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GREENHOUSE GAS METHANE AND NITROUS OXIDE PRODUCTION AND MIRCOBIAL FUNCTIONING GENE CHARACTERIZATION IN GRASSLANDS AND THE INFLUENCES BY GRAZING LAND MANAGEMENT

Maura M. Purcell

GREENHOUSE GAS METHANE AND NITROUS OXIDE PRODUCTION AND MIRCOBIAL FUNCTIONING GENE CHARACTERIZATION IN GRASSLANDS AND THE INFLUENCES BY GRAZING LAND MANAGEMENT

by

MAURA PURCELL

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Riqing Yu, Ph.D., Committee Chair

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MAURA PURCELL

has been approved for the thesis requirement on
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for the Master of Science degree

Approvals:

Thesis Chair: Riqing Yu, Ph.D.

Member: Ali Azghani, Ph.D.

Member: Kate Hertweck, Ph.D.

Member: Anil Somenahally, Ph.D.

Chair, Department of Biology

Dean, College of Arts and Sciences

Dedication

I dedicate this Thesis with love to my youngest sister, Tara Sheila Quinn Purcell, who is on my mind and in my heart every day.

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Table of Contents

List of Tables

List of Figures

Abstract

Greenhouse Gas Methane and Nitrous Oxide Production and Microbial Functioning Gene Characterization in Grassland and the Influences by Grazing Land Management

Maura Purcell

Thesis Chair: Riqing Yu, Ph.D.

The University of Texas at Tyler
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Methane and nitrous oxide gases are significantly more potent in their ability to create a greenhouse effect than CO_2. Grazing lands can either be a sink or source of GHG depending on management scenarios and climatic conditions. Management of grasslands can have a broad impact on the levels of GHG emissions, as grazing pressure, crop rotations, and levels and types of fertilization inputs can alter microbial communities and influence on GHG production. Methanogens and denitrifying microbial communities are two major groups associated with the production of GHGs. This study attempted to unravel the microbial and geochemical characteristics associated with CH_4 and N_2O production, and the interplay between the grazing pressure and the fertilizer amendments in the nitrogen fixing clover and nitrate supplemented rye cover grasslands. Using GC analysis of incubated soil samples, this study indicated that surficial soils (0-8 cm) in both clover and rye grasslands contributed the highest production of CH_4 and N_2O. CH_4 production showed significant seasonal changes. High levels of grazing intensity caused a significantly increased CH_4 yield, which was particularly true with no nitrogen fertilized lands. Quantitative PCR of methyl coenzyme M reductase (*mcrA*) *gene*, one of the genes for

methanogenesis pathway, further confirmed theses effects. Increased amendment of nitrogen and carbon of these soils showed that nitrate addition at 100 kg ha^{-1} after 9 day incubation stimulated CH_4 production. Higher nitrate addition, however, could initially suppress methanogenic activities. Organic carbon additions also significantly enhanced CH_4 production.

Chapter 1

Introduction and General Information

Methane gas (CH_4) is an important contributor to the greenhouse effect of global warming. Although the quantity of methane in the atmosphere is lower than that of CO_2, the global warming potential of CH_4 is significantly higher than that of CO_2 (*IPCC*, 2013; Nair et al., 2015). Soil represents one of the largest sources and sinks for methane gas on the global scale (Nair et al., 2015).

Within anaerobic ecosystems, methane is produced as a metabolic byproduct of a class of archaea known as methanogens. Methanogens can be characterized genetically by the methyl coenzyme reductase gene (*mcrA*) which is exclusively found in methanogens. Methanogenesis, the process of methane production by methanogens, consists of a complex series of reactions mediated by multiple different enzymes. This process requires an electron acceptor (e.g., CO_2) as a precursor and an electron donor, most commonly H_2, or formate, but many other organic compounds may also be utilized (St-Pierre et al., 2015). There are a wide range of electron accepting precursors to methane production, CO_2 being the most common (Lal, 2004). Methanol acetate and some minerals like iron may also be used as electron acceptors. Currently there are eleven known substrates for methanogens, which include three main types: CO_2-type substrates, methyl substrates, and acetotrophic substrates (St-Pierre et al., 2015). Methanogens frequently exist in a symbiotic relationship with methanotrophs, a class of aerobic bacteria capable of using methane as a carbon source. Methanotrophs can be genetically identified by the presence of particulate methane

monooxygenase gene (*pmoA*), a gene responsible for production of the enzyme associated with methane oxidation pathway that is peculiar to methanotrophs (Luke et al., 2014).

Methanogens tend to be most abundant in reduced anoxic environments (Aschenbach et al., 2013) including wetlands, bogs, and rice paddies. Much of the research on methanogens, therefore, has focused on these environments (Kruger et al., 2001, Hines et al., 2008; Ma et al., 2010). Multiple studies using in situ and or slurry incubations of soil samples combined with quantification of *mcr*A genes in soils have shown that methanogenic abundance has a direct relation to the production of methane and the biogeochemical profile of the soil, and that alterations in the environment can change both the microbial population and the production of GHGs including methane gas (Kruger et al., 2001; Ma et al., 2012; Aschenbach et al., 2013; Hines et al., 2008). Although much of the current literature on methanogens focuses on environments such as rice paddies and wetlands where they are most abundant, methanogens can be found across all soil types particularly at the deeper anaerobic levels (Aschenbach et al., 2013).

Grasslands and pasture/grazing lands cover 25% of the world's land surface (Peterson, 2017). Therefore the potential of grazing land and grassland to contribute to the global methane emissions or carbon sequestration is significant. Agricultural practices including application of livestock and cover crops can be a significant contributing factor to methane gas emissions, and changes in agricultural management can help to mitigate the negative externalities of methane production by fostering higher levels of methane consumption than production (Nair et al., 2015). Use of fertilizer and relative abundance of nitrogen and carbon sources in the soil have an important and complex role in the production of GHG

in soils (Varga et al., 1990; Praeg et al., 2014; Xun et al., 2016). In order to seek a balance between maximizing profit and minimizing environmental impact, more studies are needed to investigate the genetic profiles of microbial communities and the biogeochemical features, with special focus on their influences on the CH_4 flux of the grazing land under managements. Previous historic studies over 40 years on the same experimental plots suggested that variations of fertilization and cover crops might impact the retention of SON and SOC within the soil (Rouquette & Smith, 2010; Silveira et al., 2013), thus changing the emission rates of carbon and nitrogen to the atmosphere in the form of GHG.

The goal of this project was to explore the interplay between the GHG production and the soil microbial communities in nitrogen-fixing grass clover (*Trifolium* sp.) and nitrogen fertilized ryegrass (*Lolium multiflorum* Lam.) grazing lands. Potential production of GHGs and abundance of functioning genes *mcrA* and *pmo*A were analyzed in order to determine GHG emission and activities of methane producing and consuming microbes in the grassland soils and the effects on the soil with nutrient amendments. Bacterial and Archaeal ribosomal RNA genes and denitrification genes *nir*K, *nir*S and *nos*Z were also targeted in order to understand the overall interplay of GHGs within the microbial community.

Chapter 2

Literature Review

Greenhouse Gases and Climate Change

Since the industrial revolution, global temperatures have been increasing at a steady rate. This evidence is based not only on the historical records collected on regional temperatures but on ice cores, geology, and increasingly sophisticated climate models (Xun et al., 2016; Karmalkar & Bradley, 2017; Rochester, 2011, Karmalkar & Bradley, 2017; Praeg et al., 2014; *IPCC,* 2013; Johnson et al., 2007). Combined with experimental evidence that first began to be gathered in the turn of the 20th century, there is now a preponderance of evidence that the global temperatures are increasing and the cause of the rise is at least predominantly anthropogenic in origin (Paustian et al., 2000; *IPCC*, 2013). The potential effects of this temperature increase could have devastating consequences for the wellbeing of both human society and the world's environment. Global warming is widely considered as the most important environmental issue of the past century, due to both the global nature of the problem and the myriad of ripple effects in which even a modest change in global temperatures can cause damage on the world's ecosystems and resources.

Under normal circumstances, solar radiation in the form of ultraviolet radiation, visible light, and IR (infrared) radiation enters the Earth's atmosphere. About 30% of that solar radiation is reflected back out of the atmosphere by clouds, ice, snow or other reflective surfaces, with the remaining radiation absorbed by the ocean and land surfaces of the earth. Once absorbed, this radiation is then rereleased as IR thermal radiation by the land

and oceans back into the atmosphere where it eventually leaves (Berger & Tricot, 1992). This whole process keeps the global temperature within a habitable range to sustain life. However, an increase in greenhouse gases in the atmosphere can throw this system out of balance. Greenhouse gases (GHG) can be loosely defined as any gas that absorbs IR thermal radiation, thus preventing it from leaving the atmosphere. In addition to the natural emission of carbon dioxide, nitrous oxide, and methane (CO_2, NO_2 and CH_4 respectively), large scale industrial and agricultural applications that depend on fossil fuels produce the aforementioned gases at a high rate. These atmospheric gases create a "greenhouse" effect wherein the solar radiation enters the atmosphere, but then leaves less readily due to being trapped by greenhouse gases. Along with naturally occurring water vapor, the aggregate rise of CO_2, N_2O and CH_4 emissions since the industrial revolution's inception are most directly responsible for the increases in the greenhouse effect and of the world temperature (Nair et al., 2015). A 2013 report from the Intergovernmental Panel on Climate Change, IPCC, showed that the atmospheric levels of N_2O have increased by 15%, of CO_2 by 30% and CH_4 by a dramatic 145% since the advent of fossil fuel-based technology and industrial scale farming beginning in the mid-18th century (*IPCC*, 2013). The aggregate concentrations of CO_2 in the atmosphere are much higher than the aggregate concentrations of CH_4 and N_2O in the atmosphere. However, methane and nitrous oxide are far more potent greenhouse gases.

Carbon Dioxide

Carbon dioxide or CO_2, is the primary GHG responsible for global warming. As of 2014 CO_2 accounted for 80% of US GHG emissions. The vast majority of these

emissions, 94% in the United States, are due to the burning of fossil fuels. The remaining CO_2 in the atmosphere can be attributed to natural sources, as well as other human activities including agriculture and the burning of biofuels. Currently, non-agricultural land is a carbon sink, due to the process of photosynthesis capturing carbon from the atmosphere.

Methane Gas

Methane is a colorless, odorless gas, most of which is naturally produced in wetlands, oceans, rivers, lakes, forest fires, by vegetation and by animal digestion (Mikkela et al., 1995; Karbin, 2015; Strong, 2015). Methane is also used as a fuel source, as it is the principle component of natural gas (Strong, 2015). Indeed, along with thermogenesis, methanogenesis, is the primary way that organic matter is converted into natural gas. Methane has a significantly higher global warming potential than CO_2 due to its higher ability to absorb radiation as compared to CO_2 (Bodelier & Steenbergh, 2014; Dove, 1996; Obata & Shibata, 2012). Although methane gas is released into the atmosphere at far lower levels than CO_2, with only 1.8 ppm of CH_4 to the 390 ppm of CO_2 (Obata & Shibata, 2012; Dove, 1996), methane gas is 25 times more powerful as compared to CO_2 in its potential for trapping radiation, making it the second most threatening GHG in terms of its negative potentiality after CO_2 (Nair et al., 2015). Close to 40% of the atmospheric CH_4 levels are anthropogenic in origin (Strong, 2015). Recent climate models estimate that the concentrations of methane gas in the atmosphere are expected to increase from the current level of 1.77-1.78 ppm to 2.55 ppm within the next forty years (*IPCC*, 2013).

Nitrous Oxide

Nitrous oxide is a water soluble, non-toxic gas naturally found in the air. Nitrous oxide has a 114 year lifetime in the atmosphere, significantly longer than methane's eight year life expectancy. As of 2014, N2O represents 6% of the total man-made greenhouse gases in the atmosphere (Forster et al., 2007). Compared to CO2, nitrous oxide is roughly 300 times more potent as a greenhouse gas due to its ability to absorb radiation (Bouwman, 1996). It has an added hazard that causes the ozone depletion (Ravishankara et al., 2009). Nitrification and denitrification within the soil add close to three quarters of the N2O to the total global emission levels (Braker & Conrad, 2011; Syakila & Kroeze, 2011). Natural emissions of N2O are mainly due to bacterial respiration in the soil. The supplementation of nitrogen into the soil increases the overall output of N2O by these bacterial populations, thus increasing the output of N2O into the atmosphere (EPA, 2016).

