in ecosystems. In this study, we used 454 pyrosequencing approach coupled with Geochip functional gene array to analyze AOA communities in grassland soils subjected to 12-year elevated $CO_2$ and N deposition. Our results showed that long-term $eCO_2$, N deposition, and their interactive effects did not change the abundance of archaeal-*amoA* genes, but significantly change the overall *amoA* diversity and structure of soil AOA communities. Environmental factors including plant root biomass, soil moisture and pH were significantly correlated with the abundances of several archaeal-*amoA* taxa. This

study provides novel insights into our understanding microbial ecology of AOA communities in grassland ecosystems.

The first objective of this study was to determine whether the abundance of AOA community will be altered in response to long-term elevated $CO_2$ and N deposition in this grassland ecosystem. Based on the signal intensity of archaeal-*amoA* probes detected by GeoChip, no significant differences in AOA abundance were observed under different $CO_2$ and N conditions. This result was in accordance with previous reports showing that inorganic N fertilization significantly increases the abundance of soil ammonia oxidizing bacteria (AOB) but not AOA (Shen et al. 2008, Di et al. 2009). Contrarily, negative effects of $NO_3^-$ by fertilization on AOA community were also reported (Ying et al. 2017). It should be noted that relatively small N amendment level (4 g N $m^{-2}$ $yr^{-1}$) was carried out in this experiment, which is less than the N addition in several other studies (up to 56 g N $m^{-2}$ $yr^{-1}$) to simulate agricultural N fertilization (Zanetti et al. 1997, Reich et al. 2001). As a result, no significant differences in soil N content or pH were observed between unfertilized and fertilized soils in this study ($P > 0.05$).

Enhanced plant-derived organic matter input under elevated $CO_2$ was commonly found to stimulate belowground microbial biomass and alter community structure (He et al. 2010). However, changes in the AOA community abundance in response to the surplus of organic matter input is still debatable. Studies on AOA representatives *N. maritimus* and *N. yellowstonii* showed slow, or halted growth in the presence of additional organic substrates even at very low concentrations, suggesting a strong inhibition for AOA by organic matter (Falkowski et al. 2008). Similarly, a negative linear relationship between the $\delta^{13}C$ and archaeal-*amoA* gene was observed in the Duke Forest FACE experiment,

indicating a negative effect of elevated $CO_2$ on the growth ammonia oxidizing archaea (Long et al. 2012). However, other evidence had demonstrated that labile organic matter may have a positive effect on the growth of AOA (Stopnisek et al. 2010). Inconsistencies in these observations may relate to the potential ability of AOA to use both inorganic and organic material as C sources (Hallam et al. 2006, Zhang et al. 2010). Meanwhile, elevated $CO_2$ always increase soil moisture by increasing plant water using efficiency (Reich et al. 2006), an effect that observed in the unfertilized plots as well in this study. Studies on *amoA* gene in pristine forest soil revealed a decreasing abundance of AOA with increasing soil moisture, suggesting that AOA may sensitive to anaerobic conditions (Szukics et al. 2010). Together, the unchanged AOA abundance in response to elevated $CO_2$ in this study may be an effect of those mechanisms canceling each other. In addition, it should be noted that less than 100 archaeal-*amoA* probes were detected using GeoChip which limits its ability in quantifying the abundance of AOA in this study. Further studies such as quantitative PCR targeting the *amoA* genes are needed to confirm these results.

The results of PCoA, βMNTD and non-parametric dissimilarity tests generally agreed with each other showing that elevated $CO_2$ significantly influenced the diversity and structure of AOA community in this grassland ecosystem rather than N deposition. Long-term elevated $CO_2$ significantly decreased AOA community richness, taxonomic and phylogenetic diversity. Similar results were reported by previous studies at this site showing significantly decreased overall soil microbial richness, although no differences in overall functional diversity were observed in response to elevated $CO_2$ (He et al. 2010, He et al. 2012). However, an increase in overall functional community diversity was observed in response to elevated $CO_2$ (Chapter 3). These findings suggest the responses

of community diversity of different microbial phylogenetic and functional groups to global change factors are diverse. Meanwhile, varied responses of different taxonomic and phylogenetic groups of AOA to elevated $CO_2$ and N deposition were observed, contributing to the significant changes of the AOA community structure in response to elevated $CO_2$ and N deposition. Similarly, markedly different phylogenetic/functional structure of overall microbial community between ambient and elevated $CO_2$ were widely observed at BioCON (He et al. 2010, Deng et al. 2012, Xu et al. 2013, He et al. 2014, Tu et al. 2015). Our results also suggested that N deposition mitigated the effects of elevated $CO_2$ on the archaeal *amoA*-community. It appears that N deposition negated the elevated $CO_2$ effects, resulting in weaker $CO_2$ effects on AOA community diversity and structure at enriched than ambient N conditions. This may partially relate to the unresponsiveness of plant and soil factors to the combined effect of elevated $CO_2$ and N deposition. As community composition and structure of AOA correlate to soil nitrification potential (Gubry-Rangin et al. 2010, Radax et al. 2012, Xue et al. 2016), AOA community diversity and structure shifts under elevated $CO_2$ have the potential to alter N cycling in grassland soil.

AOA communities in this study were predominantly composed of *Nitrososphaera*, a finding consistent with other studies that have shown the dominance of this group of AOA in soils (Wessen et al. 2011, Jiang et al. 2014). In contrast, the *Nitrosotalea*-associate AOA have been observed mainly in acidic environments (Lehtovirta-Morley et al. 2011, Pester et al. 2012). Thus, it is not surprising for the presence of these groups in the slightly acidic BioCON soil, although their relative abundance were low (pH 5.3-6.3). In addition, studies on the only *Nitrososphaera* sister

cluster isolate '*Ca.* N. franklandus' revealed high ammonia tolerance and suggests potential contributions to nitrification in fertilized soils for this group of AOA (Lehtovirta-Morley et al. 2016). Consistently, significant N effect on the relative abundance of *Nitrososphaera* sister subcluster 1.1 was observed in our study where N deposition significantly increased their abundance.

Unlike AOB who rely on Calvin-Benson-Bassham (Calvin cycle) to fix carbon (Kowalchuk and Stephen 2001), AOA seems developed different carbon fixation strategies. Both autotrophic and heterotrophic life strategies were suggested through lab incubation and molecular analysis targeting AOA isolates (Ingalls et al. 2006, Agogue et al. 2008, Mussmann et al. 2011). These results raised uncertainties regarding the ecological function of AOA community in N cycling because under possible mixotrophic, even heterotrophic life styles, AOA may not gain energy through oxidation of ammonia to nitrite, and under which the ammonia oxidation potential of AOA may not be functional. Correlation test in this study revealed significant correlations between measured soil nitrification rate and individual archaeal-*amoA* phylogenetic groups, indicating soil AOA may actively involve in the nitrification process in this grassland soil.

In conclusion, this study comprehensively analyzed the abundance, diversity, and structure of ammonia oxidation archaea communities in a $CO_2$ enriched grassland ecosystem under different soil N conditions. Our results provided several valuable insights into the microbial ecology of AOA and their responses to long-term elevated $CO_2$ and/or N deposition. First, this study was conducted in a grassland ecosystem subjected to >12 years elevated $CO_2$ and N deposition treatments using multiple complementary approaches, providing reliable evidence that individual and interactive effects of these global change factors can significantly affect the diversity and structure of AOA

community but not their abundance. Second, AOA community composition was significantly correlated with selected environmental factors including plant root biomass and soil moisture. Finally, several individual archaeal-*amoA* phylogenetic groups may actively involve in the nitrification process in this grassland soil. As nitrification is the rate-limiting step in the N cycling of highly complex terrestrial ecosystems, shifts in the diversity and structure of AOA community may have a large influence on the N cycling in grassland ecosystem in the long term. However, additional experiments are needed on the potential nitrification rate and archaeal-*amoA* gene expression which could offer more detailed information about the response of AOA to global change such as elevated $CO_2$ and N deposition.

# Chapter 6: Summary and Output

The atmospheric $CO_2$ concentration is continuously increasing, and scientific research demonstrates that this leads to huge changes to the global ecosystem, such as climate change. How and by what mechanisms long-term elevated $CO_2$ affects belowground microbial communities is a critical issue for ecology and global change biology. Furthermore, the interactive effects of elevated $CO_2$ with other environmental change factors, such as nitrogen (N) deposition, on belowground microbial communities, and their feedbacks on ecosystem function have been scarcely studied. Using integrated approaches including high-throughput functional gene array (GeoChip 3.0), 454 pyrosequencing, Illumina sequencing, biolog, and stable isotope-based microbial C-sequestration and $N_2$-fixation measurements, this study obtained insights into grassland soil microbial community diversity and their interactive responses to long-term elevated $CO_2$ and N deposition (12-year). Based on the observed results, several outcomes and/or mechanisms about microbial community responses to the treatment effects were revealed.

**First**, to study the interactive effects of elevated $CO_2$ with N deposition on belowground microbial communities, we assessed changes in soil microbial communities of two plant functional groups (C3 grass and legume) under elevated $CO_2$ on and/or N deposition. C3 grasses were chosen as the model due to their strong responses to elevated $CO_2$, and legumes were chosen due to their symbiotic $N_2$-fixation capability via root symbioses which may abate soil N limitation compared to non-legumes under elevated $CO_2$ over a long period of time. Due partially to the extreme complexity of soil microbial community and limitations of conventional molecular microbial ecology approaches for characterizing them, until now, no consistent results were obtained about how elevated

$CO_2$ affects the belowground microbial community structure and diversity (Janus et al. 2005, Lipson et al. 2005, Lipson et al. 2006, Lesaulnier et al. 2008, Castro et al. 2010, Dunbar et al. 2012, Eisenhauer et al. 2012, He et al. 2012). Using advanced Illumina platform, we did large scale sequencing of microbial 16S rRNA gene amplicons to examine the diversity and structure. Metagenomic sequencing analyses reveals marked divergence in the structure and composition of soil microbial communities at elevated $CO_2$ and N deposition, and between C3 grass and legume plots. Elevated $CO_2$ increased community phylogenetic diversity in C3 grass plots, while increased richness in legume plots. N deposition, on the other hand, increased community richness but only in C3 grass plots. No $CO_2$ and N interactive effects were observed on community diversity or richness. Further investigation suggested that less abundant groups rather than dominant taxa were mainly responsible for the changed community diversity and structure. In addition, our results suggested that copiotrophic-like bacteria appear to be more abundant in the legume than in the C3 grass plots, whereas oligotrophic-like bacteria appear to be more abundant in the C3 grass than in the legume plots. Changes in community diversity and composition were closely related to plant and soil properties including plant biomass, biomass N content and C/N ratio, soil ammonium and nitrate, pH, moisture, temperature, and soil C and N contents. Because the belowground microbial community is intimately linked to aboveground biodiversity (Wardle et al. 2004) and mediates ecosystem multifunctioning (Fierer et al. 2009, Wagg et al. 2014), these changes in microbial diversity and structure may lead to considerable ecosystem consequences in the future. Our results demonstrated that elevated $CO_2$ effects are contingent on N conditions and

plant functional groups, underscoring the difficulty toward predictive modeling of soil ecosystem under future climate scenarios and necessitating more detailed studies.

**Second**, we found that 12-year elevated $CO_2$ increased belowground microbial functional gene diversity, structure, and potential activities, but the magnitude of such effect depends on soil N levels and plant functional groups. Microbial functional genes in C3 grass plots had stronger responses to elevated $CO_2$ and N deposition than those in legume plots. And microbial functional genes related to C, N, S and P cycling in low fertility soils were stimulated more by elevated $CO_2$ than fertilized ones. Particularly, microbial genes related to chemically recalcitrant C degradation (e.g. phenol_oxidase, manganese peroxidase, vanillin dehydrogenase, vanillate monooxygenase, malate synthase, isocitrate lyase, endochitinase, acetylglucosaminidase) were selectively increased with elevated $CO_2$ in non-fertilized C3 grass plot soils only. These results suggest that microbial N-mining through decomposition of soil organic substrates could have increased with elevated $CO_2$ (Priming effect) and being stronger in low than high nutrient conditions in the grassland ecosystem of this study. Furthermore, the abundance of *ureC* gene for ammonification was increased with elevated $CO_2$ in non-fertilized C3 grass plot soils, resulting in a positive effect of elevated $CO_2$ on net N mineralization in these plots. Moreover, the abundance of *nifH* genes increased by 35.5% with elevated $CO_2$ in non-fertilized C3 grass plots, suggesting more N could have been fixed in these plots. The combination of these effects may increase soil N availability and contribute to the long-term stimulation of elevated $CO_2$ on plant productivity, but persistent turnover of chemically recalcitrant C by microbes also accelerates soil C loss under elevated $CO_2$ particularly when N is limited, offering a

partial explanation for the lack of C accumulation in the C3 grass plot soils at this site despite 12 years of $CO_2$-induced increases in NPP (Reich and Hobbie 2013). N deposition, on the other hand, reduced genes involved in both labile and recalcitrant C decomposition (mainly in C3 grass plots), suggesting a decrease in SOM decomposition in these plots. N deposition also reduced the abundance of *nifH* genes. Together, N deposition may contribute to C accumulation but reduce biological N-gain through microbial N-mining and N-fixation in the long-term. To what extent, these changes in microbial functional potential will alter total C and N budgets in this grassland ecosystem requires further study.

**Third**, DNA-based technologies (e.g., RFLP, DGGE, and Geochip) are limited to directly reflecting the actual activity of active microbial populations (McGrath et al. 2010, Simon and Daniel 2011, Franzosa et al. 2015). Therefore, we further measured belowground microbial C utilization profile using cultivation-based Biolog EcoPlate. We found that community composition of soil microbes in the C3 grass plot soils was significantly different from those in the legume plot soils based on the C utilization rate. And microbial communities in legume plots had significantly higher metabolic potential than in C3 grass plots for decomposing organic substrates. Elevated $CO_2$ and N deposition didn't significantly alter metabolic potential of microorganisms in the C3 grass plots. Overall microbial metabolic activities significantly increased with elevated $CO_2$ by 20.6% in the fertilized legume plots, while there was no evidence for a $CO_2$ effect in the non-fertilized legume soils. These results demonstrated that elevated $CO_2$ effects on active microbial metabolic activities are contingent on N conditions, and such effect differs between plant functional groups. Meanwhile, PLFA analysis showed both total

microbial and bacterial biomass were significantly lower in legume than in C3 grass plots, showing an opposite trend to the microbial metabolic potential in these plots. Thus, differences in microbial metabolic potential among treatments and between plant functional groups were not due to population size (biomass) but likely attributed to changes in community structure and/or enzymatic activities of belowground microbes.

**Fourth**, the recently discovered ammonia oxidizing archaea (AOA) community has been shown to be widely distributed, more abundant than its bacterial counterparts, ammonia oxidizing bacteria (AOB), and may play an important role in the global N cycle. Yet, little is known regarding the effect of elevated $CO_2$ on their community diversity, composition, and structure. Furthermore, the interactive effects of multiple factors (i.e., elevated $CO_2$, soil N level, temperature) on the abundance and community structure of AOA remain to be demonstrated. Using barcoded 454 pyrosequencing coupled with GeoChip functional gene array targeting the archaeal *amoA* gene, the AOA community structure and diversity were investigated in this study. Although elevated $CO_2$ and N deposition alone and combined did not significantly alter the abundance of AOA. The taxonomic and phylogenetic diversity of AOA communities were significantly reduced by long-term elevated $CO_2$ but the $CO_2$ effects were confined within ambient N condition. Elevated $CO_2$ also significantly altered the community structure of AOA regardless of N deposition, but no main effect of N deposition on AOA community diversity and structure were observed. The relative abundance of several AOA taxa were significantly correlated with plant root biomass, proportional soil moisture, and pH. In addition, significant positive correlations between AOA taxa and soil nitrification rate were observed, indicating soil ammonia oxidizing archaea were actively involved in the nitrification

process in grassland soil. These results are important in furthering our understanding of the global change impacts on AOA community structure in the long term.

