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EFFECT OF PLANT DERIVED TANNINS ON NITROGEN AND CARBON  
CYCLING IN PASTURE SOILS

by

Kathryn A. Slebodnik

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Soil Science

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UTAH STATE UNIVERSITY  
Logan, Utah

2020

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## ABSTRACT

Utilization of Tannin-Containing Forages for Sustainable  
Beef Production in the Intermountain West

by

Kathryn A. Slebodnik, Master of Science

Utah State University, 2020

Major Professor: Dr. Jennifer Reeve  
Department: Plants, Soils and Climate

Pasture-finished beef has become increasingly popular, but nitrogen losses from these pastures are of concern. Legumes containing condensed tannins such as birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*) may serve as environmentally and economically viable alternative forages in pasture finishing systems due to their ability to produce competitive average daily gains in cattle and potentially reduce soil nitrogen mineralization and loss. However, it is incompletely understood how the tannins produced by these perennial legumes function in the soil to decrease nitrogen loss. The goal of this project was to understand how tannin type and concentration affects soil nitrogen cycling both in the lab and the field through three objectives: 1) comparing the physical, chemical, and biological characteristics of soil samples obtained from grazed alfalfa (*Medicago sativa*), birdsfoot trefoil, cicer milkvetch (*Astragalus cicer*), meadow brome grass (*Bromopsis biebersteinii*), sainfoin, and small burnet (*Sanguisorba minor*) pastures, 2) assessing how feces from cattle fed pure forage hays from objective 1

affect soil nitrogen cycling processes and greenhouse gas emissions using a feces-amended soil incubation study, and 3) assessing how forage tannin type and dose affects various nitrogen cycling processes using a tannin-amended soil incubation study with tannins extracted from birdsfoot trefoil and sainfoin leaves. My hypothesis that condensed tannin-containing legumes (birdsfoot trefoil and sainfoin) would cause decreases in nitrogen cycling parameters was upheld. In the field, soils under sainfoin had significantly lower rates of potential aerobic nitrogen mineralization compared to birdsfoot trefoil which were significantly correlated with forage condensed tannin content. Soils under birdsfoot trefoil also had significantly lower rates of potential aerobic nitrogen mineralization compared to the non-condensed tannin containing legume cicer milkvetch. In the lab, soil amended with tannin containing feces had significantly higher mineral nitrogen immobilization rates, and purified condensed tannins significantly decreased soil soluble nitrogen yields and nitrate concentrations. These results confirm that tannin containing legumes may serve as environmentally and economically viable alternative forages in pastures while reducing soil nitrogen mineralization and loss.

## PUBLIC ABSTRACT

Utilization of Tannin-Containing Forages for Sustainable  
Beef Production in the Intermountain West

Kathryn A. Slebodnik

Pasture-finished beef has become increasingly popular, but nitrogen losses from these pastures are of concern. Legumes containing condensed tannins such as birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*) may serve as environmentally and economically viable alternative forages in pasture finishing systems while reducing soil nitrogen loss. The goal of this project was to understand how tannin type and concentration affects soil nitrogen cycling both in the lab and the field. This thesis: 1) compared the physical, chemical, and biological characteristics of soil samples obtained from grazed grass and tannin and non-tannin containing legume pastures, 2) assessed how feces from cattle fed pure forage hays from objective 1 affect soil nitrogen cycling processes and greenhouse gas emissions using a feces-amended soil incubation study, and 3) assessed how forage tannin type and dose affected various nitrogen cycling processes using a tannin-amended soil incubation study with tannins extracted from birdsfoot trefoil and sainfoin leaves. The field and laboratory results of this thesis suggest that tannin-containing legumes can significantly lower rates of nitrogen cycling processes that promote nitrogen loss to the environment. These results confirm that tannin containing legumes may serve as environmentally and economically viable alternative forages in pasture-finished beef production systems.

## ACKNOWLEDGMENTS

I would like to extend a special thanks to my committee members, Drs. Jennifer Reeve, Karen Beauchemin, Jennifer MacAdam, and Jeanette Norton for their expertise, guidance, and support throughout the entire project. I would also like to thank Dr. Juan Villalba and members of the Holos team at Agriculture and Agri-Food Canada including Dr. Shannon Little, Dr. Aklilu Alemu, Dr. Roland Kröbel, Dr. Sarah Pogue, and Aaron McPherson for their expert opinion and collaboration in outreach activities. I send additional thanks to Danielle Phillips, Karen South, Breann McFarlane, Dr. Stephen Lee, and the staff at the USDA-ARS Poisonous Plants Research Laboratory for their assistance with the laboratory portions of this thesis. Thanks to Dr. Jennifer Reeve, Idowu Atoloye, Ryan Hodges, Bailey Shaffer, Anthony Whaley, Keenen Crummitt, and Karen South for their assistance with field work.

I would like to especially thank my family, friends, and colleagues for their unending support, humor, and encouragement as I navigated this process. I hold your love and kindness close.

Kathryn A. Slebodnik

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## CHAPTER I.

### INTRODUCTION

#### **1. Environmental challenges in beef production**

Beef production systems are at a crossroads in terms of environmental and economic sustainability. Ruminants contribute an estimated 288-348 g of methane ( $\text{CH}_4$ )/day, and pasture nitrogen (N) fertilization and ruminant excreta contribute 16-33% of all agricultural nitrous oxide ( $\text{N}_2\text{O}$ ) emissions (de Klien et al., 2008). Based on a combination of surveys and nearly 150 representative life cycle model simulations that consider typical regional beef production operations across the United States, 21.3 kg carbon dioxide equivalents ( $\text{CO}_2\text{-eq}$ ) and 155 g of reactive N are required on average to produce a kilogram of beef carcass for combined farm gate and post-gate processes (Rotz et al., 2019). Grass-fed beef is growing in popularity as an alternative to feedlot finished beef due to concerns among consumers for the environment and animal health. However, compared with feedlot-finished beef, grass-finished beef has a larger greenhouse gas (GHG) footprint per kilogram of beef due to lower feed quality and animal average daily gains (ADGs). Replacing grass in beef production with tannin-containing legume forages can significantly reduce GHG emissions in both soils and ruminants while attaining levels of production and acceptance similar to grain-finished beef (Chail et al., 2016). A legume-based beef production system, particularly one containing condensed tannins, can eliminate the need for fertilizer N, maintain pasture ecosystem services, and increase N retention in the production system.

In feedlot-finished systems, cattle are brought to concentrated feeding areas, where they are fed a high-starch feed, such as cereal grain. The high energy content of these feeds increases the ADGs of the cattle, allowing a large number of cattle to be finished in a shorter period of time on a smaller area of land. As a result, these systems generally have lower GHG emission rates on a per-kg meat basis. In pasture-finished systems, cattle are grazed in pastures where their diet consists mainly of grasses. Grasses are a lower quality feed than cereal grains, so ADGs are less. This requires a greater number of individuals and a longer finishing period to produce the same quantity of finished beef as a feedlot system, resulting in higher GHG emission rates on a per-kg meat basis. In Capper's (2012) comparison of conventional versus grass-fed beef production systems, animals in conventional systems were slaughtered 444 days after birth at an average weight of 569 kg, and produced a total of  $15,989 \times 10^3$  t CO<sub>2</sub>-eq. Animals in grass-fed systems were slaughtered after 679 days at an average weight of 486 kg, and produced a total of  $26,785 \times 10^3$  t CO<sub>2</sub>-eq. These calculations consider CH<sub>4</sub> and N<sub>2</sub>O from enteric fermentation and manure (using US EPA or IPCC methodologies and emission factors), N<sub>2</sub>O and carbon dioxide (CO<sub>2</sub>) from crop production (fossil fuel combustion, fertilizer and pesticide manufacturing and application, and manure application), and carbon (C) emissions from transportation distance and fuel efficiency. In MacAdam and Villalba's (2015) side-by-side comparison of grass-, legume- and grain-finished beef, the yield of meat from grass and grain finishing was comparable to Capper's (2012) data, while the yield of meat from legume (birdsfoot trefoil) finishing was similar to the yield from grain finishing when all three groups were finished for 16 weeks. Estimates of the number of cattle required to produce one billion kilograms of red

meat were comparable in conventional feedlot (2.75 million) and legume systems (2.89 million), in contrast to a grass-fed system (3.59 million) which required notably more.

## **2. Pasture nutrient cycling and ecosystem services**

Nutrient cycling plays a critical role in determining the environmental impact of a beef production system. Soil N cycling in particular has the potential to impact local water quality as well as the global climate. Nitrogen may be denitrified to inert  $N_2$  gas or  $N_2O$ , a potent GHG, depending on factors such as soil texture, moisture content, and C, N, and oxygen availability (Davidson and Verchot, 2000; de Klien et al., 2008; Weier et al., 1993). Inorganic N as nitrate ( $NO_3^-$ ) is mobile in the soil solution and may be readily leached into ground and surface waters (Norton and Stark, 2011). Excess N in water sources may lead to local environmental issues such as eutrophication and human health problems (Majumdar and Gupta, 2000). Different beef production systems require different types and degrees of energy and N inputs, such as fossil fuels and N fertilizer. Different systems may also support natural ecosystem nutrient cycling services to varying degrees. The combination and interaction of these inputs and services will dictate a systems final environmental impact.

Established grasslands used for cattle production provide a variety of ecosystem services which may be enhanced with proper pasture management. Soils regulate climate via C sequestration as vegetation is harvested and roots turn over, provide food and fuel, purify water resources, and mediate nutrient cycling (Baveye et al., 2016). The tight coupling of the C and N cycles in undisturbed grasslands protects soil and water quality. The development of grasslands has been shaped by the co-evolution of herbivores and plants. At low stocking densities, herbivores enhance ecosystem services such as C

sequestration, N cycling, and primary production (Soussana and Lemaire, 2014).

However, high stocking densities, poor management, and overgrazing tend to decouple the C and N cycles due to the release of C from the system as CO<sub>2</sub> and CH<sub>4</sub>, and return of N to the soil in highly concentrated urine patches (Soussana and Lemaire, 2014). As the C:N ratio of the soil is decreased, excess N exits the soil in aqueous or gaseous forms. In seeded grasslands, additional N may be applied as fertilizer to maintain grass production under irrigation or humid climates. Nitrogen fertilization is correlated with increased soil N<sub>2</sub>O emissions and may further promote N losses (Mulvaney et al., 1997). Legumes are particularly important for maintaining N cycling because they are able to biologically fix their own N or switch to using soil N as needed, therefore eliminating the need for external N inputs to support optimal yield. This prevents unnecessary N additions to the ecosystem, reduces the potential for N leaching, increases C sequestration, and minimizes GHG emissions (Soussana and Lemaire, 2014). Phenolic compounds found in some legumes may additionally protect soil organic matter (SOM) through a decrease in decomposition rates and promotion of soil aggregate formation (Halvorson et al., 2016).

### **3. Benefits of tannin-containing legume forages**

Improving the rate of gain of pasture-finished beef by replacing grass pastures with legume pastures will also improve the ability of ranch-based finishing to compete economically with traditional feedlot-finishing (Curtis et al., 2013). Tannin-containing legumes are non-bloating and can be grazed in pure stands (McMahon et al., 2000). The use of legumes with relatively low concentrations of ruminant-tolerated tannins, such as birdsfoot trefoil (*Lotus corniculatus*) (BFT) and sainfoin (*Onobrychis viciifolia*) (SFN), can increase ADGs of cattle in pasture systems due to their increased feed quality (Phelan



et al., 2015). In addition, tannin-containing forages provide other benefits to cattle such as decreased enteric CH<sub>4</sub> production, improved reproductive efficiency, and reduced effects of parasitism and alkaloids (Aboagye and Beauchemin, 2019; Lyman et al., 2012; Waghorn, 2008). Tannins bind with proteins in the rumen and decrease rumen protein degradation and therefore ammonia generation in the rumen. However, the tannins from birdsfoot trefoil and sainfoin typically do not lower the total tract digestion of the proteins they precipitate in the rumen, as the protein-complex is available post-ruminally. The process of tannin-protein binding in the rumen leads to an increased proportion of N excreted in feces and a decreased proportion of N excreted in urine. This shift in N from urine to feces may help to reduce denitrification hotspots and allow more N to be incorporated as organic matter (Crush, 1993; Waghorn, 2008). This is particularly important in pastures because urine from grazing ruminants is the major source of leached NO<sub>3</sub><sup>-</sup> (Hansen et al., 2012). The average ratio of urinary N:total excreted N in beef cattle is approximately 0.55 (Dong et al., 2014). However, a study by Grainger et al. (2009) found that percent of feed N lost to urine in dairy cows could be reduced from 39% to 22-26% after 163-244 g of condensed tannins extracted from *Acacia mearnsii* were incorporated into the diet of grazing dairy cows for 5 weeks.

