ABSTRACT

Microbial Community Structure, Function, and Assembly in Texas Prairie Soils: Insights from a Preindustrial-to-Future CO_2 Enrichment Gradient

Swastika Raut, Ph.D.

Mentor: Ryan S. King, Ph.D.

Anthropogenic activities have escalated recent increases in atmospheric CO_2 concentration and significantly contributed to global climate change since the industrial revolution. Direct and indirect effects of atmospheric CO_2 enrichment on terrestrial ecosystems have primarily focused on aboveground vegetation. Although soil microbial communities play a crucial role in nutrient cycling processes, we lack a comprehensive understanding of their sensitivity, resistance, or resilience to atmospheric CO_2 enrichment across divergent ecosystems. Furthermore, most long-term CO_2 enrichment studies only include elevated versus ambient CO_2 concentrations and rarely consider more than one soil type or plant species. My dissertation research focuses on how microbial community structure, function, and assembly processes are affected by a decade-long preindustrial-to-future CO_2 enrichment gradient (250-500 ppm) in Texas Prairie soils. First, I investigated bacterial community response to an ongoing CO_2 enrichment gradient in silty clay, clay and sandy loam soils with mixed C_3/C_4 vegetation. The findings suggest that long-term edaphic properties (soil texture/nutrient content) and soil moisture rather than CO_2 gradient had a

pronounced effect on global community structure. However, the results also illustrated soil-specific variation in community structure. For example, silty clay communities were better structured along the CO_2 gradient. Second, I assessed the effects of CO_2 enrichment gradient and its legacy on the structure and functional potential of switchgrass silty clay and clay soil communities by employing shotgun metagenome sequencing approach. Switchgrass soil microbiomes were resistant to long-term CO_2 enrichment gradient and exhibited minimal shifts in functional gene abundance linked to carbohydrate degradation, nitrogen cycling and phosphate metabolism. Finally, I examined bacterial community response to an ongoing CO_2 treatment in 2015 and their recovery after the cessation of CO_2 application in two subsequent years (2016/2017). Here, I primarily focused on overall community stability, assembly processes and cooccurrence patterns. The results from ecological null models indicate that stochastic processes dominated community assembly, but the relative influence of selection-based processes markedly varied among soil and plant categories. Nonetheless, highly interconnected cooccurrence networks revealed stable and distinct interactions among taxa across CO_2 treatment years. Taken together, these findings provide novel insights into soil microbiome stability and resilience under climate change.

Microbial Community Structure, Function, and Assembly in Texas Prairie Soils: Insights from a Preindustrial-to-Future CO_2 Enrichment Gradient

by

Swastika Raut, B.S.

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Approved by the Department of Biology

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Dwayne D. Simmons, Ph.D., Chairperson

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Approved by the Dissertation Committee

_____

Ryan S. King, Ph.D., Chairperson

_____

Sanghoon Kang, Ph.D.

_____

Joseph D. White, Ph.D.

_____

Robert S. Doyle, Ph.D.

_____

William C. Hockaday, Ph.D.

Accepted by the Graduate School

August 2021

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J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

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DEDICATION

To my mother, Manju Karki Raut for her lifetime of service as an educator and a role model in Nepal. And in the loving memory of my father, Gyanendra Man Singh Raut.

CHAPTER ONE

Introduction

Background

Atmospheric carbon dioxide (CO_2) has been increasing at an accelerated pace since the Industrial Revolution. Such increase can be largely attributed to anthropogenic activities, including fossil fuel consumption and land use changes. In recent decades, human influence on the global climate change has spurred the discussion of a new geological epoch – the "Anthropocene" (Crutzen, 2002; Waters et al., 2016). Glacial data based on gases trapped in Antarctic ice cores suggests natural fluctuation in CO_2 concentration during the past ~800,000 years (Lüthi et al., 2008; Petit et al., 1999). However, the rate of increase in atmospheric CO_2 concentration within the last century exceeds any changes observed in the past 23 million years of Earth history (Cui et al., 2020). Indeed, CO_2 concentration has increased from 280 ppm in the pre-industrial era to ~419 ppm in April 2021 (Tans and Keeling, 2021). Current projections suggest that CO_2 concentrations will exceed 550 ppm within the year 2100 (Ghannoum et al., 2010) and further rise rapidly if mitigation strategies are not implemented (Anderson et al. 2013; IPCC 2014). Because CO_2 is the most abundant greenhouse gas in the atmosphere, it also one of the main drivers of global climate change.

Soils in the terrestrial ecosystem store ~2,300 gigatons (Gt) of organic carbon (C), which represents more than combined stocks of C reserved in the atmosphere and vegetation (Amundson et al., 2015; J. Jansson, 2011; Jobbágy & Jackson, 2000). Furthermore, grasslands cover almost one third (~26%) of ice-free vegetated land surface (Foley et al., 2011; J. K. Jansson & Hofmockel, 2020) and also constitute a substantial fraction of the global C budget. Terrestrial C cycle is maintained by a delicate balance between photosynthesis and respiration. Heterotrophic soil respiration, which is one of the primary carbon fluxes between biosphere and atmosphere (Fay et al., 2021), is mediated by soil microbes. Small changes in annual flux of CO_2 in large C sinks or reservoirs like grasslands and permafrost could have significant implications on soil carbon storage, nutrient cycling processes and biosphere feedback mechanisms. Thus, the vulnerability of grasslands to climate change is intricately linked to microorganisms that inhabit the soils (Jansson & Hofmockel, 2020; Naylor et al., 2020).

Soil microorganisms play a critical role in cycling key nutrients, including soil organic carbon (SOC), nitrogen, phosphorus, and other micronutrients. They also provide essential ecosystem services and are responsible for maintaining ecosystem function. The effects of global change drivers such as atmospheric CO_2 enrichment have received wide attention in the context of global biodiversity loss or increased primary productivity in plants (Dijkstra et al., 2008). In contrast, the impact of CO_2 enrichment on soil

microorganisms and their resilience to global climate change remain poorly understood. Thus, investigating CO_2 enrichment effects on microbial diversity, structure, function and community assembly mechanisms could have significant implications in predicting ecosystem-level responses to ongoing and future climate change.

The direct effects of local CO_2 enrichment in soil microorganisms is less evident because the concentration of CO_2 within the soil microhabitat is much higher compared to that of the atmosphere (Drigo et al., 2008). However, CO_2 enrichment in the atmosphere is expected to have a cascading effect on soil microbes (Figure 1.1), through enhanced plant productivity resulting in increased plant C inputs and rhizodeposition through root exudates (Bardgett et al., 2008). These changes influence the quality and quantity of C entering the soil organic matter (SOM) pool and the fate of SOM is ultimately determined by soil microbial communities. Consequently, CO_2 induced changes in above-ground plant productivity, plant species turnover, soil water and nutrient availability could indirectly elicit shifts in microbial diversity, community structure and functional potential.

Soil microorganisms have incredibly small size and are highly diverse compared to macro-organisms. In earlier decades, it was challenging to study different aspects of microbial diversity, community structure, and function in response to climate change because most soil microbes in nature are “not-yet-cultivable” in lab environment. However, the recent advances in high-throughput next generation sequencing technologies and computational methods developed have provided a platform to investigate shifts in

microbial community composition as well as patterns in taxonomic, phylogenetic and functional diversity in greater detail.

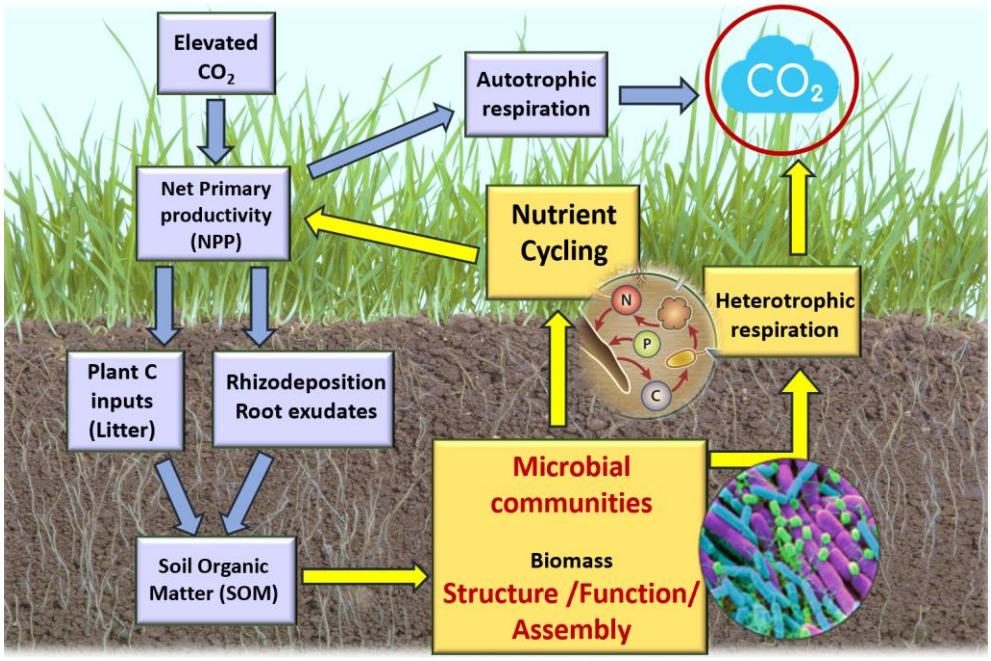

Figure 1.1. Ecosystem feedbacks and cascading effects of atmospheric CO_2 enrichment on soil microbial communities through enhanced plant productivity and C flow from plant litter and root exudates. Adapted from Bardgett et al. 2008.

Our understanding of soil microbial community responses to current and projected changes in atmospheric CO_2 concentrations have been primarily established through free-air CO_2 enrichment (FACE) experiments (Deng et al., 2016; Drigo et al., 2013; Hayden et al., 2012). While these field studies have been valuable in comparing the effects of ambient versus elevated CO_2 levels on soil microbiome, it is difficult to discern the trajectory of microbial response to various levels of CO_2 concentrations.

Ecosystem responses in most studies are also primarily focused on single soil type. However, soil properties vary across different landscapes. The physiochemical characteristics of different soil types can modulate microbial response to climate change

factors. Soil texture affects water holding capacity, cation exchange capacity (Procter et al., 2015) and nutrient availability (McLauchlan, 2006). These factors could have a significant role in regulating soil C and N dynamics, which in turn could affect shifts in microbial community composition and function (Bach et al., 2010). Thus, including soils with broad range of texture, moisture and nutrient content can further elucidate the variation in magnitude and trajectory of microbial response to global climate change in diverse ecosystems.

Project Overview and Chapter Objectives

This dissertation consists of five chapters. In Chapter one, I provide the background information, overarching goal and chapter specific aims to understand this project. Chapter two was published in Global Change Biology Journal as a primary research article. Chapters three and four are the manuscripts in preparation. The final chapter constitutes overall summary and conclusions.

In my dissertation research, I used Lysimeter CO_2 gradient (LYCOG) facility located at USDA site in Temple, TX (Figure 1.2) as a model grassland ecosystem to understand microbial community responses to a decade-long preindustrial-to-future CO_2 gradient (250-500 ppm) as well as overall community stability during and post experimental CO_2 treatment conditions. The details of the LYCOG experimental setup are illustrated in materials and methods section of Chapter two. I investigated soil microbes associated with three contrasting soils (an upland silty clay Mollisol, a low land clay Vertisol, and an alluvial sandy loam Alfisol) and two vegetation types (mixed C_3/C_4 grasses and switchgrass monocultures). The experimental design in LYCOG closely simulates the

ongoing CO_2 trends in the natural environment, and therefore provides a unique setting to study the historical trajectory of soil microbial community response.

Figure 1.2. Aerial view of Lysimeter CO_2 gradient facility located in Temple, TX showing vegetation enclosed in two longitudinal, tunnel shaped chambers.

In Chapter two, I focus on bacterial community response to preindustrial-to-future CO_2 gradient in three contrasting soils (silty clay, clay, and sandy loam) associated with mixed C_3/C_4 vegetation. I collected the soil samples (n=83) at the beginning, middle and end of growing season in 2015, which was the last year of CO_2 treatment. In this study, I implemented 16S rRNA gene amplicon sequencing and conducted multivariate statistical analysis with ancillary plant and soil data. I primarily focused on the effect of ongoing CO_2 gradient on microbial diversity and community structure by analyzing alpha and beta diversity patterns. In addition, I also examine the effects of environmental constraints on global community structure and taxon-specific response to CO_2 gradient. The results suggest that edaphic conditions rather than CO_2 gradient have a significant influence on global community structure. Nonetheless, soil specific variation in community structure

and selective enrichment of certain taxonomic families under CO_2 gradient could be important while considering microbial responses to past, present and future climate change scenario.

In Chapter three, I sought to better understand specific nutrient cycling processes and functional genes of the soil microbial communities associated with switchgrass monocultures. I utilized a subset of clay and silty clay soil samples from switchgrass monocultures (n=20) in 2015 (the last year of CO_2 gradient treatment) and 2016 (year one after the cessation of CO_2 treatment). I implemented shotgun metagenome sequencing to ascertain all the genes present in all microorganisms (bacteria, archaea, and fungi) against a protein reference database. Here, I utilized SEED classification to elucidate the functional genes involved in carbohydrate degradation, nitrogen cycling and phosphate metabolism. Shotgun sequencing also allows to detect the contribution of different microbial groups involved in these processes. Although soil type had significant influence on overall community structure, switchgrass soil microbiomes were resistant to long-term CO_2 enrichment gradient and revealed minimal changes in functional gene abundance albeit a few genes involved in nitrate reduction and starch degradation. Notably, *Micromonosporales* and *Solirubrobacterales* significantly contributed to diverse carbon, nitrogen and phosphorus metabolism categories, suggesting their key role in nutrient cycling processes specifically in switchgrass soils.

In Chapter four, I provide a comprehensive analysis of CO_2 enrichment and its legacy on the response and recovery of soil bacterial communities. Furthermore, I examined predicted functional metagenome profiles, community assembly mechanisms and cooccurrence patterns within each soil and plant category. I collected bulk soil samples

from three distinct soil types (silty clay, clay, and sandy loam) and two vegetation types (mixed C_3/C_4 grasses and switchgrass monocultures) in 2015 and 2016/2017 (n=260). 2015 was the last year of a decade-long CO_2 gradient (250-500 ppm) experiment. Thus, soil monoliths returned to ambient CO_2 conditions during the two consecutive years in 2016 and 2017 following the cessation of CO_2 application at LYCOG facility. I utilized 16S rRNA gene amplicon sequencing and combined the 2015 data set from Chapter one. I quantified community assembly processes based on ecological null models inferred from phylogenetic and taxonomic beta diversity metrics. The findings suggest that the relative influence of ecological processes governing microbial community assembly in Texas Prairie grasslands are predominantly stochastic, but the extent of influence of selection-based processes is soil-specific and plant-specific. Furthermore, CO_2 enrichment and its legacy alone may not impose a strong selective pressure in shaping the overall community structure.

Chapter five is a summary of the results from soil microbial communities investigated in LYCOG project. In essence, Chapter five includes the main conclusions from previous chapters and the implications of my findings for future research on soil microbiomes and climate change.

Author Contributions

Chapter one was written by Swastika Raut (SR) with substantial inputs on overarching goal and chapter specific aims from Dr. Sanghoon Kang (SK). Dr. Wayne Polley (WP) and Dr. Philip A. Fay (PAF) designed the Lysimeter CO_2 gradient, based upon which chapters two, three and four were prepared as primary research articles. SR conducted field soil sampling, processed the soils, performed bioinformatics/statistical

analysis, and wrote the manuscripts. SK had significant contribution in developing hypothesis and reviewing results for chapters two, three and four. WP and PAF also had significant inputs and helped in revision of manuscripts prior to journal submission. Dr. Ryan S. King (RSK) oversaw the administrative work, provided feedback on the overall dissertation, and contributed to revision as manuscript coauthor for chapters three and four. Chapter five was written by SR as a summary of the entire project and its implications for climate change research.

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CHAPTER TWO

Bacterial Community Response to a Preindustrial-to-Future CO_2 gradient is Limited and Soil-specific in Texas Prairie Grassland

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Abstract

Rising atmospheric CO_2 concentration directly stimulates plant productivity and affects nutrient dynamics in the soil. However, the influence of CO_2 enrichment on soil bacterial communities remains elusive, likely due to their complex interactions with a wide range of plant and soil properties. Here, we investigated the bacterial community response to a decade long preindustrial-to-future CO_2 gradient (250-500 ppm) among three contrasting soil types using 16S rRNA gene amplicon sequencing. In addition, we examined the effect of seasonal variation and plant species composition on bacterial communities. We found that Shannon index (*H*') and Faith's phylogenetic diversity (PD) did not change in response to the CO_2 gradient ($R^2 = 0.01$, $p > 0.05$). CO_2 gradient and season also had a negligible effect on overall community structure, although silty clay soil communities were better structured on a CO_2 gradient ($p < 0.001$) among three soils. Similarly, CO_2 gradient had no significant effect on the relative abundance of different phyla. However, we observed soil-specific variation of CO_2 effects in a few individual families. For example, the abundance of *Pirellulaceae* family decreased linearly with CO_2 gradient, but only in sandy loam soils. Conversely, the abundance of *Micromonosporaceae*

and *Gaillaceae* families increased with CO_2 gradient in clay soils. Soil water content (SWC) and nutrient properties were the key environmental constraints shaping bacterial community structure, one manifestation of which was a decline in bacterial diversity with increasing SWC. Furthermore, the impact of plant species composition on community structure was secondary to the strong influence of soil properties. Taken together, our findings indicate that bacterial communities may be largely unresponsive to indirect effects of CO_2 enrichment through plants. Instead, bacterial communities are strongly regulated by edaphic conditions, presumably because soil differences create distinct environmental niches for bacteria.

Introduction

Atmospheric CO_2 concentration has been rising since the industrial revolution at rates that are unprecedented (Anderson *et al.*, 2013; IPCC, 2014). Increase in CO_2 concentration has both direct and indirect impacts on soil C storage, plant growth, as well as nutrient cycling processes driven by soil microbes. Direct positive effects of CO_2 enrichment on aboveground vegetation have been well documented from free-air CO_2 enrichment (FACE) studies (Dijkstra *et al.*, 2010; Luo *et al.*, 2006; Nie & Pendall, 2016; Reich, 2009). For example, several studies have shown that elevated CO_2 stimulates aboveground net primary productivity (ANPP), root biomass, and C inputs into the soil (Ainsworth & Long, 2004; Pritchard, 2011). The concentration of CO_2 within the pore space of soil is several orders of magnitude higher than that in the atmosphere (Drigo *et al.*, 2008). In addition, soil CO_2 efflux is controlled by multiple biotic and abiotic factors. A substantial proportion of CO_2 in soil is released from plant root respiration and microbial decomposition of soil organic matter, while a minor fraction is derived from carbonate

weathering as CO_2 dissolves in water to form carbonic acid (Andrews & Schlesinger, 2001). Consequently, the effects of local atmospheric CO_2 enrichment on soil microbial communities is expected to be indirect, and primarily driven by plant C inputs from rhizodeposition and root exudation (Bardgett *et al.*, 2008).

Microbial responses to CO_2 enrichment have been variable across different ecosystems. Some studies have reported shifts in microbial community structure and function under elevated CO_2 treatment (Drigo *et al.*, 2013; Hayden *et al.*, 2012; He *et al.*, 2010). For example, relative abundance of certain taxonomic groups of soil bacteria, such as *Firmicutes* and *Bacteroides* phyla, increased under elevated CO_2 (Drigo *et al.*, 2013). Increase in microbial biomass and differential stimulation of functional genes involved in labile C degradation and nitrogen (N) fixation under elevated CO_2 have also been reported (He *et al.*, 2010). However, other studies have shown that elevated CO_2 has negligible effect on soil microbial communities (Ge *et al.*, 2010; Hagedorn *et al.*, 2013). Microbial communities mediate biogeochemical processes through complex interactions with plants and soil properties (Rousk & Bengtson, 2014; Singh *et al.*, 2010). Inconsistent results of elevated CO_2 effects on bacterial communities are likely due to discrepancies in analytical techniques, and variations in soil physiochemical properties and plant functional types across different experimental settings.

Most CO_2 enrichment studies compare the microbial responses at the current ambient CO_2 concentration and an elevated CO_2 concentration projected for a fixed point in the future (Deng *et al.*, 2015; Dunbar *et al.*, 2012; Ebersberger *et al.*, 2004). We used the Lysimeter CO_2 gradient (LYCOG) facility to determine microbial responses to an atmospheric CO_2 gradient spanning the preindustrial level (250 ppm) to a concentration

(500 ppm) projected by 2050 (Collins *et al.*, 2013; Forster *et al.*, 2007). Consequently, the experimental setup allows us to discern whether soil bacterial communities exhibit any threshold or non-linear patterns of response to a range of CO_2 concentrations. It also provides a unique setting to study the trajectory of soil bacterial community response to CO_2 increase since the industrial revolution. CO_2 change represents a contemporary disturbance compared to pre-existing edaphic properties. Thus, we can also elucidate the relative influence of historical contingency versus contemporary CO_2 disturbance using the LYCOG experiment.

Soil bacteria are incredibly diverse, which is supported by microhabitats from the soil physiochemical heterogeneity. Large variation in physiochemical properties among soils may elicit changes in community structure, taxonomic composition and diversity. Soil texture affects soil water holding capacity, cation exchange capacity (Krull *et al.*, 2001) and nutrient availability (McLauchlan, 2006). Fine-textured clay soils have a larger surface area, smaller aggregates, and higher water retention ability (van Gestel *et al.*, 1996), which could favor diverse groups of microbes. For example, microbial communities in soils with higher clay content have been found to be more diverse and more abundant (Sessitsch *et al.*, 2001). Conversely, sandy loam soil contains larger aggregates, particle size and pore space, which could limit the water retention ability and induce more water loss to drainage (Polley *et al.*, 2012). The question remains as to whether CO_2 effects on microbes differ among soils that vary in physiochemical properties, including texture, moisture content, and C and N content.

Our study aimed at investigating the shifts in bacterial community structure, composition, and diversity in response to a continuous CO_2 gradient (250-500 ppm) and

season of sampling among three contrasting soils. As CO_2 effects on bacteria are presumed to be indirect and mediated through effects on plants, we examined the influence of plant species relative abundances as well as soil properties on bacterial communities. Previous studies in the LYCOG system have reported increased relative abundance of the tall-grass *Sorghastrum nutans* in mixed-species plant communities at elevated CO_2 levels (Polley *et al.*, 2012) as well as soil-specific variation in CO_2 effects on above ground net primary productivity (ANPP) response and soil water availability (Fay *et al.*, 2012). Similarly, fungal community response and soil enzyme activities differed by soil type along the CO_2 gradient (Kelley *et al.*, 2011; Procter *et al.*, 2014). However, soil bacterial community responses to CO_2 gradient among different soil types have yet to be investigated. Here, we hypothesized that CO_2, soil type and associated properties, season and their interactive effects would determine bacterial community structure and taxonomic composition. Furthermore, the effects of CO_2 gradient on soil bacterial communities would be indirectly driven through corresponding changes in plant species relative abundances and ANPP. We expected that bacterial diversity would positively correlate to increasing CO_2 concentration and that the patterns of response (linear, non-linear or threshold) along the CO_2 gradient would differ by soil type. We utilized 16S rRNA gene amplicon sequencing to characterize bacterial communities.

Materials and Methods

Experimental Design

The Lysimeter CO_2 Gradient (LYCOG) facility is located in Temple, Texas, USA (31^0 05'N, $97^0$20' W) and operated by the USDA-ARS Grassland Soil and Water Research Laboratory since 2006 (Fay *et al.*, 2009). LYCOG consists of two longitudinal, tunnel-

shaped chambers (1.2 m x 1.5 m x 60 m), each divided into 10 sections (Figure S1). Intact soil monoliths were placed beneath the chambers. A continuous linear CO_2 gradient of 500 ppm - 380 ppm was maintained in the elevated chamber, where pure CO_2 was mixed with incoming ambient air at the entrance (Figure S1b). The ambient chamber had identical setup except CO_2 was not injected and ambient air was directly introduced in the chamber to initiate a CO_2 gradient of 380 ppm at the entrance and 250 ppm at the exit. Photosynthesis by enclosed vegetation progressively depleted the enriched air as it was advected through chambers using blower fans. Fan speed was regulated to maintain desired CO_2 levels at the entry and exit points. The direction of air flow in both chambers was reversed during nighttime. CO_2 levels were measured at the entrance and exit of each section at 2 min intervals and the concentration for each monolith was estimated by linear interpolation from the measured values (Fay *et al.*, 2012).

Air temperature and vapor pressure deficit were regulated near ambient values by cooling and dehumidifying air at 5-m intervals along chambers. CO_2 gradient was maintained during the portion of the year when vegetation photosynthetic capacity was adequate, typically from late April to early November (Fay *et al.*, 2015a). Irrigation regime for each soil monolith simulated the seasonal rainfall pattern in central Texas. We measured volumetric soil water content (SWC) using a calibrated neutron attenuation probe (503DR Hydroprobe, CPN International). Soil water potential (SWP) was calculated from measured values of SWC using soil specific SWP vs SWC relationships.

The vegetation on existing soil monoliths were treated with non-residual herbicide and seedlings of seven perennial species native to the Texas Blackland Prairie were transplanted into 60 of the 80 monoliths in June 2003, three years prior to CO_2 treatment

(Fay *et al.*, 2015a). C4 species included *Bouteloua curtipendula* (side-oats grama), *Schizachyrium scoparium* (little bluestem), *Sorghastrum nutans* (Indiangrass), and *Tridens albescens* (white tridens). Three forb (C3) species included *Salvia azurea* (pitcher sage), *Solidago canadensis* (Canada goldenrod), and the legume *Desmanthus illinoensis* (Illinois bundleflower). Eventual dominant species included the C4 grasses *B. curtipendula, S. nutans* and the forb species *S. canadensis* (Polley *et al.*, 2015). Specific soils series used in the facility were Houston series (a black clay Vertisol), Austin series (a high carbonate, silty clay Mollisol), and Bastsil series (an alluvial sandy loam Alfisol). All standing aboveground biomass was harvested at the end of the growing season. The biomass of individual species in each monolith was used to quantify relative plant species contributions to ANPP.

Soil Sampling and Chemical Analysis

Soil samples were collected from 31 monoliths (Figure S1c) during May, August and November of the 2015 growing season, the tenth year of CO_2 regulation. Three cores from the top 0-5 cm of monoliths and located close to vegetation were collected using mini metal cores of 1.5 cm diameter. The soil cores (~15 g) were transferred to 50 ml centrifuge tubes. Samples were transported in dry ice and immediately stored at a -80°C freezer until further analysis. Soil organic carbon and total nitrogen for samples collected in this study were measured using an elemental analyzer. After accounting for sequencing depth, and ancillary plant and soil data, a total of 83 soil samples from 2015 were used for downstream microbial analysis (n=32 for silty clay, n=24 for sandy loam, and n=27 for clay).

DNA Extraction and PCR Amplification

Community DNA from soil samples was extracted using commercial extraction kits (PowerSoil® DNA Isolation Kit, Mo Bio Laboratories). The purity of DNA samples was assessed by measuring 260/230 and 260/280 in a Nanodrop spectrophotometer. Quantification of the samples were determined by Qubit 3.0 flourometer. A two-step PCR amplification targeting V4-V5 region of 16S rRNA gene was performed. Amplicon primers (515F forward and 926R reverse) for 16s rRNA (Caporaso *et al.*, 2010) and barcode index primers for second step PCR were ordered from Integrated DNA technologies (IDT). 2X PlatinumTM Green Master Mix from Invitrogen was used as PCR master mix. Overhang adapter sequences compatible with Illumina index primers and sequencing adapters were added along with amplicon primer during the first step. PCR reaction conditions for the first step were 94 °C for 2:00 min, followed by 30 cycles of (1) 94 °C denaturation step for 45 secs, (2) 50 °C annealing step for 1:00 min, and (3) 72 °C elongation step for 1:30 min.

The full complement of unique index barcodes primers for each sample was added during 2nd step PCR so that the libraries could be pooled together for sequencing. PCR reaction conditions for the second step were 94 °C for 2:00 min, followed by 8 cycles of (1) 94 °C denaturation step for 45 secs, (2) 59 °C annealing step for 1:00 min, and (3) 72 °C elongation step for 1:30 min. After each step, the PCR products were purified using Agencourt AMPure XP beads from Beckman Coulter Life Sciences. The size of amplicon of was expected to be ~500 base pairs and was verified with gel electrophoresis. Qubit 3.0 flourometer was used for library quantification and standardization.

16S rRNA gene Amplicon Sequencing and Sequence Processing

We used Illumina MiSeq platform with v3 Reagent kit to sequence paired-end 300-bp reads targeting V4-V5 region. The fastq files for individual samples were generated by initially processing and demultiplexing raw sequences and barcodes through 16S metagenomic Pipeline in Illumina BaseSpace. Downstream processing of sequences filtered from BaseSpace was conducted through QIIME (Caporaso *et al.,* 2010). Read 1 and Read 2 from each sample were aligned using the join-fastq algorithm (Aronesty, 2013) and the aligned sequences were filtered at a Phred score of 20. We further detected chimeric sequences using the USEARCH algorithm (Edgar, 2010) and removed the sequences identified as chimeras. We implemented open reference de novo OTU picking with UCLUST (DeSantis *et al.,* 2006). Greengenes 13_8 was used as a reference database to assign taxonomy for corresponding OTUs. The samples were rarefied to a sequencing depth of 20,000 sequences.

Statistical Analyses

Statistical analyses were carried out with R statistical software v.3.4.1 (R Development Core Team, 2016) with packages (vegan, phyloseq, heatmap.plus, ggplot2 and others) and custom scripts. Unconstrained ordinations were performed using Non-metric multidimensional scaling (NMDS) on $\log_{10}(x + 1)$ transformed OTU tables with Bray-Curtis distance and UniFrac distance using vegan (Oksanen *et al.*, 2017) and phyloseq (McMurdie & Holmes, 2013) packages, respectively. To assess the effect of CO_2 gradient on community structure, ordisurf function with GAM fitting was incorporated in the NMDS ordinations. PROcrustean randomization TEST of community environment concordance (PROTEST) function with 999 permutations was used to compare UniFrac

and Bray-Curtis ordinations. The statistical test with 'procrustes' function in vegan package was used to determine the correlation between ordination configurations. Non-parametric permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were used to evaluate the significance of soil type and season groups. We constructed Redundancy analysis (RDA) model using vegan package to assess the effects of environmental variables on community structure. Model selection in RDA was performed with all the environmental variables at first. Then, the function ordistep, which uses significance test based on random permutations, was performed to select the best model. The variables that were highly collinear were eventually removed from the final model by considering their variation inflation factor (VIF). Partial RDA and partial Mantel tests were also conducted to assess the effects of soil properties alone by holding plant species composition constant and vice versa.

We used clustering analysis with heatmap.plus package (Day, 2012) to visually identify shifts in relative abundance of different taxa within specific soil types and seasons. Generalized additive model (GAM) were conducted to assess the effects of CO_2 on relative abundance of sensitive taxa within different soils. Since CO_2 was a considered a continuous explanatory variable, GAM fitting allowed us to better capture any linear, non-linear or threshold responses along the CO_2 gradient (Marra & Wood, 2011). We used Shannon index (*H*') and Faith's phylogenetic diversity (PD) to estimate the taxonomic and phylogenetic diversity, respectively. We fitted GAM with volumetric soil water content (SWC) and CO_2 gradient as linear predictors, and diversity indices as response variables Model validation was performed via visualization of residual plots. Most analyses not specified above were carried out by vegan package and R base functions.

Results

Taxonomic and Phylogenetic Diversity

Results from GAM with global fit including samples across all soil types and seasons demonstrated that CO_2 gradient did not have a significant effect on taxonomic and phylogenetic diversity (Figure 2.1). Both Shannon index *(H')* and Faith's PD did not change in response to increasing CO_2 concentrations ($p > 0.1$). Although the patterns of diversity response to CO_2 varied slightly among soil types and seasons (Appendix A: Figure A.S2), the interactive effects between CO_2 × soil and CO_2 × season were still not significant ($p > 0.1$). In contrast, soil water content (SWC) strongly affected bacterial diversity. Both Shannon index and Faith's PD (Appendix A: Figure A.S3a, b) showed a near linear decreasing trend (Adjusted $R^2 = 0.16$, $p < 0.001$) with increasing SWC. Overall diversity was higher in sandy loam soil with low SWC, and lower in clay soils with high SWC (Appendix A: Figure A.S3c, d) but seasonal variation had no significant effect on bacterial diversity.

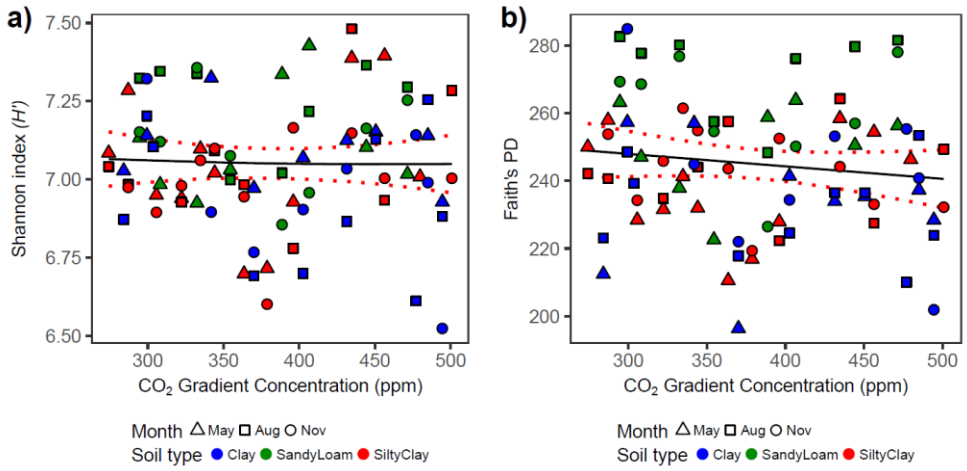

Figure 2.1 Generalized additive model (GAM) fitted with (a) Shannon index (adj. R^2= -0.01, p=0.86) and (b) Faith's PD (adj. R^2 = 0.001, p=0.243) along a preindustrial-to-future CO_2 gradient including samples across all soil types and seasons.