In addition, based on the archaeal *amoA* gene sequences obtained from this study, we also constructed high-density functional gene arrays (FGAs) containing more archaeal *amoA* genes probes for better monitoring AOA community dynamics in natural environments.

In conclusion, this study advances our understanding of (i) the diversity of grassland microbial communities, (ii) the responses of soil microbial communities to changes in atmospheric $CO_2$ and N deposition, and (iii) the mechanistic linkages among aboveground plant and belowground microbial communities and associated ecosystem processes. These results have important implications for feedback responses of ecosystems to atmospheric $CO_2$ and N deposition.

Those results from this study and other associated projects that I have involved are largely reflected in my publications (published, in press, in preparation) as they are listed below:

1. **Feifei Liu**, Kai Xue, Bo Wu, Qun Gao, Zhili He, Liyou Wu, Sarah Hobbie, Peter Reich, Jizhong Zhou. Contingency in belowground microbial functional responses to rising atmospheric $CO_2$ at contrast nitrogen conditions. *Preparing for submission.*
2. **Feifei Liu,** Bo Wu, Renmao Tian, Daliang Ning, Sarah Hobbie, Peter Reich, Jizhong Zhou. Shifts of phylogenetic composition and structure of soil microbial communities in response to elevated carbon dioxide and nitrogen deposition. *Preparing for submission.*
3. **Feifei Liu** et al. Pyrosequencing analysis of *amoA* genes for soil ammonia-oxidizing archaea communities under elevated CO2 and nitrogen deposition conditions. *Draft.*
4. **Feifei Liu** et al. Dynamic responses of soil microbial communities to elevated $CO_2$. *Draft.*
5. Michelle Q. Carter , Kai Xue, **Feifei Liu**, Liyou Wu, Jacqueline W. Louie, Robert E. Mandrell, Jizhong Zhou. Functional Metagenomics of Escherichia coli O157:H7

Interactions with Spinach Indigenous Microorganisms during Biofilm Formation. *Plos One, 05 September 2012*.

6. Kai Xue, Jianping Xie, Aifen Zhou, **Feifei Liu,** Dejun Li, Liyou Wu, Ye Deng, Zhili He, Joy Van Nostrand, Yiqi Luo, Jizhong Zhou. Warming alters expressions of microbial functional genes important to ecosystem functioning. *Front. Microbiol. 06 May 2016*.
7. Christopher Penton, Caiyun Yang, **Feifei Liu,** Liyou Wu, Qiong Wang, Jin Zhang, Yujia Qin, Ye Deng, Christopher L Hemme, Tianling Zheng, Edward A.G. Schuur, James M. Tiedje, Jizhong Zhou "NifH- Harboring Bacterial Community Composition Across an Alaskan Permafrost Thaw Gradient. *Front. Microbiol. 2016*.
8. Bo Wu, **Feifei Liu,** Lina Shen, Ye Deng, Liyou Wu, Michael Weiser, Mike E. Kaspari, Brian J. Enquist, Robert B. Waide, and James H. Brown, Shouwen Chen*, and Jizhong Zhou, and Zhili He*. The diversity and biogeographic distribution of $N_2O$-reducing microbial communities in forest soils along a temperature gradient. *Functional Ecology*. *In revision*
9. Chongqing Wen; Liyou Wu; Yujia Qin; Joy Van Nostrand; Bo Sun; Kai Xue; Ye Deng; **Feifei Liu**; Jizhong Zhou. Evaluation of the Reproducibility of Amplicon Sequencing with Illumina MiSeq Platform. *PLoS ONE. 2017*
10. Penton C.R., Yang C., Tiedje J., **Liu F**., Ma J., Yuan M., Zhang J., Xue K., Van Nostrand J., Yuan T., Wu L., He X., Schuur E.A.G., Zhou J. 2012. Diversity of soil fungal communities across a permafrost thawing gradient in an Arctic tundra. *In prep.*
11. Penton C.R., Pham A., St. Louis D., **Liu F.**, Zhang J., Schuur E.A.G., Tiedje J. 2012. Fungal community shifts due to artificial warming in grassland and tundra ecosystems. *In prep.*
12. Penton C.R., St. Louis D., Pham A., **Liu F**., Zhang J., Schuur E.A.G., Tiedje J. 2012. Changes in N functional communities as a response to warming in grassland and tundra ecosystems through pyrosequencing of *nifH*, *nirS*, *nirK*, *nosZ*, and *amoA* genes. *In prep.*

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# Appendix A: Supplementary Tables

**Table S3.1** Effects of $eCO_2$ and eN on additional plant and soil attributes.

**Table S3.2**. Unique and overlapped genes among treatments in C3 grass and legume plots.

**Table S3.3** Overall microbial functional gene diversity at different $CO_2$ and N levels.

**Table S5.1** Correlation test between phylogenetic archaeal-*amoA* groups and environmental factors and soil nitrification rate.

**Table S3.1** Effects of $eCO_2$ and eN on additional plant and soil attributes.

| | | Variables | Mean ± SE | | | |
|---|---|---|---|---|---|---|
| | | | aCaN | eCaN | aCeN | eCeN |
| C3 grass | Soil | pH | 5.65 ± 0.12 | 5.49 ± 0.10 | 5.68 ± 0.11 | 5.58 ± 0.11 |
| | | Moisture | 0.27 ± 0.03 **b** | 0.46 ± 0.11 **a** | 0.24 ± 0.03 **b** | 0.16 ± 0.03 **b** |
| | | Temperature (°C) | 20.62 ± 0.44 | 20.72 ± 0.4 | 21.08 ± 0.46 | 20.81 ± 0.45 |
| | | Total C (%) | 0.4 ± 0.15 | 0.56 ± 0.20 | 0.67 ± 0.22 | 0.52 ± 0.15 |
| | | Total N (%) | 0.04 ± 0.02 | 0.07 ± 0.02 | 0.07 ± 0.02 | 0.06 ± 0.02 |
| | Plant | Aboveground C/N | 36.25 ± 4.43 **b** | 46.17 ± 1.43 **a** | 32.98 ± 2.76 **bc** | 26.04 ± 1.50 **c** |
| | | Root C/N | 41.97 ± 5.05 **a** | 42.82 ± 3.81 **a** | 32.25 ± 2.33 **ab** | 27.59 ± 2.80 **b** |
| Legume | Soil | pH | 5.53 ± 0.14 | 5.71 ± 0.13 | 5.33 ± 0.25 | 5.62 ± 0.03 |
| | | Moisture | 0.20 ± 0.01 | 0.21 ± 0.04 | 0.20 ± 0.01 | 0.25 ± 0.05 |
| | | Temperature (°C) | 19.55 ± 0.68 | 20.12 ± 0.67 | 19.68 ± 0.54 | 18.99 ± 0.22 |
| | | Total C (%) | 0.7 ± 0.02 | 0.69 ± 0.05 | 0.86 ± 0.11 | 0.76 ± 0.06 |
| | | Total N (%) | 0.08 ± 0.01 | 0.09 ± 0.01 | 0.08 ± 0.01 | 0.09 ± 0.01 |
| | Plant | Aboveground C/N | 27.14 ± 3.63 | 22.46 ± 2.75 | 30.40 ± 4.94 | 29.54 ± 7.24 |
| | | Root C/N | 20.30 ± 0.58 | 18.11 ± 1.34 | 20.13 ± 2.00 | 15.86 ± 1.31 |

1. Values labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Shown are mean values (n=3) ± standard errors.
2. All plant and soil properties were data collected from the sampling year (2009). And soil moisture was the combination of all three measured proportions (0-17cm, 42-59cm and 83-100cm).
3. Abbreviations: aCaN: ambient $CO_2$ and ambient N; eCaN: elevated $CO_2$ and ambient N; aCeN: ambient $CO_2$ and enriched N; eCeN: elevated $CO_2$ and enriched N.

**Table S3.2** Unique and overlapped genes among treatments in C3 grass and legume plots.

| | | aCaN | eCaN | aCeN | eCeN |
|---|---|---|---|---|---|
| C3 grass | aCaN | **394(11.92%)** | 2733(51.18%) | 1929(46.96%) | 2117(48.77%) |
| | eCaN | | **1201(25.19%)** | 2328(45.03%) | 2587(48.52%) |
| | aCeN | | | **245(8.97%)** | 1948(49.50%) |
| | eCeN | | | | **363(11.52%)** |
| Legume | aCaN | **448(19.67%)** | 1261(46.31%) | 1416(49.75%) | 1558(48.92%) |
| | eCaN | | **158(9.26%)** | 1227(49.82%) | 1355(48.12%) |
| | aCeN | | | **258(13.00%)** | 1470(49.35%) |
| | eCeN | | | | **540(21.91%)** |

1. The gene only existed in one treatment was a unique gene (**Bold**), and gene existed in two treatments was overlapped gene. Abbreviations: aCaN: ambient $CO_2$ and ambient N; eCaN: elevated $CO_2$ and ambient N; aCeN: ambient $CO_2$ and enriched N; eCeN: elevated $CO_2$ and enriched N.

**Table S3.3** Overall microbial functional gene diversity at different $CO_2$ and N levels

| | | aCaN | eCaN | aCeN | eCeN |
|---|---|---|---|---|---|
| C3 grass | 1/*D* | 2648.48 ± 287.57 **b** | 3975.55 ± 176.13 **a** | 2308.92 ± 74.00 **b** | 2384.84 ± 342.10 **b** |
| | No. of probes | 2669 ± 290 **b** | 4012 ± 179 **a** | 2323 ± 77 **b** | 2406 ± 349 **b** |
| Legume | 1/*D* | 1798.94 ± 308.08 | 1380.85 ± 135.66 | 1615.25 ± 83.13 | 1962.36 ± 161.00 |
| | No. of probes | 1811 ± 311 | 1388 ± 137 | 1623 ± 84 | 1975 ± 163 |

1. Values labeled with different letters are significantly different ($p < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Shown are mean values (n=3) ± standard errors. Values labeled with stars (*) indicate significant ($p < 0.05$) F ratios: *, $p < 0.05$; **, $p < 0.01$.

**Table S5.1** Correlation test between phylogenetic archaeal-*amoA* groups and environmental factors and soil nitrification rate.

| | Aboveground biomass | | Root biomass | | pH | | Moisture (0-17 cm) | | Moisture (42-59 cm) | | Moisture (83-100 cm) | | Nitrification | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *rho* | *p* | *rho* | *p* | *rho* | *p* | *rho* | *p* | *rho* | *p* | *rho* | *p* | *rho* | *p* |
| *Nitrososphaera* subcluster 1.1 | 0.34 | 0.14 | 0.24 | **0.04** | 0.33 | 0.16 | -0.06 | 0.81 | -0.51 | **0.02** | -0.46 | **0.04** | -0.37 | 0.10 |
| *Nitrososphaera* subcluster 3.1 | -0.01 | 0.96 | 0.01 | 0.96 | 0.16 | 0.51 | 0.37 | 0.10 | -0.02 | 0.93 | -0.01 | 0.97 | 0.51 | **0.02** |
| *Nitrososphaera* subcluster 3.2 | -0.22 | 0.35 | -0.12 | 0.61 | -0.07 | 0.77 | 0.54 | **0.01** | 0.31 | **0.09** | 0.09 | 0.70 | 0.47 | **0.04** |
| *Nitrososphaera* subcluster 3.3 | -0.23 | 0.34 | 0.07 | 0.76 | -0.15 | 0.54 | 0.26 | 0.28 | -0.07 | 0.76 | 0.01 | 0.97 | 0.25 | 0.29 |
| *Nitrososphaera* subcluster 5.1 | 0.13 | 0.60 | -0.11 | 0.65 | 0.08 | 0.75 | 0.05 | 0.84 | -0.35 | **0.06** | -0.16 | 0.50 | 0.16 | 0.50 |
| *Nitrososphaera* subcluster 6.1 | 0.16 | 0.50 | 0.23 | **0.03** | 0.33 | 0.16 | -0.19 | 0.41 | -0.52 | **0.02** | 0.12 | 0.62 | 0.42 | **0.07** |
| *Nitrososphaera* subcluster 7 | 0.24 | 0.31 | -0.20 | 0.40 | -0.21 | 0.38 | 0.01 | 0.96 | -0.15 | 0.53 | -0.19 | 0.42 | -0.08 | 0.75 |
| *Nitrososphaera* subcluster 8.1 | -0.05 | 0.84 | -0.22 | **0.03** | -0.09 | 0.70 | 0.22 | 0.36 | -0.04 | 0.87 | -0.18 | 0.45 | 0.04 | 0.87 |
| *Nitrososphaera* subcluster 8.2 | -0.12 | 0.63 | 0.12 | 0.63 | 0.27 | 0.25 | 0.13 | 0.58 | 0.24 | 0.31 | -0.15 | 0.52 | 0.16 | **0.05** |
| *Nitrososphaera* subcluster 11 | 0.01 | 0.96 | -0.12 | **0.06** | -0.06 | 0.79 | 0.21 | 0.39 | -0.01 | 0.95 | -0.24 | 0.31 | -0.16 | 0.50 |
| *Nitrososphaera* cluster other | -0.01 | 0.98 | -0.34 | 0.15 | -0.05 | 0.82 | -0.27 | 0.25 | 0.02 | 0.94 | 0.27 | 0.24 | 0.33 | 0.16 |
| *Nitrososphaera* sister cluster other | -0.12 | 0.60 | -0.38 | **0.07** | 0.04 | 0.88 | 0.32 | 0.18 | 0.41 | **0.07** | 0.11 | 0.64 | -0.08 | 0.72 |
| *Nitrososphaera* sister subcluster 1.1 | -0.06 | 0.80 | 0.58 | **0.01** | -0.29 | 0.22 | -0.32 | 0.17 | -0.26 | 0.27 | 0.15 | 0.53 | -0.05 | 0.82 |
| *Nitrososphaera* sister subcluster 2 | -0.23 | 0.34 | 0.30 | **0.05** | -0.45 | **0.04** | -0.05 | 0.84 | 0.02 | 0.94 | 0.14 | 0.55 | 0.00 | 1.00 |
| *Nitrosotalea* subcluster 1.1 | 0.30 | 0.19 | -0.21 | 0.38 | 0.03 | 0.91 | -0.28 | 0.22 | -0.16 | 0.51 | -0.11 | 0.65 | 0.37 | 0.11 |
| Unclassified | 0.14 | 0.56 | 0.26 | 0.27 | 0.10 | 0.68 | 0.06 | 0.80 | -0.02 | 0.93 | -0.24 | 0.31 | 0.34 | 0.14 |

## Appendix B: Supplementary Figures

**Fig. S2.1** Changes in selected environmental variables under $eCO_2$ and eN. AB: aboveground biomass; RB: root biomass; AB-N: aboveground biomass N content; AB-C/N: aboveground biomass C/N ratio; R-N: root N content; R-C/N: root C/N ratio.

**Fig. S2.2** PCoA plot of communities of C3 grass and legume plots under four different $CO_2$ and N levels.

**Fig. S2.3** Classes with different abundance levels among treatments within C3 grass and legume plots.

**Fig. S2.4** Genera with different abundance levels among treatments within C3 grass plots.

**Fig. S2.5** Genera with different abundance levels among treatments within legume plots.

**Fig. S2.6** Spearman's rank correlation coefficient rho of selected classes (with different abundance levels among treatments) with environmental attributes.

**Fig. S2.7** Spearman's rank correlation coefficient rho of selected genera (with different abundance levels among treatments) with environmental attributes.

**Fig. S2.8** Variation partitioning analysis (VPA) of microbial community structure explained by plant properties, soil geochemical variables, $CO_2$, and N.

**Fig. S2.9** Relative difference of each genus between treatment and control as a function of genus abundance rank in control.

**Fig. S3.1** Multivariate regression tree (MRT) analysis to illustrate the relative contributions of plant functional group (C3 grass and legume), $eCO_2$ and eN in shaping microbial functional composition and structure derived from GeoChip 3.0.

