ABSTRACT

QIU, YUNPENG. Soil Microbial Responses to Climate Change Factors: Impacts on Soil N_2O Emission and Organic Carbon Decomposition (Under the direction of Shuijin Hu and H. David Shew).

The ongoing climate change resulting from human activities can significantly influences the structure and functions of terrestrial ecosystems. However, our capacity to predict the impacts of climate change on ecosystem processes is critically hampered by our limited knowledge of microbial responses to climate change factors. Climate change factors such as elevated atmospheric carbon dioxide (CO_2), ozone (O_3) and temperature can significantly alter plant growth and subsequent carbon (C) allocation belowground for soil microorganisms. However, the underlying mechanisms by which soil microbes respond to and feedback to climate change factors that alter resource availability remain poorly understood. This dissertation addresses two questions: 1) how do soil microbes mediate the impact of climate change factors on N_2O emissions? 2) how do arbuscular mycorrhizal (AM) fungi mediate plant and ecosystem responses to climate change factors? We first conducted a microcosm experiment to examine the impact of plant roots and AMF on N_2O emissions under ambient and elevated CO_2 in the presence of two distinct N forms, ammonium (NH_4^+) and nitrate (NO_3^-). Results obtained show that the form of N inputs dominated the N_2O emissions: N_2O emissions were almost undetectable with NH_4^+ input, but increased significantly with NO_3^- input. This enhanced N_2O emission was related to a change in soil denitrifier community composition. Secondly, we initiated a long-term study in a soybean agroecosystem to investigate how elevated ozone and temperature impact nitrifiers and denitrifiers and thus N_2O emissions. Results obtained demonstrate that warming significantly enhanced soil N_2O and this increased N_2O was mainly due to enhancement of denitrifiers. In

contrast, elevated O_3 had little impact on N_2O emissions because of its contrasting effects on microbes that produce or consume N_2O. Lastly, we conducted a field experiment to assess how simulated warming and elevated O_3 affect organic C decomposition. We show that elevated O_3 and temperature reduced plant biomass, root diameter and root colonization by AMF. However, they increased root length and root fragility, and stimulated residue decomposition. Also, warming and elevated O_3 significantly reduced genus *Glomus*, but increased genus *Paraglomus*. These results suggest that alterations in quantity and quality of organic C allocated belowground may alter the soil C dynamics under future climate change scenarios.

Soil Microbial Responses to Climate Change Factors: Impacts on Soil N_2O Emission and Organic Carbon Decomposition

by
Yunpeng Qiu

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APPROVED BY:

_____	_____
Dr. Shuijin Hu Chair of Advisory Committee	Dr. H. David Shew Co-chair of Advisory Committee
_____	_____
Dr. Kent O. Burkey	Dr. Richard W. Zobel

DEDICATION

To my parents, my sister and my mentor, Mr. Hua Wang over last 11 years!

BIOGRAPHY

Yunpeng Qiu was born on October 5, 1987 and grew up in a China city (Yancheng, Jiangsu Province). He developed his long interest to be a scientist when he was in high school. In 2006, he chose the major bio-medicine and studied in Xinxiang Medical University where he spent four years. After graduation, he made a decision to study ecology in Zhejiang University. There he mainly examined the interactions between predator and prey based on model and experimental method. Then, he received a four-year fellowship from China Scholarship Council with the help of Dr. Shuijin Hu and Dr. David Shew to study in North Carolina State University.

In 2013, he began to work with Dr. Shuijin Hu, a leading scientist in soil ecology and global change research, to pursue his Ph.D. degree. Over last four years, Yunpeng mainly investigated how global climate change factors impact soil nitrous oxide emission and soil carbon decomposition.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 1

Literature review

1.1 Introduction

Over the past two hundred years, human activities (e.g. fossil fuel combustion and land use change) have significantly increased the emissions of carbon dioxide (CO_2), ozone (O_3) and nitrous oxide (N_2O) into the atmosphere. These trace gases are greenhouse gases and a change in their concentration can induce significant changes in climate (Solomon *et al.*, 2007). For example, atmospheric CO_2 concentration has risen from pre-industrial levels of 280 μmol mol^{-1} to currently 400 μmol mol^{-1} and is projected to continue to increase in the next several decades (IPCC, 2013). During this period, global atmospheric ozone concentration has also increased from 38 nmol mol^{-1} to 60 nmol mol^{-1} in 2015 (Brauer *et al.*, 2015; Fuhrer *et al.*, 2016), reaching a level that is already 50% higher than the threshold set for injury to O_3-sensitive plants (Fuhrer *et al.*, 1997). Correspondingly, global surface temperature has increased by around 0.6°C from 1880 to 2012, and is predicted to continue to increase between 1.1 and 6.4°C by the year 2100 (Singh *et al.*, 2010; IPCC, 2013).

Elevated CO_2, O_3 and temperature as well as other global change factors such as nitrogen deposition, biotic invasion and changes in precipitation may significantly alter terrestrial ecosystem structure and function, and constitute a potential threat to humans, animals and plants (Vitousek *et al.*, 1997; Booker *et al.*, 2009; Cheng, 2010). Over the last several decades, ecologists have long focused on exploring how these global climate change factors affect functions and structure of terrestrial ecosystems and most work has been centered on the responses of aboveground to these climate change factors (Melillo *et al.*, 1993; Houghton, 2005; Wittig *et al.*, 2009; Ghannoum *et al.*, 2010). Soil microbes play a

critical role in determining soil physical, chemical and biological characteristics, biogeochemical cycling and terrestrial ecosystem structure and function (Coleman, 2004; Prosser, 2015) and microbial processes have long been key drivers of, and responders to, climate change (Singh *et al.,* 2010). However, our limited understanding on belowground responses, especially soil microbes and microbially-mediated processes, hampers our effort for predicting future ecosystem processes (Hu *et al.,* 1999; Bardgett *et al.,* 2008; Rousk *et al.,* 2012; Cheng *et al.,* 2012).

This chapter (chapter I) provides an overview of current knowledge of global change effects on soil microbes with the emphasis on the impacts of elevated atmospheric CO_2, O_3 and temperature.

1.2 Soil microbial responses to global climate change factors: current knowledge

1.2.1 Microbial responses to elevated atmospheric CO_2

The rising CO_2 in the atmosphere generally enhances the photosynthetic activities of C_3 plants and thus stimulates plant growth, net primary production (NPP) and net ecosystem production (NEP) (Drake *et al.,* 1997; Ainsworth & Long, 2005; Reich *et al.,* 2006; Chapin *et al.,* 2011). Consequently, elevated CO_2 often increases C inputs belowground through enhancing rhizodeposition or litter inputs (Hungate *et al.,* 1997; Hu *et al.,* 1999; van Groenigen *et al.,* 2011). Elevated CO_2 can enhance or suppress microbial activities depending on soil resource availability (Hu *et al.,* 2006; Norby & Zak, 2011). Therefore, the resulting changes in microbial biomass, activities and community composition may alter N availability

for plants and alter long-term ecosystem processes under future CO_2 scenarios (Hungate *et al.,* 1997b; Zak *et al.,* 2000).

Microbial biomass and activities

As soil microbes are generally C-limited, an increase in C input belowground may increase soil microbial biomass and activities under elevated CO_2 (Hu *et al.,* 1999; Zak *et al.,* 2000; Reich *et al.,* 2006). Many studies have been conducted to examine CO_2 effects on soil microbial biomass and activities over the past several decades (Hungate *et al.,* 1997a; Hu *et al.,* 2001; de Graaff *et al.,* 2006; Cheng *et al.,* 2012). eCO_2 has been shown to enhance, reduce or has no effect on microbial biomass (Hungate *et al.,* 1996; *Hu et al.,* 2001; Hu *et al.,* 2005; Blankinship *et al.,* 2011). Over the past two decades, several excellent reviews have summarized the effects of CO_2 on microbial biomass (Oneill, 1994; Hu *et al.,* 1999; Zak *et al.,* 2000; Hu *et al.,* 2006; Sillen & Dieleman, 2010; García-Palacios *et al.,* 2014). For example, in a synthesis, Hu *et al.* (2006) found that elevated CO_2 caused an average increase by 26% in MBC and 24% in MBN among 19 of 40 studies and 12 of 27 studies, respectively. The remaining studies showed unchanged or even decreased in microbial biomass. Sillen and Dieleman (2012) recently reviewed 77 published studies and found a slightly higher increase in microbial biomass in the combined CO_2 and N fertilization treatment compared with the single CO_2 enrichment treatment. Microbes also need N to be able to accumulate C into their biomass (Kaye & Hart, 1997; Hu *et al.,* 2001). In ecosystems with severe N limitation, microbes use the energy from decomposing easily degradable C-compounds to decompose N-richer compounds, resulting in higher microbial respiration while microbial biomass

remains constant (Sillen & Dieleman, 2012). In another review. Blankinship *et al.* (2011) found that the positive effects of elevated CO_2 on the abundance of soil biota diminished with time, suggesting trophic group, body size, and experimental approaches best explained the responses of soil biota to elevated CO_2

A large number of studies have also been conducted to investigate CO_2 effects on microbial activities, measured as microbial respiration (Zak *et al.,* 1993; Hu *et al.,* 2001; Austin *et al.,* 2009; Bader *et al.,* 2010) and enzymatic activities (Finzi *et al.,* 2006; Chung *et al.,* 2006; Dorodnikov *et al.,* 2009), and often show a CO_2-stimulatory effect. In a meta-analysis of 47 studies evaluating soil microbial respiration under elevated CO_2 (Zak *et al.,* 2000) found that elevated CO_2 generally increased microbial respiration. Another meta-analysis of 30 studies, de Graaff *et al.* (2006) showed that CO_2 enrichment enhanced microbial respiration by approximately 17%. Enzyme production and activity reflects the balance of microbial nutrient availability and specific soil microbial functions (Allison & Vitousek, 2005; Manzoni *et al.,* 2010). Kelley *et al.* (2011) conducted the first meta-analysis of 34 studies examining the responses of soil enzymatic activities to elevated CO_2. They found that the activity of N-acetylglucosaminidase (chitin-degrading enzyme) increased consistently by 12.6% under elevated CO_2. This suggests a transition towards microbes that can produce enzymes responsible for degrading more recalcitrant forms of N with increasing CO_2. In addition, they showed that other enzymes including those degrading starch, cellulose, lignin, xylan/hemicellulose and organic P and S respond more variably to CO_2 due to the wide variety of ecosystems and experimental design (Kelly *et al.,* 2011).

Mycorrhizae and AMF community structure

Mycorrhizal fungi, especially arbuscular mycorrhizal fungi belonging to *Glomeromycota*, form symbiotic associations with roots of ~80% of land plant species and obtain carbon (C) from their hosts (Smith & Read, 2008; Cheng *et al.*, 2012). In exchange, mycorrhizal fungi provide their host plants with mineral nutrients (e.g., phosphorus (P) and nitrogen (N)) and enhance the abiotic and biotic stress resistance of the host (Kiers *et al.*, 2011; Fellbaum *et al.*, 2012). eCO_2 is expected to increase mycorrhizal biomass because plant demands for N and P will increase, and plants will allocate more photosynthates belowground to the mycorrhizal fungi to help satisfy the increased nutrient demand (Fitter *et al.*, 2000; Johnson *et al.*, 2010; Antoninka *et al.*, 2011). Studies, either from microcosm experiments or field experiments across a wide range of biomes, have found that elevated CO_2 often stimulates mycorrhizal fungal growth (Fitter *et al.*, 2000; Treseder & Allen, 2000; Alberton *et al.*, 2005; Cheng *et al.*, 2012). In an earlier meta-analysis, Treseder (2004) reported that elevated CO_2 increased mycorrhizal colonization by 47% including only field studies. Later, Alberton *et al.* (2005) conducted another meta-analysis with more data and examined whether elevated CO_2 differentially affect ectomycorrhizal (ECM) fungi and arbuscular mycorrhizal (AM) fungi. They showed that CO_2 enrichment caused an average increase by 21% and 34% in the abundance of AM fungi and ECM fungi, respectively. In addition, Mohan *et al.* (2014) recently reviewed that the effects of CO_2 enrichment on AM fungal growth and reported both enhanced and reduced root colonization, depending on associated plant species and ecosystems.

Compared with abundances of AM fungi, the responses of AMF community composition to elevated CO_2 has been less studied. AM fungal community composition may shift following CO_2 enrichment. For instance, Treseder *et al.* (2003) observed a significant increase of *Scutellospora* and *Acaulospora*, but a significant decrease of *Glomus* under elevated CO_2, based on data from spore evaluation method. Klironomos *et al.* (2005) demonstrated that the relative abundance of *Gigaspore sp.* was reduced by an abrupt increase of CO_2 concentration, resulting in an immediate decline in fungal richness and a change of mycorrhizal functioning. Cotton *et al.* (2015) employed high-throughput terminal restriction fragment length polymorphism (TRFLP) analysis and revealed a significant change in AM fungal communities with a significant reduction in the abundance of *Gigasporaceae* under elevated CO_2. They suggested that changes in carbon availability are important determinants of the community dynamics of the AM fungi. With the development of next generation sequencing (Öpik *et al.*, 2010; Dumbrell *et al.*, 2011), higher resolution community characterization would help understand how AM fungi will change and how these alterations will feedback to ecosystem processes under future CO_2 scenarios.

Litter decomposition

The CO_2-induced changes in soil microbes can feed back to atmospheric CO_2, mainly through altering soil decomposition processes. Elevated CO_2 may increase the C/N ratio and recalcitrant compounds such as lignins and other phenolic compounds in plant biomass (Booker *et al.*, 2005; Bardgett *et al.*, 2008), thereby reducing soil C losses through suppressing soil microbes. For instance, Hu *et al.* (2001) found that elevated CO_2

significantly increased plant N uptake and reduced soil N availability to soil microbes at the late growing season, leading to reduced microbial activity and suppression of microbial decomposition and ultimately increased ecosystem carbon accumulation. However, Norby *et al.* (2001) reviewed that CO_2 enrichment reduced N concentrations only by 7% in naturally-senesced leaves, much less than that often observed in green leaves (Norby & Cotrufo, 1998; Couteaux *et al.,* 1999). The effects of CO_2 enrichment on carbon loss or respiration rates from litter were not significant compared with litter grown in ambient CO_2. Moreover, Hu *et al.* (2006) summarized that only 4 out of 19 studies showed direct inhibitive effect of elevated CO_2 on decomposition.

In contrast, elevated CO_2 may increase soil C losses through stimulating soil microbes, positively feeding back to the rising atmospheric CO_2 (Zak *et al.,* 1993; van Groenigen *et al.,* 2014). First, elevated CO_2 increases C/N ratio of litter and enhances decomposition due to stimulation of microbial abundance and activity, and enhances mineralization of recent and old soil organic carbon, a phenomenon known as "priming effect" (Dijkstra & Cheng, 2007; Hartley *et al.,* 2012; Cheng *et al.,* 2012). Second, elevated CO_2 could stimulate decomposition of plant-derived C such as root exudation and plant residues (Hungate *et al.,* 1997b; Phillips *et al.,* 2011). Third, studies showed that AMF can enhance plant litter decomposition under elevated CO_2 (Cheng *et al.,* 2012). One proposed mechanism involves stimulation of saprotrophic activity via the release of labile C by AMF, thereby stimulating the decomposition of plant litter (Phillips *et al.,* 2012; Cheng *et al.,* 2012; Carrillo *et al.,* 2016).

Nitrification, denitrification and N_2O emission

Nitrous oxide is the key trace gas that plays a major role in the greenhouse effect and ozone destruction in the stratosphere (Dickinson & Cicerone, 1986; IPCC, 2013). Globally, agricultural soils are a major source of anthropogenic N_2O due to the use of N fertilizer and manure (Mosier *et al.,* 1998; Davidson, 2009; IPCC, 2013). N_2O is mainly produced through two microbial transformations of N (i.e. nitrification and denitrification) (Firestone & Davidson, 1989; Conrad *et al.,* 1996; Wrage *et al.*, 2001). Nitrification is the aerobic oxidation of ammonia to nitrate via nitrite ($NH_3 \rightarrow NH_2OH/HNO \rightarrow NO_2^- \rightarrow NO_3^-$). The first step (i.e. NH_3 to NO_2^-), ammonia oxidation, is performed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Prosser, 1989; Könneke *et al.,* 2005; Brochier-Armanet *et al.,* 2008). The second step (i.e. NO_2^- to NO_3^-) is carried out by nitrite-oxidizing bacteria (NOB) (Freitag *et al.,* 1987; Hu *et al.,* 2015). The first step is believed to be the rate-limiting step for the whole nitrification processes (Kowalchuk & Stephen, 2001). Denitrification is a major microbial process that reduces oxidized mineral forms of N to the gaseous products ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) under oxygen-limited conditions (Philippot *et al.,* 2009). In order to better predict and mitigate N_2O emissions, it is critical to understand the key factors controlling soil N_2O production.

Alterations in C and N availability under elevated CO_2 may significantly influence microbial N transformations and subsequent N losses via leaching and gaseous emissions (Hu *et al.,* 2006). In an early meta-analysis of 11 studies, Barnard *et al.* (2005) found elevated CO_2 decreased nitrifying enzyme activity (NEA). Given that nitrification is

positively related with the soil NH_4^+, it is essential that we gain better insight into the eCO_2-mediated effects on NH_4^+ availability. Cheng *et al.* (2012) conducted a meta-analysis and found that elevated CO_2 reduced soil NH_4^+ by 7.9% indicating a negative effect on nitrification under CO_2 enrichment. Elevated CO_2 has also been found to increase soil water content due to reduced stomatal conductance and enhanced water use efficiency (Hungate *et al.*, 2002; Keenan *et al.* 2013). Therefore, nitrification is inhibited at high water content. In a recent meta-analysis, Liang *et al.* (2016) found that nitrification was only significantly reduced by long-term CO_2 enrichment.

In addition, Barnard *et al.* (2005) showed that CO_2 enrichment significantly decreased denitrifying enzyme activities (DEA) and soil NO_3^- over 23 and 11 studies, respectively. However, denitrification should be favored at high soil water content under elevated CO_2. The observed decreased DEA under elevated CO_2 is not consistent with effects of elevated CO_2 on soil water content. In contrast, Cheng *et al.* (2012) found that elevated CO_2 significantly enhanced soil NO_3^- by 26.7% in their study including more data, which can provide more substrate for denitrification. In addition, Liang *et al.* (2016) reported that elevated CO_2 significantly enhanced denitrification only when N was added.

Van Groenigen *et al.* (2011) conducted a meta-analysis on the effect of CO_2 enrichment on N_2O fluxes and found that CO_2 enrichment stimulated N_2O by 18.8%. There are several possible reasons for this enhanced N_2O emissions. First, elevated CO_2 increased soil water contents due to improved water use efficiency by plants (Wullschleger *et al.*, 2002; Norby & Zak, 2011). Second, soil microbial biomass has been observed to increase under elevated

CO_2 across a broad range of ecosystems (Zak *et al.*, 2000; Pendall *et al.*, 2004). Third, increased CO_2 promoted root biomass and provided more labile carbon for denitrifiers (Weier *et al.*, 1993; Morley & Baggs, 2010).

1.2.2 Microbial responses to elevated atmospheric O_3

Ozone (O_3) at the Earth's surface (troposphere) is a dynamic, short-lived air pollutant and is toxic to human health and plant growth (Mills *et al.*, 2011; Sicard *et al.*, 2016). O_3 is formed mainly as a byproduct from chemical reactions between oxides of nitrogen (NOx) and volatile organic compounds (VOC) in the presence of sunlight (Ashmore, 2005). Ozone interacts with a number of molecules within the cell wall and on the plasma membrane surface, altering plasma membrane function and leading to damage of plant leaves (Fiscus *et al.*, 2005). Therefore, elevated tropospheric O_3 generally suppresses plant photosynthesis and plant growth, reducing agricultural yields (Fuhrer & Booker, 2003; Morgan *et al.*, 2003; Ainsworth *et al.*, 2012). Accumulating evidence indicates that elevated O_3 can cause a substantial decline in plant aboveground growth and subsequent C allocation belowground (Andersen, 2003; Fiscus *et al.*, 2005; Grantz *et al.*, 2006; Booker *et al.*, 2009). The alterations in C availability belowground will potentially impact soil microbes and associated processes.

Microbial biomass and activities

Compared with the CO_2 effect, the effects of elevated O_3 on soil microbial biomass and activities have received relatively less attention. Since soil microbes are generally C-limited,

decreased C availability would be expected to suppress soil microbial growth and activities (Islam *et al.,* 2000; Andersen, 2003; Wu *et al.,* 2016). It has been reported that elevated O_3 significantly reduced soil microbial biomass in a wheat-soybean rotation experiment using open-top chambers (Islam *et al.,* 2000), in a pot experiment using wheat plants (Chen *et al.,* 2009) and in the O_3-FACE (free-air ozone enrichment) experiment of a rice-wheat rotation system (Chen *et al.,* 2015; Wu *et al.,* 2016). However, contrasting results have also been found in other studies (Scagel & Andersen, 1997; Mörsky *et al.,* 2008; Bassin *et al.,* 2015). For example, Mörsky *et al.* (2008) found that elevated O_3 increased microbial biomass in a 4-yr experiment with peatland microcosms. Bassin *et al.* (2015) showed that elevated O_3 enhanced microbial biomass N resulting from increased litter input and lower litter quality. Moreover, soil microbial biomass was observed to remain unchanged in a meadow ecosystem (Kanerva *et al.,* 2006) and a no-till wheat-soybean rotation agro-ecosystem (Cheng *et al.,* 2012).

It is also expected that microbial activities measured as microbial respiration and enzymatic activities would decrease under elevated O_3 because of reduced C allocation belowground (Andersen, 2003). For example, Edwards (1991) found that elevated O_3 reduced root and soil respiration, respectively, due to the decreased root exudation rates and rhizosphere microbial populations. However, contrasting results have also been found in other studies (Coleman *et al.,* 1996; Andersen & Scagel, 1997; Kasurinen *et al.,* 2004). Several other studies have shown that soil microbial respiration remained unaffected at elevated O_3 (Islam *et al.,* 2000; Phillips *et al.,* 2002; Cheng *et al.,* 2012). In an open-top

chamber experiment with birch trees exposed to elevated O_3 for three consecutive growing seasons, Kasurinen *et al.* (2004) showed that elevated O_3 stimulated soil CO_2 efflux possibly due to the stimulated mycorrhizal formation. Moreover, elevated O_3 has been found to reduce cellobiohydrolase and N-acetylglucosaminidase (NAG) activity in soils (Phillips *et al.*, 2002; Chung *et al.*, 2006; Edwards & Zak, 2011). However, it has also been shown that nitrifying and denitrifying enzyme activities increased significantly (Chen *et al.*, 2015) at elevated O_3. Theses alterations indicate soil microbial community structure and function under elevated O_3 may be altered (Li *et al.*, 2013; He *et al.*, 2014). In addition, the activities of many other enzymes such as 1,4- β -xylosidase, phosphatase, 1,4-α-glucosidase, peroxidase, and phenol oxidase were not altered at elevated O_3 (Reddy *et al.*, 1991; Larson *et al.*, 2002; Chung *et al.*, 2006). Therefore, it remains unclear how elevated O_3 will affect soil microbial biomass and activities due to the limited studies.

Mycorrhizae and AMF community structure

Reduced carbon allocation belowground under elevated O_3 has been suggested to alter belowground microbial community functions and structure (Kanerva *et al.*, 2008; He *et al.*, 2014). However, it remains unknown how elevated O_3 would affect mycorrhizal fungal growth and community composition (Cotton *et al.*, 2015). Published studies showed that elevated O_3 can affect mycorrhizal colonization of roots but results are not consistent (Mohan *et al.*, 2014; Wang *et al.*, 2017). For example, it has been observed that the AMF colonization rate on roots decreased significantly in tomato (McCool & Menge, 1983) and *Elymus glaucus* under elevated O_3 (Yoshida *et al.*, 2001). However, Cheng (2010) found elevated O_3 had no

effect on mycorrhizal colonization of different AMF species in *Avena fatua* L. In addition, Cui *et al.* (2013) found that elevated O_3 enhanced root colonization in wheat. These inconsistent results possibly result from the changes in plant carbon allocation belowground can be highly variable under ozone stress (Andersen, 2003) or the different experimental conditions and differnt plant species/genotype sensitivity to ozone (Wang *et al.,* 2014). In a recent meta-analysis by Wang *et al.* (2017), they summarized the results of 20 studies and reported that elevated O_3 generally lead to a substantial decrease in AM colonization rate. Compared to the studies on AM fungal growth, researches on the AM fungal communities under elevated O_3 has received less attention. Understanding the mechanisms by which these alterations of AM fungal communities will be critical for predicting how future global climate change will affect mycorrhizas and, consequently ecosystem functions. Cotton *et al.* (2015) was the first to employ molecular methods to characterize the communities of AM fungi and reported that elevated O_3 did not alter AM fungal community composition possibly due to the young plants they sampled and method sensitivity limitations. Therefore, more efforts are needed to examine how elevated O_3 might impact AM fungi.

Litter decomposition

In addition to reduced plant growth and subsequent decreased C availability (Andersen, 2003), elevated O_3 has also been reported to increase recalcitrant compounds in leaf litter (Loya *et al.,* 2003; Booker *et al.,* 2005; Kasurinen *et al.,* 2006; Meehan *et al.,* 2010). These changes would slow soil microbial decomposition processes, reducing C losses from soil (Kasurinen *et al.,* 2006; Parsons *et al.,* 2008; Cheng, 2010; Berg & sMcClaugherty, 2014).

Compared with elevated CO_2, the research on the effect of O_3 on the quality and decomposition of litter is relatively rare. Loya *et al.* (2003) have demonstrated that the combination of elevated CO_2 and O_3 significantly diminished soil carbon in the Rhinelander FACE (free-air CO_2 and O_3 enrichment) forest experiment. Later work at the Rhinelander FACE, Talhelm *et al.* (2009) showed that what Loya *et al.* (2003) observed was a transient effect and elevated O_3 had no effect on soil carbon after the 11-year experimental fumigation. Yue *et al.* (2015) conducted a meta-analysis over 17 studies and reported that elevated O_3 had no effect on litter decomposition, which possibly was due to the limited studies. However, there have also been studies showing that elevated O_3 increases root turnover and soil respiration indicating enhanced decomposition processes (Andersen & Scagel, 1997). Therefore, more studies need to be conducted to investigate how ozone will affect root morphology and then affect carbon decomposition.

Nitrification, denitrification and N_2O emission

Alterations in the relative soil C and N availability under elevated O_3 may significantly influence microbial N transformations and subsequent N losses via leaching and gaseous emissions (Holmes *et al.,* 2003; Simpson *et al.,* 2014; Chen *et al.,* 2015). Reduced substrate availability for nitrification and denitrification may affect N cycling and therefore N_2O emissions under elevated O_3 (Chen *et al.,* 2015). However, there are limited studies on the effects of elevated O_3 on N cycling (Holmes *et al.,* 2003; Kanerva *et al.,* 2006; Wu *et al.,* 2016). Those studies indicated that elevated O_3 had minor or negative effects on soil N cycling in different ecosystems. For instance, Kanerva *et al.* (2006) found no significant

effects on total N, NO_3^--N, potential nitrification and denitrification and therefore N_2O emission. Holmes *et al.* (2003) examined elevated O_3 effects on gross N transformation rates and found that elevated O_3 reduced gross N mineralization by 13% due to the decreased organic substrate inputs belowground. However, in a Soybean Free Air Concentration Enrichment (SoyFACE) experiment in Illinois after 4 years of O_3 fumigation, Pereira *et al.* (2011) found that elevated O_3 can increase the abundance of *nos*Z gene and thus denitrification driven by the high SOC. Elevated O_3 effects on N cycling can also vary by plant species and cultivars due to different sensitivity to O_3 stress (Wu *et al.,* 2016). For instance, Wu *et al.* (2016) showed that elevated O_3 significantly reduced N cycling in sensitive wheat cultivars but had no effects on tolerant wheat cultivars. Compared with elevated CO_2, our understanding of O_3 effects on soil N dynamics is still limited.

1.2.3 Microbial responses to climate warming

The average global surface temperature is projected to continue to increase by 1.5 to 4°C by the end of 21 century (IPCC, 2013). Accumulating evidence has shown that climate warming can affect the growth of microorganisms, plants and animals, and influence different ecosystems processes ranging from polar terrestrial to topical marine environments (Walther *et al.,* 2002; Root *et al.,* 2003; Deutsch *et al.,* 2008). In contrast with elevated CO_2 and O_3, warming can have both direct and indirect effects on soil microbes that feedback greenhouse gases to the atmosphere and contribute to climate warming (Bardgett *et al.,* 2008; Classen *et al.,* 2015; Mayor *et al.,* 2017). Climate warming generally enhances plant growth and productivity, thereby indirectly altering soil physicochemical conditions, subsequent

carbon allocation belowground and the composition of microbial community (Zhang *et al.*, 2005; Dumbrell *et al.*, 2010; Classen *et al.*, 2015). Climate warming can also directly impact soil microbial growth and activities, leading to changes in ecosystem functions (Blankinship *et al.*, 2011; Manzoni *et al.*, 2012; Hagerty *et al.*, 2014).

Microbial biomass and activities

Over the last several decades, a large number of studies have examined warming effects on soil microbial biomass and activities, as summarized by several excellent reviews (Rustard *et al.*, 2001; Bai *et al.*, 2013; Lu *et al.*, 2013; Zhang *et al.*, 2015; Garcia-Palacios *et al.*, 2015; Romero-Olivares *et al.*, 2017). Soil microbial biomass has been observed to increase under warming (Schindlbacher *et al.*, 2015; Zhang *et al.*, 2016), but has also been shown to remain stable or decrease (Frey *et al.*, 2008; Bradford *et al.*, 2008; Allison *et al.*, 2010; Schindlbacher *et al.*, 2011). In a recent synthesis, Lu *et al.* (2013) found that warming enhanced plant growth and microbial growth and activities resulting in a 4.9% increase in microbial biomass. They also showed that low (<1°C) and medium (1–3°C) warming magnitudes significantly increased MBC but high magnitude (>3°C) decreased MBC indicating the temperature sensitivity of microbes may decline at high temperatures or microbes may become adapt to high temperatures (Luo *et al.*, 2001; Melillo *et al.*, 2002). Zhang *et al.* (2015) synthesized 25 studies on the Tibetan Plateau and found that MBC and MBN significantly increased by 23.1% and 41.5% under climate warming, respectively, in grasslands, but had no significant effect in forests due to the different annual air temperature between the two ecosystems. However, Bai *et al.* (2013) recently conducted another meta-

analysis with 31 observations and found warming had no significant effect on microbial biomass N. Warming may reduce soil labile carbon for microbes to grow, whereas warming may stimulate N mineralization with greater N availability which could increase microbial N (Bradford *et al.,* 2008; Frey *et al.,* 2008; Yin *et al.,* 2012). Similar results were also observed in other three reviews (Wang *et al.,* 2014; García-Palacios *et al.,* 2015; Romero-Olivares *et al.,* 2017).

A positive relationship between soil microbial respiration and temperature has been well illustrated (Lloyd & Taylor, 1994). Many studies have observed that warming can directly stimulate soil microbial respiration (Jenkinson *et al.,* 1991; Davidson & Janssens, 2006; Hartley *et al.,* 2008). In an earlier meta-analysis by Rustad *et al.* (2001), they showed that warming generally enhanced soil respiration across 16 field studies. However, some studies have observed a decline in warming effects over time (Luo *et al.,* 2001; Melillo *et al.,* 2002), whereas others have also documented no significant change (Schindlbacher *et al.,* 2011). Several mechanisms have been proposed to explain this pattern, including the depletion of substrate availability (Bradford *et al.*, 2008), acclimation of individual microbes (Frey *et al.* 2013), evolutionary adaption of microbial populations (Romero-Olivares *et al.,* 2015) and microbial community changes (Zhang *et al.,* 2005; Rousk *et al.,* 2012; Treseder *et al.,* 2016). In the meta-analysis by Lu et al. (2013), they found that warming stimulated soil respiration by 9.0%, with a 7.5% increase in heterotrophic respiration and a 9.4% increase in autotrophic respiration, respectively. Similar results were also found in another meta-analysis by Zhou *et al.* (2016). However, Romero-Olivares *et al.* (2017) performed a meta-analysis based on 25

field experiments lasting more than 10 years and found that warming effects on soil respiration declined significantly with duration of warming. After 10 years, soil respiration under warming treatment declined to the control level.

Mycorrhizae and AMF community structure

The effects of climate warming on mycorrhizal fungi can be direct or, more often, indirect via temperature impacts on plant growth and soil nutrient dynamics. Warming might directly affect AMF via stimulating the growth of hyphae, but can also indirectly affect the fungi by affecting plant photosynthesis and altering carbon allocation belowground (Rillig *et al.,* 2002; Heinemeyer & Fitter, 2004) or changes in soil mineralization rates and hence N availability (Olsrud *et al.,* 2010; Melillo *et al.* 2011). Responses of AMF to increased temperature have been examined in limited studies (Mohan *et al.,* 2014). And most studies found increased temperature had a positive impact on AMF colonization because of the direct effect of temperature (Rillig *et al.,* 2002; Gavito *et al.,* 2003; Heinemeyer & Fitter, 2004; Compant *et al.,* 2010). However, in several studies, warming had either no effect or negative effect on the colonization of AMF in roots (Schroeder-Moreno *et al.,* 2012; Wilson *et al.,* 2016). Warming not only influences the abundances of AMF in plant roots, but also alters the species composition. However, warming manipulation studies also reported that warming has inconsistent results. For instance, warming has been shown to have positive effects on AM fungal richness or neutral effects on AM fungal richness and Shannon diversity index (Yang *et al.,* 2013; Kim *et al.,* 2014; Gao *et al.,* 2015). In addition, it has also been shown warming can have positive, negative, neutral effects on AM fungal extra-radical

hyphal (ERH) density (Rallig *et al.,* 2002; Gavito *et al.,* 2003; Staddon *et al.,* 2003; Yang *et al.,* 2013; Kim *et al.,* 2014; Gao *et al.,* 2015). Meanwhile, warming significantly affected the community composition of AM fungi on the Qinghai-Tibet Plateau in China (Yang *et al.,* 2013). However, most warming manipulation studies found warming had no effect on overall AM fungal community composition (Heinemeyer *et al.,* 2004; Yang *et al.,* 2013; Kim *et al.,* 2014; Gao *et al.,* 2015). AM fungal responses to elevated temperature could have significant consequences for global climate. Nevertheless, it is less clear how alterations in AM fungal community composition in response to warming might influence ecosystem carbon storage and cycling.

Litter decomposition

It was generally accepted that elevated temperature will accelerate rates of heterotrophic microbial activity and therefore enhance CO_2 emissions into the atmosphere (Davidson & Janssens, 2006, Bardgett *et al.,* 2008, Karhu *et al.,* 2014). Because the rates of soil respiration are thought to be more sensitive to temperature than primary production (Jenkinson *et al.,* 1991; Schimel *et al.,* 1994), it is predicted that climate warming will cause a positive feedback on climate change (Cox *et al.,* 2000; Davidson & Janssens, 2006). For example, several excellent meta-analyses have determined that warming generally increases decomposition (Rustad *et al.,* 2001; Lu *et al.,* 2013; García-Palacios *et al.,* 2015; Zhang *et al.,* 2015). Several mechanisms have been proposed to explain this pattern. First, the activities of enzymes involved in decomposition increase with short-term temperature sharply (Davidson & Janssens, 2006; Wallenstein *et al.,* 2009, 2010). The size of enzyme

pool is controlled by microbial production and turnover rate of extracellular enzymes which are vulnerable to temperature (Cusack *et al.,* 2010; Conant *et al.,* 2011). Second, the influence of temperature on microbial physiology has a significant impact on soil carbon dioxide emissions (Allison *et al.,* 2010). Microbial carbon use efficiency (CUE) determines the partitioning of substrate C between microbial biomass and CO_2 production, determining the fate of soil carbon (Manzoni *et al.,* 2012; Frey *et al.,* 2013). Usually CUE declined with increasing temperature in future, thus potentially decreasing C storage (Frey *et al.,* 2013). Third, it has long been thought that recalcitrant carbon pools have greater temperature sensitivity compared with young carbon pools (Knorr *et al.,* 2005; Davidson & Janssens, 2006), although contrasting results have also been shown (Luo *et al.,* 2001; Melillo *et al.,* 2002; Fang *et al.,* 2005; Conen *et al.,* 2006; Rey *et al.,* 2006).

By contrast, it has also been shown that warming can have either a negative or neutral effect on decomposition (Luo *et al.,* 2001; Melillo *et al.,* 2002). For example, high temperatures often increase evapotranspiration and lead to drought, and low soil moisture reduces the thickness of soil water films, thus inhibiting diffusion of extracellular enzymes and soluble organic-C substrates and lowering substrate availability at reaction sites (Davidson & Janssens, 2006; Allison & Treseder, 2008). Physical and chemical protection of soil organic matter can decrease substrate availability for microbial attack, thereby reducing microbial responses to warming (Oades, 1988; Six *et al.,* 2002; Davidson & Janssens, 2006). In addition, the depletion of substrate, acclimation and alterations of soil microbial communities to higher temperature and the effects of other climate change factors may also

help explain the uncertainty (Luo *et al.*, 2001; Bradford *et al.*, 2008; Frey *et al.*, 2013; Taylor *et al.*, 2017). While it is well established that temperature plays a critical role in determining soil organic matter decomposition, the nature of the relationship between temperature and heterotrophic respiration and its potential to feedback to climate change, are far from clear (Davidson & Janssens, 2006; Bardgett *et al.*, 2008).

Nitrification, denitrification and N_2O emission

Studies suggested that the optimum temperature for nitrification process depends on the soil characteristics from different climatic zones (Myers, 1975; Malhi & McGill, 1982; Dalias *et al.*, 2002). Warming climate region soils can support nitrifying microbes with an optimum temperature of 35°C (Stark, 1996; Dalias *et al.*, 2002). However, this optimum temperature will drop to 30°C and even to 20°C in colder regions (Mahendrappa *et al.*, 1966; Malhi & McGill, 1982; Dalias *et al.*, 2002). In two recent meta-analyses, Bai *et al.* (2013) and Zhang *et al.* (2016) found that climate warming significantly enhanced nitrification rates in forest ecosystems, whereas the effect was not significant in grassland/prairie ecosystems. This stronger response of nitrification to warming in forests than grasslands was attributed to the relatively dry condition in grasslands.

Denitrification is an anaerobic process influenced by temperature, oxygen, carbon availability and N substrate (Firestone & Davidson, 1987). Elevated temperature could directly and indirectly impact the process of denitrification. For example, many laboratory soil incubation and field experiments show a positive direct effect of warming on the abundances of denitrifiers, causing an increase in denitrification (Braker *et al.*, 2010;

Cantarel *et al.,* 2012; Wertz *et al.,* 2013; Keil *et al.,* 2015). Warming can also indirectly affect denitrification by changing C, N availability, soil oxygen concentration and soil moisture content (Loiseau & Soussana, 2000; Barnard *et al.,* 2005). For instance, enhanced soil labile carbon under warming could provide more C substrate for denitrifiers (Tscherko *et al.,* 2001). Increased N availability under warming through stimulating N mineralization can also increase potential denitrification (Dijkstra *et al.,* 2012). Moreover, warming effect on soil O_2 concentration through increased soil respiration should favor denitrification (Castaldi, 2000). However, drier soil associated with climate warming could promote nitrification and reduce denitrification due to the changes of soil aeration and O_2 (Smith *et al.,* 2003; Liu *et al.,* 2016).

Dijkstra *et al.* (2012) recently reviewed how N_2O fluxes responded to increased air temperature across different field experiments and found that elevated temperature impacted N_2O emissions inconsistently. There are several reasons for this inconsistent result. First, climate warming has been shown to directly enhance the growth of nitrifiers and denitrifiers, therefore increasing the potential of N_2O production (Cantarel *et al.,* 2012). However, lower soil moisture caused by warming could have the opposing effect on N_2O emission (Bijoor *et al.,* 2008; Liu *et al.,* 2016). Second, enhanced soil microbial activity and N availability through increased N mineralization could increase N_2O emissions under elevated temperature (Dijkstra *et al.,* 2012). But, warmer conditions could also stimulate plant growth and N uptake by plants, thereby reducing N availability for denitrification process (Zak *et al.* 1990; Dijkstra *et al.,* 2012). In addition, there are studies showing that the nitrification and

denitrification to N_2O emissions is sensitive to temperature in both microcosm and field experiments (Maag & Vinther, 1996; Stres *et al.,* 2008). For example, Maag and Vinther (1996) have shown that nitrification associated N_2O fluxes decreases with temperature, but denitrification associated N_2O fluxes increases. Therefore, how N_2O fluxes responded to warming is complex and uncertain and more studies should be conducted under different ecosystems.

1.3 Soil microbial responses to global climate change factors: knowledge gaps

Although there are many studies being conducted to investigate how global change components affect soil microbial responses over the last several decades, uncertainties and challenges remain. One major theme is to understand how those climate change factors interacted with soil biota to affect soil N_2O emissions. Another major theme of current global change research is to understand whether terrestrial ecosystems could act as a C sink under future climate change scenarios. Though we have made much progress in understanding the causes of changes in soil microbes under global change components over the last decade, our knowledge of how soil microbes feedback to global change factors remains poorly understood. Given that the key role microbes played in mediating soil N_2O production and soil organic C decomposition, this knowledge gap critically hampers our ability to predict microbial contributions to future greenhouse gas emissions and associated climate change.