In the soil, tannins can inhibit microbial mineralization of feces and decrease urinary N hotspots, increasing N retention (Hättenschwiler and Vitousek, 2000; Waghorn, 2008). Slowing mineralization would decrease the N leached or denitrified to N<sub>2</sub>O. Adoption of tannin-containing forage legumes could enhance ecosystem services of grazed pastures by supporting C storage and reducing N losses from the system while intensifying meat production (Soussana and Lemaire, 2014).

#### 4. Condensed tannins and soil nutrient cycling

Tannins are a heterogeneous class of polyphenolic secondary plant compounds that have been shown to bind proteins and organic N, and may account for as much as 20-60% of plant leaves and bark (Halvorson and Gonzalez, 2008). They are further classified as being condensed tannin (CT) or hydrolysable tannin (HT). Condensed tannins are composed of flavan-3-ols with C-C bonds, whereas hydrolysable tannins are composed of sugars and gallic or ellagic acids (Nierop et al., 2006a). Because they are a diverse class of compounds, they will differ in properties such as chain length, number of functional groups, glycosylation, linkages, branching, and stereochemistry (T E C Kraus et al., 2003). Functional definitions define tannins as compounds that have a high enough molecular weight (1,000-20,000 daltons) and number of hydroxyl groups to form strong complexes with proteins or other molecules such as alkaloids (Frutos et al., 2004; Halvorson and Gonzalez, 2008).

The protein binding process is dependent on pH, protein isoelectric point, the relative concentration of tannins and proteins, tannin structure, and protein structure. Protein precipitation has been observed to be highest at pH values close to the isoelectric point of a given protein. Substantial precipitation may also occur at higher pH values as long as the tannin:protein ratio is sufficiently high and the pH is below the pKa for phenolic groups ( $\text{pH} \approx 9$ ) (Adamczyk et al., 2012, 2013). Condensed tannins which are more polymerized or contain a higher proportion of three vs. two hydroxyl groups at the B-ring can precipitate more proteins, and proteins with a looser rather than tighter geometry will have a higher affinity for protein complexation (Nierop et al., 2006a; Smolander et al., 2012). Tannins rapidly complex with proteins or sorb organic

compounds by hydrogen bonding. Once the complexes are formed, they are generally resistant to N-release (Halvorson et al., 2012; Smolander et al., 2012).

In the soil, tannins interact with soil microbes and may decrease C, N, and phosphorus (P) mineralization in pasture soils, although this process is not completely understood. Tannins may enter the soil from above and belowground in plant litter or dissolved in leachate. Once they are in the soil they can be mineralized or degraded, converted to humic substances, adsorbed to clay particles, or form a chelate. Tannins that enter in solution or are decomposed into a soluble form may exit the soil as soluble dissolved organic carbon (DOC) (Hättenschwiler and Vitousek, 2000; T E C Kraus et al., 2003). Soil tannin concentrations range from 4-40 µg/g soil in mineral soils to <10 mg/g soil in humus layers, but have been documented to reach nearly 40 mg/g soil under a Canadian spruce forest (T E C Kraus et al., 2003). Tannins influence soil C and N cycling in multiple ways, such as limiting nutrient pool availability or influencing cellular level metabolic processes in microorganisms.

Tannins have been shown to influence soil nutrient cycles through a combination of physical, chemical, and biological mechanisms. Tannins will sorb to SOM, preferentially sorbing to hydrophobic molecules; this process is correlated to the organic matter's degree of humification (Halvorson et al., 2012). This has been demonstrated in studies where tannins and non-tannin phenolics were applied to soil amended with various types of organic matter. Of the various test compounds applied, the non-tannin compounds had low rates of sorption, regardless of organic matter type. Compounds that contained tannins were found to have higher sorption rates when applied to organic matter that contained humic substances, were high in amino acids, or had a high N

content (Halvorson et al., 2012). In several laboratory studies where tannins such as tannic acid or compounds purified from tannic acid, and other non-tannin phenolics were added to a soil, the C that was added by the tannin treatments was not fully recovered in subsequent cool and hot water extractions. This, accompanied by a decrease in total phenolics in the water extracts, confirms that tannins are capable of rapidly forming stable, insoluble complexes with the soil (Halvorson et al., 2012, 2009; Halvorson and Gonzalez, 2008). This reduction in recovered soluble tannin C has been shown to occur in a dose-dependent manner in studies using a purified tannic acid derivative (Halvorson et al., 2016, 2009). However, the range of effects on recovered soluble tannin C by compounds of varying complexity would suggest that the effect of tannins may decrease as they are degraded into simpler units (Halvorson et al., 2009; Halvorson and Gonzalez, 2008). The addition of tannins to a soil may affect C cycling patterns by protecting organic matter from decomposition through their recalcitrant nature, protecting proteins in stable tannin-protein complexes, surrounding other compounds and thus making them inaccessible to decomposers, or metabolically inhibiting decomposing microorganisms (T E C Kraus et al., 2003). Chemically protecting SOM may decrease decomposition rates and result in increased rates of organic matter accumulation (Halvorson et al., 2012; T E C Kraus et al., 2003). Increased SOM has been correlated to improvements in other soil properties such as cation exchange capacity, nutrient and water retention, and aggregate stability (T E C Kraus et al., 2003). However, because tannins contain C, they may serve as a substrate for microorganisms and stimulate C mineralization (T E C Kraus et al., 2003; Smolander et al., 2012). Results have been mixed however, as other studies have shown decreased C mineralization with the addition of tannins, or no effect at all

(Halvorson et al., 2009; T E C Kraus et al., 2003; Smolander et al., 2012). These differences in C cycling may be due to the tannin type. As described by Nierop et al. (2006a), the chain length of a tannin, and therefore the ease with which it may be degraded by microbes, may determine mineralization patterns. Tannins with shorter, more easily decomposed chains may serve as a substrate for microbes, while longer, less easily decomposed chains may be active in reducing mineralization. In a tannin-amended litter incubation, hydrolyzable tannins caused short, rapid increase in C mineralization, whereas condensed tannins caused temporary and less dramatic increases in C mineralization (Nierop et al., 2006a, 2006b).

Tannins can influence N cycling both independently and in response to changes in C cycling. Tannin C may act as a substrate for microorganisms and stimulate temporary N immobilization (T E C Kraus et al., 2003). Changes in net N mineralization in the absence of changes in C mineralization are indicative of tannins complexing with organic N. These complexes form rapidly and are generally resistant to N-release (Halvorson et al., 2009; T E C Kraus et al., 2003; Smolander et al., 2012). These complexes limit nutrient pool availability and may increase the ratio of dissolved organic N (DON) to mineral N (ammonium ( $\text{NH}_4^+$ ) and  $\text{NO}_3^-$ ), as they prevent the conversion of organic to mineral N in the soil (Hättenschwiler and Vitousek, 2000; T E C Kraus et al., 2003). Increasing the amount of DON in the soil is hypothesized to allow for organic N uptake by plants, but limit mineral N availability and loss (T E C Kraus et al., 2003; Northup et al., 1995; Smolander et al., 2012). This argument has been used to explain the evolution of tannin-containing plants in nutrient-limited systems. However, it is still uncertain why there is a difference in the types and distributions of secondary plant compounds among

plant species and environmental gradients.

Both condensed and hydrolyzable tannins will form complexes in soil, although factors such as tannin type, structure, and concentration, and organic matter composition can affect the influences that tannins will exert on N cycling processes (Halvorson et al., 2016, 2012; Nierop et al., 2006a, 2006b; Smolander et al., 2012). Low concentrations of tannins have been hypothesized to reduce net N mineralization by increasing microbial N immobilization. At higher concentrations, tannin-protein complex formation may dominate patterns of decreased N mineralization (Smolander et al., 2012). Besides concentration, tannin type and chemical structure may be another factor in determining changes in N cycling. In tannin-amended litter incubation experiments performed by Nierop et al. (2006a; b), tannic acid, a hydrolyzable tannin, was shown to induce a rapid, drastic, but short-term effect on C, N, and P cycling processes. When tannic acid was added to Corsican pine litter, it induced high rates of C mineralization, net N immobilization, and net P immobilization. However, after one week of incubation the net C, N, and P mineralization rates, and net nitrification rates in the tannic acid-amended samples resembled the control samples. This temporary effect suggests that the tannic acid mainly functioned as a C source. Amending samples with condensed tannins did temporarily increase rates of C mineralization, but not as greatly as those amended with tannic acid, which was attributed to decomposition inhibition. Condensed tannin treatments also produced lesser, but longer-term reductions in net N and P mineralization (Nierop et al., 2006a, 2006b). Differences in tannin structure have been shown to influence N cycling within a single class of tannins. Condensed tannins with a higher proportion of three (prodelphinidins) versus two (procyanidins) hydroxy groups at the B-

ring inhibit net N mineralization to a greater degree in the study by Nierop et al. (2006a) described above. In general, tannins appear to generally reduce net rates of N mineralization. It has been proposed that even if net N mineralization rates increase, it is likely due to decreased immobilization rather than increased mineralization (Smolander et al., 2012).

While the literature generally agrees that tannins tend to decrease rates of N mineralization, there have been more conflicting results regarding their effect on nitrification (T E C Kraus et al., 2003; Nierop et al., 2006a; Smolander et al., 2012). Some studies have shown increases, while others have reported no change or a decrease in nitrification rates. Based on these mixed results, it is uncertain whether changes in nitrification are due to direct effects of tannins on nitrifying bacteria, or due to the indirect, cascading effects of changes to other N cycling processes. Some have proposed that decreases in nitrification rates may be due to a decrease in  $\text{NH}_4^+$  availability (McCarty and Bremner, 1986). However, studies that eliminated ammonium limitation still obtained mixed results for nitrification rates (T E C Kraus et al., 2003; Smolander et al., 2012). In a 16-day soil-suspension experiment by Adamczyk et al. (2013), three concentrations of tannic acid and condensed tannins extracted from Norway spruce were added to soils from the humus layer of a silver birch forest. At the end of the study, the highest concentrations (50 mg/5.7 g dry soil) of condensed tannins and tannic acid resulted in significantly lower nitrification potential. Because there was an excess of  $\text{NH}_4^+$  during the entire study and shaking prevented loss of inorganic N via denitrification, they concluded that high concentrations of certain condensed and hydrolyzable tannins may directly inhibit nitrification. Because tannin studies span a

variety of types of substrate and tannins, their effect on nitrification may be dependent on other factors such as soil or litter type, and tannin structure and source (Smolander et al., 2012). If tannins are able to directly or indirectly decrease nitrification rates in soils, they may be able to decrease losses of inorganic N through leaching and denitrification.

Polyphenolic compounds have been shown to inhibit denitrification in addition to other N cycling processes. This may occur directly if nitrification inhibition limits the size of the  $\text{NO}_3^-$  pool, or through direct metabolic inhibition of denitrifying microorganisms. A set of studies by Bardon et al. (2014, 2016) examined the effect of secondary compounds extracted from four *Fallopia* spp. genotypes on fifteen isolated gram-negative denitrifier strains in aerobic and anaerobic bioassays as well as a soil study where unknown *Fallopia* compounds were used as amendments. They found that the *Fallopia* extracts inhibited biological denitrification more than respiration. There was also a reduction in the denitrification enzyme activity to substrate-induced respiration ratio. This ratio accounts for changes in C availability, and a change in this ratio is thought to indicate a change in the function of a microbial community. Biological denitrification inhibition was found to be dose-dependent, although the overall ratio of denitrifiers to total bacteria was not affected. In a follow-up study, proanthocyanidins (condensed tannins) from the extracts were purified and their effects on denitrification and respiration were tested on one gram-negative and one gram-positive denitrifier strain. As before, denitrification and aerobic respiration were inhibited in a dose-dependent manner, with a greater impact on denitrification. These inhibitory effects were correlated with protein precipitation capacity and concentration of proanthocyanidins of the *Fallopia* extract for denitrification rates. While much of the literature has focused on N



mineralization and nitrification processes because they are considered to be rate-limiting steps in the N cycle, these studies indicate that tannins may affect other aspects of the N cycle such as denitrification.

Like denitrification, few studies have examined the effects of tannins on N fixation. Schimel et al. (1998) describes a lab and field study which demonstrated N<sub>2</sub> fixation inhibition in alder root nodules by balsam poplar tannins. In a hydroponic laboratory study where alder tannins, poplar tannins, and poplar phenolics were added to alder nodules, the poplar tannins significantly inhibited N fixation in the alder nodules. In a follow-up field study, N<sub>2</sub> fixation rates were measured across a successional transition from alder to poplar. The authors found that N fixation rates decreased throughout the successional transition, likely due to poplar tannin's inhibitory effects once differences in soil characteristics were dismissed.