**Fig. S3.2** Elevated $CO_2$ and eN effects on genes in C3 grass plots involved in N cycle (a), C fixation and $CH_4$ processes (b), and P/S cycles (c). The processes involved in N cycle include ammonification (1), Anammox (2), assimilatory N reduction (3), denitrification (4), dissimilatory N reduction (5), nitrification (6), and N fixation (7). Bars labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment.

**Fig. S3.3** Elevated $CO_2$ and eN effects on genes in legume plots involved in N cycle (a), C fixation and $CH_4$ processes (b), and P/S cycles (c). The processes involved in N cycle include ammonification (1), Anammox (2), assimilatory N reduction (3), denitrification (4), dissimilatory N reduction (5), nitrification (6), and N fixation (7). Bars labeled with

different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment.

**Fig. S3.4** Significance of correlations between individual or all genes involved in C degradation and SCF or HR (a); genes involved in C fixation and $^{13}C$ sequestration strength (b); *nifH* gene involved in N fixation and $^{15}N$ fixation across samples at aN plots (c); genes involved in ammonification or nitrification and soil N processes (d). Significance is represented by ** when $p<0.05$ and * when $p<0.10$.

**Fig. S3.5** Quantitative $^{13}C$ and $^{15}N$ enrichment in soils from C3 grass and legume plots at $CO_2$ and N treatments.

**Fig. S4.1** Effects of $eCO_2$ and eN on microbial metabolic diversity represented by Richness, Shannon, and Evenness. Boxes labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

**Fig. S4.2** DCA analysis of microbial communities based on metabolic diversity obtained through Biolog EcoPlate after 156h incubation. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

**Fig. S5.1** UniFrac PCoA and βMNTD analyses of the archaeal-*amoA* community. Gray: aCaN; Red: aCeN; Blue: eCeN; Green: eCaN.

**Fig. S5.2** Detection frequency and relative abundances of archaeal-*amoA* genes at OTU level in the four $CO_2$ and N conditions. Phylogenetic tree was constructed based on neighbor-joining criterion. Rings from inner to outer represents aCaN, eCaN, aCeN, and eCeN conditions, respectively. * in red, blue and green represent significant $CO_2$, N and their interactive effects, respectively (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ANOVA test).

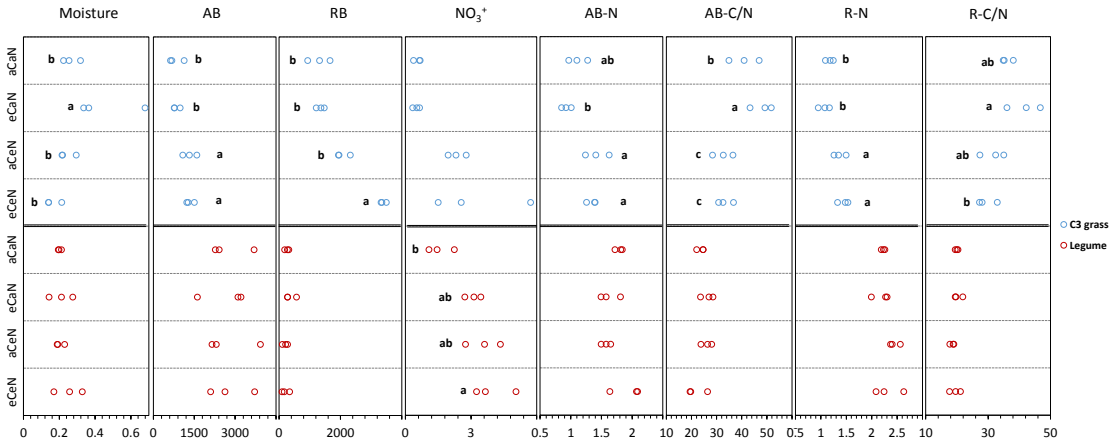



**Fig. S2.1** Changes in selected environmental variables under $eCO_2$ and eN. AB: aboveground biomass; RB: root biomass; AB-N: aboveground biomass N content; AB-C/N: aboveground biomass C/N ratio; R-N: root N content; R-C/N: root C/N ratio.

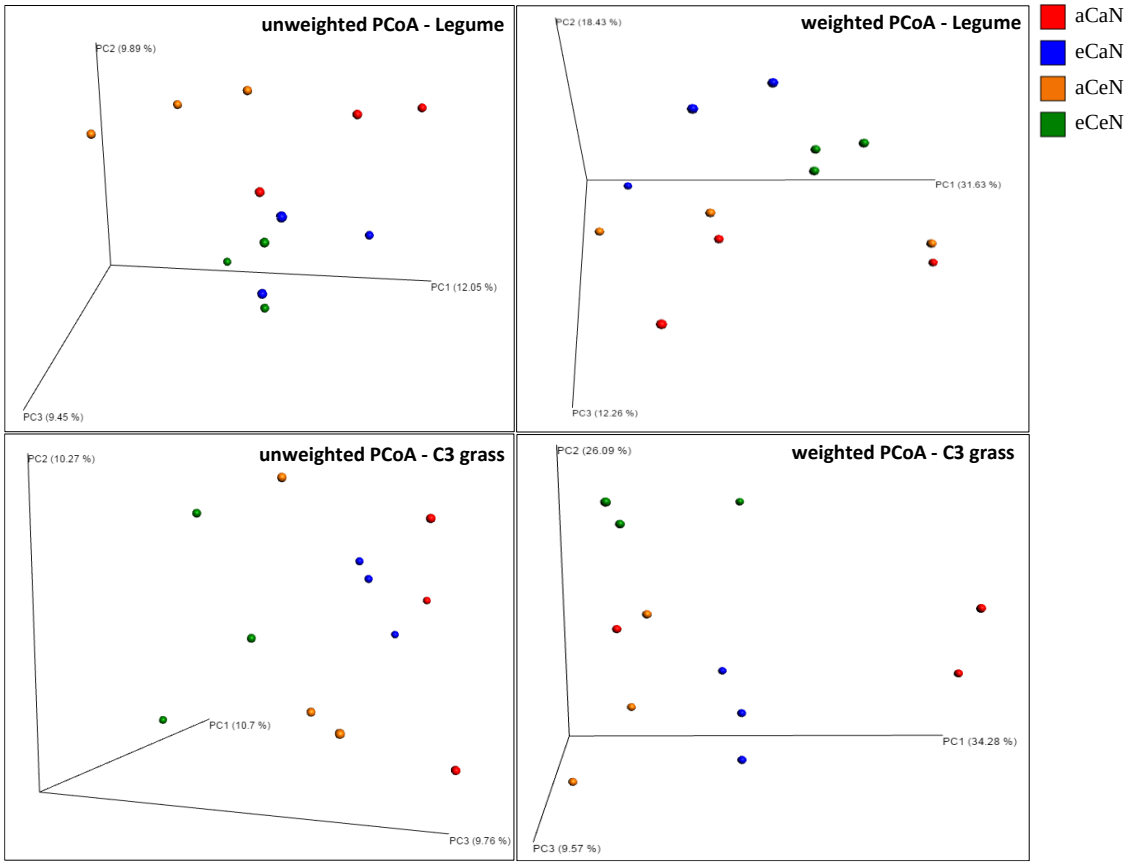



**Fig. S2.2** PCoA plot of communities of C3 grass and legume plots under four different $CO_2$ and N levels. Each point corresponds to a sample.

| | | a | ab | b | bc | c | | |
|---|---|---|---|---|---|---|---|---|
| | Class | aCaN | eCaN | aCeN | eCeN | abundance (%) | Treatment effects vs. aCaN | |
| C3 | *Actinobacteria* | | | | | 10.714% | Decreased with eCaN | |
| | *Thermoleophilia* | | | | | 8.147% | | |
| | *Acidimicrobiia* | | | | | 1.097% | Decreased with aCeN | |
| | *Planctomycetes* BD7-11 | | | | | 0.005% | | |
| | *Gammaproteobacteria* | | | | | 1.726% | Increased with eCaN | |
| | *Chlamydiae* | | | | | 0.112% | | |
| | *Verrucomicrobia* UA11 | | | | | 0.002% | | |
| | *Bellilinea* | | | | | 0.002% | | |
| | *Deltaproteobacteria* | | | | | 1.582% | None | |
| | *Phycisphaerae* | | | | | 0.666% | | |
| | *Nitrospira* | | | | | 0.353% | | |
| | *Melainabacteria* | | | | | 0.032% | | |
| | *Erysipelotrichia* | | | | | 0.029% | | |
| Le | *Deltaproteobacteria* | | | | | 1.858% | Decreased with eCaN | |
| | *Phycisphaerae* | | | | | 0.582% | | |
| | *Chloroflexi* JG30-KF-CM66 | | | | | 0.065% | | |
| | *Chloroflexi* TK10 | | | | | 0.370% | Decreased with eCeN | |
| | *Chloroflexi* Gitt-GS-136 | | | | | 0.181% | | |
| | *Actinobacteria* TakashiAC-B11 | | | | | 0.025% | | |
| | *Thermoleophilia* | | | | | 5.582% | Decreased with eCaN & eCeN | |
| | *Chloroflexi* JG37-AG-4 | | | | | 0.839% | | |
| | *Thermoplasmata* | | | | | 0.006% | | |
| | *Acidimicrobiia* | | | | | 0.963% | Decreased with aCeN | |
| | *Acidobacteria* subgroup 22 | | | | | 0.008% | Increased with eCaN | |
| | *Acidobacteria* subgroup 26 | | | | | 0.003% | | |
| | *Gammaproteobacteria* | | | | | 2.618% | Increased with eCeN | |
| | *Bacteroidetes* VC2.1 Bac22 | | | | | 0.002% | | |
| | *Latescibacteria bacterium* | | | | | 0.005% | Increased with eCaN & eCeN | |
| | *Melainabacteria* | | | | | 0.035% | Increased with aCeN | |
| | *Verrucomicrobia* Incertae Sedis | | | | | 0.006% | | |
| | *Acidobacteria* subgroup 6 | | | | | 14.982% | None | |
| | *Nitrospira* | | | | | 0.582% | | |
| | *Chlamydiae* | | | | | 0.170% | | |
| | *Caldilineae* | | | | | 0.041% | | |
| | *Planctomycetes* vadinHA49 | | | | | 0.019% | | |

**Fig. S2.3** Classes with different abundance levels among treatments within C3 grass and legume plots. The color panel under 'aCaN', 'eCaN', 'aCeN' and 'eCeN' reflects the relative abundance level at each treatment by LSD test. Red toned cells, (a); Blush toned cells, (ab); White toned cells, (b); Sapphire toned cells, (bc); and Blue toned cells, (c).

| | Kingdom | Phylum | Class | Order | Family | Genus | aCaN | eCaN | aCeN | eCeN | Abundance (%) | Treatment effects vs. aCaN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bacteria | Actinobacteria | Thermoleophilia | Solirubrobacterales | Patulibacteraceae | Patulibacter | | | | | 0.882% | Decreased with eCaN |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Virgisporangium | | | | | 0.026% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 5 | Acidobacteria Subgroup 5 | Acidobacteria Subgroup 5 | | | | | 0.092% | |
| | Bacteria | Acidobacteria | Acidobacteria | Subgroup 17 | Acidobacteria Subgroup 17 | Acidobacteria Subgroup 17 | | | | | 0.043% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Frankiales | Frankiales | | | | | 0.024% | |
| | Bacteria | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Sphingobacteriales env.OPS 17 | Sphingobacteriales env.OPS 17 | | | | | 0.018% | |
| | Bacteria | Cyanobacteria | Cyanobacteria | SubsectionIV | FamilyI | FamilyI | | | | | 0.010% | Decreased with eCeN |
| | Bacteria | Acidobacteria | Holophagae | Acidobacteria Subgroup 7 | Acidobacteria Subgroup 7 | Acidobacteria Subgroup 7 | | | | | 0.006% | |
| | Bacteria | Cyanobacteria | Cyanobacteria | SubsectionIV | FamilyI | Nostoc | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Hydrogenophilales | Hydrogenophilaceae | Hydrogenophilaceae | | | | | 0.002% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Hyphomicrobiaceae | | | | | 0.002% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Albidiferax | | | | | 0.001% | |
| | Bacteria | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Sphingobacteriales | Sphingobacteriales | | | | | 0.056% | |
| | Bacteria | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Acidimicrobiales | Acidimicrobiales | | | | | 0.017% | |
| | Bacteria | Chloroflexi | Chloroflexia | Herpetosiphonales | Herpetosiphonaceae | Herpetosiphon | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Rhodocyclales | Rhodocyclaceae | Denitratisoma | | | | | 0.002% | Decreased with eCaN & eCeN |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | Polymorphobacter | | | | | 0.002% | |
| | Bacteria | Cyanobacteria | Cyanobacteria | SubsectionIII | FamilyI | Leptolyngbya | | | | | 0.001% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 18 | Acidobacteria Subgroup 18 | Acidobacteria Subgroup 18 | | | | | 0.001% | |
| | Bacteria | Bacteroidetes | Flavobacteriia | Flavobacteriales | Cryomorphaceae | Fluviicola | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Nitrosomonadales | Nitrosomonadaceae | Nitrosomonadaceae | | | | | 0.993% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Rickettsiales Incertae Sedis | Captivus | | | | | 0.006% | Decreased with aCeN |
| | Bacteria | Proteobacteria | Betaproteobacteria | Rhodocyclales | Rhodocyclaceae | Methyloversatilis | | | | | 0.003% | |
| | Bacteria | Acidobacteria | Acidobacteria | Subgroup 4 | Acidobacteria Subgroup 4 | Acidobacteria Subgroup 4 | | | | | 0.216% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Hyphomicrobium | | | | | 0.199% | |
| | Bacteria | Firmicutes | Bacilli | Bacillales | Paenibacillaceae | Paenibacillus | | | | | 0.198% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhizobiales Incertae Sedis | Bauldia | | | | | 0.179% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Planctomyces | | | | | 0.095% | |
| | Bacteria | Firmicutes | Bacilli | Bacillales | Paenibacillaceae | Cohnella | | | | | 0.025% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Pir4 lineage | | | | | 0.022% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Deltaproteobacteria | Deltaproteobacteria | Deltaproteobacteria | | | | | 0.021% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Caulobacterales | Hyphomonadaceae | Hirschia | | | | | 0.019% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Holosporaceae | Holosporaceae | | | | | 0.018% | |
| | Bacteria | Planctomycetes | Phycisphaerae | Pla1 lineage | Pla1 lineage | Pla1 lineage | | | | | 0.017% | |
| C3 | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillales | Rhodospirillales | | | | | 0.016% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Simkaniaceae | Rhabdochlamydia | | | | | 0.013% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Ideonella | | | | | 0.012% | |
| | Bacteria | Actinobacteria | Actinobacteria | Corynebacteriales | Nocardiaceae | Smaragdicoccus | | | | | 0.007% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Burkholderiaceae | Glomeribacter | | | | | 0.006% | |
| | Bacteria | Proteobacteria | Proteobacteria TA18 | Proteobacteria TA18 | Proteobacteria TA18 | Proteobacteria TA18 | | | | | 0.005% | |
| | Bacteria | Chloroflexi | Ktedonobacteria | Ktedonobacterales | Ktedonobacterales | Ktedonobacterales | | | | | 0.005% | |
| | Bacteria | Verrucomicrobia | Verrucomicrobiae | Verrucomicrobiales | Verrucomicrobiaceae | Prosthecobacter | | | | | 0.003% | |
| | Bacteria | Planctomycetes | Phycisphaerae | Phycisphaerae WD2101 | Phycisphaerae WD2101 | Phycisphaerae WD2101 | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Brachymonas | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | Polyangium | | | | | 0.003% | |
| | Bacteria | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadetes | | | | | 0.002% | |
| | Bacteria | Verrucomicrobia | UA11 | Verrucomicrobia UA11 | Verrucomicrobia UA11 | Verrucomicrobia UA11 | | | | | 0.002% | Increased with eCaN |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Deltaproteobacteria | Deltaproteobacteria | Deltaproteobacteria | | | | | 0.002% | |
| | Bacteria | Elusimicrobia | Elusimicrobia | Lineage IIc | Lineage IIc | Lineage IIc | | | | | 0.002% | |
| | Bacteria | Chloroflexi | Bellilinea | Bellilinea | Bellilinea | Bellilinea | | | | | 0.002% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophaceae | Syntrophaceae | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Nannocystaceae | Nannocystis | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Nevskiaceae | Nevskia | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Metallibacterium | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Prosthecomicrobium | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Myxococcales | Myxococcales | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptomycetales | Streptomycetaceae | Streptacidiphilus | | | | | 0.0003% | |
| | Bacteria | Armatimonadetes | Armatimonadia | Armatimonadales | Armatimonadaceae | Armatimonas | | | | | 0.0003% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cyclobacteriaceae | Cyclobacteriaceae | | | | | 0.0003% | |
| | Bacteria | Cyanobacteria | Cyanobacteria | SubsectionIII | FamilyI | Crinalium | | | | | 0.0003% | |
| | Bacteria | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadales | Gemmatimonadaceae | Gemmatimonadaceae | | | | | 0.0003% | |
| | Bacteria | Planctomycetes | Phycisphaerae | Phycisphaerae | Phycisphaerae | Phycisphaerae | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | Defluviimonas | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Mitochondria | Mitochondria | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Rickettsiaceae | Orientia | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Desulfuromonadales | Desulfuromonadaceae | Desulfuromonas | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Chromatiales | Chromatiaceae | Nitrosococcus | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Rudaea | | | | | 0.0003% | |
| | Bacteria | Firmicutes | Bacilli | Bacillales | Thermoactinomycetaceae | Kroppenstedtia | | | | | 0.0003% | |