Bacterial Community Structure

NMDS ordinations using Bray-Curtis distance and weighted UniFrac distance revealed that CO_2 gradient had a negligible effect on bacterial community structure (Figure 2.2, Table 2.1). We fitted GAM surface on NMDS ordinations with contour lines representing the preindustrial-to-future CO_2 gradient. The fitted values computed from ordisurf GAM did not overlay well with the CO_2 gradient when samples across soil types and seasons were pooled together in the NMDS ordinations. For example, the contour lines overlaid on the ordination space depicted a narrower range (365-410 ppm) of CO_2 concentrations rather than displaying the expected gradient consistent with experimental CO_2 concentrations (250-500 ppm) for soil samples (Figure 2.2). This result was further supported by statistical analysis of ordisurf GAM fit in Table 2.1. Nonetheless, the effects of CO_2 gradient on bacterial community structure varied by soil type (Figure 2.3, Table 2.1). Further analysis of CO_2 effect within each soil type illustrated that silty clay soil communities were better structured on the CO_2 gradient ($p < 0.001$, Table 2.1). In silty clay soils, bacterial communities at sub-ambient CO_2 concentrations were clearly separated in the ordination space from those at future levels (Figure 2.3a, b). In addition, the contour lines represented a non-linear fit at higher range of CO_2 concentrations. In sandy loam soils, CO_2 gradient represented somewhat linear fit for both UniFrac and Bray-Curtis distances (Figure 2.3c, d). On the contrary, CO_2 gradient had no significant effect on communities associated with clay soil (Table 2.1) and the contour lines fitted with GAM were within narrow range of CO_2 gradient for clay soil ($p > 0.1$). Overall, the influence of CO_2 gradient on community structure with global test (Figure 2.2, Table 2.1) was marginal compared to soil-specific response (Figure 2.3, Table 2.1).

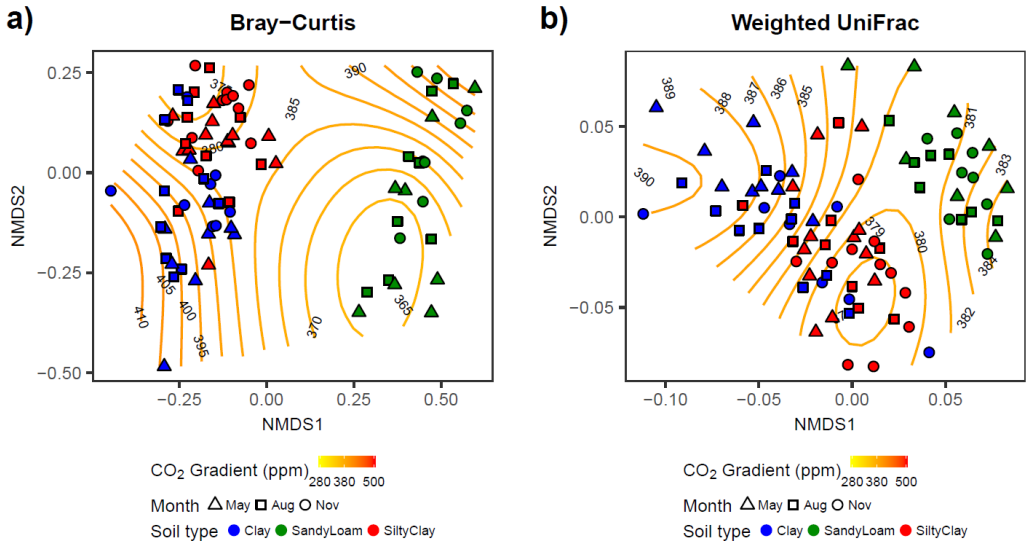

Figure 2.2 (a) Non-metric multidimensional scaling (NMDS) ordinations with Bray-Curtis distance (stress =0.09) and (b) weighted UniFrac distance (stress =0.17) representing all soil types and seasons. Contour lines overlaid in the ordination space indicate the fitted values for CO_2 gradient, which were calculated using ordisurf GAM.

Table 2.1. Statistical approaches to test the significance of a linear CO_2 gradient on bacterial community structure using ordisurf GAM.

CO_2 gradient (ordisurf)	Statistic	Bray-Curtis	Weighted UniFrac
Global (All soil types)	Adj. R^2	0.06	0.02
	p	0.09	0.23
Soil-specific			
Silty Clay	Adj. R^2	0.75	0.62
	p	<0.001**	<0.001**
Sandy Loam	Adj. R^2	0.56	0.54
	p	0.015*	0.005*
Clay	Adj. R^2	0.21	0.14
	p	0.294	0.45

*Significant ($\alpha = 0.05$).
**Significant after Bonferroni correction ($\alpha = 0.017$).

Bacterial communities were distinctly clustered based on soil type with significant overlap between silty clay and clay soil samples in the ordination space (Figure 2.2). Results from ANOSIM and PERMANOVA also revealed a strong effect of soil type on bacterial community structure ($p < 0.001$, Appendix A: Table A.S1). In addition, the

pairwise comparisons between soil types with PERMANOVA illustrated the significant variation on bacterial community structure among three soils (Appendix A: Table A.S1). Although the effect of soil type appeared to be slightly diminished with UniFrac distance (Figure 2.2b) compared to Bray-Curtis distance (Figure 2.2a), both ordination configurations were highly correlated by Procrustes analysis ($t = 0.806$, $p = 0.001$).

The season of sampling across different seasons did not have a significant effect on the overall bacterial community structure, which was confirmed by pairwise comparisons between seasons (Appendix A: Table A.S1). NMDS ordinations along with ANOSIM and PERMANOVA analysis for season grouping within soil types further revealed that season of sampling had no significant effect on silty clay and sandy loam soil communities (Appendix A: Figure A.S4 and Table A.S2). However, clay soil communities showed some variation on community structure based on the season grouping. In general, the ordination results along with multivariate statistical analyses confirmed that seasonal variation had little to no effect on bacterial community structure compared to the significant effect of soil type.

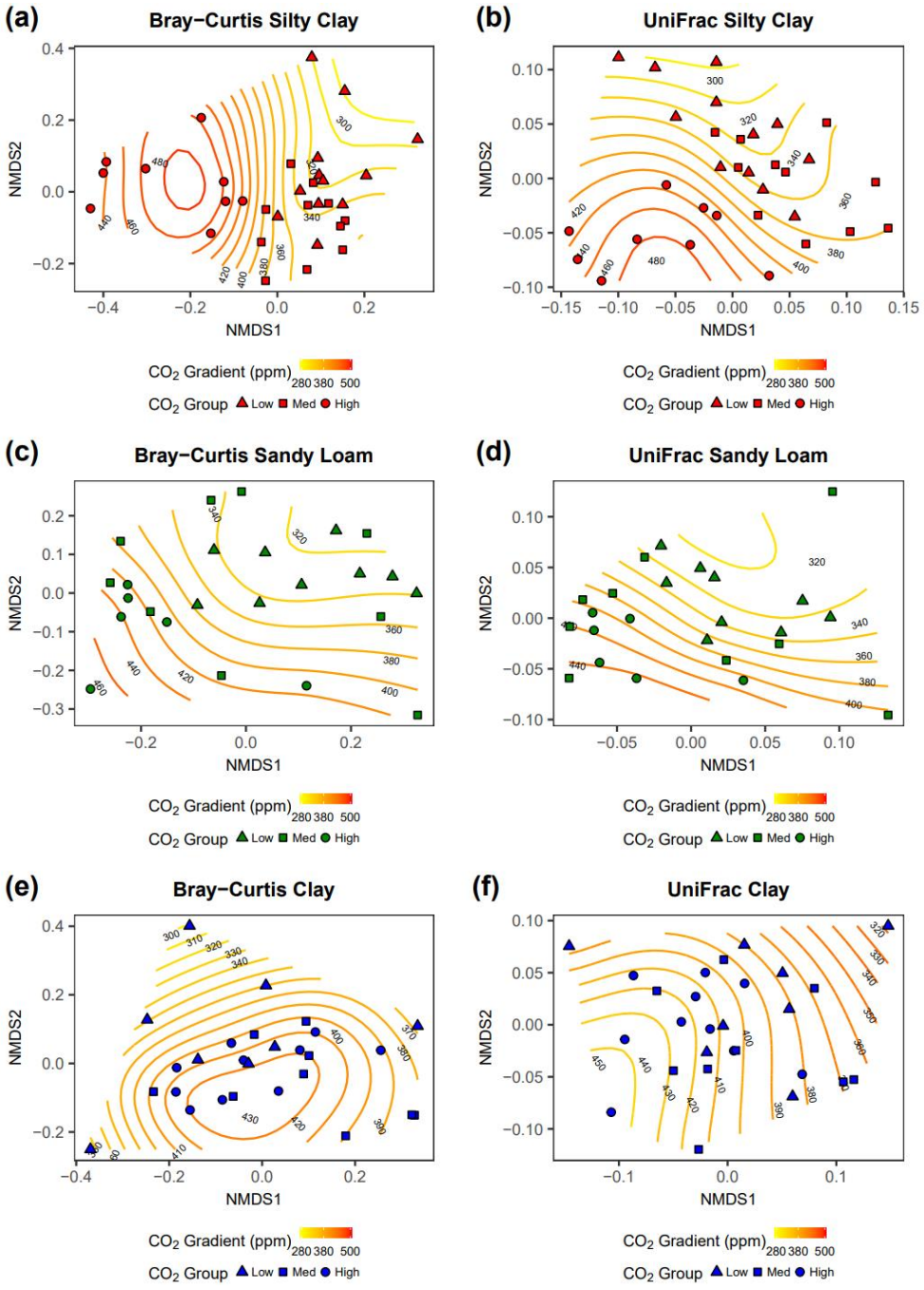

Figure 2.3. NMDS ordinations with Bray-Curtis distance and UniFrac Distance reflecting CO_2 gradient fit within specific soil types. Contour lines represent CO_2 gradient fit computed with ordisurf GAM and overlaid in the ordination space. CO_2 groups represent samples with different CO_2 gradient concentrations ranging from 250-340 ppm (Low), 341-440 ppm (Med), and 441-500 ppm (High) respectively. Yellow to red lines represent a gradient of high to low CO_2 concentration.

Taxonomic Distribution

At 97% sequence similarity, a total of 68,068 OTUs were detected. These OTUs were assigned to 47 phyla, 141 classes, 222 orders and 259 families using Greengenes database 13_8. *Proteobacteria* was the most abundant phylum (26-40%) across all samples followed by *Acidobacteria* (8-18%), *Planctomycetes* (8-18%), *Actinobacteria* (5-26%), *Bacteroidetes* (3-12%), *Chloroflexi* (1-10%), *Verrumicrobia* (2-7%) and *Firmicutes* (1-8%). *Alphaproteobacteria* was the most dominant sub-phyla within *Proteobacteria*, and *Rhodospirillaceae* and *Hyphomicrobiaceae* were consistently abundant families (Appendix A: Figure A.S5).

The overall effect of CO_2 gradient on taxonomic composition was marginal at phylum level (Appendix A: Figure A.S6). However, we detected soil-specific variation in relative abundance of some individual families within different phyla in response to CO_2 gradient (Figure 2.4). *Planctomycetes* (*Pirellulaceae* family), *Chloroflexi* (A4b family) and *Deltaproteobacteria* (*Syntrophobacteraceae* family) were most abundant in sandy loam soil among all soil types (Appendix A: Figure A.S5). Notably, the relative abundance of *Pirellulaceae* decreased linearly whereas that of A4b and *Hyphomonadaceae* (Alphaproteobacteria) increased along the CO_2 gradient in sandy loam soils (Figure 4). Some members of *Acidobacteria* (mb2424 family) and *Alphaproteobacteria* (*Sphingomonadaceae* and *Rhodospirillaceae* families) were more abundant in silty clay and clay soils (Appendix A: Figure A.S5). *Rhodospirillaceae* also slightly increased with CO_2 across all soil types (Figure 2.4). The abundance of *Actinobacteria* slightly increased with CO_2 only in clay soil (Appendix A: Figure A.S6). Within the phylum *Actinobacteria*,

the abundance of two specific families, *Micromonosporaceae* and *Gaillaceae* increased with CO_2 concentration in clay soils.

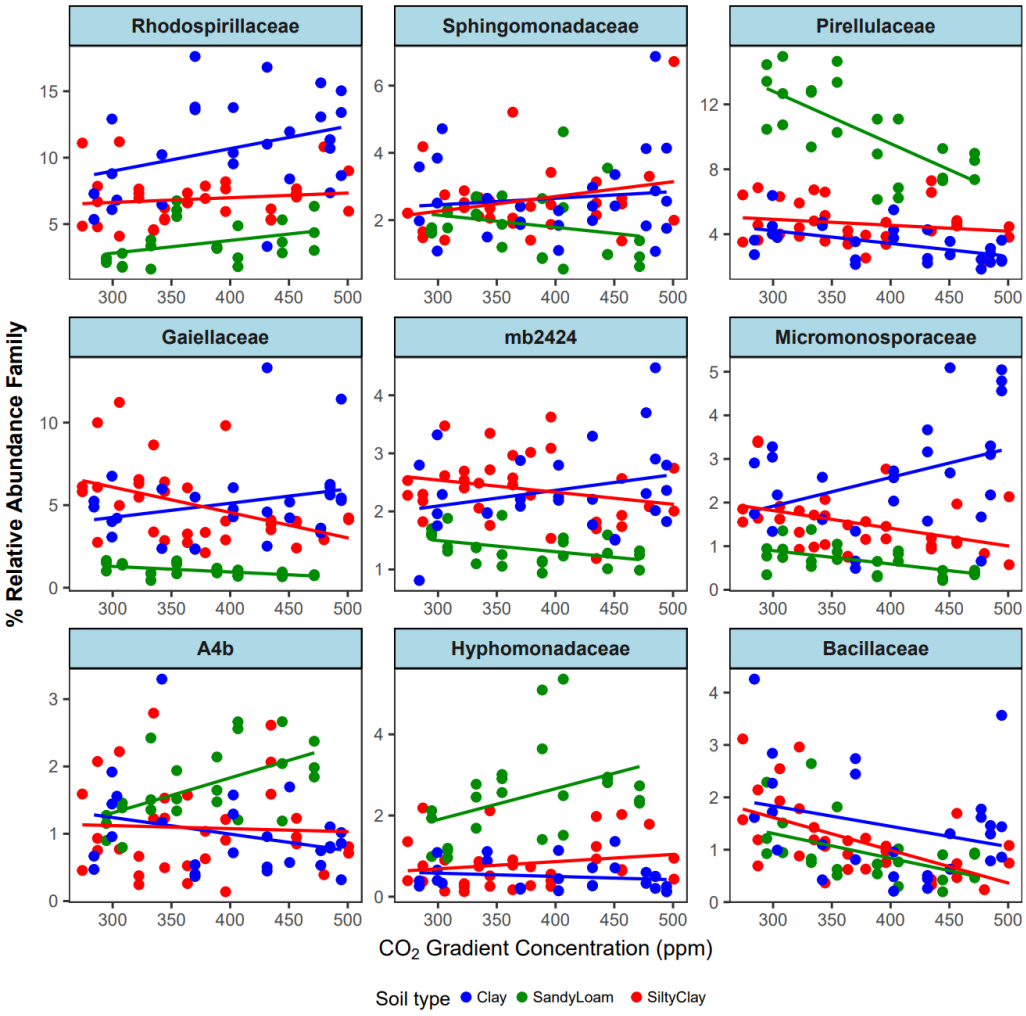

Figure 2.4. Generalized additive model (GAM) fitted illustrating soil-specific variation in individual taxa response along the CO_2 gradient. Only the taxa which were most responsive to CO_2 gradient with >1% relative abundance were selected. For these selected families which were responsive to CO_2 gradient, t-test on the slopes of lines fitted with GAMs showed that they were significantly different from zero.

Taxa associated with sandy loam soils were clustered separately, while those affiliated with silty clay and clay revealed mixed clustering, indicating similar community composition (Appendix A: Figure S5). Soil type had a significant effect on relative abundance of some phyla, including members of *Planctomycetes, Chloroflexi* and

Actinobacteria (Appendix A: Figure A.S5). In contrast, season of sampling had no effect on taxonomic composition at phylum level (Appendix A: Figure A.S6b). *Bacteroidetes* and *Firmicutes* had similar relative abundance across different soil types, seasons, and CO_2 levels (Appendix A: Figure A.S5 and A.S6).

Relative Influence of Environmental Variables on Bacterial Community Structure

To investigate the relationship between environmental factors and bacterial community structure, we constructed a redundancy analysis (RDA) model with CO_2, soil property parameters and plant species composition as constraining variables. Adjusted R^2 for the model was 0.27, where axis 1 explained 18.37 % variation and axis 2 explained 5.73 % variation (Figure 2.5). ANOVA on the RDA model revealed that the constraining soil properties had a significant effect on bacterial community structure ($p < 0.001$). Soil water content (SWC), soil water potential (SWP) and soil nutrient properties including C/N ratio and total nitrogen (TN) were the strongest constraints of bacterial community structure among all the soil properties. Soil organic content (SOC) was collinear with SWC and thus, it was removed from the model. Mantel tests also confirmed that bacterial community structure was significantly correlated with soil parameters ($r = 0.62$, $p = 0.001$, Table 2.2).

Among seven plant species, only C_4 grasses *B. curtipendula* and *S. nutans,* and C_3 forb species *S. canadensis* were correlated to bacterial community structure in the RDA model, and *B. curtipendula* had the most significant effect (Figure 2.5). Partial RDA and partial Mantel tests showed that soil properties were still very significant while controlling the effects of plants species composition ($p = 0.001$, Table 2.2). However, plant species only had a marginal effect when the effects of soil properties were held constant. The partial

tests indicated that the significant soil effect is largely independent from plant influence suggesting less than expected indirect CO_2 effect through plants to soil bacterial community. Overall, sandy loam soil had the lowest values of SWC, SOC, TN and C/N ratio, and highest values of SWP among all three soil types (Appendix A: Table A.S3). Clay soil was slightly more acidic than sandy loam and silty clay soils (Appendix A: Table S3).

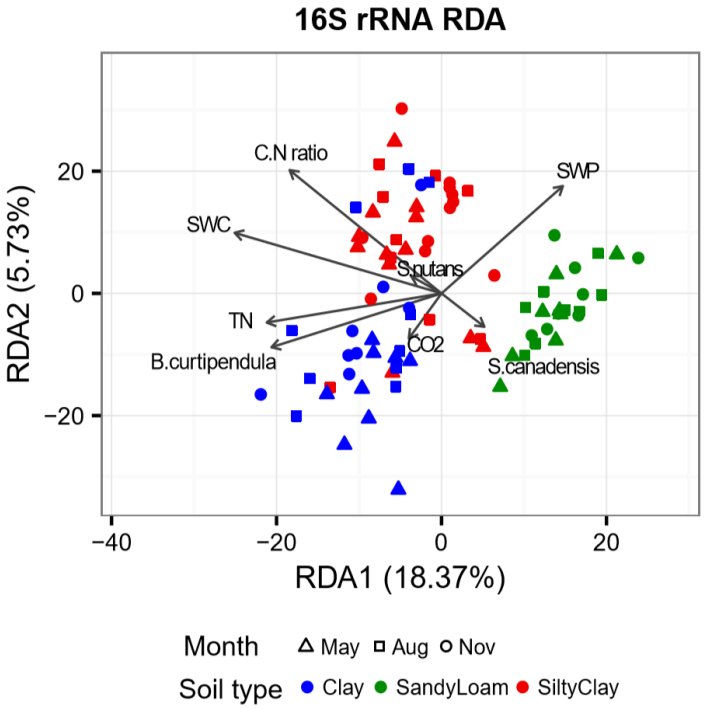

Figure 2.5. Redundancy analysis (RDA) model of bacterial communities fitted with environmental variables (arrows). The values of axes 1 and 2 are the percentages explained by the corresponding axis. SWC, SWP, TN and C.N represent soil properties including, % volumetric soil water content, soil water potential, total nitrogen, and C/N ratio respectively. *B. curtipendula, S. nutans* are C_4 grasses and *S. canadensis* is a forb species. Plant species relative abundance is considered in the RDA model.

Table 2.2. Relative influence of soil properties and plant species relative abundance on bacterial community structure.

		Soil properties	Plant species			Soil properties *Controlling for plants*	Plant species *Controlling for soil*
Mantel	*r*	0.62	0.30	Partial Mantel	*r*	0.57	0.08
	p	0.001**	0.001**		*p*	0.001**	0.03*
RDA	*F*	4.90	2.91	Partial RDA	*F*	3.32	1.61
	p	0.001**	0.001**		*p*	0.001**	0.01*

*Significant ($\alpha = 0.05$).
**Significant after Bonferroni correction ($\alpha = 0.017$).

Discussion

We hypothesized that CO_2, soil type, season and their interactive effects would determine bacterial community structure and taxonomic composition. We also expected increased bacterial diversity due to increase in labile C availability at higher CO_2 concentrations, specifically in clay soils (Procter *et al.*, 2015). Contrary to our expectations, CO_2 gradient had no significant effect on taxonomic and phylogenetic diversity. Furthermore, CO_2 gradient as well as seasonal variation had negligible effect on overall bacterial community structure and composition. For example, the relative abundance of most taxa at phylum level did not change along the CO_2 gradient or across seasons. However, our hypothesis was partially confirmed in that we detected soil-specific variation in CO_2 effects on bacterial community structure and responses of some taxa at family-level. Edaphic properties associated with different soil types were the strongest environmental constraints on bacterial community structure (Figure 2.5). We predicted that the effects of CO_2 on soil bacterial communities would be indirectly driven through corresponding changes in relative abundance of plant species or ANPP. However, plant species abundance had little influence on bacterial community structure when soil properties were controlled.

Conversely, soil properties maintained a strong influence on variation in bacterial community structure even after controlling for the plant-specific effects. Our results imply that the overall effect of a preindustrial-to-future CO_2 gradient on bacterial communities through plants was negligible although we detected soil-specific variation in community structure and responses of few taxa. Thus, our results elucidating bacterial community response to a CO_2 gradient across different soil types improve understanding of the relative influences of CO_2 enrichment, edaphic properties and their interactions on bacterial community structure and composition.

Neither taxonomic nor phylogenetic diversity in soil bacterial communities changed in response to a preindustrial-to-future CO_2 gradient when analyzed across soil types. Therefore, we did not detect any threshold or nonlinear response. In contrast, fungal OTU richness increased linearly with CO_2 in clay soils in a previous study at the LYCOG site (Procter *et al.*, 2014), which suggests that fungal communities may be more sensitive to CO_2 than bacterial communities. Similar to the results from LYCOG site, a few FACE studies also found more pronounced effects of CO_2 enrichment on richness and diversity of fungal than bacterial communities (Hayden *et al.*, 2012; Nguyen *et al*, 2011). However, the effects of elevated CO_2 on bacterial diversity in most FACE studies have been variable. Some have reported increased diversity at elevated CO_2 levels (Castro *et al*, 2010; Drissner *et al.*, 2007; Marilley *et al.*, 1999), whereas others have shown little to no change in bacterial diversity (Dunbar *et al.*, 2012; Ebersberger *et al.*, 2004; Hagedorn *et al.*, 2013; Xu *et al.*, 2013). There does not seem to be any systematic differences among these studies that account for contrasting results. However, it should be noted that elevated CO_2 level in most of the FACE study sites was > than 500 ppm (Drissner *et al.*, 2007; Ebersberger *et*

al., 2004; Hagedorn *et al.*, 2013; Xu *et al.*, 2013), the highest CO_2 concentration applied in this study. A recent study at Giessen free-air CO_2 enrichment (Gi-FACE) facility in Germany, which examined grassland communities exposed to ambient (400 ppm) and moderate (480 ppm) CO_2 levels (de Menezes *et al.*, 2016), also demonstrated negligible effect of CO_2 on bacterial communities. In agreement with our findings, they showed more pronounced effects of soil moisture than CO_2 enrichment on bacterial community structure and diversity. Although soil water release characteristics varied among soil types (Appendix A: Figure A.S7) in this study, differences in soil water content (SWC) rather than CO_2 gradient better explained the overall trend in bacterial diversity (Appendix A: Figure A.S3). One of the advantages of LYCOG system compared to several FACE studies was that a linear CO_2 gradient of 250-500 ppm allowed us to consider CO_2 as a continuous rather than a discrete variable. Thus, our gradient study was potentially more sensitive in revealing the CO_2 enrichment effect. Results from this study and that of (de Menezes *et al.*, 2016) imply that modest CO_2 enrichment will not strongly influence soil bacterial communities.

Soil microbial communities are expected to be indirectly affected by CO_2 enrichment, primarily through plant-mediated changes in productivity and resource availability (Bardgett *et al.*, 2008). Soil bacterial communities in the silty clay soil differed between pre-industrial and future levels of CO_2, whereas those on the clay soil did not (Figure 2.3). Similarly, ANPP also was more responsive to CO_2 on the silty clay and sandy loam than clay soils (Figure S8). Previous studies in the LYCOG system showed that CO_2 enrichment altered dominant plant taxa on silty clay, favoring a more productive C4 tallgrass *S. nutans* (Fay *et al.*, 2012; Polley *et al.*, 2012). Furthermore, increase in dominant

taxa biomass also contributed to a substantial increase in productivity under CO_2 enrichment (Fay *et al.*, 2015b). In this study, the abundance of *S. nutans* had a little effect on bacterial community structure. Among the three dominant plant species that were correlated to community structure, *B. curtipendula* clearly had the most significant effect (Figure 2.5). The biomass of *B. curtipendula* was significantly higher in silty clay and clay soils compared to sandy loam ($p < 0.001$). Prior research suggests that ANPP-CO_2 response on sandy loam may be driven by increased soil moisture availability with CO_2 (Fay *et al.*, 2015b). SWC slightly increased with CO_2 in sandy loam soil (Appendix A: Figure A.S8), but corresponding changes in bacterial communities were a result of SWC differences among soil types rather than a CO_2 induced effect. CO_2 effects on plant productivity, plant species relative abundances, and bacterial community structure differed among soils, but the changes associated with aboveground and belowground responses were not closely related.

Most taxa at phylum level remained largely unresponsive to CO_2 (Appendix A: Figure A.S6a). We detected soil-specific variation in CO_2 effects on the relative abundance of a few individual taxa at the family-level (Figure 2.4). For example, the relative abundance of *Micromonosporaceae* and *Gaiellaceae* families from phylum *Actinobacteria* increased along the CO_2 gradient only in clay soils. Members of *Micromonosporaceae* have previously been linked to enhanced cellulose degrading capability (Yeager *et al.*, 2017). *Gaiellaceae* are known to be chemoorganotrophic (Albuquerque *et al.*, 2011), possibly favored by environments rich with organic C. The increase in relative abundance of *Micromonosporaceae* and *Gaiellaceae* could be attributed to higher labile C availability with increasing CO_2 concentration in clay soils as shown in a previous LYCOG study

(Procter *et al.*, 2015). We did not measure microbial biomass, but results from the fourth year of CO_2 treatment at LYCOG showed that active soil microbial biomass increased most with CO_2 in the clay soils (Procter *et al.*, 2015).

The abundance of *Pirellulaceae* family significantly decreased with CO_2 in sandy loam soil, but the overall abundance of *Planctomycetes* phylum did not change with CO_2. In contrast, researchers found significant increase in *Planctomycetes* abundance with elevated CO_2 at a NZ-FACE site (Xia *et al.*, 2017). The response of individual families to CO_2 enrichment apparently is mediated by soil properties. As most phyla were clustered by soil type (Appendix A: Figure A.S5), we infer that a strong soil effect can obscure a weaker CO_2 effect. Particularly, the relative abundance of the members of *Planctomycetes*, *Chloroflexi* and *Actinobacteria* phyla significantly differed in sandy loam soil compared to silty clay and clay soils. These results could be linked to large variation in soil nutrient, moisture and texture properties across soil types (Hermans *et al.*, 2016; Kuramae *et al.*, 2012; Rughöft *et al.*, 2016).

Sandy loam soil communities were distinctly clustered, with a significant overlap between silty clay and clay soils. As anticipated, this variation was primarily due to significant differences in soil physiochemical properties among three soils (Appendix A: Table A.S3). Soil moisture, nutrient and textural differences associated with three distinct soil types strongly influenced bacterial community structure, a result consistent with several studies investigating the influence of edaphic properties on bacterial communities (Butterly *et al.*, 2016; Ge *et al.*, 2010). However, NMDS ordinations revealed that the overall soil effect may be slightly diminished with UniFrac distance compared to Bray-Curtis distance (Figure 2.2). UniFrac distance matrix also accounts for the phylogenetic

relatedness of taxa (Lozupone & Knight, 2005), and links to evolutionary relationships among samples. Thus, soil bacterial communities may be composed of closely related taxa resulting in smaller pairwise distances with UniFrac distance (Ortmann & Ortell, 2014).

Many studies have reported plant species composition and diversity as one of the key drivers of soil bacterial community structure and composition (Burns *et al.,* 2015; Leff *et al.*, 2018, 2015). We analyzed plant-specific and soil-specific effects to assess their relative influence on bacterial community structure using partial RDA and partial Mantel tests. When the soil effects were held constant, plant species abundance were poorly associated with bacterial community structure, while soil properties remained highly significant when plant effect was controlled. This is likely because our soil core samples contained mostly bulk soil, which often reflects less plant influence than does the rhizosphere soil. This result further suggests that variation in bacterial community structure through changes in plant species composition may be less evident than direct effects of soil properties.

Several studies have considered climatic factors such as temperature and precipitation as important drivers of seasonal dynamics affecting soil bacterial communities (Cruz-Martínez *et al.*, 2009; Gwosdz *et al.*, 2016; Lauber *et al*, 2013; Morales & Holben, 2013). These studies demonstrated significant impact of seasonal variation on bacterial community structure and composition. In contrast, the lack of temporal shifts in bacterial communities among the beginning, middle and end of growing season indicates that community structure did not vary with season of sampling in the current study. Both temperature and precipitation regime in this study mimicked the local seasonal patterns. For example, the daily average temperature in LYCOG facility significantly differed

between May, August, and November (Appendix A: Table A.S4). Although SWC varied seasonally across soil types, the differences in soil moisture among CO_2 treatments within soil types and season was also large (Appendix A: Figure A.S10). Herein, standalone SWC differences among sites were a better predictor of bacterial diversity (Appendix A: Figure A.S2) and community structure (Figure 2.5) than average SWC values across three sampling events during the growing season. Thus, the overall seasonal effect was negligible even though there was temporal variability in average temperature and soil water content during the growing season.

Bacterial communities were exposed to experimental conditions in the LYCOG system for a decade. Both plant communities and CO_2 gradient treatment represent contemporary changes compared to pre-existing soil conditions. A decade of CO_2 treatment had a negligible effect on overall bulk soil properties such as C/N ratio. The overall pH values changed little between the pre-CO_2 treatment year of 2002 and fourth year of CO_2 treatment in 2009 (Appendix A: Table A.S3). Initial pH differences among soil types persisted with clay soil being slightly more acidic. Procter *et al.* (2014) found that pH marginally decreased along the CO_2 gradient (p = 0.04). But, since we did not measure soil pH for all samples collected in 2015 sampling season, we could not establish a direct link between interactive effects of pH and CO_2 gradient on bacterial community structure.

Conclusions

Our results highlight that bacterial communities may be largely unresponsive to relatively short-term effects of CO_2 enrichment, possibly due to the transient effects of CO_2 on plants. Although we observed soil-specific variation in CO_2 effects on community

structure, the modest CO_2 gradient treatment may not be sufficient to induce large shifts in bacterial diversity and community structure. Instead, the long-term exposure to stable edaphic conditions such as soil texture and nutrient properties that persisted prior to CO_2 application likely created distinctive environmental niches for bacterial communities among different soil types (Ge *et al.*, 2010). Taken together, soil niche differences potentially have a greater and more direct effect on bacterial communities than changes in CO_2 treatment or plant species composition. This finding may have broader implications in ecosystem-level responses to past, present, and future climate change scenario.

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Conflict of Interest

The authors declare no conflict of interest.

Data Accessibility

The nucleotide sequences from this study have been deposited in BioProject with accession number PRJNA416942 in the NCBI BioProject database. The ancillary data related to plant and soil variables will be provided upon request.

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CHAPTER THREE

Shotgun Metagenome Analysis Reveals Minimal CO_2 Enrichment Gradient Effects on Switchgrass Soil Microbiome Structure and Functional Potential

This chapter is manuscript in preparation for submission as a primary research article: Raut, S., Polley, H. W., Fay, P. A., Kang, S. & King, R.S. (2021). Shotgun Metagenome Analysis Reveals Minimal CO2 Enrichment Gradient Effects on Switchgrass Soil Microbiome Structure and Functional Potential. In prep.

Abstract

Switchgrass (*Panicum virgatum* L) is a perennial C_4 grass and an important biomass crop for biofuel production. Although effects of rising atmospheric CO_2 concentration on C_3 grasses have been extensively studied, little is known about the complex interactions between CO_2 enrichment, soil type and legacy effects on the structure and functional potential of soil microbes associated with C_4 plants. Here, we utilized shotgun metagenome sequencing to elucidate the changes in community structure and functional gene abundance linked to carbon (C), nitrogen (N) and phosphorus (P) cycling in switchgrass silty clay and clay soils. We collected bulk soil samples from switchgrass monocultures (n=20) in 2015 (the last year of CO_2 gradient treatment) and 2016 (year one after the cessation of CO_2 treatment). We expected that long-term CO_2 enrichment would have minimal effect on community composition but significant influence on the abundance of key functional genes involved in C, N, and P cycling. On the contrary, our results showed that abundance of most genes remained largely unaffected by CO_2 enrichment except genes involved in denitrification (*nar* and *nirK/S*), dissimilatory nitrate reduction (*nap*) and one gene (glucoamylase) involved in labile C degradation. Glucoamylase increased along the long-

term CO_2 gradient (250-500 ppm) in clay soils (p=0.05) but *nap*, *nar* and *nirK/S* were significantly enriched at higher CO_2 concentration only in silty clay soils *($p<0.05$)*. Thus, the results indicate that soil x CO_2 interaction altered abundance of specific functional gene categories along the gradient even though the main effects of CO_2 gradient were minimal. We also did not detect any shifts in microbial community structure under CO_2 gradient treatment, suggesting their resistance to long-term CO_2 enrichment and its legacy. On the contrary, soil type had a strong influence on taxonomic community structure, particularly in bacteria and archaea compared to fungi although yearly variation was significant only in bacteria. At taxonomic level, members of *α-proteobacteria* and phylum *Actinobacteria* were dominant taxa involved in carbohydrate degradation, N cycling and phosphate (PO_4^{-3}) metabolism. In particular, *Micromonosporales* and *Solirubrobacterales* significantly contributed to several functional gene categories, suggesting their potential role as "core" microbiome in ecosystem functioning of switchgrass soils. These findings expand our current understanding of C, N and P cycling processes, specifically in switchgrass soil microbiome in the face of global climate change.

Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial C_4 grass, which has been proposed as a "model" crop to produce bioenergy feedstock and it could aid in substituting our dependence on fossil fuels (Wright & Turhollow, 2010). Although C_4 grasslands only account for ~18 % of vegetated land surface globally (Somerville et al., 2010; Still et al., 2003) compared to much higher contribution of C_3 grasses, they provide important ecosystem services. Among various cellulosic biomass crops, switchgrass is considered a better candidate for biofuel production because it can be grown in marginal lands with low

maintenance and minimal agricultural inputs (Jesus et al., 2016; Werling et al., 2014). Furthermore, switchgrass also contributes to lower greenhouse gas emissions than most annual crops (Oates et al., 2016). Recently, few studies have examined the role of soil microbes and diazotrophic communities in sustainable management and production of switchgrass (Bahulikar et al., 2014; Mao et al., 2014; Rodrigues et al., 2017). Despite such growing interest in switchgrass as a biomass crop, we have a limited understanding of how global change factors such as elevated CO_2 affect the overall structure and functional potential of soil microbial communities associated with switchgrass.

Rising atmospheric CO_2 concentration is expected to favor C_3 plants over C_4 plants mainly due to their differences in photosynthesis efficiency (C. Wang et al., 2012). In C_3 plants, CO_2 fixation is catalyzed by the enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase-oxygenase), which is sensitive to oxygenase activity (Ehleringer et al., 1997). Thus, increase in mesophyll CO_2: O_2 ratio due to CO_2 enrichment is expected to increase carboxylation rate and decrease photorespiration in C_3 plants (Ainsworth & Long, 2005; Fay, Polley, et al., 2012). In contrast, CO_2 is initially catalyzed by phosphoenolpyruvate carboxylase in mesophyll of C_4 plants and then released to bundle sheath cells (Sage & Kubien, 2007). The bundle sheath cells around RuBisCO are already saturated with CO_2 in C_4 plants resulting in the suppression of the oxygenase activity. Therefore, increased CO_2 concentration in the atmosphere is expected to have minimal effect in the CO_2 assimilation rate of C_4 species such as switchgrass compared to other C_3 grasses or forbs.

Although projected changes in atmospheric CO_2 fluxes could have significant implications for biosphere carbon cycle feedbacks (Dieleman et al., 2012) , CO_2 concentration within soil is significantly higher than in the atmosphere (Drigo et al., 2008).

Consequently, the direct impact of CO_2 enrichment on soil microbial communities associated with switchgrass is also less likely. However, CO_2 enrichment can reduce stomatal conductance and transpiration in C_3 or C_4 plants (Ainsworth et al. 2004). Elevated CO_2 concentration can also enhance soil net N mineralization (Dijkstra et al., 2008a; Reich et al., 2018) and increase soil moisture in C_4 plants (Leakey et al., 2009; Lecain et al., 2003), which could indirectly influenc the structure and functional potential of soil microbes.

Long-term exposure to environmental perturbations along with preexisting differences in soil properties could have a lasting impact on structure and function of soil microbial communities even after the cessation of experimental treatment (Rousk et al., 2013; Zhang et al., 2018). Thus, the effects of prior experimental conditions that persist in the subsequent seasons or years are referred to "legacies" (Cuddington, 2011). A few free-air carbon dioxide enrichment (FACE) systems (Butterly et al., 2016; He et al., 2014a) studies have primarily focused on the effects of elevated CO_2 on functional gene abundance of soil microbes in C_3 vegetation. Another study from a C_4 agroecosystem found that elevated CO_2 stimulated the key functional genes involved in C, N and P cycling (Xiong et al., 2015). These studies are important to discern how soil microbes markedly shift in their taxonomic composition/metabolic function or whether they can withstand any changes in response to an ongoing ecological disturbance such as increase in atmosphere CO_2 concentration. However, most studies only focus on a "snapshot" of response to current disturbance scenario. Consequently, evaluating the ability of soil microbes to remain resistant (communities are unchanged during perturbation), sensitive (composition/function may be significantly altered) or become resilient (recover over time

after perturbation) is necessary in the face of global climate change (Allison & Martiny, 2008; Griffiths & Philippot, 2013; Shade et al., 2012). Sampling events conducted during, and post disturbance (or termination of experimental conditions) allow us to evaluate microbiome resilience and legacy effects more precisely, which broadly benefits ecosystem management practices. Furthermore, the research on soil microbial response to CO_2 gradient treatment setting is lacking in comparison to several studies with ambient (current) versus elevated (projected increase in the future) CO_2 levels. Given the economic potential and ecological benefits of switchgrass, it is particularly important to understand interactions of C_4 plants, edaphic factors, and soil microbial communities during and after the cessation of long-term experimental conditions.

In this study, we utilized unique CO_2 gradient (250-500 ppm) to evaluate the effects of CO_2 enrichment and its legacy in switchgrass soil microbiomes associated with silty clay (an upland mollisol) and clay (a low land vertisol). The switchgrass monoliths were established on Lysimeter CO_2 gradient (LYCOG) facility located at Temple, Texas, USA in 2007. We collected the soil samples from 2015 (the last year of CO_2 treatment) and 2016 (after the experimental treatment was terminated and returned to ambient CO_2 concentration). We implemented shotgun metagenome sequencing to elucidate the changes in community structure and functional gene abundance linked to carbohydrate degradation, nitrogen (N) and phosphorus (P) cycling. Because switchgrass monocultures were well-watered with high soil moisture content and nitrogen availability (Fay, Polley, et al., 2012), we expected minimal effect CO_2 in taxonomic composition and structure. However, the cessation of CO_2 gradient could potentially affect soil nutrient dynamics, thus altering the abundance of key functional genes involved in labile carbohydrate

degradation and nitrogen cycling. We found no significant effect of long-term CO_2 gradient and its legacies on microbial community structure and abundance of most functional genes involved in C degradation, N cycling and PO_4^- metabolism. Nonetheless CO_2 x soil had some effect on abundance of glucoamylase gene involved in carbohydrate degradation and *nap*, *nar* and *nirK/S* (nitrate reductases). We also characterized putative keystone taxa (*Micromonosporales* and *Solirubrobacterales*) involved in C, N and P cycling of switchgrass soils. Identifying the taxon-specific contribution in nutrient cycling processes can further improve our prediction of their specific role in ecosystem function.

Materials and Methods

Study Site and Experimental Design

The study site is located in Temple, Texas, USA (31^0 05'N, $97^0$20'W) and Lysimeter CO_2 Gradient (LYCOG) facility was operated by USDA-ARS Grassland Soil and Water Research Laboratory since 2006 (Fay et al., 2009). LYCOG consists of two longitudinal, tunnel-shaped chambers (1.2 m x 1.5 m x 60 m), each divided into 10 sections. A daytime linear CO_2 gradient in each chamber was maintained by progressively depleting the enriched through photosynthesis by enclosed vegetation and regulating the desired CO_2 levels at the entry and exit points by controlling the speed of blower fans. The direction of air flow in both chambers was reversed during night-time. CO_2 levels were measured at the entrance and exit of each section at 2 min intervals and the concentration for each monolith was estimated by linear interpolation from the measured values (Fay, Jin, et al., 2012). Air temperature and vapor pressure deficit were regulated near ambient values by cooling and dehumidifying air at 5-m intervals along chambers. CO_2 gradient was

maintained during the portion of the year when vegetation photosynthetic capacity was adequate, typically from late April to early November. Irrigation regime for each soil monolith simulated the seasonal rainfall pattern in central Texas. The details on experimental setup and operation of these chambers are illustrated in the previous work at LYCOG (Fay et al., 2009; Raut et al., 2018).

Switchgrass monocultures were planted in May 2007 in intact soil monoliths excavated in 2002 from an upland silty clay soil (Austin series, Mollisol, Udorthentic Haplustol) and a lowland clay (Houston Black series, Vertisol, Udic Haplustert) in the Blackland Prairie region of central Texas, USA. The tillers that died at LYCOG facility after planting were replaced in May 2008. The monoliths were arranged in a stratified random design along the CO_2 gradient from 2007-2014 along with adjacent soil monoliths with mixed C_3/C_4 vegetation. The monolith positions were switched between sub-ambient and super-ambient (elevated) legs from their long-term (2007-2014) positions in 2015 (Appendix B: Figure B.S1). To study the legacy effects of CO_2 enrichment and analyze data for downstream processes, we considered long-term CO_2 gradient concentrations (Appendix B: Figure B.S1).

Soil Sampling and Chemical Analysis

A total of 20 soil samples were collected from switchgrass monoliths during August 2015 (n=10) and August 2016 (n=10) from two soil types. In 2015, switchgrass monoliths in each section were enclosed within a clear polyethylene (0.006’’/ 0.15 mm), which transmits >90% of incident light (Appendix B: Figure B.S1). Zippered openings and draft flaps in the polyethylene allowed access to monoliths during the time of sample collection in 2015. Experimental CO_2 gradient treatment in LYCOG facility was permanently

terminated and aboveground biomass were clipped in November 2015. Thereafter, switchgrass monoliths were partially enclosed in rain exclusion covers instead of polyethylene enclosures, exposing the dormant vegetation to the ambient atmospheric CO_2 levels but continuing to exclude precipitation. Thus, 2015 sampling was conducted during the last year of CO_2 enrichment and 2016 sampling was done nine months after the cessation of experimental CO_2 enrichment. Three cores from the top 0-5 cm of monoliths from bulk soil samples close to vegetation were collected using a hand-held soil auger (d. 1.5 cm). The soil cores (~15 g) were transferred to 50 ml centrifuge tubes. Samples were transported in dry ice and immediately stored at a -80°C freezer until further analysis. Soil carbon and total nitrogen for samples collected in this study were measured using an elemental analyzer. Approximately 5g of soil was mixed with 20 ml of DI water, and further analyzed for soil NO_3^--N, NH_4^+-N and PO_4^{3-} concentrations.

DNA Extraction and Shotgun Metagenome Sequencing

Total community DNA from soil samples was extracted using commercial extraction kits (PowerSoil® DNA Isolation Kit, Mo Bio Laboratories). The purity of DNA samples was assessed by measuring 260/230 and 260/280 in a Nanodrop spectrophotometer. Quantity of the DNA samples were determined by Qubit 3.0 flourometer. Total metagenomic DNA was sent to Molecular Research LP (MR DNA) located in Shallowater, TX for shotgun metagenome sequencing using Illumina HiSeq 2500 (2x150 bp paired end reads).

Sequence Processing and Analysis

Raw sequencing reads from the metagenome data had forward and reverse reads. Thus, the paired-end reads were aligned and filtered at a Phred score of 20 using the join-fastq algorithm from eautils (Aronesty, 2013) available in QIIME platform whereas the unpaired reads were eliminated before further analysis. Joined reads for each sample were then aligned against a protein reference database, NCBI-NR (NCBI Resource Coordinators, 2017) using DIAMOND version 0.9.10 (Buchfink *et al.*, 2015) in a local Linux machine. Output ".daa" files from DIAMOND were then "meganized" in MEGAN 6 community edition (CE), v.6.12.4 (Huson *et al.*, 2007) with taxonomic and functional mapping files. GI to taxonomy option was selected on the alignments for taxonomic mapping and GI to SEED option was used for functional mapping. Naïve LCA algorithm was selected for taxonomic binning (Huson *et al.*, 2016) with default parameters including a min score = 50.0, max expected = 0.01, top percent = 10.0, min support percent = 0.05.

The number of reads for 20 samples ranged from 757,177 to 1,682,277. When meganized .daa files from multiple samples were imported, the option for normalized counts was selected. The reads for each sample were then normalized to smallest sample size (~750,000) within MEGAN program considering the differences in metagenome size among samples. Taxonomic information from all samples was compared and then exported from MEGAN in tab-delimited format for overall microbial community structure and composition analysis. Functional gene abundance linked to carbohydrate degradation, N metabolism and PO_4^{3-} metabolism was extracted from SEED (level 4) within the MEGAN program (Supporting Information Table S1). To assess the contribution of different

microbial taxa in carbohydrate degradation, N and P cycling processes, first the gene of interest from SEED subsystem was selected and a separate a separate file in "*.rma"* (read-match archive) format for each sample was extracted to a new document. Then, .rma files for the individual gene was retrieved from all samples and taxonomic information was exported at phylum and order level.

Statistical Analyses

Statistical analyses were carried out with R statistical software v.3.6.3 (R Development Core Team, 2020) with packages (vegan, ggplot2, mgcv and others) and custom scripts. Unconstrained ordinations were performed on the taxa abundance tables retrieved from MEGAN using Non-metric multidimensional scaling (NMDS) available in vegan (Oksanen et al., 2017) using Bray-Curtis distance. Non-parametric permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were used to evaluate the main and interactive effects of CO_2, soil type and year on bacterial community structure.

Scatter plots with linear regression using "lm" function was implemented to test the effects of soil x CO_2 gradient on the abundance of functional genes involved in C degradation, N metabolism and PO_4^{3-} metabolism as well as relative abundance of taxa (at order level). Bar plots with standard errors were constructed to assess the main and interactive effect of soil type and year, which was ambient in 2016 but exposed to CO_2 gradient treatment in 2015. Statistics analyses were performed using two-way ANOVA and p-values were reported in the bar-plots. To assess the relationship between gene abundance and environment variables, Pearson's correlation was used. Taxonomic

contribution of different microbial groups for each gene category was illustrated in bubble plots and weighted by their relative abundance.

Results

Taxonomic Community Structure and Composition

Normalized reads aligned against NCBI-nr database in MEGAN revealed a total of 15,779 taxa for bacteria, 767 taxa for archaea and 624 taxa for fungi at the lowest taxonomic resolution. NMDS ordinations using Bray-Curtis distance showed that soil type had a strong influence on bacteria and archaea but not on fungi (Figure 3.1, Table 3.1). In contrast, long term CO_2 enrichment had no significant effect on overall microbial community structure (Figure 3.1, $p>0.05$). Similarly yearly variation had some effect on taxonomic community structure but only in bacteria (Table 3.1) Clustering patterns for archaea and fungi did not show any substantial change between two years in terms of their community structure (Figure 3.1b, c), further supporting the resistance of switchgrass soil microbiome to long term CO_2 enrichment and its legacy.

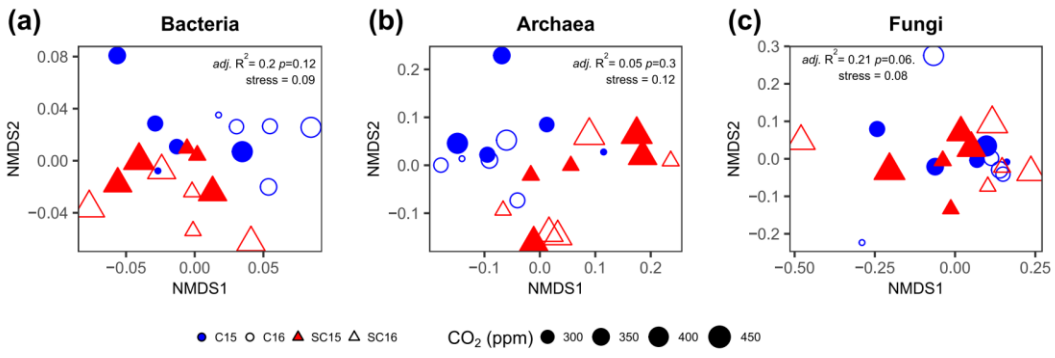

Figure 3.1 NMDS ordinations based on taxonomic community structure of Bacteria, Archaea and Fungi at species level. Soil samples were collected from silty clay (red) and clay (blue) in 2015 (the last year of CO_2 gradient treatment) and 2016 (year one after termination of experimental treatment). To evaluate the effect of long-term CO_2 enrichment, the adjusted R-squared (adj R^2) values from CO_2 gradient fit was computed with ordisurf GAM.

Table 3.1 Multivariate statistical approaches to assess the effects of soil, year and CO_2 on bacterial, archaeal, and fungal communities based on species level taxonomic resolution.

Statistic		Soil type (clay vs silty clay)			Year (ambient vs CO_2 treatment)		
		Bacteria	Archaea	Fungi	Bacteria	Archaea	Fungi
PERMANOVA	*F*	2.48	4.79	1.07	2.76	0.87	1.46
	p	**0.03***	**0.002***	0.30	**0.02***	0.45	0.14
ANOSIM	*R*	0.18	0.40	0.02	0.17	0.02	0.13
	p	**0.02***	**0.001***	0.29	**0.03***	0.26	**0.06.**

*Significant ($\alpha = 0.05$).
**Significant after Bonferroni's correction ($\alpha = 0.017$).

Further evaluation of taxonomic composition of bacterial communities at order level (>1%) also showed no significant effect of CO_2 enrichment gradient on the relative abundance of most taxa (Figure 3.2). However, a few did exhibit significant soil x CO_2 interaction (Figure 3.2). In silty clay soils, *Micrococcales* and *Geodermatophilales* showed sharp decrease in their relative abundance along the long-term CO_2 enrichment gradient of 250-500 ppm (Figure 3.2). However, none of the taxa associated with clay soils were affected by CO_2 enrichment. Similarly, we found that the effect of soil type and soil x year interaction was more frequent compared to minimal CO_2 effect (Appendix B: Figure B.S2).

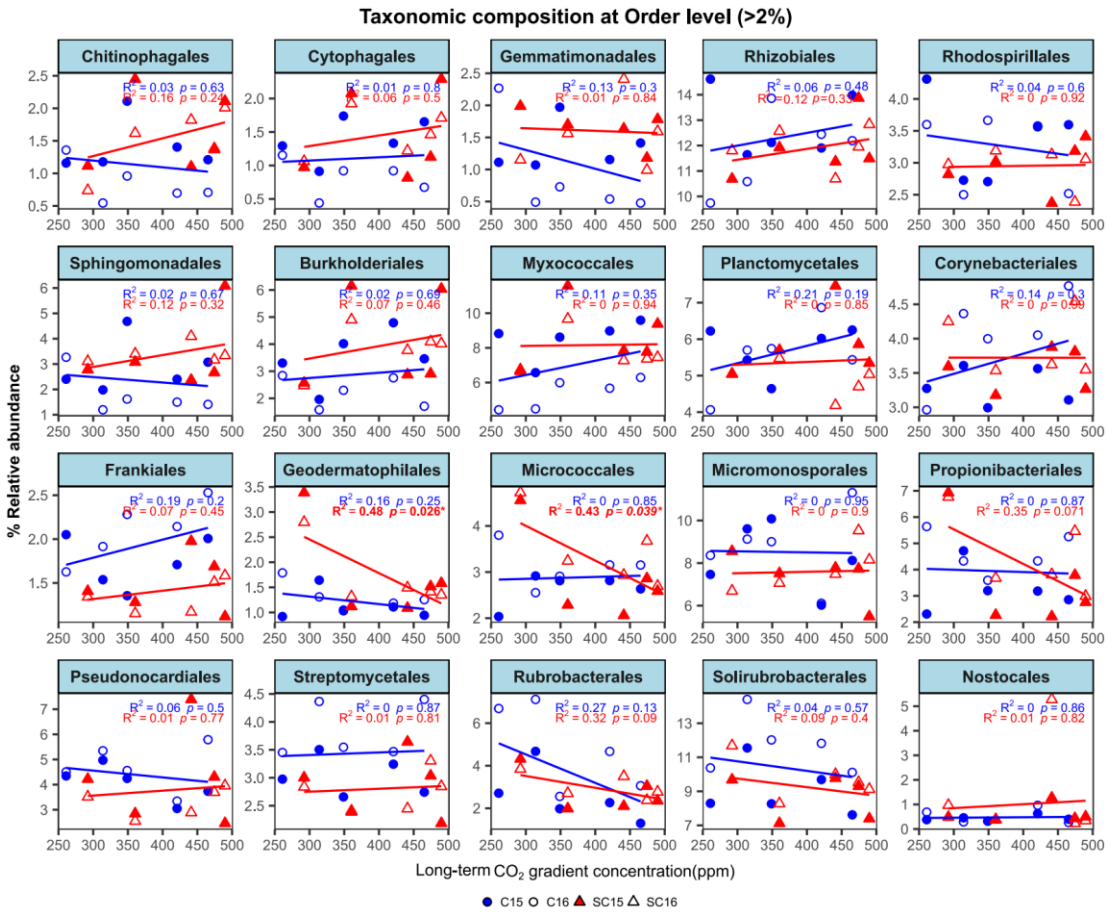

Figure 3.2. Scatter plots showing the effect of long-term CO_2 gradient treatment on the relative abundance of taxa at order level. R^2 and p-value are from the fitted linear regression models. The CO_2 concentration in x-axis indicates long-term CO_2 gradient treatment.

Effects of CO_2 Gradient, Soil type and Year on Functional Gene Abundance

Key genes involved in C degradation were selected from level 4 (individual genes) of the carbohydrate metabolism category in SEED subsystem (Appendix B: Table B.S1a). CO_2 enrichment had no significant effect on abundance of all C degradation genes except Glucoamylase (p=0.05), which marginally increased along the long-term CO_2 gradient in clay soils (Figure 3.3). The proportion of glucoamylase gene was slightly higher in clay soils compared to silty clay. We also found that the abundance of glucoamylase slightly increased in 2016 (ambient CO_2) versus 2015 (ongoing CO_2 gradient treatment) but only

in clay soils (Appendix B: Figure B.S3). Except glucoamylase, we also did not observe substantial changes in abundance of other C degradation genes in response to main effects or interactive effects between soil type and yearly variation (Figure 3.3). The proportion of glucoamylase (starch), beta-galactosidase (hemicellulose) and beta-glucosidase (cellulose) were relatively higher than other genes in all soil samples (Figure 3.3 and Appendix B: Figure B.S3).

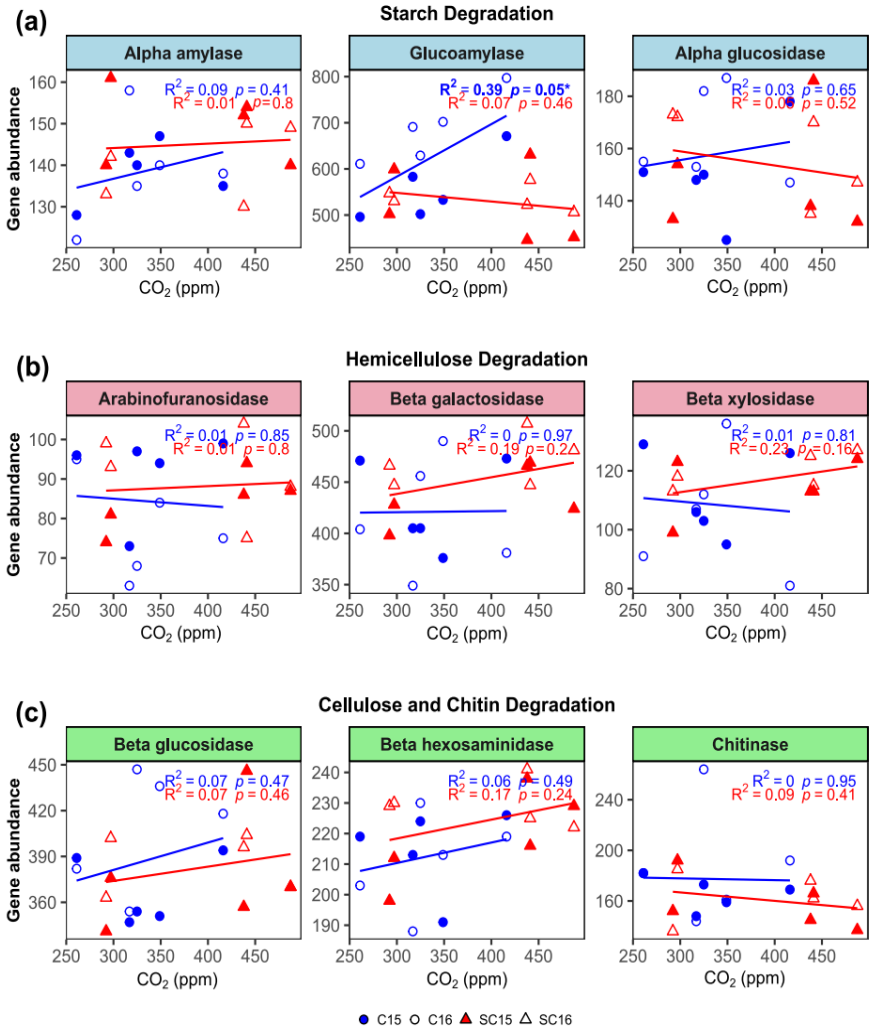

Figure 3.3 Gene abundance in the scatter plot indicates absolute gene counts for different carbohydrate substrate categories. The CO_2 concentration in x-axis indicates long-term CO_2 gradient treatment.

N cycling genes were selected from level 4 of the N metabolism category in SEED subsystem (Appendix B: Table B.S1b). Overall, long-term CO_2 enrichment gradient and its legacy had no significant effect on functional abundance of most genes involved in various nitrogen cycling processes (Figure 3.4) Nonetheless, the main effects of soil and the interactive effects between soil x year was significant in some genes (Figure 3.4 and Appendix B: Figure B.S4). Glutamate dehydrogenase (*gdh*), which is involved in mineralization of organic N into ammonia (NH_4^+), was most abundant among all N cycling genes (Appendix B: Figure B.S4). The abundance of *gdh* only increased in 2016 samples from clay soils (Appendix B: Figure B.S4).

We also detected several gene families (Appendix B: Table B.S1b) responsible for denitrification of nitrate (NO_3^-) as illustrated in the abundance of nitrate reductase (*nar*GHIJ), nitrite reductase (*nir*K/S), nitric oxide reductase genes (*nor*BC), and nitrous oxide reductase (*nos*) gene family (Figure 3.4). Among all the denitrification genes, *nar* had the highest abundance, where main effects of soil and interactive effects of CO_2 x soil was also significant (Figure 3.4). Two genes (*nar* and *nirK/S*) involved in denitrification significantly increased at higher CO_2 concentrations but only in silty clay soils ($p<0.05$). The abundance of *nas* and *nor* also increased in clay soils compared to silty clay soils (Appendix B: Figure B.S4). However, CO_2 enrichment, soil and yearly variation had no effect on the abundance of *nos*.

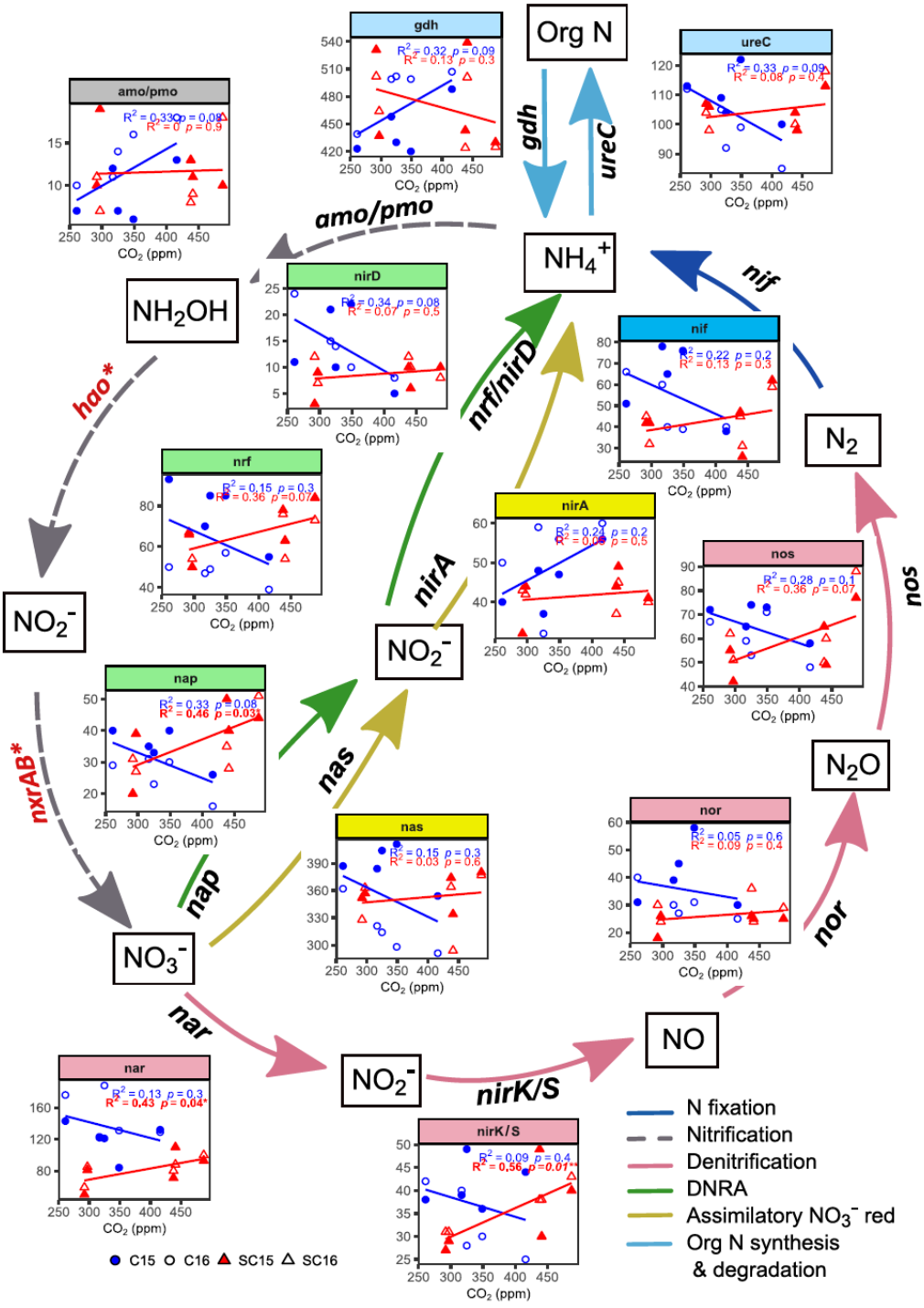

Figure 3.4 Scatter plots showing the absolute gene counts for Nitrogen cycling genes. Genes with asterisk labels (*) were either rarely detected or absent in SEED level 4 N metabolism category. The CO_2 concentration in x-axis indicates long-term CO_2 gradient treatment.

In clay soils, yearly variation had significant effect on the abundance of assimilatory nitrate reductase (*nas*) and cytochrome c nitrite reductase (*nrf*) responsible for dissimilatory nitrite reduction (Appendix B: Figure B.S4). Both *nas* and *nrf* had higher abundance 2015 clay soil samples but remained unaffected in silty clay soils (Appendix B: Figure B.S4). Another assimilatory nitrite reductase gene (*nirA*), and periplasmic nitrate reductase gene (*nap*) responsible for dissimilatory nitrate reduction were also detected. However, the abundance of only *nap* increased in silty clay soils at higher CO_2 concentration (Figure 3.4, $p<0.05$). For nitrogen fixation pathway, we selected multiple genes from nitrogenase family (*nif*). Similarly, we selected urease (*ure*C) responsible for biosynthesis of organic N. Among the nitrification genes, we also detected low gene counts for ammonia/methane monooxygenase (*amo*/*pmo*). However, CO_2 enrichment, soil type and year or their interaction had no effect on the absolute abundance of *nirA, nif, ureC* and *amo/pmo* (Figure 3.4 and Appendix B: Figure B.S4).

Microbial Contributions to Functional Gene Categories in C, N and PO_4^{3-} Metabolism

Actinobacteria was consistently the most abundant phylum across all genes involved in carbohydrate degradation (Figure 3.5 and Appendix B: Figure B.S6b). Bubble plot showed that *Micromonosporales* was significantly more abundant than other members of *Actinobacteria* at order level, particularly for the genes involved in cellulose and chitin degradation (Figure 3.5). However, taxonomic contribution to glucoamylase and other starch-degrading genes generally revealed more diverse and evenly distributed taxa within phylum *Actinobacteria* (Figure 3.5). The relative proportion of *Bacteroidetes*, *Acidobacteria* and *Firmicutes* was higher for genes involved in hemicellulose degradation (Figure 3.5). Similarly, few orders of *Proteobacteria* including *Rhizobiales*,

Rhodospirilalles and Myxococcales represented a greater proportion of amylase gene involved in starch degradation (Figure 3.5).