Elevated CO_2 can indirectly alter microbial mediated N_2O-producing processes via plants (Kammann *et al.,* 2008; Wu *et al.,* 2017). CO_2 enrichment generally enhances plant photosynthesis of C_3 plants (Ainsworth & Long, 2005) and often increases plant N uptake

(Hu *et al.*, 2001; Drake *et al.*, 2011). Most studies have shown elevated CO_2 enhanced N_2O emissions (Baggs *et al.*, 2003; Kamman *et al.*, 2008; Wu *et al.*, 2017). In a recent meta-analysis, van Groenigen *et al.* (2011) found that elevated CO_2 significantly stimulated N_2O emissions by 18.8% and attributed this increase to higher denitrification induced by CO_2-enhancement of soil labile carbon and plant water use efficiency. However, the origin of the N in the increased N_2O was not explained. Wu *et al.* (2017) first conducted a microcosm experiment with C_3 plants to examine whether the form of N critically mediate the effect of eCO_2 on N_2O production. We found that C_3 plants under elevated CO_2 may preferentially take up NH_4^+ over NO_3^- from soil, thereby enhancing N_2O emissions. However, we still do not know how elevated CO_2 will interact with soil biota, especially arbuscular mycorrhizal fungi (AMF), to affect N_2O emissions. In contrast with elevated CO_2, studies of eO_3-effects on N_2O emissions are much scarcer. Although there have been studies reported that elevated O_3 had either negative or neutral effects on N_2O fluxes (Kanerva *et al.*, 2006; Bhatia *et al.*, 2011), the mechanisms underlying O_3-mediated N_2O production remained unknown. In addition, warming has been observed to impact N_2O fluxes variably, showing a positive, negative and a neutral effect, as summarized in a recent review (Dijkstra *et al.*, 2012). Climate warming can affect multiple processes which produce opposing effects on N_2O production (Bijoor *et al.*, 2008; Dijkstra *et al.*, 2012; Liu *et al.*, 2016). Therefore, how these climate change factors affect soil N_2O production is much more complex and highly uncertain and more experiments need to be conducted to examine soil N_2O fluxes under simulated climate change conditions.

To date, most studies have emphasized on the influences of elevated CO_2, O_3 and temperature on changes in the whole soil microbial community. Few studies have ever been conducted to investigate the responses of functional groups of soil microbes that dominated the process of N_2O formation (nitrification and denitrification) (Horz *et al.,* 2004; Cantarel *et al.,* 2012; Hu *et al.,* 2016). Variations in greenhouse gas N_2O production may reflect differences in terms of the abundances and community composition of AOA (ammonium oxidizing archaea), AOB (ammonium oxidizing bacteria) and denitrifiers (Avrahami & Bohannan, 2009; Philippot *et al.*, 2010; Brown *et al.,* 2011; Cantarel *et al.*, 2012). To my knowledge, the majority of current work has centered on the effect of a single global change factor, little information is available on the causes and consequences of the impacts of multiple global change factors on soil microbes, particularly in agro-ecosystems.

AM fungi, which form symbiotic associations roots of approximately 80% of land plant species, receive a large amount of plant photosynthates (up to 20%) and deposit slowly cycling organic compounds such as chitin and glomalin (Smith & Read, 2008). These AMF-derived compounds are important binding agents for formation of soil microaggregates and AM fungal hyphae directly enmesh microaggregates into macroaggregates, protecting organic matter from microbial attack (Miller *et al.,* 1995; Wilson *et al.,* 2009). However, the magnitude of C inputs through AM hyphae and their exudates, and their rapid turnover (Staddon *et al.,* 2003a) represent a major source of labile C inputs which can serve as substrates for saprophytes. Consequently, AMF proliferation around organic residue patches likely enhances decomposition through the priming effect and increases plant N acquisition

by competing with other microbes for limited nutrients (Hodge *et al.,* 2001; Tu *et al.,* 2006; Cheng *et al.,* 2012). Emerging evidence has shown that elevated CO_2, O_3 and temperature can affect AM fungi variably (Mohan *et al.,* 2014). It remains to be examined whether and how AM fungi impact organic C decomposition under future CO_2, O_3 and temperature scenarios.

In addition, understanding the ecology of soil microbes is challenging owing to the high degree of biological diversity and the inherent temporal and spatial variation that is present at scales <1 mm in soils (Torsvik & Øvreås, 2004; Roesch *et al.,* 2007; Prosser, 2015). Our limited understanding of the mechanisms that regulate the diversity of functionally important taxa may be attributed to the lack of modern technology application (Daniel, 2005; Jones *et al.,* 2009; McGuire & Treseder, 2010). With the development of molecular techniques, improved DNA-based identification methods have allowed the characterization of soil microbial community composition in different ecosystems (Angel *et al.,* 2010; Dumbrell *et al.,* 2010; He *et al.,* 2013; Leff *et al.,* 2015). Therefore, a better understanding of complex microbial functions and structure would help predict ecosystem functioning under future climate change scenarios.

1.4 References

Ågren GI, Wetterstedt JÅM (2007) What determines the temperature response of soil organic matter decomposition? *Soil Biology and Biochemistry,* 39, 1794–1798.

Ainsworth E, Long S (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytologist,* 165, 351–371.

Alberton O, Kuyper TW, Gorissen A (2005) Taking mycocentrism seriously: mycorrhizal fungal and plant responses to elevated CO_2. *New Phytologist,* 167, 859–868.

Allison SD, Treseder KK (2008) Warming and drying suppress microbial activity and carbon cycling in boreal forest soils. *Global Change Biology,* 14, 2898–2909.

Allison SD, Vitousek PM (2005) Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biology and Biochemistry,* 37, 937–944.

Allison SD, Wallenstein MD, Bradford MA (2010) Soil-carbon response to warming dependent on microbial physiology. *Nature Geoscience,* 3, 336–340.

Andersen CP (2003) Source–sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytologist,* 157, 213–228.

Andersen CP, Scagel CF (1997) Nutrient availability alters belowground respiration of ozone-exposed ponderosa pine. *Tree Physiology,* 17, 377–387.

Angel R, Soares MIM, Ungar ED, Gillor O (2010) Biogeography of soil Archaea and bacteria along a steep precipitation gradient. *The ISME Journal,* 4, 553–563.

Antoninka A, Reich PB, Johnson NC (2011) Seven years of carbon dioxide enrichment, nitrogen fertilization and plant diversity influence arbuscular mycorrhizal fungi in a grassland ecosystem. *New Phytologist,* 192, 200–214.

Ashmore MR (2005) Assessing the future global impacts of ozone on vegetation. *Plant, Cell and Environment,* 28, 949–964.

Austin EE, Castro HF, Sides KE, Schadt CW, Classen AT (2009) Assessment of 10 years of CO_2 fumigation on soil microbial communities and function in a sweetgum plantation. *Soil Biology and Biochemistry,* 41, 514–520.

Avrahami S, Bohannan BJM (2009) N_2O emission rates in a California meadow soil are influenced by fertilizer level, soil moisture and the community structure of ammonia-oxidizing bacteria. *Global Change Biology,* 15, 643–655.

Bader M, Körner C (2010) No overall stimulation of soil respiration under mature deciduous forest trees after 7 years of CO_2 enrichment. *Global Change Biology,* 16, 2830–2843.

Baggs EM, Richter M, Hartwig UA *et al*. (2003) Nitrous oxide emissions from grass swards during the eighth year of elevated atmospheric pCO_2 (FACE). *Global Change Biology,* 9, 1214–1222.

Bai E, Li S, Xu W, Li W, Dai W, Jiang P (2013) A meta-analysis of experimental warming effects on terrestrial nitrogen pools and dynamics. *New Phytologist,* 199, 441–451.

Bardgett RD, Freeman C, Ostle NJ (2008) Microbial contributions to climate change through carbon cycle feedbacks. *ISME Joural,* 2, 805–814.

Bardgett RD, Mawdsley JL, Edwards S, Hobbs PJ, Rodwell JS, Davies WJ (1999) Plant species and nitrogen effects on soil biological properties of temperate upland grasslands. *Functional Ecolology,* 13, 650–660.

Barnard R, Le Roux X, Hungate BA, Cleland EE, Blankinship JC, Barthes L, Leadley PW (2006) Several components of global change alter nitrifying and denitrifying activities in an annual grassland. *Functional Ecology,* 20, 557–564.

Barnard R, Leadley PW, Hungate BA (2005) Global change, nitrification, and denitrification: a review. *Global Biogeochemical Cycles,* 19, 1–13.

Bassin S, Käch D, Valsangiacomo A, Mayer J, Oberholzer HR, Volk M, Fuhrer J (2015) Elevated ozone and nitrogen deposition affect nitrogen pools of subalpine grassland. *Environmental Pollution,* 201, 67-74.

Berg B, McClaugherty C (2014) Plant Litter: Decomposition, Humus Formation, Carbon Sequestration, 3rd ed., Springer, New York.

Bhatia A, Ghosh A, Kumar V, Tomer R, Singh SD, Pathak H (2011) Effect of elevated tropospheric ozone on methane and nitrous oxide emission from rice soil in north India. *Agriculture, Ecosystems and Environment,* 144, 21–28.

Bijoor NS, Czimczik CI, Pataki DE, Billings SA (2008) Effects of temperature and fertilization on nitrogen cycling and community composition of an urban lawn. *Global Change Biology,* 14, 2119–2131.

Blankinship JC, Niklaus PA, Hungate BA (2011) A meta-analysis of responses of soil biota to global change. *Oecologia,* 165, 553–565.

Booker F, Muntifering R, McGrath M *et al*. (2009) The ozone component of global change: potential effects on agricultural and horticultural plant yield, product quality and interactions with invasive species. *Journal of Integrative Plant Biology,* 51, 337–351.

Booker FL, Prior SA, Torbert HA, Fiscus EL, Pursley WA, Hu SJ (2005) Decomposition of soybean grown under elevated concentrations of CO_2 and O_3. *Global Change Biology,* 11, 685–698.

Bradford MA, Davies CA, Frey SD *et al*. (2008) Thermal adaptation of soil microbial respiration to elevated temperature. *Ecology Letters,* 11, 1316–1327.

Braker G, Schwarz J, Conrad R (2010) Influence of temperature on the composition and activity of denitrifying soil communities. *FEMS Microbial Ecology,* 73, 134–148.

Brauer M, Freedman G, Frostad J *et al.* (2015) Ambient air pollution exposure estimation for the global burden of disease 2013. *Environmental Science & Technology,* 50, 79–88.

Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P (2008) Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nature Reviews Microbiology,* 6, 245–252.

Brown JR, Blankinship JC, Niboyet A *et al.* (2011) Effects of multiple global change treatments on soil N_2O fluxes. *Biogeochemistry,* 109, 85–100.

Cantarel AAM, Bloor JMG, Pommier T, Guillaumaud N, Moirot C, Soussana J-F, Poly F (2012) Four years of experimental climate change modifies the microbial drivers of N_2O fluxes in an upland grassland ecosystem. *Global Change Biology,* 18, 2520–2531.

Caporaso JG, Lauber CL, Walters WA *et al.* (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal,* 6, 1621–1624.

Carrillo Y, Dijkstra FA, LeCain D, Pendall E (2016) Mediation of soil C decomposition by arbuscular mycorrizhal fungi in grass rhizospheres under elevated CO_2. *Biogeochemistry,* 127, 45–55.

Castaldi S (2000) Responses of nitrous oxide, dinitrogen and carbon dioxide production and oxygen consumption to temperature in forest and agricultural light-textured soils determined by model experiment. *Biology and Fertility of Soils,* 32, 67–72.

Carey JC, Tang JW, Templer PH *et al.* (2016) Temperature response of soil respiration largely unaltered with experimental warming. *Proceedings of the National Academy of Sciences of the United States of America,* 113, 13797–13802.

Chapin FS III, Matson PA, Mooney HA (2011) Principles of Terrestrial Ecosystem Ecology. Springer, New York.

Chen Z, Wang XK, Feng ZZ, Xiao Q, Duan XN (2009) Impact of elevated O_3 on soil microbial community function under wheat crop. *Water, Air, & Soil Pollution,* 198, 189–198.

Chen W, Zhang LL, Li XY *et al.* (2015) Elevated ozone increases nitrifying and denitrifying enzyme activities in the rhizosphere of wheat after 5 years of fumigation. *Plant Soil,* 392, 279–288.

Cheng L (2010) Soil microbial responses to climate change factors: Impacts on soil carbon and nitrogen dynamics, PhD thesis.

Cheng L, Booker FL, Burkey KO *et al*. (2011) Soil microbial responses to elevated CO_2 and O_3 in a nitrogen-aggrading agroecosystem. *PLoS One,* 6, e21377.

Cheng L, Booker FL, Tu C *et al*. (2012) Arbuscular Mycorrhizal Fungi Increase Organic Carbon Decomposition Under Elevated CO_2. *Science,* 337, 1084–1087.

Chung HG, Zak DR, Lilleskov EA (2006) Fungal community composition and metabolism under elevated CO_2 and O_3. *Oecologia,* 147, 143-154.

Classen AT, Sundqvist MK, Henning JA *et al.* (2015) Direct and indirect effects of climate change on soil microbial-plant interaction: What lies ahead? *Ecosphere,* 6, 1–21.

Coleman DC, Crossley DA, Hendrix PF (2004) Fundamentals of soil ecology. Second edition. Elsevier, New York, New York, USA.

Coleman MD, Dickson RE, Isebrands JG *et al*. (1996) Root growth and physiology of potted and field-grown trembling aspen exposed to tropospheric ozone. *Tree Physiology,* 16, 145–152.

Compant S, Van Der Heijden MGA, Sessitsch A (2010) Climate change effects on beneficial plant–microorganism interactions. *FEMS Microbiology Ecology*, 73, 197–214.

Conant RT, Ryan MG, Ågren GI *et al*. (2011) Temperature and soil organic matter decomposition rates – synthesis of current knowledge and a way forward. *Global Change Biology*, 17, 3392–3404.

Conrad, R. (1996). Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiological Reviews*, 60, 609–640.

Cotton TEA, Fitter AH, Miller RM, Dumbrell AJ, Helgason T (2015) Fungi in the future: inter-annual variation and effects of atmospheric change on arbuscular mycorrhizal fungal communities. *New Phytologist,* 205, 1598–1607.

Cox PM, Betts RA, Jones CD, Spall SA, Totterdell IJ (2000) Acceleration of global warming due to carbon cycle feedbacks in a coupled climate model. *Nature,* 408, 184–187.

Cui XC, Hu JL, Lin XG, Wang FY, Chen RR, Wang JH, Zhu JG (2013) Arbuscular mycorrhizal fungi alleviate ozone stress on nitrogen nutrition of field wheat. *Journal of Agricultural Science and Technology,* 15, 1043–1052.

Cusack DF, Torn MS, Mcdowell WH, Silver WL (2010) The response of heterotrophic activity and carbon cycling to nitrogen additions and warming in two tropical soils. *Global Change Biology,* 16, 2555–2572.

Dalias P, Anderson JM, Bottner P, Coûteaux MM (2001) Temperature esponses of carbon mineralization in conifer forest soils from different regional climates incubated under standard laboratory conditions. *Global Change Biology,* 7, 181–192.

Dalias P, Anderson JM, Bottner P, Coûteauxa MM (2002) Temperature responses of net nitrogen mineralization and nitrification in conifer forest soils incubated under standard laboratory conditions. *Soil Biology and Biochemistry,* 34, 691–701.

Daniel, R (2005) The metagenomics of soil. *Nature Reviews Microbiology,* 3, 470–478.

Davidson EA (2009) The contribution of manure and fertilizer nitrogen to atmospheric nitrous oxide since 1860. *Nature Geoscience,* 2, 659–662.

Davidson EA, Janssens IA (2006) Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. *Nature,* 440, 165–173.

De Graaff MA, Van Groenigen KJ, Six J, Hungate B, Van Kessel C (2006) Interactions between plant growth and soil nutrient cycling under elevated CO_2: a meta-analysis. *Global Change Biology,* 12, 2077–2091.

Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC, Martin PR (2008) Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences of the United States of America,* 105, 6668–6672.

Dickinson RE, Cicerone RJ (1986) Future global warming from atmospheric trace gases. *Nature,* 319, 09–115.

Dijkstra FA, Cheng W (2007) Interactions between soil and tree roots accelerate long-term soil carbon decomposition. *Ecology Letters,* 10, 1046–1053.

Dijkstra FA, Prior SA, Runion GB, Torbert HA, Tian H, Lu C, Venterea RT (2012) Effects of elevated CO_2 and warming on methane and nitrous oxide fluxes: evidence from field experiments. *Frontiers in Ecology and the Environment,* 10, 520–527.

Dorodnikov M, Blagodastkaya E, Blagodatsky S, Marhan S, Fangmeier A, Kuzyakov Y (2009) Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size. *Global Change Biology,* 15, 1603–1614.

Drake JE, Gallet-Budynek A, Hofmockel KS *et al*. (2011) Increases in the flux of carbon belowground stimulate nitrogen uptake and sustain the long-term enhancement of forest productivity under elevated CO_2. *Ecology Letters,* 14, 349.

Drake BG, GonzalezMeler MA, Long SP (1997) More efficient plants: a consequence of rising atmospheric CO_2? *Annual Review of Plant Physiology and Plant Molecular Biology,* 48, 609–639.

Drake JE, Macdonald CA, Tjoelker MG *et al.* (2016) Short-term carbon cycling responses of a mature eucalypt woodland to gradual stepwise enrichment of atmospheric CO_2 concentration. *Global Change Biology,* 22, 380–390.

Dumbrell AJ, Ashton PD, Aziz N *et al.* (2011) Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytologist,* 190, 794–804.

Edwards NT (1991) Root and soil respiration responses to ozone in *Pinus taeda* L. seedlings. *New Phytologist,* 118, 315–321.

Edwards IP, Zak DR (2011) Fungal community composition and function after long-term exposure of northern forests to elevated atmospheric CO_2 and tropospheric O_3. *Global Change Biology,* 17, 2184–2195.

Esperschütz J, Pritsch K, Gattinger A *et al.* (2009) Influence of chronic ozone stress on carbon translocation pattern into rhizosphere microbial communities of beech trees (*Fagus sylvatica* L.) during a growing season. *Plant Soil,* 323, 85–95.

Fang CM, Smith P, Moncrieff JB, Smith JU (2005) Similar response of labile and resistant soil organic matter pools to changes in temperature. *Nature,* 433, 57–59.

Fellbaum CR, Gachomo EW, Beesetty Y *et al.* (2012) Carbon availability triggers fungal nitrogen uptake and transport in the arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences of the United States of America,* 109, 2666–2671.

Finzi AC, Sinsabaugh RL, Long TM, Osgood MP (2006) Microbial community responses to atmospheric carbon dioxide enrichment in a warm-temperate forest. *Ecosystems,* 9, 215–226

Fiscus EL, Booker FL, Burkey KO (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant, Cell and Environment,* 28, 997–1011.

Firestone MK, Davidson EA (1989) Microbial basis of NO and N2O production and consumption in soils. In: *Exchange of trace gases between terrestrial ecosystems and the atmosphere* (eds Andreae MO, Schimel DS), pp. 7-21. John Wiley & Sons, New York.

Fitter AH, Heinemeyer A, Staddon PL (2000) The impact of elevated CO_2 and global climate change on arbuscular mycorrhizas: a mycocentric approach. *New Phytologist,* 147, 179–187.

Freitag A, Rudert M, Bock E (1987) Growth of nitrobacter by dissimilatoric nitrate reduction. *FEMS Microbiology Letters,* 48, 105–109.

Frey SD, Drijber R, Smith H, Melillo J (2008) Microbial biomass, functional capacity, and community structure after 12 years of soil warming. *Soil Biology & Biochemistry,* 40, 2904–2907.

Frey SD, Lee J, Melillo JM, Six J (2013) The temperature response of soil microbial efficiency and its feedback to climate. *Nature Climate Change,* 3, 395–398.

Fuhrer J, Booker F (2003) Ecological issues related to ozone: agricultural issues. *Environment International,* 29, 141–154.

Fuhrer J, Skarby L, Ashmore MR (1997) Critical levels for ozone effects on vegetation in Europe. *Environmental Pollution,* 97, 91–106.

Fuhrer J, Val Martin M, Mills G *et al.* (2016) Current and future ozone risks to global terrestrial biodiversity and ecosystem processes. *Ecology Evolution,* 6, 8785–8799.

Gao YC, Kim Y, Zheng W et al. (2016) Increased precipitation, rather than warming exerts strong influence on arbuscular mycorrhizal fungal community in a semiarid steppe ecosystem. *Botany,* 94, 459–469.

García-Palacios P, Vandegehuchte ML, Ashley Shaw E *et al.* (2014) Are there links between responses of soil microbes and ecosystem functioning to elevated CO2, N deposition and warming? A global perspective. *Global Change Biology,* 21, 1590–1600

Gavito ME, Schweiger P, Jakobsen I (2003) P uptake by arbuscular mycorrhizal hyphae: effect of soil temperature and atmospheric CO_2 enrichment. *Global Change Biology,* 9, 106–116.

Ghannoum O, Phillips NG, Conroy JP *et al.* 2010. Exposure to preindustrial, current and future atmospheric CO2 and temperature differentially affects growth and photosynthesis in eucalyptus. *Global Change Biology,* 16, 303–319.

Grantz DA, Gunn S, Vu HB (2006) O_3 impacts on plant development: a meta-analysis of root/shoot allocation and growth. *Plant, Cell and Environment,* 29, 1193–1209.

Hartley IP, Garnett MH, Sommerkorn M *et al.* (2012) A potential loss of carbon associated with greater plant growth in the European Arctic. *Nature Climate Change,* 2, 875–879.

Hartley IP, Hopkins DW, Garnett MH, Sommerkorn M, Wookey PA (2008) Soil microbial respiration in arctic soil does not acclimate to temperature. *Ecology Letters*, 11, 1092–1100.

Hagerty SB, Van Groenigen KJ, Allison SD *et al*. (2014) Accelerated microbial turnover but constant growth efficiency with warming in soil. *Nature Climate Change*, 4, 903–906.

Hawkes CV, Hartley IP, Ineson P, Fitter AH (2008) Soil temperature affects carbon allocation within arbuscular mycorrhizal networks and carbon transport from plant to fungus. *Global Change Biology*, 14, 1181–1190.

He Z, Xiong JB, Kent AD *et al*. (2014) Distinct responses of soil microbial communities to elevated CO_2 and O_3 in a soybean agro-ecosystem. *The ISME Journal*, 8, 714–726.

Heinemeyer A, Fitter AH (2004) Impact of temperature on the arbuscular mycorrhizal (AM) symbiosis: growth responses of the host plant and its AM fungal partner. *Journal of Experimental Botany*, 55, 525–534.

Heinemeyer A, Ridgway KP, Edwards EJ, Benham DG, Young JPW, c AH (2003) Impact of soil warming and shading on colonization and community structure of arbuscular mycorrhizal fungi in roots of a native grassland community. *Global Change Biology*, 10, 52–64.

Hodge A, Campbell CD, Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature*, 413, 297–299.

Horz HP, Barbrook A, Field CB, Bohannan BJM (2004) Ammonia oxidizing bacteria respond to multifactorial global change. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 15136–15141.

Houghton RA (2005) Aboveground forest biomass and the global carbon balance. *Global Change biology*, 11, 945–958.

Hu HW, Macdonald CA, Trivedi P *et al.* (2016) Effects of climate warming and elevated CO_2 on autotrophic nitrification and nitrifiers in dryland ecosystems. *Soil Biology & Biochemistry*, 92, 1–15.

Hu S, Chapin FS III, Firestone MK (2001) Nitrogen limitation of microbial decomposition in a grassland under elevated CO_2. *Nature*, 409, 188–191.

Hu S, Firestone MK, Chapin FS (1999) Soil microbial feedbacks to atmospheric CO_2 enrichment. *Trends in Ecology and Evolution*, 14, 433–437.

Hu S, Tu C, Chen X, Gruver JB (2006). Progressive N limitation of plant response to elevated CO2: a microbiological perspective. *Plant Soil,* 289, 47-58.

Hu S, Wu J, Burkey KO, Firestone MK (2005) Plant and microbial N acquisition under elevated atmospheric CO_2 in two microcosm experiments with annual grasses. *Global Change Biology*, 11, 223–233.

Hungate BA, Canadell J, Chapin FS (1996) Plant species mediate changes in soil microbial N in response to elevated CO_2. *Ecology,* 77, 2505–2515.

Hungate BA, Chapin FS, Zhong H, Holland EA, Field CB (1997a). Stimulation of grassland nitrogen cycling under carbon dioxide enrichment. *Oecologia,* 109, 149–153.

Hungate BA, Holland EA, Jackson RB, Chapin FS, Mooney HA, Field CB (1997b). The fate of carbon in grasslands under carbon dioxide enrichment. *Nature,* 388, 576–579.

Hungate BA, Reichstein M, Dijkstra P *et al*. (2002) Evapotranspiration and soil water content in a scrub-oak woodland under carbon dioxide enrichment. *Global Change Biology*, 8, 289–298.

Intergovernmental Panel on Climate Change (IPCC) (2013) *Climate Change 2013: The Physical Science Basis.* Contribution of Working Group I to the Fifth Assessment Report of the Intergovern–mental Panel on Climate Change. Cambridge University Press, Cambridge, UK.

Islam KR, Mulchi CL, Ali AA (2000) Interactions of tropospheric CO_2 and O_3 enrichments and moisture variations on microbial biomass and respiration in soil. *Global Change Biology*, 6, 255–265.

Jenkinson DS, Adams DE, Wild A (1991) Model estimates of CO2 emissions from soil in response to global warming. *Nature*, 351, 304–306.

Johnson NC, Angelard C, Sanders IR, Kiers ET (2013) Predicting community and ecosystem outcomes of mycorrhizal responses to global change. *Ecology Letters,* 16, 140–153.

Jones RT, Robeson MS, Lauber CL, Hamady M, Knight R, Fierer N (2009) A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME Journal,* 3, 442–453.

Kammann C, Müller C, Grünhage L, Jäger H-J (2008) Elevated CO_2 stimulates N_2O emissions in permanent grassland. *Soil Biology and Biochemistry,* 40, 2194–2205.

Kanerva T, Palojarvi A, Ramo K, Ojanpera K, Esala M, Manninen S (2006) A 3-year exposure to CO_2 and O_3 induced minor changes in soil N cycling in a meadow ecosystem. *Plant and Soil,* 286, 61–73.

Kanerva T, Palojärvi A, Rämöa K, Manninen S (2008) Changes in soil microbial community structure under elevated tropospheric O_3 and CO_2. *Soil Biology & Biochemistry,* 40, 2502–2510.

Karhu K, Auffret MD, Dungait JAJ *et al*. (2014) Temperature sensitivity of soil respiration rates enhanced by microbial community response. *Nature,* 513, 81–84.

Kasurinen A, Kokko-Gonzales P, Riikonen J, Vapaavuori E, Holopainen T (2004) Soil CO_2 efflux of two silver birch clones exposed to elevated CO_2 and O_3 levels during three growing seasons. *Global Change Biology,* 10, 1654–1665.

Kaye JP, Hart SC (1997) Competition for nitrogen between plants and soil microorganisms. *Trends in Ecology and Evolution,* 12, 139–143.

Keenan TF, Hollinger DY, Bohrer G, Dragoni D, Munger JW, Schmid HP, Richardson AD (2013) Increase in forest water-use efficiency as atmospheric carbon dioxide concentrations rise. *Nature,* 499, 324–327.

Keil D, Niklaus PA, von Riedmatten LR *et al.* (2015) Effects of warming and drought on potential N_2O emissions and denitrifying bacteria abundance in grasslands with different land-use. *FEMS Microbiology Ecology,* 91: fiv066

Kelley AM, Fay PA, Polley HW, Gill RA, Jackson RB (2011) Atmospheric CO_2 and soil extracellular enzyme activity: a meta-analysis and CO_2 gradient experiment. *Ecosphere,* 2, art96.

Kiers ET, Duhamel M, Beesetty Y *et al*. (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science,* 333, 880–882.

Kim YC, Gao C, Zheng Y *et al.* (2014) Arbuscular mycorrhizal fungal community response to warming and nitrogen addition in a semi-arid steppe ecosystem. *Mycorrhiza,* 25, 267–276.

Klironomos JN, Allen MF, Rillig MC *et al*. (2005) Abrupt rise in atmospheric CO_2 overestimates community response in a model plant-soil system. *Nature,* 433, 621–624.

Knorr W, Prentice IC, House JI, Holland EA (2005) Long term sensitivity of soil carbon turnover to warming. *Nature,* 433, 298–301.

Kowalchuk GA, Stephen JR (2001) Ammonia-oxidising bacteria: a model for molecular microbial ecology. *Annual Review of Microbiology,* 55, 485–529.

Könneke M, Bernhard AE, José R, Walker CB, Waterbury JB, Stahl DA (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature,* 437, 543–546.

Larson JL, Zak DR, Sinsabaugh RL (2002) Extracellular enzyme activity beneath temperate trees growing under elevated carbon dioxide and ozone. *Soil Science Society of America Journal,* 66, 848–1856.

Leff JW, Jones SE, Prober SM *et al*. (2015) Consistent responses of soil microbial communities to elevated nutrient inputs in grasslands across the globe. *Proceedings of the National Academy of Sciences of the United States of America,* 112, 10967–10972.

Li XY, Deng Y, Li Q *et al.* (2013) Shifts of functional gene representation in wheat rhizosphere microbial communities under elevated ozone. *The ISME Journal,* 7, 660–671.

Liang J, Qi X, Souza L, Luo Y (2016) Processes regulating progressive nitrogen limitation under elevated carbon dioxide: a meta-analysis, *Biogeosciences,* 13, 2689–2699,

Liu L, Hu C, Yang P, Ju Z, Olesen JE, Tang J (2016) Experimental warming-driven soil drying reduced N2O emissions from fertilized crop rotations of winter wheat–soybean/fallow, 2009–2014. *Agriculture, Ecosystystem & Environment,* 219, 71–82

Lloyd J, Taylor JA (1994) On the temperature dependence of soil respiration. *Functional Ecology,* 8, 315–323.

Loiseau P, Soussana JF (2000), Effects of elevated CO_2, temperature and N fertilization on nitrogen fluxes in a temperate grassland ecosystem. *Global Change Biology,* 6, 953–965.

Loya W, Pregitzer KS, Karberg NJ *et al*. (2003) Reduction of soil carbon formation by tropospheric ozone under increased carbon dioxide levels. *Nature,* 425, 705–707.

Lu M, Zhou X, Yang Q *et al.* (2013) Responses of ecosystem carbon cycle to experimental warming: a meta-analysis. *Ecology,* 94, 726–738.

Luo Y, Wan S, Hui D, Wallace L (2001) Acclimation of soil respiration to warming in a tall grass prairie. *Nature,* 613, 622–625.

Maag M, Vinther FP (1996) Nitrous oxide emission by nitrification and denitrification in different soil types and at different soil moisture contents and temperatures. *Applied Soil Ecology,* 4, 5–14.

Mahendrappa MK, Smith RL, Christiansen AT (1966) Nitrifying organisms affected by climatic region in Western United States. *Soil Science Society of America Journal,* 30, 60–62.

Malhi SS, McGill WB (1982) Nitrification in three Alberta soils: effect of temperature, moisture and substrate concentration. *Soil Biology & Biochemistry*, 14, 393–399.

Manzoni S, Taylor P, Richter A, Porporato A, Agren GI (2012) Environmental and stoichiometric controls on microbial carbon-use efficiency in soils. *New Phytologist*, 196, 79–91.

Mayor JR, Sanders NJ, Classen AT *et al.* (2017) Elevation alters ecosystem properties across temperate treelines globally. *Nature*, 542, 91–95.

McCool P, Menge J (1983) Influence of ozone on carbon partitioning in tomato: potential role of carbon flow in regulation of the mycorrhizal symbiosis under conditions of stress. *New Phytologist, 94*, 241–247.

McGuire KL, Treseder KK (2010) Microbial communities and their relevance for ecosystem models: decomposition as a case study. *Soil Biology & Biochemistry*, 42, 529–535.

Meehan TD, Crossley MS, Lindroth RL (2010) Impacts of elevated CO_2 and O_3 on aspen leaf litter chemistry and earthworm and springtail productivity. *Soil Biology & Biochemistry,* 42, 1132–1137.

Melillo JM, Butler S, Johnson J *et al*. (2011) Soil warming, carbon-nitrogen interactions, and forest carbon budgets. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 9508–9512.

Melillo JM, McGuire AD, Kicklighter DW, Moore B, Vorosmarty CJ, Schloss AL (1993). Global climate change and terrestrial net primary production. *Nature*, 363, 234–240.

Melillo JM, Steudler PA, Aber JD et al. (2002) Soil warming and carbon-cycle feedbacks to the climate system. *Science,* 298, 2173–2176.

Myers RJK (1975) Temperature effects on ammonification and nitrification in a tropical soil. *Soil Biology & Biochemistry*, 7, 83–86.

Miller RM, Reinhardt DR, Jastrow JD (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. *Oecologia*, 103, 17–23.

Mills G, Hayes F, Simpson D *et al*. (2011) Evidence of widespread effects of ozone on crops and (semi-) natural vegetation in Europe (1990–2006) in relation to AOT40 – and flux-based risk maps. *Global Change Biology*, 17, 592–613.

Mohan JE, Cowden CC, Baas P *et al*. (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: mini-review. *Fungal Ecology*, 10, 3–19.

Mosier AR, Delgado JA, Keller M (1998) Methane and nitrous oxide fluxes in an acid Oxisol in western Puerto Rico: effects of tillage, liming and fertilization. *Soil Biology & Biochemistry,* 30, 2087–2098.

Morgan PB, Ainsworth EA, Long SP (2003) How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. *Plant, Cell and Environment,* 26, 1317–1328.

Morley N, Baggs EM (2010) Carbon and oxygen controls on N_2O and N_2 production during nitrate reduction. *Soil Biology and Biochemistry,* 42, 1864–1871.

Mörsky SK, Haapala JK, Rinnan R *et al*. (2008) Long-term ozone effects on vegetation, microbial community and methane dynamics of boreal peatland microcosms in open-field conditions. *Global Change Biology,* 14, 1891–1903.

Myers RJK (1975) Temperature effects on ammonification and nitrification in a tropical soil. *Soil Biology & Biochemistry,* 7, 83–86.

Norby RJ, Cotrufo MF (1998) A question of litter quality. *Nature,* 396, 17–18.

Norby RJ, Cotrufo MF, Ineson P *et al*. (2001) Elevated CO_2, litter chemistry, and decomposition: a synthesis. *Oecologia,* 127, 153–165.

Norby R, Zak DR (2011) Ecological lessons from Free-Air CO2 Enrichment (FACE) experiments. *Annual Review of Ecology, Evolution, and Systematics,* 42, 181–203.

Oades JM (1988) The retention of organic matter in soils. Biogeochemistry 5, 35–70.

Olsrud M, Carlsson BA, Svensson BM, Michelsen A, Melillo JM (2010) Responses of fungal root colonization, plant cover and leaf nutrients to long-term exposure to elevated atmospheric CO2 and warming in a subartic birch forest understory. *Global Change Biology,* 16, 1820–1829.

Oneill EG (1994) Response of soil biota to elevated atmospheric carbon-dioxide. *Plant Soil,* 165, 55-65.

Öpik M, Vanatoa A, Vanatoa *et al.* (2010) The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist,* 188, 223–241.

Parsons WFJ, Bockheim JG, Lindoroth RL (2008) Independent, interactive, and species-specific responses of leaf litter decomposition to elevated CO_2 and O_3 in a northern hardwood forest. *Ecosystems,* 11, 505–519.

Pendall E, Bridgham S, Hanson PJ et al. (2004) Below-ground process responses to elevated CO_2 and temperature: a discussion of observations, measurement methods, and models. *New Phytologist,* 162, 311–322.

Pereira EIP, Chung H, Scow K, Sadowsky MJ, van Kessel C, Six J (2011) Soil nitrogen transformations under elevated atmospheric CO_2 and O_3 during the soybean growing season. *Environmental Pollution,* 159, 401–407

Phillips RP, Finzi AF, Bernhardt ES (2011) Enhanced root exudation induces microbial feedbacks to N cycling in a pine forest under long-term CO_2 fumigation. *Ecology Letters,* 14, 187–194.

Phillips RP, Meier IC, Bernhardt ES, Grandy AS, Wickings K, Finzi AC (2012) Roots and fungi accelerate carbon and nitrogen cycling in forests exposed to elevated CO_2. *Ecology Letters,* 15, 1042–1049.

Phillips RL, Zak DR, Holmes WE, White DC (2002) Microbial community composition and function beneath temperate trees exposed to elevated atmospheric carbon dioxide and ozone. *Oecologia,* 131, 236–244.

Philippot L, Andersson SGE, Battin TJ (2010) The ecological coherence of high bacterial taxonomic ranks. *Nature Reviews Microbiology,* 8, 523–529.

Philippot L, Čuhel J, Saby NPA *et al.* (2009) Mapping field-scale spatial patterns of size and activity of the denitrier community. *Environmental Microbiology,* 11, 1518–1126.

Prosser JI (1989) Autotrophic nitrification in bacteria. *Advances in Microbial Physiology,* 30: 125–181.

Prosser JI (2015) Dispersing misconceptions and identifying opportunities for the use of 'omics' in soil microbial ecology. *Nature Reviews Microbiology,* 13, 439–446.

Rillig MC, Wright SF, Shaw MR *et al*. (2002) Artificial climate warming positively affects arbuscular mycorrhizae but decreases soil aggregation. *Oikos,* 97, 52–58.

Reddy GB, Reinert RA, Eason G (1991) Enzymatic changes in the rhizosphere of loblolly pine exposed to ozone and acid rain. *Soil Biological & Biochemistry,* 23, 1115–1119.

Reich PB, Hungate BA, Luo Y (2006) Carbon-nitrogen interactions in terrestrial ecosystems in response to rising atmospheric carbon dioxide. *Annual Review of Ecology, Evolution and Systematics,* 37, 611–636.

Rey A, Jarvis P (2006) Modelling the effect of temperature on carbon mineralization rates across a network of European forest sites (FORCAST). *Global Change Biology*, 12: 1894–1908.

Roesch L, Fulthorpe RR, Riva A *et al.* (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal,* 1, 283–290.

Root TL, Price JT, Hall KR *et al*. (2003) Fingerprints of global warming on wild animals and plants. *Nature,* 421, 57–60.

Romero-Olivares AL, Allison SD, Treseder KK (2017) Soil Microbes and Their Response to Experimental Warming over Time: A Meta-Analysis of Field Studies. Soil *Biology and Biochemistry,* 107, 32-40.

Rousk J, Frey SD, Bååth E (2012) Temperature adaptation of bacterial communities in experimentally warmed forest soils. *Global Change Biology,* 18, 3252–3258.

Rustad LE, Campbell JL, Marion GM *et al*. (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia,* 126, 543–562.

Scagel CF, Andersen CP (1997) Seasonal changes in root and soil respiration of ozone-exposed ponderosa pine (*Pinus ponderosa*) grown in different substrates. *New Phytologist,* 136, 627–643.

Schimel DS, Braswell BH, Holland EA *et al.* (1994) Climatic, edaphic, and biotic controls over storage and turnover of carbon in soils. *Global Biogeochemical Cycles,* 8, 279–293.

Schindlbacher A, Rodler A, Kuffner M, Kitzler B, Sessitsch A, Zechmeister-Boltenstern S (2011) Experimental warming effects on the microbial community of a temperate mountain forest soil. *Soil Biology & Biochemistry,* 43, 1417–1425.

Schindlbacher A, Schnecker J, Takriti M, Borken W, Wanek W (2015) Microbial physiology and soil CO_2 efflux after 9 years of soil warming in a temperate forest–no indications for thermal adaptations. *Global Change Biology,* 21, 4265–4277.

Schroeder-Moreno MS, Greaver TL, Wang S, Hu S, Rufty TW (2012) Mycorrhizal-mediated nitrogen acquisition in switchgrass under elevated temperatures and N enrichment. *GCB Bioenergy,* 4, 266–276.

Sicard P, Serra R, Rossello P (2016) Spatiotemporal trends of surface ozone concentrations and metrics in France. *Environmental Research,* 149, 122–144.

Sillen WMA, Dieleman WIJ (2012) Effects of elevated CO_2 and N fertilization on plant and soil carbon pools of managed grasslands: a meta-analysis. *Biogeosciences,* 9, 2247–2258.

Simpson D, Arneth A, Mills G, Solberg S, Uddling J (2014) Ozone—The persistent menace: Interactions with the N cycle and climate change. *Current Opinion in Environmental Sustainability*, 9–10, 9–19.

Singh BK, Bardgett RD, Smith P, Reay DS (2010) Microorganisms and climate change: terrestrial feedbacks and mitigation options. *Nature Reviews Microbiology*, 8, 779–790.

Six J, Callewaert P, Lenders S *et al*. (2002) Measuring and understanding carbon storage in afforested soils by physical fractionation. *Soil Science Society of America Journal*, 66, 1981–1987.

Smith KA, Ball T, Conen F, Dobbie KE, Massheder J, Rey A (2003) Exchange of greenhouse gases between soil and atmosphere: interactions of soil physical factors and biological processes. *European Journal of Soil Science*, 54, 779–791.