Methanogens

Methanogens are a class of archaea that are characterized by their ability to produce methane gas as a metabolic byproduct. Methanogens can be identified by the enzyme methyl coenzyme M reductase which is entirely unique to methanogens and can be identified by targeting the *mcrA* gene which codes for methyl coenzyme M reductase (Aschenbach et al., 2013).Due to the anaerobic nature of methanogens, it was not until much later that methanogens were able to be cultured and definitively identified (Schink & Stams, 2013). In 1977 Woes and Fox discovered the entire phylum of Archaea due to their research using methanogens as a model, which at that time had not yet to be

formally classified and were infrequently worked with due to their difficulty to culture. The entire history of the establishment of a new “tree of life”, and the establishment of the phylum of Archaea ultimately owes its origin to the study of methanogens (Woese & Fox, 1977).

Archaea, with methanogens among them, are characteristically chemotrophic and frequently extremophiles (Schink & Stams, 2013). Archaea share many characteristics with both Eubacteria and Eukaryotes. Like bacteria, archaea contain circular chromosomes, lack membrane-bound organelles, and reproduce asexually or via horizontal gene transfer. Their cell membranes are composed of pseudopeptioglycans (Madigan & Martinko, 2010; Schink & Stams, 2013). All methanogens, by definition, produce methane as a byproduct of the breakdown of substrates into energy (Madigan & Martinko, 2006). Methanogenesis is the process of methane production by methanogens. It consists of a complex series of reactions mediated by multiple different enzymes. The process requires an electron acceptor as a precursor (e.g., CO_2) and an electron donor, most commonly H_2, or formate, but many other organic compounds may also be utilized (St-Pierre et al., 2015). There is a wide range of electron accepting precursors to methane production, CO_2 being the most common (Lal, 2004). Methanol acetate and some minerals like iron may also be used as electron acceptors. To date there are eleven known substrates for methanogens. These substrates fall into three main types: CO_2-type substrates, methyl substrates, and acetotrophic substrates (Schink & Stams, 2013). The potential of methanogens to produce the economically profitable methane gas fuel has not

escaped the notice of natural gas interests and is currently a lively area of research (Strong et al., 2015).

Methanogens are capable of inhabiting a wide range of anaerobic environments including the digestive tract of rumens, the cecum of cecal animals, the large intestine of monogastric animals, the hindgut of cellulolytic animals, the sediments of marshes, rice paddies, swamps, lakes, landfills, and artificial biodegradation facilities (Schink & Stams, 2013; Madigan & Martinko, 2010). Within soils methanogens are abundant and primary source of methane production (Nair et al., 2015). In order to function properly, methanogens require a source of organic carbon and an absence of oxygen, thus methanogens are thought to typically be most prolific in lower anaerobic soil levels (Demirel & Scherer, 2008; Lal, 2004; St-Pierre et al., 2015).

Methanotrophs

Methanotrophs are a class of prokaryotes that consume methane as one of their major sources of carbon. Methanotrophs require a source of oxygen to function and thus often occupy aerobic soil layers, unlike methanogens which inhabit anoxic habitats. Methanotrophs oxidize methane and some similar one-carbon molecules as a carbon source and an electron donor for energy generation. Methanotrophs possess the enzyme methane monooxygenase that produces methanol from methane and O_2 during the course of their metabolic pathway. High levels of sterols are one of the distinguishing features of methanotrophs, as these sterols are part of the internal membrane system required for methane oxidation (Madigan & Martinko, 2010).

Although initially classified according to morphology, methanotrophs are split into two groups based on their internal cell structure, metabolic pathways and phylogeny. They fall under the *Proteobacteria* phylum, specifically Gamma- and Alpha-proteobacterium also known as type I or type II respectively (Strong et al., 2015). Type I uses a RuMP pathway to metabolize carbon whereas type II utilizes a serine pathway to fix carbon (Oremland & Culbertson, 1992; Holmes et al., 1999). Methanotrophs are characterized by having methane monooxygnase (MMO) enzymes, which come in two variations, particulate (pMMO) and the less common cytoplasmic soluble sMMO. Both types oxidize methane into methanol, thus initiating the process of methane metabolism (Lieberman & Rosenzweig, 2004). The genes encoding the MMO enzymes can be identified by use of the *pmoA* gene primers (Kolb et al., 2003; Ma et al., 2010).
Like methanogens, methanotrophs are often widely distributed in wetlands, mud, sludge, rice paddies, soils, and bodies of freshwater. Due to their ability to tolerate and even thrive on oxygen, methanotrophs often are able to occupy a higher soil layer than methanogens. Methanotrophs may be thought of as a "biofilter" for the methane produced by methanogens (Karbin et al., 2015). Some studies indicated that when methane production by indigenous methanogens was subsided, methanotrophs were "primed" to begin increased consumption of atmospheric methane as a new energy source (Karbin et al., 2015)

Nitrifying and Denitrifying Microbes within Soil

Microbial denitrification and nitrification metabolisms are the primary source of N_2O generated in managed and unmanaged soils (Firestone & Davidson, 1989).

Nitrification is a fundamental metabolic step of the nitrogen cycle, whereby ammonia/ammonium is oxidized first to nitrite and then further to nitrate. Nitrification rates are controlled primarily by relative abundance of available O_2 and NH_4^+ in the soil (Firestone & Davidson, 1989). Since the oxidation of NH_4^+ requires oxygen, the rate of nitrification declines with decreasing oxygen levels. When this happens, ammonium oxidizing bacteria often use NO_2^- as an electron acceptor in lieu of O_2 (Poth & Focht, 1985). For this reason the ratio of N_2O/NO_3^- increases as soil becomes more anaerobic (Goreau et al., 1980).

Denitrification is the process by which nitrate or nitrite is reduced to NO_2, N_2 or NO. Availability of O_2, NO_3^- and organic carbon all impact the rate of denitrification by microbes within the soil (Firestone & Davidson, 1989). A wide array of soil microbes (bacteria, protozoa, nematodes, and fungi) are capable of both emitting and sequestering nitrous oxide (Butterbach-Bahl et al., 2013). It therefore simplifies the discussion of N_2O emission by soil if the focus is placed instead on enzymes associated with N_2O metabolism.

The respiratory enzyme NO reductase (NOR) is the main contributor to the production of N_2O by soil. The enzyme is commonly found in both denitrifying and some ammonia oxidizing microbes (Spiro, 2012). There are currently three known types of NOR enzymes: c-type NOR, norCB, and q-type NOR, all of which catalyze NO reduction using two electrons from either small c-type cytochrome, copper protein psudoazurin or the quionine pool respectively (Zumft, 2005). These enzymes differ in electron entry routes and their subunit structure (Spiro, 2012). A variety of non-

denitrifying bacteria use an enzyme of the flavor-diiron type to reduce NO to N_2O, and its purpose appears to be for detoxification. The implications of the activities of these enzymes are relatively minor. The final enzyme of note is flavohemoglobin Hmp, found in both denitrifying and non-denitrifying bacteria and used for detoxification purposes as well. In anoxic conditions, it is known to reduce NO and N_2O, albeit at a slower rate making it a minor contributor to the overall N_2O budget (Spiro, 2012). Unlike its production, N_2O sequestration has been thus far only linked to one enzyme: N_2O reductase (NoS) (Spiro, 2012). NoS is a periplasmic copper protein that catalyzes the reduction of N_2O.

N_2O Emission and Transformation in Soil

There are multiple sources for the formation of N_2O in the soils, including nitrate ammonification or nitrate reduction via ammonia, denitrification by organisms capable of using nitrogen oxides as alternative electron sources, co-denitrification of nitrogen compounds along with NO, autotrophic and heterotrophic nitrification of hydrorylamine, nitrification and denitrification within nitrifying microorganisms, abiotic decomposition of ammonium nitrate and chemo-denitrification of nitrate in soil on exposure to light, humidity or reacting surfaces (Firestone & Davidson, 1989, Butterbach-Bahl et al., 2013). Since moisture levels regulate the oxygen levels within soil, moisture, as a contributing factor in N_2O emissions and temperature levels, can account for about 95% of soil N_2O flux (Butterbach-Bahl et al., 2013).

Carbon Sequestration

Carbon sequestration can be defined as the long-term storage of organic carbon in soil profile. More specifically on agricultural lands, carbon sequestration is the storage of plant and microbial biomass carbon fixed through primary production, ie conversion of atmospheric CO_2 to biomass (EPA, 2016; Izaurralde et al., 2007; Johnson et al., 2007). Soil is a critical habitat of the global carbon and nitrogen cycles. Although the oceans represent the largest carbon sink on earth, roughly three quarters of the carbon storage capacity on the Earth's land surface is surface soil, as opposed to vegetation and animal life (Nair et al., 2015). Methane is an important part of the carbon cycle along with CO_2 (Fig. 1).

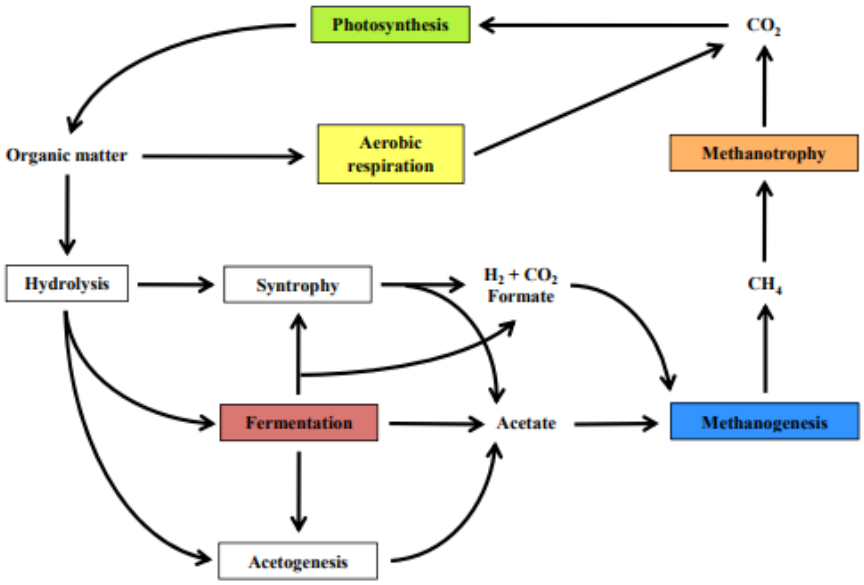

Figure 1. The Methane Cycle

Methane gas cycles through the atmosphere and terrestrial ecosystems, and activities of microbes within the soil (Nazaries et al., 2013).

Management of pasture land can have a significant impact on emission rates of greenhouse gases from soils. Carbon exchange is facilitated by the process of photosynthesis, respiration and carbon precipitation (Grace, 2001). Carbon in various forms is cycled among the atmosphere, oceans, reservoirs, land and marine biota (Fig. 1). The conversion of natural land to grazing land has caused an estimated 50 Pg of carbon to be emitted form soils into the atmosphere (Paustian et al., 2000). Use of fertilizer in agricultural practices and their subsequent influence on biogeocycling within soils are the primary causes of CO_2 production by agriculture (Nair et al., 2015). Currently, one of the feasible and effective way of removing CO_2 from the atmosphere is by enhancing carbon capture and storage, i.e., carbon sequestration and minimizing SOC loss as methane emissions. Previous studies estimated that improved management strategies could increase soil organic carbon (SOC) by 51% (Conant et al., 2001). Management strategies that improve sequestration rates include minimizing soil disturbance, enhancing soil fauna, strengthening soil health and structure, and increasing levels of biomass within the soil (Nair et al., 2015). The soil organic carbon (SOC) pool can be increased by use of mulch farming, conservation tillage, crop rotations and cover cropping in grazing land (Lal, 2004). Cover crops refer to any vegetation cultivated for the purpose of enhancing soil quality and protection of natural resources. Most of the SOC is in the form of some soil organic matter (SOM) which is comprised of plant and animal debris, microbes and carbon associated soil minerals. Although there are a variety of agronomic management

practices available for increasing SOC sequestration, our focus of this study were soil nitrogen management and grazing pressure management.

Nitrogen Fixation and Cover Crops

Most improved agriculture cultivation practices rely on synthetic N fertilizers to supplement soil N, which has negative consequences on environment and climate through nitrate leaching and nitrous oxide emissions. However, use of nitrogen fixing legumes as a nitrogen supplying cover crop is one of many examples of management practices designed to decrease N fertilizer inputs and increase levels of organic carbon in the soils (Lal, 2004). Biological nitrogen fixation is the processes by which N_2 in the atmosphere is converted by prokaryotes in soil or in legume nodules into organic nitrogen compounds, as metabolically usable substrates (Rouquette & Smith, 2010). This conversion is due to a symbiotic relationship between rhizobia prokaryotes and legumes (Rouquette & Smith, 2010). The rhizobia form nodules in the root hairs of legumes and benefit by having a host and energy source from the plant, while the plant benefits by having its nitrogen requirements met for protein formation and growth. A consequence of this is a reduced requirement for nitrogen supplementation in the form of nitrogen fertilizers as is the case with other popular cover crops such as rye grass. Fixation rates can range from 20 kg ha^{-1} to 200 kg ha^{-1} annually in plots seeded with legume cover crops (Rouquette & Smith, 2010).