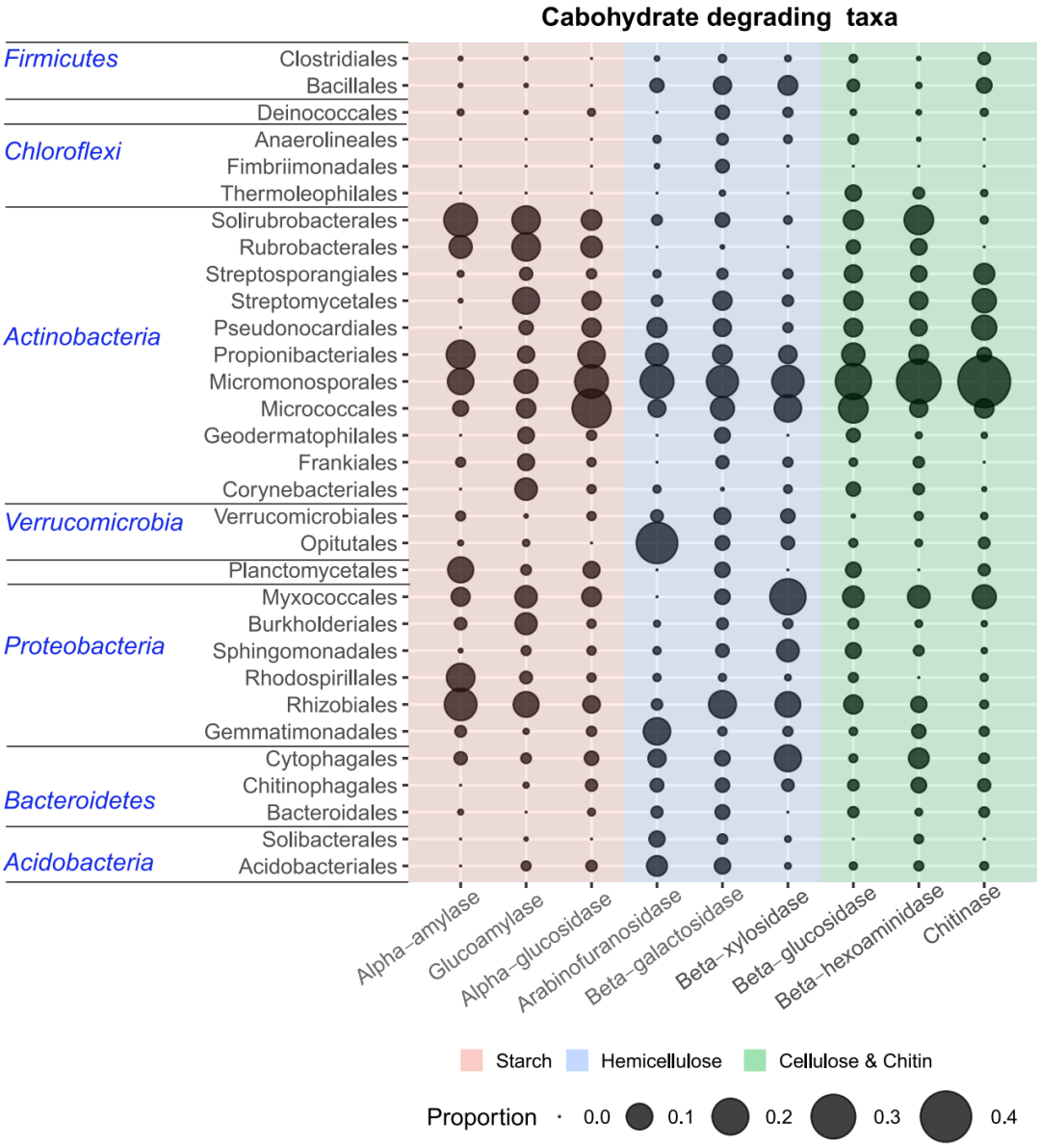

Figure 3.5 Bubble plots showing the proportion of microbial populations involved in carbohydrate degradation at order level. Size of the nodes is weighted by their relative abundance.

Most taxa involved in nitrogen cycling pathways were not evenly distributed as illustrated in the bubble plots with relative proportion at order level and absolute count at phylum level respectively (Figure 3.6 and Appendix B: Figure S6c). *Rhizobiales*, which is

a member of *α-proteobacteria* was particularly the most abundant taxa across most N cycling genes except *nar* and *gdh.* Bubble plot showed that the most abundant taxa involved in nitrogen fixation process belonged to unclassified *Acidobacteria,* followed by *Rhodospirillales* and *Rhizobiales* (Figure 3.6). The proportion of *Micromonosporales* and *Solirubrobacterales* from phylum *Actinobacteria* were relatively much higher among the taxa contributing to *nar* gene (Figure 3.6), which reduces nitrate (NO_3^-) to nitrite (NO_2^-). Taxa contributing to nitrification gene (*amo/pmo*) were less abundant and less diverse, where *Nitrosomonas* from *β-proteobacteria* was involved along with a few members from phylum *Actinobacteria* (Figure 3.6). Furthermore, *Actinobacteria* were also most dominant phyla in organic N transformation, as illustrated by their contribution to *ureC* and *gdh* (Appendix B: Figure B.S6).

CO_2 enrichment, soil type and year or their interaction had no effect on the absolute abundance of three genes (*alp, ppk* and *ppx*) detected in PO_4^{3-} metabolism (Figure 3.7a and Appendix B: Figure B.S5). Among the phyla contributing to PO_4^{-3} metabolism, the relative proportion of those involved in assimilation of P from soil organic matter, namely alkaline phosphatase (*alp),* were more diverse (Appendix B: Figure B.S6c). In contrast, only a few taxa contributed to polyphosphate transformation regulated by exopolyphosphatase *(ppx)* and polyphosphate kinase (*ppk)* gene (Figure 3.7b). For example, members of *Cyanobacteria, Acidobacteria, Bacteroidetes* and *α-proteobacteria* were more abundant and evenly distributed for *alp* whereas members of *Actinobacteria* were more abundant in *ppk* and *ppx* (Appendix B: Figure S6c). At order level, *Rhizobiales* had dominant contribution to *alp* gene abundance (Figure 3.7b). Similarly, *Solirubrobacterales* had most abundant *ppx* and *ppk* gene (Figure 3.7b).

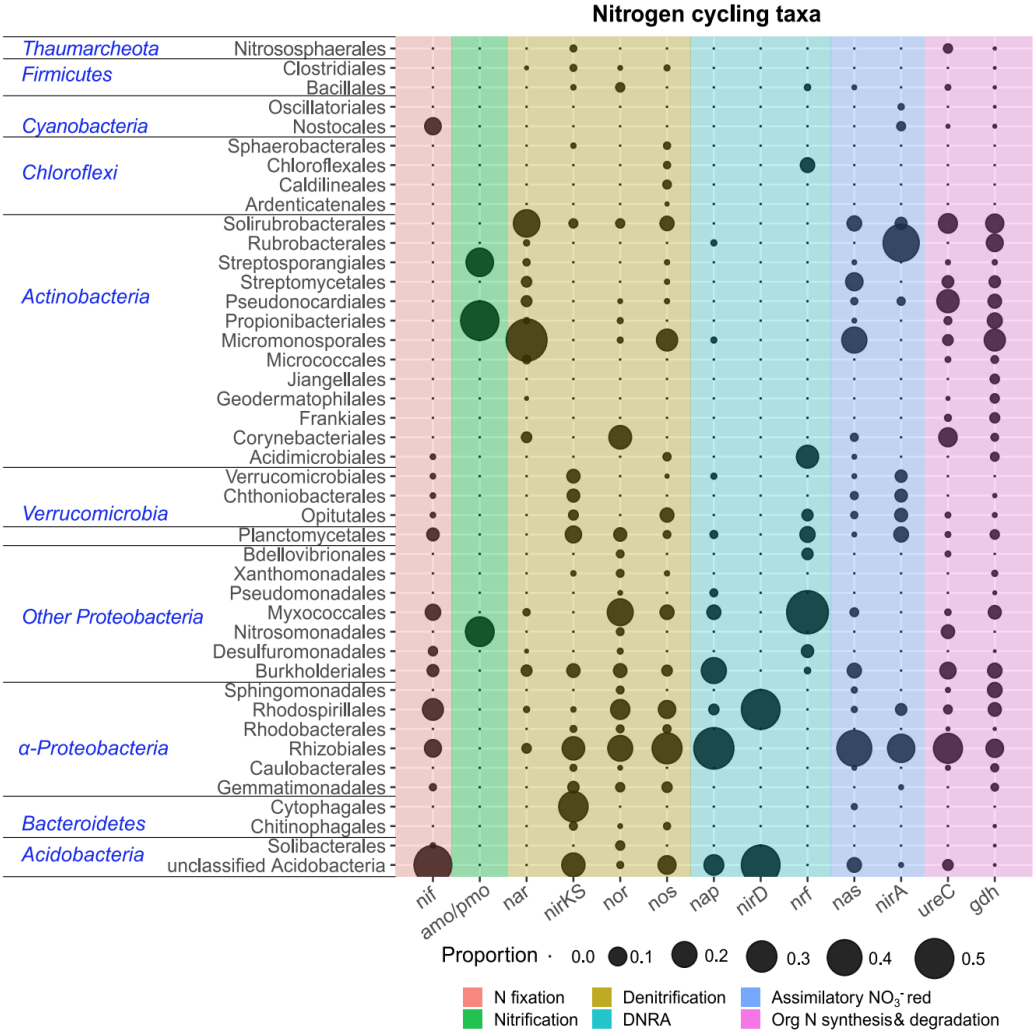

Figure 3.6 Bubble plots indicate the proportion of microbial populations involved in nitrogen cycling processes at order level. Size of the nodes is weighted by their relative abundance.

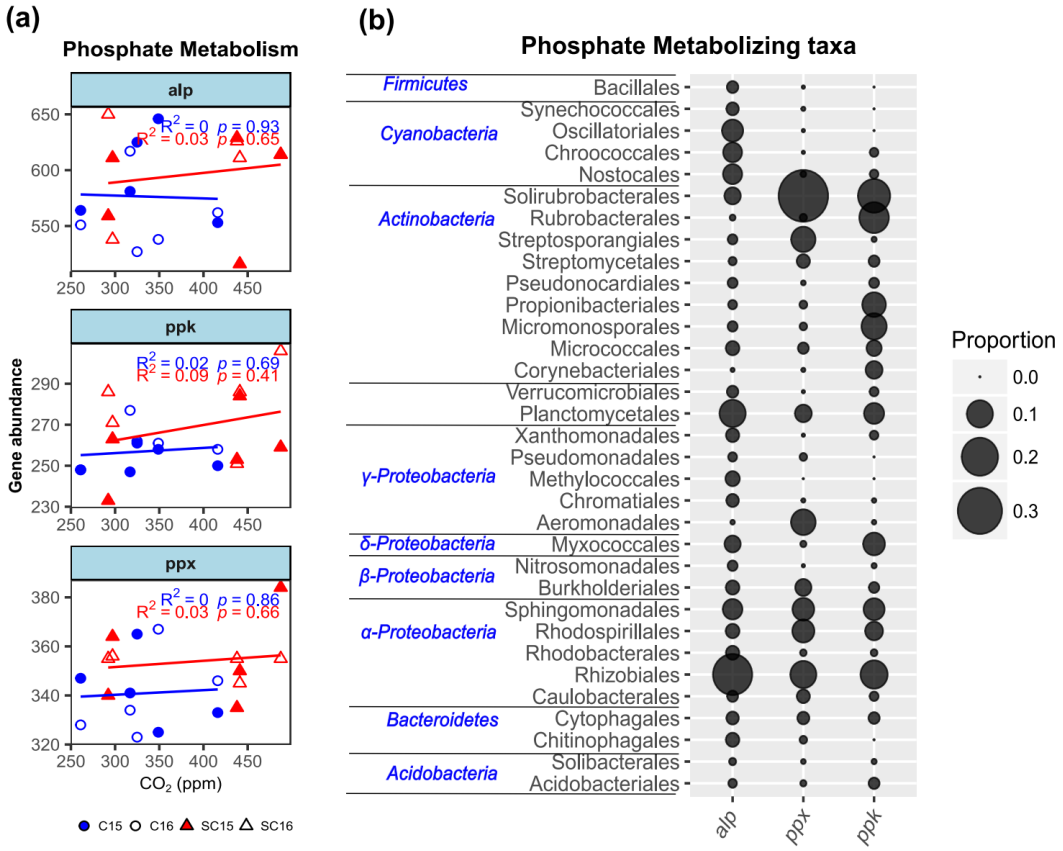

Figure 3.7 (a) Scatter plots illustrate the absolute gene counts for alkaline phosphatase (*alp*), polyphosphate kinase (ppk), and exopolyphosphatase (ppx), which represent the key genes involved in Phosphate metabolism. The CO_2 concentration in x-axis indicates long-term CO_2 gradient treatment. (b) Bubble plots showing the relative proportion of microbial populations involved in Phosphate (PO_4^{-3}) metabolism.

Soil C/N, NH_4^+, NO_3^- and PO_4^{-3} Availability

The effect of long-term CO_2 gradient was on C/N ratio was not significant. However, soil C/N ratio was markedly higher for silty clay soil than clay soil ($p<0.001$) but remained consistently stable across the years (Appendix B: Figure B.S7). We found significant correlation between nar gene abundance and C/N ratio. Furthermore, we did not find any CO_2 x soil interaction on NH_4^+-N, NO_3^--N and PO_4^{-3} availability (Appendix B: Figure B.S7). Except soil C/N ratio, there was also large variability among soil samples for NH_4^+, NO_3^- and PO_4^{-3} concentration as indicated by error bars. Soil NO_3^--N was slightly

variable between soil types and between years in clay soil, although increase may be attributed to one outlier in 2016 clay soil (Appendix B: Figure B.S7). Soil NO_3^--N availability was significantly correlated to a few genes involved in C degradation (e.g., Glucoamylase), dissimilatory nitrate reduction (*nas* and *nirA)* and organic N assimilation *(gdh)* (Supporting Information Table S2). Soil NH_4^+-N availability was also hemicellulose degradation (e.g., Beta galactosidase, Beta xylosidase), assimilatory nitrate reduction (*nap)* and PO_4^{-3} metabolism (Appendix B: Table B.S2).

Discussion

Given the critical role of soil microbial communities in regulating the nutrient cycling processes (Singh *et al*., 2010), it is important to understand how soil microbial communities respond to CO_2 enrichment. In this study, we examined the effects of linear CO_2 enrichment gradient (250-500 ppm), soil type (clay versus silty clay) and year (ambient versus CO_2 treatment) on structure and functional potential of switchgrass soil microbial communities. Furthermore, we also investigated whether the legacy effects would persist after the cessation of experimental CO_2 treatment particularly in communities sensitive to long-term CO_2 enrichment. We expected the stimulation of key functional genes involved in C, N, and P cycling under CO_2 enrichment. However, our results showed that most functional genes involved in carbohydrate degradation, nitrogen cycling pathway and phosphate metabolism remained largely unaffected under CO_2 enrichment. CO_2 alone did not have a strong influence perhaps due to the stronger soil effect. The interactive effects of soil x CO_2 were significant in glucoamylase gene responsible for starch degradation (Figure 3.3), *nar* and *nirK/S* involved in denitrification as well as *nap* responsible for dissimilatory nitrate reduction (Figure 3.4). The short-term legacy effect of

yearly variation also differed by soil type and was prevalent in some functional gene categories. Furthermore, our results identified certain degree of taxonomic specialization for substrate-specific carbohydrate degradation, N cycling pathways and phosphate metabolism. These findings expand our current understanding of C, N and P cycling processes, specifically in switchgrass soil microbes exposed to CO_2 enrichment.

Our analysis from a unique gradient of preindustrial-to-future CO_2 concentrations revealed that switchgrass soil microbial communities are generally resistant to CO_2 enrichment (Figure 3.1). Soil type, on the other hand showed strong effects on bacterial and archaeal community structure while negligible effect on fungal community structure (Figure 3.1 and Table 3.1). Negligible effect of CO_2 and strong effect of soil type on soil bacterial and archaeal communities associated with switchgrass was consistent with our previous study investigating the bacterial community response to CO_2 gradient in mixed C_3/C_4 grasses (Raut *et al*., 2018). There could be few reasons for resistance to CO_2 enrichment treatment in switchgrass soil microbiomes.

First, it has been well established that C_4 photosynthetic pathway in plants such as switchgrass is locally CO_2-saturated and thus, carboxylation rates have little scope to change under elevated CO_2 concentrations (Leakey et al., 2009; C. Wang et al., 2012). Consequently, the direct effect of CO_2 on primary productivity and cascading effects through plant C inputs on soil microbes could be less pronounced. Notably, a recent study by Peter Reich's group (Reich et al., 2018) showed the long-term trend (> 12 years) might defy this conventional understanding indicating the patterns may be drastically changed from our site with several more years of treatment. Researchers attributed this strong effect on C_4 plants to increased soil net N mineralization rates under elevated CO_2 (Reich et al.,

2018). However, both soils in our study were nutrient rich with high soil moisture (Fay, Polley, et al., 2012) and nitrogen content (Appendix B: Figure B.S7). Consequently, CO_2 enrichment induced N mineralization was not evident from our results. Indeed, a previous study from LYCOG site also showed that ANPP did not increase with CO_2 in fully established switchgrass plants although it slightly varied between soil types (Fay, Polley, et al., 2012).

Another possible reason for no significant CO_2 enrichment effects may be because the elevated CO_2 concentration (super-ambient section) in LYCOG setting was lower (Appendix B: Figure B.S1) and reflected gradient rather than much higher fixed point from most FACE studies with elevated CO_2 level greater than 550 ppm (He *et al.*, 2010; Xiong *et al.*, 2015; Yu *et al.*, 2018). Although soil monoliths were switched from their long-term position (2007-2014) in 2015 (the last year of CO_2 treatment), we did not find any legacy effects on community structure. However, abrupt changes to much higher elevated CO_2 levels could elicit more significant changes in microbial community structure when compared to moderate CO_2 level in this study (Klironomos et al., 2005). While the effect of long-term CO_2 enrichment and its legacy alone was not evident, we did observe the difference in taxonomic structure between 2015 and 2016 samples particularly in bacterial communities. These results suggest that the legacy effect of short-term yearly changes is variable among microbial groups, where bacteria tend to recover more quickly and thus may be more resilient to environmental disturbance (Allison & Martiny, 2008) compared to fungi and archaea. Furthermore, vegetation clipping conducted at the end of growing seasons between soil sampling events could have also some contributions in variation between two years (Xue et al., 2016; Zhang et al., 2005).

We expected that the abundance of labile C degradation genes would increase while recalcitrant C degradation genes will remain similar in response to CO_2 enrichment. However only glucoamylase gene involved in labile C (starch) degradation showed significant enough change along the CO_2 gradient concentration (Figure 3.3) in clay soils. Previous studies have reported increase in labile C degradation under elevated CO_2 conditions (He et al., 2010, 2014b; Xu et al., 2013) although a recent study found that the functional gene abundance involved in C degradation decreased under elevated CO_2 conditions (Butterly *et al.,* 2016). At SoyFACE study site, researchers found significant stimulation of most C degradation genes in C_4 maize agrosystem (Xiong *et al*., 2015). As shown in our previous work, this negligible effect may be attributed to the increased points for CO_2 treatment over most conventional FACE studies with single CO_2 treatment (Raut et al. 2018). Nevertheless, current understanding of CO_2 effects on the functional potential microbial communities associated with C_4 plants is still elusive and demand more studies.

Most FACE studies have demonstrated significant changes in gene abundance under elevated CO_2 (He et al., 2010, 2014b; Tu et al., 2017; Xiong et al., 2015; Xu et al., 2013; Yang et al., 2019; Yu et al., 2018). Our results showed that most genes involved in N cycling (*gdh, ureC, amo/pmo, nirA, nirD, nas, nos, nif*) remained unchanged except a few in denitrification (*nar* and *nirK/S*) process and dissimilatory nitrate reduction (*nap)* with significant soil x CO_2 interaction (Figure 3.4). In silty clay soils, *nar* and *nirK/S* significantly increased along the long-term CO_2 enrichment gradient in both 2015 and 2016 (Figure 4, $p<0.05$)., which indicates that the legacy effects of prior experimental conditions may persist only in specific N cycling processes (e.g., denitrification) for some time. The effect of soil type alone was also more pronounced on *nar* possibly due to strong correlation

with C/N ratio, resulting from soil-specific differences (Appendix B: Figure B.S7, Table B.S2). A recent study at the Giessen Free-Air Carbon Dioxide Enrichment (GiFACE) sites found increased dissimilatory nitrate reduction under elevated CO_2 levels even though soil nitrate levels were not affected by CO_2 enrichment. This result is consistent with our finding of selective stimulation of genes (*nap, nar* and *nirK/S*) involved in heterotrophic processes such as dissimilatory nitrate reduction and denitrification under CO_2 enrichment. However, comparable results on functional potential microbiome resilience and legacy effects of exposure to long-term CO_2 enrichment in C_4 soil microbes is rarely examined. Thus, understanding microbiome resilience post disturbance specifically linked to their function may require studies evaluating long-term temporal dynamics (Chen et al., 2019; Shade, 2018) compared to a short duration in our experimental setting.

Of the several factors that influence N cycling processes, soil water availability and N limitation have been considered as important constraints under elevated CO_2 (Leakey et al., 2009; Yiqi Luo et al., 2006; Norby et al., 2010; Reich et al., 2006; Reich, 2009). For example, some studies have found that elevated CO_2 could result in progressive nitrogen limitation due to increase in C input (Dijkstra et al., 2008b; Y. Luo et al., 2004; Reich & Hobbie, 2013). However, in this study CO_2 enrichment had no influence on soil C/N, NO_3^--N, and NH_4^+-N, instead the main effect of soil type was significant on C/N ratio and NO_3^--N (Appendix B: Figure B.S7). Although soil nutrient dynamics remained mostly stable in both 2015 and 2016, soil×year interaction had some effect on soil NO_3^- N levels. This result indicates that nutrient limitation was minimal at higher CO_2 concentration but slightly variable among soil types in our study. Observation of flexible stochiometric homeostasis from aquatic bacteria could potentially explain this finding (Godwin & Cotner, 2018; Scott

et al., 2012). Furthermore, in a water-limited system or drought conditions, elevated CO_2 could increase the photosynthetic water use efficiency of even C_4 plants due to reduced transpiration (Morgan *et al.*, 2011), which may affect the functional potential of N cycling genes (Xiong *et al.*, 2015). Nonetheless, our system was not water-limited, and CO_2 enrichment did not have a significant impact in water availability either (Fay et al., 2012; Raut et al., 2018), possibly resulting in negligible CO_2 effect on N cycling genes.

In general, diverse taxa particularly from phylum *Actinobacteria* and *Proteobacteria* were responsible for carbohydrate degradation, N metabolism and orthophosphate utilization (*alp*). (Figure S6). Denitrification, assimilatory NO_3^- reduction and organic N metabolism had the highest gene abundance among N cycling gene groups. Furthermore, taxa specializing in organic N metabolism (*gdh* and *ureC*) were more diverse at order level (Figure 6). Members of *α-proteobacteria*, were particularly dominant in denitrification, as well as assimilatory and dissimilatory NO_3^- reduction. This finding is consistent with a recent study evaluating taxonomic contribution of different microbial groups (Nelson et al., 2015; Tu et al., 2017).

The effects long-term CO_2 enrichment gradient and its legacy across the years only persisted in *Micrococcales* and *Geodermatophilales* from phylum *Actinobacteria*. Their abundance significantly decreased under higher CO_2 concentration in but only in silty clay soils. Prior work from LYCOG study illustrated soil-specific variation in taxa response although different families (e.g., *Micromonosporaceae* and *Gaillaceae*) of *Actinobacteria* were affected in clay soils with mixed C_3/C_4 vegetation, which suggests a the secondary role of plant species composition following the strong soil x CO_2 interaction effect (Raut et al., 2018). *Micrococcales* abundance also has been previously linked to soil water

availability (Z. Wang et al., 2020). In this study, *Micrococcales* had some genes in starch degradation (e.g., α-glucosidase) but no major role in N or PO_4^{-3} metabolism. Similarly, the overall contribution of *Geodermatophilales* in the nutrient cycling processes examined here were minimal in comparison to other taxa. However, their sensitivity to CO_2 enrichment gradient specifically in switchgrass soils is interesting since they have been only recently classified as a new order of class *Actinobacteria* in the phylogenetic tree (Sen et al., 2014).

Our finding suggests that *Micromonosporales* can utilize a broad range of C substrates (Carro *et al.*, 2018) with slight preference toward more recalcitrant C substrates like cellulose and chitin compared to labile C substrates such as starch (Figure 5). *Micromonosporales* and *Solirubrobacterales* within phylum *Actinobacteria* particularly outweighed the contribution of other taxa in nitrate to nitrite reduction by *nar* gene (Figure 6). However, both denitrifying taxa were unaffected by CO_2 gradient treatment (Figure 2), possibly because these microbial groups were detected in several functional gene categories involved in C, N and P cycling processes (Figure 3). For example, *Solirubrobacterales* also had significantly higher number of genes in polyphosphate transformation (*ppk* and *ppx*) and changes in their abundance could affect the availability of inorganic P (Li et al., 2014). However, very few metagenomic studies have examined the taxonomic contributions of microbes involved in P cycling. A recent shotgun metagenome study in a temperate stream ecosystem found significant number of *Acinetobacter* (order *Pseudomonadales*) in organophosphate functional genes grouping (LeBrun *et al.*, 2018) although we did not detect the role of these taxa in PO_4^{-3} metabolism in switchgrass soils. Switchgrass is one of Department of Energy's energy crops (Englund

et al., 2020; Wright & Turhollow, 2010) and finding of a specific taxon with abundant labile and recalcitrant C degradation genes as well as denitrification and PO_4^{-3} metabolism genes should be valuable for the continued research on bioenergy crops.

The importance of *Rhizobiales* in N fixation has been widely established in several studies. However, the contribution of lesser known diazotrophs such as in N fixation has not been extensively studied. In this study, we found significant role of *Rhodospirillales* in N fixation. A recent study in grassland with calcareous karst soils significant effect of P availability in the abundance of diazotrophs (*Rhizobiales* and *Rhodospirillales*) abundance diazotroph diversity primarily affected by P availability (Xiao et al., 2020). Although *Rhizobiales* had the highest contribution in *alp*, most taxa were evenly distributed. This suggests that decrease in the diversity and abundance could possibly influence organic P transformations in soil. The relative proportion of nitrification genes (*amo/pmo*) were less abundant and *Nitrosomonas* was one of the dominant taxa contributing to this process. Because the switchgrass monoliths in both soils were not water-limited (Fay, Polley, et al., 2012), soil environmental conditions may be more anaerobic. Consequently, nitrification activity could be limited.

Conclusions

Shotgun metagenome studies on soil microbial communities associated with C_4 plants allow us to predict their metabolic/functional potential. To our knowledge, this is the first study to report the effects of CO_2 enrichment on functional potential of switchgrass soil microbial communities. Although CO_2 enrichment alone did not have a significant impact on microbial community structure or the abundance of most genes involved in C, N and P cycling, the effect of long-term CO_2 gradient and it legacy across years on specific

taxa and functional gene categories slightly varied among soils. Because little is known about the effects of CO_2 enrichment alongside its legacy, further studies incorporating longer term (years to decade) multifactorial experimental conditions could advance our existing knowledge on the resistance and resilience of microbial communities following environmental perturbation. Metagenome analysis is not feasible in wide ranging studies because it is still more expensive than 16S rRNA gene amplicon sequencing. However, this approach advantageous in that we can characterize the taxonomic contribution of microbes associated with specific functional gene groups. For example, we found *Micromonosporales* and *Solirubrobacterales* could be putative keystone taxa or part of "core" microbiome of switchgrass soils with crucial role in community stability and broad nutrient cycling processes. Future research integrating metagenome and metatranscriptome data could provide novel insights into active and dormant communities and their role in maintaining ecosystem function in the face of global climate change.

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Conflict of Interest

The authors declare no conflict of interest.

Data Accessibility

The nucleotide sequences from this study have been deposited in BioProject with accession number PRJNA416942 in the NCBI BioProject database. The ancillary data related to plant and soil variables will be provided upon request.

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CHAPTER FOUR

Soil Bacterial Community Assembly Mechanisms and Legacy Effects of CO_2 Enrichment Gradient in Texas Prairie Grassland

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Abstract

Microbial community assembly mechanisms are simultaneously governed by deterministic and stochastic processes. Although both ecological processes are important, their relative contribution across different plant and soil types in the context of global climate change remains poorly understood. In this study, we examined microbial communities spanning three distinct soil types (silty clay, clay, and sandy loam) and two vegetation types (mixed C_3/C_4 grasses and switchgrass monocultures). We collected bulk soil samples from the last year of a decade-long CO_2 gradient (250-500 ppm) experiment and two consecutive years following the cessation of CO_2 application, where soil monoliths returned to ambient CO_2 conditions. We utilized 16S rRNA gene amplicon sequencing and quantified community assembly processes based on null model approach. Our results showed that dispersal limitation had a significant influence in stochastic assembly across different soil and plant categories. However, the proportion of homogenous selection was higher in clay soils, but variable selection had higher contribution in silty clay and sandy loam soil communities associated with mixed C_3/C_4 grasses. Phylogenetic turnover (βNTI) positively correlated to ΔCO_2 concentration ($p<0.01$) only in silty clay soils with mixed

C_3/C_4 grasses. Furthermore, the legacy effects of long-term CO_2 treatment on taxonomic community structure and predicted function were minimal in all switchgrass communities but soil-specific in those associated with mixed C_3/C_4 grasses. Cooccurrence network analysis showed clustered networks with distinct core microbiome and putative key stone taxa within each plant and soil category. Taken together, our results suggest that the relative influence of ecological processes governing microbial community assembly in Texas Prairie grasslands are predominantly stochastic, but the extent of influence of selection-based processes is soil-specific and plant-specific. Furthermore, CO_2 enrichment and its legacy alone may not impose a strong selective pressure in shaping the overall community structure.

Introduction

One of the fundamental questions in microbial ecology is how microbial communities are structured and assembled in terms of their taxonomic composition, evolutionary history, and functional traits (Bagousse-Pinguet et al., 2019; Zhou & Ning, 2017). However, deciphering the underlying drivers of microbial community assembly along with shifts in community structure and function, particularly, in the context of global climate change is challenging. Soil microorganisms play a central role in maintaining ecosystem function and stability, but they have complex relationships with each other and their environment (Shade, 2018). Thus, it is necessary to understand microbiome resilience and community assembly mechanisms in response to global change drivers across different plant species and soil type.

Based on the core concepts derived from macro-ecology, two ecological theories: (i) niche-based and ii) neutral theory, have been widely recognized to disentangle the

mechanisms of microbial community assembly. Traditional niche-related theory predicts that deterministic processes primarily control the patterns in diversity and community structure (Webb et al., 2002). Selection imposed by differences in abiotic factors (e.g., soil texture, moisture, pH, temperature, plant diversity, and nutrient heterogeneity) and biotic interactions (competition, facilitation, mutualisms, predation, and host filtering, etc.) largely mediate deterministic processes (Chase & Myers, 2011; Chesson, 2000; Gilbert & Bennett, 2010). On the contrary, neutral theory states that variation in community composition or structure is not significantly different than expected from random events due to birth, death, speciation, extinction or dispersal (Chave, 2004; Hubbell, 2001; Leibold et al., 2017). Therefore, this process is presumed to be stochastic or ecologically equivalent (Chase & Myers, 2011). Several studies over the past decade have established the significance of both stochastic and deterministic processes in microbial community assembly and asserted that they are not mutually exclusive (Caruso et al., 2011; Chase, 2010; Dong et al., 2021; Ning et al., 2020; Ofiţeru et al., 2010; Stegen et al., 2012; Tripathi et al., 2018; Vellend et al., 2014). However, the relative contribution of each process in shaping community structure across divergent ecosystems remains a central issue.

Vellend's conceptual framework broadly fits both niche-based and neutral ecological processes into four main categories of selection, drift, dispersal and diversification (Vellend, 2010). Ecological selection, which is mostly deterministic, could be homogeneous or heterogenous depending on the extent of variation in environmental conditions and interactions (synergistic or antagonistic) among species (Chase & Myers, 2011; Zhou & Ning, 2017). Although dispersal (spatial movement of species) along with diversification (speciation) are largely considered to be stochastic, both could have

deterministic components (Nemergut et al., 2013). Drift alone is inherently stochastic, but it could be more difficult to quantify (Vellend et al., 2014; Zhou & Ning, 2017). Recent advances in high throughput sequencing technology along with application of ecological null models have allowed us to translate this theoretical framework and quantify community assembly processes more precisely. Stegen et al.'s ecological null model is based on taxonomic and phylogenetic beta diversity patterns (βNTI and the RC_{bray} metric), where the underlying assumption is that strong phylogenetic signal supports niche conservatism (Stegen et al., 2013a, 2015) compared to null model expectation around neutral community assembly. In conjunction with ecological null models, cooccurrence network analysis can reveal complex interactions among taxonomically diverse group of soil microbes and identify putative keystone taxa responsible for maintaining overall community stability in the face of global climate change (Xu et al., 2019; Zhou et al., 2011).

Community stability is defined by ability of microorganisms to withstand immediate impact of disturbance (resistance) and their capacity to recover post the disturbance (resilience). Microbial response and community stability could be mediated by the intensity of disturbance and the time of recovery under different forms of environmental perturbation (Allison & Martiny, 2008; Shade et al., 2012). Previous studies have also shown significant legacy effects of drought, fire or nitrogen addition (Hawkes & Keitt, 2015; Hinojosa et al., 2019; Jurburg et al., 2017; Liu et al., 2020; Zhang et al., 2018). Rising atmospheric CO_2 is one of the major drivers of global climate change and anthropogenic ecological disturbance. To our knowledge, the legacy effects of CO_2 enrichment gradient and microbial community resilience across different soil type and plant species has not been explored yet. To address this gap, we utilized in the Lysimeter CO_2 Gradient

(LYCOG) which constitutes a unique preindustrial-to-future CO_2 enrichment gradient (250-500 ppm) and Texas prairie soils with a broad range of texture, moisture, and nutrient content. In a previous study from 2015 samples, we focused on the response of bacterial communities to ongoing CO_2 enrichment gradient (the last year of CO_2 treatment) in mixed C_3/C_4 communities associated with three contrasting soils (Raut et al., 2018). We found limited but soil specific response to CO_2 gradient in bacterial communities. As a follow-up study, here we examined soils from two subsequent years (2016/2017) after the cessation of experimental treatment, where soil monoliths were exposed to ambient CO_2 conditions. Thus, comprehensive analysis across CO_2 treatment years was useful in examining the legacy effects and microbiome resilience. Here, we also expand our previous study to include community assembly mechanisms, predicted functional metagenome profiles and biotic interactions among taxa.

The purpose of this study was to investigate the effects of a decade-long preindustrial-to-future CO_2 gradient and its legacies on soil bacterial communities associated with three distinct soils (an upland silty clay, a low land clay, and an alluvial sandy loam) and two vegetation types (mixed C_3/C_4 grasses and switchgrass monocultures). We primarily focused on three questions: (i) How do soil microbes respond to and recover from exposure to a decade-long CO_2 gradient treatment across different plant species and soil type? (ii) What is the relative importance of stochastic and deterministic processes in community assembly? (iii) Does CO_2 treatment impose a strong selective pressure? To address these questions, we utilized 16S rRNA gene amplicon sequencing and applied ecological null models along with multivariate statistical analysis. We hypothesized that mixed C_3/C_4 communities would be more responsive to CO_2 gradient

than those associated with switchgrass (C_4 only monoculture) due to inherent physiological differences in photosynthetic efficiencies among C_3 and C_4 plants. Based on the existing edaphic factors and prior studies from the same experimental setting, we expected soil-specific variation in resistance, recovery rate (resilience) and persistence of legacy effects (or sensitivity) following the cessation of experimental CO_2 gradient treatment. We also anticipated that relative influence of stochastic and deterministic processes would differ by soil and plant category. For example, there could be higher contribution of homogenous selection in switchgrass clay soils due to similar soil properties and plant species composition along with weakened effect of CO_2 enrichment.