(a)

| | Kingdom | Phylum | Class | Order | Family | Genus | aCaN | eCaN | aCeN | eCeN | Abundance (%) | Treatment effects vs. aCaN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | a | ab | b | bc | c | |
| C3 | Bacteria | Actinobacteria | Actinobacteria | Corynebacteriales | Mycobacteriaceae | Mycobacterium | | | | | 0.960% | |
| | Bacteria | Actinobacteria | Thermoleophilia | Solirubrobacterales | Solirubrobacterales | Solirubrobacterales | | | | | 0.765% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Micromonosporaceae | | | | | 0.506% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Devosia | | | | | 0.362% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Amycolatopsis | | | | | 0.301% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Dokdonella | | | | | 0.097% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Planosporangium | | | | | 0.096% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Xanthobacteraceae | Labrys | | | | | 0.096% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Kibdelosporangium | | | | | 0.084% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Thermomonosporaceae | Actinomadura | | | | | 0.045% | |
| | Bacteria | Actinobacteria | Actinobacteria | Catenulisporales | Catenulisporaceae | Catenulispora | | | | | 0.022% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Intrasporangiaceae | Intrasporangiaceae | | | | | 0.014% | |
| | Bacteria | Actinobacteria | Actinobacteria | Corynebacteriales | Corynebacteriales | Corynebacteriales | | | | | 0.012% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadales | Sphingomonadales | | | | | 0.010% | |
| | Bacteria | Verrucomicrobia | Spartobacteria | Chthoniobacterales | Chthoniobacterales | Chthoniobacterales | | | | | 0.005% | Increased with eCeN |
| | Bacteria | Actinobacteria | Thermoleophilia | Gaiellales | Gaiellales | Solirubrobacter | | | | | 0.003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Frankiaceae | Frankiaceae | | | | | 0.002% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Micrococcaceae | Micrococcaceae | | | | | 0.002% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Schlesneria | | | | | 0.002% | |
| | Bacteria | Cyanobacteria | Cyanobacteria ML635J-21 | Cyanobacteria ML635J-21 | Cyanobacteria ML635J-21 | Cyanobacteria ML635J-21 | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Hydrogenophaga | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | Thalassospira | | | | | 0.001% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Simkaniaceae | Simkaniaceae | | | | | 0.001% | |
| | Bacteria | Planctomycetes | Planctomycetes | Planctomycetes | Planctomycetes | Planctomycetes | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Corynebacteriales | Corynebacteriaceae | Corynebacteriaceae | | | | | 0.0003% | |
| | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Dysgonomonas | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Caulobacterales | Hyphomonadaceae | Hyphomonadaceae | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Aurantimonadaceae | Aurantimonadaceae | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhodobiaceae | Rhodobiaceae | | | | | 0.0003% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Simplicispira | | | | | 0.0003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Propionibacteriales | Nocardioidaceae | Kribbella | | | | | 0.092% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Gammaproteobacteria NKB5 | Gammaproteobacteria NKB5 | Gammaproteobacteria NKB5 | | | | | 0.068% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Legionellales | Legionellaceae | Legionella | | | | | 0.063% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Parachlamydiaceae | Protochlamydia | | | | | 0.029% | Increased with eCaN & eCeN |
| | Bacteria | Planctomycetes | Phycisphaerae | Phycisphaerales | Phycisphaeraceae | Phycisphaeraceae | | | | | 0.011% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 3 | Acidobacteria Subgroup 3 | Acidobacteria Subgroup 3 | | | | | 0.010% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Oligoflexales | Oligoflexaceae | Oligoflexaceae | | | | | 0.004% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadales | Xanthomonadales | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Oxalobacteraceae | Oxalobacteraceae | | | | | 0.074% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Thermomonosporaceae | Actinoallomurus | | | | | 0.069% | |
| | Bacteria | Chloroflexi | Ktedonobacteria | Ktedonobacteria | Ktedonobacteria | Ktedonobacteria | | | | | 0.012% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Dyella | | | | | 0.006% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteriales | Acidobacteriaceae Subgroup 1 | Telmatobacter | | | | | 0.006% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylocystaceae | Methylocystaceae | | | | | 0.005% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Rhodocyclales | Rhodocyclaceae | Azospira | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Alcaligenaceae | Bordetella | | | | | 0.002% | Increased with aCeN |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylocystaceae | Methylocystis | | | | | 0.002% | |
| | Bacteria | Armatimonadetes | Armatimonadia | Armatimonadales | Armatimonadales | Armatimonadales | | | | | 0.002% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Rickettsiaceae | Rickettsiaceae | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Goodfellowiella | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Legionellales | Legionellaceae | Legionellaceae | | | | | 0.001% | |
| | Archaea | esearchaeota (DHVEG | euryarchaeote | euryarchaeote | euryarchaeote | euryarchaeote | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | Serratia | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Thermoleophilia | Gaiellales | Gaiellales | Gaiellales | | | | | 0.0003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Pseudonocardia | | | | | 1.090% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | Mesorhizobium | | | | | 0.308% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Dactylosporangium | | | | | 0.236% | |
| | Bacteria | Nitrospirae | Nitrospira | Nitrospirales | Nitrospiraceae | Nitrospira | | | | | 0.215% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Geodermatophilaceae | Modestobacter | | | | | 0.168% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Micromonospora | | | | | 0.083% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | Cytophagaceae | | | | | 0.075% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Acetobacteraceae | Acidiphilium | | | | | 0.072% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Geodermatophilaceae | Blastococcus | | | | | 0.055% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Caulobacterales | Hyphomonadaceae | Woodsholea | | | | | 0.027% | |
| | Bacteria | Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Asteroleplasma | | | | | 0.026% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Arenimonas | | | | | 0.021% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Luteibacter | | | | | 0.020% | None |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Streptosporangiaceae | Nonomuraea | | | | | 0.017% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Micrococcaceae | Micrococcus | | | | | 0.012% | |
| | Bacteria | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Chitinophagaceae | Niabella | | | | | 0.012% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | Defluviicoccus | | | | | 0.012% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Lysobacter | | | | | 0.010% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Saccharopolyspora | | | | | 0.010% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Microbacteriaceae | Herbiconiux | | | | | 0.009% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Microbacteriaceae | Marisediminicola | | | | | 0.005% | |
| | Bacteria | Candidate division OP3 | uncultured bacterium | uncultured bacterium | uncultured bacterium | OP3 | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Acetobacteraceae | Rubritepida | | | | | 0.002% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Tahibacter | | | | | 0.002% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Saccharothrix | | | | | 0.001% | |

(b)

**Fig. S2.4** Genera with different abundance levels among treatments within C3 grass plots. The color panel under 'aCaN', 'eCaN', 'aCeN' and 'eCeN' reflects the relative abundance level at each treatment by LSD test. Red toned cells, (a); Blush toned cells, (ab); White toned cells, (b); Sapphire toned cells, (bc); and Blue toned cells, (c).

| | Kingdom | Phylum | Class | Order | Family | Genus | aCaN | eCaN | aCeN | eCeN | Abundance (%) | Treatment effects vs. aCaN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bacteria | Actinobacteria | Thermoleophilia | Solirubrobacterales | Solirubrobacterales | Solirubrobacterales | a | | | | 0.377% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Haliangiaceae | Haliangium | a | | | | 0.329% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | Sorangium | a | | | | 0.115% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bdellovibrionaceae | Bdellovibrio | a | | | | 0.081% | |
| | Bacteria | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Sphingobacteriales | Sphingobacteriales | a | | | | 0.055% | |
| | Bacteria | Armatimonadetes | Chthonomonadetes | Chthonomonadales | Chthonomonadaceae | Chthonomonas | a | | | | 0.052% | Decreased with eCaN |
| | Bacteria | Elusimicrobia | Elusimicrobia | Lineage IV | Lineage IV | Lineage IV | a | | | | 0.004% | |
| | Bacteria | Armatimonadetes | Armatimonadia | Armatimonadales | Armatimonadales | Armatimonadales | a | | | | 0.002% | |
| | Bacteria | Cyanobacteria | Melainabacteria | Vampirovibrionales | Vampirovibrionales | Vampirovibrionales | a | | | | 0.002% | |
| | Bacteria | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadales | Gemmatimonadaceae | Gemmatimonadaceae | a | | | | 0.002% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Thiotrichales | Thiotrichales | Thiotrichales | a | | | | 0.001% | |
| | Bacteria | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Acidimicrobiales | Acidimicrobiales | a | ab | | | 0.646% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Singulisphaera | a | ab | | | 0.166% | |
| | Bacteria | Actinobacteria | TakashiAC-B11 | TakashiAC-B11 | TakashiAC-B11 | TakashiAC-B11 | a | ab | | | 0.025% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Frankiales | Frankiales | ab | b | a | c | 0.022% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | Sporocytophaga | a | ab | | | 0.015% | Decreased with eCeN |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | Cytophaga | a | ab | | | 0.007% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | Paracocccus | a | ab | a | | 0.003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylocystaceae | Methylocystis | a | ab | b | c | 0.001% | |
| | Bacteria | Chloroflexi | Ktedonobacteria | Ktedonobacterales | Ktedonobacteraceae | Ktedonobacter | a | ab | ab | | 0.001% | |
| | Bacteria | Chloroflexi | Chloroflexi | Chloroflexi | Chloroflexi | Chloroflexi | a | ab | ab | | 0.839% | |
| | Bacteria | Planctomycetes | Phycisphaerae | Phycisphaerae WD2101 soil group | Phycisphaerae WD2101 soil group | Phycisphaerae WD2101 soil group | a | | ab | | 0.461% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Frankiaceae | Jatrophihabitans | a | | ab | | 0.306% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Gemmata | a | | ab | | 0.198% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Cryptosporangiaceae | Cryptosporangium | a | b | ab | c | 0.045% | |
| | Bacteria | Chloroflexi | Ktedonobacteria | Ktedonobacteria | Ktedonobacteria | Ktedonobacteria | a | | ab | | 0.018% | |
| | Archaea | Euryarchaeota | Thermoplasmata | Thermoplasmatales | Marine Group II | Marine Group II | a | | ab | | 0.006% | Decreased with eCaN & eCeN |
| | Bacteria | Actinobacteria | Actinobacteria | Propionibacteriales | Propionibacteriales | Propionibacteriales | a | | ab | | 0.002% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Hydrogenophaga | a | | | | 0.002% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Thermomonosporaceae | Spirillospora | a | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Frankiaceae | Frankiaceae | a | | a | | 0.001% | |
| | Bacteria | Firmicutes | Bacilli | Bacillales | Paenibacillaceae | Paenibacillaceae | a | | | | 0.001% | |
| | Bacteria | Planctomycetes | Phycisphaerae | Phycisphaerales | Phycisphaeraceae | Phycisphaeraceae | a | | | | 0.001% | |
| | Bacteria | Firmicutes | Bacilli | Lactobacillales | Carnobacteriaceae | Carnobacterium | a | | | | 0.0003% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria AT-s3-28 | Acidobacteria AT-s3-28 | Acidobacteria AT-s3-28 | a | ab | | | 0.007% | |
| | Bacteria | Armatimonadetes | Armatimonadetes | Armatimonadetes | Armatimonadetes | Armatimonadetes | ab | b | a | c | 0.005% | Decreased with eCeN |
| | Bacteria | Firmicutes | Negativicutes | Selenomonadales | Veillonellaceae | Sporomusa | a | ab | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Rhodoplanes | | a | | | 0.536% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Betaproteobacteria, | Betaproteobacteria | Betaproteobacteria | | a | | | 0.103% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | Adhaeribacter | | a | | | 0.098% | |
| Le | Bacteria | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Chitinophagaceae | Chitinophagaceae | | a | | | 0.086% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Legionellales | Coxiellaceae | Coxiella | | a | | | 0.027% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Beijerinckiaceae | Beijerinckiaceae | | a | a | | 0.024% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Holosporaceae | Holosporaceae | | a | | | 0.023% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Rhodomicrobium | | a | | | 0.017% | |
| | Bacteria | Acidobacteria | Holophagae | Holophagae Subgroup 10 | Holophagae Subgroup 10 | Holophagae Subgroup 10 | ab | a | b | c | 0.016% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Tahibacter | | a | | | 0.011% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Streptosporangiaceae | Microbispora | | a | | | 0.010% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Burkholderiales | Burkholderiales | | a | | | 0.009% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Caulobacterales | Caulobacteraceae | Asticcacaulis | | a | | | 0.009% | Increased with eCaN |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Ottowia | | a | a | | 0.006% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhizobiales | Rhizobiales | | a | | | 0.005% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Hamadaea | | a | | | 0.004% | |
| | Bacteria | Planctomycetes | Phycisphaerae | Phycisphaerae | Phycisphaerae | Phycisphaerae | | a | | | 0.004% | |
| | Bacteria | Acidobacteria | Acidobacteria Subgroup 26 | Acidobacteria Subgroup 26 | Acidobacteria Subgroup 26 | Acidobacteria Subgroup 26 | | a | | | 0.003% | |
| | Bacteria | Chloroflexi | Caldilineae | Caldilineales | Caldilineaceae | Litorilinea | | a | | | 0.003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Xanthobacteraceae | Starkeya | | a | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Bartonellaceae | Bartonella | | a | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Nocardiopsaceae | Streptomonospora | | a | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Corynebacteriales | Tsukamurellaceae | Tsukamurella | | a | | | 0.0003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Propionibacteriales | Propionibacteriaceae | Microlunatus | | a | | | 0.0003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Streptosporangiaceae | Sinosporangium | | a | | | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Bradyrhizobiaceae | Bradyrhizobiaceae | | ab | ab | a | 0.128% | |
| | Archaea | Thaumarchaeota | Thaumarchaeota (SCG) | Thaumarchaeota (SCG) | Thaumarchaeota (SCG) | Thaumarchaeota (SCG) | | ab | | a | 0.106% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Myxococcales | Myxococcales | | ab | | a | 0.089% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | Arenimonas | | | | a | 0.059% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Parachlamydiaceae | Metachlamydia | | ab | | a | 0.025% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Chlamydiales | Chlamydiales | | ab | | a | 0.018% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 3 | Acidobacteria Subgroup 3 | Acidobacteria Subgroup 3 | | | | a | 0.017% | |
| | Archaea | Thaumarchaeota | Thaumarchaeota (SCG) | Unknown Order | Unknown Family | Nitrososphaera | | | | a | 0.015% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Streptosporangiaceae | Nonomuraea | | ab | a | a | 0.013% | |
| | Bacteria | Acidobacteria | Acidobacteria Subgroup 22 | Acidobacteria Subgroup 22 | Acidobacteria Subgroup 22 | Acidobacteria Subgroup 22 | | ab | | a | 0.008% | Increased with eCeN |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bacteriovoracaceae | Bacteriovorax | | ab | a | a | 0.006% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Rhodocyclales | Rhodocyclaceae | Rhodocyclaceae | | | | a | 0.005% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhizobiales Incertae Sedis | Phreatobacter | | ab | a | a | 0.001% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 17 | Acidobacteria Subgroup 17 | Acidobacteria Subgroup 17 | | ab | a | a | 0.001% | |
| | Bacteria | Bacteroidetes | Flavobacteriia | Flavobacteriales | Flavobacteriaceae | Epilithonimonas | | | | a | 0.001% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Order Incertae Sedis | Family Incertae Sedis | Marinicella | | | | a | 0.0003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | Leisingera | | | | a | 0.0003% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteriales | Acidobacteriaceae Gp 1 | Bryocella | | | | a | 0.0003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Microbacteriaceae | Galbitalea | | | | a | 0.0003% | |