Smith SE, Read DJ (2008) Mycorrhizal symbiosis. Cambridge, UK: Academic Press.

Solomon S, Qin D, Manning M *et al*. (2007) Technical summary. In: Climate Change 2007: *The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (eds SolomonS, QinD, ManningM *et al*.), pp. 19–93. Cambridge University Press, Cambridge, UK.

Torsvik V, Øvreås L (2004) Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology*, 5, 240– 245.

Stark, JM (1996) Modeling the temperature response of nitrification. *Biogeochemistry*, 35, 433–445.

Staddon PL, Ramsey CB, Ostle N, Ineson P, Fitter AH (2003a) Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of C-14. *Science*, 300, 1138–1140.

Staddon PL, Thompson K, Jakobsen I *et al*. (2003b) Mycorrhizal fungal abundance is affected by long-term climatic manipulations in the field. *Global Change Biology*, 9, 1365–2486.

Stres B, Danevčič T, Pal L *et al.* (2008) Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. *FEMS Microbiology Ecology*, 66, 110–122.

Talhelm AF, Pregitzer KS, Zak DR (2009) Species-specific responses to atmospheric carbon dioxide and tropospheric ozone mediate changes in soil carbon. *Ecology Letters*, 12, 1219–1228.

Taylor PG, Cleveland CC, Wieder WR *et al.* (2017) Temperature and rainfall interact to control carbon cycling in tropical forests. *Ecology Letters,* 20, 779–778.

Treseder KK (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. *New Phytologist,* 164, 347–355.

Treseder KK, Allen MF (2000) Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO_2 and nitrogen deposition. *New Phytologist,* 147, 189–200.

Treseder KK, Egerton-Warburton LM, Allen MF, Cheng Y, Oechel WC (2003) Alteration of soil C pools and communities of mycorrhizal fungi in chaparral exposed to elevated C dioxide. *Ecosystems,* 6, 786–796.

Treseder KK, Marusenko Y, Romero-Olivares AL, Maltz MR (2016) Experimental warming alters potential function of the fungal community in boreal forest. *Global Change Biology,* 22, 3395–3404.

Tscherko D, Kandeler E, Jones TH (2001) Effect of temperature on below-ground N-dynamics in a weed model ecosystem at ambient and elevated atmospheric CO_2 levels. *Soil Biology & Biochemistry,* 33, 491–501.

Tu C, Booker FL, Watson DM, Chen X, Rufty TW, Shi W, Hu SJ (2006) Mycorrhizal mediation of plant N acquisition and residue decomposition: impact of mineral N inputs. *Global Change Biology,* 12, 793–803.

Van Groenigen KJ, Osenberg CW, Hungate BA (2011) Increased soil emissions of potent greenhouse gases under increased atmospheric CO_2. *Nature,* 475, 214–216.

Van Groenigen KJ, Qi X, Osenberg CW, Luo YQ, Hungate BA (2014) Faster decomposition under increased atmospheric CO2 limits soil carbon storage. *Science,* 344, 508–509.

Vitousek PM, Mooney HA, Lubchenco J Melillo JM (1997). Human domination of Earth's ecosystems. *Science,* 277, 494–499.

Wallenstein MD, Mcmahon SK, Schimel JP (2009) Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. *Global Change Biology,* 15, 1631–1639.

Wallenstein MD, Steinweg JM, Ernakovich J, Allison S, Sinsabaugh RL (2010) Controls on the temperature sensitivity of soil enzymes: a key driver of in-situ enzyme activity rates. In: *Soil Enzymology* (eds Shukla G, Varma A), pp. 245–258. Springer, New York.

Walther GR, Post E, Convey P *et al*. (2002) Ecological responses to recent climate change. *Nature,* 416, 389–395.

Wang S, Augé RM, Toler HD (2017) Arbuscular mycorrhiza formation and its function under elevated atmospheric O_3: A meta-analysis. *Environmental Pollution*, 226, 104–117.

Wang SG, Wang F, Diao XJ, He LS (2014). Effects of elevated O_3 on microbes in the rhizosphere of mycorrhizal snap bean with different O_3 sensitivity. *Canadian Journal of Microbiology*, 60, 93–103.

Wang X, Dong S, Gao Q, Zhou H, Liu S, Su X, Li Y (2014) Effects of short-term and long-term warming on soil nutrients, microbial biomass and enzyme activities in an alpine meadow on the Qinghai-Tibet Plateau of China. *Soil Biology & Biochemistry*, 76, 140–142.

Weier KL, Doran JW, Power JF, Walters DT (1993) Denitrification and dinitrogen nitrous oxide ratio as affected by soil-water, available carbon, and nitrate. *Soil Science Society of America Journal*, 57, 66–72.

Wertz S, Goyer C, Zebarth BJ, Burton DL, Tatti E, Chantigny MH, Filion M (2013) Effects of temperatures near the freezing point on N_2O emissions, denitrification and on the abundance and structure of nitrifying and denitrifying soil communities. *FEMS Microbiology Ecology*, 83, 242–254.

Wilson GWT, Rice CW, Rillig MC, Springer A, Hartnett DC (2009) Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. *Ecology Letters*,12, 452–461.

Wilson H, Johnson BR, Bohannan B, Pfeifer-Meister L, Mueller R, Bridgham SD (2016) Experimental warming decreases arbuscular mycorrhizal fungal colonization in prairie plants along a Mediterranean climate gradient. *Peer J*, 4:e2083

Wittig VE, Ainsworth E, Naidu SL, Karnosky DF, Long SP (2009) Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta-analysis. *Global Change Biology*, 15, 396–424.

Wrage N, Velthof GL, van Beusichem ML, Oenema O (2001) Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*, 33, 1723–1732.

Wu H, Li Q, Lu C *et al.* (2016) Elevated ozone effects on soil nitrogen cycling differ among wheat cultivars. *Applied Soil Ecology*, 108, 187–194.

Wu K, Chen D, Tu C *et al.* (2017) CO_2-induced alterations in plant nitrate utilization and root exudation stimulate N_2O emissions. *Soil Biology & Biochemistry*, 106, 9–17.

Wu Z, Dijkstra P, Koch GW, Peñuelas J, Hungate BA (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. *Global Change Biology*, 17, 927–942.

Wullschleger SD, Tschaplinski TJ, Norby RJ (2002) Plant water relations at elevated CO_2– implications for water-limited environments. *Plant, Cell and Environment*, 25, 319–331.

Yang W, Zheng Y, Gao C *et al.* (2013) The arbuscular mycorrhizal fungal community response to warming and grazing differs between soil and roots on the Qinghai-Tibetan plateau. *PLoS ONE*, 8, e76447.

Yin HJ, Chen Z, Liu Q (2012) Effects of experimental warming on soil N transformations of two coniferous species, Eastern Tibetan Plateau, China. *Soil Biology & Biochemistry*, 50, 77–84.

Zak DR, Holmes WE, Pregitzer KS (2007). Atmospheric CO_2 and O_3 alter the flow of ^{15}N in developing forest ecosystems. *Ecology*, 88, 2630–2639.

Zak DR, Pregitzer KS, Curtis PS *et al*. (1993) Elevated atmospheric CO2 and feedback between carbon and nitrogen cycles. *Plant and Soil*, 151, 105–117.

Zak DR, Pregitzer KS, King JS, Holmes WE (2000) Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: a review and hypothesis. *New Phytologist*, 147, 201–222.

Zhang W, Parker KM, Luo Y, Wan S, Wallace LL, Hu S (2005) Soil microbial responses to experimental warming and clipping in a tallgrass prairie. *Global Change Biology*, 11-266–277.

Zhang XM, Johnston ER, Li LH, Konstantinidis KT, Han XG (2016) Experimental warming reveals positive feedbacks to climate change in the Eurasian Steppe. *The ISME Journal*, 11, 885–895.

Zhang XZ, Shen ZX, Fu G (2015) A meta-analysis of the effects of experimental warming on soil carbon and nitrogen dynamics on the Tibetan Plateau. *Applied Soil Ecology*, 87, 32–38.

Zhou L, Zhou X, Shao J *et al.* (2016) Interactive effects of global change factors on soil respiration and its components: a meta-analysis. *Global Change Biology*, 22, 3157–3169.

Chapter 2

Effects of arbuscular mycorrhizal fungi and plant roots on N_2O emissions depend on the form of nitrogen species and the level of CO_2

2.1 Abstract:

Elevated atmospheric CO_2 (eCO_2) often increases soil N_2O emissions, which has been attributed to CO_2-enhancement of soil labile carbon (C) and water use efficiency. This labile C increase mainly stems from eCO_2 stimulation of roots and their symbiotic microbes such as mycorrhizae. However, direct impacts of plant roots and mycorrhizae on N_2O emissions under elevated CO_2 have not been examined. We conducted a microcosm experiment assessing the effects of plant roots and arbuscular mycorrhizal fungi (AMF) on N_2O emissions under ambient and elevated CO_2, in the presence of two distinct N forms, ammonium (NH_4^+) and nitrate (NO_3^-). We found that the form of N inputs dominated the N_2O emissions: N_2O emissions were almost undetectable with NH_4^+ inputs, but increased significantly with NO_3^- inputs. The enhanced N_2O emission was more related to a change in soil denitrifier community composition. We determined the abundance of key genes responsible for N_2O production (*nir*K and *nir*S) and N_2O consumption (*nos*Z), and found that eCO_2 significantly increased the ratio of ((*nir*K+*nir*S)/*nos*Z) under NO_3^- fertilization. In addition, plant roots and AMF significantly reduced N_2O emission under eCO_2 when NO_3^- was applied. While AMF reduced N_2O emission through improved N nutrition retention in microbial biomass, roots reduced N_2O emission through increased plant N uptake. Our results suggest that plant roots and their associated AMF may play a more important role in modulating soil N_2O emissions under future CO_2 scenarios.

Keywords: elevated CO_2, AMF, nitrous oxide (N_2O), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrification, denitrification, microbial community

2.2 Introduction:

CO_2 and N_2O are two major greenhouse gases, accounting for ~65% and ~6% warming potential, respectively (WMO, 2014; Weller *et al.*, 2016). Experimental studies over the last three decades showed that elevated CO_2 (eCO_2) often enhanced N_2O emissions (Baggs *et al.*, 2003; Kammann *et al.*, 2008; Lam *et al.*, 2011; Wu *et al.*, 2017), raising the possibility of a positive feedback loop between eCO_2 and N_2O under future climate conditions. The mechanisms that underlie eCO_2 stimulation of N_2O are still unclear, but higher denitrification induced by CO_2-enhancement of soil labile C and plant water use efficiency have been proposed as the primary mechanisms (van Groenigen *et al.*, 2011). However, the origin of the N in the enhanced N_2O remains unknown.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous, form associations with roots of approximately 80% of land plant species and obtain C from their host plants in return for mineral nutrients (Smith & Read, 2010; Kiers *et al.*, 2011). Atmospheric CO_2 enrichment generally increases plant growth (Drake *et al.*, 1997; Ainsworth & Long, 2005) and allocates more photosynthates to symbiotic microbes (particularly mycorrhizae) (Treseader, 2004; Alberton *et al.*, 2005). Plant roots and AMF may have contrasting effects on N cycling and N_2O emissions. On one hand, they increase plant N uptake (Hu *et al.*, 2001; Drake *et al.*, 2011) and reduce N for nitrification and subsequent denitrification, therefore reducing N_2O production (Bender *et al.*, 2014, 2015; Wu *et al.*, 2017). On the other hand, more root exudates and labile C may stimulate denitrifiers as most of them are heterotrophs and require

labile C as the energy source and thus enhance N_2O emissions (van Groenigen *et al.*, 2011; Storer, 2013).

Elevated CO_2 and the form of N input may critically mediate the impact of plant roots and AMF on plant N uptake and N_2O emissions (Wu *et al.*, 2017). Cheng *et al.* (2012) conducted a meta-analysis and reported that eCO_2 enhanced soil NO_3^- by 26.7%, but decreased soil NH_4^- by 7.6%, suggesting that elevated CO_2 may have reduced plant N uptake and increased soil N availability. Several studies have shown that nitrate assimilation in C_3 plants was slower under elevated than ambient CO_2, leading to lower plant N utilization while receiving NO_3^- than NH_4^+ as a sole N (Bloom *et al.*, 2010, 2014; Asensio *et al.*, 2015). Together, these studies suggest that C_3 plants under elevated CO_2 may preferentially take up NH_4^+ over NO_3^- from soil, contributing to the stimulation of N_2O emissions. Wu *et al.* (2017) first directly illustrated that the form of N inputs dominates the eCO_2 effect on soil N_2O emissions. N_2O emission was significantly higher under elevated than ambient CO_2 when NO_3^- was applied, but was not detectable when NH_4^+ was applied under ambient and elevated CO_2. However, it is not known whether the presence of plant roots and AMF will modify the impact of elevated CO_2 on N_2O emissions and their combined impact remains unknown.

We conducted a microcosm experiment with wheat (*Triticum aestivum* L.) to assess the impact of plant roots, AMF and eCO_2 on N_2O emissions. We hypothesized that 1) eCO_2 increases N_2O emissions when NO_3^- was applied, 2) the presence of roots and AMF reduces

N_2O emission, and 3) eCO_2 effects on N_2O emissions are related to alteration in denitrifying microbes.

2.3 Materials and methods

Experimental design and treatments

This experiment was conducted in the USDA-ARS Plant Science Research CO_2 facility at North Carolina State University (Raleigh, NC, USA). The facility consisted of 8 continuously – stirred tank reactors (CSTR) for the exposure of plants to CO_2 (Wu *et al.*, 2017). Each CSTR is a cylindrical chamber covered with Teflon and measured 1.2 m in diameter by 1.4 m tall (Cheng *et al.*, 2012; Wu *et al.*, 2017). Compressed CO_2 was mixed with air and dispensed to chambers 24 hours daily using rotameters to maintain CO_2 concentrations at a target level. To monitor CO_2 concentrations, chamber air was measured using infrared analyzers (model 6252, LiCOR Inc., Lincolin, NE USA).

This experiment was a split-split plot design with whole-plots randomly assigned into each of the four blocks. Two adjacent CSTR chambers were formed into one block. The whole-plot treatments were two atmospheric CO_2 levels (ambient: 400 μmol CO_2 mol^{-1} and elevated CO_2: 680 μmol CO_2 mol^{-1}) with four replicates. The split-plot treatments were two N species (NH_4^+ and NO_3^-), which were further split into three mycorrhizal treatments.

In each CSTR chamber, we have two plexi-glass microcosms and each microcosm was divided into six compartments with each compartment measuring 13×14×15cm (width × depth × height) (Tu *et al.*, 2006). Three compartments in a row were designated as HOST

compartments (containing host plants and AM fungi) and the other three adjacent compartments were designated as TEST compartments to determine the N_2O emission. The HOST and TEST compartments were separated by a replaceable 0.45 μm, 20 μm or 1.6 mm mesh (Tetko/Sefar mesh, Sefar America, NY) that controlled the penetration of plant roots or AM fungal hyphae into the TEST compartments (Hodge *et al.,* 2001). Therefore, this experiment had three mycorrhizal treatments. In the control treatment (CK), the HOST and TEST compartments were separated by a fine mesh screen (0.45 μm). In the AMF treatment, a 20 μm mesh screen was used to allow AM fungal hyphae, but no plant roots, to grow into the TEST compartment. In the root treatment, a 1.6 mm mesh screen was used to allow both AM fungal hyphae and plant roots to grow into the TEST compartment.

In this experiment, the multiple AM fungal species were trap-cultured from an agricultural soil, collected from the Center for Environmental Farming Systems, NC, and were then pot-cultured to increase fungal biomass in the greenhouse. Twelve AM fungal species were identified and characterized according to the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM). AMF inoculum consisted of culture media containing spores, hyphae, and colonized root pieces (Tu *et al.,* 2006; Cheng *et al.,* 2012).

Planting, plant sampling and analysis

Each compartment of the microcosm was filled with a 3.3 kg sandy loam soil collected from the Lake Wheeler Research Farm of North Carolina State University, Raleigh, NC, USA (35° 43' N, 78° 40' W; elevation 120 m) (Wu *et al.,* 2017). The soil is well drained with

a pH of 6.02, and contained 17 g C kg^{-1} and 1.5 g N kg^{-1} soil prior to the experiment. The HOST compartments were inoculated with 150 g inoculum of a mixture of AMF species. Twenty seeds of wheat were sown into HOST compartments and then thinned after emergence to ensure the uniform density. Plants received CO_2 fumigation immediately following seed germination. Each compartment received 20 mg N kg^{-1} soil was added at the 4^{th} week after seedling emergence. Microcosms were watered with deionized water regularly. Plants were grown for 15 weeks. The aboveground of plants were cut at the soil surface. Roots were carefully separated from the soil and washed thoroughly with tap water.

Root colonization by mycorrhizal fungi was measured using the gridline-intersect method (Giovannetti & Mosse, 1980). Thoroughly washed root samples (cut into about 1 cm in length) were cleared in 10% (w/v) KOH, acidified in 1% (v/v) HCl for 2 hours, and then stained with acidic glycerol-trypan blue solution (Phillips & Hayman, 1970). The stained roots were then spread on a Petri dish with gridlines and examined for infection using a dissecting microscope at ×40 magnification. Results obtained were expressed as percentage root length colonized (PRLC) (Tu *et al.*, 2006).

The shoots and roots were oven-dried (65°C) and weighed. The oven-dried shoots were finely ground to powder for measuring total C, and biomass N. Shoot biomass C and N concentrations were determined with CHN elemental analyzer (Cara Erba and model 2400, Perkin Elmer Co., Norwalk, CT, USA).

CO_2 and N_2O flux measurement

Nitrogen fertilization: CO_2 and N_2O sampling was conducted twice during this experiment. After the 8^{th} and 12^{th} week of plant growth, each TEST compartment was watered to 80% water-filled pore space (WFPS) with deionized water mixed with a fertilizer [NH_4^+ as $(NH_4)_2SO_4$ or NO_3^- as KNO_3] pulse corresponding 40 kg N ha^{-1}. Potassium was balanced with K_2SO4. TEST compartments were watered to maintain the soil moisture to 80% WFPS to ensure an environment conducive for denitrification. We employed a Campbell Scientific HS2 Hydrosense II probe (Campbell Scientific, Logan, UT, USA) to measure soil volume water contents (VWC, %) to 12 cm depth and then converted the VWC into the water-filled pore space (WFPS). Water addition was terminated when N_2O emission was not detectable.

CO_2 and N_2O sampling and measurements: At the 8^{th} week of plant growth, gas emissions were sampled starting at 12, 24, 48, 72, 96 and 120 h after fertilizer-N addition, resulting in 6 flux measurements. Again, at the 12^{th} week of plant growth, gas emissions were sampled starting at 12, 24, 36, 48, 72, 96, 120 until 312 h after fertilizer - N addition, resulting in 16 fluxes measurements. CO_2 and N_2O flux measurements were taken in all TEST compartments using a modified static chamber method (Cavagnaro *et al.*, 2012). The chambers were closed with a lid, and fitted with a rubber septum to allow gas sampling via syringes. Gas samples (5 ml) were taken with 20 ml PE syringes (Beceton Dickinson Franklin Lakes, NJ, USA) and immediately injected into N_2-preflushed 12mL vials at time 0 and 30 min after the closure of lids. Air temperature and soil WFPS were recorded during sampling period. Gas samples were analyzed within 24 h on a gas chromatograph fitted with

an electron capture detector (ECD) and a flame ionization detector (FID) (Shimadzu GC – 2014, Kyoto, Japan) with an autosampler (Shimadzu AOC-5000 Auto-Injector). The CO_2 (μg CO_2-C m^{-2} h^{-1}) or N_2O (μg N_2O-N m^{-2} h^{-1}) fluxes were calculated using the formulas by Wu *et al.* (2017):

$$CO_2 \text{ or } N_2O \text{ flux} = \rho \times (P/760) \times (V/A) \times (\Delta C/\Delta t) \times [273/(273 + T)]$$

Where ρ is the density of N_2O at 0 °C and 760 mm Hg (1.25 kg m^{-3}), V is the chamber volume (m^3), A is the area of the gas sampling static chamber (m^2), $\Delta C/\Delta t$ is the rate of gas accumulation in the chamber, T is the chamber air temperature (°C) and *P* (mm Hg) is the air pressure of the experimental site (mm Hg). The altitude of the experimental site for this study is very close to sea level, so P/760 close to 1. Mean CO_2 or N_2O emissions of a TEST compartment were calculated by averaging the fluxes from each time point during the sampling period.

Soil sampling, soil and microbial analyses

Soil samples from TEST compartments were taken after plants were harvested and stored in 4°C refrigerator for analysis. MBC and MBN were determined using a fumigation-extraction method (Vance *et al.*, 1987). Briefly, 20-g dry weight soil samples were fumigated with ethanol-free chloroform for 48 h and then extracted with 50 mL of 0.5 M K_2SO_4 by shaking for 30 min. Another 20-g samples of nonfumigated soil were extracted with 50 mL of 0.5 M K_2SO_4 shaking for another 30 min. Soil extractable organic C in both fumigated and non-fumigated extracts was determined using a TOC analyzer (Shimadzu TOC-5050A,

Shimadzu Co., Kyoto, Japan). Soil extractable inorganic N referred to the sum of NH_4^+-N and NO_3^--N in the extracts of non-fumigated soils. MBN was determined following alkaline persulfate oxidation of the fumigated and non-fumigated K_2SO_4 extracts (Cabrera & Beare, 1993). The concentration of NO_3^- and NH_4^+ was quantified on the Lachat flow injection analyzer (Lachat Instruments, Milwaukee, WI, USA). The differences in extractable organic C and inorganic N between fumigated and non-fumigated soils were used to calculate MBC and MBN using a conversion factor of 0.33 (k_{EC}) and 0.45 (k_{EN}), respectively (Vance *et al.*, 1987; Tu *et al.*, 2006).

Gene copy numbers

To investigate how eCO_2, roots and AMF and N forms affect denitrification bacterial communities, copy numbers of key genes involved in denitrification and N_2O production (*nir*K, *nir*S and *nos*Z encoding the copper and cytochrome cd1 nitrite reductases and the nitrous oxide reductase, respectively) were quantified.

Soils (0.5 g) were weighed for genomic DNA extraction using a FastDNA SPIN kit (MP Bio, Solon, OH, USA). Soil DNA quality and size were checked by electrophoresis on a 1% agarose gel. The quantity of the DNA extractions was determined with a nanodrop spectrophotometer (Thero Scientific, Wilmington, DE, USA). The copy numbers of *nir*S, *nir*K, and *nos*Z genes were performed on each soil DNA sample using quantitative real-time polymerase chain reaction (PCR) (CFX96 Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). The primers for the three genes in the soils are given in Appendix 1, which were considered as the surrogates of the abundances of bacterial denitrifiers. Each

reaction system was performed in a 20 µL volume involving 14 µL 1*SsoAdvanced ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 2 µL of template DNA, and 2 µL of each primer. All the qPCR reactions were conducted using an initial denaturation at 98 °C for 3 min, followed by a number of touchdown cycles and/or regular PCR cycles as specified in Appendix 1. These reactions were performed in triplicate for each bacterial denitrifying gene.

The standard curve for determining the gene copy number was developed with the agarose gel-purified PCR products based upon the method of Chen *et al.* (2015). Briefly, PCR products were run on a 1% low-melting agarose gel and gel bands corresponding to the expected amplicon sizes were excised. Then, target DNA was extracted with a gel extraction kit (Qiagen, Valencia, CA, USA). The quantity of the DNA extractions was determined with a nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Number of the gene copies per µL of the standard was calculated by the equation: $\frac{A \times B}{C \times D}$, where A is the concentration of the PCR product (ng μL^{-1}), B is the Avogadro number (i.e., 6.023×10^{23} copies mol^{-1}), C is the average molecular weight of a DNA base pair (i.e., 6.6×10^{11} ng mol^{-1}), and D is the respective PCR amplicon size listed in Appendix 1. Serial dilutions of the PCR products (i.e., the standard) were amplified in triplicate together with samples and an average value was calculated. A standard curve was obtained by plotting the logarithm of the copy numbers against the mean threshold cycle (Ct) value for each standard dilution.

Statistical analyses

Data were subjected to analysis of variance (ANOVA) using the Mixed Model procedure (Proc Mixed) of SAS 9.4. All the data sets of this experiment (split-split-plot design) were analyzed using linear mixed-effects model, which tested the effects of CO_2 (whole-plot), N form (split-plot), mycorrhizae (split-split-plot) and their interactions on all the variables. Statistical significance is indicated for $P < 0.001$, $P < 0.01$, or $P < 0.05$, and actual P values are presented where $0.05 < P < 0.1$.

2.4 Results

Plant biomass

eCO_2 significantly stimulated the growth of plant (Fig. 2.1, Appendix 2.2). The form of N has no effect on plant biomass (Fig. 2.1, Appendix 2.2). AMF had no impact on plant shoot and total biomass (Fig. 2.1a, 2.1c), but tended to reduce root biomass by 12% (P=0.1, Fig. 2.1b). When plant roots penetrated the TEST plot, plant biomass increased significantly (Fig. 2.1).

There was no significant $CO_2 \times N$ interaction on plant biomass (Fig. 2.1, Appendix 2.2). But there was a significant N × mycorrhizae interaction on plant shoot and total biomass (Fig. 2.1a, 2.1c, Appendix 2.2). Under eCO_2, NH_4^+ also enhanced plant biomass when plant roots penetrated into the TEST compartments, but the application of NO_3^- had no effect (Fig. 2.1). There was a significant $CO_2 \times N \times$ mycorrhizae interaction on plant growth (Fig. 2.1, Appendix 2.2).

Mycorrhizal colonization

The mycorrhizal infection rate in all treatments was relatively low (Appendix 2.3). Yet, eCO_2 significantly increased AMF colonization rate (Appendix 2.2, 2.3). Neither the form of N nor the penetration of roots or AMF into the TEST compartments significantly affected mycorrhizal colonization of wheat roots (Appendix 2.2, 2.3).

Shoot biomass nitrogen and C:N ratio

eCO_2 significantly increased shoot biomass N (Appendix 2.2, 2.4). N forms had no effect on total shoot biomass of N (Appendix 2.2, 2.4a). Plant roots significantly increased shoot biomass N ($P<0.05$, Appendix 2.4a), but, AMF has no effect ($P>0.05$, Appendix 2.4a). Under eCO_2, plant roots significantly increased shoot biomass N when NH_4^+, not NO_3^- was applied ($P<0.05$, Appendix 2.4a). There was a significant $CO_2 \times N \times$ mycorrhizae interaction on shoot biomass N (Appendix 2.2, 2.4a).

Both CO_2 concentrations and N forms significantly affected shoot biomass C:N ratio (Appendix 2.2, 2.4b). eCO_2 increased biomass C:N ratio by 12.3%. Also, shoot biomass C:N ratio was significantly higher under NO_3^- than NH_4^+ was applied (Appendix 2.2, 2.4b). Neither plant roots nor AMF had effect on shoot biomass C:N when NH_4^+ was applied (Appendix 2.2 and 2.4b). However, plant roots significantly decreased and AMF tended to reduce biomass C:N ratio when NO_3^- was applied ($P=0.01$ and $P=0.07$, respectively; Appendix 2.4b). There was a marginal mycorrhizae × N interaction on shoot biomass C:N ratio (Appendix 2.2, 2.4b).

Soil extractable inorganic nitrogen and nitrate concentration

We found that CO_2 enrichment had no significant on total soil extractable inorganic N and nitrate concentration (Fig. 2.2, Appendix 2.2). The application of NH_4^+ significantly increased soil extractable inorganic N and nitrate concentration compared with NO_3^- fertilization (Fig. 2.2, Appendix 2.2). Either AMF or plant roots significantly reduced soil extractable inorganic N and nitrate concentration. (Fig. 2.2, Appendix 2.2). And a significant N × AMF interaction was observed (Fig. 2.2, Appendix 2.2). AMF significantly reduced soil extractable inorganic N and nitrate concentration with NH_4^+, not NO_3^- fertilization (Fig. 2.2). Under ambient CO_2, AMF had no effect on soil extractable inorganic N and nitrate concentration (Fig. 2.2). However, under elevated CO_2, there was a significant decrease of soil extractable inorganic N and nitrate concentration when only AMF penetrated into the TEST compartment by 30.5% and 26.2%, respectively (Fig. 2.2). This reduction was mainly induced under NH_4^+ application (Fig. 2.2).

Soil extractable organic carbon (C)

Neither CO_2 nor N forms had a significant effect on total soil extractable organic C (Appendix 2.2, 2.5). However, there was a significant decrease in soil extractable organic C when plant roots penetrated into TEST compartment (Appendix 2.5), while, AMF had no significant effect on soil extractable organic C (Appendix 2.5).

Microbial biomass carbon and nitrogen

We did not observe any significant difference on soil MBC among all the treatments (Appendix 2.2, 2.6b). NO_3^- application significantly enhanced MBN by 21.4% (Appendix

2.6a). eCO_2 had no impact on MBN. There was also a $CO_2 \times N \times AMF$ interaction on soil MBN (Appendix 2.2, 2.6a). Under elevated CO_2, AMF significantly increased MBN by 40.9% compared with control when NO_3^- was applied ($P<0.05$, Appendix 2.6b). In addition, NO_3^- fertilization significantly altered microbial biomass C to N ratio (MBC/MBN), leading to a decrease from 6.85 to 5.68 (Appendix 2.2, 2.6c) indicating a significant change of microbial community.

Soil CO_2 and N_2O emissions

Both soil CO_2 and N_2O emissions were low in all treatments after 8 weeks of plant growth when plants were small (Appendix 2.7, 2.8). While, four weeks later, soil CO_2 emissions were enhanced significantly, varying from 116.1 to 5338.3 mg C m^{-2} d^{-1} (Fig. 2.3). N_2O emissions increased significantly only when NO_3^- was applied (Fig. 2.4c, 2.4d), but were still close to the background when $NH4^+$ was applied (Fig. 2.4a, 2.4b). The peak of N_2O flux in the NO_3^- treatment was significantly higher under elevated CO_2 than ambient CO_2 (Fig. 2.4c, 2.4d). When NO_3^- was applied, eCO_2 enhanced the peak N_2O emission fluxes by 86.7% (Fig. 2.4c, 2.4d).

Elevated CO_2 tended to enhance the mean CO_2 emission by 25.3% (Fig. 2.5a, Appendix 2.2). N forms significantly affected mean CO_2 emission. NH_4^+ application significantly increased average CO_2 emission by 26.7% compared with NO_3^- application (Fig. 2.5a, Appendix 2.2). There was no significant $CO_2 \times N$ interaction on CO_2 emission (Fig. 2.5a, Appendix 2.2). Plant roots significantly increased soil mean CO_2 emission by 44.1%

compared with control (P<0.05, Fig. 2.5a, Table 2.1). However, AMF has no effect on soil mean CO_2 emission (P>0.05, Fig. 2.5a, Table 2.1).

Elevated CO_2 significantly increased the mean N_2O emission by 35.9% and this increase was mainly caused by the fertilization of NO_3^- (Fig 2.5b, Appendix 2.2). N forms also significantly affected mean N_2O emission (Fig 2.5b, Appendix 2.2). Average N_2O emission was significantly different among control, AMF and Root treatments (Fig 2.5b, Appendix 2.2). AMF and plant roots significantly reduced soil mean N_2O emission from TEST compartments by 25.2% and 37.4%, respectively. Under ambient CO_2 conditions, AMF had no effect on N_2O emissions, but AMF could significantly reduce N_2O emissions under CO_2 elevated when NO_3^- was applied (Fig 2.5b, Appendix 2.2).

Denitrification gene copy numbers

We found that there was not any significant effect on the gene copy numbers of *nir*S and *nos*Z among any treatments (Fig. 2.6b, 2.6c, Appendix 2.2). While, we observed that the application of NO_3^- tended to increase the gene copy numbers of *nir*K by 42.1% (P=0.08, Fig. 2.6a, Table 2.1). And plant roots significantly reduced the copy numbers of *nir*K, but AMF had no effect (Fig. 2.6a, Table 2.1). eCO_2 tended to increase *nirK* gene copy numbers by 23.7% when NO_3^- was applied (P=0.08, Fig. 2.6, Table 2.1). There was a significant CO_2 × N interaction on the gene copy numbers of *nir*K (Fig. 2.6a, Appendix 2.2).

NO_3^- application significantly enhanced the ratio of (*nir*K+*nir*S)/*nos*Z (Fig. 2.6d, Appendix 2.2). Plant roots significantly increased the ratio (P<0.05, Fig. 2.6d, Table 2.1), but

AMF had no effect (P>0.05, Fig. 2.6d, Table 2.1). There was also a significant CO_2 × N interaction on the (*nir*K+*nir*S)/*nos*Z ratio (Fig. 2.6d, Appendix 2.2).

2.5 Discussion:

It has long been recognized that N_2O emissions are highly sensitive to changes in atmospheric CO_2 concentrations (van Groneigen *et al.,* 2011), and there is growing evidence that CO_2 enrichment is leading to an increase in root biomass and AM fungi (Treseder, 2004; Chen *et al.,* 2007). However, the effects of plant roots and AMF on soil N_2O fluxes under future CO_2 scenarios are virtually unknown. Here, we first demonstrated in a microcosm experiment that the effects of plant roots and AMF on soil N_2O emission is both modulated and strongly controlled by nitrogen forms and CO_2 conditions. Our results showed that there was a significant CO_2-stimulation of N_2O emissions in the presence of NO_3^- rather than NH_4^+ indicating that the form of N inputs critically mediates the effects of eCO_2 on denitrification that dominates N_2O emissions. And AMF had no significant effect on N_2O emissions under aCO_2, but, significantly reduced it when NO_3^- was provided under eCO_2. While, plant roots significantly reduced soil N_2O emissions under ambient and elevated CO_2 when NO_3^- was applied after 12 weeks of plant growth.

Denitrifiers are very diverse and consist up to 5% of all soil microbes (Tiedje, 1988; Henry *et al.,* 2006). Most of them are heterotrophs and obtain energy from organic compounds to convert NO_3^- to N_2O and other products (Firestone & Davidson 1989; Wrage *et al.,* 2001). Elevated CO_2 generally increases belowground allocation of plant photosynthates for roots and AMF and soil labile carbon (Hungate *et al.,* 1997; Treseder,

2004; Chen *et al.*, 2007), therefore stimulating heterotrophic denitrifying microbes and N_2O emissions (van Groenigen *et al.*, 2011; Knohl & Veldkamp, 2011). Wu *et al.* (2017) reported that the effect of eCO_2 on wheat biomass strongly depended on the N forms. eCO_2 significantly increased wheat shoot and total biomass when NH_4^+ was applied, but reduced them when NO_3^- was applied. Also, NO_3^- fertilization significantly reduced root biomass under eCO_2. In addition, they found eCO_2 significantly reduced total shoot biomass N when NO_3^- was applied. Recent studies showed that eCO_2 inhibits nitrate assimilation in C_3 plants (Bloom *et al.*, 2010, 2012), suggesting that the enhanced N_2O emission resulted from eCO_2-constraints on plant NO_3^- utilization in wheat.

In this study, we found that eCO_2 significantly stimulated the growth of plant and shoot biomass N, but the form of N had no effect on them (Appendix 2.2). Also, eCO_2 had no effect on soil inorganic N and nitrate concentration (Appendix 2.2). At the end of experiment, we found that NO_3^- application significantly reduced soil extractable N, but increased gene copy numbers of *nir*K and the ratio of (*nir*K+*nir*S)/*nos*Z, indicating a change in the community composition of denitrifiers under NO_3^- fertilization. It has been shown that a relative reduction in denitrifying organisms containing *nosZ* gene can lead to enhanced N_2O emissions (Philippot *et al.*, 2011). Hence, these observations suggest that the alterations in soil denitrifier community composition contribute to the increased N_2O emissions in the presence of NO_3^- under eCO_2.

Until now, very few studies addressed a potential effect of plant roots and AMF on emissions of N_2O, especially under elevated CO_2 conditions. Bender *et al.* (2014) found that

AMF can reduce N_2O emissions under ambient CO_2 condition. One proposed mechanism involves AMF transfer of soil mineral nutrients, especially N, to their host plants, suggesting that they can reduce substrate availability for denitrifying organisms (Smith & Read, 2010; Bender *et al.*, 2015). In our study, plant roots significantly increased shoot biomass N and decreased soil NO_3^- when NO_3^- was fertilized, resulting the lower N_2O emission. However, AMF had no effect on them (Appendix 2.2). One possible reason was that AMF may have a minor role in nutrient transfer with high N concentrations (Reynolds *et al.*, 2005; Johnson *et al.*, 2015) as we applied 20 mg N kg^{-1} to each plant compartment after seed germination. While, there was a significant decrease of both soil extractable inorganic N and NO_3^- by AMF under elevated CO_2 only when NH_4^+ was provided, suggesting that plants rely more on AMF for N-acquisition when NH_4^+ is the dominating N form (Yoshida & Allen, 2001; Hodge & Storer, 2015). This implies an additional involvement of a mechanism other than improved plant N nutrition by AMF to prevent N_2O emissions under NO_3^- fertilization.

It has been reported that soil MBN was significantly higher under NO_3^- than NH_4^+ application (Wu *et al.*, 2017) indicating enhanced plant competition for NH_4^+, but not NO_3^-. One of the reasons was that both plants and microbes must reduce NO_3^- to NH_4^+ before assimilating it (Gavrichkova & Kuzyakov, 2008; Wang *et al.*, 2015). High energy demands during NO_3^- assimilation by roots and microbes result in enhanced soil microbial biomass and activity (Wang *et al.*, 2015). Bender *et al.* (2014) found that the microbial biomass N (MBN) was higher in AMF treatment and suggested that increased N immobilization by the soil microbial biomass may have contributed to the reduced N_2O emissions. In this

experiment, we observed that AMF increased MBN significantly with the fertilization of NO_3^- under elevated CO_2 (Appendix 2.6b). Thus, our results suggest that eCO_2 significantly increased AMF abundance, although the colonization rate was low, and this resulting change of AMF helped improve microbial N nutrition in the presence of high NO_3^-concentrtion resulting in lower N_2O emissions. Also, there was a tendency for AMF to reduce root biomass (P=0.1), which can help explain the reduced N_2O emissions in AMF treatment. Many studies showed that mycorrhizal fungi can act as facultative parasites (Reynolds *et al.*, 2005; Johnson & Graham, 2013; Smith and Smith, 2013). AMF are known as obligate biotrophs relying on C provided by their host plant. It is generally considered that the main benefit to the plant from mycorrhizae is its improved nutrition, while the cost is the C expended in growth and maintenance of the fungus. Williams *et al.* (2016) showed that allocation of C to AMF increased linearly with increasing levels of N fertilization and this corresponded with a significant decline in barley shoot biomass. Moreover, AMF have been shown to uptake substantial quantities of soil N for their own growth (Hodge & Fitter, 2010). AMF plants should grow less than their non-AMF controls when mycorrhization results in higher C expenditure by the plants on their fungal associates. The low colonization rate of wheat root and high application of N in our study further supported this explanation.

Results from our experiment provide the first direct evidence illustrating that the mediation of roots and AMF on N_2O emissions depends on the form of N inputs and CO_2 conditions. Based on these findings, we suggested that soil labile carbon and microbial community composition play a more important role in mediating N_2O emissions. These

findings potentially have several important implications. First, our results suggest that microbial conversion of NO_3 to N_2O will accelerate under future CO_2 conditions. Second, plant roots and AMF can reduce N_2O emissions from soil. The abundance of AMF in soil depends on soil nutrient availability and declines with fertilization (Treseder, 2004). There is a tendency of increasing N fertilizers as agricultural production continues to intensify to meet the increasing food demand in the future (Howden *et al.,* 2007; Reay *et al.,* 2012). The results obtained here suggest that a reduction of AMF by high fertilization in future may initiate a cascade of below-ground interactions that further enhance N_2O emissions from soil. Third, this effect exerted by the AMF symbiosis of reducing N_2O has a more pronounced effect under elevated CO_2 and are the results of complex interaction between fungi and plants, which warrants further investigations.

2.6 References

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. *New Phytologist,* 165, 351–371.

Alberton O, Kuyper TW, Gorissen A (2005) Taking mycocentrism seriously: mycorrhizal fungal and plant responses to elevated CO2. *New Phytologist,* 167, 859–868.

Asensio JSR, Rachmilevitch S, Bloom AJ (2015) Responses of Arabidopsis and wheat to rising CO_2 depend on nitrogen source and nighttime CO_2 levels. *Plant Physiology,* 168, 156–163.

Baggs EM, Richter M, Hartwig UA, Cadisch, G (2003) Nitrous oxide emissions from grass swards during the eighth year of elevated atmospheric pCO_2 (Swiss FACE). *Global Change Biology,* 9, 1214–1222.

Bender SF, Conen F, van der Heijden MGA (2015) Mycorrhizal effects on nutrient cycling, nutrient leaching and N_2O production in experimental grassland. *Soil Biology & Biochemistry,* 80, 283–292.

Bender SF, Plantenga F, Neftel A *et al.* (2014) Symbiotic relationships between soil fungi and plants reduce N_2O emissions from soil. The *ISME Journal,* 8, 1336–1345.

Bloom AJ, Burger M, Asensio JSR, Cousins AB (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. *Science,* 328, 899–902.

Bloom AJ, Burger M, Kimball BA, Pinter PJJ (2014) Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. *Nature Climate Change,* 4, 477–480.

Cabrera M, Beare M (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. *Soil Science Society of America Journal*, 57, 1007-1012.