At a cellular level, tannins generally affect the metabolic functioning of soil microorganisms through cell membrane interference, enzyme inhibition, and limitation of metal availability (Adamczyk et al., 2013; Kraus et al., 2003a; McDonald et al., 1996; Mila et al., 1996; Ultee et al., 2002). Tannins have been shown to destabilize the cell membrane which ultimately results in the death of the cells through a reduction in the pH gradient and redox potential across the membrane (Alberto et al., 2001; Ultee et al., 2002). Tannins inhibit the production and movement of enzymes both in the soil and in the cell. Enzymes bound in tannin complexes are not available for microbial functioning and cell membrane destabilization inhibits exoenzyme movement in and out of the cell (Adamczyk et al., 2013; T E C Kraus et al., 2003; Smolander et al., 2012; Ultee et al., 2002). In addition to proteins and enzymes, phenolic compounds will complex with

metals such as copper (II), iron (III), and zinc (II) and make them unavailable, thus limiting the availability of essential micronutrients to microbial communities (McDonald et al., 1996; Mila et al., 1996). Tannins are generally difficult for microorganisms to degrade unless they produce phenol oxidase or tannase (Hättenschwiler and Vitousek, 2000; T E C Kraus et al., 2003).

Tannins are not the only secondary plant compounds that are able to influence soil N cycling. Other compounds such as saponins which are found in alfalfa have been known to create similar effects as tannins, such as increased N immobilization rates and decreased ammonification and N mineralization rates (Levanon et al., 1982). Saponins are not phenolic compounds, but are comprised of triterpenes, glycosylated steroids, and steroidal alkaloids (Haralampidis et al., 2002). Like tannins, saponins are found in forage species such as alfalfa which contains triterpene glycoside saponins (Lu and Jorgensen, 1987). Saponins face similar fates as tannins in the soil such as incorporation into microbial biomass and mineralization to CO<sub>2</sub>, or adsorption to humic acids (Okumura et al., 1999). Like tannins, increased N immobilization and denitrification rates likely due to sugar structures in saponins. Alfalfa saponins, which contain medicagenic acid, can also inhibit enzyme activity in rhizosphere bacteria strains and inhibit fungal communities, leading to decreased N mineralization rates (Hoagland et al., 2001; Levanon et al., 1982).

## **5. Need for further research**

Many past tannin litter and soil studies have focused on forested environments, used tannins extracted from forest species, or used manufactured tannins. Tannin structure and effects can vary among plant species, and commercial tannic acid used in many studies has been identified as having a chemical structure that is different from

other naturally occurring tannins (T E C Kraus et al., 2003). Because I am specifically interested in the effects of tannins in pasture soils, it is critical to use tannins from my species of interest. Additionally, nutrient cycling dynamics and plant communities in pastures are distinct from those in forested environments. Tannin concentrations in forages are much lower than in forest species. Tannins account for 1-4% of dry matter in birdsfoot trefoil or 3-8% of dry matter in sainfoin, whereas tannins account for up to 40% of dry weight in tree leaves and bark (T E C Kraus et al., 2003; MacAdam and Villalba, 2015). Pastures also typically have a much greater soil N surplus than forests. Therefore, it is unclear how tannins from forage species specifically affect soil N cycling in pastures. Past studies have explored aspects of this topic, but there are still several details that remain unknown.

A series of laboratory and field studies examined the effects of dung from sheep fed condensed tannin-containing big trefoil (*Lotus pedunculatus*) compared with the dung from sheep fed perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) on pasture soil N cycling. Although all dung treatments led to an increase in soil nitrate, the authors observed nitrification inhibition under the dung treatments containing big trefoil tannins (Crush, 1993). Ammonification rate did not appear to be inhibited, suggesting that the tannins bound to nitrite in the soil. In a subsequent soil incubation amended with big trefoil or white clover herbage, big trefoil again had lower nitrification rates than the clover treatment (Crush and Keogh, 1998). However, when the big trefoil herbage was incubated with soil that had grown big trefoil for one vs. three years, nitrification rates were higher in the soil that had grown Lotus for three years. The authors hypothesized that this was due to a shift in the soil microbial community to

organisms which could decompose the big trefoil (Crush and Keogh, 1998). As described in previous sections, several studies by Halvorson and others examined the effects of a commercial tannin and a purified derivative on C and N cycling in both forest and pasture soils. While they demonstrated stable complexation of these compounds to the soil through a reduction in soluble C and N, further similar studies are needed using tannins isolated from the particular species of interest.

In order to understand tannin function in pasture systems, it is critical to determine if purified tannins extracted from forage species impact soil C and N cycling under controlled conditions, because tannin structure heavily affects function. Secondly, it is necessary to examine if low doses of tannins, introduced to the pasture system via above and belowground plant inputs and fecal matter, affect soil C and N cycling in the field. Finally, it is necessary to understand if tannins in inputs such as fecal matter have a persistent impact on soil C and N cycling under controlled conditions.

By understanding how tannin type and concentration affect N cycling, land managers can create management recommendations for beef producers that will increase N retention and decrease losses via ammonia volatilization, GHG emissions, and nitrate leaching. This information could increase the profitability for farmers interested in local finishing and marketing of cattle by reducing inputs and energy usage, and increasing forage quality. As of 2017, Utah was home to 6,508 beef farms, raising 338,572 beef cows (USDA National Agricultural Statistics Service, 2017a). Finishing cattle on tannin-containing legumes may provide the opportunity for a regional cottage industry as an economically and environmentally viable alternative to grass or feedlot finished beef. In 2017, the market value of cattle and calves sold in Utah was worth \$3.8 million (USDA

National Agricultural Statistics Service, 2017b). The addition of tannin-containing legume forages could further increase the profitability of beef production through decreased input costs, increased ADGs, and increased beef profitability, while reducing N loss via leaching and gaseous emissions (Curtis et al., 2013). This would ultimately enhance the quality of farmer's lives as well as that of local communities by enabling farmers to increase the sustainability of their operations by reducing GHG emissions, and facilitate their assessment of the environmental impact of alternative management decisions. At a global level, adoption of tannin-containing legumes will improve air and water quality, enhance the ecosystem services provided by grasslands, and decrease competition between cattle and humans for cereal grain.

## **6. Goals and objectives**

This thesis will address one goal of the funded USDA NIFA grant 2016-67019-25086 awarded to Utah State University:

1. Assess the ecosystems services (including C and N storage, nutrient cycling, and climate regulation) provided by soils under polyphenolic-containing legume pastures, as compared to grass pastures, in pasture-finished beef production systems.

The specific objectives and hypotheses of this thesis are:

### **Objective 1**

To compare physical, chemical, and biological characteristics of soil samples obtained from grazed alfalfa (*Medicago sativa*, ALF), BFT, cicer milkvetch (*Astragalus cicer*, CMV), MBG, and SFN pastures on a seasonal basis.

## Hypothesis 1

Legume treatments (ALF, BFT, CMV, SFN) will have higher soil total nitrogen (TN) than the non-legume treatment (MBG) due to their ability to fix nitrogen.

Condensed tannin-containing legume treatments (BFT and SFN) will have reduced concentrations of soil  $\text{NH}_4^+$  and  $\text{NO}_3^-$  and values of potential N mineralization due to the ability of tannins to inhibit N mineralization, and non-tannin-containing legume treatments (ALF and CMV) will have greater  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations and values of potential N mineralization due to their N fixing abilities. Because condensed tannins may inhibit microbial activity and complex with proteins, condensed tannin-containing legume treatments (BFT and SFN) will have reduced values of soil respiration, microbial biomass, and dehydrogenase enzyme activity (DHA). Because of this decreased microbial activity or protein complexation, condensed tannin-containing legume treatments (BFT and SFN) will have greater concentrations of total organic carbon (TOC) due to a combination of decreased decomposition rates or chemical protection of organic matter. This accumulation of organic matter will lower bulk density, increase soil moisture, increase readily mineralizable carbon, and cation exchange capacity. Due to their root secretions and nutrient uptake needs, legume treatments (ALF, BFT, CMV, SFN) will have greater concentrations of phosphorus (P) and lower soil potassium (K) and pH. There will be no significant differences between treatments for soil texture, electrical conductivity, or micronutrients.

## Objective 2

To assess how feces from cattle fed pure forage hays listed in objective 1, as well as pure small burnet (*Sanguisorba minor*, SBN) hay, affect soil N cycling processes and

GHG emissions using a feces-amended soil incubation study.

## Hypothesis 2

At the end of the incubation, the condensed tannin-containing legume treatments (BFT and SFN) will yield reduced concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , and volatilized N per initial concentration of total N, and rates of  $\text{N}_2\text{O}$  and  $\text{CO}_2$  production due to a combination of protein complexation and inhibition of microbial mineralization, denitrification, and respiration. The control treatment will have the next greatest values of these variables as it does not contain manure or tannins, while treatments without condensed tannins (ALF, CMV, and MBG), or those containing hydrolyzable tannins (SBN), will have the greatest concentrations of the measured variables due to the addition of manure as a microbial substrate without condensed tannins to inhibit microbial processes.

## Objective 3

To assess how forage tannin type and dose affects various N cycling processes using a tannin-amended soil incubation study with tannins extracted from the leaves of BFT and SFN plants.

## Hypothesis 3

At the end of the incubation the SFN High and BFT High, SFN Low and BFT Low, and control treatments (soil alone) will have increasing concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ , volatilized N, and autoclave citrate extractable (ACE) proteins, respectively, reflecting the decreasing tannin concentrations (and therefore potential for microbial inhibition and protein precipitation) in each set of treatments. All treatments will yield low concentrations of extractable tannins at the end of the incubation because they are

expected to complex with proteins and alkaloids present in the soil.



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## CHAPTER II.

### INFLUENCE OF POLYPHENOLIC COMPOUNDS ON N-CYCLE DYNAMICS IN PASTURE SOILS

#### 1. Introduction

Grass-fed beef is growing in popularity in response to concerns regarding animal health and the environmental impact of feedlots. However, Capper's 2012 comparison of feedlot vs grass-fed beef production systems found that grass-fed beef had a larger greenhouse gas (GHG) footprint than feedlot finished beef. In grass-fed systems, the low feed quality of grass decreases average daily gains (ADGs). According to Capper (2012), grass-fed cattle took longer to finish and weighed less at slaughter than feedlot cattle.

In addition to higher GHG emissions, pasture systems are prone to N loss. In poorly managed grazing systems, the soil C:N ratio decreases due to the release of biomass carbon (C) via animal respiration and enteric methane (CH<sub>4</sub>) production, while N is returned to the system in concentrated urine and fecal patches (Soussana and Lemaire, 2014). As the C and N cycles become decoupled, excess N is lost from the system through leaching to ground and surface waters or to the atmosphere as dinitrogen (N<sub>2</sub>) gas or greenhouse gases such as nitrous oxide (N<sub>2</sub>O) through denitrification. An alternative legume-based beef production system using legumes that contain plants with condensed tannins such as birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*) may decrease GHG emissions and N losses in pastures through eliminating the need for N fertilizer, altering soil C and N cycling, and maintaining pasture ecosystem services.

Tannins are a heterogeneous class of polyphenolic secondary plant compounds characterized by a molecular weight of 1,000-20,000 daltons (Frutos et al., 2004; Halvorson and Gonzalez, 2008). Condensed tannins are characterized by flavan-3-ols and C-C bonds as well as their ability to form strong complexes with other molecules (Nierop et al., 2006a). Tannins may enter the soil in plant residue, leachate, or feces deposited by animals grazing on plants containing tannins. In the soil they face a variety of fates, including being mineralized, transforming into humic substances, adsorbing to clay particles, or forming a chelate (Hättenschwiler and Vitousek, 2000; T E C Kraus et al., 2003).

In soils, tannins may limit N losses by i) altering the availability of nutrient pools to plants and microbial communities, and ii) altering microbial activity. Tannins alter N availability by complexing with proteins and enzymes during decomposition, therefore increasing the ratio of dissolved organic N (DON) to mineral N by limiting the contribution of proteins to the mineral N pool (T E C Kraus et al., 2003; Smolander et al., 2012). Sorption of tannins and other phenolics to soil particles has also been shown to decrease soluble soil C and has been proposed as a method of building and storing SOM (Halvorson et al., 2012; T E C Kraus et al., 2003). This process may be influenced by factors such as pH, tannin structure, protein structure, isoelectric point, and the ratio of tannins to proteins (Adamczyk et al., 2012, 2013; Nierop et al., 2006a; Smolander et al., 2012).

Tannins can also stimulate or inhibit microbial communities. The substantial C content of tannins may act as a substrate and stimulate microbial activity, resulting in a short-term immobilization of soil N (T E C Kraus et al., 2003). However, tannins may

inhibit microbial metabolism by interfering with the cell membrane, complexing with enzymes, and limiting certain metal availabilities (Adamczyk et al., 2013; T E C Kraus et al., 2003; McDonald et al., 1996; Mila et al., 1996; Ultee et al., 2002). These mechanisms have been documented to reduce decomposition rates, increase SOM accumulation, and reduce net N mineralization rates (Halvorson et al., 2009, 2012; Hättenschwiler and Vitousek, 2000; T E C Kraus et al., 2003; Nierop et al., 2006b, 2006a; Smolander et al., 2012).