Materials and Methods

Study Site and Soil Sampling

The study site is located in Temple, Texas, USA (31^0 05'N, $97^0$20'W) and Lysimeter CO_2 Gradient (LYCOG) facility was operated by the USDA-ARS Grassland Soil and Water Research Laboratory since 2006 (Fay et al., 2009). In the super-ambient/elevated leg, a daytime linear CO_2 gradient inside the longitudinal chamber was maintained by injecting CO_2 at the entry point and photosynthesis by enclosed vegetation progressively depleted to the desired CO_2 levels at exit point. Same approach was implemented in sub-ambient leg except ambient CO_2 was introduced at the entry point and the exit point CO_2 concentration was maintained at 250 ppm by regulating the blower fan speed. The direction of air flow in both chambers was reversed during night-time. CO_2 levels until 2015 were measured at the entrance and exit of each section at 2 min intervals and the concentration for each monolith was estimated by linear interpolation from the

measured values (Fay et al., 2012). Air temperature and vapor pressure deficit were closely monitored. CO_2 gradient was maintained during the portion of the year when vegetation photosynthetic capacity was adequate, typically from late April to early November. Average annual precipitation at LYCOG facility is ~880 mm and the mean monthly temperature vary between 15.3°C and 35.4°C in January and August, respectively (Polley et al., 2020). The details on experimental setup and operation of these chambers are illustrated in the previous studies from same experimental setting (Fay et al., 2009; Raut et al., 2018).

LYCOG facility was permanently shut off in November 2015 and aboveground biomass were clipped. In 2015, intact soil monoliths in each section were enclosed within a clear polyethylene and only opened during the time of sample collection. In 2016 and 2017, soil monoliths were partially enclosed in rain exclusion covers instead of polyethylene enclosures, exposing the dormant vegetation to the ambient atmospheric CO_2 levels, temperature and precipitation.

Three cores from the top 0-5 cm of monoliths and located close to vegetation were collected using mini metal cores of 1.5 cm diameter. The soil cores (~15 g) were transferred to 50 ml centrifuge tubes. Samples were transported in dry ice and immediately stored at a -80°C freezer until further analysis. Bulk soil samples exposed to a decade long preindustrial-to-future CO_2 gradient (250-500 ppm) were collected from 2015 (last year of CO_2 treatment) and 2016/2017 (post the cessation of CO_2 treatment) from the same site as illustrated in Appendix C: Figure C.S1. A total of 260 samples collected across all CO_2 treatment years (2015-2017) constituted silty clay mixed C_3/C_4 (n= 80), clay mixed C_3/C_4

(n= 66), sandy loam mixed C_3/C_4 (n=53), silty clay switchgrass (n=28) and clay switchgrass (n=33).

DNA Extraction and 16S rRNA gene Amplicon Sequencing

Total community DNA from soil samples was extracted using commercial extraction kits (PowerSoil® DNA Isolation Kit, Mo Bio Laboratories). The purity of DNA samples was assessed by measuring 260/230 and 260/280 in a Nanodrop spectrophotometer. Quantification of the samples were determined by Qubit 3.0 flourometer. A two-step PCR amplification targeting V4-V5 region of 16S rRNA gene was performed. PCR conditions (Raut et al. 2018). We used Illumina MiSeq platform with v3 Reagent kit to sequence paired-end 300-bp reads.

Bioinformatic Analysis

Raw sequence reads for 2016/2017 samples, subsequently exposed to ambient conditions, were initially processed and demultiplexed using 16S metagenomic pipeline in Illumina BaseSpace. 2015 samples analyzed in a previous study with QIIME from the tenth year of CO_2 treatment were also simultaneously reprocessed and combined with 2016/2017 samples using QIIME2 pipeline to examine the legacy effects and community assembly. QIIME2 is an upgraded version of QIIME (Bolyen et al., 2019). However, similar process from the previous work (Raut et al. 2018) was implemented for joining reads, filtering chimeric sequences, and picking OTUs, albeit some minor updates while assigning taxonomy and phylogeny. First, Read 1 and Read 2 from each sample were aligned using the join-fastq algorithm (Aronesty, 2013) and the aligned sequences were filtered at a Phred score of 20. We further detected chimeric sequences using the VSEARCH algorithm and

removed the sequences identified as chimeras (Rognes et al., 2016). The samples were rarefied to a sequencing depth of 15,000 sequences to generate abundance table for operational taxonomic units (OTUs) at 97 % sequence similarity.

Predicted Functional Gene profiling

In addition to taxonomic analysis, we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) to predict functional metagenome profiles (Douglas et al., 2019) based on 16S rRNA sequences and phylogenetic tree constructed from closed reference OTU clustering. Greengenes 13_8 was used as a reference database for closed reference OTU clustering. The metagenome profiles with predicted functions were generated from the Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (Kanehisa et al., 2016) in QIIME2 pipeline using q2-picrust2 module. The final output for downstream analysis was similar to species abundance table but with predicted functional gene abundance of KEGG orthology (KO) metagenomes inferred from 16S rRNA gene sequences for each sample.

Quantification of Community Assembly Processes

To quantify the relative contribution of stochastic and deterministic community assembly processes, we applied a null-modeling-based approach (Stegen et al., 2013b, 2015). First, between-community mean-nearest-taxon-distance (βMNTD) was used to measure the observed phylogenetic turnover between a pair of samples within each soil type and plant category (Fine & Kembel, 2011; Webb et al., 2008). Empirical βMNTD was calculated using "comdistnt" function in "picante" package. Next, taxa labels and their relative abundances were randomly shuffled across the tips of phylogenetic tree, a process

which was repeated 999 times to generate a null distribution of randomized βMNTD. Then, between-community nearest taxon index (βNTI) was used to measure the deviation between observed $\beta MNTD_{obs}$ and the mean of $\beta MNTD_{null}$. βMNTD is a phylogenetic distance metric among closest relatives and the underlying assumption is that a strong phylogenetic signal approximates ecological similarity between communities (Zhou & Ning, 2017). Thus, βNTI > +2 or < -2 indicates a significant deviation from the null distribution, where selection governed by deterministic factors is expected to be dominant (Stegen et al., 2012).

Subsequently, a taxonomic β-diversity metric (RC_{bray}) was used to further disentangle stochastic community assembly processes (|βNTI|<2) under the assumption of weak selection and random dispersal. RC_{bray} calculates the deviation between observed Bray-Curtis dissimilarity and null distribution of Bray-Curtis dissimilarities (999 randomizations) while maintaining the local species richness and taking into account their relative abundance across all communities (Chase & Myers, 2011). Based on βNTI and RC_{bray} metric for a given pairwise comparison, the relative contributions of stochastic and deterministic processes are quantified into five categories. If the communities are phylogenetically more similar (βNTI < -2) or less similar (βNTI > +2) than expected from null distribution, they are assembled by (i) homogeneous or (ii) variable selection, respectively. Similarly, if the communities are taxonomically more similar (RC_{bray}< -0.95) or less similar (RC_{bray}< +0.95) and have an absolute βNTI of <2, they are assembled by (iii) homogeneous dispersal or (iv) dispersal limitation, respectively. The remaining fraction (i.e., |βNTI| <2 and $|RC_{bray}|$ <0.95) is categorized as (v) undominated processes, which could be attributed to drift, weak selection or weak dispersal. We quantified the

phylogenetic alpha diversity within each sample using net relatedness index (NRI) and the nearest-taxon-index (NTI) and implementing similar null model approach in package 'picante'.

Coocccurence Networks and Statistical Analyses

Statistical analyses were carried out with R statistical software version 3.6.3 (R Core Team, 2019) with packages (vegan, phyloseq, ggplot2, picante, mgcv and others) and custom scripts. We considered robust positive (spearman's $\rho > 0.7$) and significant ($p < 0.01$) correlations among OTUs. We filtered OTUs <10 occurrence (absolute counts) to reduce the complexity of networks and effect of rare taxa. We used i-graph package (Csardi et al., 2006) to calculate the global topological properties of networks within each subcategory of plant species and soil type. We further calculated node specific topological features as well as within and among module connectivity. We utilized Gephi 0.9.2 to visualize the network with nodes and edges (Bastian et al., 2009).

We performed unconstrained ordinations using nonmetric multidimensional scaling (NMDS) on log10(x + 1) transformed OTU tables with Bray–Curtis distance and UniFrac distance using VEGAN (Oksanen et al., 2017) and phyloseq (McMurdie & Holmes, 2013) packages, respectively. To assess the effect of CO_2 gradient on community structure, we implemented ordisurf function with GAM fitting in the NMDS ordinations similar to previous analysis (Raut et al., 2018). PROcrustean randomization test (Procrustes test) of community environment concordance (PROTEST) function with 999 permutations along with mantel test was used to compare NMDS ordinations among taxonomic, phylogenetic, and predicted functional community structure. Non-parametric permutational multivariate analysis of variance (PERMANOVA) and analysis of

similarities (ANOSIM) were used to evaluate the main effect of soil type, CO_2 treatment year, and plant type.

Results

Global Community Structure and Predicted Function

We characterized global community structure of all 260 samples using NMDS ordinations and multivariate statistics. Beta-diversity patterns of 16s rRNA gene OTUs showed clear segregation of microbial communities associated with sandy loam soils from silty clay and clay soils (Figure 4.1a), indicating dominant impact of soil type on taxonomic community structure. The difference between the last year of CO_2 treatment (2015) and two subsequent years exposed to ambient CO_2 conditions (2016/2017) also contributed to shifts in taxonomic composition, although the overall influence of soil type was much stronger (Table 4.1). NMDS ordinations for predicted functional genes (PICRUSt2) revealed some overlap among clusters of sandy loam communities with silty clay and clay soil communities (Figure 4.1b). PERMANOVA analysis further illustrated that yearly variation between CO_2 gradient treatment (2015) and ambient conditions (2016/2017) shifted the predicted functional profiles of microbial communities to a greater extent than soil type (Table 4.1). Nonetheless, mantel test ($r= 0.64$, $p<0.001$) and Procrustes analysis ($t = 0.70$, $p = 0.001$) indicated strong correlation between global taxonomic community structure and predicted functional profiles of all samples. In addition, both phylogenetic beta-diversity metrics (βMNTD and UniFrac) showed similar clustering patterns and the results were in line with Bray–Curtis metrics from 16s rRNA OTUs and PICRUSt2 respectively (Appendix C: Figure C.S1). In terms of alpha diversity, Shannon index (*H*')

of 16s rRNA OTUs and predicted functional genes were also significantly correlated (Appendix C: Figure C.S2).

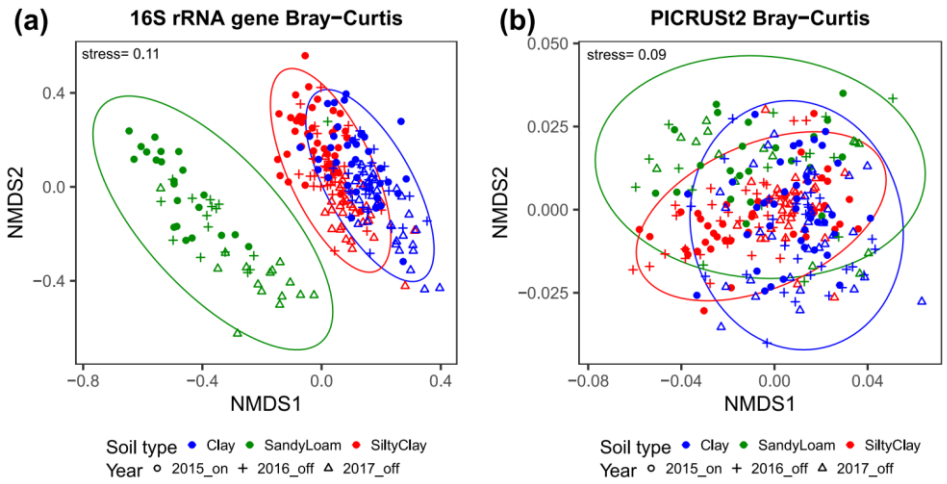

Figure 4.1. Nonmetric multidimensional scaling (NMDS) ordinations of (a) taxonomic community structure (stress = 0.09) and (b) predicted functional gene profiles or PICRUSt2 (stress = 0.11) using Bray–Curtis distance metric. Predicted functional genes (KOs) were generated from PICRUSt2 pipeline following 16S rRNA gene amplicon sequencing. The ordinations represent the global community structure with all 260 samples. The color and shape indicate different soil type and CO_2 treatment years respectively. 2015 was the last year of a decade-long CO_2 gradient treatment in the two subsequent years (2016 and 2017) following the cessation of long-term CO_2 gradient treatment, samples were exposed ambient CO_2 conditions.

Table 4.1. Multivariate statistical analyses to test the effect of soil type and yearly variation in CO_2 treatment on global community structure in terms of taxonomic beta diversity (16s rRNA OTUs) and predicted functional gene profiles (PICRUSt2) using Bray-Curtis distance.

Statistics (Global dataset)		**Soil type**		**CO_2 treatment Year**	
		16S rRNA gene (OTUs)	PICRUSt2 (predicted genes)	16S rRNA gene (OTUs)	PICRUSt2 (predicted genes)
ANOSIM	*R*	0.53	0.24	0.16	0.24
	p	0.001**	0.001**	0.001**	0.001**
PERMANOVA	*F*	31.69	25.54	11.32	32.02
	p	0.001**	0.001**	0.001**	0.001**

*Significant ($\alpha = 0.05$).
**Significant after Bonferroni's correction ($\alpha = 0.017$).

Soil-specific Legacy Effects of CO_2 and Plant type

We further elucidated the effect of plant type, CO_2 enrichment and its legacy, and yearly variation within each soil. NMDS ordinations based on Bray-Curtis dissimilarities among 16S rRNA gene OTUs show more clustered distribution of switchgrass communities than mixed C_3/C_4 communities (Figure 4.2a, c). However, PICRUSt2 data reveal that plant species had a greater effect on the predicted functional profiles of clay soil than silty clay soils (Figure 4.2b, d and Appendix C: Table C.S1). Overall effect of CO_2 gradient on community structure and function was minimal in switchgrass communities associated with both clay soils and silty soils (Figure 4.2a-d). The contour lines overlaid on NMDS ordinations also depicted a narrower range of CO_2 concentrations and did not fit the decade-long CO_2 gradient (250–500 ppm) from the LYCOG experiment (Figure 4.2). Sandy loam soils only had mixed C_3/C_4 vegetation, so the effect of plant species was not evident.

In silty clay mixed C_3/C_4 communities, ordisurf analysis and NMDS ordinations showed that CO_2 gradient had a significant effect on taxonomic structure (Figure 4.2a and Appendix C: Table C.S2) and had some influence on predicted function (Figure 4.2d) of soil microbes. Yearly variation across CO_2 treatment years also had a significant effect on both taxonomic structure and predicted function of silty clay mixed C_3/C_4 communities (Appendix C: Table C.S1). Pairwise comparison with PERMANOVA between CO_2 treatment years further showed greater degree of variation between 2015, last year of CO_2 gradient treatment, and 2017, two years after return to ambient CO_2 conditions (Appendix C: Table C.S1). However, communities within 2017 samples were functionally more clustered, but they maintained taxonomic variation along the CO_2 gradient (Figure 4.2a, b). Thus, in silty clay mixed C_3/C_4 communities, legacy effects of long-term CO_2 persisted

to some extent on taxonomic community structure, but the impact gradually diminished on predicted function.

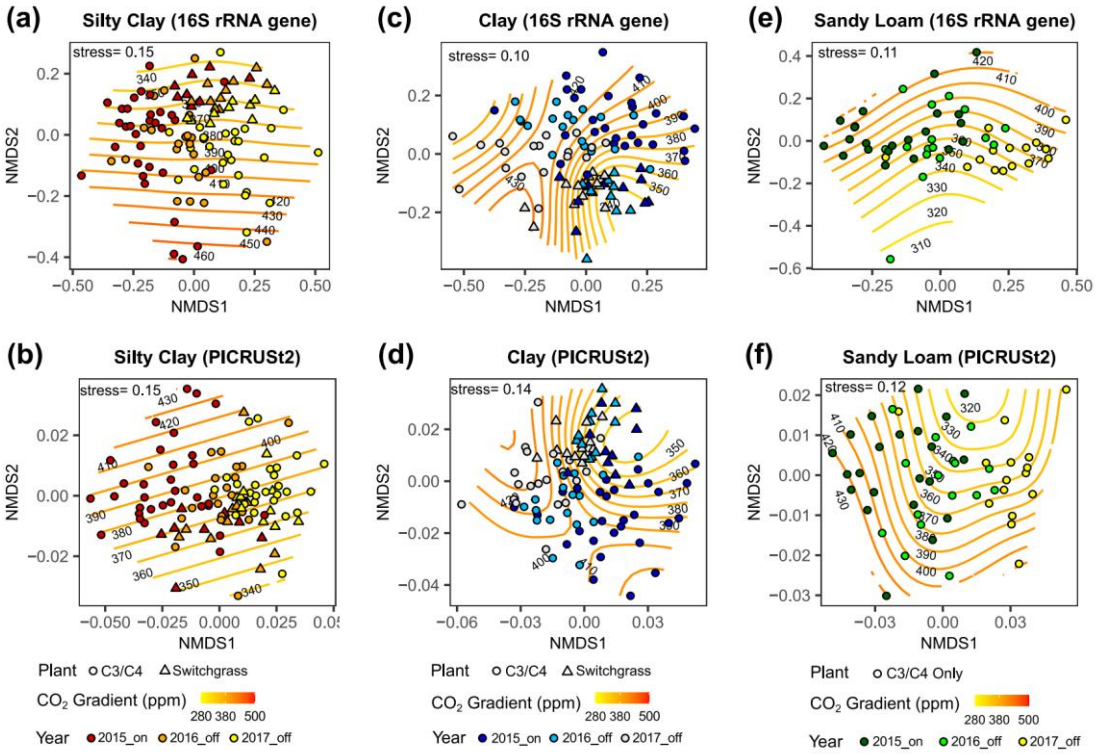

Figure 4.2. NMDS ordinations with Bray–Curtis distance reflecting the distribution of sites by plant type and CO_2 treatment year within each soil. Contour lines represent CO_2 gradient fit computed with ordisurf GAM and overlaid in the ordination space. Yellow to red lines represent a gradient of high to low CO_2 concentration. Top panel figures (a, c, e) are based on 16S rRNA gene OTUs and bottom panel figures (b, d, f) are based on predicted functional gene profiles (PICRUSt2).

Among soils associated with mixed C_3/C_4 vegetation, clay soil communities were least responsive to CO_2 gradient (Figure 4.2c and Appendix C: Table C.S2). Ordisurf analysis also confirm the minimal effect of CO_2 gradient on predicted functional metagenome profile of clay soil mixed C_3/C_4 communities (Figure 4.2d). However, the pairwise comparison of both taxonomic structure and PICRUSt2 metagenome profiles between 2015 and 2017 showed significant difference due to yearly variation (Appendix

C: Table C.S1) even though the effect of long-term CO_2 gradient was negligible (Appendix C: Table C.S2).

CO_2 gradient had some effect on taxonomic structure of sandy loam soil microbial communities associated with mixed C_3/C_4 vegetation (Figure 4.2e), albeit to a lesser extent than in silty clay mixed C_3/C_4 communities (Appendix C: Table C.S2). Nonetheless, sandy loam mixed C_3/C_4 communities were functionally better structured along the CO_2 gradient as shown in ordisurf analysis (Appendix C: Table C.S2) and NMDS ordinations from PICRUSt2 metagenome profiles (Figure 4.2f). Yearly variation across sandy loam mixed C_3/C_4 soils had a strong effect on both predicted function and taxonomic composition (Appendix C: Table C.S1). Furthermore, the distribution of sites in NMDS ordinations and pairwise comparisons with PERMANOVA also showed significant variation in functional community structure both within and between CO_2 treatment years (Figure 4.2f and Appendix C: Table C.S1). In particular, communities within 2017 samples appeared functionally more divergent in sandy loam than in silty clay mixed C_3/C_4 soils along the CO_2 gradient, indicating higher degree of legacy effects on predicted function in sandy loam communities.

Soil and Plant-specific CO_2 *Gradient Effects on Phylogenetic Turnover*

To infer soil and plant-specific ecological processes, we split the global data with all 260 samples into five sub-categories of plant and soil type and used βNTI to assess the phylogenetic turnover between a pair of samples. Most of the βNTI values across the soil types, plant species and CO_2 treatment year were between +2 and -2 (Figure 4.3). Except in silty clay C_3/C_4 communities (Figure 4.3a), we found no significant correlation between difference in CO_2 concentration (Δ CO_2) and βNTI (Figure 4.3b-e). βNTI positively

correlated to increasing difference in CO_2 concentration (r= 0.15, p=0.001) across CO_2 treatment years in silty clay mixed C_3/C_4 soils. Overall, pairwise comparisons of βNTI values within and between 2015 (the last year when CO_2 gradient treatment was on) and 2016/2017 samples (two subsequent years following the termination of experimental CO_2 treatment) did not exhibit any consistent pattern on the phylogenetic turnover (Figure 4.3).

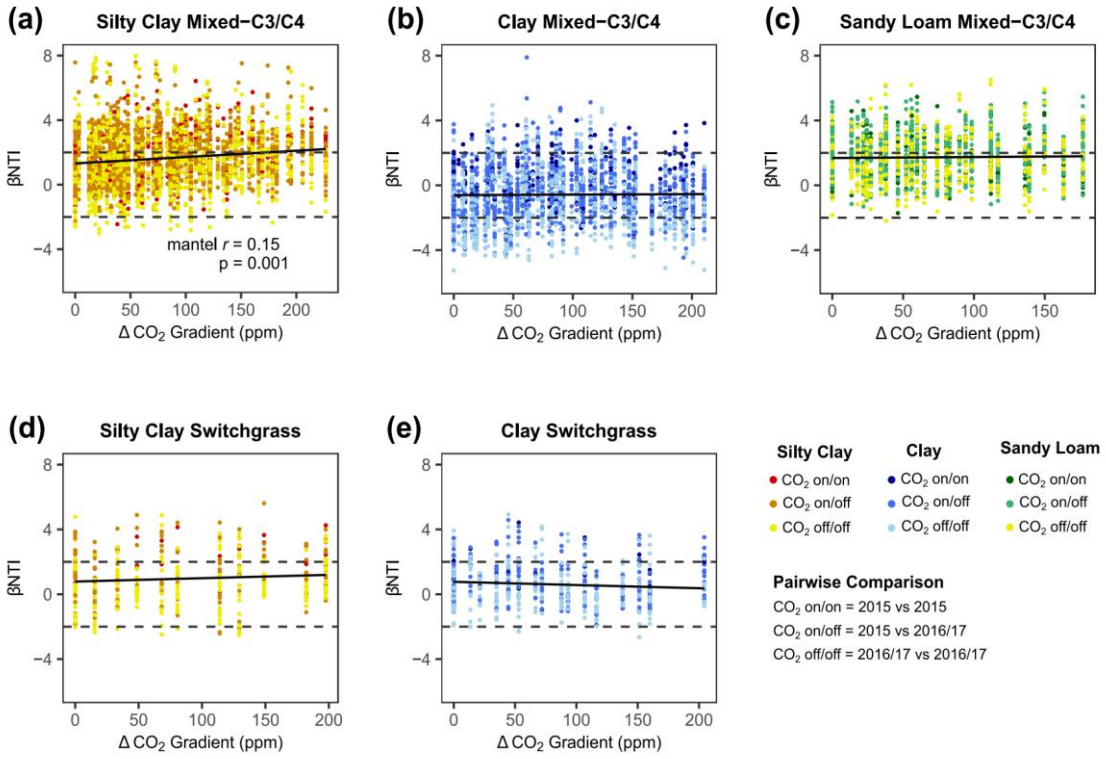

Figure 4.3. Relationship between beta-Nearest Taxon Index (βNTI) and change in CO_2 concentration (Δ CO_2) within five subcategories of plant and soil. In 2015, soil monoliths were exposed to CO_2 gradient but in 2016/2017, they returned to ambient conditions after the termination of CO_2 treatment. Each point represents a βNTI value between a pair of samples and the colors indicate pairwise comparison between CO_2 treatment year of those two samples. The horizontal dotted line above +2 or below -2 reflect the 95% confidence intervals around the expectation under neutral community assembly and indicate greater (or less) than expected phylogenetic turnover, respectively. The difference in CO_2 concentration (Δ CO_2) is depicted as Euclidean distance between each pair of sampling sites.

Variation in phylogenetic alpha-diversity within sample was calculated using nearest taxon index (NTI) and mean relatedness index (NRI). The results showed that NRI values across all plant and soil categories were between +2 and -2 (Appendix C: Figure

C.S3), indicating lesser than expected turnover in phylogenetic alpha-diversity. NTI values also were mostly between +2 and -2 even though we observed slightly higher phylogenetic clustering for mixed C_3/C_4 communities compared to switchgrass communities in clay soils (Appendix C: Figure C.S4). Overall CO_2 gradient and yearly variation had marginal impact on phylogenetic alpha-diversity derived from the null models even though the patterns of response slightly varied among plant and soil categories (Appendix C: Figure C.S3, C.S4).

Community Assembly Processes

We examined the relative contribution of stochastic and deterministic processes in microbial community assembly based on the βNTI and the RC_{bray} metric inferred from null models. Stochastic processes were relatively more dominant than deterministic processes (Figure 4.4, Appendix C: Table C.S3), accounting for >60% community assembly across all plant and soil sub-categories. However, the magnitude of contribution of dispersal-based (stochastic) versus selection-based (deterministic) processes varied significantly by plant and soil type. Dispersal limitation accounted for substantial fraction of stochastic assembly process with slightly higher proportion in sandy loam and clay soil mixed C_3/C_4 communities (>46%). On the contrary, the fraction of homogenizing dispersal was relatively low except in clay soil microbial communities associated with switchgrass (>26%). Undominated processes, characterized by drift along with weak selection and dispersal, also contributed to stochastic community assembly albeit to a higher degree (>26%) in silty clay and clay soil communities associated with switchgrass. Relative influence of variable selection was higher in community assembly of soil microbes associated with mixed C_3/C_4 vegetation in silty clay and sandy loam communities (Figure 4.4). In contrast, clay soil mixed C_3/C_4 communities had the lowest proportion of variable

selection and highest proportion of homogeneous selection (>20%) among all plant and soil categories.

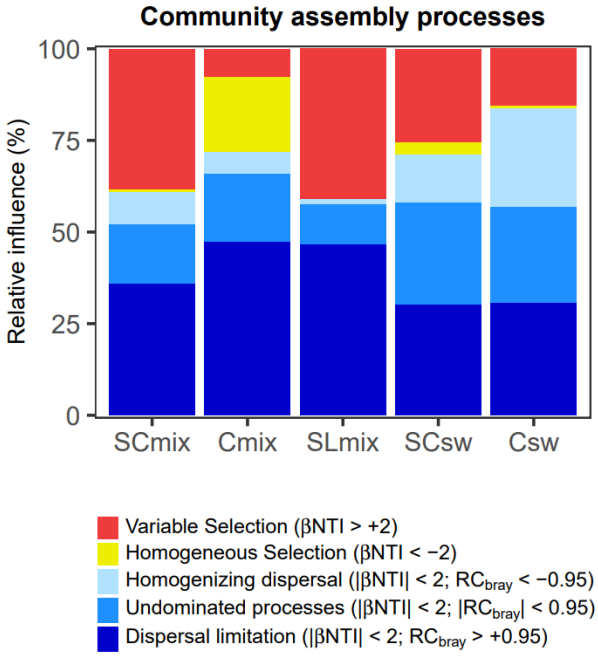

Figure 4.4. Relative influence of community assembly processes depicted in stacked bar graphs. Based on βNTI and RC_{bray} metric from null models, deterministic processes are indicated by heterogenous or homogenous selection (red/yellow) and stochastic (blue) processes are reflected through dispersal or drift in combination. SC_{mix}, C_{mix}, SL_{mix} indicate silty clay, clay and sandy loam soil communities associated with mixed C_3/C_4 plant category, respectively. Similarly, SC_{sw} and C_{sw} represent silty clay and clay soils associated with switchgrass monocultures, respectively.

Cooccurrence Networks

We considered robust positive (spearman's $\rho > 0.7$) and significant ($p < 0.01$) correlations among OTUs to identify mutually coexisting microbial taxa. Cooccurrence network analysis performed within the sub-categories of distinct plant and soil types exhibited modular structure (Figure 4.5, Table 4.2), with overall modularity values >0.4. Global topological properties of individual networks differed among plant and soil types (Table 4.2). The total number of nodes were slightly higher in sandy loam mixed C_3/C_4 and the number of edges was highest for clay soil communities associated with switchgrass

(Figure 4.5, Table 4.2). Node-level topological properties showed high degree of betweenness centrality in silty clay mixed C_3/C_4, sandy loam mixed C_3/C_4 and clay switchgrass networks (Appendix C: Figure C.S5). In silty clay mixed C_3/C_4, closeness centrality and average node degree was significantly higher (Table 4.2 and Appendix C: Figure C.S5), revealing a highly interconnected network with closer relationships among neighboring OTUs. Among the five sub-categories, silty clay switchgrass soils had significantly lower number of edges, average node degree and clustering coefficient (Table 4.2).

Table 4.2. Global topological properties of co-occuring networks from five sub-categories of plant and soil.

	Silty clay mixed C_3/C_4	**Clay mixed C_3/C_4**	**Sandy loam mixed C_3/C_4**	**Silty clay switchgrass**	**Clay switchgrass**
Nodes	638	631	898	556	878
Edges	2301	1846	2253	783	2517
Clustering coefficient	0.46	0.45	0.32	0.29	0.38
Average pathlength	7.58	4.16	8.33	5.20	9.83
Modularity	0.63	0.77	0.68	0.78	0.52
Network diameter	19.00	14.00	26.00	15.00	24.00
Average degree	7.21	5.85	5.02	2.71	5.73

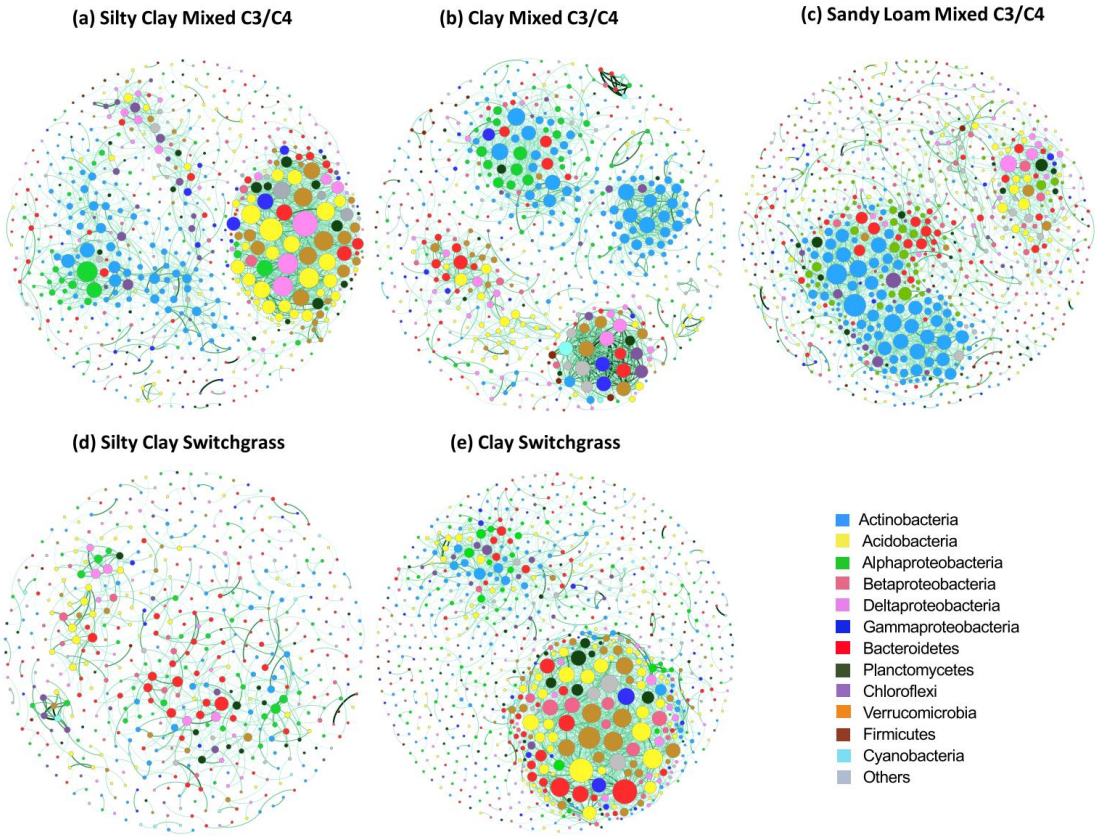

Figure 4.5. Bacterial co-occurrence networks from five sub-categories of plant and soil calculated with a magnitude of spearman correlation > 0.7. Each node (dot) represents a unique OTU and the size of the node is weighted by node degree (i.e., number of connections with other OTUs). The color of the node corresponds to a single phylum or class. The edges are represented by green lines and dark green color weighted by thickness represents stronger correlation between OTUs.

Majority of OTUs identified at phylum/class level were distributed into 12 phyla but were mostly unique within each plant and soil category (Figure 4.5). *Acidobacteria* had more frequent interactions within modules in silty clay mixed C_3/C_4 (Figure 4.5a) and clay switchgrass soils (Figure 4.5e) whereas *Actinobacteria* had higher number of connections with other OTUs and were more dominant within the network of sandy loam mixed C_3/C_4 (Figure 4.5c). *Proteobacteria* were detected across all cooccurrence networks although the relative number of interactions varied among individual networks. Overall, within-module connectivity (Z_i) and among-module connectivity (P_i) plots show similar frequency of

peripheral taxa (specialist) and connectors (generalists) across all networks (Appendix C: Figure C.S6). A few putative keystone taxa, that formed the network hubs (supergeneralists), were present in sandy loam C_3/C_4 soils but entirely absent in silty clay switchgrass soil (Figure 4.5 and Appendix C: Figure C.S6).