(a)

| | Kingdom | Phylum | Class | Order | Family | Genus | aCaN | eCaN | aCeN | eCeN | Abundance (%) | Treatment effects vs. aCaN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | a | ab | b | bc | c | |
| Le | Bacteria | Acidobacteria | Acidobacteria | Subgroup 4 | Acidobacteria Subgroup 4 | Acidobacteria Subgroup 4 | | | | | 5.806% | Increased with eCaN & eCeN |
| | Bacteria | Acidobacteria | Holophagae | Holophagae | Holophagae | Holophagae | | | | | 0.044% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | Phyllobacteriaceae | | | | | 0.037% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillales | Rhodospirillales | | | | | 0.021% | |
| | Bacteria | Verrucomicrobia | Spartobacteria | Chthoniobacterales | Chthoniobacterales | Chthoniobacterales | | | | | 0.020% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Legionellales | Coxiellaceae | Coxiellaceae | | | | | 0.008% | |
| | Bacteria | Elusimicrobia | Elusimicrobia | Lineage IIa | Lineage IIa | Lineage IIa | | | | | 0.007% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Gammaproteobacteria | Gammaproteobacteria | Gammaproteobacteria | | | | | 0.005% | |
| | Bacteria | Latescibacteria | Latescibacteria | Latescibacteria | Latescibacteria | Latescibacteria | | | | | 0.005% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylocystaceae | Methylocystaceae | | | | | 0.004% | |
| | Bacteria | Actinobacteria | Actinobacteria | Corynebacteriales | Mycobacteriaceae | Mycobacterium | | | | | 0.876% | Increased with aCeN |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Oxalobacteraceae | Noviherbaspirillum | | | | | 0.260% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Amycolatopsis | | | | | 0.108% | |
| | Bacteria | Actinobacteria | Thermoleophilia | Gaiellales | Gaiellales | Gaiellales | | | | | 0.030% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | Pantoea | | | | | 0.026% | |
| | Bacteria | Cyanobacteria | Melainabacteria | Obscuribacterales | Obscuribacterales | Obscuribacterales | | | | | 0.025% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Pseudorhodoferax | | | | | 0.016% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Acetobacteraceae | Rhodopila | | | | | 0.008% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Alphaproteobacteria | Alphaproteobacteria | Alphaproteobacteria | | | | | 0.008% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Microbacteriaceae | Marisediminicola | | | | | 0.006% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Leptothrix | | | | | 0.006% | |
| | Bacteria | Verrucomicrobia | Verrucomicrobia Incertae Sedis | Unknown Order | Unknown Family | Methylacidiphilum | | | | | 0.006% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Oxalobacteraceae | Oxalicibacterium | | | | | 0.005% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Rubrivivax | | | | | 0.004% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhodobiaceae | Rhodoligotrophos | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhizobiaceae | Shinella | | | | | 0.003% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Brucellaceae | Brucellaceae | | | | | 0.002% | |
| | Bacteria | Cyanobacteria | Cyanobacteria | SubsectionIII | FamilyI | FamilyI | | | | | 0.002% | |
| | Bacteria | Firmicutes | Bacilli | Bacillales | Bacillaceae | Oceanobacillus | | | | | 0.002% | |
| | Archaea | Thaumarchaeota | SAGMCG-1 | Unknown Order | Unknown Family | Nitrosotalea | | | | | 0.001% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Nostocoida | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Propionibacteriales | Nocardioidaceae | Nocardioidaceae | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Pseudonocardiales | Pseudonocardiaceae | Thermocrispum | | | | | 0.001% | |
| | Bacteria | Verrucomicrobia | Spartobacteria | Chthoniobacterales | Chthoniobacterales DA101 soil | Chthoniobacterales DA101 soil | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Tepidimonas | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | Schlegelella | | | | | 0.0003% | |
| | Bacteria | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadales | Gemmatimonadaceae | Gemmatimonas | | | | | 0.538% | None |
| | Bacteria | Nitrospirae | Nitrospira | Nitrospirales | Nitrospiraceae | Nitrospira | | | | | 0.483% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Actinoplanes | | | | | 0.462% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Rhizobiales Incertae Sedis | Bauldia | | | | | 0.155% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Pedomicrobium | | | | | 0.150% | |
| | Bacteria | Acidobacteria | Acidobacteria | Subgroup 5 | Acidobacteria Subgroup 5 | Acidobacteria Subgroup 5 | | | | | 0.090% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Micromonospora | | | | | 0.076% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Microbacteriaceae | Curtobacterium | | | | | 0.063% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Caulobacterales | Hyphomonadaceae | Woodsholea | | | | | 0.054% | |
| | Bacteria | Actinobacteria | Actinobacteria | Frankiales | Geodermatophilaceae | Blastococcus | | | | | 0.038% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Parachlamydiaceae | Protochlamydia | | | | | 0.038% | |
| | Bacteria | Actinobacteria | Actinobacteria | Streptosporangiales | Thermomonosporaceae | Actinoallomurus | | | | | 0.037% | |
| | Bacteria | Chlamydiae | Chlamydiae | Chlamydiales | Parachlamydiaceae | Parachlamydiaceae | | | | | 0.036% | |
| | Bacteria | Actinobacteria | Acidimicrobiia | Acidimicrobiales | Acidimicrobiaceae | CL500-29 marine group | | | | | 0.024% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | Polyangiaceae | | | | | 0.021% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Bdellovibrionales | Bacteriovoracaceae | Peredibacter | | | | | 0.017% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | Defluviicoccus | | | | | 0.015% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Microbacteriaceae | Frigoribacterium | | | | | 0.009% | |
| | Bacteria | Proteobacteria | Deltaproteobacteria | Oligoflexales | Oligoflexales | Oligoflexales | | | | | 0.008% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Rickettsiales Incertae Sedis | Captivus | | | | | 0.007% | |
| | Bacteria | Planctomycetes | Planctomycetacia | Planctomycetales | Planctomycetaceae | Blastopirellula | | | | | 0.006% | |
| | Bacteria | Actinobacteria | Actinobacteria | Kineosporiales | Kineosporiaceae | Kineosporiaceae | | | | | 0.005% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rickettsiales | Mitochondria | Pseudogymnoascus | | | | | 0.005% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 15 | Acidobacteria Subgroup 15 | Acidobacteria Subgroup 15 | | | | | 0.005% | |
| | Bacteria | Verrucomicrobia | Verrucomicrobiae | Verrucomicrobiales | Verrucomicrobiaceae | Roseimicrobium | | | | | 0.004% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cyclobacteriaceae | Cyclobacteriaceae | | | | | 0.004% | |
| | Bacteria | Firmicutes | Bacilli | Bacillales | Planococcaceae | Planococcus | | | | | 0.003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Cellulomonadaceae | Cellulomonadaceae | | | | | 0.003% | |
| | Bacteria | Firmicutes | Clostridia | Thermoanaerobacterales | Thermodesulfobiaceae | Coprothermobacter | | | | | 0.003% | |
| | Bacteria | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Dysgonomonas | | | | | 0.003% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micromonosporales | Micromonosporaceae | Pilimelia | | | | | 0.002% | |
| | Bacteria | Proteobacteria | Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | Serratia | | | | | 0.002% | |
| | Bacteria | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Sphingobacteriaceae | Solitalea | | | | | 0.002% | |
| | Bacteria | Acidobacteria | Acidobacteria | Acidobacteria Subgroup 18 | Acidobacteria Subgroup 18 | Acidobacteria Subgroup 18 | | | | | 0.001% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Flammeovirgaceae | Amoebophilus | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | Aquamicrobium | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | Azospirillum | | | | | 0.001% | |
| | Bacteria | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | Flexibacter | | | | | 0.001% | |
| | Bacteria | Actinobacteria | Actinobacteria | Micrococcales | Micrococcales | Micrococcales | | | | | 0.001% | |
| | Bacteria | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | Prosthecomicrobium | | | | | 0.001% | |

(b)

**Fig. S2.5** Genera with different abundance levels among treatments within legume plots. The color panel under ‘aCaN’, ‘eCaN’, ‘aCeN’ and ‘eCeN’ reflects the relative abundance level at each treatment by LSD test. Red toned cells, (a); Blush toned cells, (ab); White toned cells, (b); Sapphire toned cells, (bc); and Blue toned cells, (c).

| | pH | Moisture | Tm (°C) | TC (%) | TN (%) | $NO_3^+$ (mg $kg^{-1}$) | $NH_4^-$ (mg $kg^{-1}$) | AB (g $m^{-2}$) | RB (g $m^{-2}$) | AB-N (%) | AB-C/N | RB-N (%) | RB-C/N | RIB (g $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Acidimicrobiia* | | | -0.46* | | | | -0.48* | 0.71*** | -0.73*** | 0.68*** | -0.69*** | 0.63** | -0.63** | -0.84*** |
| *Acidobacteria* | | | -0.55** | | | 0.43* | -0.49* | 0.66*** | -0.77*** | 0.61** | -0.64** | 0.64*** | -0.66*** | -0.76*** |
| *Actinobacteria* | | | 0.52** | | | | 0.57** | | 0.47* | | | | | 0.46* |
| *Alphaproteobacteria* | 0.44* | | | | | | | | | | | | | |
| *Bacilli* | | | -0.50* | | 0.43* | | -0.49* | 0.43* | -0.56** | 0.43* | -0.44* | | | -0.54** |
| *Betaproteobacteria* | | | | | | 0.51* | | | | | | | | |
| *Chlamydiae* | | | | | | 0.52* | | 0.49* | | 0.48* | -0.51* | 0.46* | -0.45* | |
| *Chloroflexi* | 0.41* | | | | | | | 0.61** | -0.42* | 0.54** | -0.52** | | -0.43* | -0.43* |
| *Chthonomonadetes* | | | | | | | | | | | | | | |
| *Cyanobacteria* | | 0.58** | 0.45* | | | -0.69*** | | -0.82*** | 0.53** | -0.75*** | 0.76*** | -0.83*** | 0.83*** | 0.58** |
| *Cytophagia* | | | -0.41* | | 0.42* | | | 0.43* | -0.60** | 0.51* | -0.56** | 0.49* | -0.50* | -0.58** |
| *Deltaproteobacteria* | | | | | | | | | | | | | | |
| *Erysipelotrichia* | | 0.41* | 0.56** | | | -0.67*** | | -0.86*** | 0.69*** | -0.72*** | 0.73*** | -0.83*** | 0.85*** | 0.69*** |
| *Gammaproteobacteria* | | | -0.59** | | | 0.68*** | | 0.73*** | -0.67*** | 0.64*** | -0.71*** | 0.78*** | -0.80*** | -0.59** |
| *Gemmatimonadetes* | | | | | | | | | | | | | | |
| *Holophagae* | | | | | | | | | | | | | | |
| *Ktedonobacteria* | | | 0.53** | | | -0.54** | 0.42* | -0.73*** | 0.72*** | -0.68*** | 0.70*** | -0.78*** | 0.78*** | 0.74*** |
| *Melainabacteria* | | | | | | | | | | | | | | |
| *Nitrospira* | | | -0.55** | | | | -0.55** | 0.51* | -0.65*** | 0.49* | -0.52* | 0.44* | -0.48* | -0.66*** |
| *Phycisphaerae* | | 0.45* | | | | -0.56** | | | | | 0.46* | -0.61** | 0.58** | |
| *Planctomycetacia* | | -0.41* | | | | 0.41* | | 0.44* | | | -0.43* | | | |
| *Soil Crenarchaeotic Group* | | | -0.57** | | | | -0.48* | | -0.52** | | -0.42* | | -0.43* | -0.55** |
| *Spartobacteria* | | | | 0.43* | 0.43* | 0.59** | | 0.51* | -0.45* | 0.45* | -0.50* | 0.46* | -0.48* | |
| *Sphingobacteriia* | | | -0.44* | | | | | | -0.43* | | | | | |
| *TakashiAC-B11* | | | | | | | | | | | | | | |
| *Thermoleophilia* | | | 0.65*** | | | | 0.49* | -0.71*** | 0.80*** | -0.63** | 0.68*** | -0.62** | 0.66*** | 0.77*** |

**Fig. S2.6** Spearman's rank correlation coefficient rho of selected classes (with different abundance levels among treatments) with environmental attributes. Coral toned cells represent significant positive correlations; Cyan toned cells represent significant negative correlations. Only classes with different abundance levels among treatments were subjected to this test. *P* values have been adjusted for multiple comparisons by FDR. The first seven columns correspond to correlations of each phylum with soil attributes including pH, moisture, temperature (Tm), total C (TC) and N (TN) content, nitrate, and ammonium. The next seven columns correspond to correlations of each phylum with plant attributes including aboveground biomass (AB), root biomass (RB) ( 0-20 cm, fine roots), aboveground biomass N (AB-N) and C/N (AB-C/N), root biomass N (RB-N) and C/N (RB-C/N), and root ingrowth biomass (RIB). Soil properties were data collected from the sampling year (2009). All plant properties were calculated as the sum of six harvests measured in both June and August from 2007-2009.