Cavagnaro T, Barrios-Masias F, Jackson L (2012) Arbuscular mycorrhizas and their role in plant growth, nitrogen interception and soil gas efflux in an organic production system. *Plant and Soil,* 353, 181-194.

Chen X, Tu C, Burton MG, Watson DM, Burkey KO, Hu SJ (2007) Plant nitrogen acquisition and interactions under elevated carbon dioxide: impact of endophytes and mycorrhizae. *Global Change Biology,* 13, 1238–1249.

Cheng L, Booker FL, Tu C *et al*. (2012) Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO_2. *Science,* 337, 1084–1087.

Drake JE, Gallet-Budynek A, Hofmockel KS *et al.* (2011) Increases in the flux of carbon belowground stimulate nitrogen uptake and sustain the long-term enhancement of forest productivity under elevated CO_2. *Ecology Letters,* 14, 349–357.

Drake BG, GonzalezMeler MA, Long SP (1997) More efficient plants: a consequence of rising atmospheric CO_2? *Annual Review of Plant Physiology and Plant Molecular Biology,* 48, 609–639.

Firestone MK, Davidson EA (1989) Microbial basis of NO and N_2O production and consumption in soils. In: *Exchange of trace gases between terrestrial ecosystems and the atmosphere* (eds Andreae MO, Schimel DS), pp. 7-21. John Wiley & Sons, New York.

Gavrichkova O, Kuzyakov Y (2008) Ammonium versus nitrate nutrition of Zea mays and Lupinus albus: effect on root-derived CO_2 efflux. *Soil Biology & Biochemistry,* 40, 2835–2842.

Giovannetti M, Mosse B (1980) Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist,* 84, 489–500.

Henry S, Bru D, Stres B, Hallet S, Philippot L (2006) Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of

16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Applied and Environmental Microbiology*, 72, 5181–5189.

Hodge A, Campbell CD, Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Nature (London)*, 413, 297–299.

Hodge A, Fitter AH (2010) Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 13754–13759.

Hodge A, Storer K (2015) Arbuscular mycorrhiza and nitrogen: implications for individual plants through to ecosystems. *Plant and Soil*, 386, 1–19.

Howden SM, Soussana JF, Tubiello FN, Chhetri N, Dunlop M, Meinke H (2007) Adapting agriculture to climate change. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 19691–19696.

Hu S, Chapin FS III, Firestone MK (2001) Nitrogen limitation of microbial decomposition in a grassland under elevated CO_2. *Nature*, 409, 188–191.

Hungate BA, Holland EA, Jackson RB, Chapin FS, Mooney HA, Field CB (1997) The fate of carbon in grasslands under carbon dioxide enrichment. *Nature*, 388, 576–579.

Jenkinson DS, Brookes PC, Powlson DS (2004) Measuring soil microbial biomass. *Soil Biology & Biochemistry*, 36, 5–7.

Johnson NC, Graham JH (2013) The continuum concept remains a useful framework for studying mycorrhizal functioning. *Plant and Soil*, 363, 411–419.

Johnson NC, Wilson GW, Wilson JA, Miller RM, Bowker MA (2015) Mycorrhizal phenotypes and the Law of the Minimum. *New Phytologist*, 205, 1473–1484.

Kammann C, Müller C, Grünhage L, Jager HJ (2008) Elevated CO_2 stimulates N_2O emissions in permanent grassland. *Soil Biology & Biochemistry*, 40, 2194–2205.

Kiers ET, Duhamel M, Beesetty Y *et al.* (2011) Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science*, 333, 880–882.

Knohl A, Veldkamp E (2011) Global change: indirect feedbacks to rising CO_2. *Nature*, 475, 177–178.

Lam SK, Lin E, Norton R, Chen D (2011) The effect of increased atmospheric carbon dioxide concentration on emissions of nitrous oxide, carbon dioxide and methane from a

wheat field in a semi-arid environment in northern China. *Soil Biology & Biochemistry*, 43, 458-461.

Philippot L, Andert J, Jones CM, Bru D, Hallin S (2011) Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N_2O emissions from soil. *Global Change Biolpgy*, 17, 1497–1504.

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society*, 55, 158–161.

Reynolds HL, Hartley AE, Vogelsang KM, Bever JD, Schultz PA (2005) Arbuscular mycorrhizal fungi do not enhance nitrogen acquisition and growth of old-field perennials under low nitrogen supply in glasshouse culture. *New Phytologist,* 167, 869–880.

Smith SE, Read DJ (2010) Mycorrhizal Symbiosis. Academic Press, San Diego, CA.

Smith FA, Smith SE (2013) How useful is the mutualism–parasitism continuum of arbuscular mycorrhizal functioning? *Plant and Soil,* 363, 7–18.

Storer KE (2013) Interactions between arbuscular mycorrhizal fungi and soil greenhouse gas fluxes. PhD thesis, University of York.

Tiedje JM (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: *Biology of anaerobic microorganisms* (ed. Zehnder, AJB), pp. 179-244. John Wiley & Sons, New York, NY, USA.

Treseder KK (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. *New Phytologist*, 164, 347–355.

Tu C, Booker FL, Watson DM, Chen X, Rufty TW, Shi W, Hu SJ (2006) Mycorrhizal mediation of plant N acquisition and residue decomposition: impact of mineral N inputs. *Global Change Biology,* 12, 793–803.

Tu C, Louws FJ, Creamer NG et al. (2006) Responses of soil microbial biomass and N availability to transition strategies from conventional to organic farming systems. Agriculture, Ecosystems and Environment, 113, 206–215.

Vance E, Brookes P, Jenkinson D (1987) An extraction method for measuring soil microbial biomass C. *Soil Biology & Biochemistry*, 19, 703-707.

Van Groenigen KJ, Osenberg CW, Hungate BA (2011) Increased soil emissions of potent greenhouse gases under increased atmospheric CO_2. *Nature*, 475, 214–221.

Wang YS, Cheng SL, Fang HJ *et al.* (2015) Contrasting effects of ammonium and nitrate inputs on soil CO2 emission in a subtropical coniferous plantation of southern China. *Biology and Fertility of Soils,* 51, 815–825.

Weller S, Janz B, Jörg L *et al.* (2016) Greenhouse gas emissions and global warming potential of traditional and diversified tropical rice rotation systems. *Global Change Biology,* 22, 432–448.

Williams A, Manoharan L, Rosenstock NP, Olsson PA, Hedlund K (2017) Long-term agricultural fertilization alters arbuscular mycorrhizal fungal community composition and barley (Hordeum vulgare) mycorrhizal carbon and phosphorus exchange. *New Phytologist,* 213, 862–873

WMO (2014) World Meteorological Organization Greenhouse Gas Bulletin: the state of greenhouse gases in the atmosphere based on observations through 2013. Available at: http://www.wmo.int/pages/prog/arep/gaw/ghg/GHGbulletin.html (accessed 8 September 2014).

Wrage N, Velthof G, Van Beusichem M, Oenema O (2001) Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology & Biochemistry,* 33, 1723-1732.

Wu K, Chen D, Tu C *et al.* (2017) CO_2-induced alterations in plant nitrate utilization and root exudation stimulate N_2O emissions. *Soil Biology &Biochemistry,* 106, 9-17.

Yoshida LC, Allen EB (2001) Response to ammonium and nitrate by a mycorrhizal annual grassland native shrub in Southern California. *American Journal of Botany*, 88, 1430–1436.

2.7 Table

Table 2.1 Effects of CO_2 concentrations, plant roots and AMF on mean CO_2, mean N_2O emission, *nir*K, *nir*S, and *nos*Z gene abundances and ratio of (*nir*K+*nir*S)/*nos*Z (means and standard errors are presented; n=4) under two distinct N.

			Mean CO_2 ($\mu g\ C\ m^{-2}\ d^{-1}$)	Mean N_2O ($\mu g\ N\ m^{-2}\ d^{-1}$)	Copy numbers of *nir*K (10^8 copy per g soil)	Copy numbers of *nirS* (10^7 copy per g soil)	Copy numbers of *nosZ* (10^7 copy per g soil)	(*nir*K+*nir*S)/*nos*Z
		CK	939.5±30.3	14.9±4.11	4.26±1.79	3.14±1.03	7.70±4.70	21.99±14.01
	NH_4^+	AMF	869.6±90.4	9.68±3.40	2.95±1.76	1.78±0.60	6.22±3.73	14.50±9.76
		Root	1408±138.8	11.5±2.33	3.09±2.02	1.96±0.65	6.16±3.28	7.24±3.68
aCO_2		CK	697.2±71.8	465.7±16.5	5.33±2.19	1.96±0.82	6.48±4.28	33.88±20.16
	NO_3^-	AMF	889.3±47.7	359.6±72.8	3.39±1.73	2.46±0.77	6.99±3.63	12.21±5.46
		Root	931.6±111.8	278.5±33.0	2.30±1.68	2.10±0.62	9.24±3.55	5.47±3.01
		CK	1114±135.5	17.6±2.91	2.02±1.24	2.78±1.29	22.40±17.47	6.58±4.13
	NH_4^+	AMF	1024±23.6	10.6±3.54	2.46±1.57	2.18±0.78	7.89±3.54	6.63±3.64
		Root	1867±250.5	10.8±6.86	3.40±2.52	1.52±0.71	9.16±3.92	6.07±2.41
eCO_2		CK	1004±44.7	634.2±56.0	6.62±1.96	2.33±0.96	8.27±4.03	87.71±78.45
	NO_3^-	AMF	976.8±97.3	467.4±59.0	3.41±2.05	2.88±1.06	5.73±3.62	53.01±46.47
		Root	1202±167.5	408.7±45.6	2.97±1.97	2.64±0.98	4.82±2.78	20.13±15.56

aCO_2: ambient CO_2 concentration; eCO_2: elevated CO_2 concentration.

2.8 Figures

Fig. 2.1 Effects of CO_2 concentrations, plant roots and AMF on shoot, root and total biomass under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^-fertilization. Values are means ± 1 SE (n=4). (a) Shoot biomass (CO_2 effect: $P<0.001$; Mycorrhizae effect: $P<0.001$; CO_2 × Mycorrhizae effect: $P=0.07$, N × Mycorrhizae effect: $P<0.001$; CO_2 × N × Mycorrhizae effect: $P<0.01$). (b) Root biomass: (CO_2 effect: $P<0.01$; Mycorrhizae effect: $P<0.01$; CO_2 × N × Mycorrhizae effect: $P<0.05$). (c) Total biomass: (CO_2 effect: $P<0.001$; Mycorrhizae effect: $P<0.001$; CO_2 × Mycorrhizae effect: $P=0.08$, N × Mycorrhizae effect: $P<0.01$; CO_2 × N × Mycorrhizae effect: $P<0.01$).

Fig. 2.2 Effects of CO_2 concentrations, plant roots and AMF on total soil extractable nitrate and inorganic N under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). (a) Soil extractable nitrate (N effect: $P<0.05$; Mycorrhizae effect: $P<0.001$; CO_2 × Mycorrhizae effect: $P=0.07$; N × Mycorrhizae effect: $P<0.05$). (b) Soil extractable inorganic N: (N effect: $P<0.01$; Mycorrhizae effect: $P<0.001$; CO_2 × Mycorrhizae effect: $P<0.05$; N × Mycorrhizae effect: $P<0.01$).

Fig. 2.3 Effects of CO_2 concentrations, plant roots and AMF on CO_2 fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 12^{th}

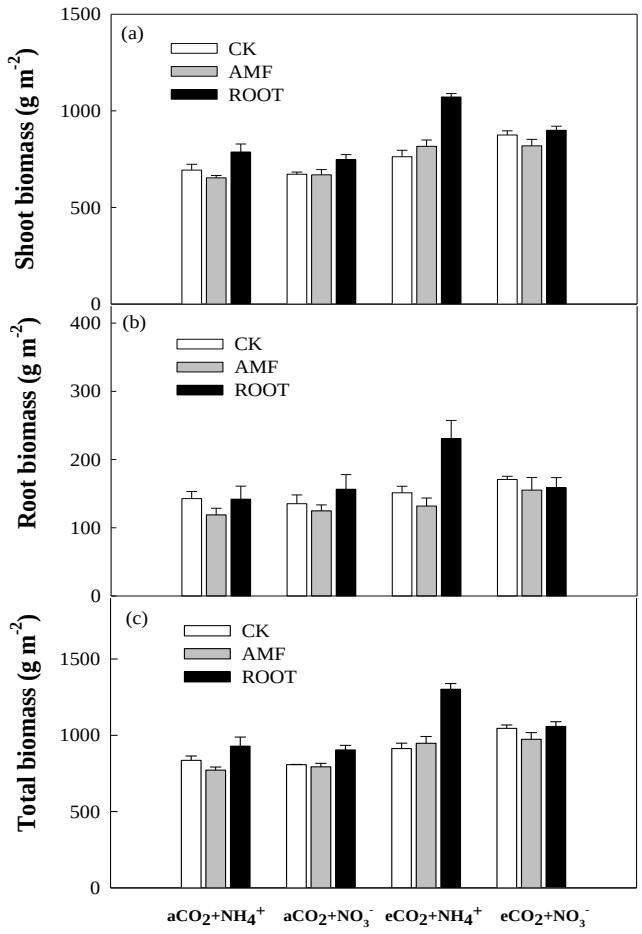
week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4) at any given time point.

Fig. 2.4 Effects of CO_2 concentrations, plant roots and AMF on N_2O fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 12^{th} week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4) at any given time point.

Fig. 2.5 Effects of CO_2 concentrations, plant roots and AMF on mean N_2O and CO_2 emissions under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 12^{th} week of plant growth. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^-fertilization. Values are means ± 1 SE (n=4). (a) Mean CO_2 emission (CO_2 effect: P=0.090; N effect: P<0.001; Mycorrhizae effect: P<0.001; N × Mycorrhizae effect: P<0.01). (b) Mean N_2O emission (CO_2 effect: P=0.044; N effect: P<0.001; Mycorrhizae effect: P<0.001; CO_2 × N effect: P<0.036; N × Mycorrhizae effect: P<0.001).

Fig. 2.6 Effects of CO_2 concentrations, plant roots and AMF on abundances and the ratio of denitrification genes under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+

fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). Only the main effect of mycorrhizae is significant ($P<0.05$). (a) gene copy numbers of *nir*K (log-transformed) (N effect: $P<0.05$; Mycorrhizae effect: $P<0.05$; $CO_2 \times N$ effect: $P<0.05$; N × Mycorrhizae effect: $P<0.05$). (b) gene copy numbers of *nir*S (log-transformed) (N × Mycorrhizae effect: $P<0.05$); (c) gene copy numbers of *nos*Z (log-transformed) (No significant effect was observed); (d) ratio of (*nir*K+*nir*S)/*nos*Z (log-transformed) (N effect: $P<0.05$; $CO_2 \times N$ effect: $P<0.05$).



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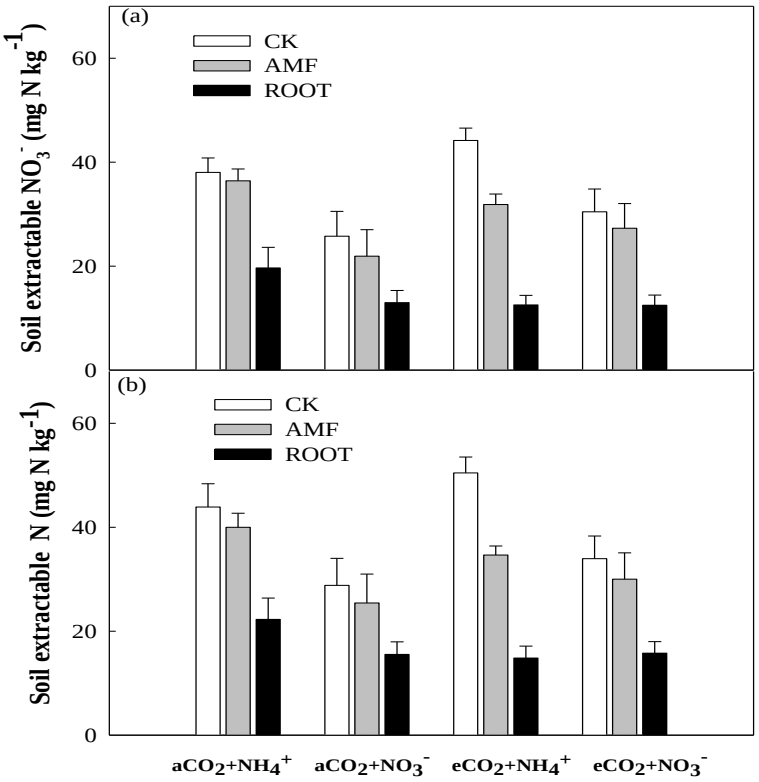


Fig. 2.2 Effects of CO_2 concentrations, plant roots and AMF on total soil extractable nitrate and inorganic N under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). (a) Soil extractable nitrate (N effect: $P<0.05$; Mycorrhizae effect: $P<0.001$; CO_2 × Mycorrhizae effect: $P=0.07$; N × Mycorrhizae effect: $P<0.05$). (b) Soil extractable inorganic N: (N effect: $P<0.01$; Mycorrhizae effect: $P<0.001$; CO_2 × Mycorrhizae effect: $P<0.05$; N × Mycorrhizae effect: $P<0.01$).

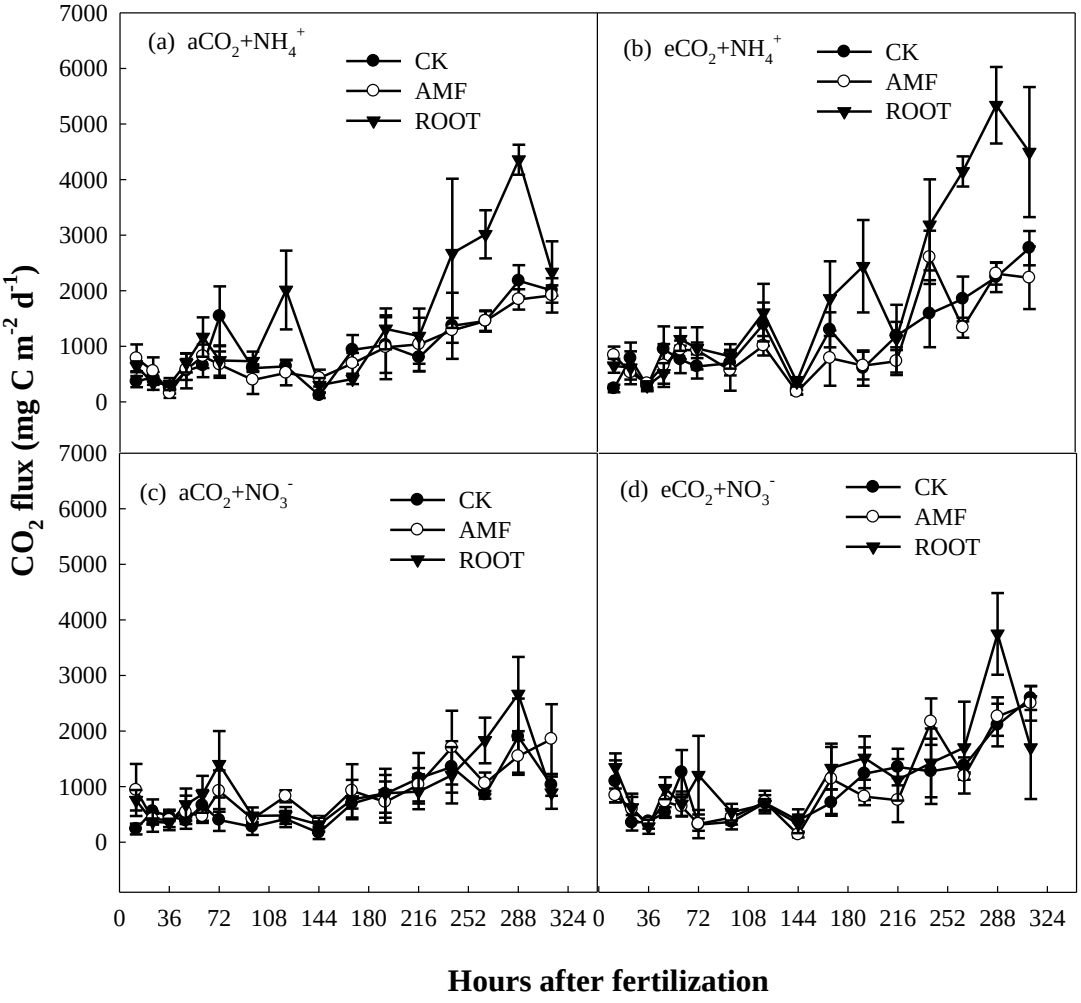


Fig. 2.3 Effects of CO_2 concentrations, plant roots and AMF on CO_2 fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 12th week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4) at any given time point.

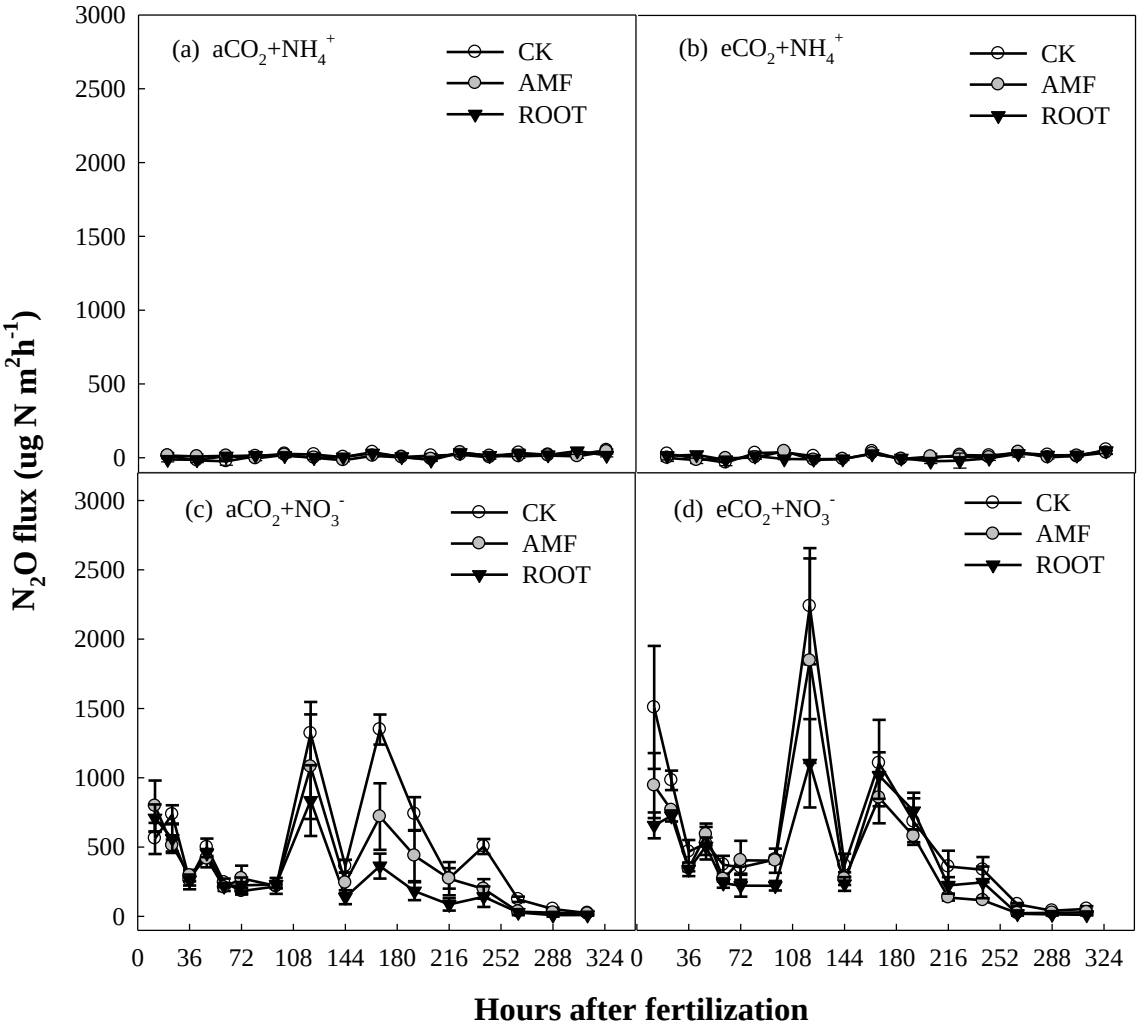


Fig. 2.4 Effects of CO_2 concentrations, plant roots and AMF on N_2O fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 12th week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4) at any given time point.

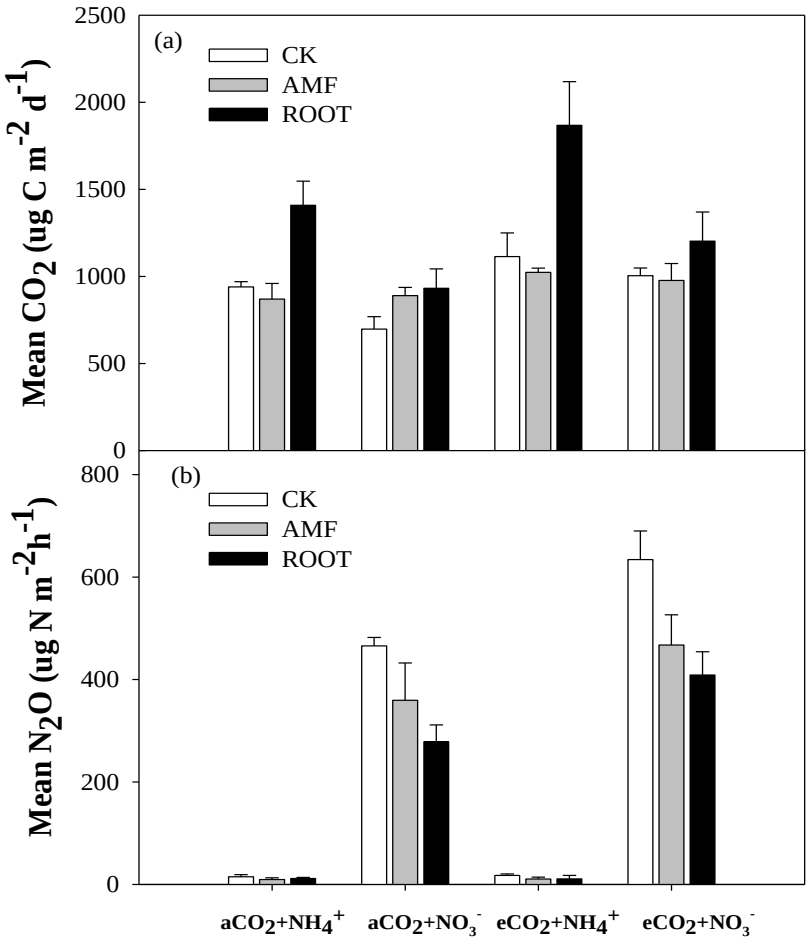


Fig. 2.5 Effects of CO_2 concentrations, plant roots and AMF on mean N_2O and CO_2 emissions under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 12th week of plant growth. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^-fertilization. Values are means ± 1 SE (n=4). (a) Mean CO_2 emission (CO_2 effect: P=0.090; N effect: P<0.001; Mycorrhizae effect: P<0.001; N × Mycorrhizae effect: P<0.01). (b) Mean N_2O emission (CO_2 effect: P=0.044; N effect: P<0.001; Mycorrhizae effect: P<0.001; CO_2 × N effect: P<0.036; N × Mycorrhizae effect: P<0.001).

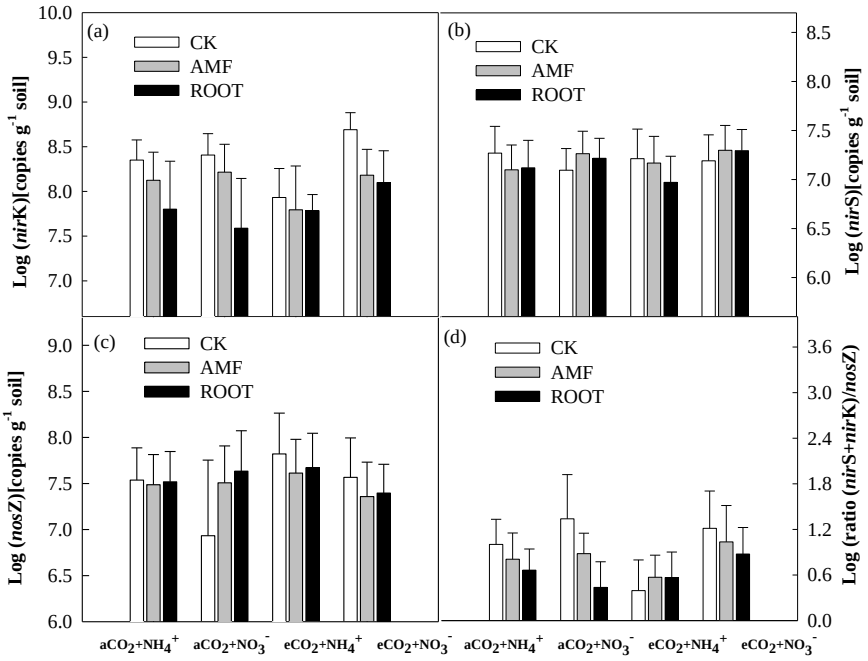


Fig. 2.6 Effects of CO_2 concentrations, plant roots and AMF on abundances and the ratio of denitrification genes under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). Only the main effect of mycorrhizae is significant (P<0.05). (a) gene copy numbers of *nir*K (log-transformed) (N effect: P<0.05; Mycorrhizae effect: P<0.05; CO_2 × N effect: P<0.05; N × Mycorrhizae effect: P<0.05). (b) gene copy numbers of *nir*S (log-transformed) (N × Mycorrhizae effect: P<0.05); (c) gene copy numbers of *nos*Z (log-transformed) (No significant effect was observed); (d) ratio of (*nir*K+*nir*S)/*nos*Z (log-transformed) (N effect: P<0.05; CO_2 × N effect: P<0.05).

2.9 Appendix

Appendix 2.1 Primers and qPCR conditions for the real-time PCR quantifications of *nir*S, *nir*K, and *nos*Z genes extracted from soils.

Appendix 2.2 *P* values of ANOVA for CO_2, N and mycorrhizae effects, and all interactions over the experimental period.

Appendix 2.3 Effects of CO_2 concentrations, plant roots and AMF on root colonization under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^-fertilization. Values are means ± 1 SE (n=4). Only the main effect of CO_2 is significant ($P<0.05$).

Appendix 2.4 Effects of CO_2 concentrations, plant roots and AMF on shoot biomass N and C:N ratio under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). (a) Shoot biomass N (CO_2 effect: $P<0.05$; N effect: $P=0.08$; Mycorrhizae effect: $P<0.001$; N × Mycorrhizae effect: $P=0.05$; CO_2 × N × Mycorrhizae effect: $P<0.05$). (b) Shoot biomass C:N: (CO_2 effect: $P<0.01$; N effect: $P<0.01$; CO_2 × Mycorrhizae effect: $P=0.06$; N × Mycorrhizae effect: $P=0.08$).

Appendix 2.5 Effects of CO_2 concentrations, plant roots and AMF on total soil extractable carbon (C) under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization;

$eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). Only the main effect of mycorrhizae is significant (P<0.05).

Appendix 2.6 Effects of CO_2 concentrations, plant roots and AMF on microbial biomass C, N and C:N. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). (a) Microbial biomass C (N × Mycorrhizae effect: P<0.05). (b) Microbial biomass N: (N effect: P<0.05; CO_2 × Mycorrhizae effect: P<0.05; CO_2 ×N × Mycorrhizae effect: P<0.05). (c) Microbial biomass C:N: (N effect: P<0.05).

Appendix 2.7 Effects of CO_2 concentrations, plant roots and AMF on CO_2 fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 8^{th} week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^--N fertilization. Values are means ± 1 SE (n=4) at any given time point.

Appendix 2.8 Effects of CO_2 concentrations, plant roots and AMF on N_2O fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 8^{th} week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d)

eCO_2+NO_3^-: elevated CO_2 and NO_3^--N fertilization. Values are means ± 1 SE (n=4) at any given time point.

Appendix 2.1 Primers and qPCR conditions for the real-time PCR quantifications of *nir*S, *nir*K, and *nos*Z genes extracted from soils

Target genes	Primer	Sequence	qPCR conditions	References
*nir*S	nirSCd3aF	AACGYSAAGGARACSGG	Six TD CL—98 °C for 10 s, 63 °C for 30 s, and 72 °C for 30 s with AT dropped by 1 °C CL−1 to 58 °C; 40 CL—98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s	Kandeler *et al.*, 2006
	nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA		
*nir*K	nirK876	ATYGGCGGVAYGGCGA	Same as *nir*S	Henry *et al.*, 2004
	nirK1040	GCCTCGATCAGRTTRTGGTT		
*nos*Z	nosZ1F	WCSYTGTTCMTCGAGCCAG	Six TD CL—98 °C for 10 s, 67 °C for 30 s, and 72 °C for 30 s with AT dropped by 1 °C CL−1 to 62 °C; 40 CL—98 °C for 10 s, 62 °C for 30 s, and 72 °C for 30 s	Henry *et al.*, 2006
	nosZ1R	ATGTCGATCARCTGVKCRTTYTC		

CL, TD, AT are short for cycles, touchdown, and annealing temperature, respectively

References:

Kandeler E, Deiglmayr K, Tscherko D, Bru D, Philippot L (2006) Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. *Applied and Environmental Microbiology*, 72, 5957–5962.

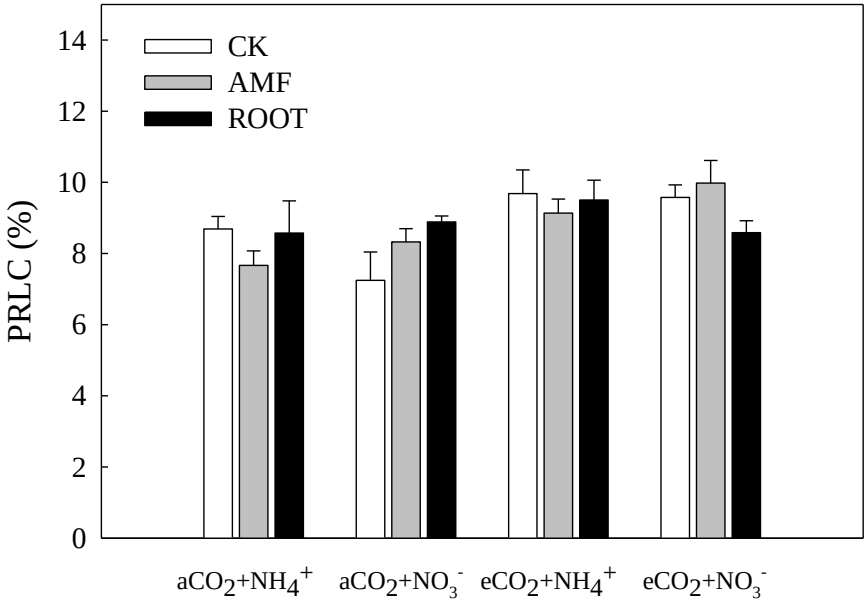
Henry S, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A, Philippot L (2004) Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *Journal of Microbiological Methods*, 59, 327–335.

Henry S, Bru D, Stres B, Hallet S, Philippot L (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. *Applied and Environmental Microbiology*, 72, 5181-5189.

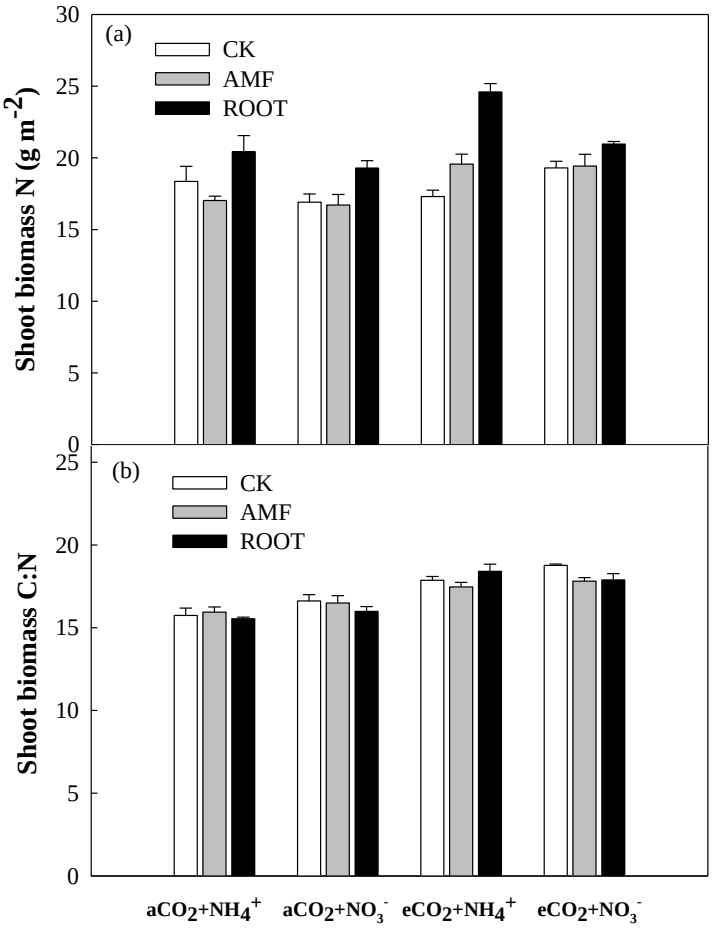
Appendix 2.2 *P* values of ANOVA for CO_2, N and mycorrhizae effects, and all interactions over the experimental period.

Variable	CO_2	N	Mycorrhizae	CO_2 ×N	Mycorrhizae ×CO_2	Mycorrhizae ×N	CO_2×N ×Mycorrhizae
Mean CO_2	*0.090*	**<0.001**	**<0.001**	0.570	0.336	**0.006**	0.612
Mean N_2O	**0.044**	**<0.001**	**<0.001**	**0.036**	0.778	**<0.001**	0.804
SB	**<0.001**	0.237	**<0.001**	0.880	*0.071*	**<0.001**	**0.003**
RB	**0.002**	0.801	**0.003**	0.517	0.430	0.105	**0.019**
TB	**<0.001**	0.276	**<0.001**	0.599	*0.077*	**0.001**	**0.002**
Shoot C	0.325	*0.056*	**0.023**	0.382	**0.002**	0.181	*0.068*
Shoot N	**0.012**	*0.077*	**<0.001**	0.412	0.111	*0.052*	**0.034**
Shoot C/N	**<0.001**	**0.004**	0.225	*0.099*	*0.056*	*0.083*	0.439
AMF infection	**0.02**	0.658	0.963	0.817	0.165	0.151	0.250
Etrx-N	0.878	**0.005**	**<0.001**	0.270	**0.037**	**0.005**	0.247
NO_3^--N	0.834	**0.008**	**<0.001**	0.299	**0.035**	**0.025**	0.233
Etrx-C	0.763	0.187	**0.006**	0.873	0.880	0.252	0.256
MBN	0.187	**0.010**	0.378	0.880	**0.042**	0.960	**0.045**
MBC	0.576	0.753	0.372	0.435	0.729	**0.029**	0.891
MBC/MBN	0.103	**0.013**	0.277	0.152	0.107	0.296	0.120
nirK	0.9932	*0.071*	**0.024**	*0.059*	0.544	0.783	0.874
nirS	0.948	0.130	0.354	0.273	0.602	**0.010**	0.401
nosZ	0.232	0.249	0.726	0.758	*0.070*	0.269	0.243
(K+S)/Z	0.782	**0.032**	*0.065*	**0.032**	0.263	0.321	0.790

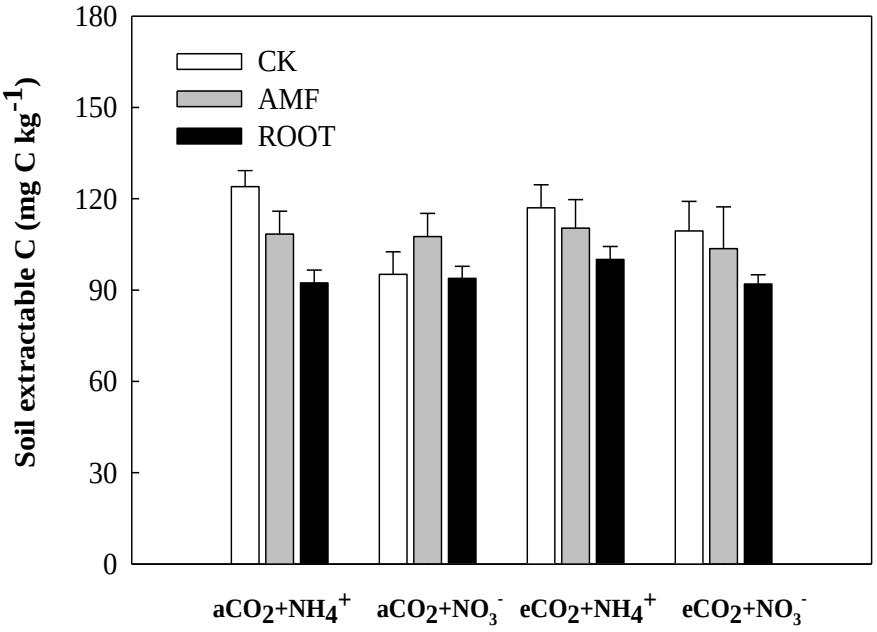
Significant effects (*** (*P*<0.01) and *(*P*≤0.05)) are shown in bold and marginal effects (0.05<*P*<0.1) are shown in italic text. SB, shoot biomass; RB, root biomass; Extr-C, soil extractable organic carbon; Extr-N, soil extractable inorganic nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; (K+S)/Z, (*nir*K+*nir*S)/*nos*Z.