Discussion

We examined the assembly processes along with the response of soil bacterial communities to a unique CO_2 enrichment gradient and its legacy across soils with a broad range of textures, nutrient content and plant species. Our results suggest that the ecological processes governing bacterial community assembly in Texas prairie soils are predominantly stochastic. However, the extent of influence of selection-based processes particularly varied among microbial communities associated with contrasting soil types and plant species. Although CO_2 enrichment may not impose a strong selective pressure, its legacy may persist in taxonomic composition. Silty clay soils communities were most sensitive to CO_2 enrichment gradient but functionally they appeared resilient within two years of cessation of CO_2 treatment. In contrast, switchgrass soils as well as clay soil communities were more resistant, which indicates soil-specific variation in CO_2 effects and it legacy. Furthermore, highly connected networks and co-occurrence patterns show stable but distinct core taxa within each plant and soil category, which may have significant role in ecosystem function in the face of global change.

Based on differences on soil texture and edaphic properties, we expected that the relative influence of stochastic and deterministic processes would vary across soil type. Our results showed that stochastic processes had a significant influence (>60%), where dispersal limitation was one of the major drivers of overall community assembly across

plant and soil categories (Figure 4.4). However, undominated processes categorized by drift also contributed to stochastic assembly albeit to a lesser extent than dispersal limitation. Recent studies in naturally restored ex-arable lands (Chen et al., 2020), intact permafrost soils (Bottos et al., 2018; Doherty et al., 2020) and groundwater-filled granite fractures (Beaton et al., 2016) also revealed significant contribution of dispersal limitation. However, these results reflect the stochastic community assembly processes in natural ecosystems. In this study, the experimental design possibly did not favor dispersal due to restriction in spatial movement of microbial communities between intact soil monoliths although the linear chambers were interconnected to maintain CO_2 enrichment gradient. Indeed, the relative proportion of homogenizing dispersal was only slightly higher in switchgrass clay soil communities compared to other plant and soil categories. Thus, dissimilarities among communities resulting from dispersal limitation within each soil and plant category may not be entirely stochastic in LYCOG setting even though we had spatio-temporal variation in soil sampling.

Notably, the influence of selection-based processes significantly varied among soils. In mixed C_3/C_4 communities, the impact of homogenous selection was highest in clay soils whereas variable selection had greater contribution on silty clay and sandy loam soil (Figure 4.4). We expected that the relative influence of homogenous selection in switchgrass clay soils would be greater due to homogeneity in soil properties and plant species composition. On the contrary, we found significant contribution of homogenous selection only in mixed C_3/C_4 clay soil communities. This result suggests that homogenous selection has a greater influence in fine-textured clay soils with higher water retention ability and nutrient content although very few studies have includes differences in soil

texture or aggregate size while examining community assembly processes (Dong et al., 2021; Xun et al., 2019). Furthermore, priority effects due to historical contingencies of initial edaphic conditions (Feng et al., 2018; Fukami, 2015) could play an important role in deterministic community assembly. Thus, the degree of influence of selection even within a partially closed system and similar experimental conditions may vary by soil type. Prior studies have shown significant to minimal CO_2 enrichment effect on soil microbial community diversity, structure and function (de Menezes et al., 2016; Deng et al., 2016; Dunbar et al., 2012; Simonin et al., 2017; Xiong et al., 2015; Yu et al., 2021). These studies provide an immediate response of microbial communities to an ongoing elevated/ambient CO_2 experiment but rarely including multiple soil types (Butterly et al., 2019) or CO_2 gradient approach. Furthermore, the role of CO_2 enrichment gradient specifically in deterministic assembly and the underlying community assembly processes across different soil and plant categories remain elusive.

Our results show that difference in CO_2 concentration between a pair of samples had a minimal effect on overall phylogenetic turnover (βNTI) compared to null model expectations and thus, did not significantly impose a strong selective pressure. We found positive correlation between ΔCO_2 and βNTI only in silty clay mixed C_3/C_4 communities (Figure 4.3a). This finding implies that incremental changes in CO_2 gradient concentration may not result in significant environmental filtering compared to larger differences in other factors such as warming (Ning et al., 2020), soil pH (Barnett et al., 2020; Fan et al., 2018; Shen et al., 2019) or soil organic matter (Feng et al., 2018). Our finding is consistent with other studies which conclude that the degree of change in environmental variables primarily mediate the relative contribution of stochastic or deterministic community

assembly processes (Chase, 2010; Dini-Andreote et al., 2015; Langenheder & Lindström, 2019). Greater variation in environmental constraints support variable selection whereas highly similar abiotic conditions could favor similar taxonomic profiles and phylogenetic clustering resulting in homogenous selection. However, CO_2 level in this study may not have been sufficient to elicit such strong selective pressures across different plant and soil categories.

In addition to community assembly processes, we also examined global and soil-specific community structure in terms of their taxonomic composition as well as predicted functional profiles across CO_2 treatment years. We found that soil type had pronounced effect on global community structure (Figure 4.1a), indicating that taxonomic community structure is primarily mediated by differences in soil properties. In the previous study from the same experimental setting for 2015 samples, we found soil moisture and nutrient content as important environmental factors in shaping global community structure albeit the differences within contrasting soils were subject to minimal changes (Raut et al., 2018). The effect of soil type slightly diminished on predicted functional profiles compared to taxonomic structure (Figure 4.1a). However, the correlation between taxonomic diversity and predicted functional gene diversity for all samples was also significant. This suggests limited functional redundancy, where diverse groups of taxa across soil types and plant species may have similar functional metagenome profiles (Miki et al., 2014; Wohl et al., 2004). The overall effect of yearly variation on global community structure and predicted function was also significant. The CO_2 gradient as well as temperature and precipitation were closely regulated and monitored in 2015, where the vegetation were completely enclosed in polyethylene covers from May through November (Raut et al., 2018).

Conversely, precipitation and temperature along with CO_2 concentrations mimicked ambient conditions in 2016/2017 and were only partially enclosed in rain covers after the cessation of experimental CO_2 treatment. Thus, the yearly variation in community structure could be attributed to these differences in experimental conditions as well as annual vegetation clipping the end of growing season between each sampling year (Guo et al., 2018; Xue et al., 2016).

Overall, CO_2 was not a major determinant of global community structure and its effects were outweighed by significant differences in soil properties, yearly variation and plant species composition. However, bacterial community resistance, resilience and sensitivity to long-term CO_2 enrichment gradient and its legacy varied significantly by soil and plant type. In the silty clay soils, which were most sensitive to CO_2 enrichment, the legacy effects of long-term CO_2 persisted on taxonomic community structure, suggesting soil bacterial communities may take longer to recover back and adapt to ambient conditions (Figure 4.2a). However, they became functionally more resilient to changes in CO_2 concentration after the cessation of long-term CO_2 treatment. Pairwise comparison between 2015 (CO_2 gradient) and 2017 (ambient CO_2) revealed marked shifts on predicted functional profiles of silty clay communities (Figure 4.2b). Similarly, CO_2 enrichment gradient had no significant effect on the taxonomic structure or predicted functional profiles of clay soil communities across the years, indicating that they are resistant to any incremental changes in CO_2 concentration. Structural equation modeling from a previous LYCOG study on eight years of soil CO_2 efflux (J_{CO2}) data also suggested soil specific differences in the trajectory of J_{CO2} responses (Fay et al., 2021). For example, J_{CO2} response in silty clay soils was asymptotic, where multiple constraints including soil water potential

and plant species turnover resulted in positive feedbacks along with concurrent negative feedback from plant species diversity. In sandy loam soils, both plant species diversity and aboveground net primary productivity (ANPP) reinforced positive feedbacks in J_{CO2} response. In contrast, turnover mediated by ANPP was the main driver of J_{CO2} responses in clay soil. Thus, our results also indicate that multiple constraints may be attributed to pronounced bacterial community response and functional resilience in silty clay soil as opposed to a single limiting factor in clay soils responses (Fay et al., 2021). Only few studies have investigated the legacy effects of CO_2 on above ground communities (Bain & Day, 2019; Stiling et al., 2013). Thus, longer term studies with multiple global change drivers may be required to disentangle the underlying drivers of soil microbiome resistance to ongoing environmental perturbation and resilience or recovery after the cessation of such perturbation.

Soil bacterial communities associated with switchgrass were resistant to CO_2 treatment but had distinct taxonomic composition than mixed C_3/C_4 in both soils. Shot-gun metagenome sequencing analysis for a subset of switchgrass samples (Unpublished data) also showed that CO_2 enrichment had no significant effect on the abundance of most functional genes involved in carbohydrate degradation, nitrogen cycling and phosphate metabolism. Thus, this result was in line with our expectation of minimal CO_2 effect on microbial community structure of switchgrass soils due to inherent physiological differences between carboxylation rates under elevated CO_2 concentrations (Leakey et al., 2009; Wang et al., 2012). A recent study showed the long-term trend (> 12 years) might result in reversal of C_3 versus C_4 response to CO_2 enrichment, although this could be attributed to corresponding changes in nitrogen mineralization rates (Reich et al., 2018).

Nonetheless, our study revealed significant effect of plant species composition on bacterial community structure and predicted functional profiles even although soil effect was predominant.

Co-occurrence networks has been frequently used to identify interactions among different groups of microbes and keystone taxa, which play a major role in community stability and function (Banerjee et al., 2018; Barberán et al., 2012). For example, a long-term FACE study at the Cedar Creek Ecosystem in Minnesota showed that global change drivers such as CO_2 could have significant influence on microbial interaction (Zhou et al., 2011). In contrast, our study suggests that strong correlation and cooccurrence patterns among OTUs across all CO_2 treatment years within each plant and soil category. Cooccurrence networks analysis from Maintenance of Exotic vs. Native Diversity (MEND) experiment in Vertisol soils at the adjacent site in USDA, Temple Texas also revealed strong effect of plant communities and abiotic factors such as soil depth and irrigation (Upton et al., 2020). Thus, our findings indicate that contrasting plant and soil type harbor highly interconnected stable networks, but each have their unique core taxa or putative keystone taxa regardless of differences in experimental CO_2 conditions between years.

Conclusions

Taken together, our study provides a unique perspective on the relative influences of ecological processes that govern bacterial community assembly mechanisms exposed to a decade-long CO_2 enrichment gradient. We utilized ecological null models, PICRUSt2 and correlation network analysis among different OTUs to decipher community assembly mechanisms, predicted functional gene profiles and microbial interactions respectively.

However, these approaches are statistical proxies for investigating ecological processes, functional potential and biotic interactions. Nonetheless, it was applicable in identifying soil and plant specific differences in relative contribution of selection-based processes within the same experimental setting. Our study suggests CO_2 gradient treatment may not be enough to induce large shifts in deterministic community assembly, structure or function. Furthermore, microbiome resilience studies may need to incorporate longer term spatio-temporal data. Understanding the how soil microbial communities respond to and recover from global change factors such as CO_2 enrichment is crucial to predicting their role in ecosystem function and biosphere feedback mechanisms. Thus, future research should couple community assembly processes to functional traits based on active/dormant microbial populations with multiple environmental constraints.

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Conflict of Interest

The authors declare no conflict of interest.

Data Accessibility

The nucleotide sequences from this study have been deposited in BioProject with accession number PRJNA416942 in the NCBI BioProject database. The ancillary data related to plant and soil variables will be provided upon request.

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CHAPTER FIVE

Conclusion

Bacterial Community Response to Preindustrial-to-Future CO_2 Gradient

Chapter two established that CO_2 gradient had no significant effects on bacterial taxonomic and phylogenetic diversity across different soil types and season of sampling. Although decade-long exposure to CO_2 gradient had minimal effect on global community structure compared to the strong influences of soil properties, we did observe soil specific variation in community structure patterns and change in relative abundance of few individual families. Notably, silty clay soil communities associated with mixed C_3/C_4 vegetation were better structured on a CO_2 gradient ($p < 0.001$) among three soils. The relative abundance of *Pirellulaceae* family decreased linearly with CO_2 gradient, but only in sandy loam soils. Conversely, the abundance of *Micromonosporaceae* and *Gaillaceae* families increased with CO_2 gradient in clay soils. In addition, the impact of plant species composition on community structure was secondary to the strong influence of soil properties (soil moisture and nutrient content). Nonetheless, soil specific as well as taxa specific responses to CO_2 enrichment gradient confirms the necessity to include multiple soil types in future climate change studies and long-term field experiments evaluating the impact of anthropogenic disturbances on soil microbial communities.

Functional Potential of Switchgrass Soil Microbiome

In chapter three, we utilized shotgun metagenome sequencing which provided a broad overview of CO_2 enrichment effects on the whole community (bacteria, archaea and

fungi) as well as the functional potential of switchgrass soil microbiome. In this study, we found that the abundance of most functional genes remained largely unaffected by CO_2 enrichment except genes involved in denitrification (*nar* and *nirK/S*), dissimilatory nitrate reduction (*nap*) and one gene (Glucoamylase) involved in labile C degradation. Although *nap, nar* and *nirK/S* were significantly enriched at higher CO_2 concentration only in silty clay soils *($p<0.05$),* Glucoamylase increased along the long-term CO_2 gradient (250-500 ppm) in clay soils (p=0.05). Thus, the results indicate soil x CO_2 interaction in abundance of specific functional gene categories even though the main effects of CO_2 gradient were minimal. We further identified taxonomic contribution of diverse microbial groups to various nutrient cycling processes. Interestingly, two taxa, *Micromonosporales* and *Solirubrobacterales,* significantly contributed to several functional gene categories. These findings suggesting their potential role as "core" microbiome in ecosystem functioning of switchgrass soils. Because of the significance of switchgrass as a bioenergy crop, overall resistance of its soil microbiome to long-term CO_2 enrichment and legacy effects can have significant implications in predicting microbial responses of C_4 plants under future climate change scenario. However, other studies on C_4 plants have shown that nutrient limitation and water availability can significantly alter these responses.

Community Assembly Mechanisms and Legacy effects of CO_2 Enrichment

In chapter four, we provide a comprehensive analysis of all soil samples collected in this project to assess microbial community stability, assembly processes and cooccurrence patterns. Significant contribution of dispersal limitation across different soil and plant categories could be attributed to LYCOG experimental design, which largely restricted dispersal between intact soil monoliths. Interestingly, the proportion of

homogenous selection and variable selection significantly differed between soil types. Phylogenetic turnover (βNTI) positively correlated to ΔCO_2 concentration ($p<0.01$) only in silty clay soils with mixed C_3/C_4 grasses. Furthermore, the legacy effects of long-term CO_2 treatment on taxonomic community structure and predicted function were minimal in all switchgrass communities but soil-specific in those associated with mixed C_3/C_4 grasses. Cooccurrence network analysis showed clustered networks with distinct core microbiome and putative key stone taxa within each plant and soil category. Taken together, our results suggest that the relative influence of ecological processes governing microbial community assembly in Texas Prairie grasslands are predominantly stochastic, but the extent of influence of selection-based processes is soil-specific and plant-specific. Furthermore, CO_2 enrichment and its legacy alone may not impose a strong selective pressure in shaping the overall community structure.

Summary

Our results did not show a strong or direct impact of CO_2 enrichment gradient on soil microbial communities. Instead, our findings indicate that modest CO_2 gradient treatment (250- 500 ppm) may not be enough to induce large shifts in bacterial diversity and community structure. Our results from switchgrass soil microbiome also identified certain degree of taxonomic specialization for substrate-specific carbohydrate degradation, N cycling pathways and phosphate metabolism. In summary, long-term physiochemical properties (e.g., soil texture and nutrient content), soil moisture content and plant community composition had a pronounced effect on microbial community composition and predicted function as depicted in the conceptual model (Figure 5.1). Although CO_2 enrichment could indirectly alter dominant taxa and stimulate net primary productivity

indirectly through increased soil water content, it appears to have minimal effect on long-term stable edaphic properties specifically in ecosystems that are not nutrient limited. Nonetheless, the degree of community stability (resistance/resilience) or sensitivity under CO_2 enrichment along with relative contribution of stochastic and deterministic community assembly processes significantly varied by soil type and plant community composition.

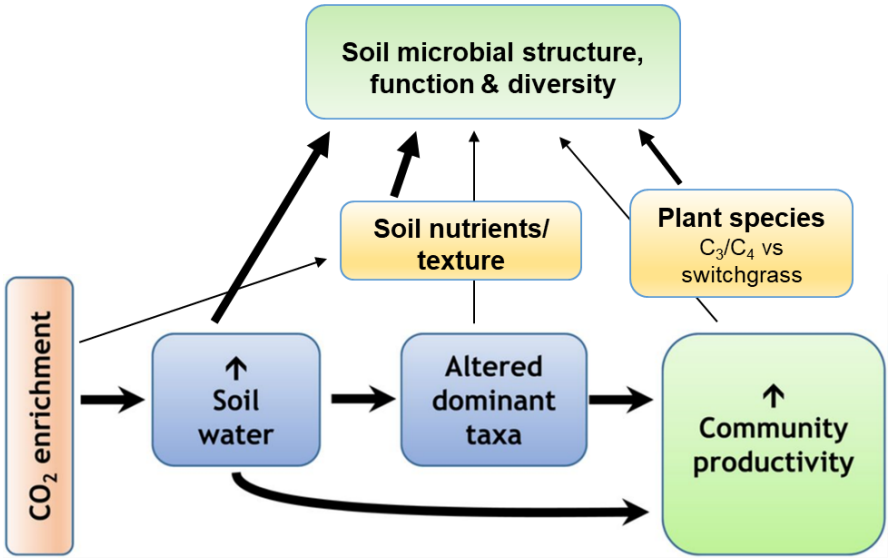


Figure 5.1 Conceptual model summarizes the finding that soil water content, soil physiochemical properties and plant species composition had stronger effect on soil microbial communities than CO_2 enrichment gradient.

Future Research Implications

This dissertation provides novel insights into how soil microbial communities respond to an ongoing preindustrial-to-future CO_2 gradient treatment and recover after the cessation of those experimental conditions. 16S rRNA gene amplicon sequencing was useful in discerning patterns in taxonomic and phylogenetic diversity. However, linking shifts in diversity and community composition to ecosystem function requires a more

rigorous analysis. In this study, I also incorporated shotgun sequencing of soil microbial communities associated with switchgrass but could only infer to their functional potential based on the abundance of genes involved in carbohydrate degradation, nitrogen cycling and phosphate metabolism. However, metagenome sequencing is still comparatively more expensive, and the assembly and annotations of entire genomes of all organisms require more computational resources. Thus, ecological null models and PICRUSt2 were valuable tools in deciphering microbial community assembly mechanisms and predicting functional metagenome profiles, respectively. In the next decade, "meta- omics" approach (e.g., metagenomics, metatranscriptomics, metaproteomics) can provide useful information on dormant and active microbial taxa as well as their functional roles in various nutrient cycling processes. Furthermore, this empirical data could be included in ecosystem models to predict how microbial diversity is related to broader functional traits and ecosystem processes. Thus, future research integrating compositional data and microbial activity, or gene expressions along with ecosystem process rates such as soil respiration could expand our current understanding of the link between biodiversity and ecosystem function in the face of global climate change.

Because the degree of sensitivity, resistance and resilience could be affected by the intensity of disturbance and vary across ecosystems, multifactorial experiments can advance our existing knowledge on the stability of microbial communities. Similarly, investigating the priority effects based on historical contingency can provide a baseline and thus, better assess the recovery rate of soil microbial communities under various forms of environmental perturbation. Since little is known about the effects of CO_2 enrichment on microbial community resilience, further studies incorporating longer term (years to decade)

before, during and after experimental CO_2 treatment is necessary. Taken together, system biology approach and multidisciplinary research is integral to predict ecosystem-scale responses to various global change drivers and their long-term impact on overall ecosystem function as well as biosphere feedback mechanisms.

APPENDICES

APPENDIX A

Supplementary Information (Chapter Two)

Supplementary Figures

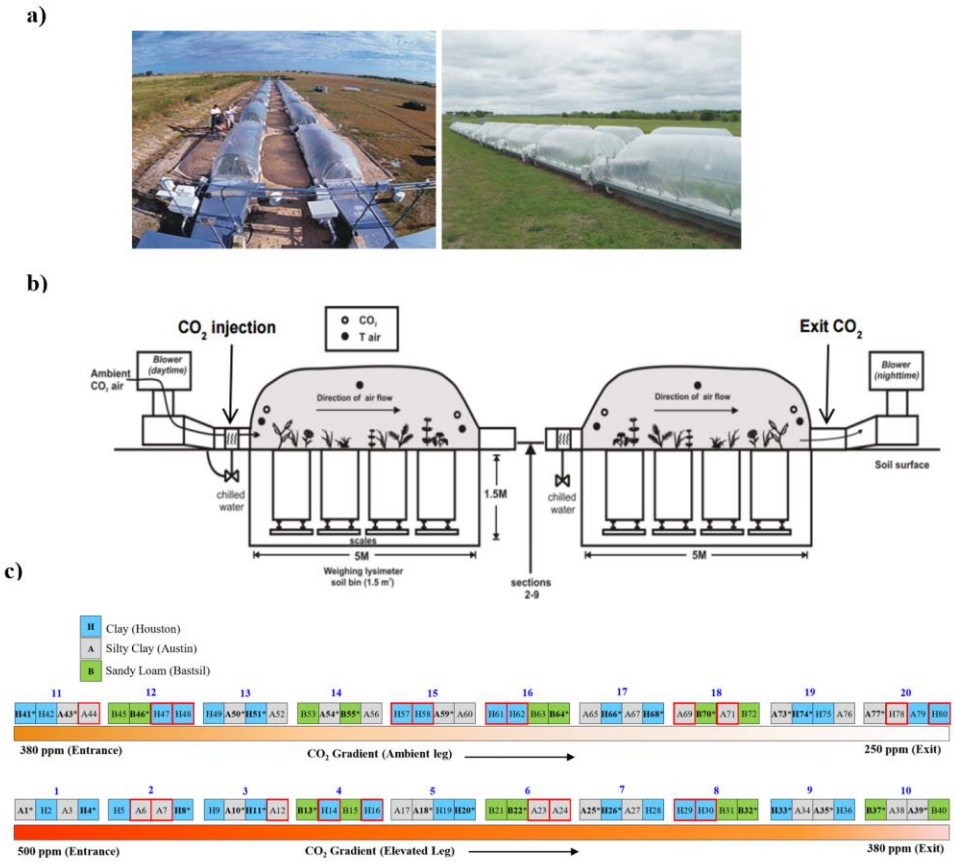


Figure A.S1. Schematic diagram of Lysimeter CO_2 gradient (LYCOG) facility at USDA-ARS, Temple TX (Adapted from Fay et al. 2009). a) Pictures showing continuous CO_2 gradient maintained in the longitudinal chambers, with vegetation enclosed inside the clear polyethylene. b) Of the two chambers, elevated leg showing the CO_2 injection point at the entrance. Chilled water-cooling coils between sections (1-10) control the air temperature. In the ambient chamber (not shown here), CO_2 was not injected. **c)** Arrangement of 80 soil monoliths. Red boxes denote 20 monoliths which were replaced with *Panicum virgatum* L., switchgrass (not considered in this study) after grub damage in 2007. Remaining 60 constitute mixed C3/C4 grasses, of which 31 monoliths shown here in asterisk (*), were considered for bacterial community analysis.

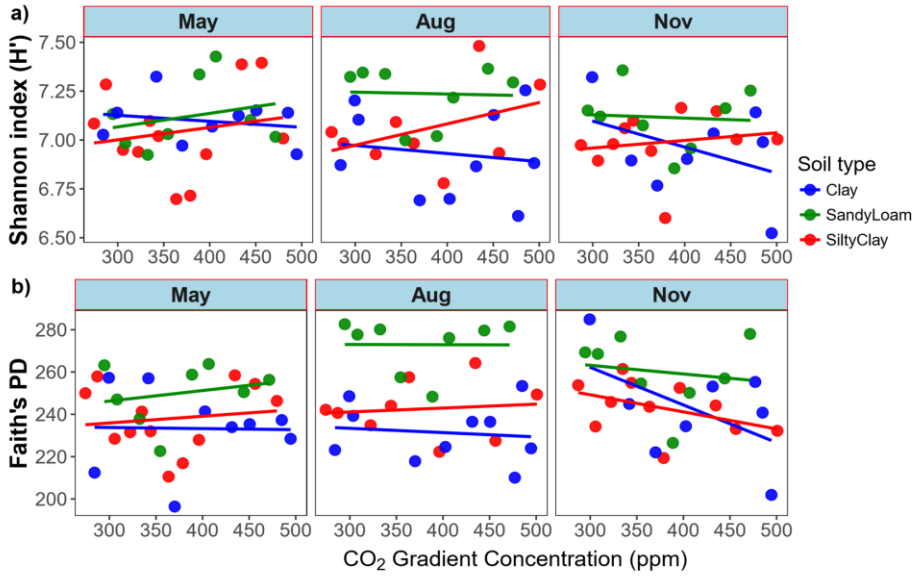


Figure A. S2. GAM fitted with Shannon Index (a) and Faith's PD (b) along a preindustrial-to-future CO_2 showing diversity response within specific soil types per season. ANOVA on GAM models with CO_2 x soil and CO_2 x season revealed that the interactive effects were also not significant (p>0.1)

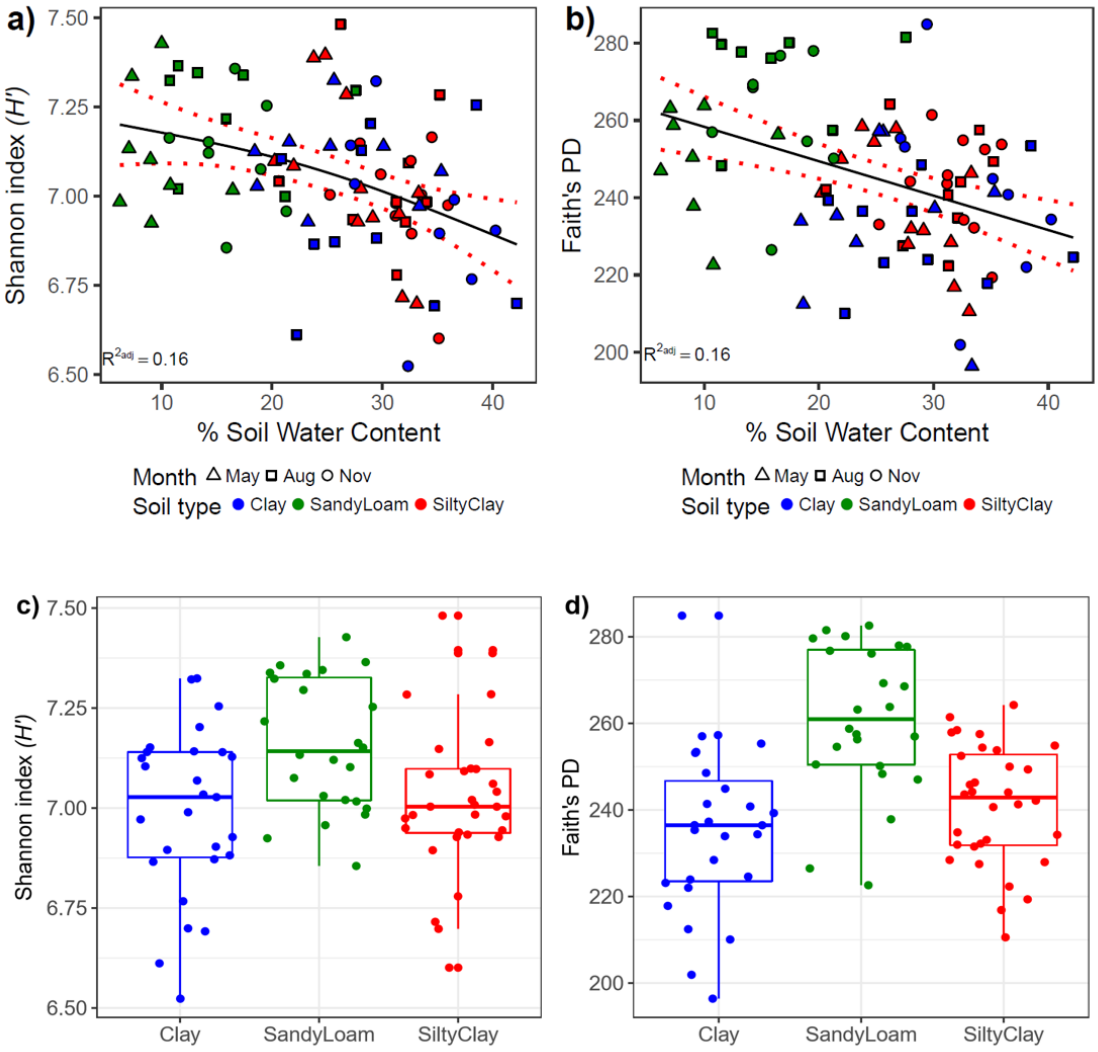


Figure A.S3 **(a).** Generalized additive model (GAM) fitted with Shannon index (*H'*) and % soil water content across all soil types and seasons (R^2_{adj} = 0.16, $p < 0.001$). **(b).** GAM fitted with Faith's PD and % soil water content across all soil types and seasons (R^2_{adj} = 0.16, $p < 0.001$). **(c)** and **(d).** Box plots showing the significant differences in bacterial diversity indices ($p<0.01$) among three soil types.

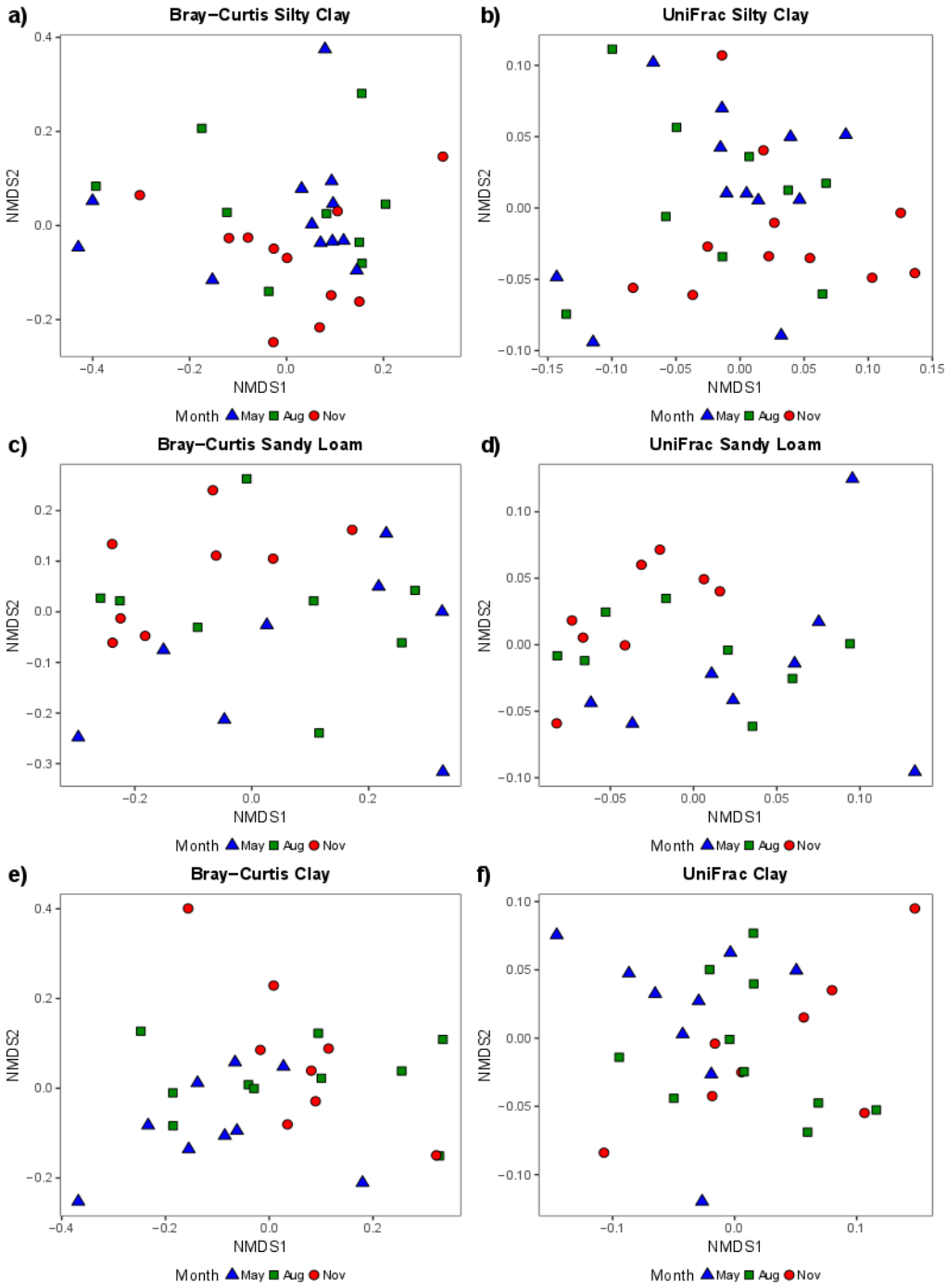


Figure A.S4. Non-metric multidimensional scaling (NMDS) ordinations with Bray-Curtis distance and UniFrac distance reflecting the effect of season grouping within specific soil types.