While the literature generally agrees that tannins reduce net N mineralization, their effect on nitrification rates are conflicting (T E C Kraus et al., 2003; Nierop et al., 2006a; Smolander et al., 2012). It is uncertain if changes in nitrification rates are due to direct inhibition of nitrifying bacteria, or the cascading effects of reduced N mineralization rates decreasing mineral N pools (Adamczyk et al., 2013; McCarty and Bremner, 1986). These mechanisms are likely complicated by other factors such as soil type, litter quality, and tannin source and structure (Adamczyk et al., 2012, 2013; Halvorson et al., 2012; Nierop et al., 2006a, 2006b; Smolander et al., 2012). Like nitrification, the effects of tannins on denitrification rates may be due to reduced mineral N pools or direct inhibition of denitrifying microorganisms, as Bardon et al. (2014, 2016) concluded.

Tannin-containing legumes have been documented to provide other ancillary benefits in pasture systems, such as increased feed quality, reduced enteric methane production, a shift of urinary N to feces, improved reproductive efficiency, and reduced effects of parasites and alkaloids (Crush, 1993; Lyman et al., 2012; Phelan et al., 2015; Waghorn, 2008). If tannin-containing legumes are able to successfully increase soil N

retention in pastures, producers may be able to decrease N losses and GHG emissions as well as compete economically with feedlot systems.

While tannins have been widely studied in forest systems, there is a lack of information regarding the effect of tannins in managed pasture systems. Halvorson et al. (2012) identified that in agricultural soils tannins had higher sorption rates when the SOM contained higher levels of humic substances, amino acids, or N. Other studies by this group spanning a range of forest, pasture, and cultivated soils concluded that certain tannins and their derivatives were able to decrease recovered soluble tannin C and soil N in a dose-dependent manner. These decreases may be influenced by the complexity of these compounds, or the compound's degree of degradation (Halvorson et al., 2012, 2009; Halvorson and Gonzalez, 2008). In another study examining the effects of tannin and non-tannin-containing sheep dung on pasture soil N cycling, the authors concluded that although dung did increase soil nitrification rates, dung that contained tannins from *Lotus pedunculatus* inhibited nitrification to a degree (Crush, 1993). This result was consistent with a follow up study where soil incubated with *Lotus* herbage had lower nitrification rates than soil incubated with white clover (*Trifolium repens*) herbage. However, this trend may change over time as the soil microbial community adjusts to forages containing tannins (Crush and Keogh, 1998). In order to further understand tannin function in pasture systems, research will need to focus on the effect of tannins found specifically in forage species and their implications for managing systems prone to N loss. This will include determining if purified tannins extracted from forage species impact soil C and N cycling under controlled conditions, examining if low doses of tannins input through biomass and feces affect C and N cycling in the field, and if tannin-

containing fecal inputs have a persistent impact on soil C and N cycling. If tannin-containing legumes can increase soil N retention in pastures, these systems may provide an economically and environmentally viable alternative to feedlot finished beef.

To address this knowledge gap, I compared the effect of different legume forages on soil N dynamics in grazed pasture systems. The specific objectives of this study included: 1) quantifying the effect of condensed tannin vs non-condensed tannin-containing forages on soil N mineralization and microbial activity in a grazed pasture setting, and 2) quantifying the effect of fecal additions derived from tannin vs non-condensed tannin-containing hays to a pasture soil on N mineralization and denitrification processes over the course of an 84-day soil incubation. I hypothesized that field soils under established legume pastures containing condensed tannins would have lower concentrations of inorganic N, values of aerobic N mineralization, soil respiration and dehydrogenase enzyme activity, and increased concentrations of total organic C (TOC) and readily mineralizable C. I also hypothesized that incubated soils treated with feces containing condensed tannins would yield lower concentrations of mineral N and N<sub>2</sub>O production.

## **2. Methods**

### **2.1. Field experiment one**

I sampled two separate field experiments located at the Utah Agricultural Experiment Field Station in Lewiston, Utah (41°57'4" N, 111°52'26" W). The first field experiment included three treatments: alfalfa (ALF) (*Medicago sativa*, var. *Vernal*), birdsfoot trefoil (BFT) (*Lotus corniculatus*, var. *Langille*), and sainfoin (SFN)



(*Onobrychis viciifolia*, var. *Shoshone*) (Fig. 1). The experiment consisted of a randomized complete block design (RCBD) established in 2016 with 3 blocks and 3 treatments per block. Soils at this site consisted of i) Kidman fine sandy loam (coarse-loamy, mixed, superactive, mesic Calcic Haploxeroll) and ii) Lewiston fine sandy loam (coarse-loamy, mixed, superactive, mesic Aquic Calcixeroll). The first field experiment was sampled over one year with samples collected in August 2017. Samples were taken from 0-90 cm in 30 cm increments using a 4.3 cm diameter soil coring probe. Two cores were taken per plot for bulk density and three cores were taken and homogenized for soil physical, chemical, and biological properties. Five samples were taken from 0-10 cm with a 16 mm diameter step-in soil corer.

Forage biomass was measured pre- and post-grazing during each period in June and August of 2017. Sixty readings were made using a rising plate pasture meter (Electronic Plate Meter Jenquip EC-10, Agriworks Ltd, NZ). Calibration curves were created for each forage during each period by taking readings at different herbage heights pre- and post-grazing. For calibration samples, the forage was cut to ground-level using a 0.10 m<sup>2</sup> quadrat, the same area as the plate. The cut forage was oven-dried at 60°C to constant weight to obtain forage dry matter. Dry matter and plate meter readings were correlated for each treatment and period using linear equations. Biomass samples collected in June and August of 2017 were analyzed for total condensed tannin content. Samples were analyzed in triplicate by the butanol-HCl-acetone spectrophotometric method described in Grabber et al. (2013) using a Thermo Fisher Spectronic BioMate 3 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) against condensed tannin standards isolated from sainfoin and birdsfoot trefoil.

## 2.2. Field experiment two

A separate second experiment included 4 treatments: birdsfoot trefoil (BFT) (var. *Langille*), cicer milkvetch (CMV) (*Astragalus cicer*, var. *Monarch*), and meadow bromegrass (MBG) (*Bromus commutatus*, var. *Cache*) (Fig. 2). The experiment consisted of a RCBD established in 2012 with 5 replications and three treatments. A forb, small burnet (SBN) (*Sanguisorba minor*, var. *Delar*), was planted as a ten-foot strip through the center of BFT pastures. This site consisted of Lewiston fine sandy loam (coarse-loamy, mixed, superactive, mesic Aquic Calcixeroll). The second field experiment was sampled over two years with samples collected after the grazing season ended in August 2017 and August 2018. In 2017, five soil cores were sampled from 0-60 cm in 30 cm increments in each plot using a step-in corer, with five additional samples taken from 0-10 cm and 10-30 cm. Sampling occurred again in August 2018 with samples taken from 0-10 cm, and 0-90 cm in 30 cm increments following the same protocol described in field experiment one. All soil samples from both field experiments were transported to the lab on ice and stored at 4°C. Samples were sieved to 2 mm prior to analysis.

Forage biomass was measured in each BFT, CMV, and MBG plot using a calibrated Farmworks (Feilding, New Zealand) rising plate meter at the start and end of each grazing period in 2017 and 2018. Pre- and post-grazing forage biomass was measured on 5 June (period 1), 3 July (period 2), and 31 July (period 3) in 2017 and on 20 June (period 1), 19 July (period 2), and 9 August (period 3) in 2018. Harvested pre-grazing biomass from each date in 2017 was analyzed for total condensed tannin content by the method described in Grabber et al. (2013) using a Thermo Fisher Scientific BioMate 3 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples

were analyzed in triplicate against condensed tannin standards isolated from birdsfoot trefoil (Hagerman, 2011).

### 2.3. Soil physical, chemical, and biological analyses

Bulk density was analyzed on field-moist soils for field experiment one in 2017 and field experiment two in 2018 by drying intact cores at 105 °C until dry core weight was constant (Castle, 2019). Soils sampled in 2017 were prepared for texture analysis with pre-treatment for carbonate removal according to Gavlak et al. (2005) (Method S-14.10) and analyzed using the simplified clay fraction method according to Gee and Or (2002) to accommodate the high sand content. Total  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were extracted using 2M potassium chloride (KCl) (Gavlak et al., 2005 Method S-3.50). Aerobic N mineralization was performed according to Schmidt and Belser (1994) where 10 g of soil was adjusted to 37% moisture in falcon tubes and sealed with parafilm. Aerobic N mineralization samples were incubated for 21 days at 35°C. After 21 days, samples were extracted with 2M KCl for total  $\text{NH}_4^+$  and  $\text{NO}_3^-$  according to the Gavlak et al. (2005) method described above. Aerobic N mineralization was then calculated using the equation:

$$x = \text{ammonium} - N_{\text{final}} + \text{nitrite} - N_{\text{final}} + \text{nitrate} - N_{\text{final}} \quad (1)$$

All KCl extracts were analyzed in duplicate using a Lachat Quikchem 8500 Flow Injector analyzer (Lachat Instruments, Loveland, CO, USA) according to Harbridge (2007a) for  $\text{NH}_4^+$  and Harbridge (2007b) for  $\text{NO}_3^-$ . Soil cation exchange capacity (CEC), Olsen phosphorus (P) and potassium (K), and micronutrients iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) were analyzed according to Gavlak et al. (2005) methods S-

10.10, S-4.10, and S-6.10, respectively. Soil pH and electric conductivity (EC) were measured in a 1:2 soil:water suspension. Soil pH was analyzed using an Orion Research Expandable Ion Analyzer EA 920 (Orion Research Incorporated, Jacksonville, FL, USA) and EC was analyzed using a Thermo Scientific Orion 3 Star Conductivity Benchtop Analyzer with the Orion 013005MD Conductivity Cell probe (Thermo Fisher Scientific, Waltham, MA, USA). Total N was analyzed using a Skalar Primacs SN Total Nitrogen Analyzer (Skalar, Breda, Netherlands) and normalized per cubic centimeter of soil. Total organic carbon (C) was analyzed for finely ground air-dried soil using a Skalar Primacs SLC Carbon Analyzer (Skalar, Breda, Netherlands) and calculated by the difference in total and inorganic C and normalized per cubic centimeter of soil. Dehydrogenase enzyme activity was analyzed in triplicate according to Tabatabai (1994) where 2.5 g of 0-10 cm soil was adjusted to 22% moisture. Samples were incubated overnight at 25°C. The next day, 0.5 mL of 3% triphenyl tetrazolium chloride and 1.0 mL of 2%  $\text{CaCO}_3$  solution was added to each sample and incubated at 37°C for 24 hours. At the end of the incubation, the triphenylformazan (TPF) product was extracted with 10 mL of methanol and the absorbance was analyzed at 490 nm with a Molecular Devices SpectraMax M2 plate reader (Molecular Devices, San Jose, CA, USA). The  $\mu\text{g TPF g dry soil}^{-1}$  was calculated using a standard curve with subtracted blanks. Mineralizable C (RMC), soil respiration (SR) and microbial biomass (MB) were analyzed in duplicate on 0-10 cm samples according to Davidson et al. (1987) and Anderson and Domsch (1978). These variables were analyzed for field experiment one 2017 samples and field experiment two 2018 samples. Five grams of soil was adjusted to 22% moisture for optimum water content. Samples were incubated in screw-top vials fitted with rubber septa in darkness at

20°C for 11 days. Mineralizable C was analyzed by measuring the total CO<sub>2</sub> evolved (mg CO<sub>2</sub>-C kg soil<sup>-1</sup>) during the incubation period using an infrared gas analyzer (Model LI-6251 LICOR Biosciences, Lincoln, NB, USA). Caps were then removed from the samples and vials were sealed with parafilm and stored overnight at 20°C. On day 12 the vials were flushed with moisture saturated air, capped, and basal respiration (mg CO<sub>2</sub>-C kg soil<sup>-1</sup> hr<sup>-1</sup>) was measured two hours after capping. Caps were then removed from the samples and vials were sealed with parafilm and stored overnight at 20°C. Microbial biomass was measured on day 13 from substrate-induced respiration. Each sample received 250 µL of 6% D-Glucose anhydrous solution dissolved in distilled di-ionized water (DDI). Samples were capped and CO<sub>2</sub> evolution was analyzed exactly two hours after the substrate addition. Microbial biomass C was calculated using the equation  $x = 40.4y + 3.7$  where x is microbial biomass C (µg microbial C g soil<sup>-1</sup>) and y is the maximum respiration rate (µL CO<sub>2</sub> g soil<sup>-1</sup> hr<sup>-1</sup>) (Anderson and Domsch, 1978). The metabolic quotient of each sample was determined by calculating the ratio of respiration to microbial biomass C (Anderson and Domsch, 1993).