In grazing lands, cover cropping is the use of plants such as legumes or small grains like ryegrass as a foraging source for grazing animals, or in between, the growth of other crops is in a sequence of crop rotations. Cover crops and/or crop rotations improve soil

structure and health by adding plant biomass to the soil. However, root depth and growth rates are higher in grasses as compared to legumes, and can add higher root biomass and increase the sequestration potential in grasses as compared to legumes (Lal et al., 1999). Many cover crops, either nitrogen fixing, fertilized or some combination thereof, are planted in order to provide a foraging source for livestock, and potentially to increase soil health by increasing sequestration of carbon and nitrogen within the soil. When soils are rich in nitrogen, microbes are better able to convert SOC into CO_2 (Lagomarsino et al., 2007). Thus although necessary for plant growth, nitrogen in excess can lead to not only high CO_2 levels but high N_2O levels as well (Curtin et al., 2000).

Grazing Intensity and GHG Emission Rates

The intensity of grazing pressure by cattle can have an impact on greenhouse gas production beyond just the foraging grasses that the animals require. Grazing can reduce the levels of plant residues that contribute to SOM and thus lower carbon sequestration capacity. This is particularly true when the abundance and quality of carbon returned as manure is quickly mineralized, which is normally the case. This is precisely the reason for 'hot spot and hot moments' effect on GHG emissions in grazing lands, where higher microbial activity and GHG emissions are observed on fresh manure deposited areas. Thus management of grazing lands must aim to optimize grazing pressure on vegetation to maintain both productivity and environmental stewardship (Rouquette & Smith, 2010). One option for managing grazing pressure is through cattle stocking management to mitigate the adverse effects on vegetation stand and soil quality (Wright et al. 2004). There is some evidence that moderate grazing intensity tends to increase soil organic

levels (Derner et al., 1997; Schuman et al., 1999). The reason for this may have to do with cattle packing down plant residue within soil (Fales et al., 1996). Still other studies indicated grazing had little effect on SOC (Milchunas & Laurenroth, 1993). In a long term study on SOC and SN (soil nitrogen) levels in Bermuda grass pastures covered by either clover of rye grass under low and high grazing intensity, SN and SOC levels were significantly lower in plots under high grazing pressure compared to low or moderately grazed plots (Silvera et al., 2013). The authors attributed this to higher levels of plant and fecal turnover and soil disturbance in the highly grazed plots (Silvera et al., 2013).

The cover crop used for foraging can also make a difference on SOC and SN. Clover, for example, is preferred by cattle over most other cover crops (Freer, 1981; Buxton et al., 1996; Dove, 1996; Silvera et al., 2013). Carbon and nitrogen in the clover covered plots were recycled efficiently, partly due to higher turnover and partly due to preferential grazing (Varga et al., 1990; Waldo et al., 1990; Dove, 1996). A consequence of this enhanced biocycling of SOC and SN is the potential for higher N_2O emissions from manure additions (Floate, 1981; Limmer & Steele, 1983; Schimel et al., 1986). Silvera et al. (2013) found that Bermuda grass plots mixed with clover covers sequestered less SOC and SON under high grazing intensity, as compared to those mixed with rye but not at more moderate grazing intensity levels (Silveira et al., 2013). Coupled with the effect of preferential grazing pressure, the higher nitrogen content of clover may have led to a higher turnover of SN and SOC as compared to ryegrass. The result was that high grazing intensity factor combined with clover cover led to lower SOC and SN sequestration,

higher CO_2 emissions and nitrogen mineralization when compared to rye mixed plots under equal grazing pressure (Silvera et al., 2013).

Chapter 3

Materials and Methods

Study sites, geochemical characteristics, and sampling methods

The study sites are located in the grasslands affiliated with Texas A&M Argrilife Research Extension Center, Overton, Texas. The sites serve as the experimental grazing plots which have been historically maintained under different grazing rates and fertilization for more than 40 years (Wright et al., 2004). Soil samples were taken from the the grazing plots from August of 2015 to April 2017. Forage vegetation covers of the experimental plots consist of a mix of either Bermuda grass seeded with clover (*Trifolium* sp.) or Bermuda grass seeded with ryegrass (*Lolium multiflorum* Lam.). The study sites are alternately aligned with three plots of clover-dominated (designated as 'C') and three rye-dominated grasslands (designated as 'R') (Fig. 2). To maintain the forage cover on the plots, the rye-seeded plots have been supplemented with nitrogen base fertilizer (urea or ammonium nitrate) at rate of approximately 100kg ha^{-1} to 300 kg ha^{-1} annually. However, the clover-seeded plots have not been fertilized with any synthetic N. These grass plots have been consistently subjected to the grazing at three different grazing intensities (at high, moderate and low rates) starting from 1969 (Silvera et al., 2013). Stocking rates on the plots, i.e., the means to implement the grazing rates, have been on average of about 2, 3.5 and 5 cow calf pairs per hectare with 685 kg of head equivalent to one pair (Silvera et al., 2013). Grazing treatments were denoted in this study in terms of grazing intensity 1-3 (1 being the highest whereas 3 being the lowest). Each plot with the same grazing intensity was split into four replicate subplots. Layout of the plots can be seen below (Fig. 2).

Sampling Methods

Surface samples were taken using a hand probe of 5 cm (2'') diameter from the first replicate subplot. In each subplot, 12-15 repeated samples at a same depth were randomly taken from an area of a radius of about 2.5 meters. The samples taken with sterile gloves were homogenized thoroughly in clean plastic zip bags on the sites, and immediately brought back to store at 4 °C prior to incubation processing. Deep soil samples, those exceeding 15 cm in depth, were taken in duplicate from each replicate plot using a hydraulic probe of 7.5 cm (3'') diameter at a depth of 60 cm (24''). In situ water used for incubations was obtained from a nearby freshwater creek and was sterilized and deoxygenated using N_2 gas flushing prior to incubation.

Geochemical Analysis of Soil Samples

Soil samples for chemical measurements were taken from varied depth layers at different sites and frozen at -80 °C prior to analysis. Upon analysis, 5 g of crushed soil samples were subjected to water extraction by dissolving the sample in approximately 25 ml of Millipore deionized water within 50 ml Falcon tubes. Soil samples were then shaken thoroughly for one hour and centrifuged 10 minutes prior to water extraction. The water extraction samples were filtered through Whatman #42 filter paper using a vacuumed flask.

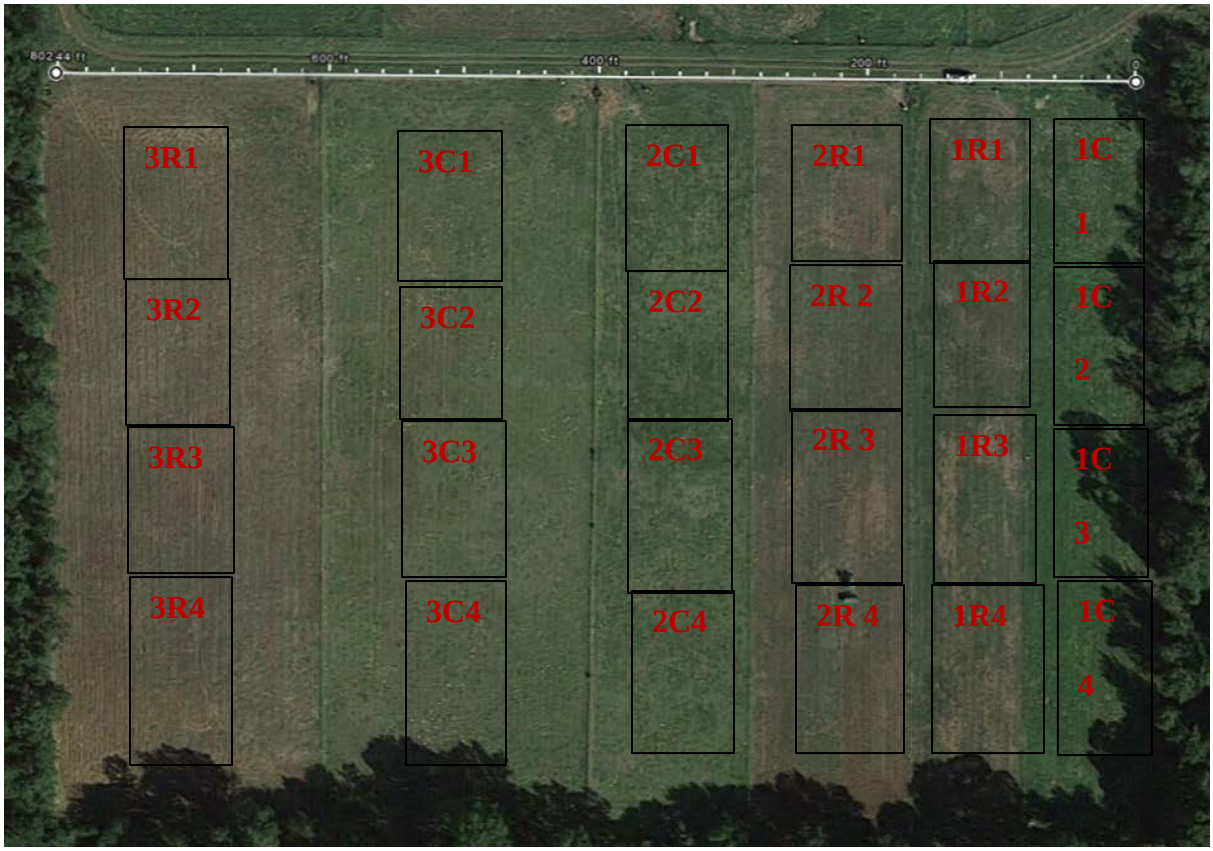

Figure 2. Experimental Grazing Plots

Sampling sites of clover and rye grassland at Texas A&M Agrilife research Extension in Overton, TX.

After water extraction, dissolved organic carbon and dissolved organic nitrogen content of the samples were analyzed using a Shimadzu TOC-VCSH Total Organic Carbon Analyzer with TNM-1 Total Nitrogen Measuring Unit. Water extraction samples were further filtered using a filter pipette, and anion and cation levels in the filtered samples were then analyzed by a Dionex ICS 5000+ ion chromatography (Thermo Scientific). pH measurements were performed using a calibrated Thermo Scientific Orion Star A215 Benchtop pH/Conductivity meter. Approximately 10g of soil samples were mixed with

di-H_2O at a 1:2 ratio. Total organic nitrogen and total organic carbon of select samples were quantified using an Elementar Vario Macrototal Combustion Analyzer on samples of approximately 0.5 g. Approximately 1-2 g of soil samples were weighed prior to heating for 24-48 hours at 105 °C in aluminum weigh boats. Dry weigh was measured after heating and roughly 5 minutes of cooling within a desiccation chamber.

Microcosm Incubation Preparations for GHG Assay

All soil incubation preparations were carried out in an anaerobic chamber flushed with balanced N2 and a mixture of CO_2 (20 %) and H2 (5%). Within 36 hours of sampling, soil samples from each treatment plot were first well homogenized again in the chamber. Seven grams of the soil samples were added in a 20 ml glass vial (MicroLiter Wheaton) mixed with 3 ml of sterilized deoxygenated in-situ water from one creek near the grassland plots. The vials with soil slurry were sealed with 20mm Gray Butyl stoppers (Microliter) and incubated at 25 °C under dark conditions in the incubator within the anaerobic chamber. The vials were shaken once a day during the incubation period.

Time Range and Depth Assays

Based on the preliminary tests, surface soils for incubation time range finding were sampled at 0-15 cm depths from the 1C1 and 1R1 plots, and were incubated in triplicate for periods of 2, 4, 6, 9 and 12 days to determine the proper incubation period as for the endpoint time of GHG production assay. For the depth assay, soil samples at different depths (0-5, 5-15, 15-30, 30-45 and 45-60 cm) were taken from the 1C1, 1R1 and 3C1 plots. Duplicates of cores from each treatment were homogenized prior to incubation.