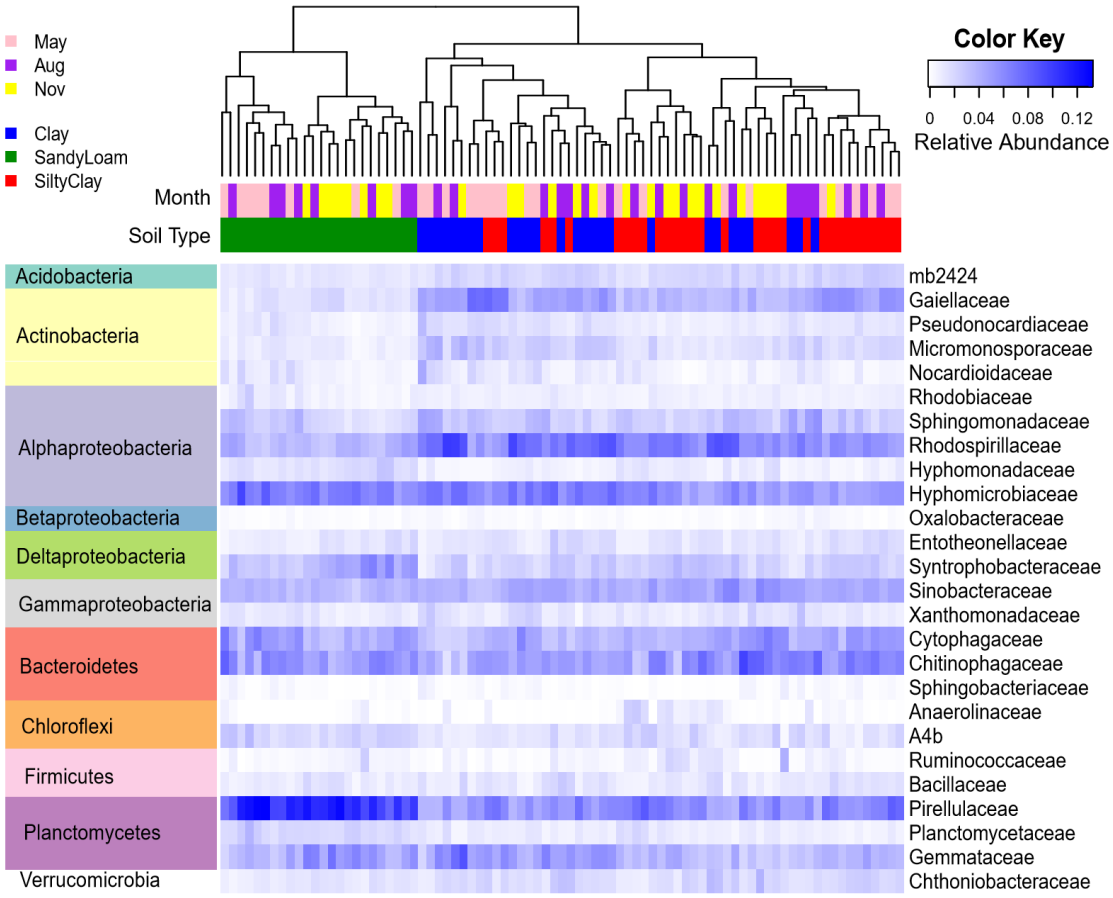
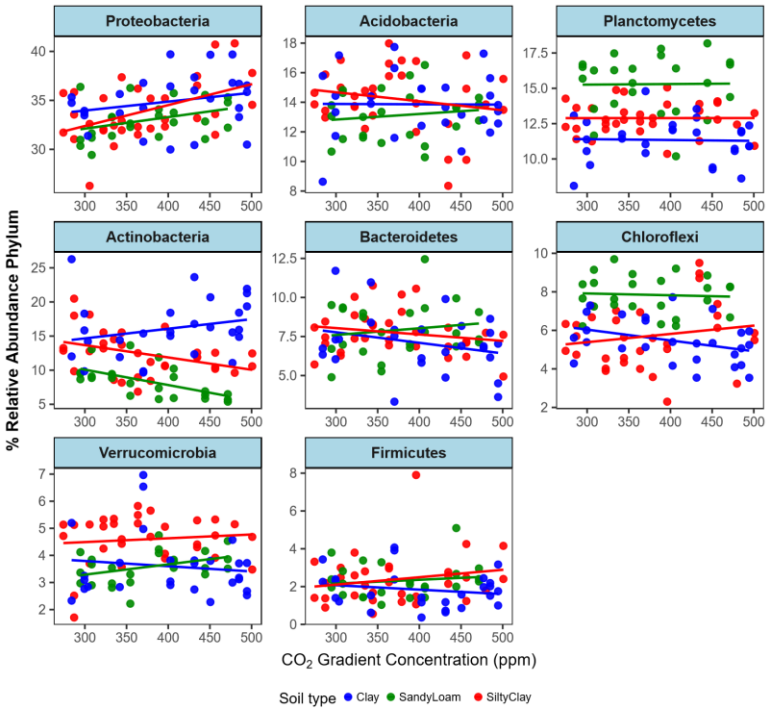


Figure A.S5. Heatmap representing the taxonomic distribution at family level per phylum. Taxa with >2% relative abundance were considered.

a)



b)

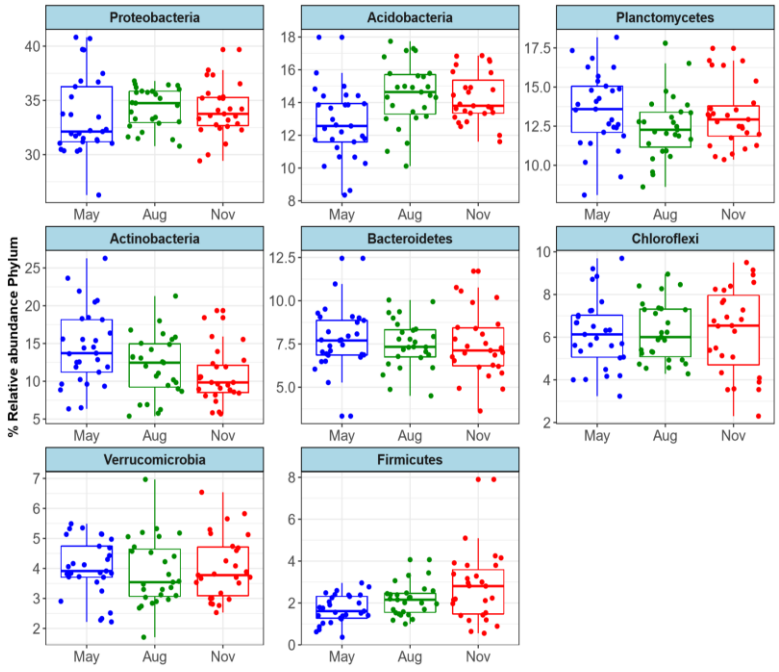


Figure A.S6(a). Relative abundance of most abundant phyla (>1%) along a CO_2 gradient for different soil types fitted with GAM. **(**b). Relative abundance of most abundant phyla (>1%) by season represented in boxplots.

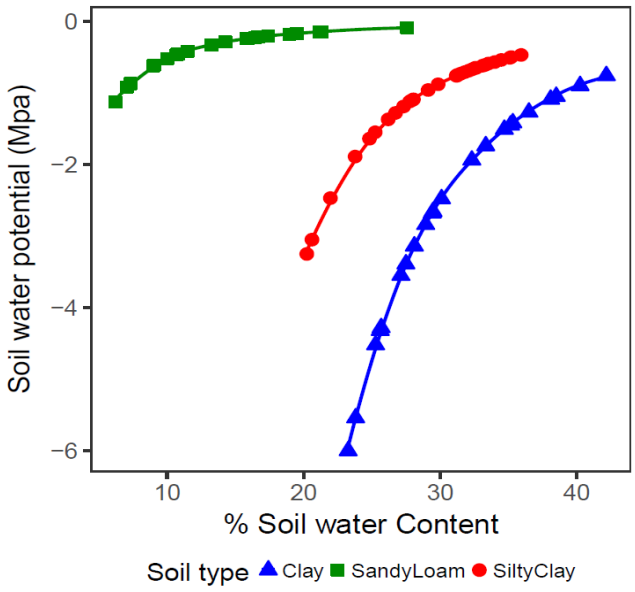


Figure A.S7. Soil water release curves with the estimated soil water potential for the given values of volumetric soil water content.

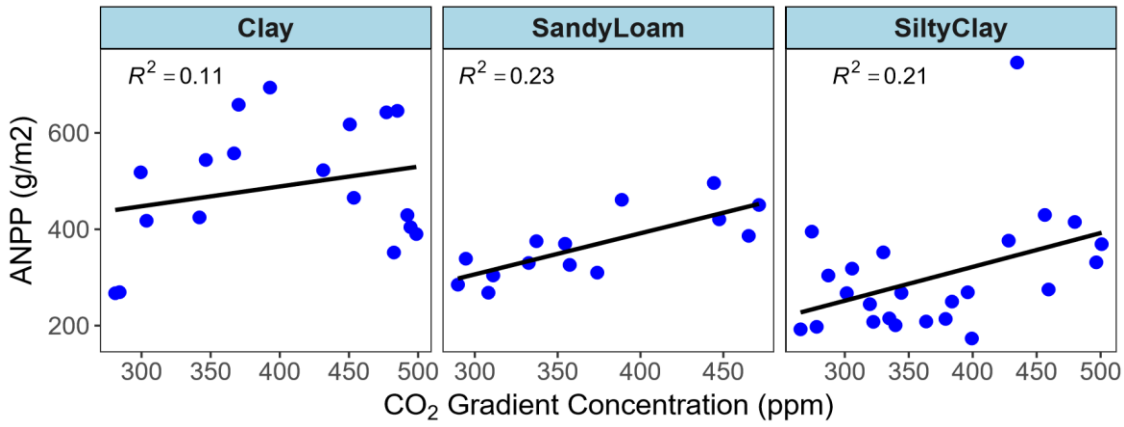


Figure A.S8**.** Relationships between ANPP and CO_2 concentration in different soil types fitted with GAM. The data points represent all soil monoliths with mixed C_3/C_4 grasses. The plots show overall trends in ANPP response, which is comparable to prior year results. Some monoliths with significant grub damage have been excluded.

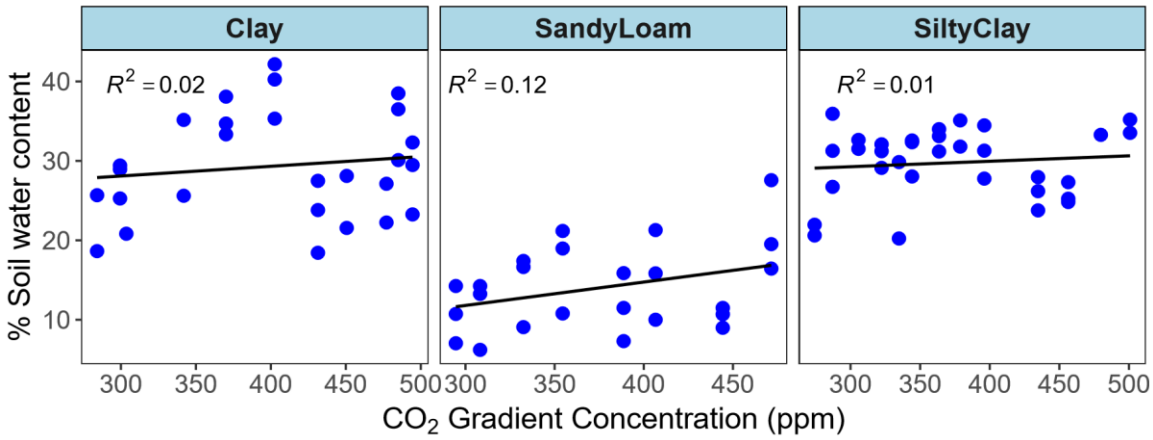


Figure A.S9. Relationships between soil water content and CO_2 concentration in different soil types fitted with GAM. The data points represent the soil monoliths that were sampled for microbial analysis during May, August and November of 2015.

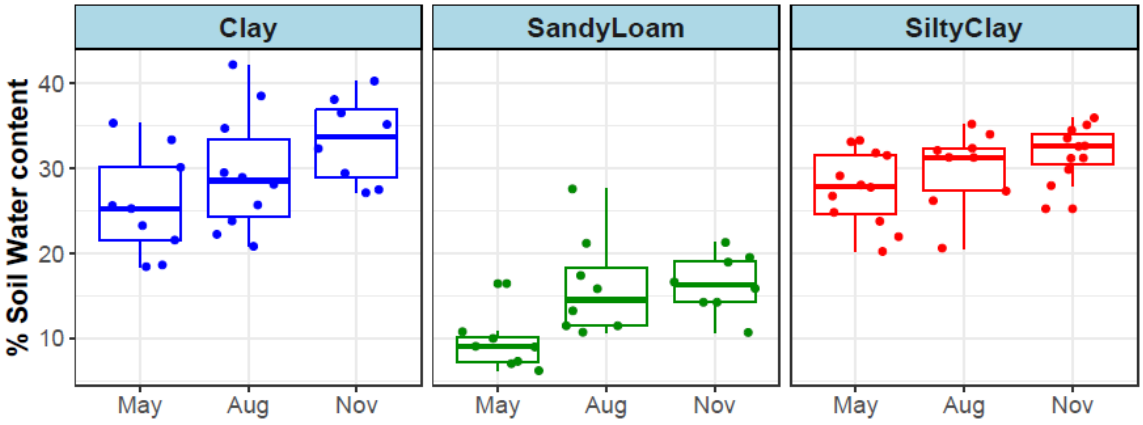


Figure A.S10. Trends in soil water content during May, August and November of 2015 growing season for each of the three soil types.

Supplementary Tables

Table A.S1. Multivariate statistical approaches to assess the significance of soil type and season grouping on bacterial community structure with global and pairwise comparisons.

	PERMANOVA		**ANOSIM**	
	F	*p*	*R*	*p*
Soil type (Global)	11.68	0.001**	0.606	0.001**
Clay vs Silty clay	5.41	0.001*		
Clay vs Sandy loam	15.62	0.001**		
Silty clay vs Sandy loam	15.06	0.001**		
Season (Global)	0.019	0.082	0.396	0.073
May vs August	1.23	0.06.		
May vs November	1.94	0.56		
August vs November	0.96	0.98		

*Significant (α = 0.05). **Significant after Bonferroni correction (α = 0.017)

Table A.S2. Multivariate statistical analyses illustrating the effect of seasonal variation within specific soil type.

Statistics		**Silty Clay**	**Sandy Loam**	**Clay**
ANOSIM				
(Season grouping)	*R*	0.01	0.026	0.123
	p	0.29	0.278	0.01*
PERMANOVA				
(Season grouping)	*F*	0.99	1.14	1.64
	p	0.46	0.167	0.005*

*Significant (α = 0.05). **Significant after Bonferroni correction (α = 0.017)

Table A.S3. Soil physiochemical characteristics.

Soil properties	**Silty Clay**	**Sandy Loam**	**Clay**
Volumetric Soil Water Content (% SWC)	20.2-35.9	6.2-27.6	18.4-42.2
Soil Water Potential (Mpa ψ)	-0.47 to -3.25	-0.09 to -1.13	-0.76 to -5.54
Total N (%)	0.27	0.14	0.30
Organic C (%)	9.77	1.98	6.64
Organic C/Total N	36.07	13.66	22.52
% Sand (se)	12 (0.4)	72.8 (0.6)	11.2 (0.5)
% Silt (se)	45.9 (0.7)	19.7 (0.4)	39.5 (1.0)
% Clay (se)	41.9 (0.6)	7.5 (0.4)	49.2 (1.2)
Soil pH			
(2002, prior to CO_2 treatment)	8.03	8.06	7.6
(2009, 4th year of CO_2 gradient)	-	8.09*	7.98*

Nutrient properties (SOC, TN, C: N ratio), soil water content (% SWC) and soil water potential (SWP) were measured for all samples collected during May, August and November of 2015 growing season. Soil texture values for individual soil types are from 0-10 cm of soil profile (Table 2, Fay et al. 2009) and were measured in 2002 prior to CO_2 gradient treatment. Although we did not measure soil pH for 2015 samples, pH was measured in 2002 for all soil types. The soil pH data for 2009 samples (*) represent the average values for clay and sandy loam soils only (Table S3, Procter *et al*. 2015).

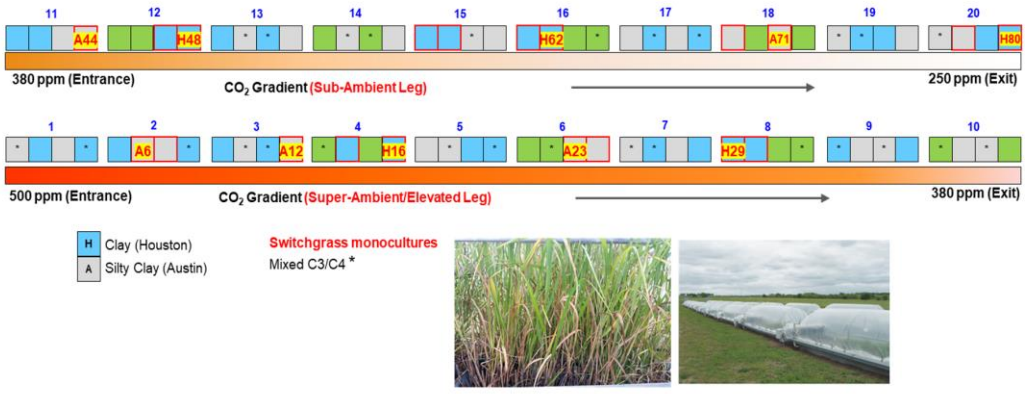
Table A.S4. Average values of ambient temperature (degrees Celsius) recorded at LYCOG site, Grassland Soil and Water Research Center in Temple, TX, USA for the Year 2015.

	2015 Growing Season		
Sampling Date	May 19	August 11	November 2
Mean Ambient Temperature (°C)	24.08	29.13	17.08

APPENDIX B

Supplementary Information (Chapter Three)

Supplementary Figures



Monolith (2015 #position)	Soiltype	2015 CO_2 (ppm)	2006-2014 CO_2 (ppm)	2016_CO_2 (ppm)	2015 Section (leg)	2006_2014 Section (leg)	2016
H16	Clay	465.3	324.9	410	Super_Ambient	Sub_Ambient	Ambient
H29	Clay	420.8	316.9	410	Super_Ambient	Sub_Ambient	Ambient
H48	Clay	348.9	261	410	Sub_Ambient	Sub_Ambient	Ambient
H62	Clay	314	416.1	410	Sub_Ambient	Super_Ambient	Ambient
H80	Clay	261	348.9	410	Sub_Ambient	Super_Ambient	Ambient
A6	SiltyClay	489.8	437.9	410	Super_Ambient	Super_Ambient	Ambient
A12	SiltyClay	474.4	297.1	410	Super_Ambient	Sub_Ambient	Ambient
A23	SiltyClay	441.1	292.2	410	Super_Ambient	Sub_Ambient	Ambient
A44	SiltyClay	360.4	487.4	410	Sub_Ambient	Super_Ambient	Ambient
A71	SiltyClay	292.2	441.1	410	Sub_Ambient	Super_Ambient	Ambient

Figure B.S1. Schematic diagram of Lysimeter CO_2 gradient (LYCOG) facility at USDA-ARS, Temple TX (Adapted from Fay et al. 2009). Red boxes denote soils monoliths which were replaced with *Panicum virgatum* L., switchgrass in 2007 along the linear CO_2 gradient as depicted in the two longitudinal sections in the picture below. Soil samples were collected from 2015 (the last year of CO_2 treatment) and 2016 (ambient conditions after termination of experimental CO_2 treatment). However, intact soil monoliths in 2015 were switched from their long-term position (2006-2014) and exposed different CO_2 concentration as illustrated below. To analyze the legacy effects of CO_2 enrichment, we used the long-term CO_2 gradient concentrations.

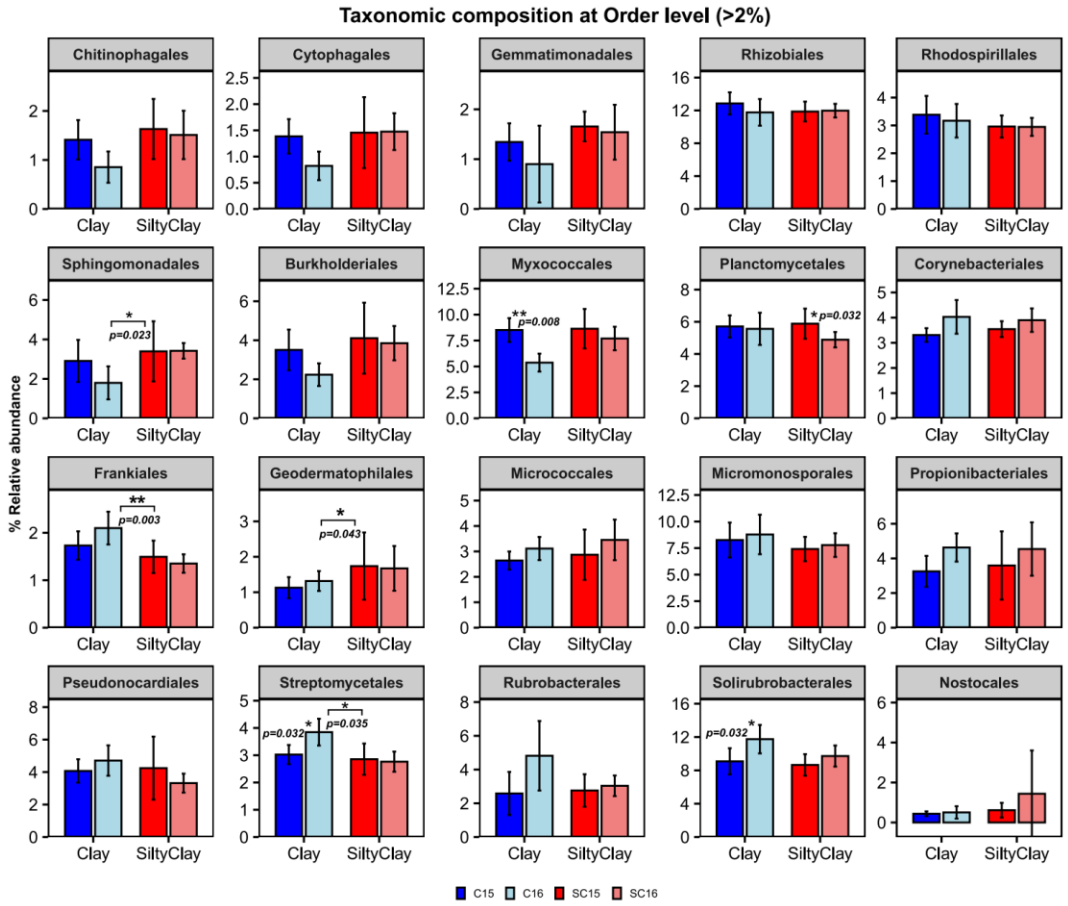


Figure B.S2. Bar graphs showing the main effect of soil type and interactive effects of soil x year on the relative abundance of all taxa at order level. *Significant (α = 0.05). **Significant after Bonferroni correction (α = 0.017).

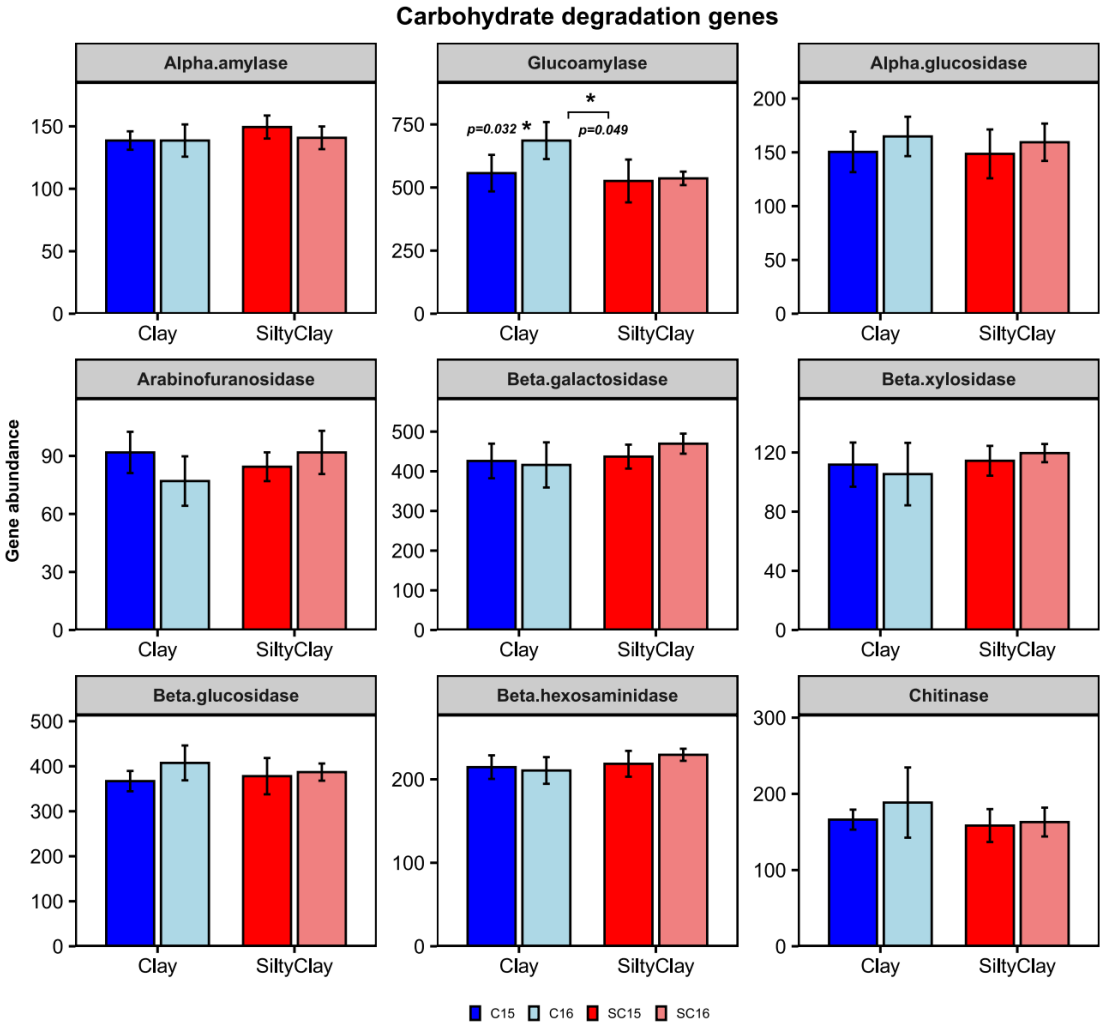


Figure B.S3. Bar graphs showing the main effect of soil type and interactive effects of soil x year on carbohydrate degradation gene abundance (absolute gene count). *Significant (α = 0.05). **Significant after Bonferroni correction (α = 0.017).

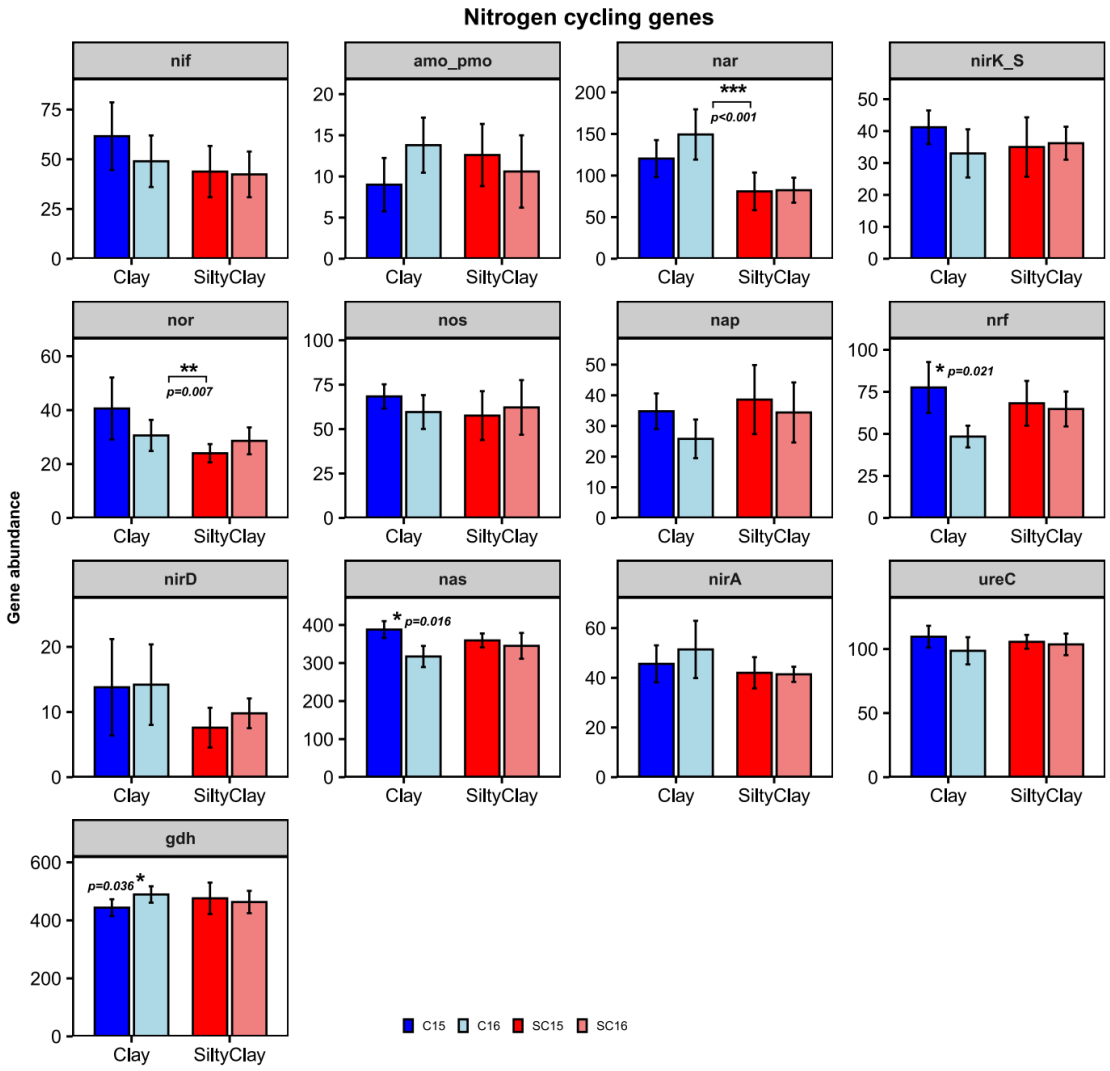


Figure B.S4**.** Bar plots showing the main effect of soil type and interactive effects of soil x year on nitrogen cycling gene abundance (absolute gene count). *Significant (α = 0.05). **Significant after Bonferroni correction (α = 0.017).

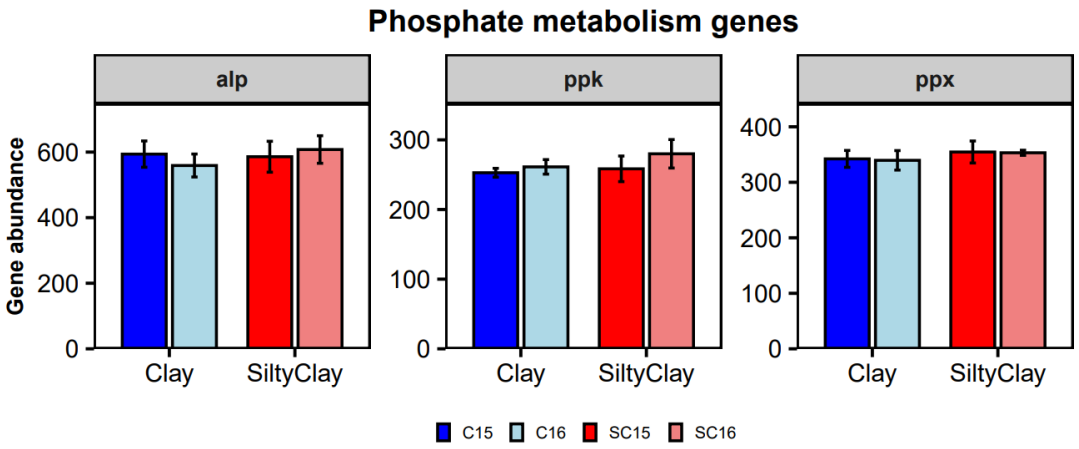


Figure B.S5. Bar plots showing the main effect of soil type and interactive effects of soil x year on phosphate metabolism gene abundance (absolute gene count).