Appendix 2.3 Effects of CO_2 concentrations, plant roots and AMF on root colonization under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^-fertilization. Values are means ± 1 SE (n=4). Only the main effect of CO_2 is significant (P<0.05).



Appendix 2.4 Effects of CO2 concentrations, plant roots and AMF on shoot biomass N and C:N ratio under two distinct N forms. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). (a) Shoot biomass N (CO_2 effect: $P<0.05$; N effect: $P=0.08$; Mycorrhizae effect: $P<0.001$; N × Mycorrhizae effect: $P=0.05$; CO_2 × N × Mycorrhizae effect: $P<0.05$). (b) Shoot biomass C:N: (CO_2 effect: $P<0.01$; N effect: $P<0.01$; CO_2 × Mycorrhizae effect: $P=0.06$; N × Mycorrhizae effect: $P=0.08$).



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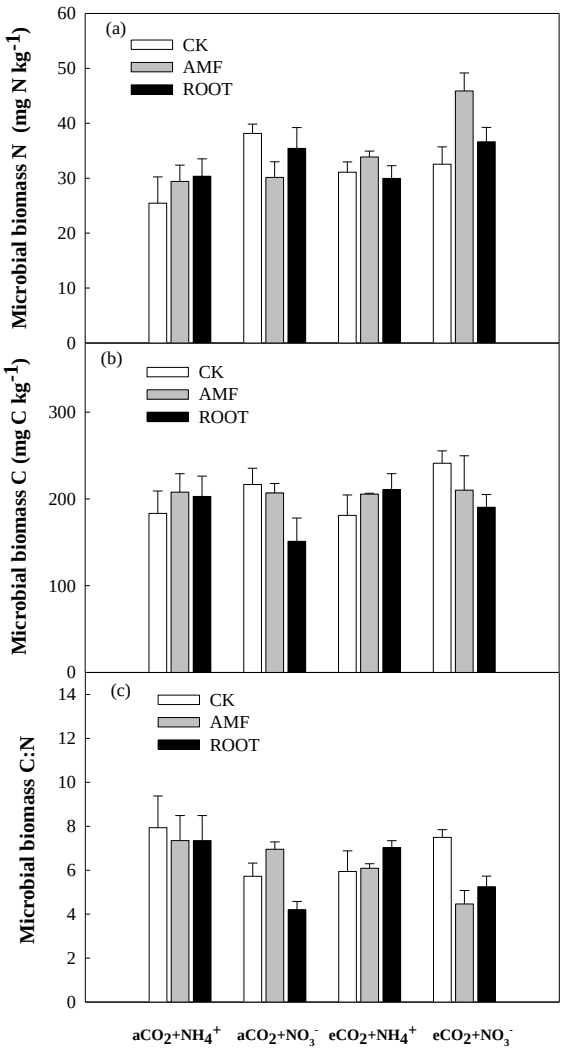
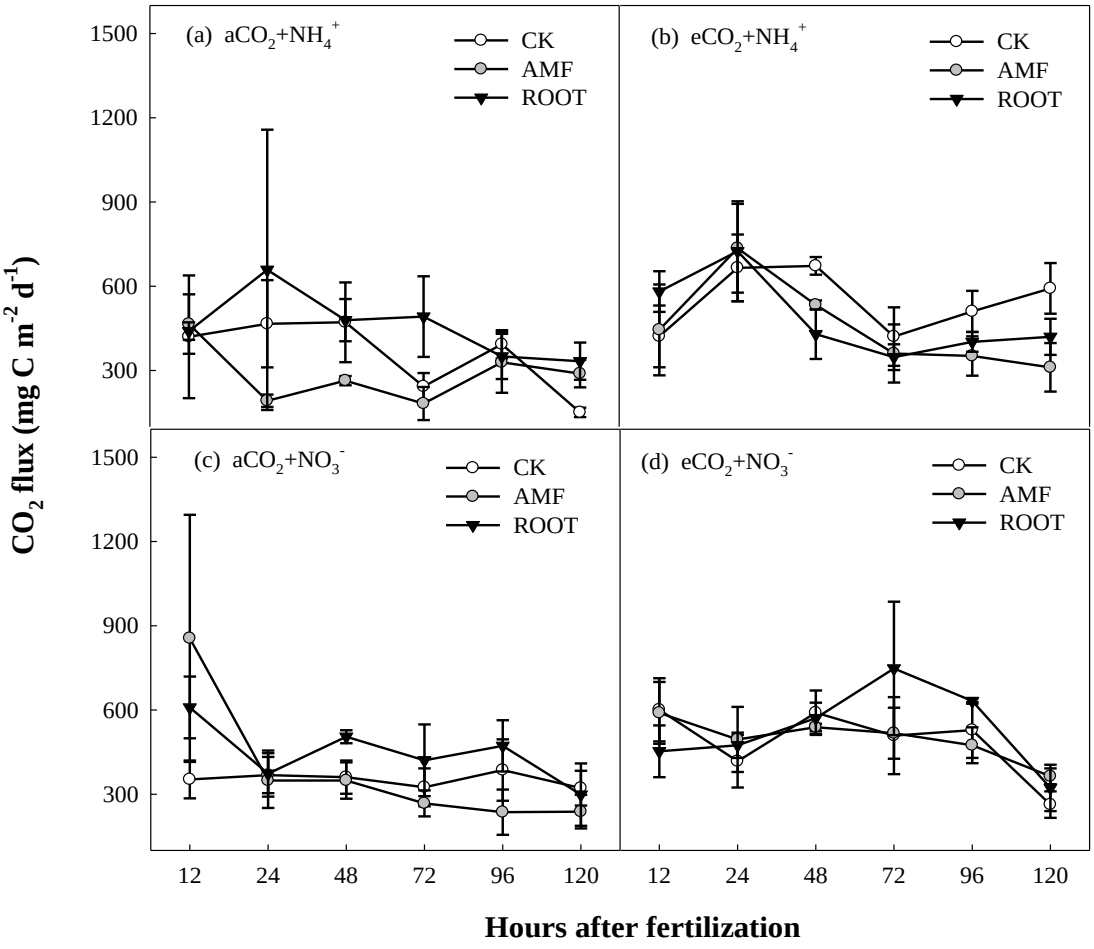
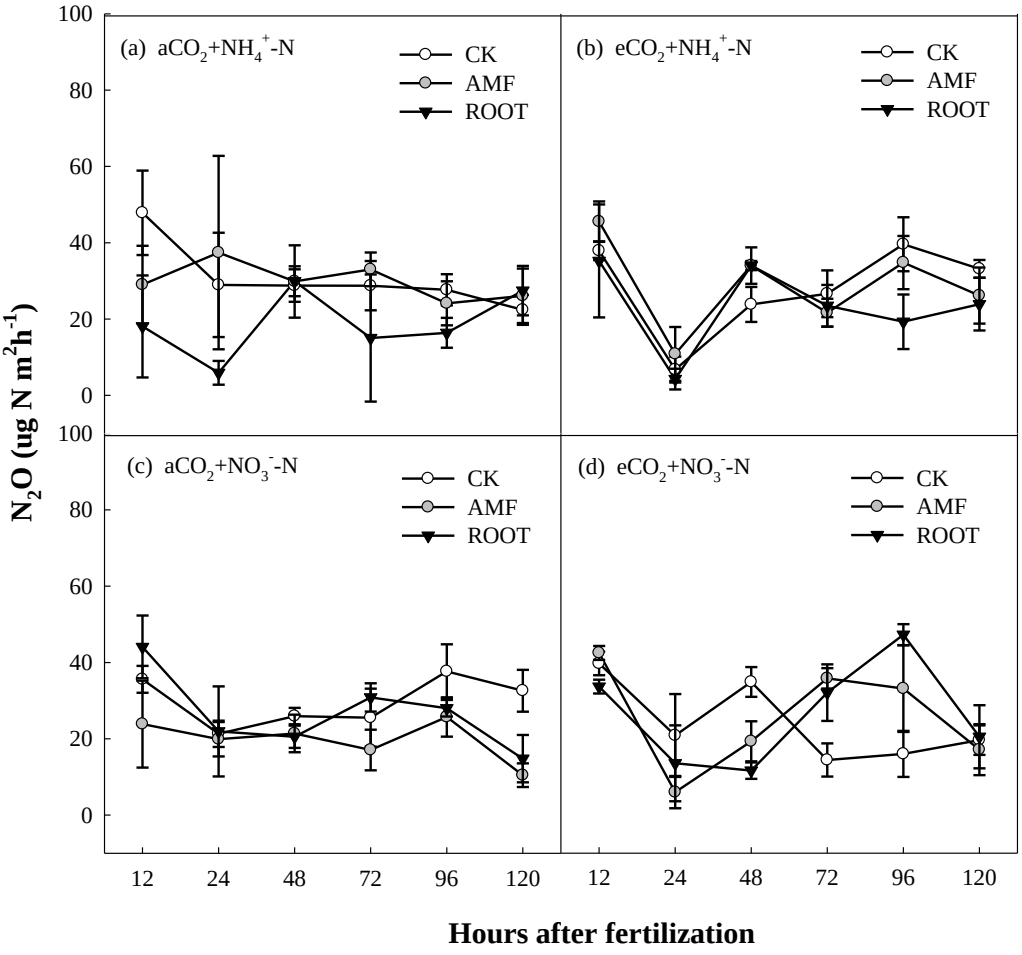


Appendix 2.6 Effects of CO_2 concentrations, plant roots and AMF on microbial biomass C, N and C:N. $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; $eCO_2+NO_3^-$: elevated CO_2 and NO_3^- fertilization. Values are means ± 1 SE (n=4). (a) Microbial biomass C (N × Mycorrhizae effect: $P<0.05$). (b) Microbial biomass N: (N effect: $P<0.05$; CO_2 × Mycorrhizae effect: $P<0.05$; CO_2 ×N × Mycorrhizae effect: $P<0.05$). (c) Microbial biomass C:N: (N effect: $P<0.05$).



Appendix 2.7 Effects of CO_2 concentrations, plant roots and AMF on CO_2 fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 8^{th} week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^--N fertilization. Values are means ± 1 SE (n=4) at any given time point.



Appendix 2.8 Effects of CO_2 concentrations, plant roots and AMF on N_2O fluxes under two distinct N forms after a water and fertilization pulse corresponding to 40 kg N ha^{-1} at 8^{th} week of plant growth. (a) $aCO_2+NH_4^+$: ambient CO_2 and NH_4^+ fertilization; (b) $eCO_2+NH_4^+$: elevated CO_2 and NH_4^+ fertilization; (c) $aCO_2+NO_3^-$: ambient CO_2 and NO_3^- fertilization; (d) $eCO_2+NO_3^-$: elevated CO_2 and NO_3^--N fertilization. Values are means ± 1 SE (n=4) at any given time point.

Chapter 3

Climate warming and ozone differentially influence N_2O fluxes in a soybean agro-ecosystem

3.1 Abstract

Nitrous oxide (N_2O) in the atmosphere is a major greenhouse gas and a depleter of stratospheric ozone. N_2O largely originates from two microbial transformation processes of soil mineral N (i.e., nitrification and denitrification). Climate change factors such as temperature increase and ozone affect N_2O fluxes but the magnitude of these effects and the underlying mechanisms remain unclear. We examined the impact of simulated warming (control + 3.6 ^oC) and elevated ozone (control + 45 ppb) on soil N_2O fluxes in a soybean agro-ecosystem in Raleigh, NC. Results obtained showed that warming significantly increased the rate and the sum of N_2O emissions by 2 to 3 fold, while elevated O_3 had no significant effect. Warming also significantly enhanced soil labile carbon, and microbial biomass carbon and nitrogen but elevated O_3 significantly reduced these parameters. In addition, we quantified the abundances of key genes responsible for nitrification (*amoA* of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), and denitirication (*nir*S, *nir*K and *nos*Z). Warming marginally enhanced AOA abundance and significantly increased the abundances of all three denitrification-related genes. In contrast, elevated O_3 slightly reduced AOB abundance and significantly decreased both N_2O production genes (*nir*S, *nir*K) and N_2O consumption gene *(nos*Z). The correlation analysis showed strong positive correlations between N_2O emissions and the abundances of denitrifiers. Together, our findings suggest that warming may stimulate N_2O mainly through increasing denitrifiers, while elevated O_3 may have little impact on N_2O emissions because of its contrasting effects on microbes that produce or consume N_2O.

Keywords: warming, ozone, nitrous oxide (N_2O), nitrification, denitrification, microbial community

3.2 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas with a global warming potential 300 times greater than that of carbon dioxide (CO_2) (Wuebbles, 2009; IPCC, 2013), and is the single most important depleter of stratospheric ozone (Ravishankara *et al.,* 2009). Globally, agricultural soils are a major source of anthropogenic N_2O emissions owing to N fertilizer and manure applications, and represent nearly 70% of the annual N_2O budget worldwide (Mosier, 1998; IPCC, 2013). N_2O is mainly produced in soils via two microbial transformation processes: nitrification (i.e., oxidation of NH_4^+ into NO_3^-) and denitrification (i.e., reduction of NO_3^- to gaseous forms, N_2O, NO and N_2) (Firestone & Davidson, 1989; Wrage *et al.*, 2001). The emission of N_2O is highly spatially and temporally variable because of the intricacy of nitrification and denitrification processes and the complex regulation of their controlling factors (Firestone & Davidson, 1989; Butterbach-Bahl *et al.,* 2013).

Global climate change factors may impact nitrification and denitirification directly or indirectly, thereby altering N_2O production (Barnard & Leadley, 2005; Brown *et al.*, 2012; Bai *et al.,* 2013; Butterbach-Bahl *et al.,* 2013). Experimental results have shown that warming can affect N_2O emission fluxes very differently, with an increase, a decline or even a neutral effect on N_2O production under different conditions (Maag & Vinther, 1996; Avrahami & Bohannan, 2009; Hu *et al.,* 2010; Dijkstra *et al.*, 2012). These inconsistent results may stem from the fact that warming impacts multiple processes, some of which may have contrasting effects on N_2O production. Climate warming may affect N_2O emissions through impacting the growth and activities of N_2O –producing microbes, availability of N

sources, and soil moisture that critically mediates nitrification and denitrification processes (Barnard & Leadley, 2005; Dijkstra *et al.,* 2012). On one hand, climate warming may stimulate nitrifiers and/or denitirifiers that produce N_2O (Horz *et al.,* 2004; Cantarel *et al.,* 2012). Also, warming may significantly enhance N mineralization and nitrification (Meillo *et al.,* 2002; Bai *et al.,* 2013), increasing the N source for N_2O emissions (Cantarel *et al.,* 2011). On the other hand, warming may reduce nitrifiers or denitrifiers through reducing N substrates or altering soil moisture. Warming can enhance plant growth and N uptake, thereby reducing soil NH4+ and/or NO3 (Zak *et al.,* 1990; Dijkstra *et al.,* 2012). Higher temperature stimulates evapotranspiration and plant NO_3^- uptake along with water, reducing soil moisture and suppressing denitrificaiton (Zhang *et al.,* 2005; Bijoor *et al.,* 2008; Dijkstra *et al.,* 2013). In contrasting to the direct effect of warming on nitrifiers and denitrifiers, elevated tropospheric ozone (eO_3) affects microbial processes of nitrification and denitrification mainly through its effect on plant growth and plant-mediated processes (Decock *et al.,* 2012), because O3 itself hardly penetrates into soil to directly affect soil microbes (Andersen, 2003). Elevated O_3 often leads to a substantial decline in plant aboveground biomass, subsequent carbon (C) allocation belowground and associated substrate availability for soil microbes (Anderson, 2003; Fiscus *et al.,* 2005; Feng *et al.,* 2008; Zak *et al.,* 2011; Ainsworth *et al.,* 2012). All these changes may alter functional diversity, composition and structure of soil microbial community with the potential influences on emissions of N_2O (Cheng *et al.,* 2011; Tang *et al.,* 2013; He *et al.,* 2014). However, very few experiments have so far examined the effect of elevated O_3 on N_2O emissions. Kanerva *et al.* (2007) reported that 3-yr exposure to elevated O_3 reduced the N_2O

flux rate in a meadow ecosystem, and then attributed this decrease to reduced denitrification resulting from lower soil labile C and microbial biomass N.

Recently, information on N_2O emission and microbial activities subjected to individual climate component has become increasingly available (Larsen *et al.,* 2011; Cantarel *et al.,* 2012; Dijkstra *et al.,* 2013). Yet, data on N_2O emissions under multiple and simultaneous environmental changes remain scarce. The scarcity of experimental data of interactive effects of multiple climate change factors on nitrification, denitirfication, and subsequent N_2O emissions prohibits any generalization (Decock *et al.,* 2012; Tang *et al.,* 2013). We initiated a long-term experimental warming and ozone experiment in a soybean agro-ecosystem to understand how elevated temperature, ozone and their interactions affect plant growth and soil C and N cycling. Taking advantage of this field study, we examined the impact of warming and ozone on N_2O fluxes, soil labile C and N pools, and the abundances of nitrifiers and denitrifiers that are primarily responsible for N_2O emissions.

3.3 Materials and Method:

Site description

We initiated a long-term experiment in June 2013 to investigate the response of a soybean agroecosystem to elevated atmospheric O_3 and temperature conditions using an air exclusion system (AES, 3m × 10m). The experimental site is located at the Lake Wheeler Experimental Station, 5 km south of North Carolina State University, Raleigh, North Carolina, USA (35° 43′N, 78° 40′W; elevation 120 m). The soil is an Appling sandy loam

(fine, kaolinitic, thermic Typic Kanhapludult), well drained with a pH of 6.2, and contained 12.5 g C and 0.94 g N kg^{-1} soil when the experiment started. Before O_3 and warming treatments were initiated, the soil was repeatedly turned-over using a disc implement and rotovator.

This experiment was a 2×2 factorial design with four treatments assigned into three blocks. Four different treatments were: (a) charcoal-filtered air and ambient temperature (CF); (b) charcoal-filtered air plus 3.6ºC increase in temperature (+T); (c) charcoal-filtered air plus ambient temperature and 1.4 times ambient ozone (+O_3); (d) charcoal-filtered air + warming +1.4 times ambient ozone (+T+O_3). The purpose of filtration of ambient air with activated charcoal was to reduce ambient O_3 concentrations to levels considered nonphytotoxic to soybeans. The seasonal daily average concentrations (12-h) of O_3 and degree of temperature over the experimental duration are shown in Table 3.1.

Soybean (cultivar Jake) was planted in the first week of June each year into AES plots. O_3 was generated from O_2 by corona discharge (model TG-20, Ozone Solutions, Hull, IA, USA). The O_3 concentration in each plot was monitored at canopy height with a UV photometric O_3 analyzer (model 49C, Thermo Environmental Instruments Co., Franklin, MA, USA). Air was warmed by electrical resistance heaters and solar water heaters. Each warmed plot used four heater elements that operate 24 h a day and two water-to-air exchangers. The water circulates through an array of black polyethylene pipes during the daylight hours, which is heated by the sun. The water then passes through the heat exchangers as forced air moves across the metal surfaces, increasing the air temperature by

convection. The addition of warm, humidified air dispensed into the treatment plot to achieve temperature increases. Air temperature and relative humidity were monitored using HOBO sensors (model U23-001, HOBO Pro V2 Temp/RH Datalogger, Onset Computer Corporation, Bourne, MA, USA). Soil temperature was measured with an Enviropro dielectric soil probe (model EP100D, Apcos Pty Ltd, Adelaide, Australia).

During soybean growing seasons, plants were irrigated with drip lines to prevent water stress. There were a total of three harvests of aboveground biomass in August, September and October and two harvests of root biomass in August and September each year. Aboveground biomass was harvested at soil surface. Three soil cores of 25 cm in diameter were taken to a 20-cm depth in each plot to harvest three plant roots. Roots were washed with tap water. Aboveground and root biomass were weighed after oven drying at 70 °C for 72 h.

N_2O measuring

Each AES plot has 8 rows with a total width of 3 meters with two static gas chambers randomly installed into all the plots. Gas samples were taken within 24-48 hours following an irrigation event approximately every 10 days. The chambers (sampling volume is 6392 ml) were closed with a lid, and fitted with a rubber septum to allow gas sampling via syringe. Gas samples (5 ml) were taken with 20 ml PE syringes (Beceton Dickinson Franklin Lakes, NJ, USA) and immediately injected into N_2-preflushed 12 mL vials at time 0 and 30 min after the closure of lids. Air temperature and soil moisture were also recorded during each sampling period. Gas samples were analyzed within 24 h on a gas chromatograph fitted with an electron capture detector (ECD) and a flame ionization detector (FID) (Shimadzu GC –

2014, Kyoto, Japan) with an autosampler (Shimadzu AOC-5000 Auto-Injector). The N_2O fluxes and cumulative N_2O emissions were calculated using formulas described chapter 2.

Soil sampling

We used a 5-cm diameter soil corer to take six to seven soil cores to 15 cm depth after each harvesting. Soil cores were separated into 0-7.5 cm and 7.5-15 cm depth fractions. All soil samples were sealed in plastic bags, stored in a cooler and transported to the laboratory.

Soil samples were mixed thoroughly and sieved through a 2-mm mesh within 24 h of the field sampling and we carefully removed all visible residues and plant roots. Subsamples (~ 20 g) were then taken immediately and stored at -20 °C for DNA analysis and the remainder soils were stored at 4 °C for other microbial and chemical analyses. A 10-g soil subsample was oven-dried at 105 °C for 48 h and weight for the determination of the water content.

Soil sample analysis

Soil microbial biomass C and N. Soil microbial biomass C (MBC) and biomass N (MBN) were measured using the fumigation-extraction method (Vance *et al.,* 1987). 20-g dry weight soil samples were fumigated with ethanol-free chloroform for 48 h and then extracted with 50 mL of 0.5 M K_2SO_4 by shaking for 30 min. Another 20-g sample of nonfumigated soil were extracted with 50 mL of 0.5 M K_2SO_4 shaking for another 30 min. Soil extractable organic C in both fumigated and non-fumigated extracts was determined using a TOC analyzer (Shimadzu TOC-5050A, Shimadzu Co., Kyoto, Japan). Soluble inorganic N in the

extracts of fumigated and non-fumigated soils was quantified on the Lachat flow injection analyzer (Lachat Instruments, Milwaukee, WI, USA) after digestion with alkaline persulfate (Cabrera & Beare, 1993). The differences in extractable organic C and inorganic N between fumigated and non-fumigated soils were used to calculate MBC and MBN using a conversion factor of 0.33 (k_{EC}) and 0.45 (k_{EN}), respectively (Vance *et al.,* 1987; Tu *et al.,* 2006).

Soil extractable C and N. The concentration of organic C in non-fumigated soil extracts was used to represent soil extractable C. The extractable inorganic N referred to the sum of NH_4^+ and NO_3^- in non-fumigated soil extracts (Cheng *et al.,* 2012).

Soil microbial respiration. An incubation-alkaline method was used to determine soil heterotrophic respiration (Coleman *et al.,* 1978). Briefly, 20-g dry soil samples were weighed in a 200-mL beaker, adjusted to a moisture level of 60% water holding capacity and placed in 1-L Mason jars. Then, 5 mL of 0.25 M NaOH were added in a 50-mL beaker and suspended in each jar. All jars were incubated at 25 °C in the dark for 2 weeks. Each week, respired CO_2 was trapped in NaOH. After the first week incubation, NaOH solutions were replaced with fresh solutions. The respired CO_2 from the soil was titrated with 0.125 M HCl to determine the soil microbial respiration (SMR). SMR rate was expressed as mg CO_2 kg^{-1} soil d^{-1} by averaging the data across two1-wk incubations (Cheng *et al.,* 2011).

Net soil N mineralization. Potential N mineralization was determined following a 4-wk incubation at 25 °C in the dark. After incubation, soil NH_4^+ and NO_3^- were extracted with 50 mL of 0.5 M K_2SO_4 by shaking for 30 min. Inorganic N concentrations were also measured

on the Lachat flow injection analyzer. Net mineralized N (NMN) was then calculated by the difference in extractable total inorganic N between un-incubated and incubated soil subsamples.

Abundances of functional genes responsible for nitrification and denitrification in soil. Fresh soil (0.50 g) was extracted for nucleic acid using FastDNA SPIN kit (MP Bio, Solon, OH, USA) according to the manufacturer's instructions. Soil DNA quality and size were checked by electrophoresis on a 1% agarose gel. The copy numbers of AOA *amoA*, AOB *amoA*, *nirS*, *nirK* and *nosZ* genes of each soil DNA sample were determined using quantitative real-time polymerase chain reaction (PCR) (CFX96 Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA) with the primers given in Appendix 3.1. The final qPCR reaction volume was 20 µl, with a of 2 µl of template DNA, 14 µl 1× SsoAdvancedTM SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and 2 µl of each primer. The standard curve for determining the gene copy number was developed with the agarose gel-purified PCR products based upon the method of Chen *et al.* (2015b). All the qPCR reactions followed the conditions shown in Appendix 3.1.

Statistical analysis

We examined results for the entire growing season in 2015 and used the linear mixed model (Littell *et al.*, 2006) to test the main effects of temperature, O_3 and the interaction of temperature and O_3, and whether these changed over time. We employed a set of covariance structures including compound symmetric model (CS), the first-order autoregressive model [AR (1)], and autoregressive with random effect to reduce autocorrelation. The *P* values for

treatments and interaction terms were reported based on the covariance structure that minimized Akaike information criterion (AIC) and Bayesian information criterion (BIC) (Littell *et al.*, 2006). Data for plant biomass, soil and microbial parameters and gene copy numbers were subjected to the analysis of variance using the mixed model. All statistical analyses were performed using the SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). For all tests, the significant differences were determined at the 95% probability level.

3.4 Results

Characteristics of climate change treatments

During the experiment period, the difference in mean daily temperature between control and elevated temperature treatments (+T and +T+O_3) was 3.6 ± 0.07 ^oC (Table 3.1, Appendix 3.3a). Mean daily (12-h) O_3 differences between the control and elevated ozone treatments (+O_3 and +T+O_3) was 45.6 ± 1.0 ppb (Table 3.1, Appendix 3.3b). Meteorological variables (i.e., air humidity, soil moisture and soil temperature) recorded on days of sampling gas indicated higher soil temperature in the warming treatments (+T and +T+O_3) compared with the control (CF). No significant differences in air humidity and soil moisture were found for the sampling dates during the experimental study (Table 3.1).

Plant biomass

Over the growing season, warming significantly reduced aboveground biomass by 11% (P<0.05, Fig. 3.1a), while marginally increased root biomass by 11% (P=0.055, Fig. 3.1b). However, elevated O_3 reduced both aboveground and belowground biomass by 7%

(P=0.066, Fig. 3.1a) and 17% (P<0.05, Fig. 3.1b), respectively. There were also significant effects of the warming×O_3 interaction on both aboveground (P<0.05, Fig. 3.1a) and belowground biomass (P<0.05, Fig. 3.1b).

Soil extractable organic C and N

Warming had no significant effect on soil extractable organic C at the surface soil layer (0-7.5 cm), but significantly enhanced it at the subsoil layer (7.5-15 cm) (Appendix 3.2, 3.4). Elevated O_3 reduced soil extractable organic C (Appendix 3.2, 3.4). Compared to the charcoal-filtered plots (CF, +T), soil extractable organic C under elevated O_3 (+O_3, +T+O_3) was 12% (P<0.05) and 10% (P=0.09) lower at the surface soil and subsoil soil layer, respectively. No significant effect of the warming × O_3 interaction on soil extractable C was observed at both soil layers (Appendix 3.2, 3.4)

In general, warming tended to increase concentrations of total extractable inorganic N (Appendix 3.2, 3.5). Soil extractable N in warming plots increased by, on average, 10% (P>0.1) and 11% (P=0.08), respectively, at the surface soil and subsoil layer over the growing season (Appendix 3.2, 3.5). However, neither the O_3 treatment nor the warming×O_3 interaction were statistically significant (Appendix 3.2, 3.5).

Microbial biomass C and N

Warming enhanced MBC with an increase of 11% (P<0.05) and 16% (P=0.08) at the surface soil and subsoil layer, respectively (Fig. 3.2a, 3.2b, Appendix 3.2). Similar to the effect on MBC, warming tended to enhance MBN at both soil layers with an increase of 19%

(*P*=0.1) and 29% (*P*=0.09), respectively (Fig. 3.2c, 2d, Appendix 3.2). However, elevated O_3 significantly reduced both MBC and MBN at both soil layers (Fig. 3.2, Appendix 3.2). And the interaction of warming × O_3 was only statistically significant for the top soil layer on MBC and no significant effect of the warming × O_3 interaction on MBN was observed (Fig. 3.2, Appendix 3.2)

Soil microbial respiration (SMR)

Over the experimental period, warming significantly increased SMR rates only in the surface soil layer, leading to an average increase of 24% (Appendix 3.2, 3.6). However, this increase resulted primary from warming-induced enhancement in the late growing season of the experiment (Appendix 3.6a). While, elevated O_3 significantly reduced SMR rates in both soil layers (Appendix 3.2, 3.6). Compared with charcoal-filtered air, SMR under elevated O_3 was 18% (*P*<0.01) and 22% (*P* <0.05) lower at the surface soil and the subsoil layer, respectively. And the interaction of warming × O_3 was only statistically significant for the top soil layer (Appendix 3.2, 3.6a).

Net soil N mineralization

No significant warming effect on net soil N mineralization at both surface soil and subsoil layers was observed (Appendix 3.2, 3.7). However, a significant warming×O_3 interaction was observed at the surface soil layer (Appendix 3.2, 3.7). Compared with the CF treatment, the warming treatment (+T) alone significantly increased net soil N mineralization

rate on average by 20% (Appendix 3.7a). Additionally, elevated O_3 significantly reduced net soil N mineralization at the subsoil layer (Appendix 3.2, 3.7b).

N_2O emissions

Over the course of the study, N_2O fluxes ranged from -0.47 to 5.74 mg N m^{-2} d^{-1} across the experimental treatments and showed significant variation (Fig. 3.3). Overall, warming significantly increased N_2O emission, while O_3 has no significant effect (Fig. 3.3). Cumulative N_2O was 49.9, 126.0, 52.8 and 77.8 mg N m^{-2} in the CF, +T, +O_3 and +T+O_3 treatments, respectively (Fig. 3.3). Compared with CF treatment, the warming treatment significantly increased cumulative N_2O, on average, by 2 to 3 folds (P<0.05, Fig. 3.3) and the +T+O_3 treatment enhanced it by 56% (P<0.05, Fig. 3.3), while +O_3 treatment had no impact on cumulative N_2O over the study period (P>0.05, Fig. 3.3).

Gene abundances of ammonia-oxidizing microbes

In general, warming tended to increase AOA abundance (Table 3.2, Appendix 3.8). There were no significant effects of O_3 and no interaction of warming × O_3 on AOA abundance (Table 3.2). However, elevated O_3 tended to reduce AOB abundance (Fig. S6, Table 2). Neither the warming treatments nor the warming × O_3 interaction had significant effects on AOB abundance (Table 3.2).

Abundances of functional genes that control denitrification

Warming significantly increased the copy numbers of *nir*K and *nir*S, key genes involved in denitrification and N_2O production, while elevated O_3 significantly reduced them (Table 3.2, Appendix 3.9). Compared with CF treatment, the warming (+T) treatment alone significantly increased *nir*K and *nir*S gene abundance, on average, by 82% and 38%, respectively, while the elevated O_3 treatment ($+O_3$) significantly reduced them by 47% and 45%, respectively (Table 3.2, Appendix 3.9). However, there were no significant interactions of warming × O_3 on the two denitrification gene abundances (Table 3.2).

Similar to the effects on *nir*K and *nir*S, warming also significantly enhanced *nos*Z gene abundance, while elevated O_3 significantly reduced it (Table 3.2, Appendix 3.9). The mean copy numbers of nitrous oxide reducers (*nos*Z) was estimated at 5.81×10^7, 6.07×10^7, 2.56×10^7, 7.05×10^7 in the CF, +T, $+O_3$ and $+T+O_3$ treatments, respectively. Compared with CF treatment, elevated O_3 ($+O_3$) treatment alone significantly reduced the copy numbers of *nos*Z gene (Table 3.2, Appendix 3.9). Neither elevated temperature (+T) nor the combination of elevated temperature and ozone ($+T+O_3$) had any significant effect on *nos*Z (Table 3.2, Appendix 3.9).

Relationships between field N_2O fluxes, plant biomass, soil characteristics and N-transforming gene abundances

As shown in Table 3.3, the copy numbers of *nir*K and *nir*S were significantly positively related to the field N_2O emission. Soil temperature, NO_3^-concentration and microbial biomass was also significantly positively correlated with N_2O emission. Significant

correlations were also observed between soil extractable organic C, microbial biomass and the abundances of *nir*K and *nir*S.

3.5 Discussion

Soil N_2O emissions are the dominant source to the atmosphere and are primarily controlled by two microbial processes, nitrification and denitrification (Firestone & Davidson, 1989; Butterbach-Bahl *et al.,* 2013). Also, plants compete against nitrifiers and denitrifiers for the N source, leading to complex plant-microbial interactive controls over N_2O emissions. Although global climate change factors such as warming, elevated CO_2 and O_3 can affect soil N_2O emissions (van Groenigen *et al.,* 2011; Decock *et al.,* 2012; Ward *et al.,* 2013; Wu *et al.*, 2017), the primary drivers and major pathways through which N_2O emissions are modulated are not fully understood.

Our results showed that warming increased N_2O emissions and this stimulation was positively correlated with microbial biomass C and N, suggesting that the increased N_2O emission was caused by warming-induced changes in soil microbial biomass and community composition. It is generally accepted that warming enhanced plant biomass and productivity and consequently increased C input through enhancing rhizodeposition or litter inputs, increasing C availability to soil microbes (Rustad *et al.,* 2001; Wu *et al.,* 2011; Lu *et al.,* 2013). As soil microbes are commonly C-limited, an increase in below-ground carbon input could increase soil microbial biomass and activities under future climate change conditions (Hu *et al.,* 1999; Zak *et al.,* 2000; Wu *et al.,* 2011). Results obtained in this study showed

warming enhanced soil microbial biomass C and N (Fig. 3.2), possibly due to increased root exudates and secretions in the rhizosphere.

AOA and AOB regulate the rate-control step of nitrification that produce N_2O under aerobic conditions (Schreiber *et al.,* 2012; Stieglmeier *et al.,* 2014). AOB are traditionally believed to be autotrophic and do not have the capacity or need for energy from organic matter in soil (De Boer & Kowalchuk, 2001; Xia *et al.,* 2011). However, there is increasing evidence suggesting that AOA are mixotrophic and utilize both inorganic and organic C (Hallam *et al.,* 2009; Zhang *et al.,* 2010). Therefore, increased plant root biomass under warming (Fig. 3.1b) could stimulate the growth of AOA. Higher temperature could also provide beneficial environment through directly stimulating AOA metabolic activity (Karhu *et al.,* 2014; Hu *et al.,* 2016) and stimulate organic matter mineralization and enhance nitrogen availability (Melillo *et al.,* 2002; Bai *et al.,* 2012; Frey *et al.,* 2013), as evidenced by 20% increases of mineralization by warming (+T) at the surface soil layer in our experiment (Appendix 3.7). More broadly, warming stimulation of N mineralization and nitrification may elicit higher N_2O emission through denitrification, particularly when temperature is in the early and late growing season while plant N need is low. Our results showed that warming increased AOA *amoA* abundance by 42% (P=0.076), but had no effect on the abundance of AOB *amoA* (P=0.148), indicating that AOAs are more responsive to elevated temperature than AOBs (Tourna *et al.,* 2008; Hu *et al.,* 2016).

Most denitrifiers are heterotrophs that rely on organic compounds for energy, warming stimulation of plant growth may enhance denitrification when oxygen is limiting. Because

denitrifiers are highly diverse, it is almost impossible to quantify the species composition at this stage (Wallenstein *et al.,* 2006). However, the function genes that encodes enzymes for nitrite reductase (Nir) have been well established for studies of denitrifier diversity (Braker *et al.,* 1998; Wallenstein *et al.,* 2006). Nitrite reductase (Nir) is the key enzyme in the dissimilatory denitrification process (Braker *et al.,* 1998). There are two structurally different enzymes catalyzing nitrite reduction: the copper and cytochrome cd_1- nitrite reductase encoded by two function gene *nir*K and *nir*S, respectively (Braker *et al.,* 1998, 2001). Our results showed that warming had a positive impact on the abundance of both *nir*K and *nir*S. Some studies have shown that *nir*K-type denitrifiers were more sensitive to the alterations in temperature (Szukics *et al.,* 2010; Cantarel *et al.,* 2012; Wertz *et al.,* 2013). However, Cui *et al.* (2016) conducted an incubation experiment using alkaline soils and reported that the abundance of *nir*S-type denitrifiers, not *nir*K-type denitrifers increased significantly with increasing temperature. This might be because of different response patterns of these two-type denitrifiers to environmental factors due to the niche differentiation between them (Jones & Hallin, 2010). In addition, we observed that the abundances of *nos*Z denitrifiers increased significantly under warming (Table 2).

Moreover, N_2O emissions through the growing seasons enhanced significantly under warming in our study. Both nitrifiers and denitrifiers may be involved in soil N_2O emissions (Wertz *et al.,* 2013). In our study, N_2O emissions were correlated with soil microbial biomass and NO_3^- concentration but not with soil NH_4^+, which suggests that denitrification was the major source of N_2O. The strong positive correlations between N_2O emissions and the

abundances of denitrifiers suggested the direct effect of stimulating soil denitrifier activity enhanced N_2O production under warming (Table 3). This aligns well with results from the previous experiments in grassland ecosystems through promoting substrate availability under climate warming (Cantarel *et al.*, 2012; Keil *et al.*, 2015). However, Liu *et al.* (2016) showed that warming significantly reduced N_2O fluxes across five years in agroecosystems due to lower soil water content. Previous warming experiments reported that higher temperature leads to higher evapotranspiration with rapid soil drying, which could reduce soil microbial turnover (Allison & Treseder, 2008). Therefore, the denitrification process would be limited in a dry environment (Dijkstra *et al.*, 2012). In this study, we did not observe significant difference on soil moisture during the experimental period among all the treatments (Table 3.1). Taken together, our results indicated that higher temperature increased the abundance of denitirfiers, while had no significant effect on the abundance of nitrifiers, which could result in a positive response of soil N_2O emission to warming. Therefore, how N_2O fluxes respond to warming is complex and should be studied carefully under different conditions.

Elevated O_3 often suppresses plant growth, productivity and yield (Andersen *et al.*, 2003, Fiscus *et al.*, 2007; Ainsworth *et al.*, 2012). In the current study, elevated O_3 ($+O_3$, $+T+O_3$) significantly reduced belowground biomass by 17% (Fig. 3.1, $P<0.05$), while marginally decreased aboveground biomass by 7% (Fig. 3.1, $P=0.066$) indicating that soybean roots may be more seriously affected than shoots after long-term exposure to elevate O_3. This response is consistent with the shifts in carbon allocation away from belowground

toward aboveground biomass that other O_3 exposure studies have previously observed (Cooley & Manning, 1987; Grantz *et al.,* 2006; Tang *et al.,* 2015)

It has been proposed that because O_3 does not penetrate into the soil beyond a few milimeters to affect soil microbial communities directly, the effects of O_3 on the belowground processes should be indirectly mediated by plant genotype or the diversity of plant communities (Talhelm *et al.,* 2009; Li *et al.,* 2013; He *et al.,* 2014). However, few studies have been focused on the effect of elevated O_3 on microbial community functions. For example, no detectable effects of O_3 on any soil microbial parameters were observed in a no-till wheat-soybean rotation agro-ecosystem (Cheng *et al.,* 2011), whereas the nitrifying and denitrifying enzyme activities were significantly affect by O_3 but the abundances of some key functional genes (AOA *amoA*, AOB *amoA*, *nir*K and *nir*S) involved in N cycling in a rice-wheat rotation system were not altered (Chen *et al.,* 2015a). Recently, a few studies indicated that elevated O_3 altered the functional diversity of soil microbial communities (Holmes *et al.,* 2006; Chung *et al.,* 2006; Li *et al.,* 2013; He *et al.,* 2014). Here we showed that elevated O_3 significantly reduced MBC, MBN and soil microbial respiration rate (Fig. 3.2, Appendix 3.6). Accordingly, elevated O_3 marginally reduced AOB abundances (P=0.069, Table 3.2) and significantly decreased the abundances of the key genes involved in N_2O production (*nirK* and *nir*S, P<0.05, Table 3.2) and consumption (*nos*Z, P<0.05, Table 3.2).

Interestingly, no effects of elevated O_3 on N_2O emissions were observed in our study. This is consistent with results from previous experiments in a soybean agro-ecosystem

(Decock & Six, 2012; Decock *et al.*, 2012), which provided mechanistic evidence that elevated O_3 had no effect on soil moisture, NH_4^+ and NO_3^- concentration and C availability. Nevertheless, Kanerva *et al.* (2007) first reported that elevated O_3 reduced N_2O emissions, which was attributed to reduced carbon input after three 3-year exposure to eO_3. In contrast, the effect of elevated O_3 on N_2O was still lacking in our field experiment, despite observed decreases in root biomass, soil microbial biomass C and N, and the abundances of nitrifiers and denitrifiers. The unresponsiveness of N_2O emissions in our study might be caused by counteracting effects on the production and consumption gene that control N_2O emissions.