| | pH | Moisture | Tm (°C) | TC (%) | TN (%) | $NO_3^+$ (mg $kg^{-1}$) | $NH_4^-$ (mg $kg^{-1}$) | AB (g $m^{-2}$) | RB (g $m^{-2}$) | AB-N (%) | AB-C/N | RB-N (%) | RB-C/N | RIB (g $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Metachlamydia* | | | | | | 0.64*** | | 0.48* | -0.49* | 0.47* | -0.45* | 0.55** | -0.51* | |
| *Nitrososphaera* | | | -0.57** | | | | -0.48* | | -0.52** | | -0.42* | | -0.43* | -0.55** |
| *Protochlamydia* | | | | | | 0.64*** | | 0.48* | -0.49* | 0.47* | -0.45* | 0.55** | -0.51* | |
| *Acidimicrobiales* | | | -0.41* | | | | | 0.75*** | -0.69*** | 0.79*** | -0.77*** | 0.70*** | -0.70*** | -0.74*** |
| *Acidiphilium* | | | | | | | 0.50* | | | | | | | 0.52** |
| *Acidobacteria Subgroup 17* | | | | | | | | | | | | | | |
| *Acidobacteria Subgroup 3* | | | | -0.44* | -0.54** | | | -0.42* | | | | -0.50* | 0.44* | 0.45* |
| *Acidobacteria Subgroup 4* | | | -0.53** | | | 0.45* | -0.47* | 0.67*** | -0.78*** | 0.60** | -0.64** | 0.65*** | -0.66*** | -0.76*** |
| *Acidobacteria Subgroup 5* | | 0.49* | | | | | | | | | | | | |
| *Actinoallomurus* | | | 0.42* | | | | 0.48* | | | | | | | 0.43* |
| *Actinomadura* | | | | | | | 0.53** | | 0.42* | | | | | 0.44* |
| *Actinoplanes* | | | 0.52** | | | | 0.41* | | | | | | | |
| *Adhaeribacter* | | | | | | 0.46* | | 0.58** | -0.55** | 0.64*** | -0.68*** | 0.65*** | -0.65*** | -0.62** |
| *Amycolatopsis* | | | 0.49* | | | | 0.58** | | 0.59** | | | | | 0.66*** |
| *Arenimonas* | | | -0.42* | | | | -0.42* | | -0.61** | | | | | -0.48* |
| *Asteroleplasma* | | 0.41* | 0.56** | | | -0.67*** | | -0.86*** | 0.69*** | -0.72*** | 0.73*** | -0.83*** | 0.85*** | 0.69*** |
| *Bauldia* | | | -0.42* | | | 0.50* | | 0.73*** | -0.70*** | 0.73*** | -0.75*** | 0.70*** | -0.70*** | -0.65*** |
| *Bdellovibrio* | | | -0.58** | 0.42* | | | | 0.55** | -0.68*** | 0.63** | -0.63** | 0.62** | -0.63** | -0.72*** |
| *Beijerinckiaceae* | | | | | | | | | | | | | | |
| *Betaproteobacteria, other* | | | | | | | | | | | | | | |
| *Blastococcus* | | | 0.49* | | | | | | | | | | | |
| *Bradyrhizobiaceae* | | | | | | | 0.48* | -0.62** | 0.59** | -0.51* | 0.54** | -0.51* | 0.52* | 0.63** |
| *Candidatus Protochlamydia* | | | | | | | | | | | | | | |
| *Catenulispora* | | | | | | | | | 0.44* | | | | | 0.45* |
| *Chitinophagaceae* | | | | | | | | | -0.52** | | -0.47* | 0.50* | -0.51* | -0.50* |
| *Chlamydiales* | -0.41* | | | | | | | | | | | | | |
| *Chloroflexi* | 0.41* | | | | | | | 0.61** | -0.42* | 0.54** | -0.52** | | -0.43* | -0.43* |
| *Chthoniobacterales* | | | | 0.43* | 0.43* | 0.59** | | 0.51* | -0.45* | 0.45* | -0.50* | 0.46* | -0.48* | |
| *Chthonomonas* | | | | | | | | | | | | | | |
| *CL500-29 marine group* | | | | | | -0.60** | | | | -0.44* | 0.44* | -0.55** | 0.56** | |
| *Cohnella* | | | | | | | -0.55** | | -0.45* | | | | | -0.51* |
| *Corynebacteriales* | -0.42* | | | | | | | | | | | | | |
| *Coxiella* | | | -0.46* | | | 0.43* | -0.46* | 0.66*** | -0.56** | 0.54** | -0.59** | 0.58** | -0.58** | -0.61** |
| *Cryptosporangium* | | -0.48* | | | | | | 0.53** | -0.55** | 0.55** | -0.61** | 0.69*** | -0.66*** | -0.60** |
| *Curtobacterium* | | | | 0.43* | 0.41* | | | | | | | | | |
| *Cytophagaceae* | | | | | | | | | -0.44* | | | | | -0.42* |
| *Dactylosporangium* | | | 0.66*** | | | | 0.46* | -0.49* | 0.71*** | -0.52* | 0.57** | -0.54** | 0.60** | 0.68*** |
| *Defluviicoccus* | | | | | | | | | | | | | | |
| *Deltaproteobacteria GR-WP33-30* | | | | | | | -0.66*** | | | | | | | -0.43* |
| *Devosia* | | -0.45* | -0.46* | 0.50* | 0.42* | 0.72*** | | 0.75*** | -0.63*** | 0.80*** | -0.82*** | 0.82*** | -0.82*** | -0.57** |
| *Dokdonella* | | | | | | | 0.73*** | | 0.51* | | | | | 0.64*** |
| *FamilyI* | | 0.58** | 0.45* | | | -0.69*** | | -0.82*** | 0.53** | -0.75*** | 0.76*** | -0.83*** | 0.83*** | 0.58** |
| *Frankiales* | | | | | | | | | | | | | | |
| *Gaiellales* | | | | | | | | | | | | | | |
| *Gammaproteobacteria NKB5* | | | -0.53** | | | | | 0.51* | -0.47* | 0.49* | -0.57** | 0.45* | -0.48* | -0.43* |
| *Gemmata* | | | | | | | | | | | | | | |
| *Gemmatimonas* | | | | | | | | | | | | | | |
| *Haliangium* | | | | | | -0.42* | | -0.57** | 0.54** | -0.46* | 0.56** | -0.62** | 0.59** | 0.61** |
| *Hirschia* | | | -0.47* | | | | | 0.48* | -0.60** | 0.48* | -0.50* | 0.41* | -0.45* | -0.54** |
| *Holophagae* | | | | | | | | | | | | | | |
| *Holophagae Subgroup 10* | | | 0.47* | | | | | | | | | | | |
| *Holosporaceae* | | 0.47* | | | | | | | | | | | | |
| *Hyphomicrobium* | | | -0.46* | | | | | | | | | | | |
| *Ideonella* | | | | | | | | | | | | | | |
| *Intrasporangiaceae* | | -0.48* | | | | | | | | | | | | |
| *Jatrophihabitans* | | | 0.42* | | | | | | | | | | | |
| *Kibdelosporangium* | | | 0.56** | | | | 0.49* | -0.46* | 0.71*** | | | -0.50* | 0.50* | 0.73*** |
| *Kribbella* | | -0.55** | | | | 0.70*** | | 0.52** | | | -0.45* | 0.58** | -0.55** | |
| *Ktedonobacteria* | | | 0.53** | | | -0.54** | 0.42* | -0.73*** | 0.72*** | -0.68*** | 0.70*** | -0.78*** | 0.78*** | 0.74*** |
| *Labrys* | | | | | | | | | | | | | | |
| *Legionella* | | | | | | | | | | | | | | |
| *Luteibacter* | | | | | | 0.64*** | | 0.52** | -0.46* | 0.51* | -0.55** | 0.72*** | -0.66*** | -0.45* |
| *Lysobacter* | | | -0.43* | | | | | 0.59** | -0.61** | 0.44* | -0.48* | 0.48* | -0.50* | -0.62** |
| *Mesorhizobium* | | | 0.44* | | | | | | | | | | | |
| *Microbispora* | | | 0.68*** | | | -0.45* | | -0.74*** | 0.81*** | -0.72*** | 0.78*** | -0.77*** | 0.80*** | 0.83*** |
| *Micrococcus* | | | | | | | | | | | | | | |
| *Micromonospora* | | | | | | | | | | | | | | |
| *Micromonosporaceae* | | | 0.62** | | | | 0.48* | | 0.58** | | 0.41* | | | 0.51* |
| *Modestobacter* | | | 0.48* | | | | 0.51* | | 0.44* | | | | | |
| *Mycobacterium* | | -0.56** | | | | | 0.47* | | | | | | | |
| *Myxococcales* | | | -0.49* | | | | | 0.47* | -0.51* | 0.64*** | -0.58** | 0.47* | -0.52** | -0.49* |

(a)

| | pH | Moisture | Tm (°C) | TC (%) | TN (%) | $NO_3^+$ (mg $kg^{-1}$) | $NH_4^-$ (mg $kg^{-1}$) | AB (g $m^{-2}$) | RB (g $m^{-2}$) | AB-N (%) | AB-C/N | RB-N (%) | RB-C/N | RIB (g $m^{-2}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Niabella* | | | | | | | 0.42* | | | | | | | 0.49* |
| *Nitrosomonadaceae* | | | | | | | | | | | | | | |
| *Nitrospira* | | | -0.55** | | | | -0.55** | 0.51* | -0.65*** | 0.49* | -0.52* | 0.44* | -0.48* | -0.66*** |
| *Nonomuraea* | | | | | | | | | | | | | | |
| *Noviherbaspirillum* | | | | | | 0.52** | | | | | | | | |
| *Obscuribacterales* | | | | | | | | | | | | | | |
| *Oxalobacteraceae* | | | -0.44* | 0.48* | 0.46* | 0.62** | | 0.53** | -0.55** | 0.57** | -0.60** | 0.71*** | -0.66*** | -0.45* |
| *Paenibacillus* | | | -0.50* | | 0.42* | | -0.49* | 0.44* | -0.56** | 0.46* | -0.47* | 0.42* | -0.43* | -0.54** |
| *Pantoea* | | | | | | | | 0.44* | -0.57** | 0.46* | -0.47* | 0.48* | -0.48* | -0.53** |
| *Parachlamydiaceae* | | | | | | 0.64*** | | 0.48* | -0.49* | 0.47* | -0.45* | 0.55** | -0.51* | |
| *Patulibacter* | | | 0.65*** | | | | 0.43* | -0.55** | 0.67*** | -0.48* | 0.53** | -0.46* | 0.50* | 0.60** |
| *Pedomicrobium* | 0.45* | | | | | | | | | | | | | |
| *Peredibacter* | | | -0.55** | | | | | 0.50* | -0.70*** | 0.64*** | -0.62** | 0.49* | -0.48* | -0.62** |
| *Phycisphaeraceae* | | | | | | | | | | | | | | |
| *Phycisphaerae WD2101 soil group* | | | | | | -0.60** | | -0.43* | | -0.43* | 0.49* | -0.61** | 0.59** | |
| *Phyllobacteriaceae* | 0.52** | | | | | | | | | | | | | |
| *Pir4 lineage* | | | | | | | | 0.53** | -0.45* | | -0.45* | | | -0.53** |
| *Pla1 lineage* | | | | | | | | | | | | | | |
| *Planctomyces* | | | -0.63** | | | 0.43* | | 0.62** | -0.60** | 0.60** | -0.63** | 0.50* | -0.55** | -0.55** |
| *Planosporangium* | | | 0.58** | | | | 0.58** | -0.48* | 0.72*** | -0.45* | 0.46* | -0.50* | 0.50* | 0.72*** |
| *Polyangiaceae* | | | 0.58** | | | -0.49* | 0.45* | -0.71*** | 0.64*** | -0.63** | 0.65*** | -0.70*** | 0.73*** | 0.70*** |
| *Pseudonocardia* | | -0.48* | | | | | 0.51* | | | | | | | |
| *Pseudorhodoferax* | | | | | 0.44* | | | | | | | 0.44* | -0.41* | -0.46* |
| *Rhabdochlamydia* | | | | | | 0.64*** | | 0.48* | -0.49* | 0.47* | -0.45* | 0.55** | -0.51* | |
| *Rhodomicrobium* | | | | | | | | -0.41* | 0.48* | | | | | |
| *Rhodoplanes* | | | | | | -0.46* | | -0.76*** | 0.76*** | -0.74*** | 0.75*** | -0.76*** | 0.73*** | 0.75*** |
| *Rhodospirillales* | | | | | | | | | -0.61** | | | | | -0.46* |
| *Saccharopolyspora* | | | 0.44* | -0.43* | -0.55** | | 0.60** | -0.43* | 0.62** | -0.51* | 0.52** | -0.49* | 0.48* | 0.70*** |
| *Singulisphaera* | | | | | | | | | | | | | | |
| *Solirubrobacterales* | | | 0.62** | | | | 0.46* | -0.68*** | 0.87*** | -0.65*** | 0.68*** | -0.72*** | 0.75*** | 0.85*** |
| *Sorangium* | | | 0.58** | | | -0.49* | 0.45* | -0.71*** | 0.64*** | -0.63** | 0.65*** | -0.70*** | 0.73*** | 0.70*** |
| *Sphingobacteriales* | | | -0.43* | | | | | | | | | | | |
| *Sphingobacteriales env.OPS 17* | | | | | | | | | -0.52** | | -0.47* | 0.50* | -0.51* | -0.50* |
| *Sphingomonadales* | | -0.65*** | | | | 0.53** | | 0.55** | | 0.53** | -0.54** | 0.61** | -0.61** | |
| *Sporocytophaga* | | | | | | | | 0.44* | -0.57** | 0.55** | -0.55** | 0.43* | -0.42* | -0.58** |
| *Tahibacter* | | | -0.42* | | | | -0.41* | 0.42* | -0.61** | | | 0.45* | -0.46* | -0.49* |
| *TakashiAC-B11* | | | | | | | | | | | | | | |
| *Thaumarchaeota SCG* | | | -0.57** | | | | -0.48* | | -0.52** | | -0.42* | | -0.43* | -0.55** |
| *Virgisporangium* | | -0.41* | 0.49* | | | | | | | | | | | |
| *Woodsholea* | | | 0.49* | | | -0.48* | | -0.75*** | 0.69*** | -0.79*** | 0.79*** | -0.78*** | 0.80*** | 0.71*** |

(b)

**Fig. S2.7** Spearman's rank correlation coefficient rho of selected genera (with different abundance levels among treatments) with environmental attributes. Coral toned cells represent significant positive correlations; Cyan toned cells represent significant negative correlations. Only genera with different abundance levels among treatments were subjected to this test. *P* values have been adjusted for multiple comparisons by FDR. The first seven columns correspond to correlations of each phylum with soil attributes including pH, moisture, temperature (Tm), total C (TC) and N (TN) content, nitrate, and ammonium. The next seven columns correspond to correlations of each phylum with plant attributes including aboveground biomass (AB), root biomass (RB) ( 0-20 cm, fine

roots), aboveground biomass N (AB-N) and C/N (AB-C/N), root biomass N (RB-N) and C/N (RB-C/N), and root ingrowth biomass (RIB). Soil properties were data collected from the sampling year (2009). All plant properties were calculated as the sum of six harvests measured in both June and August from 2007-2009.

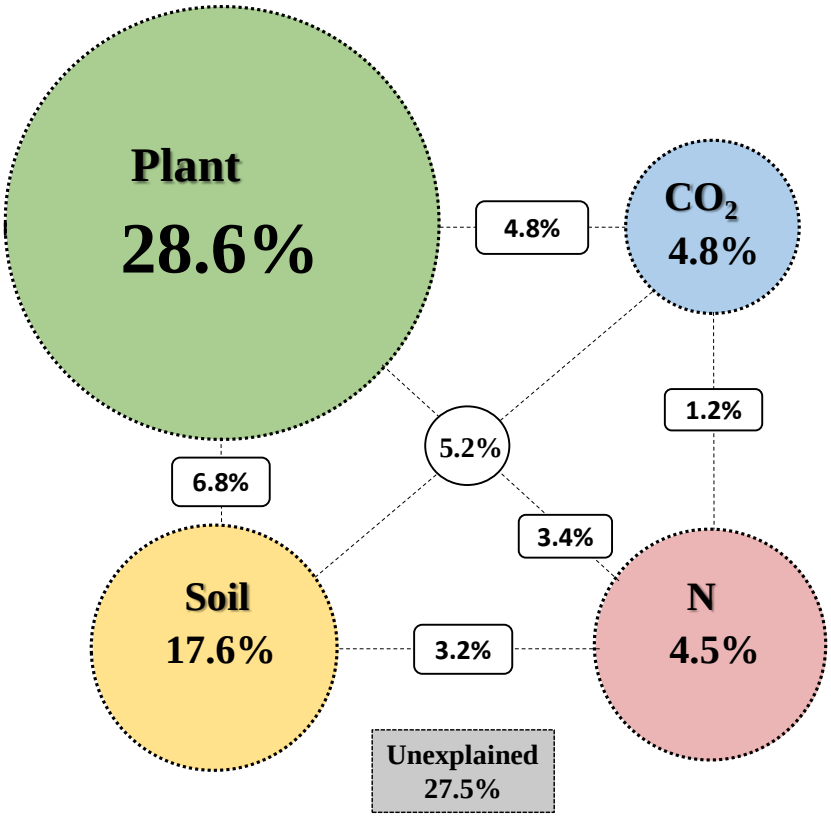



**Fig. S2.8** Variation partitioning analysis (VPA) of microbial community structure explained by plant properties, soil geochemical variables, $CO_2$, and N. Each diagram represents the biological variation partitioned into the relative effects of each factor or a combination of factors. Only contribution of variation larger than 1% were shown. The same sets of plant and soil properties screened for Mantel tests were used. The concentrations of $CO_2$ are 368 p.p.m. for ambient and 560 p.p.m. for elevated plots; the N addition are 0 $g \cdot m^{-1} \cdot yr^{-1}$ for ambient and 4 $g \cdot m^{-1} \cdot yr^{-1}$ for elevated plots.