#### 2.4. Incubation study

Feces from cattle fed two different condensed tannin (BFT and SFN) and 4 different non-condensed tannin- (ALF, CMV, MBG, SBN) containing hays were each added to a uniform soil and incubated for 84 days. The soil was a live Kidman fine sandy loam (coarse-loamy, mixed, superactive, mesic Calcic Haploxeroll) collected from 0-10 cm under a grass alleyway at the Utah Agricultural Experiment Field Station in Lewiston, Utah, and sieved to 2 mm. Soil samples were analyzed prior to incubation for particle

size analysis, pH, EC, sodium adsorption ratio, Olsen P and K, soil organic matter, and total C and N by Utah State University Analytical Laboratories (Table 8). Fecal samples were collected in September and October of 2016 from cattle fed ALF, BFT, CMV, MBG, SFN, and SBN hays produced in June of 2016. A soil control treatment with no fecal addition was also included for a total of seven treatments. Fecal samples were collected fresh after deposit, freeze-dried in a Labconco FreeZone freeze dryer (Labconco Corporation, Kansas City, MO, USA), and milled to pass through a 1 mm screen using a Wiley Model 4 mill (Thomas Scientific, Swedesboro, NJ, USA). Fecal treatments were added to the soil to achieve an equivalent total C content (2.4 %C) on a dry matter basis to assess the effect of varied total N content (0.14-0.17 %N) among those treatments (Table 7). Once combined, soil and feces were adjusted to 15% moisture content. Fifteen grams (oven dry equivalent) of each combined soil and fecal treatment were weighed into 32 oz glass mason jars with 36 replicates of each treatment for triplicate analysis at each time point, with four replicates for headspace analysis to be kept intact for the duration of the study.

Rates of N mineralization and immobilization were determined by analyzing samples in triplicate for  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations using the 2M KCl extraction and analyzed in duplicate using a Lachat Quikchem 8500 Flow Injector analyzer (Lachat Instruments, Loveland, CO, USA) described above on days 0, 7, 14, 21, 28, 42, and 84 (Gavlak et al., 2005 Method S-3.50; Harbridge, 2007a; b). Headspace samples were collected from jars fitted with rubber septa using a syringe for carbon dioxide ( $\text{CO}_2$ ) and nitrous oxide ( $\text{N}_2\text{O}$ ) analysis on days 0, 7, 14, 21, 28, 42, and 84. Carbon dioxide was analyzed using a HP 6890 Series Gas Chromatograph System with a thermal conductivity

detector (Hewlett-Packard, Palo Alto, CA, USA) at 50°C with a 80/100 Chromosorb 12 6 ft x 1/8 in (2.1 mm) SS column. Nitrous oxide was analyzed using an Agilent Technologies 6850 Series II Network GC System (Agilent Technologies, Santa Clara, CA, USA) with an electron capture detector at 55°C with an 80/100 Chromosorb 102 6 ft x 1/8 in (2.1 mm) SS column. Jars were flushed to ambient conditions between sampling time points.

Each treatment included an additional 3 replicates for an irrigation treatment on days 0, 21, 42, and 84 which mimicked typical conditions during regular pasture irrigation events. During irrigation, the additional samples were brought to approximate field-moist capacity at 29% moisture and incubated for an additional 48 hours. Headspace samples were taken at 2, 24, and 48 hours after irrigation and analyzed for CO<sub>2</sub> and N<sub>2</sub>O concentrations. Samples were extracted with 2M KCl after 48 hours for NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations.

## 2.5. Statistical analysis

Statistical analysis was performed separately for each field experiment using a mixed linear model and RCBD for analysis of variance with the MIXED procedure in SAS Studio University Edition (version 9.4, SAS Institute, Cary, NC, USA). Results were significant at p<0.10 for potential aerobic N mineralization and at p<0.05 for all other parameters. Parameters were analyzed for the main effects of treatment, depth, month and their interaction using the Tukey method for means separation, where year was accounted for a repeated measure in field experiment two. In field experiment one, NO<sub>3</sub><sup>-</sup> concentration, TN, and biomass total condensed tannin content were log-

transformed to meet the assumptions of normality, and CEC was analyzed using a non-parametric ranking procedure. In field experiment two, forage biomass was square root-transformed. Nitrate concentration, TC, Olsen P, and Olsen K, were log-transformed, and EC was reciprocally-transformed to meet the assumptions of normality. Ammonium, clay content, Cu, and Mn were analyzed using a non-parametric ranking procedure. Outliers were removed by assessing residuals. Single outliers were removed if their removal was critical in creating a normally distributed dataset. Outliers were kept in boxplots and graphs. Results were significant at  $p < 0.05$  for all parameters. Pearson correlation analysis was performed among field parameters and performed separately for each field experiment using the CORR procedure in SAS Studio University Edition (version 9.4, SAS Institute, Cary, NC, USA) at  $p < 0.05$ .

Statistical analysis was performed separately for the irrigated and non-irrigated incubation samples using a mixed linear model and a RCBD for analysis of variance with the MIXED procedure in SAS Studio University Edition (version 9.4, SAS Institute, Cary, NC, USA) at  $p < 0.05$ . Parameters were analyzed for the main effects of fecal treatment and day using the Tukey method for means separation, where day was accounted for as a repeated measure. For non-irrigated samples, total and cumulative  $\text{CO}_2$  production were log-transformed to meet the assumptions of normality. Carbon dioxide production rate,  $\text{N}_2\text{O}$  production rate, cumulative  $\text{N}_2\text{O}$  production, and ammonium and nitrate concentrations were analyzed using a non-parametric ranking procedure. A  $\text{N}_2\text{O}$  headspace sample was not obtained for one replicate of the BFT treatment on day 42. A linear model based on the other two replicates of the BFT treatment was used to estimate total  $\text{N}_2\text{O}$  production value for this sample. For irrigated samples,  $\text{CO}_2$  production rate,



total CO<sub>2</sub> and N<sub>2</sub>O production, and NO<sub>3</sub><sup>-</sup> concentration were log-transformed to meet the assumptions of normality. Nitrous oxide production rate and NH<sub>4</sub><sup>+</sup> concentrations were analyzed using a non-parametric ranking procedure.

Statistical analysis was performed to compare CO<sub>2</sub> and N<sub>2</sub>O production rates among irrigated and non-irrigated incubation samples using a mixed linear model and a RCBD for analysis of variance with the MIXED procedure in SAS Studio University Edition (version 9.4, SAS Institute, Cary, NC, USA) at  $p < 0.05$ . Parameters were analyzed for the main effects of fecal treatment, irrigation and day using the Tukey method for means separation, where day was accounted for as a repeated measure. Irrigated and non-irrigated greenhouse gas production rates were compared on the four days when irrigation events took place (days 0, 21, 42, and 84). Greenhouse gas production rates were calculated by dividing the total CO<sub>2</sub> or N<sub>2</sub>O produced over the 48-hour incubation period for irrigated samples or total CO<sub>2</sub> or N<sub>2</sub>O produced from the start of the incubation up to the sampling point of interest for the non-irrigated samples. The total CO<sub>2</sub> or N<sub>2</sub>O produced was then divided by the incubation time for the respective samples and the kg of soil incubated. Both CO<sub>2</sub> and N<sub>2</sub>O production rates were analyzed using a non-parametric ranking procedure to meet the assumptions of normality.

Pearson correlation analysis was performed among all non-irrigated incubation experiment parameters using the CORR procedure in SAS Studio University Edition (version 9.4, SAS Institute, Cary, NC, USA) at  $p < 0.05$ .

### 3. Results

#### 3.1. Field experiment one

There were significant differences in potential aerobic N mineralization and ammonium concentration among treatments, suggesting condensed tannins do inhibit N mineralization. Potential aerobic N mineralization rates differed significantly among treatments ( $p=0.0765$ ) with higher mineralization under BFT than SFN ( $p=0.0856$ ), with ALF as an intermediate ( $p=0.9241$ ,  $p=0.1392$ ) (Fig. 3). This could indicate complexation of SFN CTs with organic N. Total CT content differed significantly among treatments ( $p<0.0001$ ) with SFN having significantly higher CT concentrations than BFT ( $p=0.0045$ ) or ALF ( $p<0.0001$ ), followed by BFT which had greater CT concentrations than ALF ( $p=0.0001$ ) with no effect of month ( $p=0.1714$ ) (Fig. 4). Sainfoin had a similar average biomass to BFT ( $p=0.0567$ ), but lower forage N content than BFT in June 2017 ( $p=0.0104$ ) (Fig. 5a-b,6). Although BFT did not have significantly higher average biomass than SFN it appeared to be trending in that direction. This increase in organic matter and biomass N could also create higher potential N mineralization rates much like CT complexation. However, Pearson correlations revealed a significant ( $p=0.0251$ ) negative relationship between forage total condensed tannin content and potential aerobic N mineralization, but no significant relationship between forage biomass and potential aerobic N mineralization ( $p=0.1149$ ) (Table 1). This would suggest that complexation was the primary mechanism in reducing potential aerobic N mineralization. There was a significant treatment  $\times$  depth interaction for  $\text{NH}_4^+$  concentrations ( $p=0.0009$ ) (Fig. 7a-b). Ammonium concentrations were significantly higher under BFT than ALF ( $p=0.0096$ ) or SFN ( $p=0.0041$ ) from 0-10 cm (Fig. 7a), indicating higher N mineralization rates.

However, this is likely due to accidental sampling in urine patches, as evidenced by one high value and a high standard error. There were no differences in  $\text{NH}_4^+$  concentration among treatments at other depths. There were also no differences between treatments for  $\text{NO}_3^-$  (Fig. 7b) or TN among treatments at any depth despite a significant treatment  $\times$  depth interaction ( $p=0.0314$ ) (Table 2).

Differences in carbon cycling parameters were potentially due to several factors, including low sample size and high variability in pasture nutrient deposition and perennial root distribution. Mineralizable C ( $p=0.0337$ ) and respiration ( $p=0.0282$ ) differed significantly among treatments (Fig. 8a-b). Values of both parameters were higher under BFT than ALF ( $p=0.0304$  and  $p=0.0213$ , respectively), with SFN as an intermediate ( $p=0.1610$ ,  $p=0.7059$  and  $p=0.2865$ ,  $p=0.3674$ , respectively). This could be related to the increased  $\text{NH}_4^+$  concentrations under BFT, where elevated concentrations of C or N may have stimulated mineralization of the other. There were no differences among treatments for microbial biomass, metabolic quotient, DHA, or TOC, suggesting that differences were due to variations in C or N availability rather than a shift in microbial community or function (Tables 3 and 4). Birdsfoot trefoil biomass did not differ from ALF or SFN. There were no significant differences in pH, EC, CEC, micronutrients, Olsen P and K, or physical characteristics between treatments (Tables 3 and 4).

### 3.2. Field experiment two

Like field experiment one, differences were observed among treatments for inorganic N concentrations and potential aerobic N mineralization rates. Soil  $\text{NO}_3^-$  concentration varied with a significant treatment  $\times$  depth interaction ( $p=0.0013$ ; Fig. 9a-

b). Meadow bromegrass had significantly lower  $\text{NO}_3^-$  than BFT or CMV treatments at 0-10 cm ( $p<0.0001$ ,  $p<0.0001$ ), 0-30 cm ( $p<0.0001$ ,  $p<0.0001$ ), and 30-60 cm ( $p<0.0001$ ,  $p<0.0001$ ) depths, possibly as a result of high soil inorganic N uptake and a greater biomass C:N ratio. Potential aerobic N mineralization also differed significantly among treatments ( $p=0.0003$ ; Fig. 10). Potential aerobic N mineralization was lower under the CT-containing BFT treatment than the non-CT-containing CMV ( $p=0.0028$ ). Potential aerobic N mineralization under BFT did not differ from MBG ( $p=0.3757$ ), which had the lowest potential aerobic N mineralization rates. Low mineralization rates under MBG were likely due to the higher uptake of inorganic N and higher biomass C:N ratio of grasses. There was a significant treatment  $\times$  month interaction ( $p=0.0215$ ) for forage total CT content, but there were no major crosses in the data so the interaction was dismissed and the main effects of treatment and month were analyzed. There were significant differences in forage total CT content among treatment ( $p<0.0001$ ) with BFT having higher total CT concentrations than CMV ( $p<0.0001$ ) and MBG ( $p<0.0001$ ), followed by CMV having higher total CT concentrations than MBG ( $p<0.0001$ ) (Fig. 11a). Forage total CT concentrations were significantly ( $p=0.0006$ ) higher in grazing periods 2 and 3 than grazing period 1 ( $p=0.0009$ ,  $p=0.0035$ ) (Fig. 11b). Like field experiment one, lower potential aerobic N mineralization under the BFT treatment corresponded to significantly lower BFT biomass than CMV biomass ( $p=0.0001$ ) making it difficult to discern the effect of biomass vs tannin content (Fig. 12). Unlike field experiment one, there were no significant Pearson correlations between potential aerobic N mineralization and biomass or potential aerobic N mineralization and forage total CT concentration, making it difficult to determine the mechanism reducing potential aerobic N mineralization (Table

5). There were no differences in  $\text{NH}_4^+$  concentration or TN among treatments (Fig. 9a, Table 6).