Seasonal Change Assays

For the seasonal change assay, surface soil samples from the first subplot of each treatment plot (i.e., 1C1, 1R1, 2R1, 2C1, 3C1, 3R1) were taken during the months of August, September, October, November and December of 2016 and February and April of 2017. Except for the July and August 2016 samples (see appendix A), samples in all other months were taken from two layers including the first 0-5 cm and 5-15 cm of surface soil from each plot. All treatments (or aforementioned layers) were incubated in triplicate and allowed to incubate for a period of nine days along with a duplicate of gas blanks and a triplicate of kill controls. Kill controls were made by autoclaving freshly-prepared slurry incubation of surface samples (1C1 or 1R1) at 120°C for 50 min (killing all microbes active in the samples). After incubation periods, all samples were stored at -80°C to terminate microbial activities prior to GC analysis.

Nutrient Manipulation Assays

Surface soil samples from 0-15 cm in depth were taken in February 2017 and March 2017 from 1C1 and 1R1 plots. All soil slurry samples were prepared under anaerobic conditions and incubated as the methods described above. According to the historical levels of fertilizer application on these plots, ammonium nitrate levels equivalent of 100 kg ha^{-1} (around 0.41 mM) and 300 kg ha^{-1} (around 1.23 mM) of fertilizer were spiked in the soil slurry by using 100× concentrated nitrate stock (prepared with sterilized deoxygenated water). Samples incubated for nine days were triplicated. Native soil samples without nitrate addition were prepared to serve as additional controls along with the killed controls. The nitrate amendment experiments were repeated with the

samples taken in March with more time points for gas analyses (with addition of four and six days of incubation periods in triplicate). Organic carbon amendments were prepared by the similar methods by adding sterilized fresh cow manure in 2% and 5% of the wet soil weight, respectively.

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Gas Chromatography

Frozen incubation samples in 20ml glass vials were shipped overnight to the Grazing Lands Research Laboratory in St. El Reno, OK for GHG analysis. Methane, carbon dioxide, and nitrous oxide levels were measured from the headspace of each vial

by using a Shimadzu 2014 gas chromatograph (Kyoto, Japan) with flame ionization (FID), thermal conductivity (TCD), and electron capture (ECD) detectors, which was equipped with a Shimadzu AOC-5000 auto sampler with a 2.5 mL gastight syringe. Chromatograms were analyzed by integrating the peaks at known retention times and comparing them to the linear regression of integrals of known calibration gases run at the beginning of each analysis.

DNA Extraction, and qPCR Analysis

DNA samples from all soils were extracted using PowerLyser PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol, modified by starting with initial soil aliquant of approximately 0.5 g and by use of a beadbeater for cell lysing. Quality and concentration of extracted DNA were determined spectrophotometrically using a ND-1000 nanodrop (Thermo Scientific).

The qPCR analysis was performed using a Corbett Rotor-Gene (model RG-6000) and Rotor-Gene 6000 Series Software 1.7.75. Detection of *NosZ*, *nirK*, Bacterial *16S* and *nirS* genes was prepared according to Harter et al. (Harter *et al.*, 2014) with minor modifications. The following primers were used to target genes including *mcrA*, *pmoA*, Archaeal *16S*, Bacterial *16S*, and the denitrifying genes *nirK*, *norS*, *nirS* (Table 2). Loading of samples for qPCR analyses was performed using a Corbett CAS1200 robot after GC analysis was performed. Standards were made via serial dilution of a gBock synthetic DNA sequence manufactured by ITD DNA Technologies, Inc. (Coralville, Iowa). Spikes were composed of equal parts sample and high standard DNA. All standards and NIC were run as triplicate while the samples, check standards and controls were run as duplicate.

Reactions targeting Bac*16S* genes were diluted 10× for all samples. The initial *pmoA* reactions from the nitrate amendment and partial seasonal sets as mentioned above were diluted from 1 to 10×. All *nirK* and *nirS* reactions were also subjected to a 10× dilution. All samples were prepared according to the protocol referenced therein.

Table 1. Target genes and primers used in qPCR.

Gene	*Primer Sequences*	*Source*
*mcr*A	mcrAlas 5'-GGTGGTGTMGGTTCACMCARTA-3' mcrArev 5'-CGTTCATGGGACTTCTGG-3'	Steinberg & Regan, 2009
*pmo*A	A189f 5'-GGN GAC TGG GAC TTC TGG-3' A682r 5'-GAA SGC NGA GAA GAA SGC-3'	Holmes et al., 1999; Luke et al., 2014; Luke et al., 2014
*nir*K	nirK_Sm_F 5'-TCTGAGCAATTCCAGATGAC-3' nirK_Sm_F 5'-ATCAGATCGTCGTTCCAGT-3'	Harter et al., 2014
*nir*S	nirS_Re_F 5'-CATTGCCGCTCTCACTCT-3	Harter et al., 2014
*nos*Z	nirS_Re_R 5'-GTTATAGGCGTTGAACTTGC-3' nosZ_Sm_F 5'-TCAAACGAAGAAACCAAGAT-3' nosZ_Sm_R 5'-CTTCATCTCCATGTGCATC-3'	Harter et al., 2014
Arch16S	Arch 967F 5'-AATTGGCGGGGGAGCAC-3' Arch-1060R 5'-GGCCATGCACCWCCTCTC-3'	Cadillo-Quiroz et al., 2006; Bengtson et al., 2012
Bac16S	Bac16S, F 5'-TGGAGCATGTGGTTTAATTCGA-3' Bac16S, R 5'-TGCGGGACTTAACCCAACA-3'	(Fierer et al., 2005; Bengtson et al., 2012)

Chapter 4

Results and Discussion

Biogeochemical Data

There were only slight variations in the TOC/TN ratios in the nitrate amended samples. Overall, the rye covered samples had higher levels of carbon relative to comparable clover covered samples. This ratios declined in both treatment types as the enrichment level of ammonium nitrate increased. It should be noted that nitrate concentrations in soil samples were measured after incubation (Table 2).

Time Range and Depth Profile Assay

Time range assays of GHG emission from the grassland soil samples were conducted to establish optimal incubation time periods for the gas emission analyses. Nine days of incubation was determined to be optimal for the GHG emission analyses from both clove and rye soils (Fig. 3). Although 12 days of incubation in the 1C1 and 1R1 plots produced the highest methane production, the standard deviations of analyses varied largely, mainly due to high output outliers of single measurements. These data points are particularly relevant since accuracy of methane production data is of higher priority than the other greenhouse gases (Fig. 3). The optimal time period for accurate CO_2 emission analyses appeared to be around six to nine days.

The CH_4 and N_2O production assay across 70 cm of soil depth showed that methane production potentials in both 1C1 and 1R1 soil sample subplots were all the highest in the top surface soil layer (0-8 cm) (Fig. 4). Beyond this top layer, there was a marked drop in

methane production. Levels of CH_4 yield in the case of the rye plot decreased along the soil depth. N_2O emission also displayed a clear depth profile, with the highest production on the top soil (especially in clover covered plots).

Seasonal Changes of GHG Levels and Functioning Gene Abundance

A seasonal comparison of GHG emission across all soil sampling plots revealed that, from September of 2016 to February of 2017, there was a general trend of increasing levels of methane production potential across all plots as the year progressed, with peaks in either December 2016 (1C1) or February 2017 (1R1). This general increases in CH_4 production over time were not reflected as strongly in other plots. Indeed, in the 2R1 surface plot the reverse seasonal changes seems to be in effect (Fig. 5).

Heavily grazed plots (e.g., 1C1 or 1R1) generally had higher methane production levels than minimally grazed plots (Fig. 5). The high level of grazing pressure had a clear impact on the production of methane gas in the top layer of the soil after incubation, below which the CH_4 production rates decreased dramatically. These high CH_4 production plots (1C1 and 1R1) also increased production levels steadily as the season progressed.

Clover cover plots generally had higher production levels as compared to rye cover plots. The top 5 cm of clover cover plots at high grazing intensity producted the highest levels of methane particularly during the month of December 2016. This was in contrast to the rye plot under the same pressure where the peak of production occurred during Feburay of 2017. The CH_4 production rates in the surface samples from the 1C1 plots were

overwhelmingly and consistantly the highest across all months from September 2016 to Febuary 2017 (Fig. 5).

Based on the qPCR quantification results of *mcrA* gene copies and archaeal 16S rRNA gene copies, copies of *mcrA* gene (unique genes for methanogens) and archaeal cell numbers in the incubated 1C1 or 1R1 soil samples were most abundant in December, although the native 1C1 soil in October contained the second highest gene copies of *mcrA* genes. The result indicated that methanogen abundance increased in the anoxic incubation conditions with addition of only sterilized site water in the soil (Fig. 6). As reflected by qPCR analysis of the functioning gene *pmo*A of methane oxidation, methanotrophs seemed to be most abundant in the native soil and slurry samples after incubation at site 1C1 in December. Abundance of denitrifying bacteria in seasonal samples was highly variable. Changes of gene abundance upon incubation of samples were not noticeable in the denitrifying bacteria (Fig. 6).

Nitrate and Nutrient Manipulation

Addition of ammonium nitrate at the equivalent of high (300 kg ha^{-1}) and low (100 kg ha^{-1}) fertilization rates initially suppressed the production of methane at Day 2 in both 1C1 and 1R1 slurry samples. However, low nitrate addition (100 kg ha^{-1}) significantly stimulated CH_4 production compared with the normal incubation samples without nitrate amendment in the two repeated experiments after 9 d incubation (Fig. 7 A and B). The effects of high nitrate addition (300 Kg Ha^{-1}) varied among the two grass types and repeated experiments. The differences in methane production between the two incubation time

periods (Day 2 and 9) across all samples were statistically significant ($p < 0.05$, a two-way t-test).

From qPCR estimates, nitrate addition (100 kg ha^{-1}) stimulated the highest level of *mcrA* gene expression of methanogens and archaeal biomass in 1C1 incubated soil (Fig. 8), which corresponded to the peaks of methane production in the samples (Fig. 7). It seemed that nitrate addition at two levels significantly enhanced active methanotroph cells estimated by *pmoA* gene abundance in 1C1 soil, while higher nitrate levels inhibited their cell densities in 1R1 samples (Fig. 8).

Organic Carbon Nutrient Manipulation

Addition of sterilized organic carbon in the form of fresh cow manure at 2% and 5% of the total surface soil sample volume (March 2017) significantly increased the methane production levels as compared to the unamended control soil samples ($p< 0.05$, Fig. 9). The CH_4 yield comparisons between the different cover treatments and the different levels of organic carbon were not statistically significant. The levels of organic carbon added appeared to show some effects between the rye cover samples. Standard deviations on all treatments were high, creating ambiguity in comparisons amongst the carbon treated samples. Along with the organic carbon amendment, the levels of N_2O decreased inversely with the increase in CH_4, to a level at or barely above that of the gas blank (Fig. 9).

Addition of organic carbon significantly increased *mcrA* gene abundance and archaeal densities in the incubated 1C1 and 1R1 samples in comparison with the unamended samples (Fig. 10). Significant increase of *pmoA* genes was only observed in the 1R1 soil

due to organic carbon enrichment. Functioning genes of denitrifying bacteria showed little to no variability across the carbon treatments (Fig. 10).

Discussion

Seasonal Changes: The methane production potential changed across the time period of September 2016 to February 2017 with measurements peaking in December 2016 in the case of clover cover crops and February 2017 in the case of rye. This is intriguing since much of the existing literature shows little sensitivity to seasonal variations (Kruger et al., 2005; Scavino et al., 2013). Moisture levels and average rainfall were higher during the winter months of 2017 than the other time points. Methanogens are sensitive to temperature and moisture changes, and the higher rainfall during the months of December and February could have created anoxic niches within the soil pores that increased survivability and activity of methanogens (Angel et al., 2011; Czepiel et al., 1995; Sitaula et al., 1995). However, since the incubations occurred under highly controlled conditions the pre-existing added moisture probably had minimal impact on methanogenesis. Other conditions that were not accounted for could have been the driving force behind this change in methane production across the sampling period.

High grazing intensity of pastureland, irrespective of cover crop or level of fertilization, yielded a higher output of methane gas upon soil sample incubation as compared to soil under lower grazing pressure. A thirty years' study on these grazing lands by Wright 2004 showed that SOC and SN levels were negatively impacted by high grazing intensity, and the grazing intensity of the cattle increased nutrient cycling. SOC can be lost in the form

of methane gas, this phenomenon may have been occurring in the soil incubations as well. Assessment of the TOC in the nitrate manipulation samples showed that the rye plots generally retained more carbon as compared to clover samples under the same treatment, and this could be a factor in the discrepancy between methane production in clover or rye covered plots. Cattle activity, due to physical agitation of the soil and addition of carbon to the soil via fecal matter, has been shown to increase GHG production. High grazing intensity with higher cattle activity are likely the primary causes for the high levels of CH_4 emission. The exact mechanism behind the high emissions from clover cover plots are not entirely clear, but they could be linked to fluctuations in TOC and to a lesser extent to pre-existing moisture levels.