Our results showed that the combination of warming and ozone reduced plant biomass compared with charcoal filtered (CF) treatment (Fig. 3.1), suggesting that warming and O_3 can interact to negatively affect plant growth. While, elevated O_3 tended to decrease soil extractable C and N, and microbial parameters under warming over the course of experiment. This indicated that added O_3 prevented a portion of warming-induced stimulation in these parameters (Fig. 3.2, Appendix 3.4, 3.5, 3.6, 3.7). In addition, we found warming promoted abundance of some eO_3-suppressed genes to ambient, or even to warming-induced levels. For example, the abundances of AOB *amoA*, *nir*K and *nir*S gene exhibited charcoal filtered (CF) control treatment levels under $+T+O_3$. And the abundance of *nos*Z were returned to warming level under $+T+O_3$ treatment. Moreover, elevated O_3 tended to reduce N_2O emission under warming (Fig. 3.3). This indicated that added O_3 reduced a portion of the warming-induced stimulation in N_2O production. Our results suggest that the interactive effects by warming and O_3 are much more complex and highly uncertain.

To the best of our knowledge, this work is the only one study that reported effects of eO_3 and warming + eO_3 on nitrification, denitrification and N_2O emissions. Therefore, more experiments are needed to predict and interpret responses of N_2O emissions to elevated O_3 in different ecosystems and under a broader range of climate change conditions.

3.6 References:

Ainsworth EA, Yendrek CR, Sitch S, Collins WJ, Emberson LD (2012) The effects of tropospheric ozone on net primary productivity and implications for climate change. *Annual Review of Plant Biology,* 63, 637–661.

Allison SD, Treseder KK (2008) Warming and drying suppress microbial activity and carbon cycling in boreal forest soils. *Global Change Biology,* 14, 2898–2909.

Andersen CP (2003) Source–sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytologist,* 157, 213–228.

Avrahami S, Bohannan BJM (2009) N_2O emission rates in a California meadow soil are influenced by fertilizer level, soil moisture and the community structure of ammonia-oxidizing bacteria. *Global Change Biology,* 15, 643–655.

Bai E, Li SL, Xu WH, Li W, Dai WW, Jiang P (2013) A meta-analysis of experimental warming effects on terrestrial nitrogen pools and dynamics. *New Phytologist,* 199, 441-451.

Barnard R, Leadley P (2005) Global change, nitrification, denitrification: a review. *Global biogeochemical cycles*, 16, GB1007.

Bijoor NS, Czimczik CI, Pataki DE, Billings SA (2008) Effects of temperature and fertilization on nitrogen cycling and community composition of an urban lawn. Global Change Biology, 14, 2119–2131.

Braker G, Fesefeldt A, Witzel KP (1998) Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples. Applied and Environmental Microbiology, 64, 3769–3775.

Braker G, Schwarz J, Conrad R (2010) Influence of temperature on the composition and activity of denitrifying soil communities. *FEMS Microbiology Ecology,* 73,134–148.

Braker G, Zhou ZJ, Wu LY, Devol AH, Tiedje JM (2000) Nitrite reductase genes (nirK and *nir*S) as functional markers to investigate diversity of denitrifying bacteria in Pacific northwest marine sediment communities. Applied and Environmental Microbiology, 66, 2096–2104.

Brown JR, Blankinship JC, Niboyet A *et al*. (2012) Effects of multiple global change treatments on soil N2O fluxes. *Biogeochemistry*, 109, 85–100.

Butterbach-Bahl K, Baggs EM, Dannenmann M, Kiese R, Zechmeister-Boltenstern S (2013) Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368, 20130122.

Cabrera ML, Beare MH (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. *Soil Science Society of American Journal*, 57, 1007–1012.

Cantarel AM, Bloor JMG, Deltroy N, Soussana JF (2011) Effects of climate change drivers on nitrous oxide fluxes in an upland temperate grassland. *Ecosystems*, 14, 223–233.

Cantarel AAM, Bloor JMG, Pommier T, Guillaumaud N, Moirot C, Soussana JF, Poly F (2012) Four years of experimental climate change modifies the microbial drivers of N_2O fluxes in an upland grassland ecosystem. *Global Change Biology*, 18, 2520–253.

Carter MS, Ambus P, Albert KR *et al*. (2011) Effects of elevated atmospheric CO_2, prolonged summer drought and temperature increase on N_2O and CH_4 fluxes in a temperate heathland. *Soil Biology & Biochemistry*, 43, 1660–1670.

Chen H, Mothapo NV, Shi W (2015a) Fungal and bacterial N_2O production regulated by soil amendments of simple and complex substrates. *Soil Biolology & Biochemistry*, 84, 116–126.

Chen W, Zhang LL, Li XY *et al.* (2015b) Elevated ozone increases nitrifying and denitrifying enzyme activities in the rhizosphere of wheat after 5 years of fumigation. *Plant and Soil*, 392, 279–288.

Cheng L, Booker FL, Burkey KO *et al*. (2011) Soil microbial responses to elevated CO_2 and O_3 in a nitrogen-aggrading agroecosystem. *PLoS One*, 6, e21377.

Cheng L, Booker FL, Tu C *et al.* (2012) Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO_2. *Science*, 337, 1084–1087.

Chung H, Zak DR, Lilleskov EA (2006) Fungal community composition and metabolism under elevated CO_2 and O_3. *Oecologia*, 147, 143–154.

Coleman DC, Anderson RV, Cole CV, Elliott ET, Woods L, Campion MK (1978) Trophic interactions in soils as they affect energy and nutrient dynamics .4. Flows of metabolic and biomass carbon. *Microbial Ecology,* 4, 373–380.

Cooley DR, Manning WJ (1987) The impact of ozone on assimilate partitioning in plants: a review. *Environmental Pollution,* 47, 95–113.

Cui P, Fan F, Yin C, Song A, Huang P, Tang Y, Liang Y (2016) Long-term organic and inorganic fertilization alters temperature sensitivity of potential N2O emissions and associated microbes. *Soil Biology &Biochemistry,* 93, 131–141.

Davidson EA, Janssens IA (2006) Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. *Nature,* 440, 165–173.

De Boer W, Kowalchuk GA (2001) Nitrification in acid soil: micro-organisms and mechanisms. *Soil Biology & Biochemistry,* 33, 853–866.

Decock C, Chung H, Venterea R, Gray SB, Leakey ADB, Six J (2012) Elevated CO_2 and O_3 modify N turnover rates, but not N_2O emissions in a soybean agroecosystem. *Soil Biology & Biochemistry,* 51, 104–114.

Decock C, Six J, 2012. Effects of elevated CO_2 and O_3 on N-cycling and N_2O emissions: a short-term laboratory assessment. *Plant and Soil,* 351, 277-292.

Dijkstra FA, Morgan JA, Follett RF *et al*. (2013) Climate change reduces the net sink of CH_4 and N_2O in a semiarid grassland. *Global Change Biology,* 19, 1816–1826.

Dijkstra FA, Prior SA, Runion GB, Torbert HA, Tian H, Lu C, Venterea RT (2012) Effects of elevated carbon dioxide and increased temperature on methane and nitrous oxide fluxes: evidence from field experiments. *Frontiers in Ecology and the Environment,* 10, 520–527.

Feng ZZ, Kobayashi K, Ainsworth EA (2008) Impact of elevated ozone concentration on growth, physiology and yield of wheat (*Triticum aestivum* L.): a meta-analysis. *Global Change Biology,* 14, 2696–2708.

Firestone MK, Davidson EA (1989) Microbial basis of NO and N_2O production and consumption in soils. In: *Exchange of trace gases between terrestrial ecosystems and the atmosphere* (eds Andreae MO, Schimel DS), pp. 7-21. John Wiley & Sons, New York.

Fiscus EL, Booker FL, Burkey KO (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant, Cell and Environment,* 28, 997–1011.

Frey SD, Lee J, Melillo JM, Six J (2013) The temperature response of soil microbial efficiency and its feedback to climate. *Nature Climate Change*, 3, 395-398.

Grantz DA, Gunn S, Vu HB (2006) O3 impacts on plant development: a meta-analysis of root/shoot allocation and growth. Plant, Cell and Environment, 29, 1193–1209.

Hallam SJ, Mincer TJ, Schleper *et al.* (2009) Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. *PLoS Biology*, 4, e95.

Hatzenpichler R (2012) Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. *Applied and Environmental Microbiology*, 78, 7501–7510.

He Z, Xiong JB, Kent AD *et al.* (2014) Distinct responses of soil microbial communities to elevated CO_2 and O_3 in a soybean agro-ecosystem. *The ISME Journal*, 8,714–726.

Holmes WE, Zak DR, Pregitzer KS, King JS (2006) Elevated CO_2 and O_3 alter soil nitrogen transformations beneath trembling aspen, paper birch, and sugar maple. *Ecosystems*, 9, 1354–1363.

Horz HP, Barbrook A, Field CB, Bohannan BJM (2004) Ammoniaoxidizing bacteria respond to multifactorial global change. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 15136–15141.

Hu HW, Chen D, He JZ (2015) Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiology Reviews*, 39, 729–749.

Hu HW, Macdonald CA, Trivedi P *et al.* (2016) Effects of climate warming and elevated CO_2 on autotrophic nitrification and nitrifiers in dryland ecosystems. *Soil Biology & Biochemistry*, 92, 1–15.

Hu S, Firestone MK, Chapin FS (1999) Soil microbial feedbacks to atmospheric CO_2 enrichment. *Trends in Ecology & Evolution*, 14, 433–437.

Hu Y, Chang X, Lin X *et al*. (2010) Effects of warming and grazing on N_2O fluxes in an alpine meadow ecosystem on the Tibetan Plateau. *Soil Biology & Biochemistry*, 42, 944–952.

IPCC (2013) Climate Change 2013: The Physical Science Basis. *Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.* (eds Stocker TF, Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley PM), pp. 659–740. Cambridge University Press, Cambridge, UK and New York, NY, USA.

Jones CM, Hallin S (2010) Ecological and evolutionary factors underlying global and local assembly of denitrifiers communities. *The ISME Journal,* 4, 633–641.

Kanerva T, Regina K, Rämö K, Ojanperä K, Manninen S (2007) Fluxes of N_2O, CH_4 and CO_2 in a meadow ecosystem exposed to elevated ozone and carbon dioxide for three years. *Environmental Pollution,* 145, 818–828.

Karhu K, Auffret MD, Dungait JAJ *et al.* (2014) Temperature sensitivity of soil respiration rates enhanced by microbial community response. *Nature,* 513, 81-84.

Keil D, Niklaus PA, von Riedmatten LR, Boeddinghaus RS, Dormann CF, Scherer-Lorenzen M, Marhan S (2015) Effects of warming and drought on potential N_2O emissions and denitrifying bacteria abundance in grasslands with different land-use. *FEMS Microbiology Ecology,* 91, fiv066.

Larsen KS, Andresen LC, Beier C *et al*. (2011) Reduced N cycling in response to elevated CO_2, warming, and drought in a Danish heathland: synthesizing results of the CLIMAITE project after two years of treatments. *Global Change Biology,* 17, 1884–1899.

Li X, Deng Y, Li Q *et al.* (2013). Shifts of functional gene representation in wheat rhizosphere microbial communities under elevated ozone. *The ISME Journal,* 7, 660–671.

Littell RC, Milliken GA, Stroup WW et al. (1996) SAS System for Mixed Models. SAS Institute, Cary.

Liu L, Hu C, Yang P, Ju Z, Olesen, JE, Tang, J (2016) Experimental warming-driven soil drying reduced N_2O emissions from fertilized crop rotations of winter wheat-soybean/fallow, 2009–2014. *Agriculture, Ecosystems and Environment,* 219, 71–82.

Lu M, Zhou X, Yang Q *et al.* (2013) Responses of ecosystem carbon cycle to experimental warming: a meta-analysis. *Ecology,* 94, 726–738.

Maag M, Vinther FP (1996) Nitrous oxide emission by nitrification and denitrification in different soil types and at different soil moisture contents and temperatures. *Applied Soil Ecology,* 4, 5–14.

Marsh K, Sims G, Mulvaney R (2005) Availability of urea to autotrophic ammonia-oxidizing bacteria as related to the fate of ^{14}C-and ^{15}N-labeled urea added to soil. *Biology Fertility of Soils,* 42, 137–145.

Melillo JM, Steudler PA, Aber JD *et al*. (2002) Soil warming and carbon-cycle feedbacks to the climate system. *Science,* 298, 2173–2176.

Mosier A, Kroeze C, Nevison C, Oenema O, Seitzinger S, van Cleemput O (1998) Closing the global N_2O budget: nitrous oxide emissions through the agricultural nitrogen cycle inventory methodology. *Nutrient Cycling in Agroecosystems,* 52, 225–248.

Niboyet A, Brown JR, Dijkstra P *et al.* (2011) Global change could amplify fire effects on soil greenhouse gas emissions. *PLoS ONE,* 6, e20105.

Ravishankara AR, Daniel JS, Portmann RW (2009) Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science,* 326, 123–125.

Rustad LE, Campbell JL, Marion GM *et al.* (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia,* 126, 543–562.

Schreiber F, Wunderlin P, Udert KM, Wells GF (2012). Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological path-ways, chemical reactions and novel technologies. *Frontier in Terrestrial Microbiology,* 3, 372.

Stieglmeier M, Mooshammer M, Kitzler B, Wanek W, Zechmeister-Boltenstern S, Richter A, Schleper, C (2014) Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *The ISME Journal,* 8, 1135-1146

Stres B, Danevčič T, Pal L, Fuka, MM *et al.* (2008) Influence of temperature and soil water content on bacterial, archaeal and denitrifying microbial communities in drained fen grassland soil microcosms. *FEMS Microbiology Ecology*, 66, 110–122.

Szukics U, Abell GCJ, Höld V, Mitter B, Sessitsch A, Hackl E, Zechmeister-Boltenstern S (2010) Nitrifiers and denitrifiers respond rapidly to changed moisture and increasing temperature in a pristine forest soil. *FEMS Microbiology Ecology,* 72, 395–406.

Talhelm AF, Pregitzer KS, Zak DR (2009) Species-specific responses to atmospheric carbon dioxide and tropospheric ozone mediate changes in soil carbon. *Ecology Letters,* 12, 1219–1228.

Tang HY, Liu G, Zhu, JG, Kobayashi K (2015) Effects of elevated ozone concentration on CH_4 and N_2O emission from paddy soil under fully open-air field conditions. *Global Change Biology*, 21, 1727-1736

Tourna M, Freitag TE, Nicol GW, Prosser JI (2008) Growth, activity and temperature responses of ammonia oxidising archaea and bacteria in soil microcosms. *Environmental Microbiology,* 10, 1357–1364.

Tu C, Louws FJ, Creamer NG et al. (2006) Responses of soil microbial biomass and N availability to transition strategies from conventional to organic farming systems. Agriculture, Ecosystems and Environment, 113, 206–215.

Van Groenigen KJ, Osenberg CW, Hungate BA (2011) Increased soil emissions of potent greenhouse gases under increased atmospheric CO_2. *Nature,* 475, 214–216.

Vance ED, Brooks PC, Jenkinson DS (1987) An extraction method for measuring soil microbial biomass C. *Soil Biology & Biochemistry,* 19, 703–707.

Wallenstein MD, Myrold DD, Firestone M, Voytek M (2006) Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological Applications,* 16, 2143–2152.

Ward SE, Ostle NJ, Oakley S, Quirk H, Henrys PA, Bardgett RD (2013) Warming effects on greenhouse gas fluxes in peatlands are modulated by vegetation composition. *Ecology Letters,* 16, 1285–1293.

Wertz S, Goyer C, Zebarth BJ, Burton DL, Tatti E, Chantigny MH, Filion M (2013) Effects of temperatures near the freezing point on N_2O emissions, denitrification and on the abundance and structure of nitrifying and denitrifying soil communities. *FEMS Microbiology Ecology,* 83, 242–254.

Wrage N, Velthof G, Van Beusichem M, Oenema O (2001) Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology & Biochemistry*, 33, 1723-1732.

Wu KK, Chen DM, Qiu YP, Burkey KO, Reberg-Horton SC, Peng SL, Hu SJ (2017) CO_2-induced alterations in plant nitrate utilization and root exudation stimulate N_2O emissions. *Soil Biology & Biochemistry,* 106, 9-17.

Wu Z, Dijkstra P, Koch GW, Peñuelas J, Hungate BA (2011) Responses of terrestrial ecosystems to temperature and precipitation change: a meta-analysis of experimental manipulation. *Global Change Biology,* 17, 927–942.

Wuebbles DJ (2009) Nitrous oxide: no laughing matter. *Science,* 326, 56-57.

Xia WW, Zhang CX, Zeng XW *et al.* (2011). Autotrophic growth of nitrifying community in an agricultural soil. *The ISME Journal,* 5, 1226–1236.

Zak DR, Groffman PM, Pregitzer KS *et al.* (1990) The vernal dam: plant-microbe competition for nitrogen in northern hardwood forests. *Ecology,* 71 (2), 651–656.

Zak DR, Pregitzer KS, King JS, Holmes WE (2000) Elevated atmospheric CO_2, fine roots and the response of soil microorganisms: a review and hypothesis. *New Phytologist,* 147, 201–222.

Zak DR, Pregitzer KS, Kubiske ME, Burton AJ (2011) Forest productivity under elevated CO_2 and O_3: positive feedbacks to soil N cycling sustain decade-long net primary productivity enhancement by CO_2. *Ecology Letters*, 14, 1220–1226.

Zhang LM, Offre PR, He JZ, Verhamme DT, Nicol GW, Prosser JI (2010). Autotrophic ammonia oxidation by soil thaumarchaea. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 17240–17245.

Zhang W, Parker KM, Luo Y, Wan S, Wallace LL, Hu S (2005) Soil microbial responses to experimental warming and clipping in a tallgrass prairie. Global Change Biology, 11, 266–277.

3.7 Tables

Table 3.1 Daily mean air temperature, ozone concentration (12-h), air humidity, soil moisture and temperature during the study period for experimental climate treatments. Means and standard errors are presented (n=3).

Climate treatments				Mixed Anova		
CF	+T	+O_3	+T+O_3	Warming	O_3	Warming ×O_3
Air temperature (ºC)						
24.3±0.0	28.1±0.1	24.4±0.0	27.7±0.1	**<0.001**	**0.017**	**0.016**
O_3 (ppb)						
16.1±0.3	15.5±0.4	62.3±0.7	60.5±2.3	0.266	**<0.001**	0.590
Air humidity (%)						
78.9±0.2	68.6±0.2	79.3±0.2	70.0±0.2	**<0.001**	**0.001**	**0.018**
Soil moisture (V%)						
22.6± 1.3	19.5 ± 1.0	21.2 ± 0.6	20.3 ± 1.3	0.117	0.795	0.332
Soil temperature (ºC)						
22.5±0.0	24.5±0.1	22.4±0.0	24.2±0.1	**<0.001**	0.795	0.331

CF, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Significant *P* values (*P*<0.05) are shown in bold, ANOVA mixed model.

Table 3.2 Effects of elevated air temperature and O_3 on the AOA, AOB, *nir*K, *nir*S, and *nos*Z gene abundances (means and standard errors are presented; n=3) in the 0-7.5 cm soil layer. Results from linear mixed ANOVA testing the effects of climate change treatments and their interaction are presented.

Climate treatments				Mixed Anova		
CF	+T	+O_3	+T+O_3	Warming	O_3	Warming× O_3
(a) Mean copy numbers of ammonia-oxidizing archaea (AOA) (10^8 copy per g of dry soil)						
1.06 ± 0.19	1.38 ± 0.19	0.71 ± 0.07	1.14± 0.25	*0.076*	0.116	0.688
(b) Mean copy numbers of ammonia-oxidizing bacteria (AOB) (10^7 copy per g of dry soil)						
0.81 ± 0.16	1.29 ± 0.14	0.47 ± 0.09	0.83± 0.41	0.148	*0.069*	0.863
(c) Mean copy numbers of *nir*K (10^7 copy per g of dry soil)						
7.83± 0.83	14.26± 3.08	4.18± 0.25	8.22± 1.66	**0.002**	**0.003**	0.775
(d) Mean copy numbers of *nir*S (10^7 copy per g of dry soil)						
10.01 ± 0.89	13.82± 1.76	5.47± 0.18	9.89± 1.48	**0.002**	**0.002**	0.195
(e) Mean copy numbers of *nos*Z (10^7 copy per g of dry soil)						
5.81± 1.49	6.07± 0.96	2.56± 0.23	7.05± 1.42	**0.007**	**0.049**	**0.013**

CF, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Significant *P* values (*P*<0.05) are shown in bold and marginal *P* values (0.1>*P*>0.05) are in italic.

Table 3.3 Correlation coefficients (Pearson) between field N_2O fluxes, plant biomass, soil characteristics and gene abundances pooled across experimental treatments and dates.

	N_2O	AGB	BGB	ST	SM	NH_4^+	NO_3^-	MBN	Extr-C	MBC	NMN	AOA	AOB	*nir*K	*nir*S	*nos*Z
N_2O		-.415	.494	**.848****	-.323	.271	**.608***	**.698***	.463	**.646***	**.687***	*.551*	.495	**.656***	**.569***	*.545*
AGB			.102	-.450	.396	.079	-.478	.040	-.001	-.049	.035	-.117	-.106	-.241	-.191	*-.538*
BGB				.436	-.0851	.062	.181	**.691***	**.751****	**.778****	**.667***	.398	**.622***	**.651***	*.564*	.226
ST					*-.518*	.163	.293	*.524*	.309	.426	.444	*.558*	.409	**.683***	**.669***	**.573***
SM						-.113	.161	-.342	-.123	-.289	-.163	-.364	-.186	-.347	-.388	-.365
NH_4^+							.008	.203	.386	.110	.177	.490	.209	.263	.103	.089
NO_3^-								.159	.082	.359	*.471*	-.012	.132	.113	-.094	.045
MBN									**.783****	**.899****	**.716****	**.738***	**.608***	**.778***	**.785***	*.531*
Extr-C										**.708****	**.586***	**.646***	.453	**.700***	**.666***	.407
MBC											**.684***	**.568***	**.745***	**.756***	**.648***	.448
NMN												.316	.282	.342	.305	.039
AOA													**.683***	**.853****	**.874****	**.791****
AOB														**.783****	**.649***	**.653***
*nir*K															**.934****	**.799****
*nir*S																**.830****
*nos*Z																

Significant effects (*** (P<0.01) and *(P≤0.05)) are shown in bold and marginal effects (0.05<P<0.1) are shown in italic text. AGB, aboveground biomass; BGB, belowground biomass; ST, soil temperature; SM, soil moisture; Extr-C, soil extractable organic carbon; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; NMN, net mineralizable nitrogen.

3.8 Figures

Fig. 3.1 Effects of warming and ozone on plant biomass during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3.Error bars represent SE of the means among replicate treatment plots (n=3). (a) Aboveground biomass (Repeated measures mixed model; warming effect: $P<0.01$; O_3 effect: $P=0.066$; Warming×O_3 effect: $P<0.05$; time effect: $P<0.001$). (b) Belowground biomass (Repeated measures mixed model; warming effect: $P=0.055$; O_3 effect: $P<0.01$; Warming×O_3 effect: $P<0.05$; time effect: $P<0.001$).

Fig. 3.2 Effects of warming and ozone on microbial biomass C and N during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Microbial biomass C: (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P<0.05$; O_3 effect: $P<0.01$; Warming×O_3 effect: $P<0.05$; time effect: $P>0.1$) and (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P=0.090$; O_3 effect: $P<0.001$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$). Microbial biomass N: (c) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P<0.05$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect $P<0.001$) and (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P=0.090$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$).

Fig. 3.3 Effects of warming and ozone on N_2O emission during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3. Panels

inside the figure were cumulative N_2O under different treatments. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

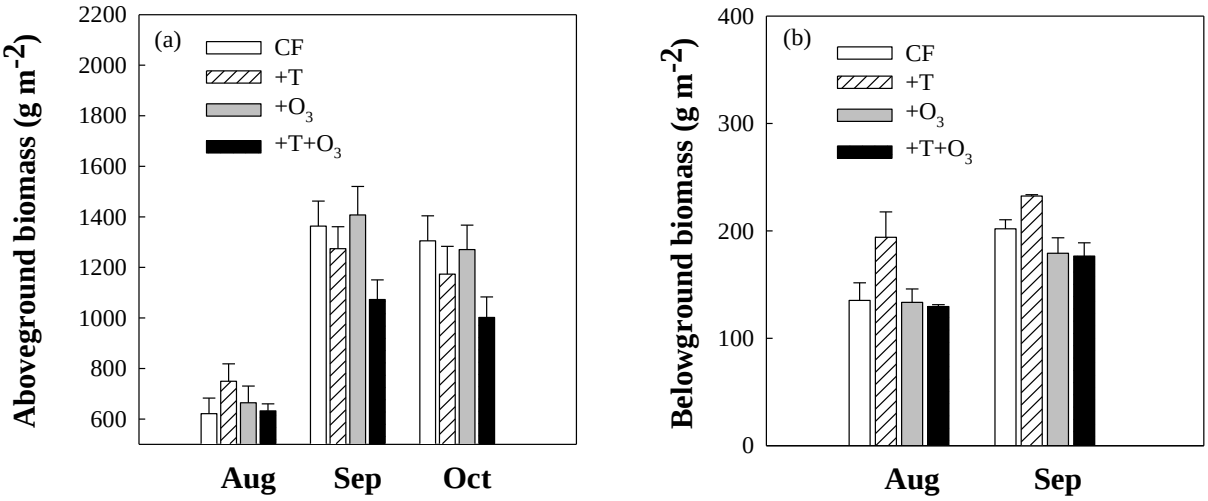


Fig. 3.1 Effects of warming and ozone on plant biomass during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3.Error bars represent SE of the means among replicate treatment plots (n=3). (a) Aboveground biomass (Repeated measures mixed model; warming effect: $P<0.01$; O_3 effect: $P=0.066$; Warming×O_3 effect: $P<0.05$; time effect: $P<0.001$). (b) Belowground biomass (Repeated measures mixed model; warming effect: $P=0.055$; O_3 effect: $P<0.01$; Warming×O_3 effect: $P<0.05$; time effect: $P<0.001$).

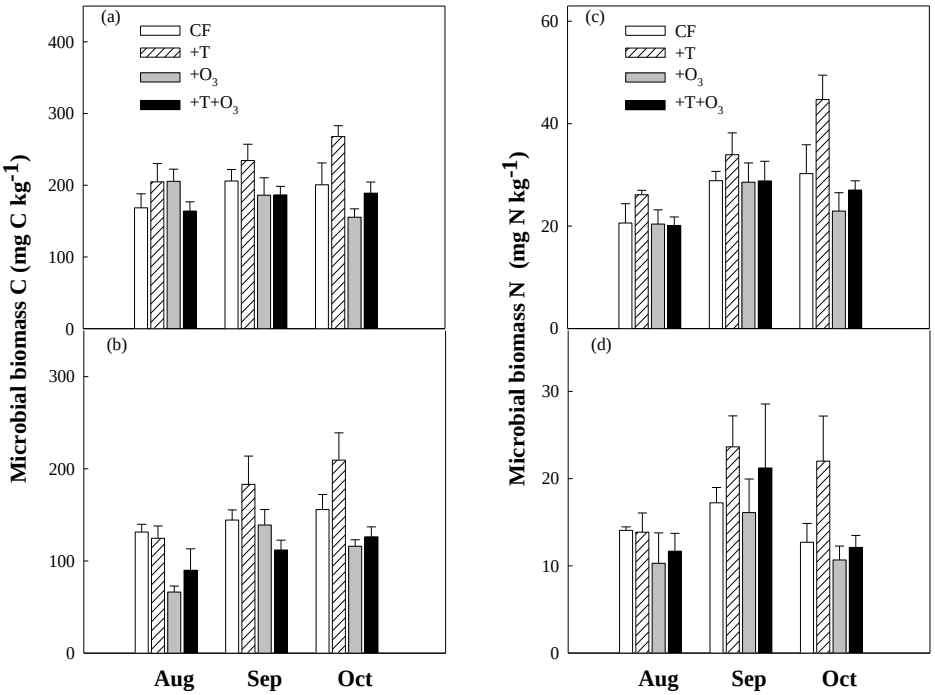


Fig. 3.2 Effects of warming and ozone on microbial biomass C and N during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O$_3$**, elevated O_3. **+T+O$_3$**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Microbial biomass C: (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P<0.05$; O_3 effect: $P<0.01$; Warming×O_3 effect: $P<0.05$; time effect: $P>0.1$) and (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P=0.090$; O_3 effect: $P<0.001$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$). Microbial biomass N: (c) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P<0.05$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect $P<0.001$) and (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P=0.090$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$).

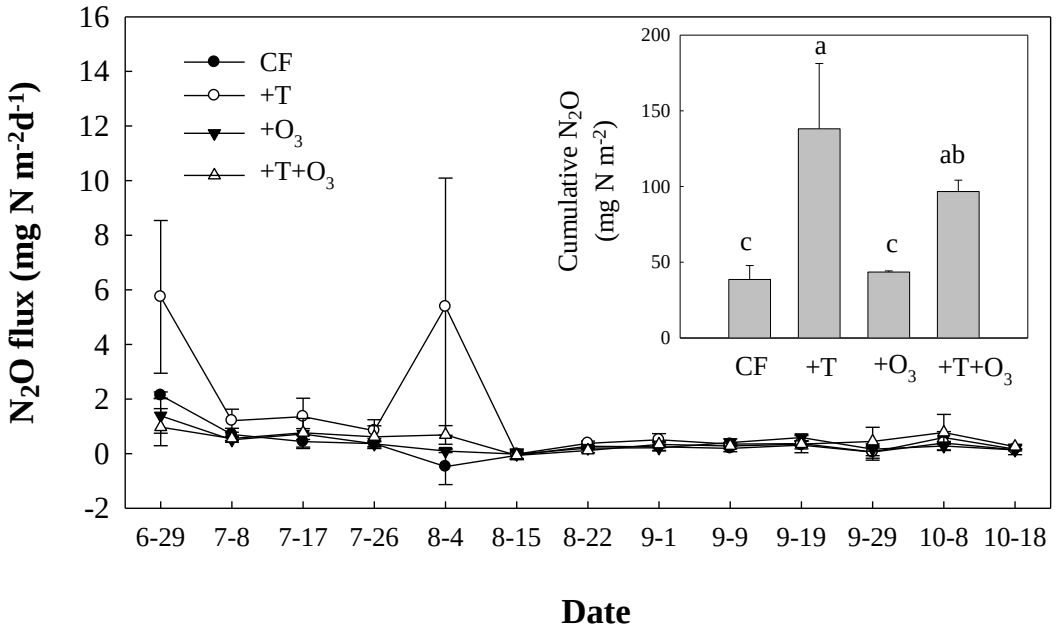


Fig. 3.3 Effects of warming and ozone on N_2O emission during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Panels inside the figure were cumulative N_2O under different treatments. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at P= 0.05 level under different treatments.

3.9 Appendices

Appendix 3.1 Primers and qPCR conditions for the real-time PCR quantifications of AOA *amoA*, AOB *amoA*, *nir*S, *nir*K, and *nos*Z genes extracted from soils.

Appendix 3.2 *P* values of analyses of repeated measures linear mixed models of warming, O_3 and time effects, and all interactions over the experimental period.

Appendix 3.3 Daily air temperature (a) and 12-h mean ozone concentration (b) during the study period. The control (**CF**) is given by gray line, whereas the warming and ozone treatment is presented by black line (**+T**, full line; **+O_3**, dashed line; **+T+O_3**, dotted line).

Appendix 3.4 Effects of warming and ozone on soil extractable organic C. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P<0.05$; O_3 effect: $P=0.087$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$).

Appendix 3.5 Effects of warming and ozone on soil extractable inorganic N. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P>0.1$; Warming×O_3 effect: $P>0.1$; time effect $P>0.1$). (b) 7.5-15 cm soil layer (Repeated measures mixed model;

warming effect: P=0.081; O_3 effect: P>0.1; Warming×O_3 effect: P>0.1; time effect: P<0.001).

Appendix 3.6 Effects of warming and ozone on soil microbial respiration (SMR). **CF**, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: P<0.01; O_3 effect: P<0.01; Warming×O_3 effect: P<0.01; time effect: P>0.1). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: P>0.1; O_3 effect: P<0.05; Warming×O_3 effect: P>0.1; time effect: P>0.1).

Appendix 3.7 Effects of warming and ozone on net soil N mineralization. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: P>0.1; O_3 effect: P>0.1; Warming×O_3 effect: P<0.05; time effect: P>0.1). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: P>0.1; O_3 effect: P<0.05; Warming×O_3 effect: P>0.1; time effect: P=0.098).

Appendix 3.8 Effects of warming and ozone on the (a) AOA and (b) AOB *amoA* gene copy numbers during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O_3**, elevated O_3. **+T**+**O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at P = 0.05 level under different treatments.

Appendix 3.9 Effects of warming and ozone on the (a) *nirK*, (b) *nirS* and (c) *nosZ* gene copy numbers during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Appendix 3.1 Primers and qPCR conditions for the real-time PCR quantifications of AOA *amoA*, AOB *amoA*, *nir*S, *nir*K, and *nos*Z genes extracted from soils.

Target genes	Primer	Sequence	qPCR conditions	References
AOA *amoA*	CrenamoA23f	ATGGTCTGGCTWAGACG	40 CL: 98 °C for 10 s, 60 °C for 30 s, & 72 °C for 30 s	Tourna et al., 2008
	CrenamoA616r	GCCATCCATCTGTATGTCCA		
AOB *amoA*	amoA-1F	GGGGTTTCTACTGGTGGT	40 CL: 98 °C for 10 s, 56 °C for 30 s, & 72 °C for 30 s	Rotthauwe et al., 1997
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC		
*nir*S	nirSCd3aF	AACGYSAAGGARACSGG	Six TD CL—98 °C for 10 s, 63 °C for 30 s, and 72 °C for 30 s with AT dropped by 1 °C CL−1 to 58 °C; 40 CL—98 °C for 10 s, 58 °C for 30 s, and 72 °C for 30 s	Kandeler et al., 2006
	nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA		
*nir*K	nirK876	ATYGGCGGVAYGGCGA	Same as nirS	Henry et al., 2004
	nirK1040	GCCTCGATCAGRTTRTGGTT		
*nos*Z	norZ1F	WCSYTGTTCMTCGAGCCAG	Six TD CL—98 °C for 10 s, 67 °C for 30 s, and 72 °C for 30 s with AT dropped by 1 °C CL−1 to 62 °C; 40 CL—98 °C for 10 s, 62 °C for 30 s, and 72 °C for 30 s	Henry et al., 2006
	norZ1R	ATGTCGATCARCTGVKCRTTYTC		

CL, TD, AT are short for cycles, touchdown, and annealing temperature, respectively

References:

Henry S, Baudoin E, López-Gutiérrez JC, Martin-Laurent F, Brauman A, Philippot L (2004) Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *Journal of Microbiological Methods*, 59, 327–335.

Henry S, Bru D, Stres B, Hallet S, Philippot L (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. *Applied and Environmental Microbiology*, 72, 5181-5189.

Kandeler E, Deiglmayr K, Tscherko D, Bru D, Philippot L (2006) Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. *Applied and Environmental Microbiology*, 72, 5957–5962.

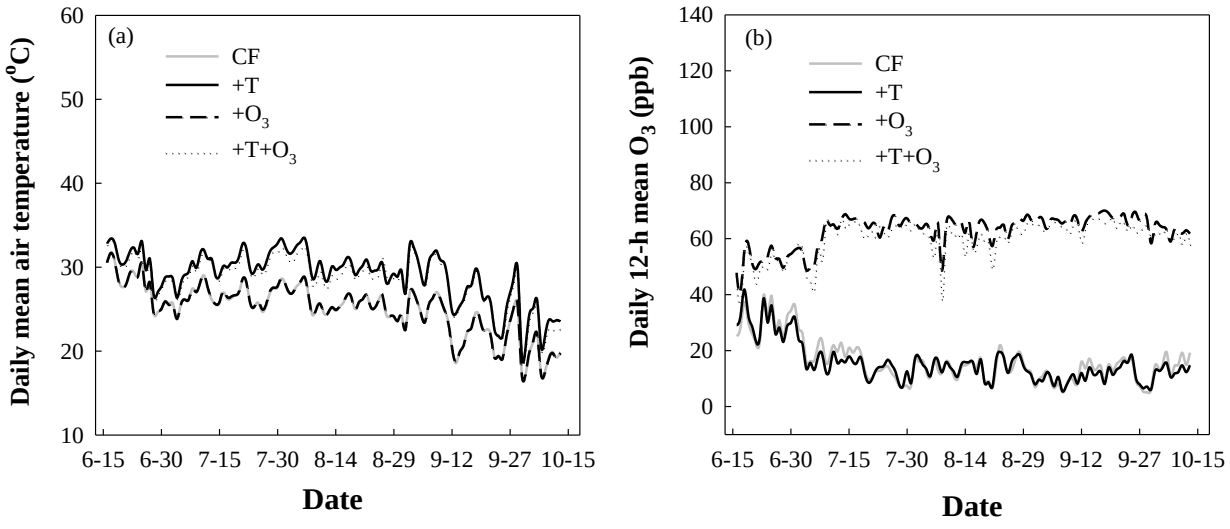
Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology*, 63, 4704–4712.

Tourna M, Freitag TE, Nicol GW, Prosser JI (2008) Growth, activity and temperature responses of ammonia oxidising archaea and bacteria in soil microcosms. *Environmental Microbiology*, 10, 1357–1364.

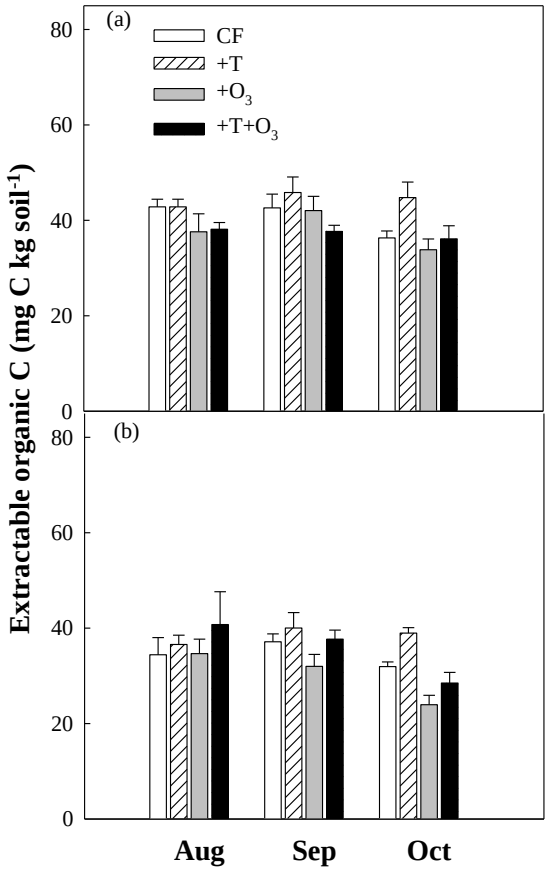
Appendix 3.2 *P* values of analyses of repeated measures linear mixed models of warming, O_3 and time effects, and all interactions over the experimental period.

Variable	Depth (cm)	Warming	O_3	Time	Warming ×O_3	Time× Warming	Time ×O_3	Time× Warming×O_3
Extr-C	0-7.5	0.442	**0.033**	**0.048**	0.32	0.176	0.981	0.624
	7.5-15	**0.033**	*0.087*	**0.021**	0.712	0.914	*0.06*	0.732
Extr-N	0-7.5	0.374	0.327	0.592	0.348	0.829	0.579	0.52
	7.5-15	*0.081*	0.185	**<0.001**	0.912	0.107	0.27	0.304
MBC	0-7.5	**0.033**	**0.003**	0.328	**0.020**	0.141	*0.093*	0.597
	7.5-15	*0.090*	**<0.001**	**0.010**	0.128	0.721	0.486	0.148
MBN	0-7.5	*0.098*	**0.048**	**<0.001**	0.206	0.181	0.041	0.761
	7.5-15	*0.09*	**0.049**	**0.013**	0.776	0.677	0.75	0.551
SMR	0-7.5	**0.002**	**0.003**	0.46	**0.003**	0.51	0.601	0.521
	7.5-15	0.330	**0.030**	0.957	0.175	0.637	0.277	0.999
NMN	0-7.5	0.198	0.750	0.104	**0.046**	*0.094*	0.130	0.830
	7.5-15	0.748	**0.024**	*0.097*	0.293	0.283	0.942	0.879

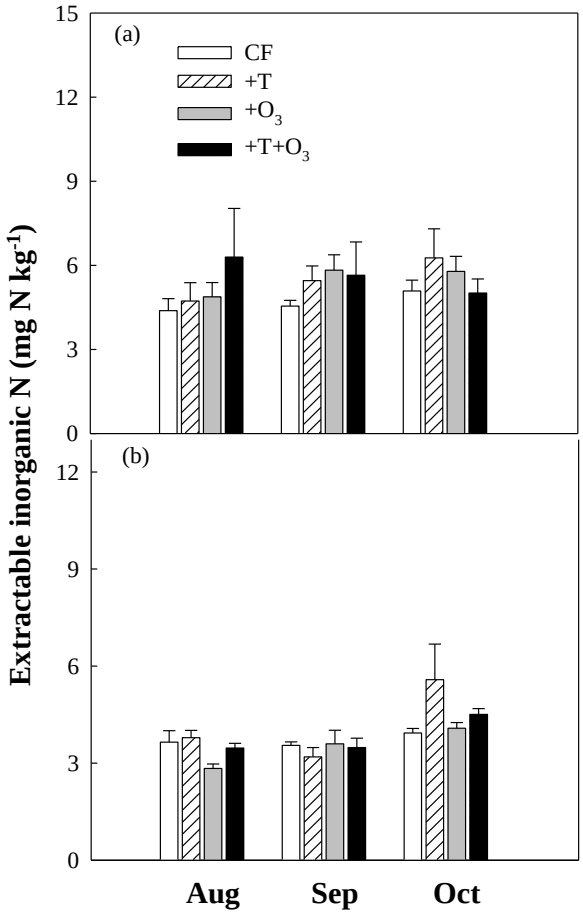
Significant effects ($P<0.05$) are shown bold and marginal effect are shown in italic text. Extr-C, soil extractable organic carbon; Extr-N, soil extractable inorganic nitrogen; MBC, microbial biomass carbon; MBN, microbial biomass nitrogen; SMR, soil microbial respiration; NMN, net mineralizable nitrogen.