Unlike field experiment one, the results from field experiment two did not provide evidence of greater readily available C under CT legumes. There were no treatment differences in TOC, mineralizable C, respiration, microbial biomass, or metabolic quotient (Tables 6 and 7). Dehydrogenase enzyme activity was the only C cycling indicator that varied significantly ( $p=0.0058$ ) among treatments (Fig. 13). Dehydrogenase enzyme activity was lower under CMV legume than MBG ( $p=0.0045$ ) with BFT as an intermediate ( $p=0.1015$ ,  $p=0.1256$ ). Significant treatment effects were observed for pH ( $p=0.0040$ ), Zn ( $p=0.0270$ ), and moisture ( $p=0.0261$ ) (Tables 6 and 7). Soils under MBG had significantly higher pH than CMV ( $p=0.0349$ ) or BFT ( $p=0.0034$ ) which was expected due to the pH lowering ability of legumes. Unexpectedly, MBG also had significantly higher Zn than BFT ( $p=0.0262$ ) contrary to trends in Zn availability along the pH gradient. Soil moisture was significantly higher under CMV compared to BFT ( $p=0.0234$ ). There were no treatment differences for any other soil chemical or physical characteristics (Tables 6 and 7).

### 3.3. Incubation study

The results of the incubation study revealed a pattern of fecal substrate use and subsequent N immobilization by microbes, with evidence of CT complexation or microbial inhibition. Equal amounts of fecal C were added to soil in all treatments, but fecal N concentrations ranged from  $16.81 \text{ g kg}^{-1}$  (MBG) to  $32.66 \text{ g kg}^{-1}$  (SBN). Inorganic N concentrations throughout the incubation revealed that substrate C:N ratio primarily influenced soil N cycling dynamics, but secondary compounds also appeared to be

important. There were significant treatment  $\times$  day interactions for  $\text{NH}_4^+$  ( $p < 0.0001$ ) and  $\text{NO}_3^-$  ( $p < 0.0001$ ) concentrations (Fig. 14a-b). For  $\text{NH}_4^+$  concentration, the control initially had significantly lower  $\text{NH}_4^+$  concentrations than the rest of the treatments ( $p < 0.0001$  for ALF, BFT, CMV, MBG, SBN, and SFN) (Table 10). This was likely due to an addition of N through the fecal substrate. The higher initial  $\text{NH}_4^+$  concentrations in the fecal treatments compared to the control lasted between 7-28 days. The initial difference in  $\text{NH}_4^+$  concentrations between the control and fecal treatments disappeared the quickest in the BFT (day 2  $p = 0.0021$ , day 7  $p = 0.0003$ ) and SFN (day 2  $p = 0.0015$ , day 7  $p = 0.0319$ ) treatments after 7 days. This was expected as the condensed tannins found in the BFT and SFN treatments are known to complex with soil N and thus increase N immobilization rates. The higher initial  $\text{NH}_4^+$  concentrations in the ALF treatment (day 2  $p < 0.0001$ , day 7  $p = 0.0001$ , day 14  $p = 0.0210$ ) disappeared after 14 days, taking longer than for the CT-containing legume treatments BFT and SFN. Higher initial  $\text{NH}_4^+$  concentrations in the CMV (day 2  $p < 0.0001$ , day 7  $p < 0.0001$ , day 14  $p = 0.0002$ , day 28  $p = 0.0380$ ) and SBN (day 2  $p < 0.0001$ , day 7  $p = 0.0066$ , day 28  $p = 0.0016$ ) treatments vs the control disappeared after 28 days, taking the longest. On day 2, the CMV treatment also had higher  $\text{NH}_4^+$  concentrations than the MBG treatment ( $p = 0.0029$ ). Immobilization was likely delayed in the ALF, CMV, and SBN treatments due to their lack of condensed tannins. However, saponins found in ALF may have produced a similar effect to the condensed tannins and increased N immobilization rates relative to the CMV and SBN treatments, which do not contain secondary compounds. Higher  $\text{NH}_4^+$  concentrations in the MBG treatment vs the control only lasted through day 0 (Table 10), but occurred again at the end of the incubation on day 84 where MBG had higher  $\text{NH}_4^+$  concentrations

than the control ( $p=0.0025$ ), CMV ( $p=0.0358$ ), and BFT ( $p=0.0300$ ) treatments.

The treatment  $\times$  day interaction for  $\text{NO}_3^-$  was more complex. The fecal treatments demonstrated patterns of N mineralization, immobilization, and subsequent mineralization, while  $\text{NO}_3^-$  steadily increased over time in the control. Initially there were no differences among treatments, as the N added through the feces had likely not yet been mineralized. On day 2,  $\text{NO}_3^-$  concentrations in the grass MBG and forb SBN treatments declined while the legume and control treatments mineralized such that the ALF ( $p=0.0002$ ), BFT ( $p=0.0025$ ), CMV ( $p<0.0001$ ), and SFN ( $p<0.0001$ ) treatments had higher  $\text{NO}_3^-$  concentrations than the SBN treatment. This suggests a first wave of substrate degradation in the non-legume MBG and SBN treatments, likely due to their high substrate C:N ratio. All treatments provided evidence of immobilization on day 7, while  $\text{NO}_3^-$  concentrations continued to increase in the control, suggesting a second wave of microbial substrate degradation. The control treatment had significantly greater concentrations of soil  $\text{NO}_3^-$  than all treatments except for the non-CT legume CMV treatment ( $p<0.0001$  for ALF, BFT, MBG, SBN, SFN). The non-CT ALF treatment had higher  $\text{NO}_3^-$  concentrations than the non-legume MBG ( $p<0.0001$ ) and SBN ( $p=0.0229$ ) treatments, while CMV had higher  $\text{NO}_3^-$  concentrations than the MBG ( $p<0.0001$ ), SBN ( $p<0.0001$ ), and BFT ( $p=0.0002$ ) treatments. This suggests that secondary compounds found in the ALF, BFT, and SFN legume treatments increased N immobilization rates, with the condensed tannins found in the BFT and SFN treatments increasing immobilization rates the most. On days 14, 21, and 28,  $\text{NO}_3^-$  concentrations in the control remained significantly higher than all other treatments ( $p<0.0001$  for ALF, BFT, CMV, MBG, SBN, SFN, all 3 days) as the N from fecal substrates was immobilized. On day 21,

the non-CT legume CMV treatment had higher  $\text{NO}_3^-$  concentrations than the BFT ( $p=0.0157$ ), MBG ( $p=0.0157$ ), and SBN ( $p=0.0157$ ) treatments. This pattern continued on day 28, as both the CMV and ALF treatments had higher  $\text{NO}_3^-$  concentrations than the BFT ( $p<0.0001$ ,  $p=0.0086$ ), SFN ( $p<0.0001$ ,  $p<0.0001$ ), MBG ( $p<0.0001$ ,  $p<0.0001$ ), and SBN ( $p<0.0001$ ,  $p=0.0398$ ) treatments, respectively. These patterns throughout the second immobilization wave also suggest that condensed tannins increase N immobilization rates, with a similar, but shorter-lasting effect by secondary compounds found in the ALF treatment. By day 42 several of the fecal treatments had begun to mineralize, although the control remained significantly higher than all treatments except for CMV, the non-CT-containing legume (ALF  $p=0.0251$ , BFT  $p<0.0001$ , MBG  $p<0.0001$ , SBN  $p<0.0001$ , SFN  $p<0.0001$ ). Nitrate concentrations remained higher in the CMV treatment than the BFT ( $p=0.0040$ ), SFN ( $p<0.0001$ ), MBG ( $p<0.0001$ ), and SBN ( $p=0.0108$ ) treatments, suggesting a continued increase in immobilization rates by CT and high substrate C:N ratios. The high substrate C:N ratio of the MBG treatment likely accounted for its significantly lower  $\text{NO}_3^-$  concentration compared to the ALF ( $p=0.0002$ ), CMV ( $p<0.0001$ ), and SBN ( $p=0.0269$ ) treatments. By the end of the incubation all fecal and control treatments had mineralized to a large degree except for the grass MBG treatment, which had significantly lower  $\text{NO}_3^-$  concentrations than all other treatments ( $p<0.0001$  for ALF, BFT, CMV, Control, SBN, SFN). While the substrate C:N ratio appears to have a more lasting effect on N mineralization and immobilization patterns, secondary compounds, particularly CT, appear to be capable of increasing soil N immobilization rates.



There was no difference in N<sub>2</sub>O production rates among treatments, but production differed among days ( $p < 0.0001$ ) and peaked on days 7 and 14 (Fig. 15a). Cumulative N<sub>2</sub>O production differed among treatments ( $p = 0.0153$ ) with significantly higher cumulative N<sub>2</sub>O production in the BFT ( $p = 0.0399$ ) and SFN ( $p = 0.0046$ ) treatments than the control (Fig. 15b). This could account for the lower NO<sub>3</sub><sup>-</sup> concentrations observed for these treatments as opposed to increased N immobilization via tannin-nitrogen complexation. However, total N<sub>2</sub>O produced over the entire incubation was similar among treatments with no significant difference by day 84 (Fig. 15c). This suggests that over a long period of time the additions did not stimulate significantly greater N<sub>2</sub>O production than the control. Nitrous oxide production rates were significantly negatively correlated with NO<sub>3</sub><sup>-</sup> concentration ( $p = 0.0166$ ) and positively correlated with CO<sub>2</sub> production rate and cumulative CO<sub>2</sub> production ( $p = 0.0308$ ). Cumulative ( $p < 0.0001$ ) and total N<sub>2</sub>O production ( $p = 0.0155$ ) were positively correlated with cumulative CO<sub>2</sub> production as well (Table 11). Both cumulative and total N<sub>2</sub>O production were significantly positively correlated with total CO<sub>2</sub> production ( $p = 0.0031$ ,  $p < 0.00001$ ), total soil and feces C ( $p = 0.0009$ ,  $p < 0.0001$ ), and N ( $p = 0.0011$ ,  $p < 0.0001$ ), and total soil and feces tannin content ( $p = 0.0095$ ,  $p < 0.0001$ ), respectively (Table 11). This suggests that the production of greenhouse gases in soil is related, and influenced by nutrient availability such as C and N. These results also suggest that nitrate was quickly denitrified in tannin containing treatments rather than remaining in mineral forms. However, when concentrations of the various forms of measured N were averaged across the entire 84-day incubation, the most N was found as mineral NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup>, with much lower amounts found as N<sub>2</sub>O (Table 9). This suggests that N was more likely

to be lost through leaching in mobile mineral forms rather than as greenhouse gases.