Time and Depth Range Assays: Methanogenic activity generally increased over the incubation time as evidenced by changes of methane gas production levels. Although it would appear that methanogens were active during the longest time period of 12 days, the variability between the triplicate samples was high. Therefore, a less variable period of nine days was selected for the standard incubation time of GHG emission assays, which is also consistent with the chosen 9 day incubation period in CH_4 emission assays employed in previous studies (Hines et al., 2008).

After the initial 3-6 days, CO_2 emission levels decreased as methane levels increased (Fig. 3). This trend is particularly noticeable in the clover cover samples, which had higher methane emissions in general than the rye cover samples. This trend may be due to the methanogens utilizing the CO_2 as a carbon source as they became more active and abundant (Nazaries et al., 2013; Ferry, 1999; Deppenmire, 2002). Similar results were observed in a

study on acetate degradation in Alaskan wetlands, wherein CH_4 production outpaced CO_2 production in slurry samples over time (Hines et al., 2008).

In the depth profile assay, both CH_4 and N_2O production rates were higher in the surface samples. This is contrary to the assumption that methanogens preferentially inhabit deeper soil layers with the low oxygen. However, there is ample evidence that methanogens can be found in all soil layers (or types), aerobic soil and aquatic surface biofilm of floating mats (Angel et al., 2011; Angel et al., 2012; Ganzert et al., 2014). Preliminary qPCR data indicated that all microbes including methanogens were less populous as the soil depth increased. We inferred that the methanogens were more active and abundant at the surface soil due to higher levels of available carbon sources. Micro and macro-aggregates in surface soil can create anaerobic niche in surface soil to some extent, and may facilitate the survival of methanogens originating from animal manure. The seasonal data also support this finding as even the difference between 0-8 cm and 8-15 cm of depth had a noticeable impact on both methane production and the abundance of methanogens across all treatment types.

Depth profile assays had overall lower emissions rates than those in the time range assays, and these discrepancies can be explained by the time assays conducted with soil samples taken in the winter month (January). Methane gas production by the clover cover plots was generally lower than that of the rye cover plots, while the N_2O levels were generally higher in the rye cover sample as compared to the clover cover samples (Fig. 4).

Nitrate Amendments: The addition of nitrate at levels equivalent to 100 kg ha^{-1} and 300 kg ha^{-1} to the grassland soils caused significant initial suppression of

methanogens. The inhibition on methanogenesis by the nitrate addition is likely dictated by the redox potential effects, considering that nitrate as an electron acceptor acts more readily than carbon dioxide thus stimulating activity of nitrate reducing bacteria (Kluber & Conrad, 1998; Loic Nazaries, 2013; Nazaries et al., 2013; Kluber & Conrad, 1998). However, after nine days this suppression dissipated and the activity of methanogens revived in comparison with the untreated control. This trend was particularly true in the mid-range treatment of 100 kg ha^{-1}. These results suggest that, added nitrate could be depleted after sufficient time and the methanogens rebounded and became more metabolically active. The discrepancy in the methane production rates between the higher and lower level of ammonium nitrate addition was probably due to the still existing nitrate inhibition at nine days in the higher level treatment. Gene abundance analysis based on qPCR on soil samples after incubation indicated that methanogens are more abundant after nine days of incubation and particularly in the 100 kg ha^{-1} treatment samples, corresponding precisely to what the GC results showed. The noticeable increase in methanogenic activity in the samples spiked with 100 kg ha^{-1} relative to the untreated controls suggested that there was a metabolic interaction mechanism at play between the processes of denitrification and methanogenesis.

Organic Carbon Amendments: Livestock manures are broadly used in agriculture in order to enhance organic carbon and improve overall soil quality (Nair et al., 2015). According to Kim et al. (Kim et al., 2014), application of fresh cattle manures to rice paddies significantly increased CH_4 emission compared with chemical fertilization and use of swine manure, mainly due to the significantly higher dissolved organic carbon and direct

transferring of cow manure-specific methanogens such as *Methanomicrobiaceae* to the soil. In this study, higher levels of carbon in the form of sterilized cattle manures was able to significantly stimulate methane production in clover and rye grassland soils. A corresponding increase in methanogenic abundance (*mcrA* genes) was also observed (Fig. 10). The difference of CH_4 synthesis between the two levels, either 2% or 5% increase in preexisting carbon levels, was not statistically significant. Our results suggest that, without introducing cow manure-related methanogens, the increase of an organic carbon source could foster methanogen activities under anoxic condition. Estimates of *pmoA* indicated that methanotrophs were more abundant in the rye soil treatment spiked with a higher organic carbon. However, higher methanotroph density in the soil slurry, indicated by *pmoA* genes, did not subdue the significant increase of net CH_4 production, possibly due to their capability to use an alternative carbon source rather than CH_4. Even if methanotrophy was occurring, methanogenesis may have been occurring at a higher rate. As the production of methane rose the production of nitrous oxide fell, suggesting that nitrate substrates were depleted, which corresponds well to the results of the nitrate amendment.

Table 2: Nitrogen Manipulations Total Organic Carbon and Total Nitrogen

Sample	***Total Organic Carbon (ppm)***	***Total Nitrogen (ppm)***	***Total Organic Carbon/Total Nitrogen***
1C1 control	14.35	3.7	3.88
1C1 0 k h^{-1} NO_{3-}	16.83	3.78	4.46
1C1 2d 100 k h^{-1} NO_{3-}	26.22	6.2	4.23
1C1 2d 300 k h^{-1} NO_{3-}	25.12	7.0	3.59
1C1 9d k h^{-1} NO_{3-}	30.88	6.25	4.94
1C1 9d 100 k h^{-1} NO_{3-}	26.28	5.98	4.4
1C1 9d 300 k h^{-1} NO_{3-}	31.14	7.99	3.9
1R1 control	23.5	5.87	4.0
1R1 2d k h^{-1} NO_{3-}	67.54	11.18	6.0
1R1 2d 100 k h^{-1} NO_{3-}	46.25	9.24	5.0
1R1 2d 300 k h^{-1} NO_{3-}	57.99	12.63	4.59
1R1 9d k h^{-1} NO_{3-}	57.34	9.42	6.08
1R1 9d 100 k h^{-1} NO_{3-}	56.02	9.68	5.79
1R1 9d 300 k h^{-1} NO_{3-}	89.9	17.08	5.26

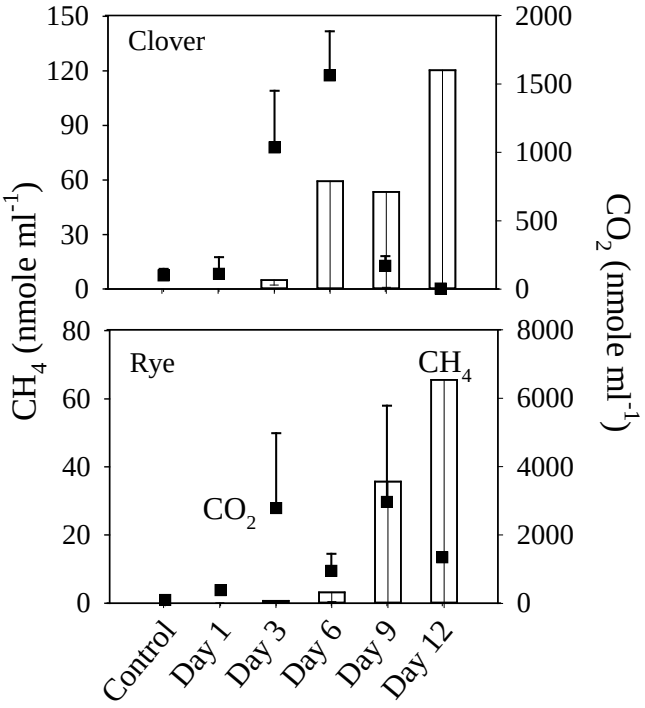

Figure 3. Time Range Assay

CH_4 (bars) and CO_2 (black boxes) production of Clover (1C1) and Rye (1R1) grassland soils within 12 days of incubation. Control: Killed control was selected for CH_4 while the gas blank was used for CO_2 analysis. Error bars indicate standard deviation, downward for methane and upward for CO_2.

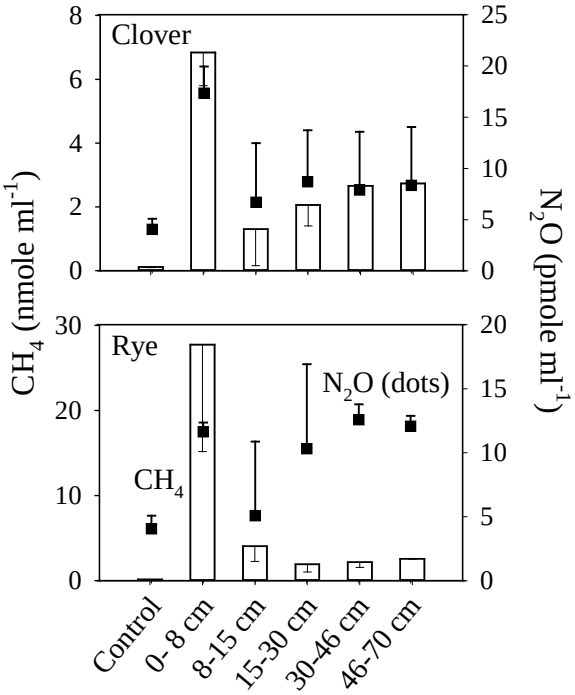

Figure 4. Depth Range Assay

CH_4 (bars) and N_2O (black boxes) production along the depth profile of Clover and Rye grassland soils (Feb.). Control: Killed control was selected for CH_4 measurements while the gas blank was used for N_2O analysis. Samples were incubated nine days. Error bars indicate standard deviations.

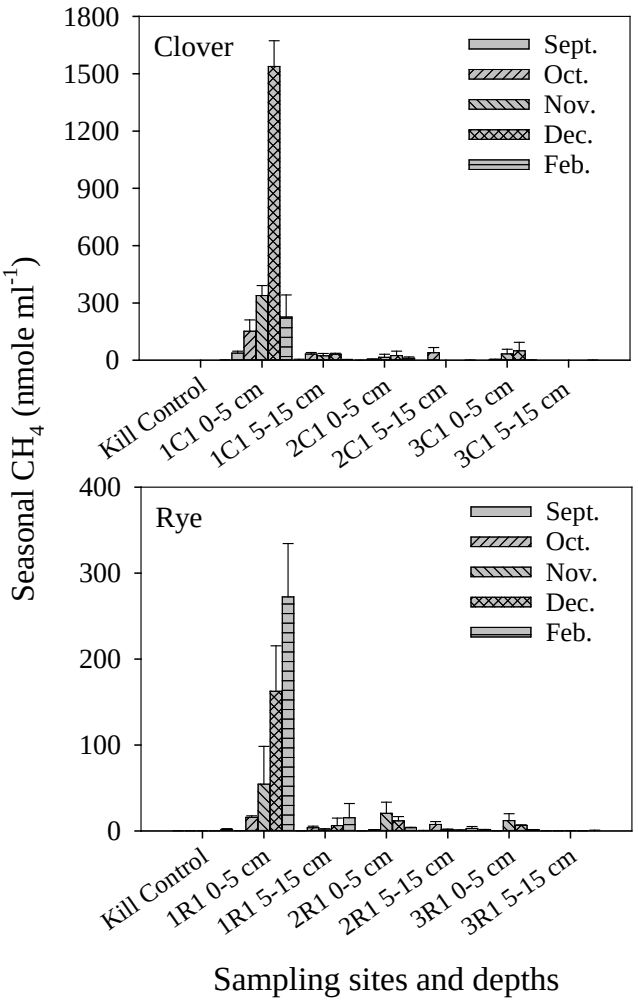

Figure 5. Seasonal Change in Methane Production

Seasonal CH_4 production changes over different sampling sites at two depths (0-5 and 5-15 cm) in Clover (C) and Rye (R) grassland soils with different grazing intensities. The initial number in the sample label, e.g., 1C1 or 1R1, stands for the grazing intensity; and '1' of the initial number represents the highest whereas '3' represents the lowest grazing rate.