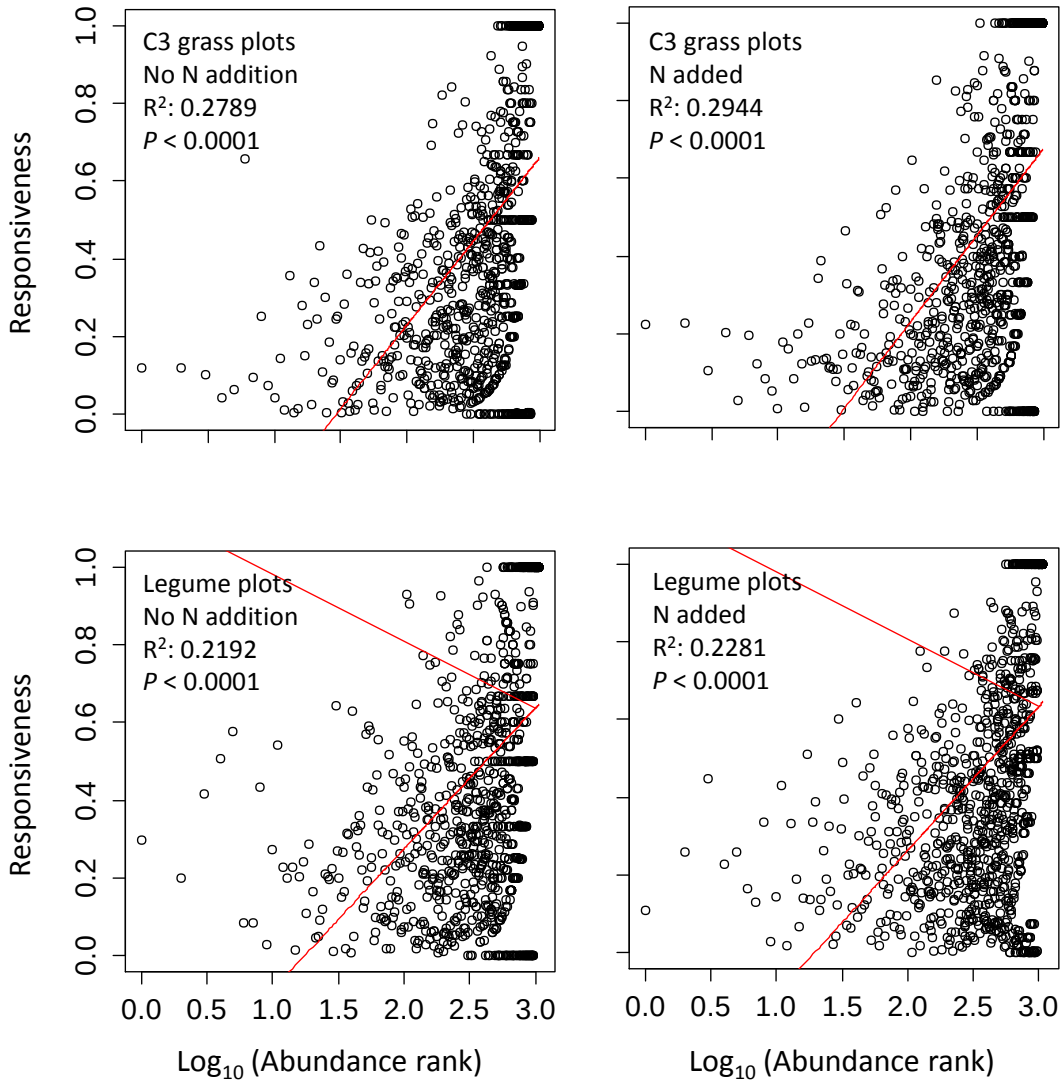



**Fig. S2.9** Relative difference of each genus between treatment and control as a function of genus abundance rank in control. The relative differences are calculated as $|R_{treatment}-R_{control}|/Max\{R_{treatment}, R_{control}\}$, where $R_{treatment}$ is the relative abundance of certain genus in treatment plots, $R_{control}$ is the relative abundance of the same genus in control plots receiving no elevated $CO_2$ and no N addition. Each circle in the plot represents a detected genus. R square values and P values are based on linear regression analysis.

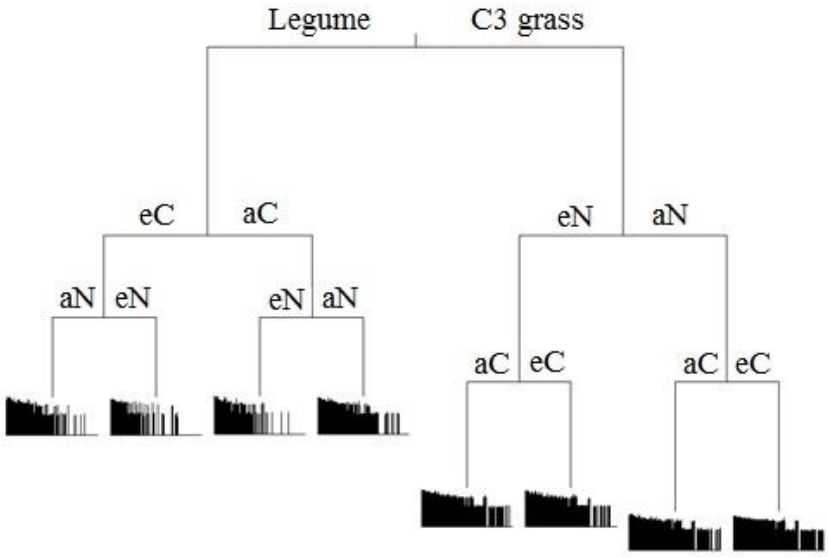



**Fig. S3.1** Multivariate regression tree (MRT) analysis to illustrate the relative contributions of plant functional group (C3 grass and legume), $eCO_2$ and eN in shaping microbial functional composition and structure derived from GeoChip 3.0.

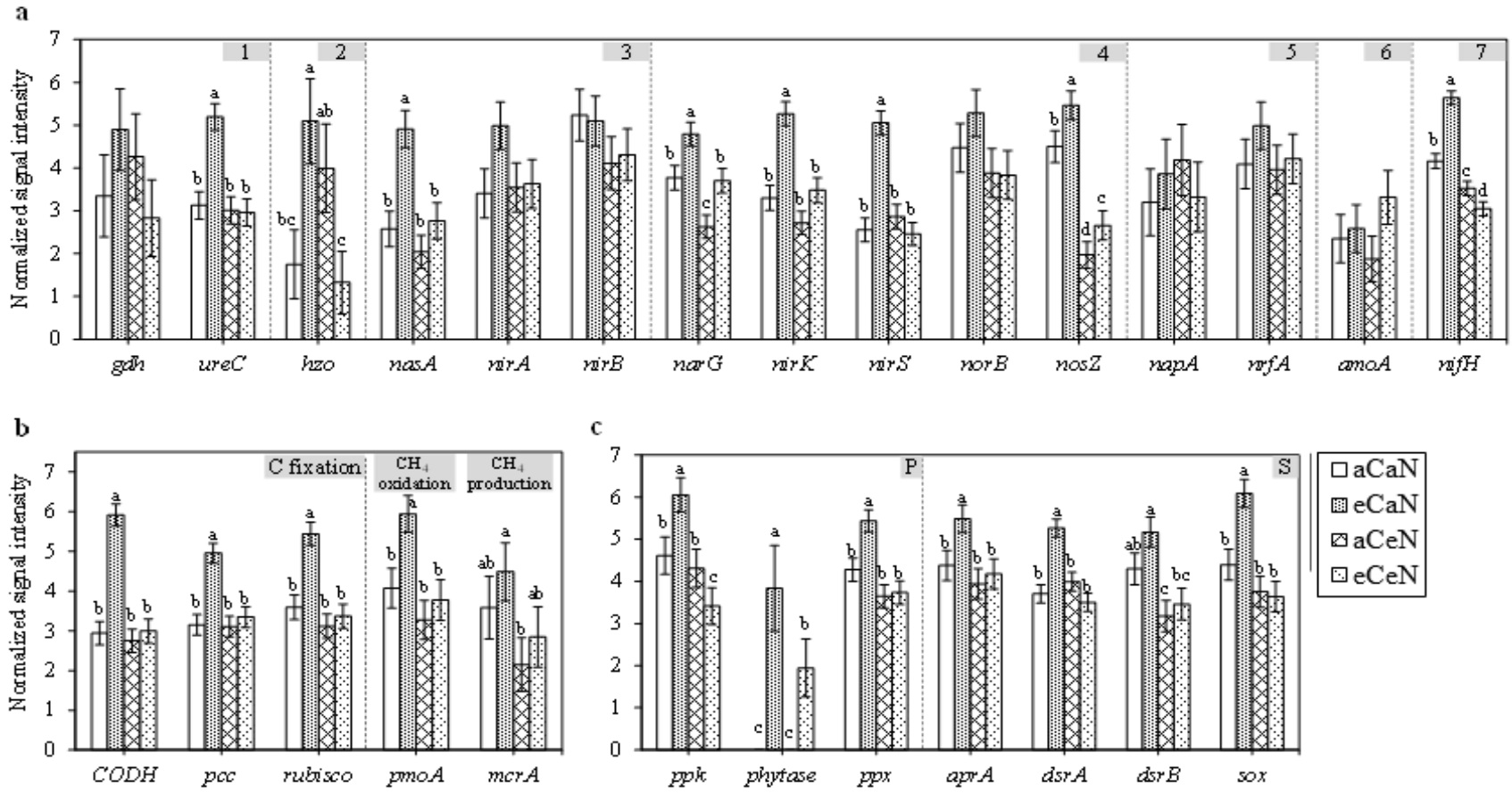



**Fig. S3.2** Elevated $CO_2$ and eN effects on genes in C3 grass plots involved in N cycle (a), C fixation and $CH_4$ processes (b), and P/S cycles (c). The processes involved in N cycle include ammonification (1), Anammox (2), assimilatory N reduction (3), denitrification (4), dissimilatory N reduction (5), nitrification (6), and N fixation (7). Bars labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment.

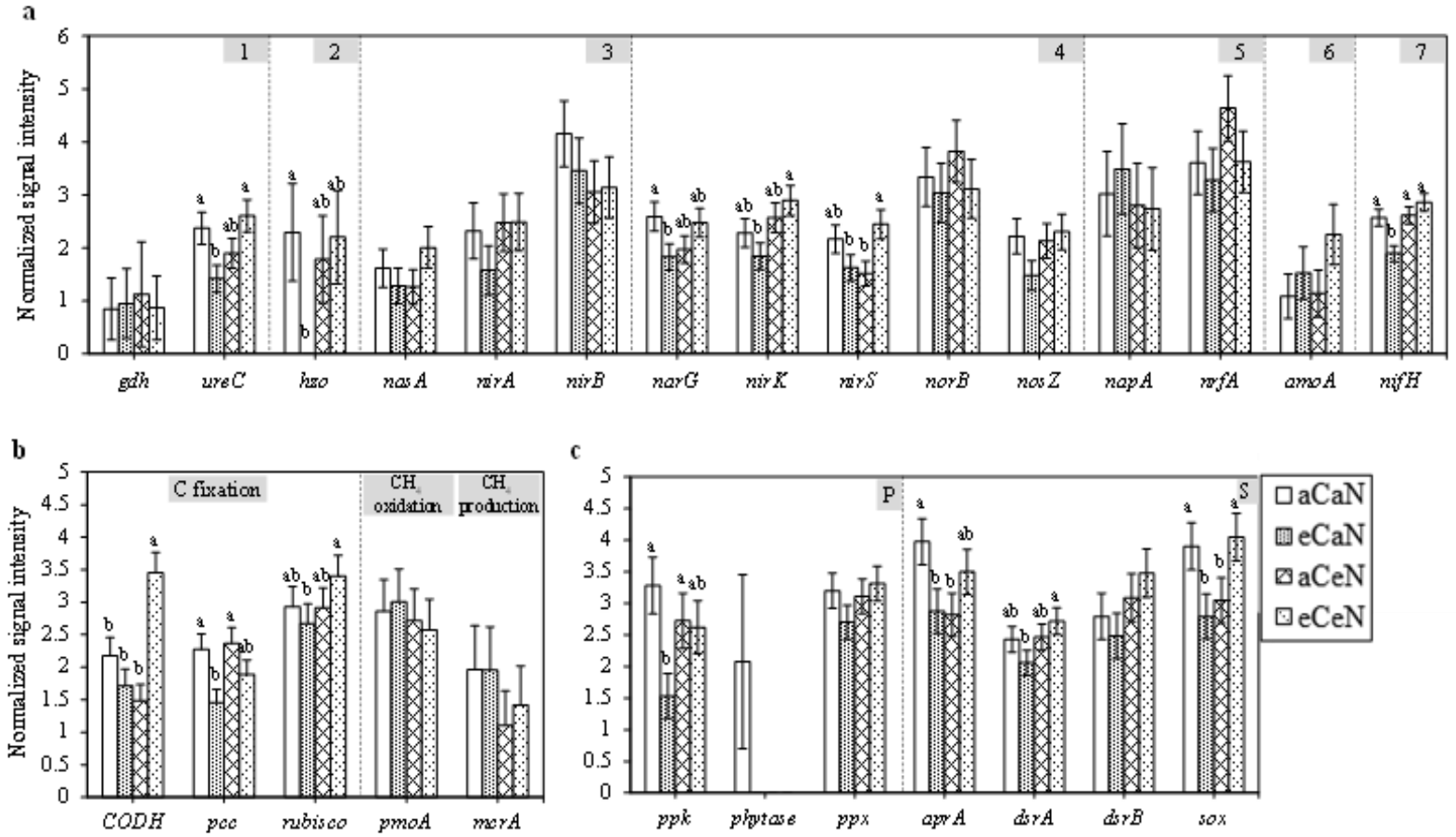



**Fig. S3.3** Elevated $CO_2$ and eN effects on genes in legume plots involved in N cycle (a), C fixation and $CH_4$ processes (b), and P/S cycles (c). The processes involved in N cycle include ammonification (1), Anammox (2), assimilatory N reduction (3), denitrification (4), dissimilatory N reduction (5), nitrification (6), and N fixation (7). Bars labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment.

(a)

| C degradation genes | Detected gene No. | SCF[a] | HR[b] |
|---|---|---|---|
| **All** | **692** | ** | ** |
| Starch | 131 | * | |
| Hemicellulose | 85 | | |
| Cellulose | 51 | ** | ** |
| Pectinase | 9 | | ** |
| Chitin | 68 | | ** |
| Aromatics | 282 | ** | ** |
| Lignin | 66 | ** | * |

(b)

| C fixation genes | Detected gene No. | $^{13}$C sequestration |
|---|---|---|
| **All** | **234** | ** |
| CODH | 68 | ** |
| *pcc* | 95 | * |
| Rubisco | 71 | * |

(c)

| N fixation gene | Detected gene No. | $^{15}$N fixation |
|---|---|---|
| *nifH* | **235** | * |

(d)

| Processes | Genes | Detected gene No. | Mantel test |
|---|---|---|---|
| Ammonification rate | **All** | **69** | ** |
| | *ureC* | 62 | *** |
| | *gdh* | **7** | |
| Nitrification rate | **All** | **17** | ** |
| | bacterial *amoA* | 6 | ** |
| | archaeal *amoA* | 10 | |
| | *hao* | 1 | |

[a] Soil C flux.
[b] Heterotrophic respiration.