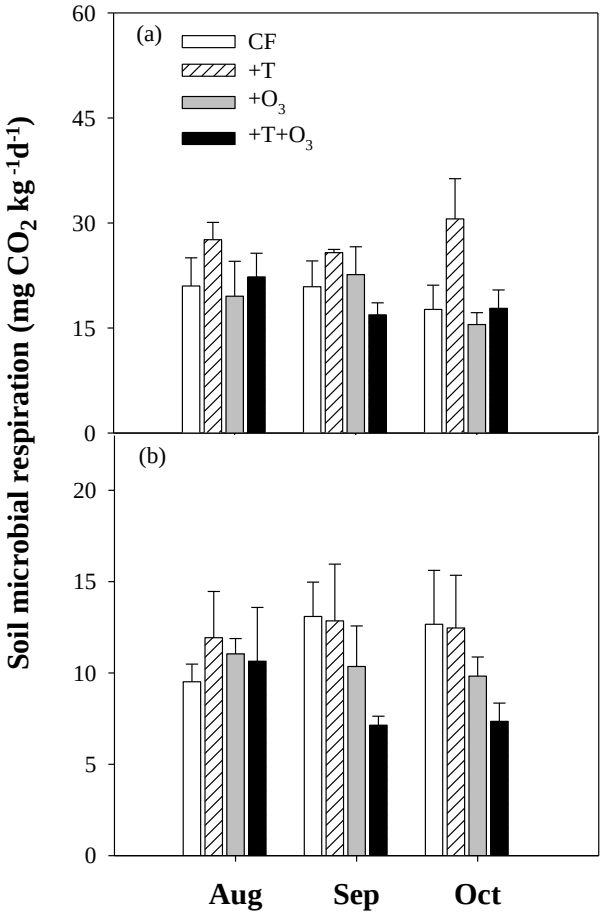
Appendix 3.3 Daily air temperature (a) and 12-h mean ozone concentration (b) during the study period. The control (**CF**) is given by gray line, whereas the warming and ozone treatment is presented by black line (**+T**, full line; **+O_3**, dashed line; **+T+O_3**, dotted line).



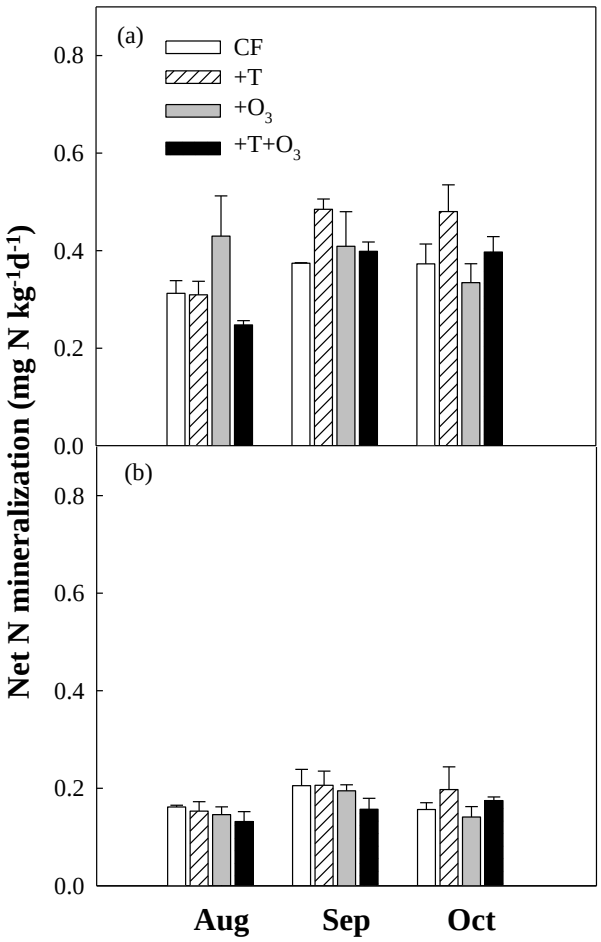
Appendix 3.4 Effects of warming and ozone on soil extractable organic C. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T+O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P<0.05$; O_3 effect: $P=0.087$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.05$).



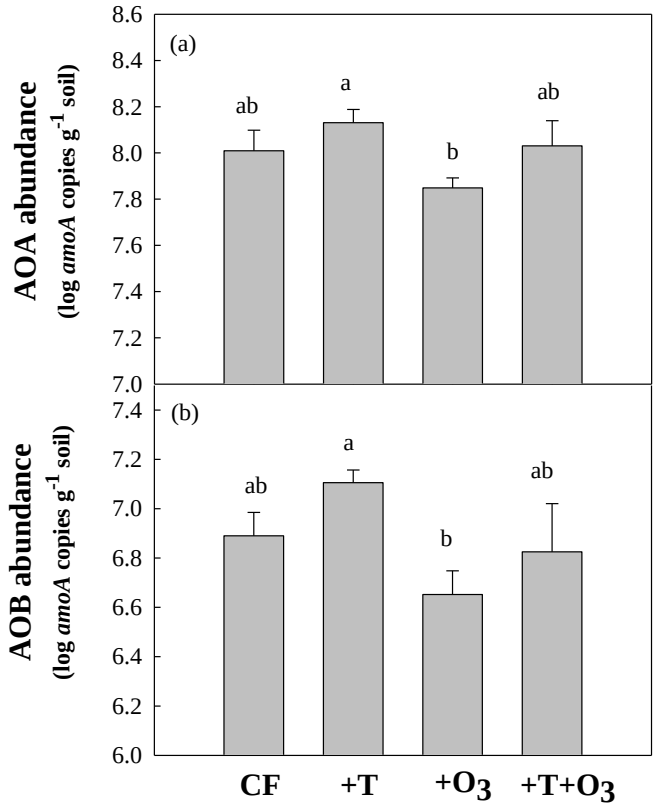
Appendix 3.5 Effects of warming and ozone on soil extractable inorganic N. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P>0.1$; Warming×O_3 effect: $P>0.1$; time effect $P>0.1$). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P=0.081$; O_3 effect: $P>0.1$; Warming×O_3 effect: $P>0.1$; time effect: $P<0.001$).



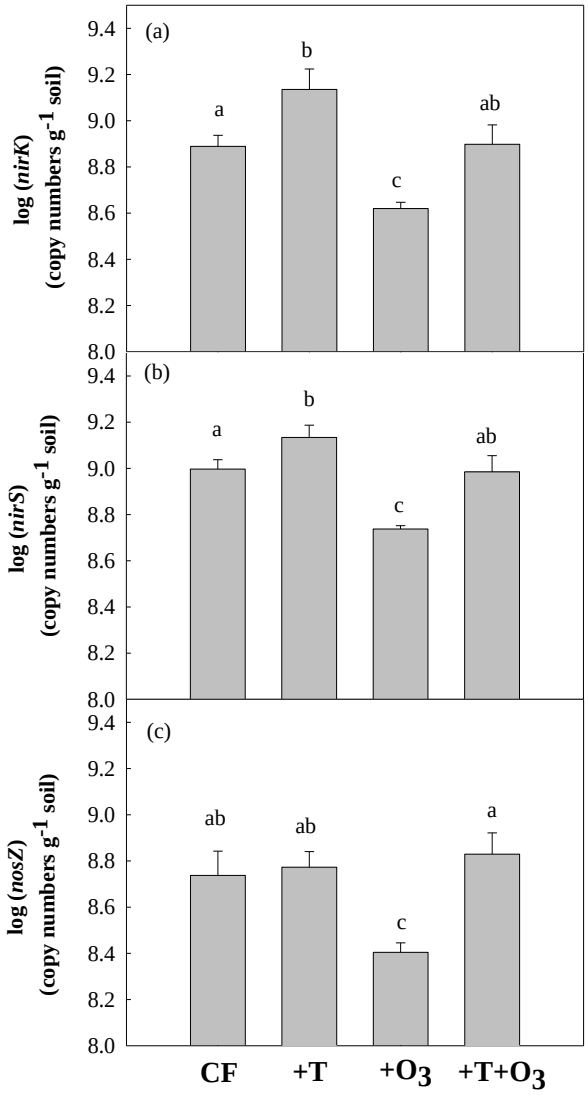
Appendix 3.6 Effects of warming and ozone on soil microbial respiration (SMR). **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P<0.01$; O_3 effect: $P<0.01$; Warming×O_3 effect: $P<0.01$; time effect: $P>0.1$). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect: $P>0.1$).



Appendix 3.7 Effects of warming and ozone on net soil N mineralization. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). (a) 0-7.5 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P>0.1$; Warming×O_3 effect: $P<0.05$; time effect: $P>0.1$). (b) 7.5-15 cm soil layer (Repeated measures mixed model; warming effect: $P>0.1$; O_3 effect: $P<0.05$; Warming×O_3 effect: $P>0.1$; time effect: $P=0.098$).



Appendix 3.8 Effects of warming and ozone on the (a) AOA and (b) AOB *amoA* gene copy numbers during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T**+**O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.



Appendix 3.9 Effects of warming and ozone on the (a) *nirK*, (b) *nirS* and (c) *nosZ* gene copy numbers during the experimental period. **CF**, charcoal-filtered ambient air. **+T**, warming. +**O3**, elevated O_3. **+T+O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Chapter 4

Mycorrhizal and root mediation of organic carbon decomposition under simulated climate warming and elevated O_3

4.1 Abstract:

Both climate warming and elevated O_3 (eO_3) can significantly affect plant growth and potentially alter ecosystem processes. However, little is known about the impact of concurrent warming and eO_3 on plant-microbial interactions and rhizosphere processes. Here we show that simulated warming (control + 3 ^oC) and eO_3 (control + 45 ppb) reduce soybean root biomass, root diameter and root colonization by arbuscular mycorrhizal fungi (AMF). However, these treatments increase root length and root fragility, and stimulate residue decomposition in the rhizosphere. Also, warming and eO_3 significantly alter the composition of AMF community by reducing the abundance of genus *Glomus*, but increasing genus *Paraglomus.* Together, our results indicate that concurrent warming and eO_3 reduce plant photosynthate allocation belowground, while stimulating soil organic carbon turnover. These findings suggest that warming and elevated O_3 can elicit a tradeoff between roos and AMF and alter the quantity and quality of C belowground allocation, stimulating soil organic C turnover and release.

Keywords: elevated temperature, elevated ozone (eO_3), Arbuscular mycorrhizas, microbial community, 18S rDNA, Illumia MiSeq, diversity, decomposition

4.2 Introduction

Greenhouse gases such as carbon dioxide (CO_2), ozone (O_3) and nitrous oxide (N_2O) in the atmosphere have been increasing because of human activities, potentially modifying plant growth and terrestrial ecosystem processes. Global surface temperature has experienced an increase by 0.74 °C from the preindustrial level and, is predicted to continue to increase between 1.8 and 3.6 °C by the year 2100 (IPCC, 2007). Simultaneously with climate warming, the tropospheric ozone concentrations have more than doubled since the Industrial Revolution (IPCC, 2007; Monks *et al.,* 2015). These climate changes are expected to affect the terrestrial carbon (C) cycle. High temperature might initially accelerate rates of heterotrophic microbial activity and therefore enhance CO_2 emissions into the atmosphere (Davidson & Janssens, 2006, Bardgett *et al.,* 2008, Karhu *et al.,* 2014). This could lead to soil C losses and an overall positive feedback to global warming (Romero-Olivares *et al.,* 2017). eO_3 has been reported to reduce plant growth, subsequent decreased C allocation belowground (Andersen, 2003; Ainsworth *et al.,* 2012) and increase recalcitrant compounds in leaf litter (Loya *et al.,* 2003; Booker *et al.,* 2005; Meehan *et al.,* 2010). These changes would slow soil microbial decomposition processes, reducing C losses from soil (Parsons *et al.,* 2008; Berg & McClaugherty, 2014).

Arbuscular mycorrhizal fungi (AMF) form symbiotic associations with roots of over 80% land plant species and acquire their carbon solely from their host plant (Smith & Read, 2010; Cheng *et al.,* 2012). In return, AM fungi enhance plant nutrient uptake (Rillig & Mummey, 2006) and stress resistance and tolerance (Smith & Read, 2010). Warming and

eO_3 can affect plant photosynthesis and further photosynthate allocation belowground to rhizosphere microbes, symbiotic microbes in particular, mycorrhizal fungi (Andersen, 2003; Hawkes *et al.*, 2008). Plant roots and AMF may have contrasting effects on C cycling and decomposition. On one hand, plant root exudates are known to stimulate microbial activity, therefore C decomposition. On the other hand, AMF might expand soil C pools through slowly depositing cycling compounds such as chitin and glomalin (Rillig *et al.*, 2001; Gleixner *et al.*, 2002), thus potentially increasing soil C sequestration. So far, however, few studies have quantitatively assessed the role of plant roots and mycorrhizal fungi, especially AM fungi, in soil organic C decomposition.

Warming and eO_3 will likely be concurrent under future climate change scenarios and it is virtually unknown whether their combination will enhance or alleviate the acuteness of individual effect on plant roots and AMF, therefore soil C decomposition. Taking advantage of an existing 3-yr field manipulation experiment, we investigated the effects of warming, eO_3 and their combination on soybean roots and their associated AMF. In particular, we quantified the AMF composition and community diversity using next generation sequencing. We predicted that AMF and plant roots would stimulate soil organic C decomposition and resulting changes in AM fungal community composition and root morphology may be the driving force of this change.

4.3 Materials and Methods

The study site and experimental manipulations

We initiated a long-term field experiment to investigate the response of soybean agroecosystem to elevated temperature and O_3 using an air exclusion system (AES). The experimental site is located at the Lake Wheeler Experimental Station, 5 km south of North Carolina State University, Raleigh, North Carolina, USA (35˚43′N, 78˚40′W; elevation 120m). The soil is an Appling sandy loam (fine, kaolinitic, thermic Typic Kanhapludult), well drained with a pH of 6.2, and contained 10.0 g C and 0.9 g N kg^{-1} soil. Before O_3 and warming treatments were initiated, the soil was repeatedly turned-over using a disc implement and rotovator.

This experiment was a 2×2 factorial design with four treatments assigned into three blocks. Four different treatments were: (a) charcoal-filtered air and ambient temperature (CF); (b) charcoal-filtered air plus 2.6^{o}C increase in temperature (+T); (c) charcoal-filtered air plus ambient temperature and1.4 times ambient ozone (+O_3); (d) charcoal-filtered air + warming +1.4 times ambient ozone (+T+O_3). The purpose of filtration of ambient air with activated charcoal was to reduce ambient O_3 concentrations to levels considered nonphytotoxic to soybean plant. The seasonal daily average concentrations (12-h) of O_3 and degree of temperature over the experimental duration are shown in Table 4.1.

Soybean (cultivar Jake) was planted into AES plots (3m × 10m) in June, 2016. O_3 was generated by corona discharge in dry O_2 (model TG-20, Ozone Solutions, Hull, IA, USA). The O_3 concentration in each plot was monitored at canopy height with a UV photometric O_3 analyzer (model 49C, Thermo Environmental Instruments Co., Franklin, MA, USA). Air was warmed by electrical resistance heaters and solar water heaters. Each warmed plot used four

heater elements that operate 24 hours a day and two water-to-air exchangers. The water circulates through an array of black polyethylene pipes during the daylight hours, which is heated by the sun. The water then passes through the heat exchangers as forced air moves across the metal surfaces, increasing the air temperature by convection. The addition of warm, humidified air dispensed into the treatment plot to achieve temperature increases up to + 3 °C. Air temperature and relative humility were monitored using HOBO sensors (model U23-001, HOBO Pro V2 Temp/RH Datalogger, Onset Computer Corporation, Bourne, MA, USA). Soil temperature was measured with an Enviropro dielectric soil probe (model EP100D, Apcos Pty Ltd, Adelaide, Australia).

A non-hyphae (pore size: 0.45 µm), hyphae (pore size: 20 µm) and root (pore size: 1.8 mm) ingrowth core were installed into the plots to assess the influence of fungal hyphae and root on organic C decomposition, respectively. The ingrowth cores were constructed of PVC cylinders (10 cm tall × 4 cm i.d.) sealed at one end with a plastic cover. Two windows were cut into each cylinder with 50% of the surface area, and covered with the three-size nylon mesh. Each filled with a mixture of 1.8 g of dual $^{15}N/^{13}C$ labeled dried and chopped (ca. 1 cm length) residues of switchgrass [6.7 mg N (0.28 mg ^{15}N), 804 mg C (8.8 mg ^{13}C)] and 180.0 g of soil [169 mg N (0.64 mg ^{15}N), 2250 mg C (24.3 mg ^{13}C)], and mixed thoroughly. After three years from the start of the long-term warming and ozone experiment (June 28, 2016), we randomly buried one non-hyphae-, hyphae-, and root- ingrowth core into each treatment plot within two rows of soybean plants.

Plant sampling, soil sampling and chemical, microbial analyses

Soybeans of approximately the same age were sampled from 12 plots in September 2016 (13-wk after planting). Above ground biomass was collected by cutting at the soil surface from each plot. Three plant roots were sampled from each plot and were washed to remove all surface soil. One-g subsample of the total roots from each root system (cut to 1 cm length) was collected and stored in FAA (formaldehyde alcohol acetic acid, 10%:50%:5% + 35% water) solution for further root morphology analysis. The shoots and remaining roots were separately oven-dried and weighed.

At the end of experiment (Oct 27, 2016), all the ingrowth cores were collected immediately. Ten (10.0) grams of soil-residue mixture samples were taken from each core, oven-dried at 65 ^{o}C and ground into fine powder. Total C and ^{13}C was measured using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at University of California, Davis (Cheng *et al.,* 2012). Isotope ratios (δ) of C were expressed relative to Pee Dee Belemnite (PDB) standard by

$$\delta^{13}\mathrm{C}\ (‰) = \left(\frac{\mathrm{R}sample}{\mathrm{R}PDB} - 1\right) \times 1,000 \qquad (1)$$

where R is the molar isotope ratio of ^{13}C/^{12}C. Sample ^{13}C contents were calculated from isotope ratios and total C content. The mycorrhizally-, and root-enhanced total C losses (MCL and RCL, mg C kg^{-1} d^{-1}) during 16-week growing period were calculated based on the method by Cheng *et al.* (2012) using the following equation:

MCL (RCL) (mg C kg^{-1} d^{-1}) = (net C losses in mycorrhizal (root) samples – net C losses in non-mycorrhizal samples)/ incubation days (2)

At the same time, soil samples were collected from each field plot and stored at 4 °C for chemical and microbial analyses. MBC and MBN were determined using a fumigation-extraction method (Vance et al., 1987). Briefly, twenty grams (20 g) dry weight soil samples were fumigated with ethanol-free chloroform for 48 h and another 20-g soil samples were not fumigated as a control (i.e., the non-fumigated control). Both fumigated and non-fumigated soils were then extracted with 50 mL of 0.5 M K_2SO_4 by shaking for 30 min. MBN was determined following alkaline persulfate oxidation of the fumigated and non-fumigated K_2SO_4 extracts (Cabrera & Beare 1993). Soil extractable organic C in both fumigated and non-fumigated extracts was determined using a TOC analyzer (Shimadzu TOC-5050A, Shimadzu Co., Kyoto, Japan). The concentrations of NH_4^+-N and NO_3^--N in the extracts were determined on a Lachat flow injection analyzer (Lachat Instruments, Milwaukee, WI, USA). The differences in extractable organic C and inorganic N between fumigated and non-fumigated soils were used to calculate MBC and MBN using a conversion factor of 0.33 (kEC) and 0.45 (kEN), respectively (Vance et al., 1987; Tu et al., 2006). Soil extractable organic C and NH_4^+-N and NO_3^--N was determined using the non-fumigated soil extracts.

Root diameters and length analysis

The one-g subsample of roots was scanned in a tray with 200 mL Reverse Osmosis water and spread out for uniformity. Roots were scanned in positive transparency mode and 16-bit grey scale at approx. 58 p mm^{-1} (1400 dpi). WinRhizo (WR) was used to determine

root diameter class length from the scanned and digital images on a DellXPS710 (Zobel *et al.,* 2007; Zobel, 2013). Diameter class size was set to the pixel size of the scanned image (58 p mm^{-1} resolution = 0.017 mm p^{-1}). In total, we have 100 diameter classes. We used a normal Gaussian model

$$y = \mathrm{a} + \mathrm{bEXP}[\frac{-1}{2}\left(\frac{\mathrm{x}-\mathrm{c}}{d}\right)^2] \qquad (3)$$

to model the very fine roots from the WR data (Zobel, 2013).

Mycorrhizal colonization

To calculate mycorrhizal colonization, dry root samples (cut into about 1 cm in length) were cleared in 10% KOH solution overnight at room temperature, acidified in 1% (v/v) HCl for 2 hours, and then stained with acidic glycerol-trypan blue solution (Giovannetti and Mosse, 1980). The stained roots were then spread on a Petri dish with gridlines and examined for infection using a dissecting microscope at ×40 magnification. Results obtained were expressed as percentage root length colonized (PRLC) and total root length colonized (TRLC) by AMF (Tu *et al.,* 2006).

Root DNA extraction, PCR and Illumine Sequencing

Air-dried subsamples of roots were ground in a Tecator Cyclotec mill fitted with a 1-mm screen (FOSS, Eden Prairie, MN, USA). Total community DNA was extracted from a 0.05 g (dry) subsample of fine roots with a PowerPlant DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions.

The primer set AMV4.5NF (5´-AAGCTCGTAGTTGAATTTCG-3´)/AMDGR (5´ CCCAACTATCCCTATTAATCAT-3´) was used to amplify the 18S rRNA gene (Lumini *et al.,* 2010) on the Illumina Miseq platform (Shanghai Personal Biotechnology Co., Ltd). Each forward primer included a 7-bp barcode unique to each sample. PCR reactions were performed in 25-uL reaction mixtures containing: 5 × reaction buffer 5μL, 5 × GC buffer 5μL, dNTP (2.5mM) 2μL, forward-primer (10μM) 1μL, reverse-primer (10μM) 1μL, DNA template 2μL, ddH_2O 8.75μL, Q5 DNA Polymerase 0.25μL. Thirty cycles were performed at 98 °C for 2 min, denaturation at 98 °C for 15 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 30 s with a final extension at 72 °C for 7 min. Triplicate reaction mixtures per sample were pooled together and purified using an agarose gel DNA purification kit (AP-GX-250G; Axygen, Union City, CA, USA) and quantified with a NanoDrop ND-1000 (Thermo Scientific, USA). The PCR products were normalized to equimolar amounts, and then the DNA samples were sequencing with the Illumina MiSeq System platform following the manufacturer's protocols.

Bioinformatical analyses

Raw high-throughput sequencing data were processed by using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit (Caporaso *et al.*, 2010). We excluded sequences below the quality score of 25 and fewer than 200 bp in length or sequences with ambiguous nucleotides. Potential chimeras were detected using UCHIME (Edgar *et al.,* 2011). Potentially similar sequences were clustered into operational taxonomic units (OTUs) using the seed-based UCLUST algorithm at a 97% identity threshold (Edgar, 2010). The most

abundant sequence from each OTU was selected as the representative sequence. The representative sequences were checked against the MaarjAM AMF database (Öpik *et al.*, 2010; http://maarjam.botany.ut.ee/).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to examine the effects of warming and ozone effect on plant biomass, total root length, soil chemical and microbial parameters and AMF colonization of roots. Treatment was used as a fixed effect and block as a random effect. Data were log transformed as necessary to meet normality assumptions. These analyses were conducted in R v.3.1.2 (R Development Core Team, 2014). To compare AM fungal community diversity among the treatments, the reciprocal Simpson's index and Margalef's index were calculated using R package vegan (Oksanen *et al.*, 2014). Significant differences between treatments were compared using Tukey's HSD test at $P < 0.05$.

To fit the very fine root data pattern from the root diameter class length distribution, Matlab R2017a (MathWorks lnc, Natick, MA) was used to do the non-linear fit to an appropriate Gaussian model using the Levenberg-Marquardt algorithm.

The structure of AMF communities was compared using the proportion of different VT reads as a proxy for the relative abundance of AMF taxa per sample (Opik *et al.*, 2009). In order to visualize differences in AM fungal communities across experimental treatments, we used non-metric multidimensional scaling (NMDS) ordination with the "vegan" package in R with 1000 uteratuibs. Bray-Curtis similarity matrices were created using the relative

abundance of AMF taxa (Anderson, 2001; Oksanen *et al.*, 2010). To test for the effects of the experimental treatments on AM fungal composition, we performed two-way permutational multivariate analyses of variance (PERMANOVA) using the default settings, including climate change treatments as the fixed effect and block as the random effect.

4.4 Results:

Plant biomass and root morphological changes

No significant warming effect was detected on root biomass of plants (Fig. 4.1). However, ozone alone reduced root biomass by 23% and the combination of warming and ozone reduced root biomass by 43% (Fig. 4.1). There was no significant effect on plant aboveground biomass across our experimental treatments (Table 4.2).

Either warming or eO_3 significantly increased total root length (Fig. 3.2). The combination of warming and eO_3 also increased total root length (Fig 3.2). The scanned images with WinRhizo were used for the assessment of root diameters and length. As indicated by Zobel *et al.* (2007), the shape of the diameter class length distribution (DCLD) curve from a whole root system is very similar to a non-linear Gaussian model (Appendix 4.2). Fitted parameters were shown in (Appendix 4.1). Modeled curves across the treatments illustrated that root length increased significantly at the diameter of 0.218 mm (Appendix 4.2). NMDS ordination and PERMANOVA revealed a significant effect of climate change treatments on plant root diameter class length distribution (Pseudo-F=3.1, P=0.02). There

was a clear separation in root morphology between control and warming, eO_3 or the combination of warming and eO_3 treatment (Appendix 4.6).

Arbuscular mycorrhizal fungi: root colonization, and community composition and diversity

Either warming or eO_3 significantly reduced the proportion of root length colonized by AMF (Fig. 4.3). The combination of warming and eO_3 also significantly reduced AMF colonization in roots (Fig. 4.3).

A total of 879183 reads (averaged length 250 bp excluding primers) were obtained, 693147 sequences were kept for subsequent analysis after quality check and chimera detection. From those sequences, 693130 (78.84% of reads) matched Glomeromycota SSU rRNA gene sequences from the MaarjAM database (similarity 97%) corresponding to 167 VT. Eleven AM fungal genera were presented in the sequencing data (*Acaulospora, Ambispora, Archaeospora, Claroideoglomeraceae, Diversispora, Geosiphon, Gigaspora, Glomus, Paraglomus, Scutellospora, Septoglomus*) (Appendix 4.5). Compared with control, elevated temperature and O_3 significantly reduced the relative abundance of genus *Glomus*, while significantly increased the relative abundance of genus *Paraglomus* (Fig. 4.4). The same results occurred when elevated temperature and O_3 was combined. While, ambient O_3 had no significant effect.

NMDS ordination and PERMANOVA revealed a significant effect of climate change treatments on the taxonomic composition of AMF communities (Pseudo-F=2.0, P=0.027). There was a clear separation in the AMF communities between control and warming or the

combination of warming and ozone treatment (Appendix 4.6). Root biomass, root length, root surface area and NO_3^- were significantly correlated with the ordination obtained, thus, being the most important soil properties affecting AMF communities (Appendix 4.2, 4.6)

Arbuscular mycorrhizal fungal richness and evenness

Warming and eO_3 has so significant effect on AM fungal community evenness (Simpson's diversity of index) and richness (Margalef's index) (Appendix 4.4).

Soil chemistry

The mycorrhizally- and root enhanced losses of the total C (calculated using the total C losses from mycorrhizal samples minus those from non-mycorrhizal samples) were higher under either warming or eO_3 treatment (Fig. 4.5). A stable isotope mixing model was used to estimate the proportion of residue-derived C in the loss of total soil C from hyphae and root ingrowth bags. Results showed that either warming or eO_3 caused a marked increase in the loss of residue-derived C due to the mycorrhizal and root effect (Fig. 4.6).

Compared with charcoal-filtered air control, warming significantly reduced soil NO_3^--N. Warming and eO_3 together also significantly reduced soil NO_3^--N (Table. 4.2, Appendix 4.7). However, eO_3 had no effect on soil NO_3^--N. The soils under eO_3 and the combination of warming and eO_3 manipulations had significantly lower soil labile carbon (Table. 4.2, Appendix 4.8). However, warming had no effect on soil labile carbon.

4.5 Discussion:

Results from our experiment showed that either AMF or plant roots can stimulate soil organic C decomposition under warming and eO_3 in field setting (Fig. 4.5, 4.6). eO_3 and warming significantly reduced the relative abundance of *Glomus* and increased the relative abundance of *Paraglomus* based on Illumina MiSeq sequencing (Fig. 4.4). Also, eO_3 and warming significantly reduced AM fungal root colonization (Fig. 4.3). In contrast, we observed that root length increased significantly and root tended to be thinner under warming and eO_3 treatments (Fig. 4.2). Our findings suggest that plants may reduce investment in AM fungal associations through growing fast with longer and thinner roots under future warming and eO_3 scenarios, thereby altering AM fungal communities. These concurrent changes may further alter soil C dynamics.

As O_3 is difficult to peFnetrate into the soil directly affecting AMF, it can indirectly impact AMF by affecting plant growth, such as changed root morphology, belowground carbon allocation and root exudation (Anderson *et al.,* 2003). In this study, eO_3 reduced AM fungal colonization of roots (Fig. 4.3) and decreased root biomass (Fig. 4.1). This finding agrees with a sizeable body evidence which shows that eO_3 may reduce AMF fungal colonization of plant roots and decrease root biomass due to reduced carbon allocation belowground (McCool & Menge, 1984; Anderson, 2003; Wang *et al.,* 2017). Although most studies suggested that ozone reduces below-ground plant growth and root length through suppressing plant growth (Kumari *et al.,* 2013; He *et al.,* 2014), we observed that eO_3 increased root length by 25% compared with the charcoal-filtered control. Our results

suggest that plants may reduce investment in AM fungal associations through growing fast with longer and thinner roots under eO_3 to explore a large soil volume for water and nutrients uptake. This experiment presents the first-ever result that eO_3 can alter *in planta* AM fungal community composition in the field. Using Illumina MiSeq, we consistently observed that there was a significant reduction in the relative abundance of *Glomus* and a significant increase in *Paraglomus* under eO_3 (36% and 55%) compared with charcoal-filtered air control (69% and 25%) after a 3-year exposure. This is consistent with other studies. For example, the colonization of *Glomus aggregatum* was found to be reduced by an increase of ozone concentration to 80 ppb (Wang *et al.*, 2015), and Yoshida *et al.* (2001) also found that eO_3 significantly reduced the abundance of *Glomus intraradices* and this effect depended on the genotype of the plants examined. While, *Cotton et al.* (2015) first conducted a FACE experiment to investigate whether ozone affected AM fungal communities and no effects of eO_3 on AM fungal community composition, richness and evenness were observed. The plants they sampled were young (around 8 weeks) and their exposure to eO_3 was limited to affect AM fungi. Their results are in consistent with other studies. For example, the relative abundance of *Glomus fasciculatum* were not affected by ozone after 6 wk, but significantly reduced after 9 - wk growth (McCool & Menge 1984). In this study, we sampled soybeans after about 13 - wk growth and the AM fungal communities of older plants are more likely to be altered by ozone.

Understanding the mechanisms by which these alterations occur will be critical for predicting how future global change will affect mycorrhizas and, consequently, ecosystem

functions. Different fungi have distinct life history strategies that are likely to produce different responses to carbon availability under eO_3 and charcoal filtered air. Cultural *Glomus* species are thought to be r-strategists, growing and providing phosphorus to their host plants quickly (Boddington & Dodd, 1999; Sýkorová *et al.*, 2007). It is therefore that the members of *Glomus* are less capable of using the carbon under eO_3 to provide plants with less nutrients, consequently obtaining less carbon and reducing their relative abundance. This is consistent with what we observed that eO_3 significantly reduced soil labile carbon and marginally decreased root biomass (Table 4.2). The combination of eO_3 and temperature also significantly reduced root biomass of soybeans compared with the charcoal-filtered control (Table 4.2). *Drigo et al.* (2010) showed that under eCO_2, the *Glomeraceae* obtained the most recently fixed photosynthate using a stable isotope probing (SIP) study. These results strongly suggest that changes in the carbon alteration to roots caused the AM fungal community changes detected in this study. Furthermore, our NMDS result showed that AM fungal communities are structured by root morphological traits of the plants (Appendix 4.6). It has been proposed that root traits can impact soil C and nutrient cycling indirectly through influencing the composition of the soil microbial community (Bardgett *et al.*, 2014). AM fungi, especially *Glomus* can produce a glycoprotein (glomalin) increasing soil C storage and stability (Miller & Jastrow, 1990; Rillig *et al.*, 2001). Thus, the reduced colonization of AMF and decreased abundance of *Glomus* can alleviate soil C protection under future eO_3. Thus, the alterations in AM community composition via the change in root morphology may accelerate soil organic C decomposition and have significant impacts on ecosystem biogeochemical processes under future O_3 scenarios.

Warming may have altered *in planta* AM fungal community composition by directly affecting hyphal growth or indirectly affecting plant photosynthesis, root morphology (Björk *et al.,* 2007; Yin *et al.,* 2012) and nitrogen-availability, subsequent leading to changes in plant allocation to AM fungi (Heinemeyer & Fitter, 2004; Rillig *et al.,* 2003; Sheldrake *et al.,* 2017). Dominance of the genus *Glomus* (70%) in AM fungal communities from our charcoal-filtered control plot was in agreement with previous studies for agricultural ecosystems (Helgason *et al.,* 1998; Daniell *et al.,* 2001). Warming consistently reduced the relative abundance of *Glomus* (29%) and increased the relative abundance of *Paraglomus* (66%). In a multispecies of AM fungal community, individual species can respond differently to temperature, with some more successful than others under coder or warmer condition (Hawkes *et al.,* 2008). The below-ground biomass was not altered and mycorrhizal colonization was significantly reduced in this study (Fig. 4.1, 4.3), indicating that elevated temperature can directly affect AMF thereby altering AM fungal community composition. Our results suggest the genus *Paraglomus* will dominate this soybean agro-ecosystem under future warming scenarios.

We also observed that plant roots became longer and tended to grow thinner under warming. Given that the SRL (specific root length) reflects the potential for plants to exploit the soil (Björk et al., 2007), this result indicates that plants can enhance their potential for nutrient and water uptake under warming condition. Consistent with our result, Pregitzer *et al.* (2000) reviewed that root length of tree fine roots is positively related to soil temperature. In addition, we showed that warming significantly reduced the amount and availability of soil

NO_3^- (Table 4.1), suggesting that N-availability to plants had decreased. Nitrogen concentrations of AM fungal hyphae are much higher than those of plant tissues (Hodge & Fitter, 2010) indicating that low N availability may be more likely to suppress fungal growth than plant growth. This is consistent with our results that root colonization was significantly lower under warming, however root biomass was reduced with no significant effect (Fig. 4.1, 4.3). Considering that different AM fungi are known to vary in their growth strategy and vary in the translocation of N to plant partners, reduced N availability under warming may have selected for low-N AM fungal specialists (Veresoglou *et al.*, 2012; Sheldrake *et al.*, 2017). Alternatively, warming may increase AM fungal competition for a limited resource, therefore altering AM fungal communities.

The effect of elevated temperature on AM fungal composition has been examined in several field studies of different ecosystems, however, most of them reported no significant effects of elevated temperature on AM fungal community composition. For example, in grassland ecosystems (Heinemeyer *et al.*, 2004; Kim *et al.*, 2015) and an alpine meadow ecosystem in China (Yang *et al.*, 2013), elevated temperature has no significant effect on the composition of the AMF community. This could be because the analysis method used in these studies were not sufficiently sensitive to detect the changes and other environmental conditions might also have played a crucial role in determining the warming effect. Higher resolution community characterization, for example using next-generation sequencing, as employed in Dumbrell *et al.* (2011), Öpik *et al.* (2010) and Williams *et al.* (2017), would help solve this issue. Dumbrell *et al.* (2011) showed different AM fungal composition

between winter and summer and attributed this difference to both increased resource supply and high soil temperature. However, they also suggested that a major caveat remains to be that temperature might not be the only factor causing these seasonal changes in AM fungal communities and other variables, such as soil moisture, will co-vary with it. In our study, warming had no significant effect on soil moisture. Thus, the composition of AMF communities will be affected by the combination of biotic (plant root morphology) and abiotic characteristics (soil N availability). Yang *et al.* (2017) first reported that warming significantly altered AM fungal community composition using a greenhouse experiment, and therefore involved extremely artificial edaphic conditions and spatial constraints on root and fungal growth, and did not investigate the long-term effects of warming treatments. By using an AES experiment, we studied more realistic simulations of conditions which plants and AM fungi will experience in the future.

Our results showed that climate warming and eO_3 altered plant root growth by enhancing root length, while reducing AMF colonization of roots. These results suggest that climate warming and eO_3 may elicit a tradeoff between roots and AMF. Also, climate warming and eO_3 significantly affected the AMF community composition in host roots by favoring the genus *Glomus* over *Paraglomus*. Moreover, alterations in root morphology and AMF communities under climate warming and eO_3 facilitated residue decomposition, likely through increasing labile C inputs and reducing AMF protection of residues from microbial attacks. Together, these findings illustrate that combination of climate warming and eO_3 can

induce a cascade of events that alter roots and AMF communities, thereby stimulating root turnover and organic C decomposition.

4.6 References

Ainsworth EA, Yendrek CR, Sitch S, Collins WJ, Emberson LD (2012) The effects of tropospheric ozone on net primary productivity and implications for climate change. *Annual Review of Plant Biology*, 63, 637–661.

Andersen CP (2003) Source–sink balance and carbon allocation below ground in plants exposed to ozone. *New Phytologist*, 157, 213–228.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, 26, 32–46.

Bardgett RD, Freeman C, Ostle NJ (2008) Microbial contributions to climate change through carbon cycle feedbacks. *ISME Joural*, 2, 805–814.

Björk RG, Majdi H, Klemedtsson L, Lewis-Jonsson L, Molau U (2007) Long-term warming effects on root morphology, root mass distribution, and microbial activity in two dry tundra plant communities in northern Sweden. *New Phytologist*, 176, 862–873.

Berg B, McClaugherty C (2014) Plant Litter: Decomposition, Humus Formation, Carbon Sequestration, 3rd ed., Springer, New York.

Bobbink R, Hornung M, Roelofs JGM (1998) The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation – a review. *Journal of Ecology*, 86, 717–738.

Boddington CL, Dodd JC (1999) Evidence that differences in phosphate metabolism in mycorrhizas formed by species of Glomus and Gigaspora might be related to their life-cycle strategies. *New Phytologist*, 142, 531–538.

Booker FL, Prior SA, Torbert HA, Fiscus EL, Pursley WA, Hu SJ (2005) Decomposition of soybean grown under elevated concentrations of CO_2 and O_3. *Global Change Biology*, 11, 685–698.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335–336.

Cabrera M, Beare M (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. *Soil Science Society of America Journal*, 57, 1007-1012.

Cheng L, Booker FL, Tu C *et al.* (2012) Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO_2. *Science*, 337, 1084–1087.

Compant S, van der Heijden MGA, Sessitsch A (2010) Climate change effects on beneficial plant–microorganism interactions. *FEMS Microbiology Ecology*, 73, 197–214.

Cotton TEA, Fitter AH, Miller RM, Dumbrell AJ, Helgason T (2015) Fungi in the future: inter-annual variation and effects of atmospheric change on arbuscular mycorrhizal fungal communities. *New Phytologist,* 205, 1598–1607.

Daniell TJ, Husband R, Fitter AH, Young JPW (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. *FEMS Microbiology Ecology,* 36, 203–209.

Dumbrell AJ, Ashton PD, Aziz N *et al.* (2011) Distinct seasonal assemblages of arbuscular mycorrhizal fungi revealed by massively parallel pyrosequencing. *New Phytologist,* 190, 794–804.

Edgar, RC (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics,* 26, 2460–2461.