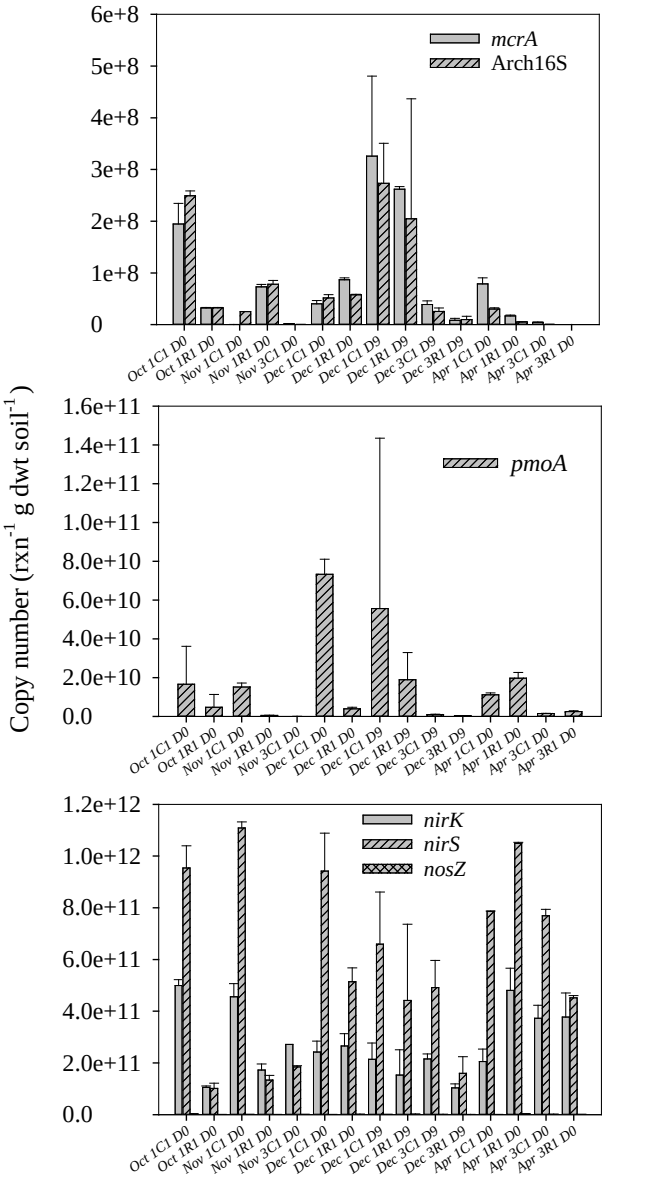

Figure 6. Seasonal Changes of Functioning Genes

Seasonal changes of gene abundance related with methanogenesis (*mcrA*) and methane consumption (*pmoA*), denitrification (*nirK* and *nirS*) and N_2O reduction (*nosZ*).

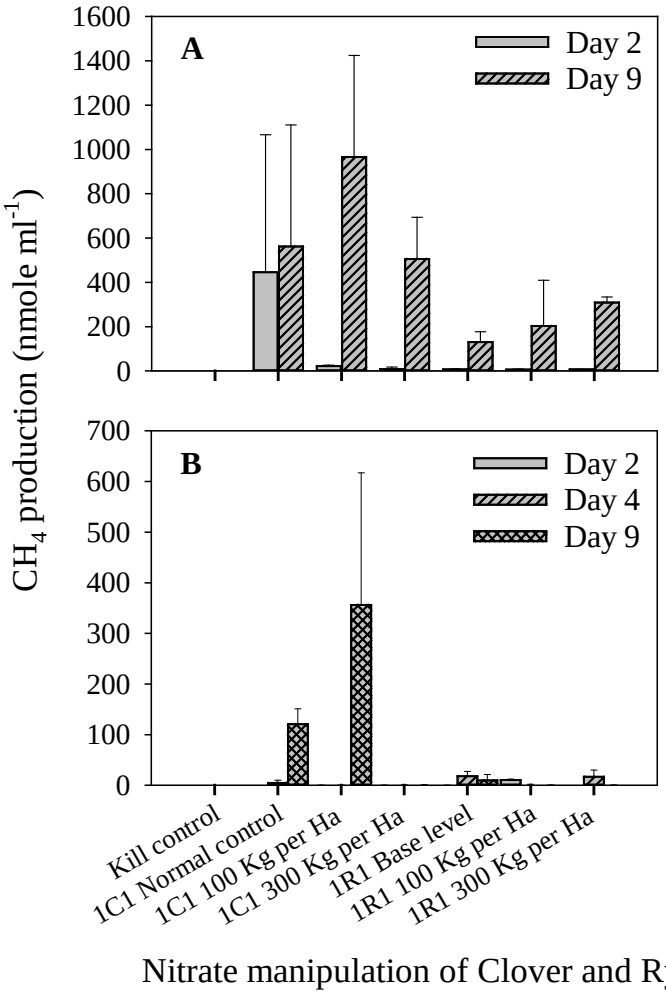

Figure 7. Nitrate Manipulation of Clover and Rye

Methane production influenced by ammonium nitrate amendments in Clover (C) and Rye (R) grassland soil incubations (A and B represent two repeated experiments on February and March 2017, respectively). Control: Gas blanks were selected for CH_4 measurements in panel A while killed controls were selected for the panel B analysis.

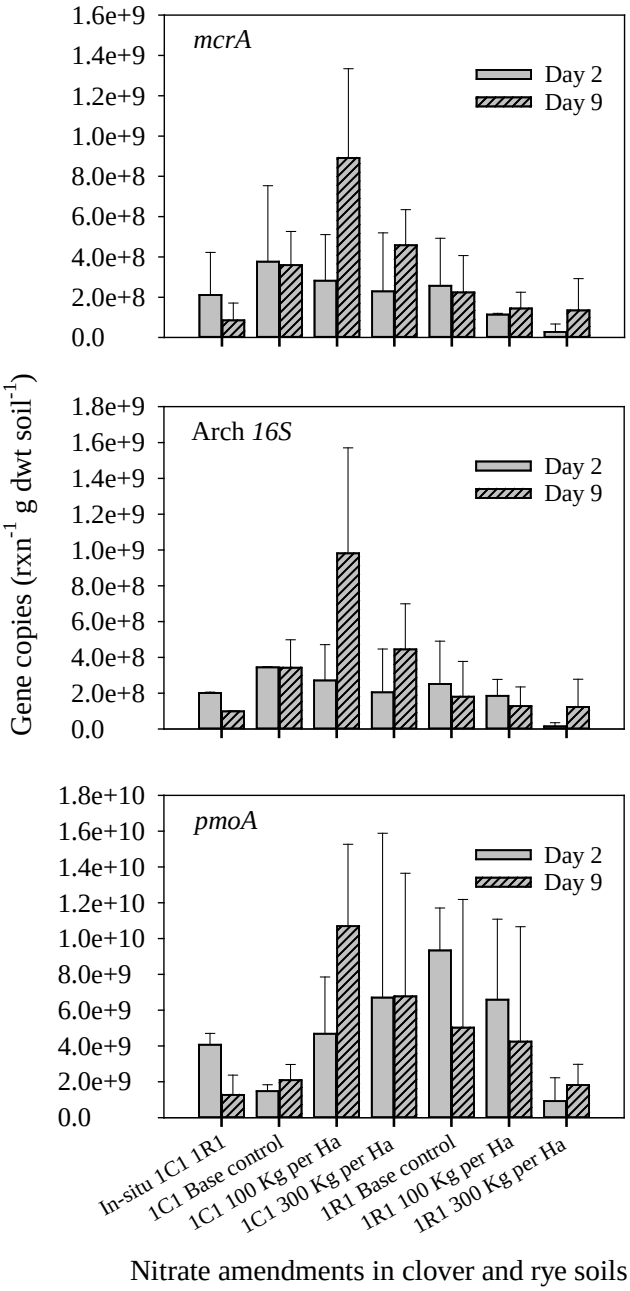

Figure 8. Gene abundance changes responded to nitrate amendments

(Experiment A in Fig. 7) in clover and rye soils. In-situ 1C1 1R1 represent the initiate un-incubated top soils from 1C1 and 1R1 grassland soils.

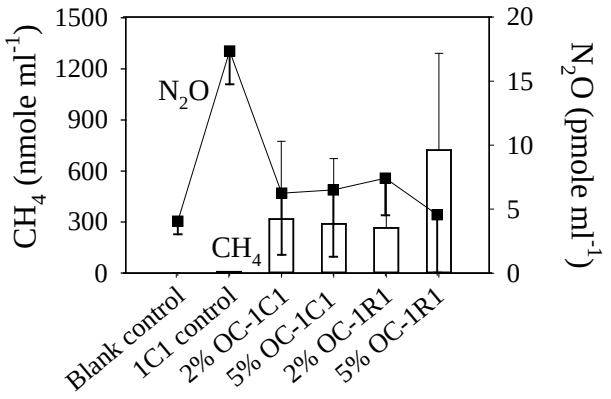

Figure 9. Organic Carbon Manipulation

CH_4 (bars) and N_2O (black boxes) production under organic carbon (OC, sterilized cow manure) amendments in surface grassland soils (0-8 cm) of Clover (1C1) and Rye (1R1). Blank control: Killed controls were selected for CH_4 while the gas blanks were used for N_2O analysis.

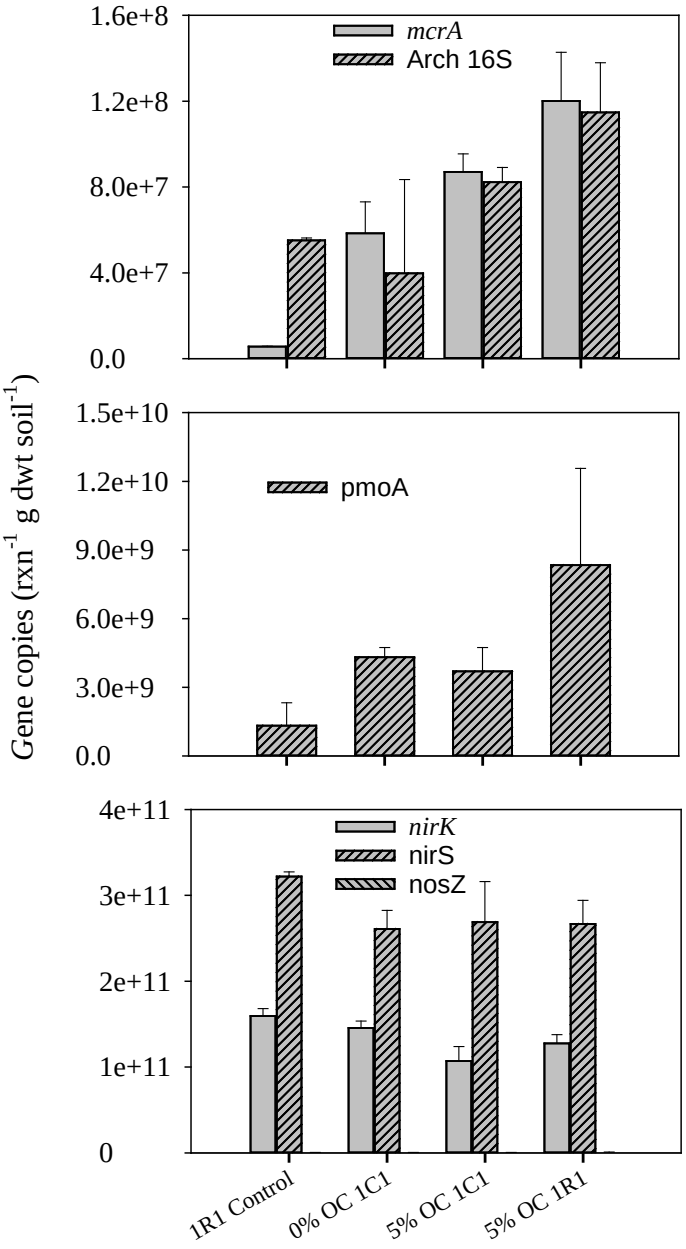

Figure 10. Gene abundance responded to organic carbon amendments.

1R1 control represents the initial in-situ 1R1 top soil (0-8 cm). All other soils for treatments were top soils.

Chapter 5

Conclusions and Recommendations

This study explored the GHG emission and sequestration by soil microbial communities, their seasonal changes, and the potential application of these results in agricultural management on temperate grazing lands in East Texas. Few studies that focused on methogens and methanotrophs have looked into this particular ecosystem. However, the large expanse of grazing land distributed globally makes this study necessary considering the fact that the soils of this type can have emitted large amount of GHGs. What this study has shown is that this particular environment is like so many others, susceptible to damage by over grazing. My results clearly showed that high grazing intensity was linked to higher emissions of methane. This loss of carbon in the form of CH_4 not only means that the greenhouse gas levels increased, but also that the carbon that plants and animals required for nutrients is being depleted. Thus overgrazing not only has the long term negative externality of contribution to global warming, but the more immediate externality to decrease soil health that plants, animals and human societies required for nutrition and energy.