While the substrate addition increased CO<sub>2</sub> production in all fecal treatments, there was also evidence of CO<sub>2</sub> production inhibition by secondary compounds (Fig. 16a-c). There were significant treatment  $\times$  day interactions for CO<sub>2</sub> production rates ( $p < 0.0001$ ) and cumulative CO<sub>2</sub> production ( $p = 0.0085$ ; Fig. 16a-b). On day 2, the control had significantly lower CO<sub>2</sub> production rates than the non-CT, low C:N ratio, CMV ( $p = 0.0134$ ) and SBN ( $p = 0.0017$ ) treatments. On days 7 and 14, the control treatment had lower CO<sub>2</sub> production rates than all other treatments (days 7 and 14 ALF, BFT, CMV, MBG, SBN, SFN  $p < 0.0001$ ) likely due to substrate addition, and MBG had lower CO<sub>2</sub> production rates than the CMV treatment (day 7  $p = 0.0098$ , day 14  $p = 0.0072$ ). By days 21 and 28, the control treatment only had significantly lower CO<sub>2</sub> production rates than the CMV (day 21  $p = 0.0283$ , day 28  $p = 0.0325$ ) and SFN (days 7 and 14  $p = 0.0429$ ) treatments. The disappearance of all treatment differences in CO<sub>2</sub> production by day 28 implies that microbes utilized the fecal additions as a substrate and degraded the majority of labile substrate within the first four weeks. The interaction for cumulative CO<sub>2</sub> production appeared to be random with no obvious crossing of treatments, so the main effects of treatment ( $p < 0.0001$ ) and day ( $p < 0.0001$ ) were analyzed (Fig. 16b). All fecal treatments had significantly higher cumulative CO<sub>2</sub> production than the control (ALF, BFT, CMV, MBG, SBN, SFN  $p < 0.0001$ ), indicating increased substrate availability. As expected, the non-CT CMV treatment had significantly higher cumulative CO<sub>2</sub> production than all treatments except for SBN (ALF  $p = 0.0194$ , BFT  $p = 0.0016$ , MBG  $p = 0.0003$ , SFN  $p = 0.0095$ ) which was an intermediate among all fecal treatments. By the end of the incubation, the control had lower ( $p < 0.0001$  for ALF, BFT, CMV, MBG,

SBN, SFN) total CO<sub>2</sub> production than all fecal treatments, and the CMV treatment had the highest (ALF  $p=0.0011$ , BFT  $p=0.0005$ , Control  $p<0.0001$ , MBG  $p<0.0001$ , SBN  $p=0.0044$ , SFN  $p=0.0014$ ) total CO<sub>2</sub> production (Fig. 16c). This suggests that CMV was more readily degradable by soil microbes, with the SBN treatment trending similarly. Carbon dioxide production rate was significantly positively correlated with N<sub>2</sub>O production rate ( $p<0.0001$ ) and negatively correlated with NO<sub>3</sub><sup>-</sup> concentration ( $p=0.0031$ ). Cumulative CO<sub>2</sub> production was negatively correlated ( $p=0.0308$ ) with N<sub>2</sub>O production rate (Table 11), while cumulative and total CO<sub>2</sub> production were both significantly positively correlated with cumulative ( $p<0.0001$ ,  $p=0.0031$ ) and total N<sub>2</sub>O production ( $p=0.0155$ ,  $p<0.0001$ ), total soil and feces C ( $p=0.0024$ ,  $p<0.0001$ ) and N ( $p=0.0019$ ,  $p<0.0001$ ), respectively (Table 11). Total CO<sub>2</sub> production was also significantly positively correlated with tannin content ( $p=0.0003$ ) (Table 11). Like measures of N<sub>2</sub>O production, these correlations suggest that CO<sub>2</sub> production is related to the stimulation of the microbial community through nutrient inputs through tannins and fecal treatments, and CO<sub>2</sub> production increases as these nutrients are immobilized in microbial biomass.

Carbon and N cycling processes can be affected by environmental factors such as soil moisture. We irrigated a sub-set of samples on days 0, 21, 42, and 84 to investigate the effect of CT in fecal additions under typical irrigation practices. Patterns of inorganic N concentrations in irrigated samples reflected trends seen in the non-irrigated samples. The main effects of treatment ( $p<0.0001$ ) and day ( $p<0.0001$ ) were both significant for ammonium concentrations in the irrigated samples (Fig. 17a). Ammonium concentrations were significantly higher in all fecal treatments compared to the control (ALF  $p<0.0001$ ,

BFT  $p=0.0021$ , CMV  $p<0.0001$ , MBG  $p<0.0001$ , SBN  $p<0.0001$ , SFN  $p<0.0001$ ), likely due to the additional N contained in the fecal treatments. Ammonium concentrations were significantly higher on day 0 compared to the rest of the irrigated sampling points (day 21  $p<0.0001$ , day 42  $p<0.0001$ , day 84  $p=0.0023$ ).

There was a significant ( $p<0.0001$ ) treatment  $\times$  day interaction for  $\text{NO}_3^-$  concentrations in the irrigated samples (Fig. 17b). On day 0, the CMV samples had the highest  $\text{NO}_3^-$  concentrations, and were significantly higher than the control ( $p=0.0197$ ), MBG ( $p<0.0001$ ), and SBN ( $p<0.0001$ ) treatments. The SFN treatment had the next highest  $\text{NO}_3^-$  concentrations, and were significantly higher than the control ( $p=0.0294$ ), MBG ( $p<0.0001$ ), and SBN ( $p<0.0001$ ) treatments. The BFT, ALF, and control treatments had the next highest  $\text{NO}_3^-$  concentrations, and were significantly higher than the MBG (BFT  $p<0.0001$ , ALF  $p=0.0005$ , control  $p=0.0461$ ) and SBN (BFT  $p<0.0001$ , ALF  $p<0.0001$ , control  $p<0.0001$ ) treatments. The MBG treatment had significantly higher  $\text{NO}_3^-$  concentrations than the SBN treatment ( $p<0.0001$ ), which had the lowest concentration. By day 21, the control had significantly higher  $\text{NO}_3^-$  concentrations than the rest of the treatments (ALF, BFT, CMV, MBG, SBN, SFN  $p<0.0001$ ). On day 42, the control still had significantly higher  $\text{NO}_3^-$  concentrations than all treatments but CMV (ALF  $p=0.0085$ , BFT  $p<0.0001$ , MBG  $p<0.0001$ , SBN  $p<0.0001$ , SFN  $p<0.0001$ ). Cicer milkvetch, which had the next highest  $\text{NO}_3^-$  concentration, was significantly higher than BFT ( $p<0.0001$ ), MBG ( $p<0.0001$ ), SBN ( $p=0.0002$ ), or SFN ( $p<0.0001$ ). Cicer milkvetch was followed by ALF, which had significantly higher concentrations than BFT, MBG, or SFN ( $p<0.0001$ , all treatments). The higher  $\text{NO}_3^-$  concentrations in the non-CT legume vs the CT legume treatments suggests that the secondary compounds

increased N immobilization through tannin-protein complexation. Small burnet had significantly higher concentrations than the remaining BFT ( $p=0.0006$ ), MBG ( $p<0.0001$ ) or SFN ( $p<0.0001$ ) treatments. Birdsfoot trefoil had significantly higher concentrations than SFN ( $p=0.0006$ ) and MBG ( $p<0.0001$ ). Higher  $\text{NO}_3^-$  concentrations in the BFT treatment than the SFN treatment may have been due to higher concentrations of secondary compounds found in SFN compared to BFT, increasing N immobilization to a larger degree. By day 84, MBG had significantly lower  $\text{NO}_3^-$  concentrations than the rest of the treatments (ALF, BFT, CMV, control, SBN, SFN  $p<0.0001$ , all treatments), and ALF had significantly higher concentrations than BFT ( $p=0.0366$ ).

Like the non-irrigated samples, the main effect of treatment was not significant for irrigated  $\text{N}_2\text{O}$  production rates or total  $\text{N}_2\text{O}$  production (Table 12). Nitrous oxide production rates were significantly higher on day 0 than day 84 ( $p=0.0005$ ), and total  $\text{N}_2\text{O}$  production was significantly higher on days 21 ( $p=0.0089$ ) and 0 ( $p=0.0147$ ) than 84. For  $\text{CO}_2$  production rates the main effect of day ( $p<0.0001$ ), but not treatment, was significant in the irrigated samples (Table 12). Carbon dioxide production was highest on day 21, followed by 0, 42, and 84. There was a significant treatment  $\times$  day interaction for total  $\text{CO}_2$  production, but the interactions appeared to be random with no obvious crosses. The main effect of treatment was significant ( $p<0.0001$ ) for total  $\text{CO}_2$  production at the end of each irrigated 48-hour incubation period (Fig. 18). All fecal treatments had significantly higher total  $\text{CO}_2$  production than the control (ALF  $p=0.0060$ , BFT  $p=0.0003$ , CMV  $p=0.0008$ , SBN  $p=0.0027$ , SFN  $p<0.0001$ ) except for MBG, which was an intermediate ( $p=0.0715$ ). This would suggest that the addition of C as feces acted as a microbial substrate. Substrate C:N ratio appeared to influence C mineralization more

strongly than secondary compound content. The main effect of day was also significant ( $p < 0.0001$ ) for total  $\text{CO}_2$  production where, like  $\text{CO}_2$  production rate, total  $\text{CO}_2$  production was highest on day 21. The results of the irrigated samples confirm that although substrate C:N ratio is the primary driver of soil N cycling, secondary compounds may also play an important role in increasing soil N retention.

When  $\text{N}_2\text{O}$  production rates were compared among irrigated and non-irrigated samples, it appeared that fecal treatment, day, and irrigation status all impacted  $\text{N}_2\text{O}$  production rates based on significant treatment  $\times$  irrigation ( $p = 0.0033$ ) and day  $\times$  irrigation ( $p < 0.0001$ ) interactions (Fig. 19a-b). Based on the treatment  $\times$  irrigation interaction the non-irrigated control had the lowest  $\text{N}_2\text{O}$  production rates, and were significantly lower than the non-irrigated SFN ( $p = 0.0124$ ) and irrigated CMV ( $p = 0.0377$ ) treatments, which had the highest and second-highest  $\text{N}_2\text{O}$  production rates, respectively. Based on the day  $\times$  irrigation interaction, the non-irrigated samples had the highest rates of  $\text{N}_2\text{O}$  production on day 21, with significantly higher production rates than the non-irrigated samples on days 0 ( $p < 0.0001$ ) and 84 ( $p < 0.0001$ ). When compared within each day, irrigated samples were significantly higher than non-irrigated samples ( $p = 0.0006$ ) on day 0. These results suggest that lower C:N legume fecal treatments may increase  $\text{N}_2\text{O}$  production rates, although this may be complicated by other conditions such as moisture status. As we have demonstrated, irrigation events and increased soil and feces moisture can significantly increase  $\text{N}_2\text{O}$  production rates.

When  $\text{CO}_2$  production rates were compared among irrigated and non-irrigated samples, the results were more complex. There was a significant ( $p = 0.0309$ ) three-way interaction for the main effects of fecal treatment, irrigation, and day (Fig. 20). When the

results were compared within each day, there was no clear pattern of the irrigated treatments producing consistently higher or lower CO<sub>2</sub> production rates than their non-irrigated counterparts. The timing of peak CO<sub>2</sub> production also appeared to be variable for each non-irrigated treatment with no clear relation to secondary compound content or plant type. Irrigated samples appeared to reach peak CO<sub>2</sub> production at the beginning or end of the incubation on days 0 or 84, but again did not clearly relate to secondary compound content or plant type. As expected, all non-irrigated fecal treatments had significantly higher CO<sub>2</sub> production rates than the non-irrigated control throughout the experiment (ALF, BFT, CMV, SBN, SFN  $p < 0.0001$  all days, MBG  $p < 0.0001$  days 0, 21, 42,  $p = 0.0003$  day 84), with the exception of the CMV treatment on days 0 ( $p < 0.0001$ ) and 42 ( $p < 0.0001$ ) when CMV was significantly lower. Interestingly, CO<sub>2</sub> production in the irrigated treatments did not appear to exceed production of the non-irrigated control until later in the experiment. All irrigated treatments were equal to the non-irrigated control on day 0, and only the irrigated control exceeded the non-irrigated control on day 21 ( $p = 0.001$ ). However, by day 42 several irrigated treatments including ALF ( $p < 0.0001$ ), control ( $p < 0.0001$ ), MBG ( $p < 0.0001$ ), SBN ( $p < 0.0001$ ), and SFN ( $p < 0.0001$ ) had significantly higher rates of CO<sub>2</sub> production than the non-irrigated control. Production rates in the irrigated samples tended to decrease towards the end of the incubation, with only the irrigated ALF ( $p = 0.0006$ ) and BFT ( $p = 0.033$ ) treatments exceeding the non-irrigated control by day 84. Fewer samples had significantly different CO<sub>2</sub> production from the irrigated control. Only the samples with the highest CO<sub>2</sub> production rates on each day significantly exceeded the irrigated control, including the non-irrigated SBN ( $p < 0.0001$ ) and MBG ( $p < 0.0001$ ) treatments on day 0, the irrigated

SBN ( $p=0.0073$ ) treatment on day 21, the non-irrigated SBN ( $p<0.0001$ ) treatment on day 42, and the non-irrigated MBG ( $p<0.0001$ ), SBN ( $p<0.0001$ ), and SFN ( $p<0.0001$ ) treatments on day 84. Non-irrigated secondary compound-containing treatments ALF, BFT, and SFN did not differ significantly from any other non-irrigated treatments. The irrigated secondary compound-containing treatments did not differ in  $\text{CO}_2$  production rates from other irrigated samples except for the irrigated control on day 21 which had significantly higher production than the irrigated ALF ( $p<0.0001$ ), BFT ( $p=0.0015$ ), and SFN ( $p<0.0001$ ) treatments, as well as the irrigated MBG treatment which exceeded the irrigated SFN treatment ( $p=0.0024$ ). These results once again suggest that in non-irrigated samples, fecal treatments can increase  $\text{CO}_2$  production rates compared to a control. However, irrigation appears to increase system complexity and this increased variability likely masks clear patterns in  $\text{CO}_2$  production.