Fiscus EL, Booker FL, Burkey KO (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. *Plant, Cell and Environment,* 28, 997–1011.

Gavito ME, Schweiger P, Jakobsen I (2003) P uptake by arbuscular mycorrhizal hyphae: effect of soil temperature and atmospheric CO_2enrichment. *Global Change Biology,* 9, 106–116.

Giovannetti M, Mosse B (1980) Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytologist,* 84, 489–500.

Gleixner G, Poirier N, Bol R, Balesdent J (2002) Molecular dynamics of organic matter in a cultivated soil. *Organic Geochemistry,* 33, 357–366.

Grantz DA, Gunn S, Vu HB (2006) O_3 impacts on plant development: a meta-analysis of root/shoot allocation and growth. *Plant, Cell and Environment,* 29, 1193–1209.

Hawkes CV, Hartley IP, Ineson P, Fitter AH (2008) Soil temperature affects carbon allocation within arbuscular mycorrhizal networks and carbon transport from plant to fungus. *Global Change Biology,* 14, 1181–1190.

Heinemeyer A, Fitter AH (2004) Impact of temperature on the arbuscular mycorrhizal (AM) symbiosis: growth responses of the host plant and its AM fungal partner. *Journal of Experimental Botany,* 55, 525–534.

Heinemeyer A, Ridgway KP, Edwards EJ, Benham DG, Young JPW, Fitter AH (2004) Impact of soil warming and shading on colonization and community structure of arbuscular mycorrhizal fungi in roots of a native grassland community. *Global Change Biology*, 10, 52–64.

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? *Nature*, 394, 431.

Hodge A, Fitter AH (2010) Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 13754–13759.

IPCC (2007) Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL, eds. Climate change 2007: the physical science basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. Cambridge, UK: Cambridge University Press.

Jansa J, Smith FA, Smith SE (2008) Are there benefits of simultaneous root colonization by different arbuscular mycorrhizal fungi? *New Phytologist*, 177, 779–789.

Jenkinson DS, Brookes PC, Powlson DS (2004) Measuring soil microbial biomass. *Soil Biology and Biochemistry*, 36, 5–7.

Karhu K, Auffret MD, Dungait JAJ *et al*. (2014) Temperature sensitivity of soil respiration rates enhanced by microbial community response. *Nature*, 513, 81–84.

Kumari S, Agrawal M, Tiwari S (2013) Impact of elevated CO_2 and elevated O_3 on *Beta vulgaris* L.: pigments, metabolites, antioxidants, growth and yield. *Environmental Pollution*, 174, 279–288.

Kim YC, Gao C, Zheng Y *et al.* (2015) Arbuscular mycorrhizal fungal community response to warming and nitrogen addition in a semiarid steppe ecosystem. *Mycorrhiza*, 25, 267–276.

Loya W, Pregitzer KS, Karberg NJ *et al*. (2003) Reduction of soil carbon formation by tropospheric ozone under increased carbon dioxide levels. *Nature*, 425, 705–707.

McCool PM, Menge JA (1984) Interaction of ozone and mycorrhizal fungi on tomato as influenced by fungal species and host variety. *Soil Biology and Biochemistry*, 16, 425–427.

Meehan TD, Crossley MS, Lindroth RL (2010) Impacts of elevated CO_2 and O_3 on aspen leaf litter chemistry and earthworm and springtail productivity. *Soil Biology & Biochemistry*, 42, 1132–1137.

Mohan JE, Cowden CC, Baas P *et al.* (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: mini-review. *Fungal Ecology*, 10, 3–19

Monks PS, Archibald AT, Colette A *et al.* (2015) Tropospheric ozone and its precursors from the urban to the global scale from air quality to short-lived climate forcer. *Atmospheric Chemistry and Physics*, 15, 8889-8973.

Oksanen J, Kindt R, Legendre P *et al.* (2014) Vegan: Community ecology package. R package version 2.0-10.

Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier Ü, Zobel M (2010) The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist*, 188, 223–241.

Olsrud M, Carlsson BA, Svensson BM, Michelsen A, Melillo JM (2010) Responses of fungal root colonization, plant cover and leaf nutrients to long-term exposure to elevated atmospheric CO_2 and warming in a subarctic birch forest understory. *Global Change Biology*, 16, 1820-1829.

Parsons WFJ, Bockheim JG, Lindoroth RL (2008) Independent, interactive, and species-specific responses of leaf litter decomposition to elevated CO_2 and O_3 in a northern hardwood forest. *Ecosystems*, 11, 505–519.

Pregitzer KS, King JS, Burton AJ (2000) Responses of tree fine roots to temperature. New Phytologist 147, 105–115.

R Development Core Team (2014) R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure. *New Phytologist*, 171, 41–53.

Rillig MC, Wright SF, Nichols KA, Schmidt WF, Torn MS (2001) Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. *Plant and Soil*, 233, 167–177.

Rillig MC, Wright SF, Shaw MR, Field CB (2002) Artificial climate warming positively affects asbuscular mycorrhizas but decreases soil aggregates water stability in an annual grassland. *Oikos*, 97, 52–58.

Romero-Olivares AL, Allison SD, Treseder KK (2017) Soil Microbes and Their Response to Experimental Warming over Time: A Meta-Analysis of Field Studies. Soil *Biology and Biochemistry*, 107, 32-40.

Singh BK, Bardgett RD, Smith P, Reay DS (2010) Microorganisms and climate change: terrestrial feedbacks and mitigation options. *Nature Reviews Microbiology, 8*, 779–790.

Sheldrake M, Rosenstock NP, Revillini D *et al.* (2017) Arbuscular mycorrhizal fungal community composition is altered by long-term litter removal but not litter addition in a lowland tropical forest. *New Phytologist,* 214, 455–467.

Smith SE, Read DJ (2010) Mycorrhizal Symbiosis. Academic Press, San Diego, CA.

Staddon PL, Gregersen R, Jakobsen I (2004) The response of two Glomus mycorrhizal fungi and a fine endophyte to elevated atmospheric CO_2, soil warming and drought. *Global Change Biology,* 10, 1909–1921.

Stevens CJ, Dise NB, Mountford JO, Gowing DJ (2004) Impact of nitrogen deposition on the species richness of grasslands. *Science,* 303, 1876–1879.

Sýkorová Z, Ineichen K, Wiemken A, Redecker D (2007) The cultivation bias: different communities of arbuscular mycorrhizal fungi detected in roots from the field, from bait plants transplanted to the field, and from a greenhouse trap experiment. Mycorrhiza 18, 1–14.

Tilman D (1987) Secondary succession and the pattern of plant dominance along experimental nitrogen gradients. *Ecological Monographs,* 57, 189–214.

Tu C, Booker FL, Watson DM, Chen X, Rufty TW, Shi W, Hu S (2006) Mycorrhizal mediation of plant N acquisition and residue decomposition: impact of mineral N inputs. *Global Chang Biology,* 12, 793–803.

Vance E, Brookes P, Jenkinson D (1987) An extraction method for measuring soil microbial biomass C. *Soil Biology & Biochemistry,* 19, 703-707.

Veresoglou SD, Chen BD, Rillig MC (2012) Arbuscular mycorrhiza and soil nitrogen cycling. *Soil Biology & Biochemistry,* 46, 53–62.

Wagg C, Jansa J, Stadler M, Schmid B, van der Heijden MGA (2011) Mycorrhizal fungal identity and diversity relaxes plant–plant competition. *Ecology,* 92, 1303–1313

Wang SG, Wang F, Diao XJ, He LS (2014) Effects of elevated O_3 on microbes in the rhizosphere of mycorrhizal snap bean with different O_3 sensitivity. *Canadian Journal of Microbiology,* 60, 93–103.

Wang SG, Diao XJ, Li YW, Ma LM (2015) Effect of *Glomus aggregatum* on photosynthetic function of snap bean in response to elevated ozone. *Journal of Agricultural Science,* 153, 837–852.

Wilson H, Johnson BR, Bohannan B, Pfeifer-Meister L, Mueller R, Bridgham SD (2016) Experimental warming decreases arbuscular mycorrhizal fungal colonization in prairie plants along a Mediterranean climate gradient. *Peer J,* 4:e2083

Yang R, Cai X, Li X, Christie P, Zhang J, Gai J (2017) Temperature-mediated local adaptation alters the symbiotic function in arbuscular mycorrhiza. Environmental Microbiology. Accepted Author Manuscript. doi:10.1111/1462-2920.13737

Yang W, Zheng Y, Gao C *et al.* (2013) The arbuscular mycorrhizal fungal community response to warming and grazing differs between soil and roots on the Qinghai-Tibetan Plateau. *PLoS One,* 8:e76447

Yin HJ, Chen Z, Liu Q (2012) Effects of experimental warming on soil N transformations of two coniferous species, Eastern Tibetan Plateau, China. *Soil Biology & Biochemistry*, 50, 77–84.

Yoshida LC, Gamon JA, Andersen CP (2001) Differences in above- and below-ground responses to ozone between two populations of a perennial grass. *Plant and Soil,* 233, 203–211.

Zobel RW, Kinraide TB, Baligar VC (2007) Fine root diameters can change in response to changes in nutrient concentrations. *Plant and Soil,* 297, 243–254.

Zobel RW (2013) Lolium perenne L. root systems are a collection of Gaussian curve shaped meso diameter class length distributions. *Plant and Soil,* 363,113–121

4.7 Tables

Table 4.1 The seasonal daily average (12h) O_3 concentration and temperature at canopy height during the experiment period. CF, charcoal-filtered ambient air. +T, warming. $+O_3$, elevated O_3. $+T+O_3$, warming$+O_3$. Error bars represent SE of the means among replicate treatment plots (n=3).

	Temperature (°C)	**Ozone (ppb)**
CF	24.9±0.00	18.1±0.28
+T	27.4±0.11	20.8±0.11
+O_3	24.9±0.03	66.4±0.81
+T+O_3	27.7±0.30	65.2±0.05

Table 4.2 Comparison of plot air temperature, ozone concentration, plant biomass and other soil properties among treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

	Shoot biomass ($g\ m^2$)	Root biomass ($g\ m^2$)	NH_4^+-N ($mg\ kg^{-1}$)	NO_3^--N ($mg\ kg^{-1}$)	DOC ($mg\ kg^{-1}$)	MBC (mg kg-1)	MBN (mg kg-1)	Soil moisture (%V)
CF	1190.7±40.5a	72.0±11.2a	0.57±0.03ab	5.92±0.80a	40.5±2.5a	255.0±18.3a	44.0±2.12a	24.7±0.6a
+T	1146.7±52.5a	63.4±3.4a	0.53±0.10ab	**3.68±0.64b**	41.3±3.4a	**330.6±17.0b**	51.1±3.5ab	23.9±0.4a
+O_3	1205.7±84.1a	55.3±10.8ab	**0.44±0.02b**	5.20±0.45a	**25.5±1.5b**	233.3±11.1a	40.6±1.6a	23.4±0.5a
+T+O_3	1178.9±40.7a	**40.5±8.1b**	0.54±0.01ab	**3.95±0.55b**	**26.8±1.1b**	258.4±15.3a	42.7±3.6a	23.7±0.2a

4.8 Figures

Fig. 4.1 Total root biomass of soybeans among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Fig. 4.2 Total root length among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Fig. 4.3 Root colonization of arbuscular mycorrhizal fungi in soybean roots among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Fig. 4.4 Relative abundance of *Glomus* and *Paraglomus* in soybean roots among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Fig. 4.5 Mycorrhizally- and root enhanced total C losses from ingrowth cores among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Fig. 4.6 Mycorrhizally- and root enhanced residue C losses from ingrowth cores among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

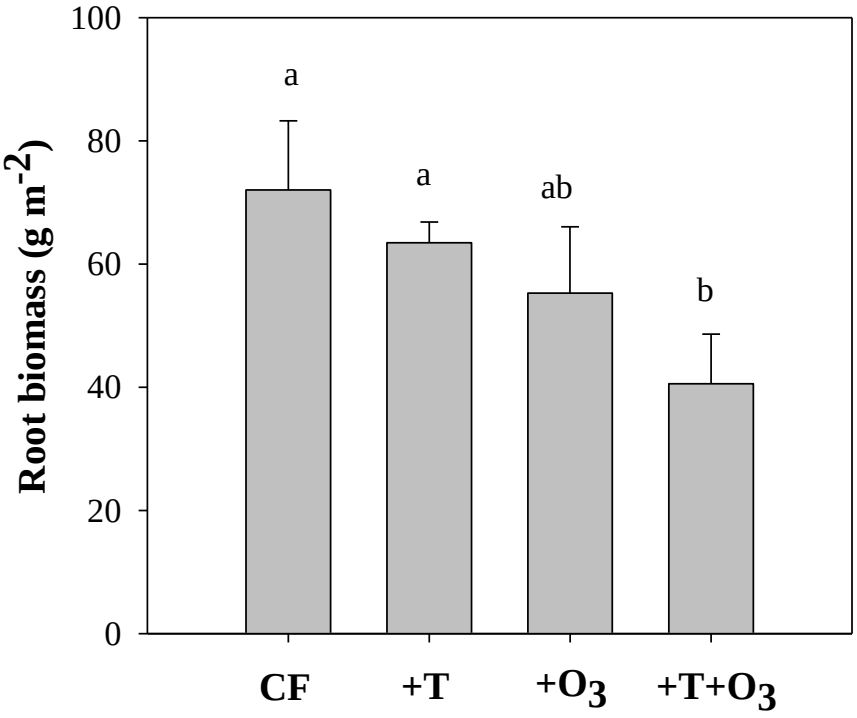


Fig. 4.1 Root biomass of soybeans among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

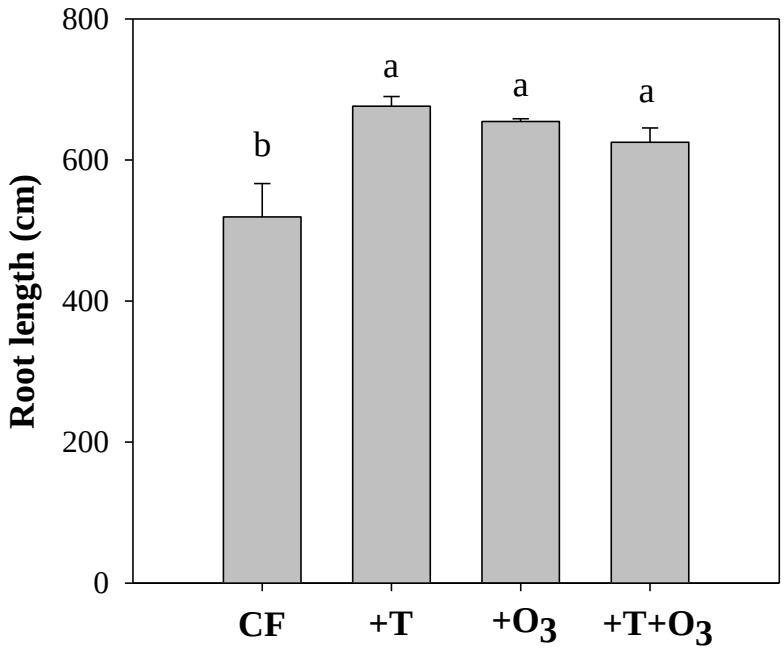


Fig. 4.2 Total root length among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

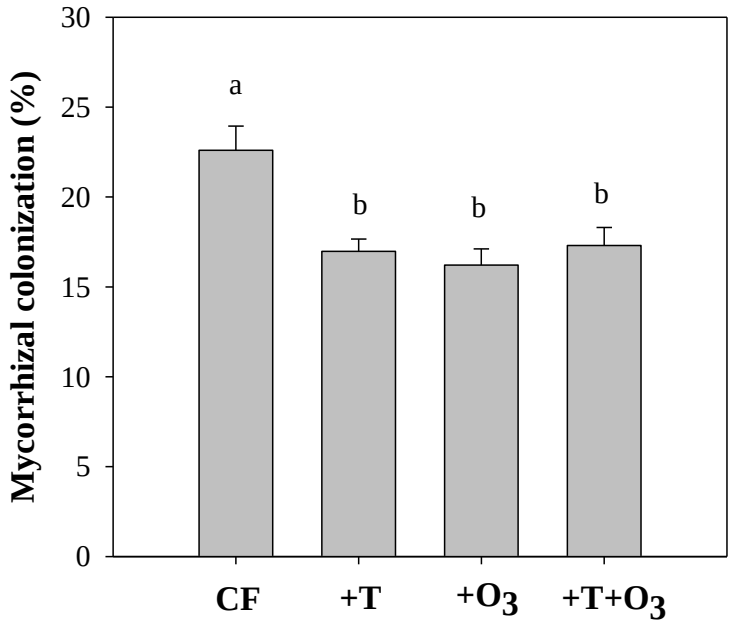


Fig. 4.3 Root colonization by arbuscular mycorrhizal fungi in soybean roots among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

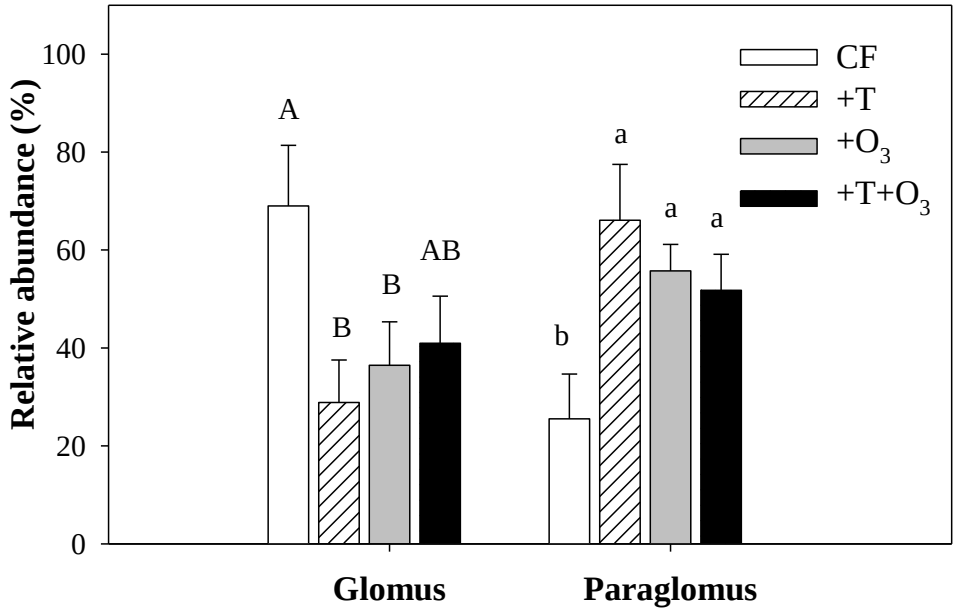


Fig. 4.4 Relative abundance of *Glomus* and *Paraglomus* in soybean roots among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at P = 0.05 level under different treatments.

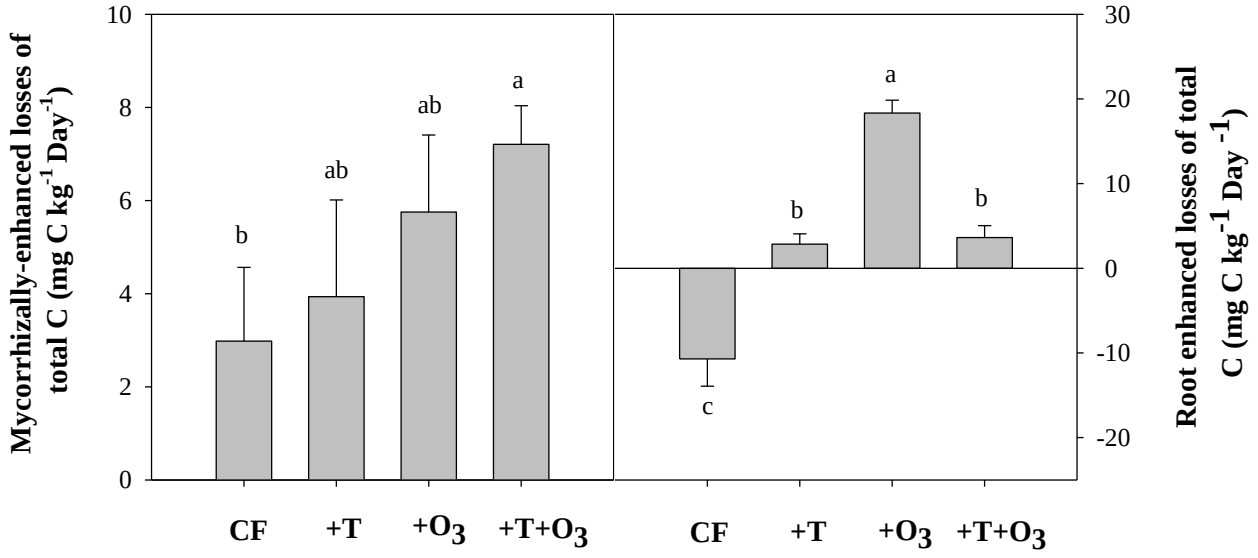


Fig. 4.5 Mycorrhizally- and root enhanced total C losses from ingrowth cores among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O3**, elevated O_3. **+T+O3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

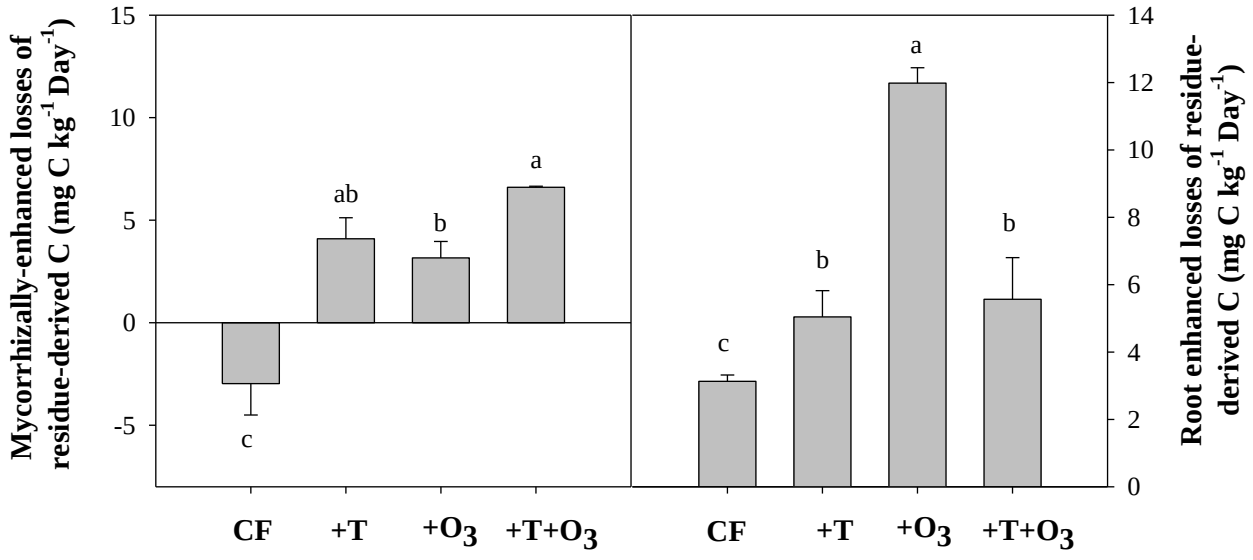


Fig. 4.6 Mycorrhizally- and root enhanced residue C losses from ingrowth cores among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

4.9 Appendices

Appendix 4.1 A presentation of the parameters from the Gaussian models of the Diameter Class Length Distribution of the images presented in Fig. 4.3 among different climate change treatments.

Treatments	a	b	c	d	r^2
CF	0.776	29.28	0.2227	-0.1124	0.9233
+T	1.088	38.43	0.2186	-0.11	0.9239
$+O_3$	0.9202	37.36	0.2221	-0.1122	0.9335
$+T+O_3$	1.129	36.99	0.2178	0.1023	0.9224

Appendix 4.2 Correlation coefficients and significance value for AMF colonization, plant biomass, root morphological parameters, and soil chemical properties and NMDS axes

	NMDS1	NMDS2	r^2	*P*
Colonization	0.418	-0.908	0.176	0.500
Shoot biomass	0.196	-0.981	0.013	0.948
Root biomass	0.657	0.754	0.500	**0.046***
Root length	-0.978	0.207	0.629	**0.027***
Root surface area	-0.982	0.190	0.534	**0.042***
Root volume	-0.986	0.151	0.349	0.143
DOC	0.324	0.946	0.435	0.058
NH_4^+	-0.698	0.716	0.007	0.964
NO_3^-	0.877	-0.480	0.533	**0.031***
Soil moisture	0.725	-0.693	0.274	0.241

The variables with the highest values are in bold; *, $P<0.05$.

Appendix 4.2 Histograms of the origin data of root diameter length class distribution (a) and data generated by the non-linear model (b) among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3.

Appendix 4.3 Nonmetric multidimensional scaling (NMDS) ordination plot showing changes in soybean diameter range composition. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3.

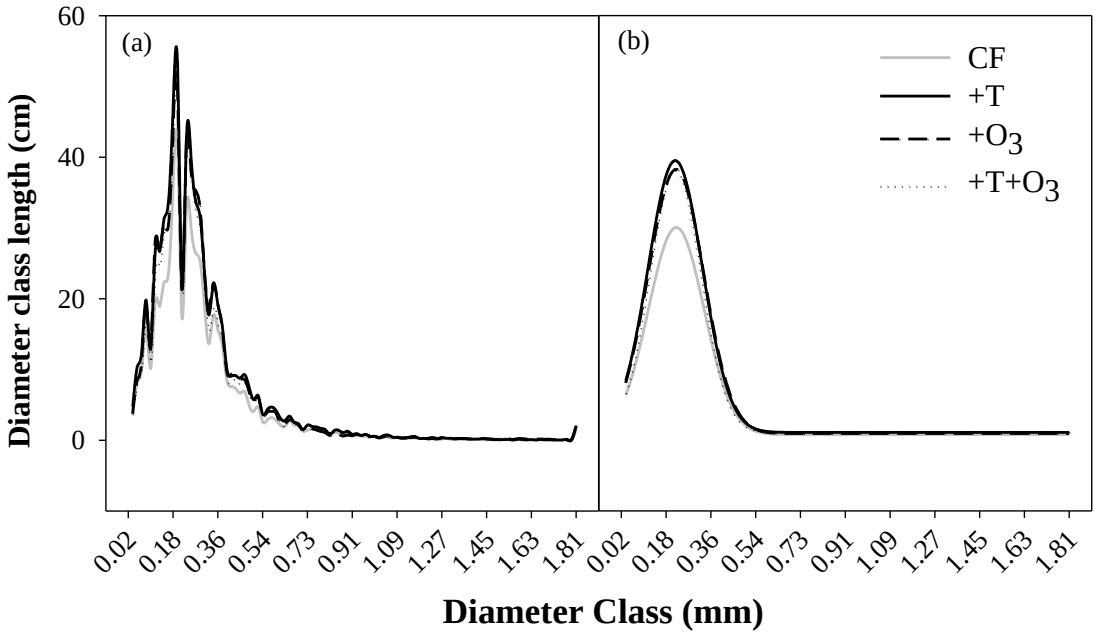
Appendix 4.4 Arbuscular mycorrhizal fungal diversity (reciprocal Simpson's index) and richness (Margalef's index) of communities among the five atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

Appendix 4.5 Mean relative abundance of arbuscular mycorrhizal (AM) fungal genera in soybean root samples among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3.

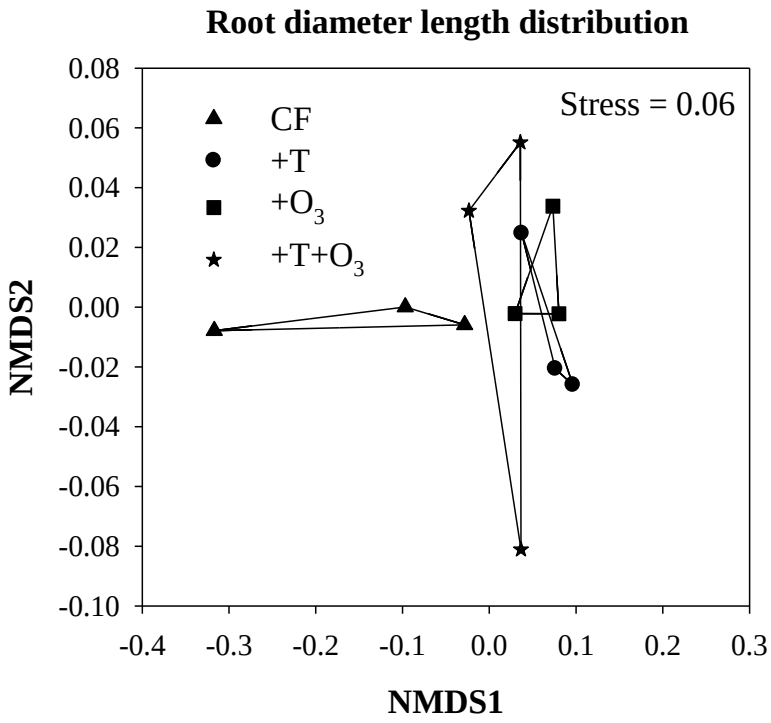
Appendix 4.6 Nonmetric multidimensional scaling (NMDS) ordination plot showing changes in arbuscular mycorrhizal (AM) fungal community composition at species level. Different variables with significant correlation with ordination are shown. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3).

Appendix 4.7 Soil extractable NH_4^+, NO_3^- at the end of experiment among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.

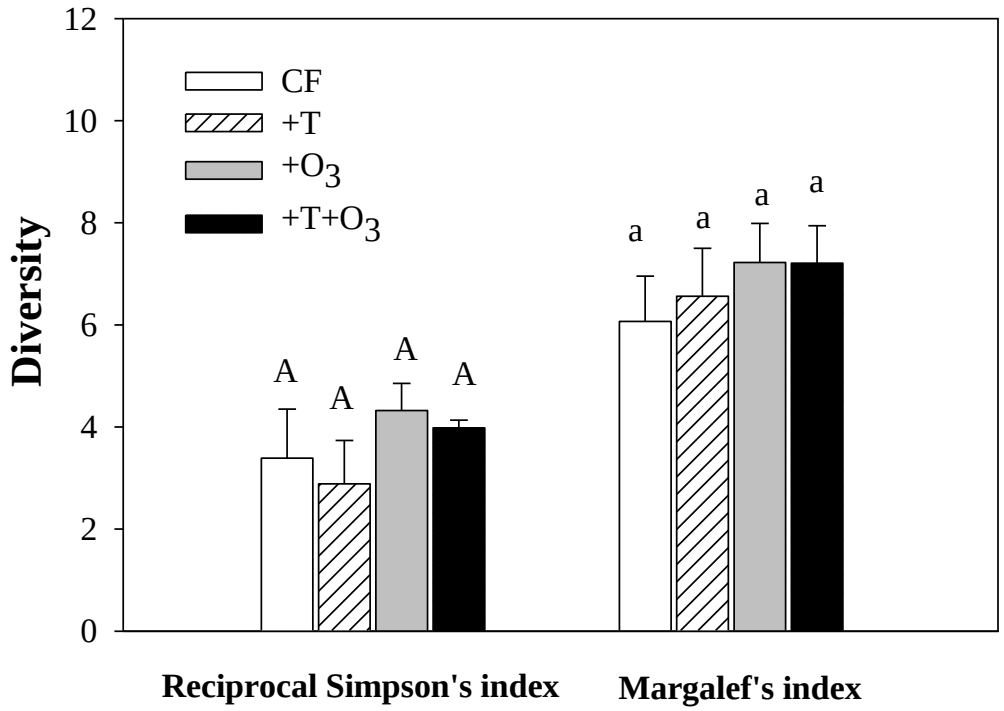
Appendix 4.8 Soil extractable organic carbon at the end of experiment among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.



Appendix 4.2 Histograms of the origin data of root diameter length class distribution (a) and data generated by the non-linear model (b) among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3.



Appendix 4.3 Nonmetric multidimensional scaling (NMDS) ordination plot showing changes in soybean diameter range composition. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3



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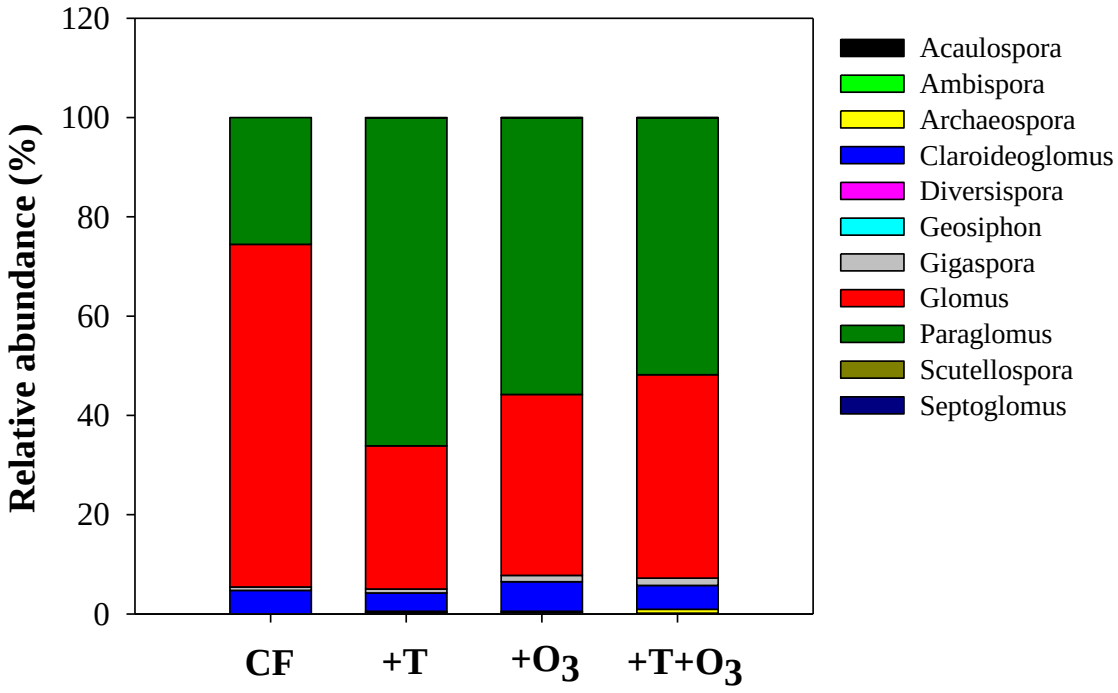
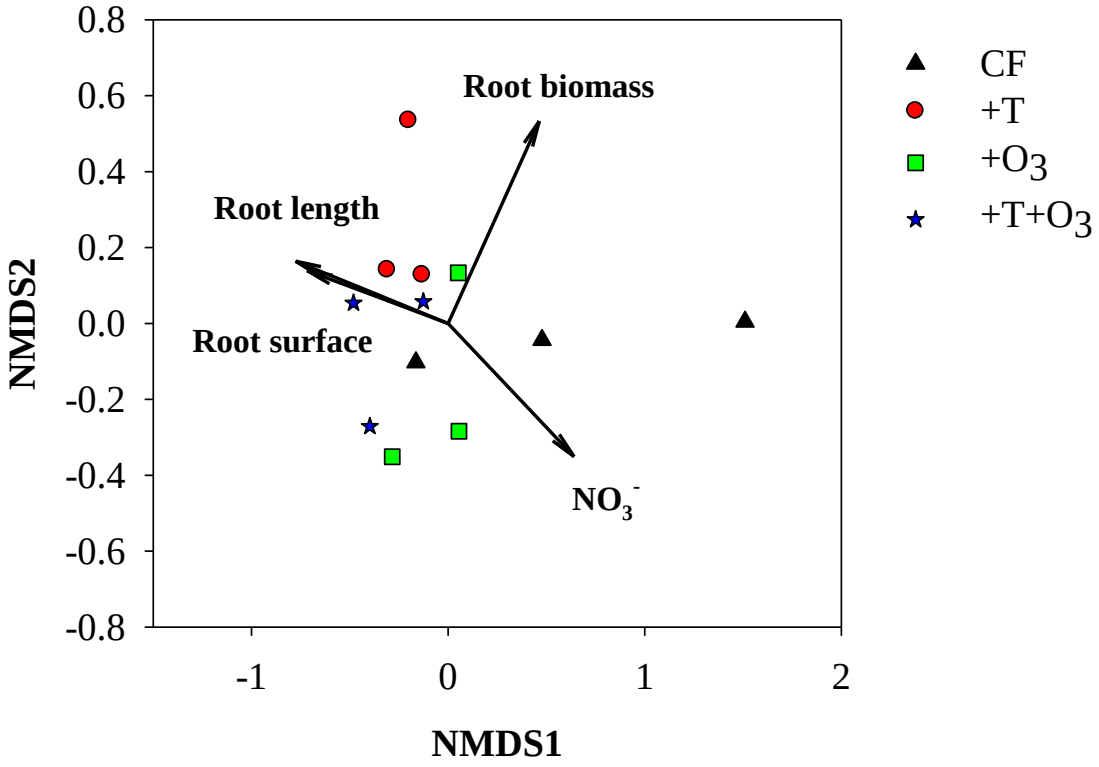
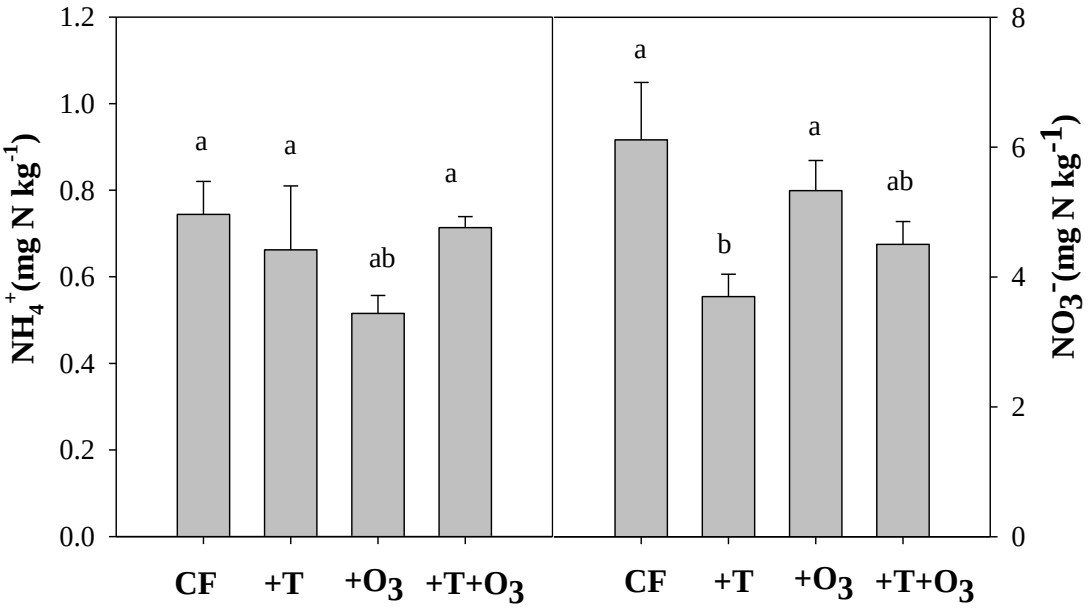


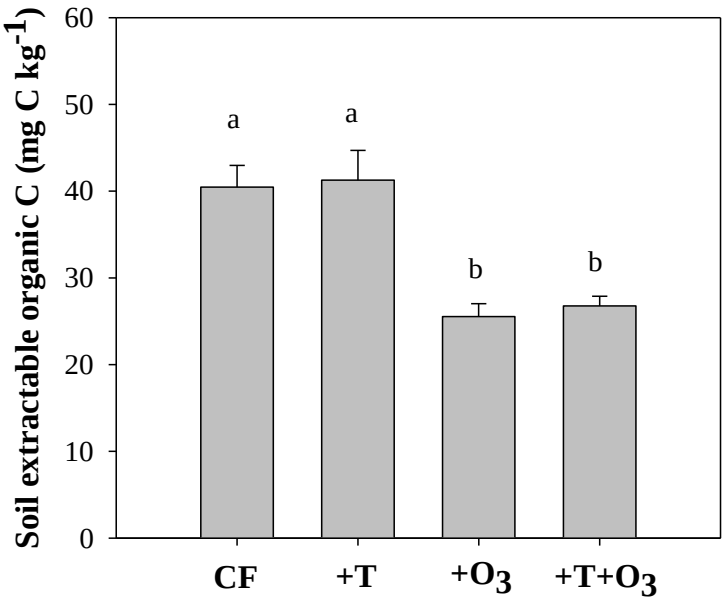
Appendix 4.5 Mean relative abundance of arbuscular mycorrhizal (AM) fungal genera in soybean root samples among the four atmospheric treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3.



Appendix 4.6 Nonmetric multidimensional scaling (NMDS) ordination plot showing changes in arbuscular mycorrhizal (AM) fungal community composition at species level (stress = 0.09). Different variables with significant correlation with the ordination are shown. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3).



Appendix 4.7 Soil extractable NH_4^+, NO_3^- at the end of experiment among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.



Appendix 4.8 Soil extractable organic carbon at the end of experiment among the four experimental treatments. **CF**, charcoal-filtered ambient air. **+T**, warming. **+O_3**, elevated O_3. **+T+O_3**, warming+O_3. Error bars represent SE of the means among replicate treatment plots (n=3). Different letters mean significant differences at $P = 0.05$ level under different treatments.