**Fig. S3.4** Significance of correlations between individual or all genes involved in C degradation and SCF or HR (a); genes involved in C fixation and $^{13}$C sequestration strength (b); *nifH* gene involved in N fixation and $^{15}$N fixation across samples at aN plots (c); genes involved in ammonification or nitrification and soil N processes (d). Significance is represented by ** when $p<0.05$ and * when $p<0.10$.

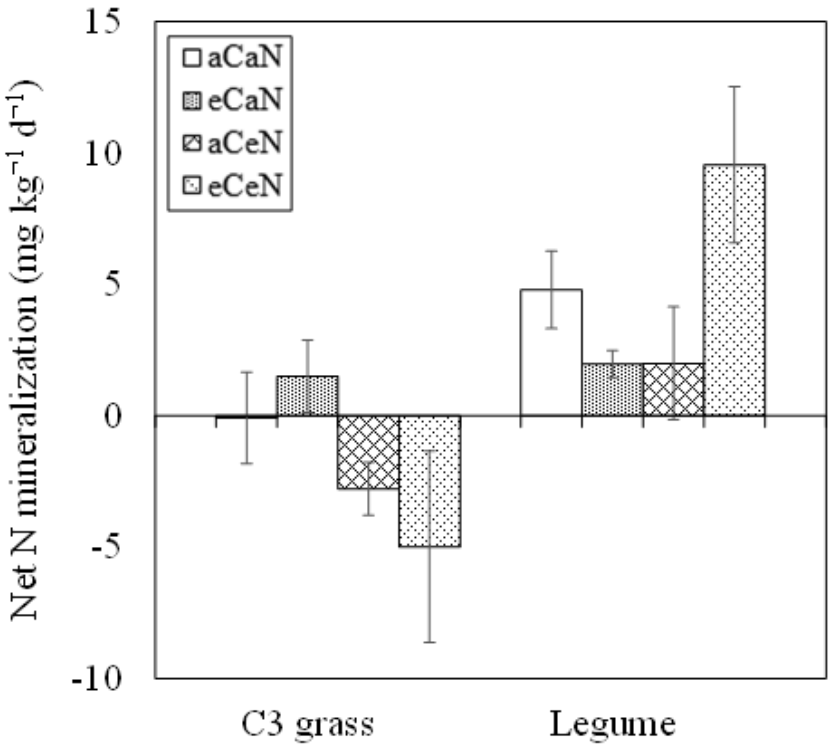



**Fig. S3.5** Quantitative $^{13}C$ and $^{15}N$ enrichment in soils from C3 grass and legume plots at $CO_2$ and N treatments.

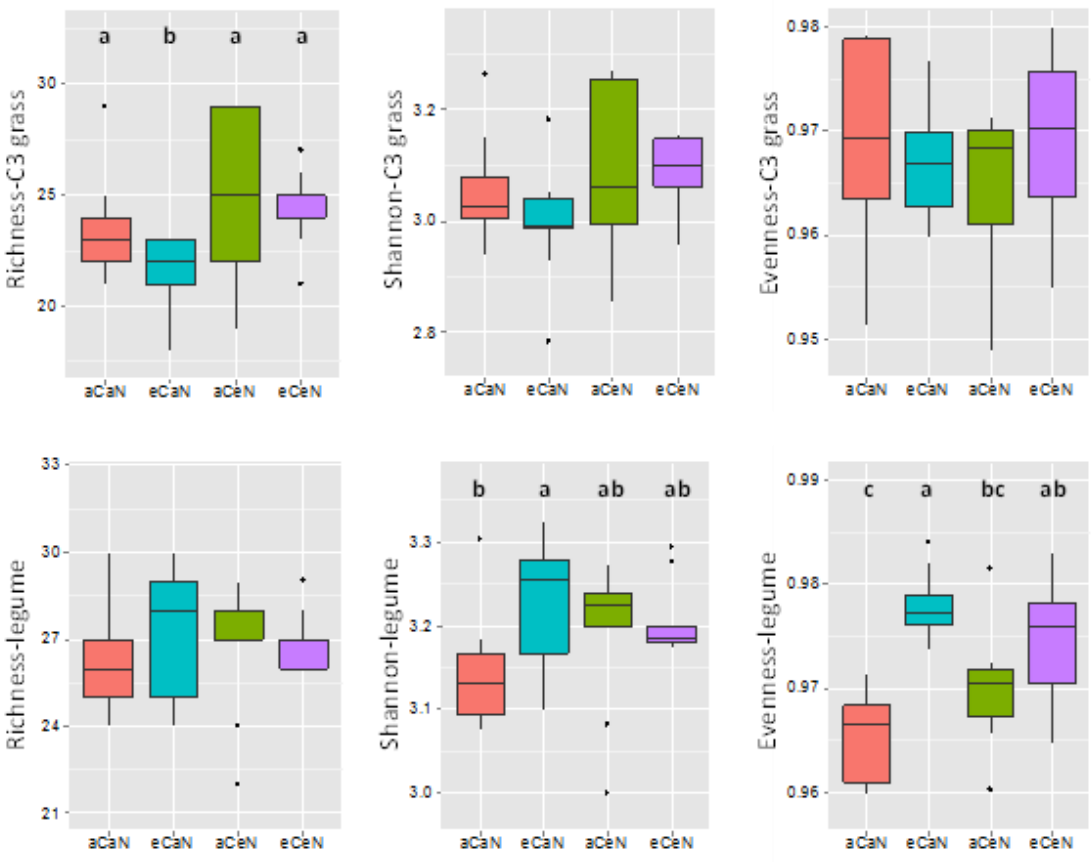



**Fig. S4.1** Effects of $eCO_2$ and eN on microbial metabolic diversity represented by Richness, Shannon, and Evenness. Boxes labeled with different letters are significantly different ($P < 0.05$) according to ANOVA, followed by Fisher's least significant difference (LSD) test with Holm-Bonferroni adjustment. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

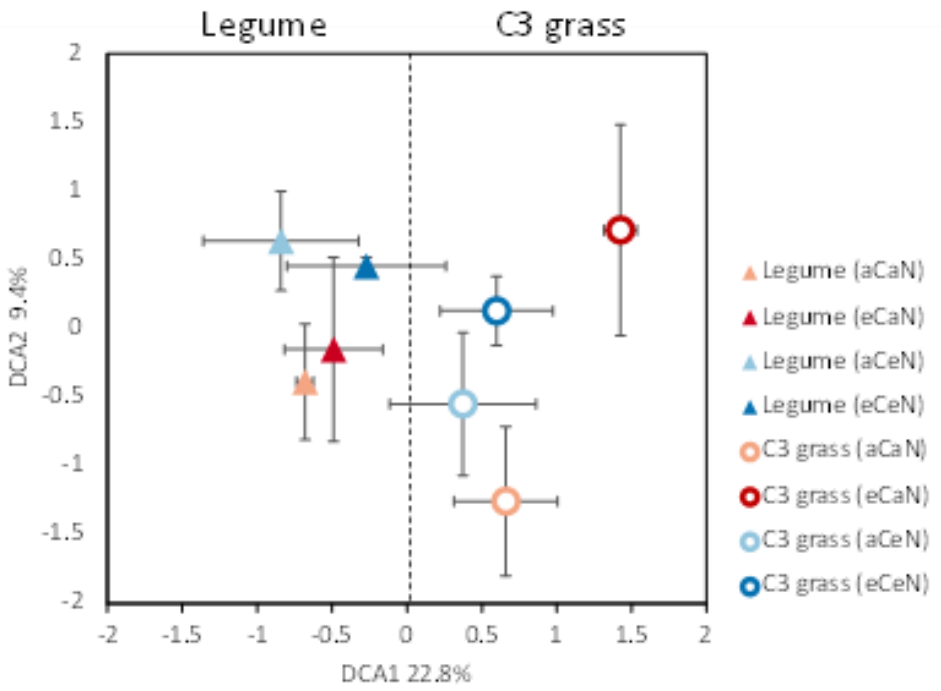



**Fig. S4.2** DCA analysis of microbial communities based on metabolic diversity obtained through Biolog EcoPlate after 156h incubation. Abbreviations: aCaN: ambient $CO_2$ and no fertilization; eCaN: elevated $CO_2$ and no fertilization; aCeN: ambient $CO_2$ and fertilized; eCeN: elevated $CO_2$ and fertilized.

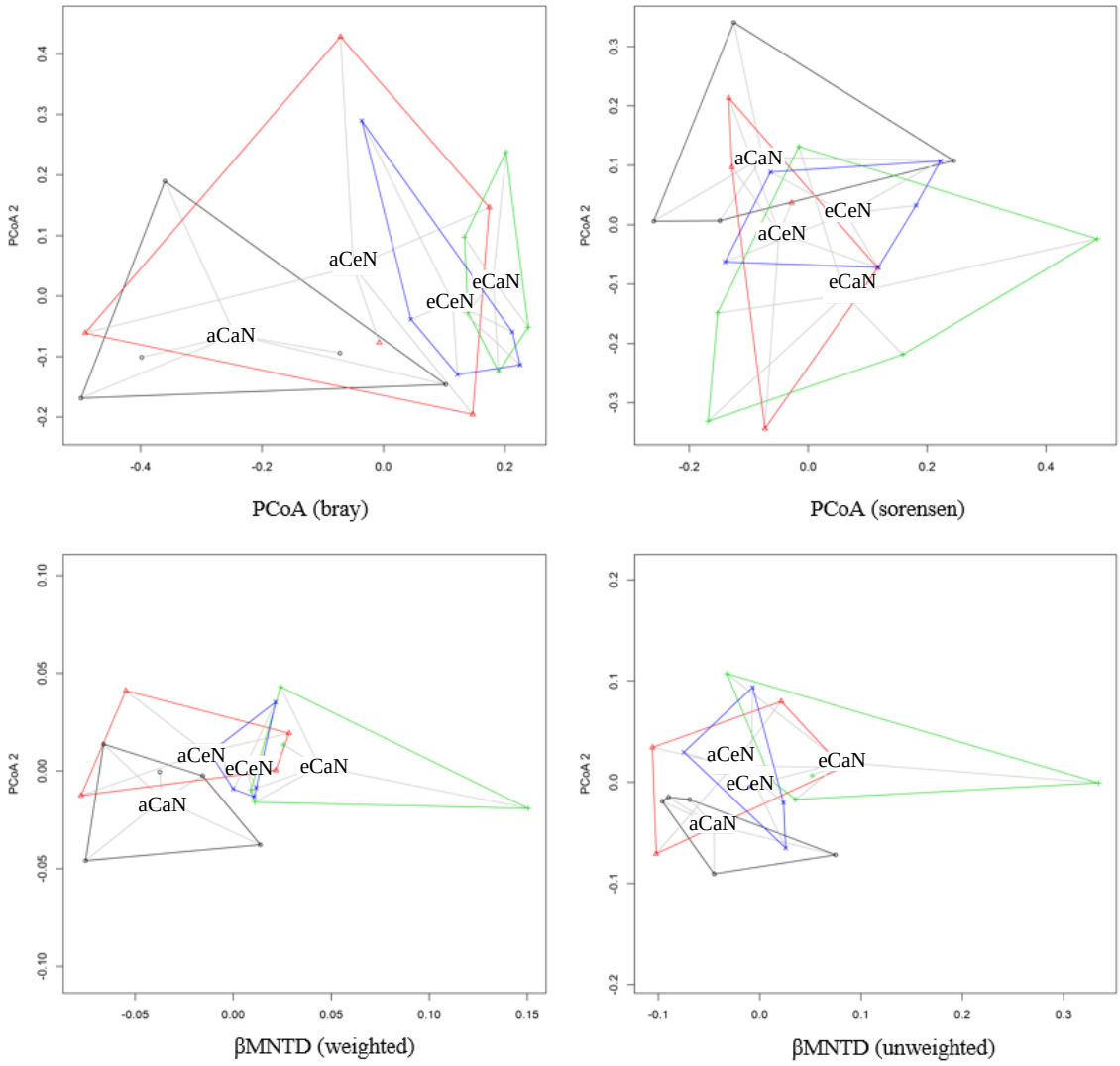



**Fig. S5.1** UniFrac PCoA and βMNTD analyses of the archaeal-*amoA* community. Gray: aCaN; Red: aCeN; Blue: eCeN; Green: eCaN.

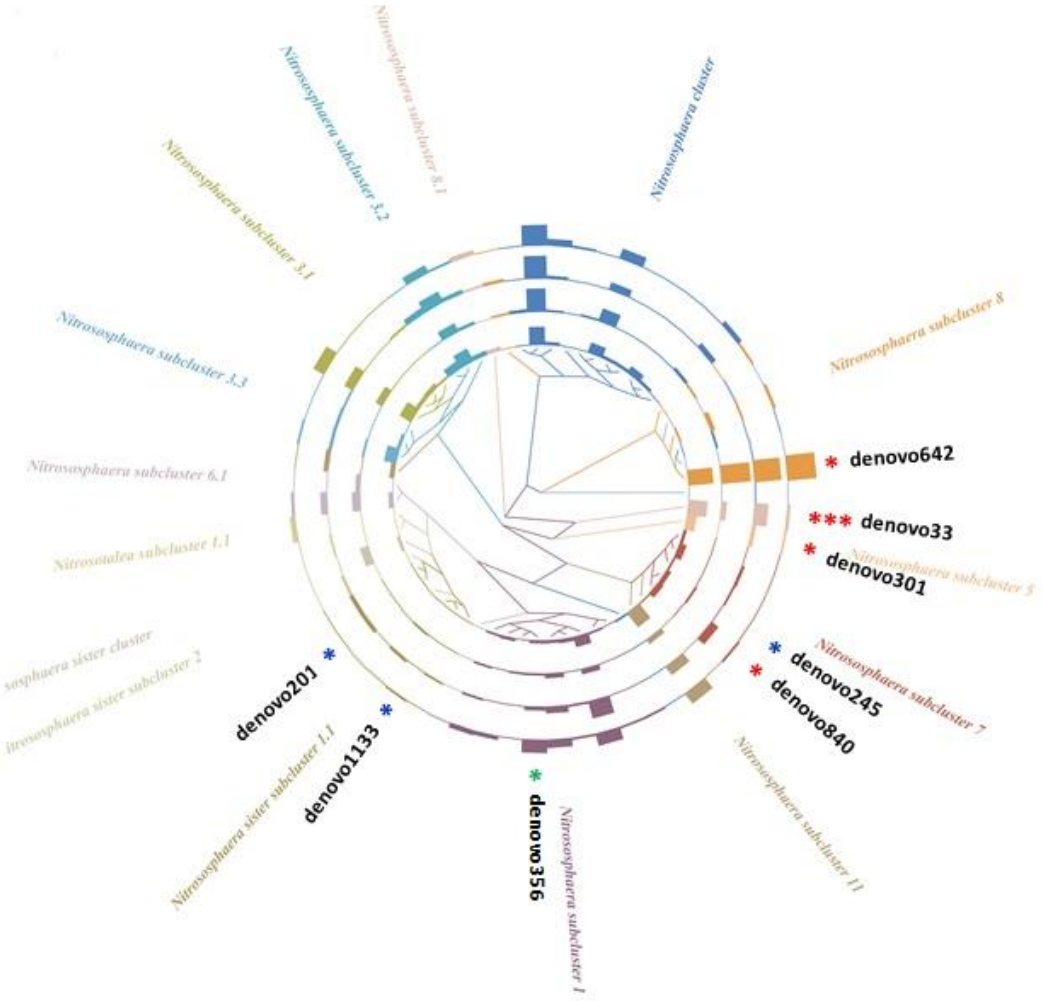



**Fig. S5.2** Detection frequency and relative abundances of archaeal-*amoA* genes at OTU level in the four $CO_2$ and N conditions. Phylogenetic tree was constructed based on neighbor-joining criterion. Rings from inner to outer represents aCaN, eCaN, aCeN, and eCeN conditions, respectively. * in red, blue and green represent significant $CO_2$, N and their interactive effects, respectively (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ANOVA test).