The discrepancy between the methane emissions of organic (clover) and inorganic (rye) soils has raised questions about its cause, which might guide future research in this direction, as this may have important implications not only for land stewardship but also for our understanding of how cover crops change soil conditions for GHG emission and gene responses from the functional microbial communities. Manipulation of soils with organic carbon and nitrate showed that both additions could lead to an increase in

methane gas emission. This finding not only helps to inform management policy, but it has shed some light on the complex interplay between methanogens, methanotrophs, and denitrifying bacteria in this unique soil environment. In the effort to better understand the implications of climate change and improve the means to address the changing climate, novel research like this will become more important and necessary.

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Appendix A. Greenhouse Gas Methane and Nitrous Oxide Production and Microbial Functioning Gene Characterization in Grassland and the Influences by Grazing Land Management

Supplemental Methods

Next Generation Sequencing

Prokaryotic amplicons were generated using primers 519F (5'-CAGCMGCCGCGGTAA-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') that amplify the V4 region of the 16S locus (Wang & Qian, 2009, Klindworth et al., 2012). Paired-end sequence data was generated on an Illumina MiSeq instrument using v3 600 cycle kits (Illumina, San Diego, CA) as described in the Illumina 16S Metagenomic Sequencing Library Preparation protocol, except that dual 6 bp instead of 8 bp index sequences were attached to each amplicon during indexing PCR.

The raw sequencing reads were processed by a third party with a combination of QIIME (Caporaso et al., 2010) and USEARCH (Edgar, 2010) software packages, as well as custom python scripts. 16S sequences were compared to the Greengenes 13.8 reference database (DeSantis et al., 2006) and AMF sequences were compared to the Silva 128 database (Quast et al., 2013) using UCLUST (Edgar, 2010) in order to pick referenced-based Operational Taxonomic Units (OTUs) at 97% similarity, and to provide taxonomic assignments for each sequence read. The sequencing dataset was rarified to an equal sequence count for each sample by randomly subsampling sequences without replacement

to provide even measures of microbial alpha- and beta-diversity and to have equal sequencing depth for the production of all figures, tables, and statistical analyses. The results of these analyses are depicted in Figures S6, S7 and S8.

Statistical Analysis of Microbial Taxa: Unweighted unifrac distance metrics were used in the calculation of diversity measures (Lozupone & Knight, 2005). In order to determine if microbial community composition was significantly different between samples, PERMANOVA was conducted using the QIIME package (Anderson, 2001; Anderson et al., 2011).

Supplemental GC and qPCR Results

Preliminary seasonal methane emission assays on samples taken in July and August 2016: As one of our preliminary experiments, CH_4 in the soil samples taken in July were overall low since the soil samples for slurry preparation were not fresh (left in low temperature storage for a period before using for slurry preparation) (Fig. S1). However, the site-specific changes of methane production between soil samples were consistent with trends that emerged in subsequent seasonal assays (Fig. 5). Samples in Aug. 2016 showed higher CH_4 emission although the highest yield appeared in 2C1 (Fig. S2). Both samples taken in these two months were mixed surface soil from 0-15 cm, which were different from all other seasons with two separated layers for the surface soil (i.e., 0-5 and 5-15 cm).

Nitrous Oxide Production Levels: Nitrous oxide emission was also analyzed on soil samples taken in October 2016. Emission rates in parts per million overall were

substantially lower than that of CO_2 or CH_4. The only apparent peak in production was in the minimally grazed clover soil sample 3C1 (Fig. S3). However, high standard deviations on these samples were observed. There is no clear correlation between peaks in N_2O and peaks in the other GHGs in these samples.

Methyl coenzyme M reductase (*mcrA*) gene detection in grassland soils: Conventional PCR was performed on genomic DNA extracted from soil samples in July 2016 and Sept. 2016 from the following sites (partial sites): 1C1 0-3”, 3-6”; 3C1 0-3’’, 3-6’’; 1R1 0-3’’,3-6’’; 2R1 0-3’’, 3-6’’; 3R1 0-3’’, 3-6’’ ; and September 2016: : 1C1 0-3”, 3-6”; : 2C1 0-3”, 3-6”; : 3C1 0-3”, 3-6”; 1R1 0-3’’,3-6’’; 2R1 0-3’’, 3-6’’; 3R1 0-3’’, 3-6’’(Figs. S4, S5). Presence of methanogens in the soils was analyzed by using the mlas/mcrA-rev primer sets to detect their functioning gene mcrA (encoding alpha subunit of methyl coenzyme M reductase) (Steinberg and Regan, 2008; Ma et al., 2012; Kim et al., 2014). PCR was carried out by using a Biorad MyCyler ™ thermocycler (Bio-Rad, Hercules, CA) according to the protocol in Steinburg and Regan et al. (2009). Amplicons of *mcrA* genes were run on an agarose gel for approximately 45 minutes and viewed under UV light.

PCR detection showed that *mcrA* genes as the unique functioning genes of methanogens were found in most sampling sites in July and Sept. 2016. The results provided the preliminary evidence to conduct the methane emission assay and qPCR analysis of these genes.

Appendix A (Continued)

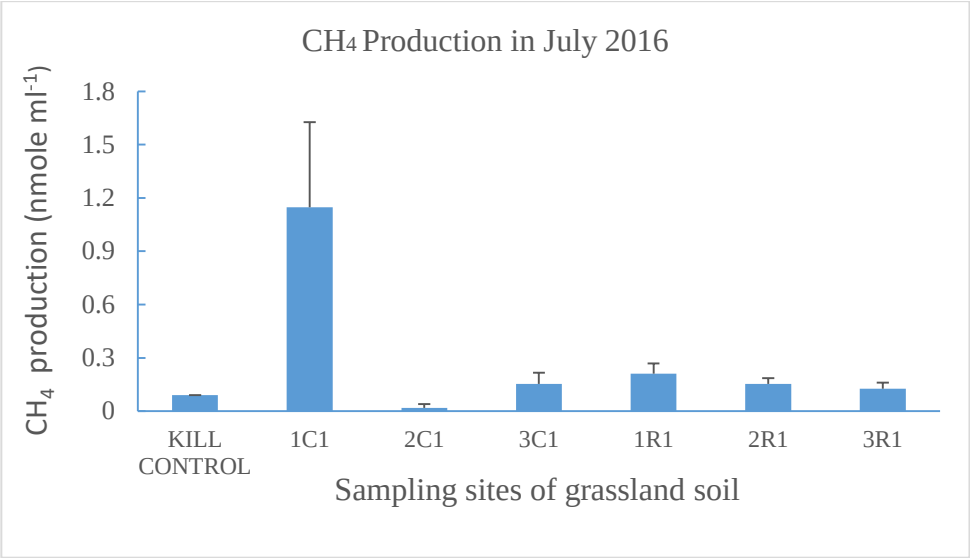

Figure S1. Preliminary methane production analysis in surface soil samples (0-15 cm) taken in July 2016 from the grassland, Overton, TX.

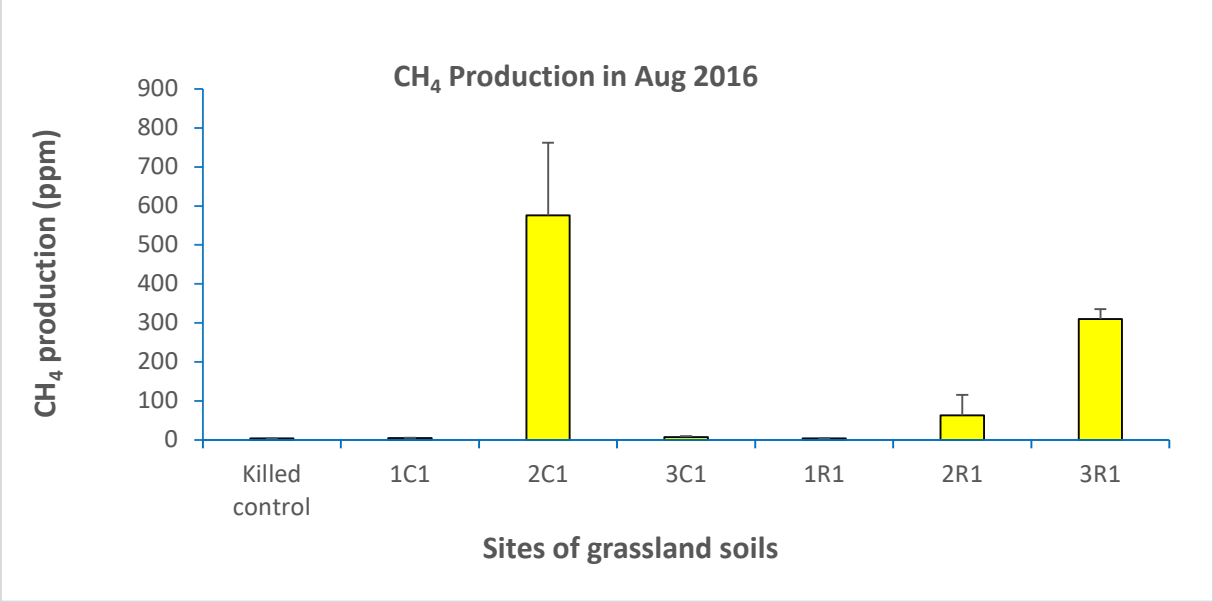

Figure S2. Methane production potential in surface soil samples (0-15 cm) taken in August 2016.

Appendix A (Continued)

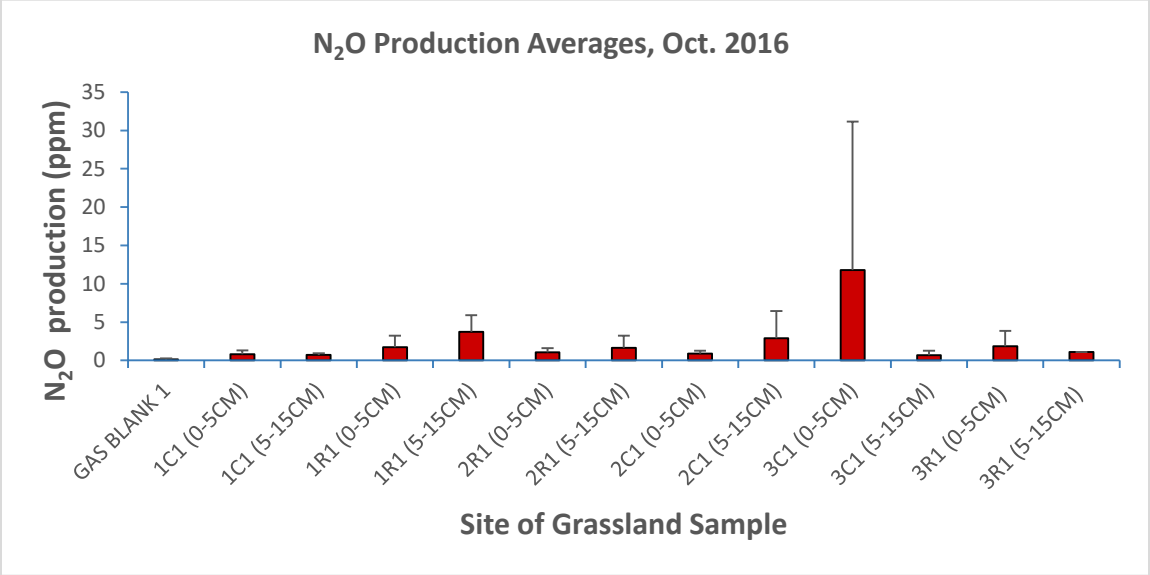

Figure S3. Nitrous oxide production potential of surface soil samples (0-15cm) taken in October 2016.

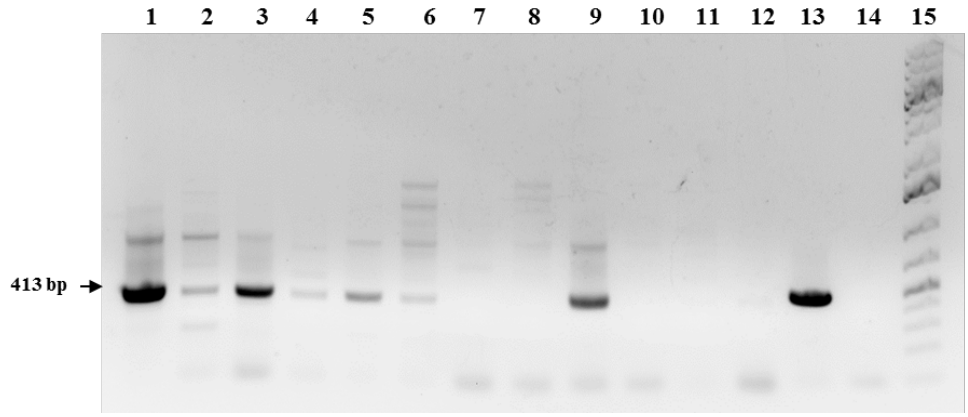

Figure S4 mcrA genes in grassland soil taken in July 2016 from east Texas. July samples: 1. 1C1; 2.1R1; 3. 2C1; 4. 2R1; 5. 3C1; 6. 3R1; 7. ; 8. ; 9. ; 10. ; 11. ; 12. ; 13.*Methanospirillum hungatei*; 14. negative control; 15. Ladder.