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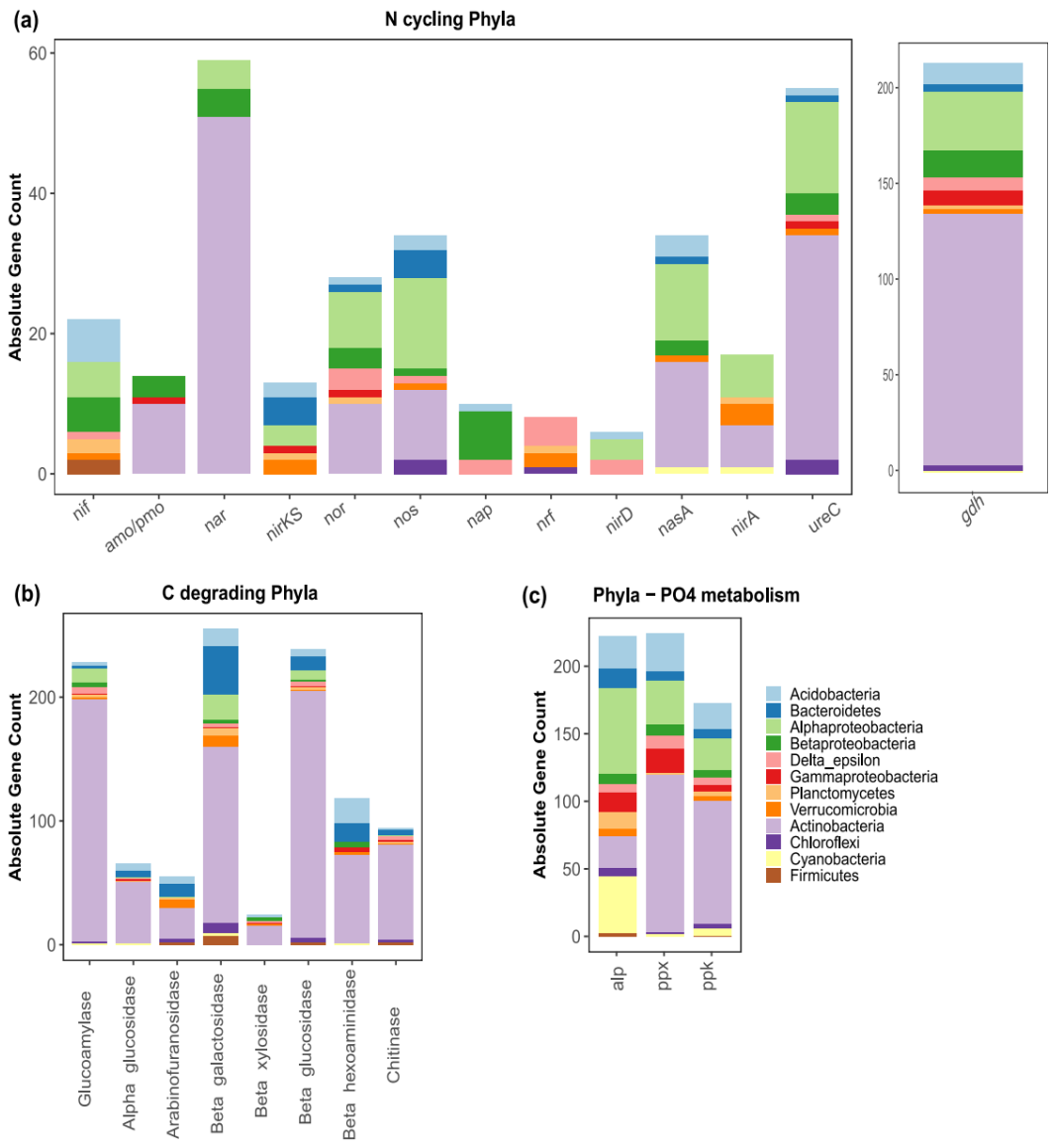

Figure B.S6. Bar graphs showing the taxonomic contribution of microbial groups (at phylum level) to different functional genes involved in (a) nitrogen cycling, (b) carbohydrate degradation and (c) phosphate metabolism

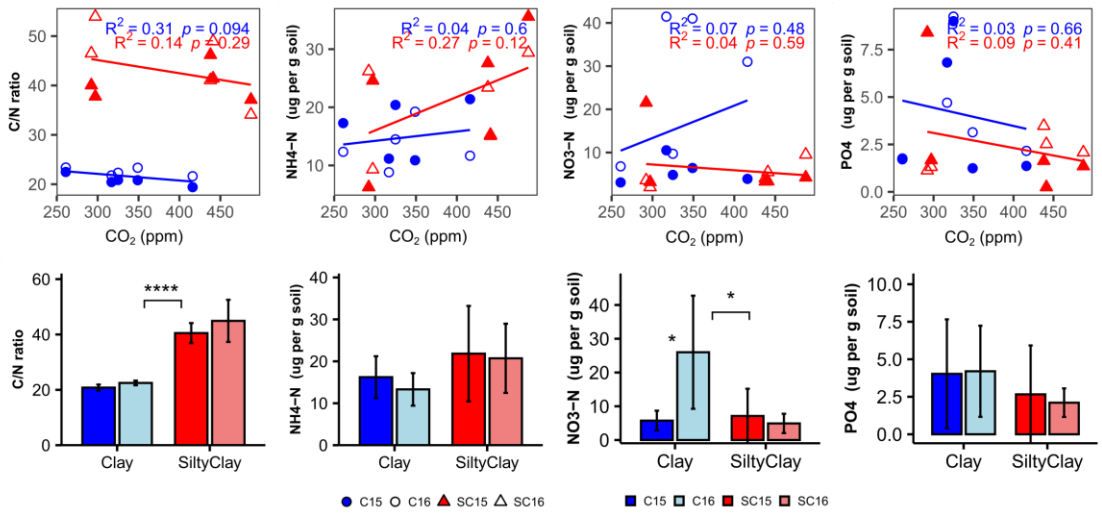

Figure B.S7. Scatterplots showing the effect of long-term CO_2 gradient treatment and bar graphs showing the main effect of soil type and interactive effects of soil x year on soil nutrient dynamics. *Significant ($\alpha = 0.05$). **Significant after Bonferroni correction ($\alpha = 0.017$).

Supplementary Tables

Table B.S1a-c. List of gene/protein nomenclature assigned to each SEED functional grouping at SEED level 4 classification).

Table B.S1a.

Carbohydrate Substrate	**C degradation genes description (SEED level 4)**
Starch	Alpha-amylase (EC 3.2.1.1)
	Glucoamylase (EC 3.2.1.3)
	Alpha-glucosidase (EC 3.2.1.20)
Hemicellulose	Alpha-N-arabinofuranosidase (EC 3.2.1.55)
	Beta-galactosidase (EC 3.2.1.23)
	Beta-xylosidase (EC 3.2.1.37)
Cellulose	Beta-glucosidase (EC 3.2.1.21)
Chitin	Beta-hexosaminidase (EC 3.2.1.52)
	Chitinase (EC 3.2.1.14)

Table B.S1b.

Nitrogen cycle	Gene	Description (SEED level 4)
Nitrogen Fixation	*nifF*	Nitrogenase (molybdenum-iron) alpha chain (EC 1.18.6.1)
	nifD	Nitrogenase (molybdenum-iron) beta chain (EC 1.18.6.1)
	nifH	Nitrogenase (molybdenum-iron) reductase and maturation protein NifH
	nifA	Nitrogenase (molybdenum-iron)-specific transcriptional regulator NifA
	nifX	Nitrogenase FeMo-cofactor carrier protein NifX
	nifE	Nitrogenase FeMo-cofactor scaffold and assembly protein NifE
	nifN	Nitrogenase FeMo-cofactor scaffold and assembly protein NifN
	nifB	Nitrogenase FeMo-cofactor synthesis FeS core scaffold and assembly protein NifB
	nifQ	Nitrogenase FeMo-cofactor synthesis molybdenum delivery protein NifQ
Nitrification	*amo/pmo*	Ammonia/Particulate methane monooxygenase
	hao	Hydroxylamine reductase
Denitrification	*narG*	Respiratory nitrate reductase gamma chain (EC 1.7.99.4)
	narH	Respiratory nitrate reductase beta chain (EC 1.7.99.4)
	narI	Respiratory nitrate reductase alpha chain (EC 1.7.99.4)
	narJ	Respiratory nitrate reductase delta chain (EC 1.7.99.4)
	nirK	Copper-containing nitrite reductase (EC 1.7.2.1)

	nirS	Cytochrome cd1 nitrite reductase (EC 1.7.2.1)
	norD	Nitric oxide reductase activation protein NorD
	norE	Nitric oxide reductase activation protein NorE
	norQ	Nitric oxide reductase activation protein NorQ
	norB	Nitric-oxide reductase subunit B (EC 1.7.99.7)
	norC	Nitric-oxide reductase subunit C (EC 1.7.99.7)
	nosX	Nitrous oxide reductase maturation periplasmic protein NosX
	nosD	Nitrous oxide reductase maturation protein NosD
	nosF	Nitrous oxide reductase maturation protein NosF (ATPase)
	nosR	Nitrous oxide reductase maturation protein NosR
	nosL	Nitrous oxide reductase maturation protein, outer-membrane lipoprotein NosL
	nosY	Nitrous oxide reductase maturation transmembrane protein NosY
	nosZ	Nitrous-oxide reductase (EC 1.7.99.6)
Assimilatory nitrate reduction	*nasC*	Assimilatory nitrate reductase large subunit (EC:1.7.99.4)
	nasD	Nitrate ABC transporter, ATP-binding protein
	nasF	Nitrate ABC transporter, nitrate-binding protein
	nasE	Nitrate ABC transporter, permease protein
	nirA	Ferredoxin--nitrite reductase (EC 1.7.7.1)
Dissimilatory nitrate reduction	*napF*	Ferredoxin-type protein NapF (periplasmic nitrate reductase)

	napG	Ferredoxin-type protein NapG (periplasmic nitrate reductase)
	napD	Periplasmic nitrate reductase component NapD
	napE	Periplasmic nitrate reductase component NapE
	napA	Periplasmic nitrate reductase precursor (EC 1.7.99.4)
	napH	Polyferredoxin NapH (periplasmic nitrate reductase)
	napB	Nitrate reductase cytochrome c550-type subunit
	nirD	Heme d1 biosynthesis protein NirD
	nirF	Heme d1 biosynthesis protein NirF
	nirG	Heme d1 biosynthesis protein NirG
	nirH	Heme d1 biosynthesis protein NirH
	nirJ	Heme d1 biosynthesis protein NirJ
	nirL	Heme d1 biosynthesis protein NirL
Dissimilatory nitrite reduction	*nrfH*	Cytochrome c nitrite reductase, small subunit NrfH
	nrfE	Cytochrome c-type heme lyase subunit nrfE, nitrite reductase complex assembly
	nrfA	Cytochrome c552 precursor (EC 1.7.2.2)
Organic N biosynthesis and degradation	*ureC*	Urease alpha subunit (EC 3.5.1.5)
	glnA	Glutamine synthetase type I (EC 6.3.1.2)
	GS_e	Glutamine synthetase type II, eukaryotic (EC 6.3.1.2)

	glnN	Glutamine synthetase type III, GlnN (EC 6.3.1.2)
	gdh-NAD	NAD-specific glutamate dehydrogenase (EC 1.4.1.2)
	gdh-NAD	NAD-specific glutamate dehydrogenase, large form (EC 1.4.1.2)
	gdhA	NADP-specific glutamate dehydrogenase (EC 1.4.1.4)

Table B.S1c.

Phosphorus cycle	**Gene**	**Description (SEED level 4)**
Phosphate metabolism	*alp*	Alkaline phosphatase (EC 3.1.3.1)
	ppx	Exopolyphosphatase (EC 3.6.1.11)
	ppk	Polyphosphate kinase (EC 2.7.4.1)

Table B.S2. Pearson's correlation statistics between gene abundance and environmental variables.

Gene of Interest	**C/N**		**NH_4^+-N**		**NO_3^--N**		**PO_4^{3-}**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***
Alpha-amylase	0.25	0.28	0.03	0.89	0.15	0.53	-0.10	0.68
Glucoamylase	-0.45	**0.04***	-0.42	0.06	0.63	**0.002****	-0.02	0.92
Alpha-glucosidase	0.05	0.82	-0.11	0.65	0.08	0.74	-0.08	0.75
Arabinofuranosidase	0.16	0.49	0.39	0.08	-0.59	**0.006****	-0.47	**0.04***
Beta-galactosidase	0.38	0.10	0.53	**0.02***	-0.37	0.11	-0.28	0.24
Beta-xylosidase	0.24	0.30	0.57	**0.009****	-0.17	0.47	-0.24	0.32
Beta-glucosidase	0.02	0.94	-0.07	0.76	0.10	0.68	-0.16	0.50
Beta-hexosaminidase	0.44	**0.05***	0.60	**0.005****	-0.51	**0.022***	-0.11	0.64
Chitinase	-0.24	0.30	-0.25	0.29	-0.09	0.70	0.32	0.17
nif	-0.52	**0.02***	0.03	0.91	0.00	1.00	0.22	0.36
nar	-0.71	**<0.001*****	-0.23	0.32	0.19	0.43	0.23	0.34
nirKS	-0.14	0.54	0.39	0.09	-0.31	0.19	-0.02	0.95
nor	-0.52	**0.02***	-0.11	0.64	-0.13	0.58	0.06	0.81
nos	-0.27	0.24	0.37	0.10	-0.03	0.91	-0.02	0.94
nap	0.18	0.43	0.64	**0.002****	-0.40	0.08	-0.38	0.10
nrf	0.02	0.93	0.40	0.07	-0.45	**0.05***	-0.01	0.98
nirD	-0.43	0.05	-0.23	0.33	-0.01	0.96	0.06	0.80
nas	-0.11	0.63	0.22	0.35	-0.55	**0.01***	0.01	0.96
nirA	-0.36	0.11	-0.20	0.40	0.53	**0.02***	-0.43	0.06
ureC	-0.12	0.61	0.21	0.36	-0.26	0.26	-0.16	0.51
gdh	-0.13	0.59	-0.44	**0.05***	0.45	**0.05***	0.16	0.50
alp	0.17	0.48	0.45	**0.04***	-0.18	0.44	0.06	0.79
ppk	0.34	0.13	0.27	0.25	-0.03	0.89	-0.32	0.17
ppx	0.32	0.16	0.59	**0.006****	-0.05	0.82	-0.17	0.47

*Significant ($\alpha = 0.05$).
**Significant after Bonferroni correction ($\alpha = 0.017$)

APPENDIX C

Supplementary Information (Chapter Four)

Supplementary Figures

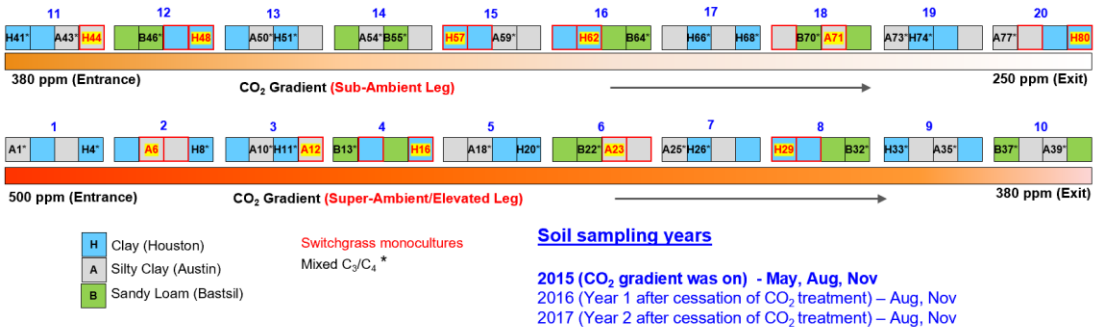

Figure C.S1. Schematic diagram of the linear CO_2 gradient as depicted in the two longitudinal sections from Lysimeter CO_2 gradient (LYCOG) facility at USDA-ARS, Temple TX (Supplemental Information, Raut et al. 2018). Red boxes denote soils monoliths which were replaced with *Panicum virgatum* L., switchgrass in 2007, of which 10 monoliths highlighted here were considered. Remaining 60 constitute mixed C_3/C_4 grasses, of which 31 monoliths shown here in asterisk (*) were considered for the comprehensive analysis of community assembly mechanisms and legacy effects of CO_2 enrichment gradient. A total of 260 samples were used for downstream analysis after collecting the soil cores from three sampling events in May, August and November of 2015 (the last year of CO_2 treatment) and four more sampling events in May and August of 2016 and 2017 (ambient conditions after termination of experimental CO_2 treatment). Intact soil monoliths in 2015 were switched from their long-term position (2006-2014) and exposed different CO_2 concentration as illustrated previously (Appendix B: Figure B.S1) but only in switchgrass soils.

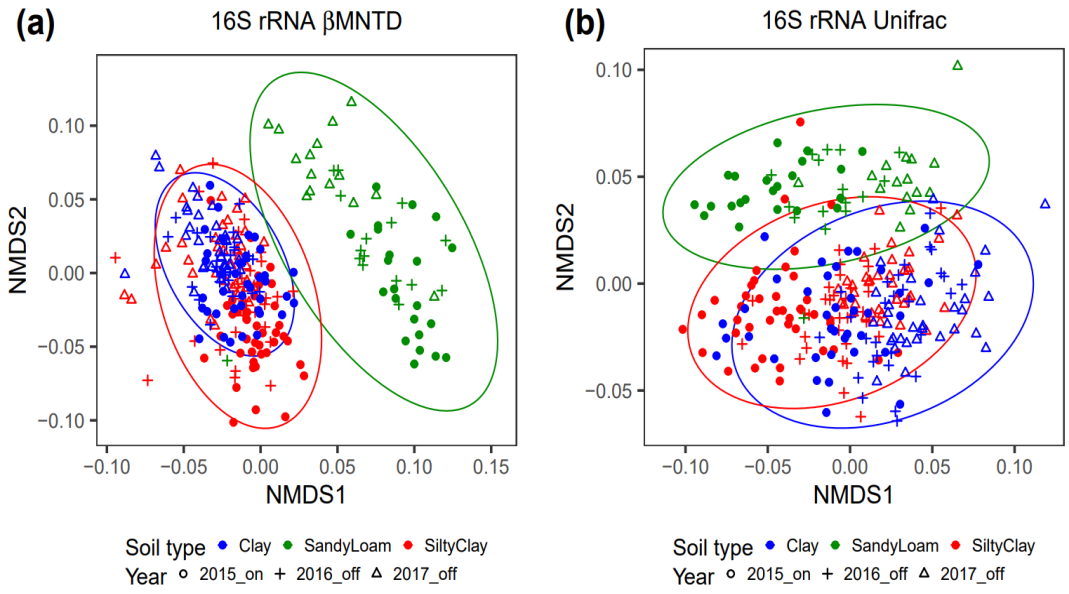

Figure C.S2. Phylogenetic community structure based on βMNTD and Unifrac Distances.

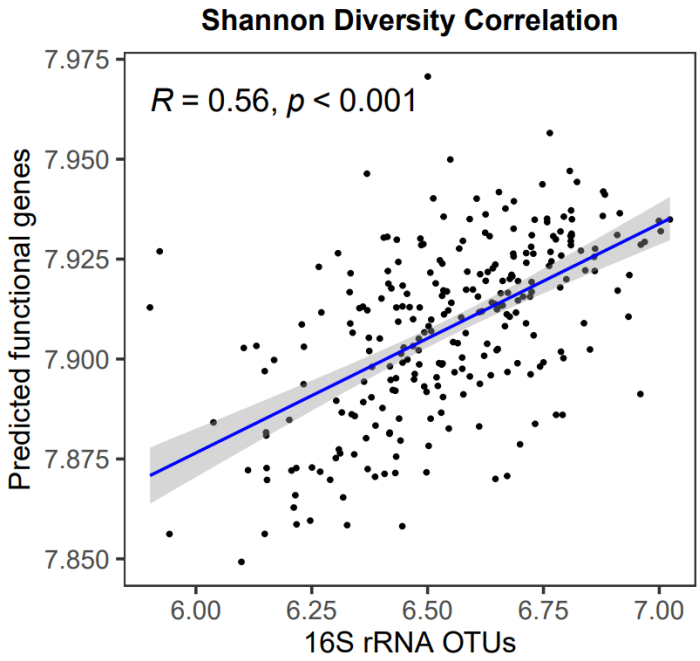

Figure C.S2. Correlation between Shannon diversity of predicted functional genes and 16S rRNA OTUs.

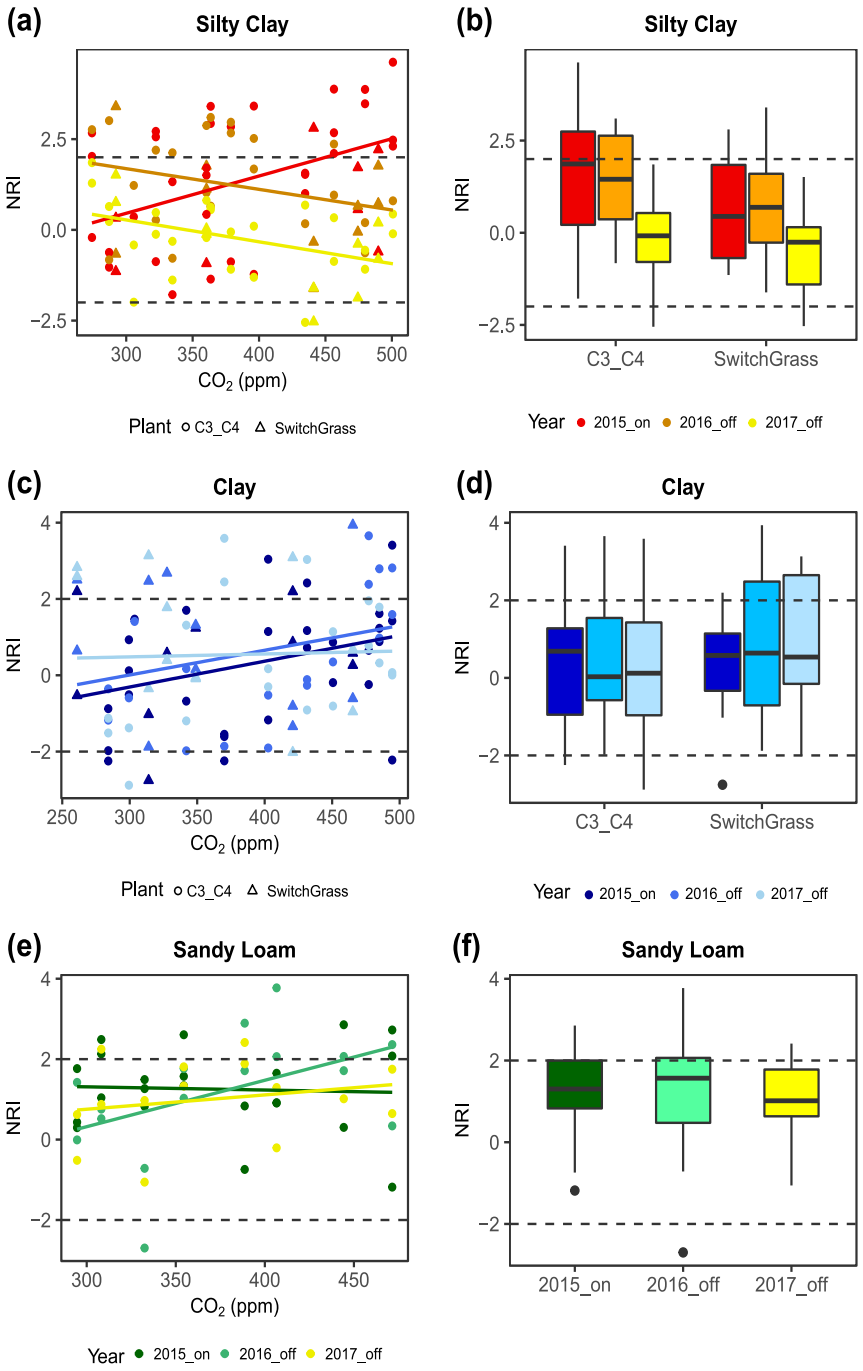

Figure C.S3. Net relatedness index (NRI) within different soil and plant categories. Left panel (a, c, e) reflects variation in NRI values along the CO_2 gradient and right panel (d, b, f) highlights the variation linked to CO_2 treatment year and plant type. The horizontal dotted line above +2 or below -2 indicate statistically significant values around the expectation under neutral community assembly.

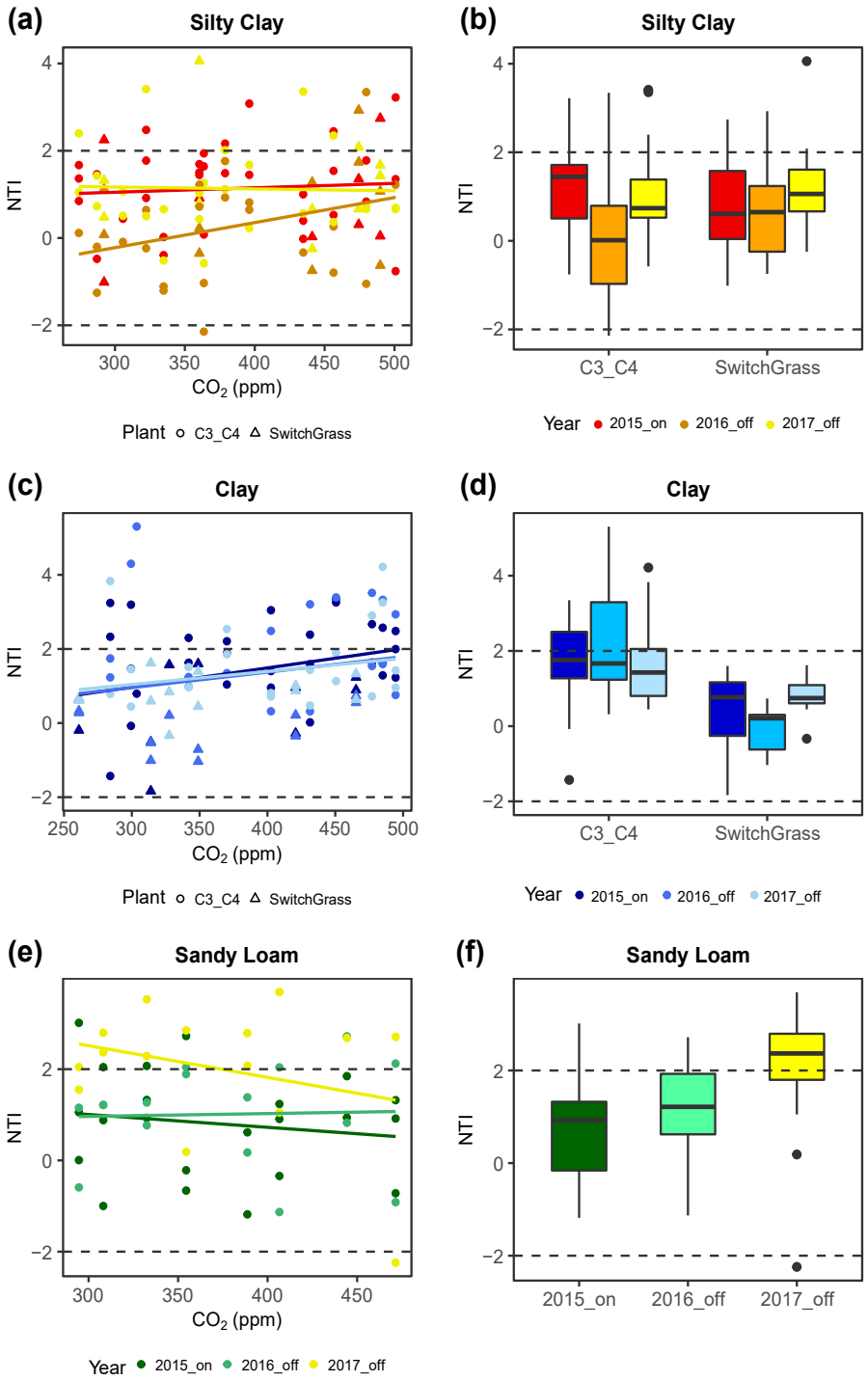

Figure C.S4. Nearest taxon index (NTI) within different soil and plant categories. Left panel (a, c, e) reflects variation in NTI values along the CO_2 gradient and right panel (d, b, f) highlights the variation linked to CO_2 treatment year and plant type. The horizontal dotted line above +2 or below -2 indicate statistically significant values around the expectation under neutral community assembly.

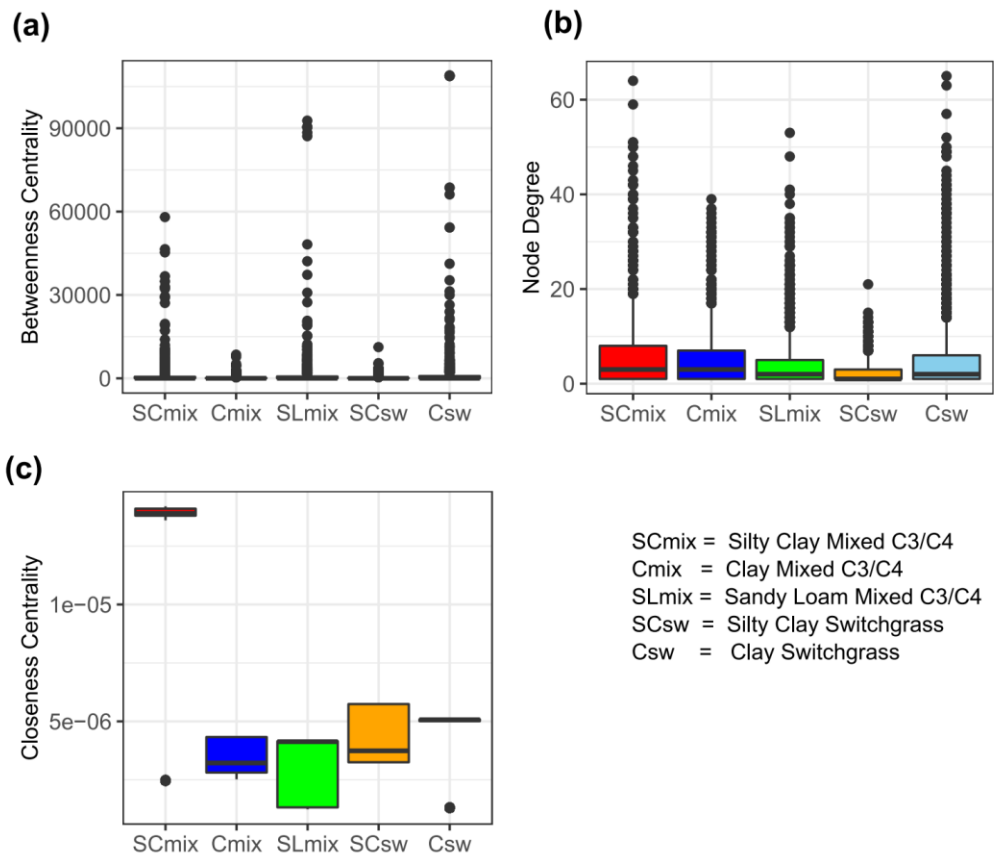

Figure C.S5. Node-level topological properties of co-occurrence networks.

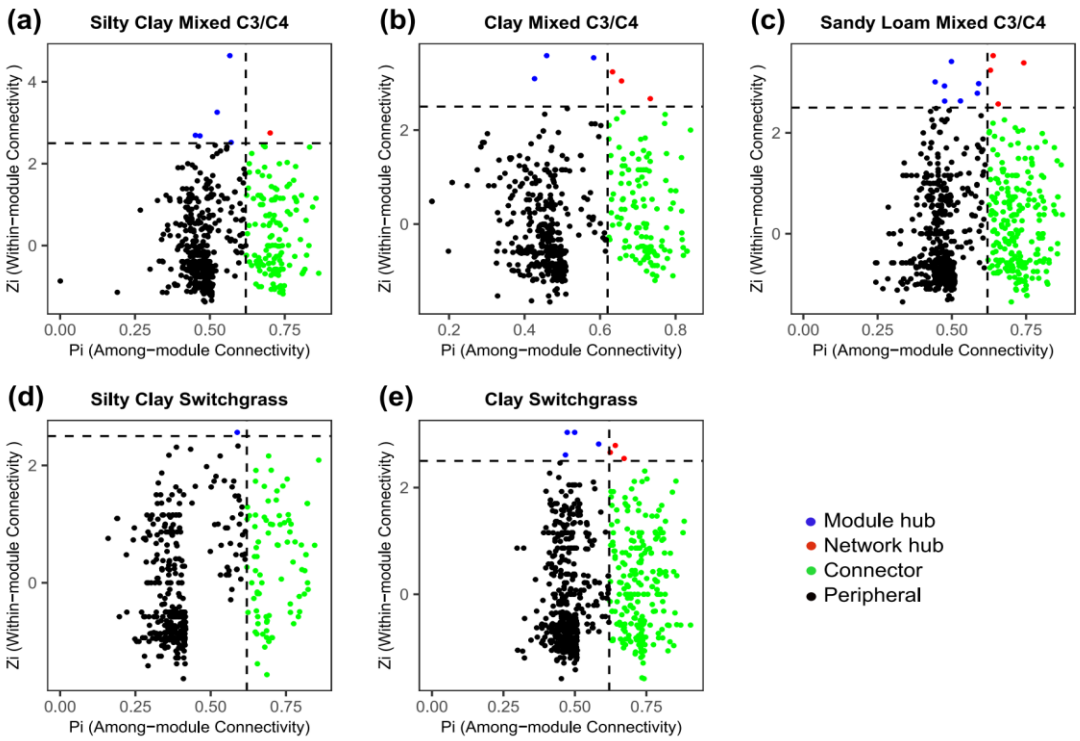

Figure C.S6. Network module analysis with Zi-Pi plot showing the distribution of OTUs based on the within-module connectivity (Zi) and among-module connectivity (Pi) scores across different plant and soil type. OTUs were further classified into either of the four sub-categories as (i) module hubs – ($Pi < 0.62$, $Zi > 2.5$), (ii) network hubs – ($Pi > 0.62$, $Zi > 2.5$), (iii) connectors – ($Pi > 0.62$, $Zi < 2.5$) or (iv) peripheral taxa – ($Pi < 0.62$, $Zi < 2.5$).

Supplementary Tables

Table C.S1. Multivariate statistical analysis illustrating the effect of plant type and CO_2 treatment year within each soil.

	16S rRNA gene (OTUs)				**PICRUSt2** (Predicted functional genes)			
	PERMANOVA		ANOSIM		PERMANOVA		ANOSIM	
	F	*p*	*R*	*p*	*F*	*p*	*R*	*p*
Silty Clay								
Plant type	8.39	0.001**	0.12	0.004**	4.38	0.019**	0.01	0.57
Year (global)	8.05	0.001**	0.23	0.001**	24.05	0.001**	0.37	0.001**
2015 vs 2016	4.96	0.001**			10.08	0.001**		
2015 vs 2017	14.71	0.001**			45.29	0.001**		
2016 vs 2017	3.92	0.001**			15.45	0.001**		
Clay								
Plant Type	9.64	0.001**	0.14	0.001**	13.38	0.001**	0.13	0.003**
Year (global)	4.74	0.001**	0.14	0.001**	11.82	0.001**	0.18	0.001**
2015 vs 2016	3.39	0.001**			8.41	0.001**		
2015 vs 2017	8.20	0.001**			22.11	0.001**		
2016 vs 2017	1.98	0.02*			3.34	0.029**		
Sandy Loam								
Year (global)	4.49	0.001**	0.34	0.001**	14.19	0.001**	0.44	0.001**
2015 vs 2016	2.36	0.001**			4.47	0.003**		
2015 vs 2017	8.37	0.001**			26.80	0.001**		
2016 vs 2017	3.52	0.001**			10.63	0.001**		

*Significant ($\alpha = 0.05$).
**Significant after Bonferroni correction ($\alpha = 0.017$)

Table C.S2. Statistical approaches to test the significance of CO_2 gradient on taxonomic community structure and predicted function using ordisurf GAM.

CO_2 gradient (ordisurf GAM)	**Statistic**	**16S rRNA gene (OTUs)**	**PICRUSt2 (predicted genes)**
Silty Clay mixed C_3/C_4	*Adj. R^2*	0.52	0.20
	p	<0.001**	<0.001**
Clay mixed C_3/C_4	*Adj. R^2*	0.03	0.07
	p	0.22	0.10
Sandy Loam mixed C_3/C_4	*Adj. R^2*	0.41	0.30
	p	<0.001**	<0.001**

*Significant ($\alpha = 0.05$).

**Significant after Bonferroni correction ($\alpha = 0.017$)

Table C.S3. Relative proportion (%) of selection and dispersal-based processes in overall community assembly.

	Homogeneous Selection	Homogenizing Dispersal	Undominated Processes	Dispersal limitation	Variable Selection
Silty Clay mixed C_3/C_4	0.82	8.67	16.33	35.73	38.45
Clay mixed C_3/C_4	20.42	6.01	18.51	47.27	7.79
Sandy Loam mixed C_3/C_4	0.07	1.45	10.89	46.59	41
Silty Clay switchgrass	3.17	13.23	27.78	30.16	25.66
Clay switchgrass	0.76	26.89	26.33	30.49	15.53

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