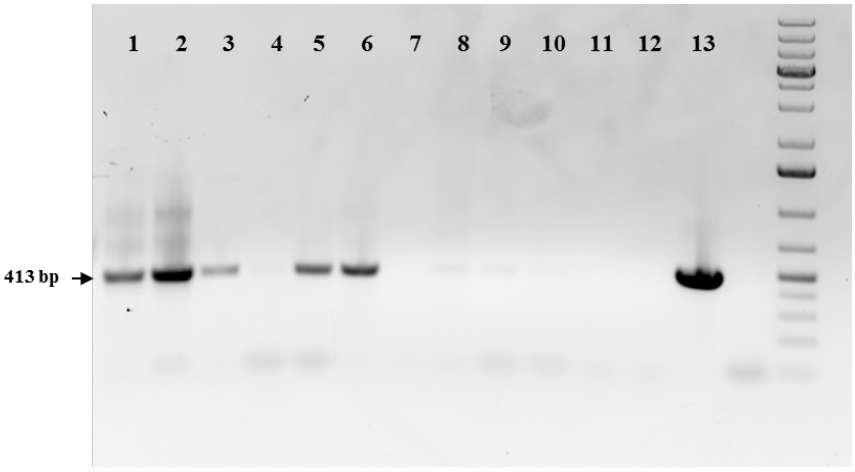

Figure S5 mcrA genes in grassland soil taken in Sept. 2016 from east Texas. Sample order: 1. 1C1-0-5 cm; 2.1C1-5-15cm; … 13. Methanospirillum hungatei; 14. negative control; 15. Ladder.

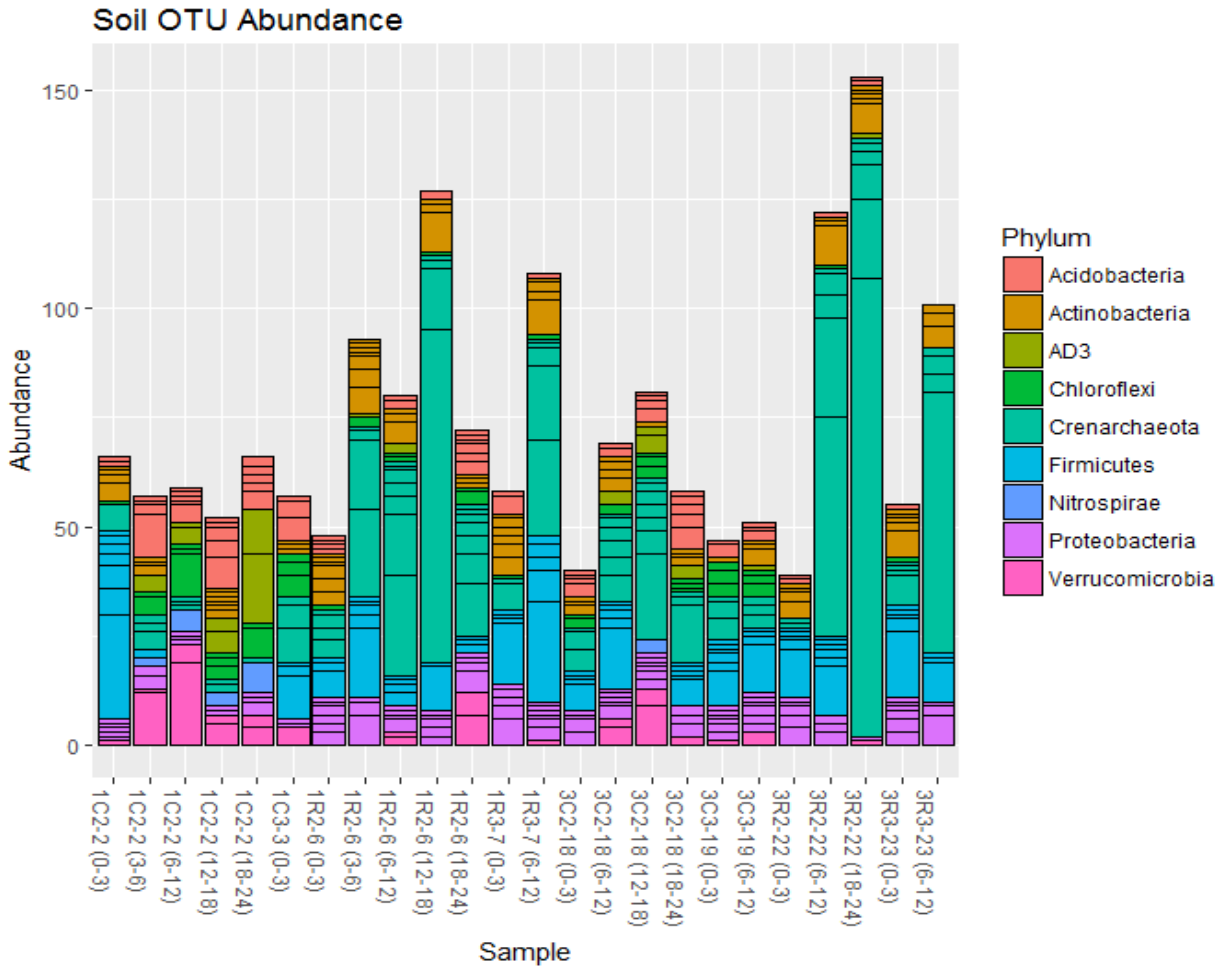

Figure S6. Microbial community composition shown by OTU abundance of grassland soil samples taken in July 2016.

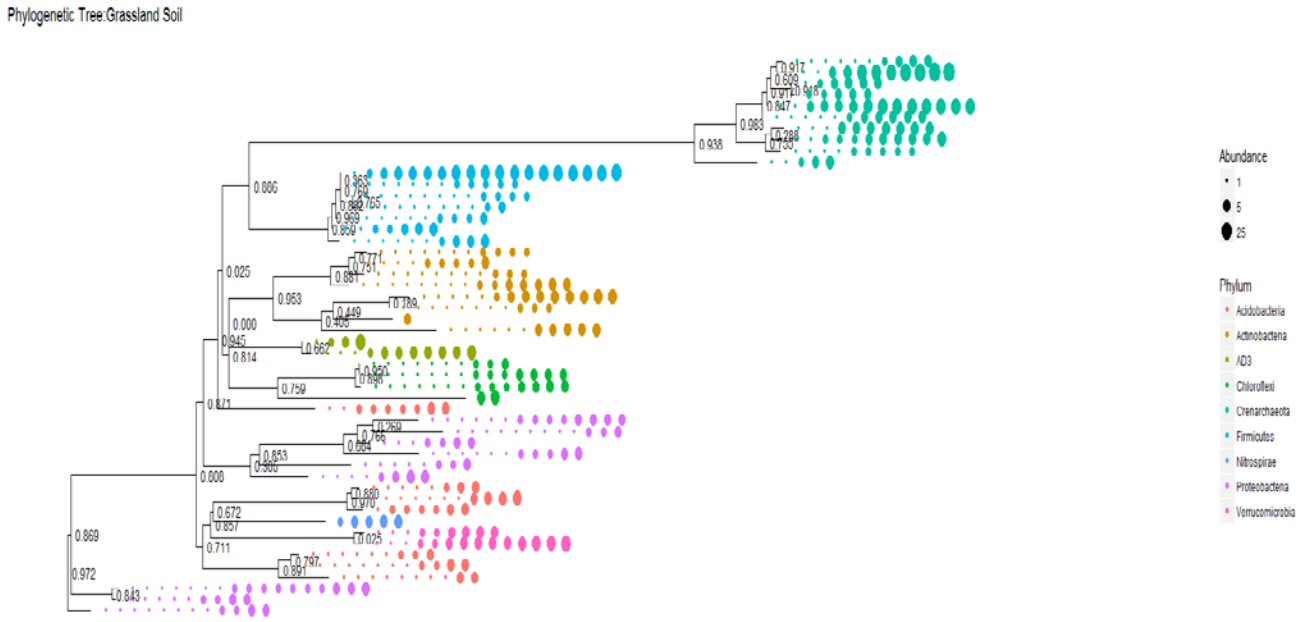

Figure S7. Phlyogenetic tree of bacterial and archaeal communities in grassland soil samples, Overton, TX.

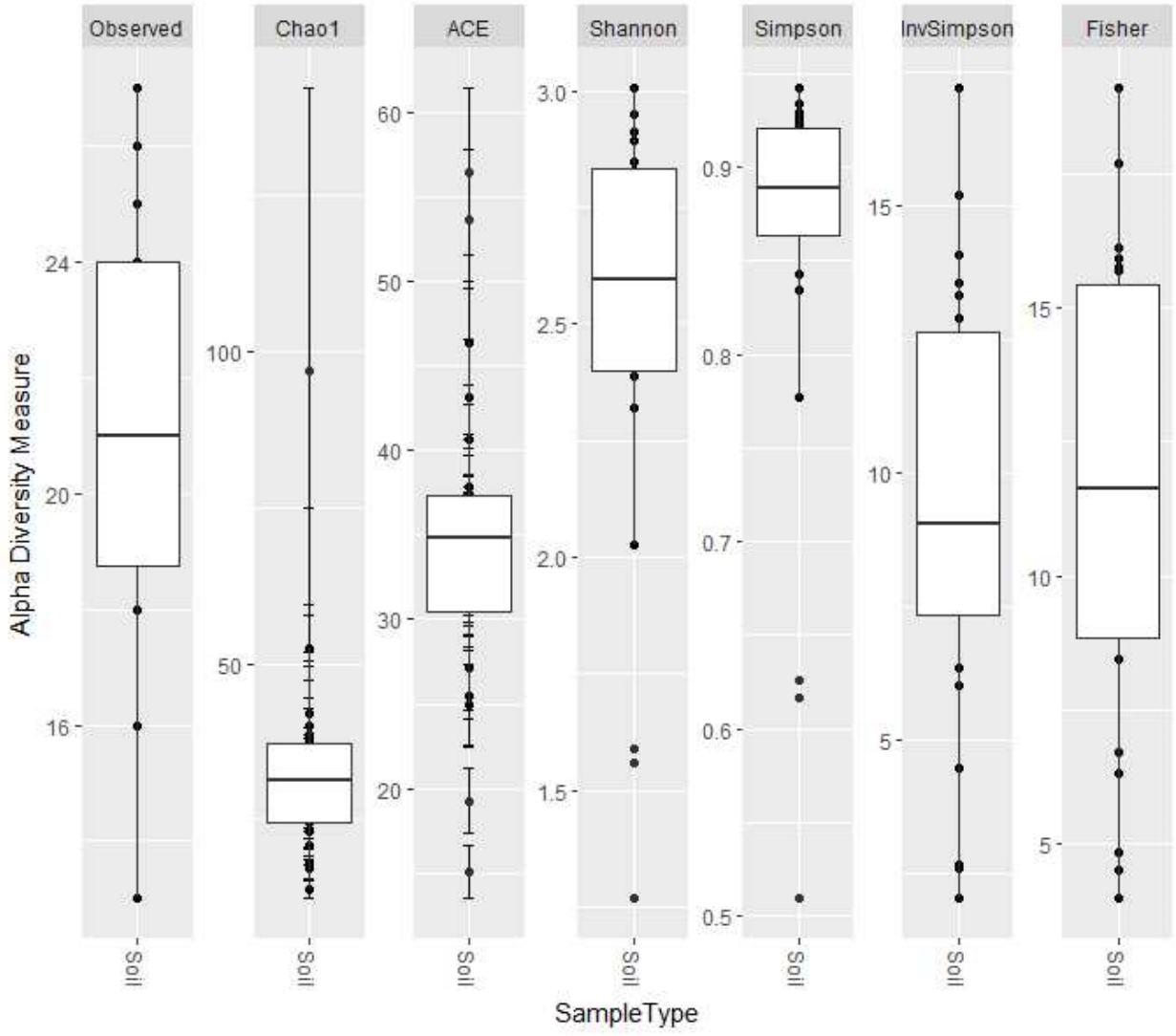

Figure S8. Diversity of bacterial and archaeal communities in grassland soil samples, Overton, TX.

Appendix A (Continued)

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