#### **4. Discussion**

In this study, I compared the effect of different legume forages on soil N mineralization and indicators of soil microbial activity in grazed pastures, and the effect of different fecal additions and irrigation on pasture soil N mineralization and greenhouse gas production during an 84-day incubation study. I hypothesized that CT-containing legumes and fecal treatments would decrease microbial activity and soil C and N mineralization compared to non-CT-containing legumes or non-N fixing, low C:N ratio forbs. As a result, these CT-containing treatments would produce similar results as a high C:N ratio grass treatment. As tannins may decrease decomposition rates, I hypothesized that soils under tannin-containing legumes would have increased organic C and but decreased readily mineralizable C. Additionally, I hypothesized that pasture soils



incubated with tannin-containing feces would have decreased concentrations of inorganic N and N<sub>2</sub>O production. Finally, I hypothesized that the addition of moisture during irrigation events would increase GHG production as a result of reduced oxygen diffusion through the sample. Across all three studies, I found that the C:N ratio of the forages and apparent C:N ratio as a result of equal C additions in fecal treatments appeared to be the primary driver of C and N mineralization patterns. However, I did find evidence of CT legumes decreasing N mineralization and increasing N immobilization. Consistent with my hypothesis, I found that potential aerobic N mineralization rates were lower in field soils under SFN in field experiment one, and under BFT in field experiment two. This was confounded by lower biomass in both cases as lower amounts of biomass would mean lower amounts of N entering the soil through decomposition which could potentially be mineralized, although this was dismissed after follow-up Pearson correlations. In the incubation study, I found increased ammonium and nitrate immobilization rates in CT treatments as well as treatments such as alfalfa which contained different secondary compounds (i.e., saponins). However, significant Pearson correlations such as a negative relationship between N<sub>2</sub>O production rate and NO<sub>3</sub><sup>-</sup> concentration as well as a positive correlation between N<sub>2</sub>O production and soil and feces tannin content suggest that this NO<sub>3</sub><sup>-</sup> may have been quickly denitrified. Despite this possible rapid denitrification in CT-containing treatments, I did not find any differences in total N<sub>2</sub>O production over the course of the incubation. I did observe evidence of irrigation events increasing N<sub>2</sub>O production rates when compared to non-irrigated samples. Carbon cycling indicators were less consistent with my hypothesis. I did not find any CT-related differences in C cycling parameters in either field experiment. In the

incubation experiment, differences in C mineralization as measured by CO<sub>2</sub> production appeared to be related to general substrate availability rather than effects of CT-related mineralization inhibition as evidenced by positive Pearson correlations between cumulative and total CO<sub>2</sub> production with soil and feces total C and N. While I did observe a significant positive correlation between soil and feces tannin content and total CO<sub>2</sub> production, the correlation was not confirmed by treatment differences in total CO<sub>2</sub> production by the end of the incubation. I also did not find any CT-related differences in CO<sub>2</sub> production rates in the comparison of irrigated and non-irrigated samples. Also contrary to my hypothesis, irrigated samples did not clearly show increased CO<sub>2</sub> production rates compared to their non-irrigated counterparts within treatments.

In the field, inorganic N concentrations in soils under CT legumes were equal to non-CT legumes (ALF in field experiment one, CMV in field experiment two). In field experiment two, NO<sub>3</sub><sup>-</sup> concentrations were lower under MBG than BFT or CMV from 0-60 cm, likely due to MBG's high C:N ratio and fine roots which could cause rapid N uptake. Aerobic N mineralization rates were significantly lower under SFN than non-CT ALF, and lower under BFT than CMV. Because there were no differences in field soil organic C or TN among treatments, this indicates that N may have been prevented from mineralizing in the short-term. However, biomass was also significantly lower in SFN and BFT treatments. After follow-up analysis there were not significant correlations between forage biomass and potential aerobic N mineralization in either field experiment, suggesting that biomass did not drive N mineralization rates. Additionally, there was a significant negative Pearson correlation between forage total condensed tannin content and potential aerobic N mineralization in field experiment one, confirming that

condensed tannin content was in fact driving N mineralization patterns. This was further confirmed by significantly lower CT content of the BFT forage compared to the SFN forage in field experiment one, and lower CT content of CMV and MBG compared to BFT in field experiment two. In the incubation study, patterns of N mineralization and immobilization were typical of C:N ratio-driven microbial growth and turnover with underlying evidence of CT complexation. The added N in the fecal treatments likely drove the initial increases in  $\text{NH}_4^+$  in the fecal treatments. However, the rate at which those pools of  $\text{NH}_4^+$  disappeared in the fecal treatments appeared to be due to increased N immobilization rates in secondary compound-containing treatments. Condensed tannins appeared to increase immobilization the most, with saponins in the ALF treatment having a weaker effect. In the irrigated samples, differences in  $\text{NH}_4^+$  concentrations appeared to be driven by substrate addition as all fecal treatments had higher concentrations than the control. In the non-irrigated samples, differences in fecal substrate C:N ratios appeared to create waves of  $\text{NO}_3^-$  immobilization. The first wave occurred in the non-legume fecal treatments and the second wave occurred in the legume and control treatments likely due to their lower C:N ratio, with the C:N ratio of the soil being the C:N ratio of the control treatment. The C:N ratio effects continued throughout the incubation as the high C:N ratio MBG treatment had low  $\text{NO}_3^-$  concentrations through day 84. Despite the influences of C:N ratio, I found evidence of secondary compound complexation with N in both the irrigated and non-irrigated samples. Throughout the incubation,  $\text{NO}_3^-$  concentrations were higher in the CMV treatment than the BFT and SFN treatments.

My finding that both C:N ratio and CT complexation drive N cycling are supported by the literature. The C:N ratio of material has been well-known to drive soil N

immobilization and mineralization rates. These findings have been previously summarized by studies such as Enwezor (1976), Janssen (1996), and Bengtsson et al. (2003). Nitrogen mineralization rates tend to be inversely correlated with C:N ratio, with low C:N substrates having increased mineralization rates. Condensed tannins have been shown to produce longer-term reductions in net N mineralization by complexing with proteins and organic N, or create shorter-term N immobilization by acting as a high C substrate. Studies by Crush (1993) and Crush and Keogh (1998) observed CT driven decreases in soil nitrate formation under CT-containing dung or incubated with CT-containing herbage. Additional studies such as Northup et al. (1995), Schimel et al. (1998), and Nierop et al. (2006a; b) have come to similar conclusions of CTs reducing N mineralization rates in soil and litter experiments. This combination of C:N ratio and CT content would explain why I saw rapid decreases in mineralization rates and increases N immobilization rates in the CT-containing BFT and SFN treatments, followed by secondary compound-containing ALF, and finally non-secondary compound-containing, low C:N ratio CMV and SBN treatments. A study by Halvorson et al. (2012) found that tannin sorption was higher in soils which were amended with biosolids or manure, supporting the idea that CT complexation decreased N mineralization in my incubation study. In both the field and incubation experiments, the ALF treatment was repeatedly an intermediary between the non-CT and CT-legume treatments, suggesting that saponins in that treatment had a similar, but weaker effect as the condensed tannins. Halvorson et al. (2009) suggested that the effect of tannins may change as they degrade, which may help to explain the shorter-lived effects of other secondary compounds such as saponins on N immobilization rates in my incubation study.

The lack of differences in N<sub>2</sub>O production rates and total production in the irrigated and non-irrigated samples is important, because differences in inorganic N concentrations, especially those likely due to rapid denitrification, would be expected to cause differences in total denitrification. It is possible that differences in the total N added (ranging from 0.7 g to 1.0 g) by the different treatments were not large enough to produce differences in total N<sub>2</sub>O production. It is also possible that the incubation conditions and periodic aeration supported complete denitrification of NO<sub>3</sub><sup>-</sup> to N<sub>2</sub>. The denitrification process is very responsive to environmental conditions such as moisture, C availability and O<sub>2</sub> availability, so small variations among samples could create high levels of variation and mask potential differences among treatments (Wallenstein et al., 2006). However, there was evidence for lower C:N ratio treatments and increased moisture increasing N<sub>2</sub>O production when irrigated and non-irrigated samples were compared directly. These patterns have been widely observed in the literature. As soil C content decreases, the ability of N to be immobilized and makes it more likely to be lost as mineral or gaseous N. Additionally, elevated soil moisture is able to create anaerobic conditions by decreasing the ability of oxygen to diffuse through a media. Since denitrification is an anaerobic process, increased soil moisture is more highly conducive to N<sub>2</sub>O production (Firestone and Davidson, 1989; Sahrawat and Keeney, 1986; Weier et al., 1993). The multitude of positive correlations among measures of N<sub>2</sub>O and CO<sub>2</sub> productions was unsurprising and supported by the literature, where production of the two greenhouse gases have been consistently linked (Eaton and Patriquin, 1989; Lou et al., 2007; Toma and Hatano, 2007).

Carbon cycling parameters showed less evidence of CT-related inhibition than N cycling parameters. Although field soils under BFT had greater mineralizable C and respiration rates than ALF in field experiment one, this is likely due to the high  $\text{NH}_4^+$  concentrations seen under BFT at 0-10 cm from likely sampling in a urine patch. There were no differences in any other C cycling parameters to substantiate a shift in microbial community or functioning. In field experiment two, the only difference in C cycling parameters was lower dehydrogenase enzyme activity under CMV than MBG, with BFT as in intermediate. This is likely related to the low C:N ratio of legume biomass compared to high C:N grass. Although these results are not consistent with my hypothesis, past reviews of the topic such as Kraus et al. (2003a) concluded that changes in soil N cycling in the absence of changes in C cycling are indicative of CT complexation with organic N. Smolander et al. (2012) suggested that low concentrations of tannins may act as a substrate and decrease N mineralization via increased N immobilization, while higher concentrations may reduce N mineralization via complexation. If pasture soils under CT legumes have sufficiently high concentrations of CTs, it may explain decreased potential aerobic N mineralization rates and lack of differences in C cycling. Although originally proposed in relation to N cycling, Crush and Keogh (1998) suggested that the microbial community may adapt to the presence of CTs over time. Higher nitrification rates in soils that had grown CT-containing *Lotus pedunculatus* for multiple years vs one year led the authors to propose a microbial shift favoring communities that can degrade *Lotus* (Crush and Keogh, 1998). It is possible that microbial communities in soils under CT legumes had sufficient time to shift and similarly select for populations capable of readily decomposing tannins. I cannot explain

why the soil under MBG had significantly higher Zn concentrations than the SBN or BFT treatments despite a higher pH. I can speculate that the MBG litter may have had higher Zn concentrations which would increase soil Zn, or that MBG had lower Zn requirements than the other forage treatments.

Carbon mineralization patterns in the incubation study could be attributed to a combination of substrate C:N ratio and CT-driven inhibition. Differences in CO<sub>2</sub> production rates only lasted for the first four weeks of the incubation, suggesting that many of the differences were due to substrate additions and that the substrates were readily decomposed (Nierop et al., 2006a, 2006b; Schimel et al., 1998). However, within those four weeks the control treatment had significantly lower CO<sub>2</sub> production rates than the non-CT, low C:N ratio CMV and SBN treatments on day 2. During the first two weeks the MBG treatment had lower CO<sub>2</sub> production rates than CMV, but likely due to a higher C:N ratio. These results were supported by cumulative and total CO<sub>2</sub> production. All fecal treatments had significantly higher cumulative and total CO<sub>2</sub> production than the control, likely due to increased substrate availability as evidenced by positive correlations with soil and feces total C and N contents and negative correlations with NO<sub>3</sub><sup>-</sup> concentrations. By the end of the incubation, CMV had significantly higher cumulative and total CO<sub>2</sub> production than all other treatments, and SBN was an intermediate for all fecal treatments for cumulative CO<sub>2</sub> production. This pattern suggests that CT legume treatments inhibited CO<sub>2</sub> production just as well as the high C:N grass treatment despite increased production across all treatments and a positive correlation with soil and feces tannin content. There were no differences in CO<sub>2</sub> production rates or total production each day in the irrigated samples. The comparison of irrigated and non-

irrigated samples also suggested that irrigation did not consistently increase CO<sub>2</sub> production nor was production clearly affected by fecal treatment or its secondary compound content. These mixed results are not surprising as the effect of tannins on C mineralization historically has been mixed (T. E.C. Kraus et al., 2003; Smolander et al., 2012). It is surprising that irrigation did not produce a clear pattern of increased CO<sub>2</sub> production, as soil wetting has been linked to increased CO<sub>2</sub> production in the literature (Inglima et al., 2009; Sponseller, 2007). It is possible that the extent of our irrigation was not sufficient or that our non-irrigated soils were too moist to produce such drastic results. Studies such as Muhr et al., (2008) did not find that wetting soils produced elevated CO<sub>2</sub> concentrations compared to a consistently moist soil. It is also possible that irrigation increased sample heterogeneity, where variation among samples due to soil microsites could mask potential differences among treatments, irrigation, and day.

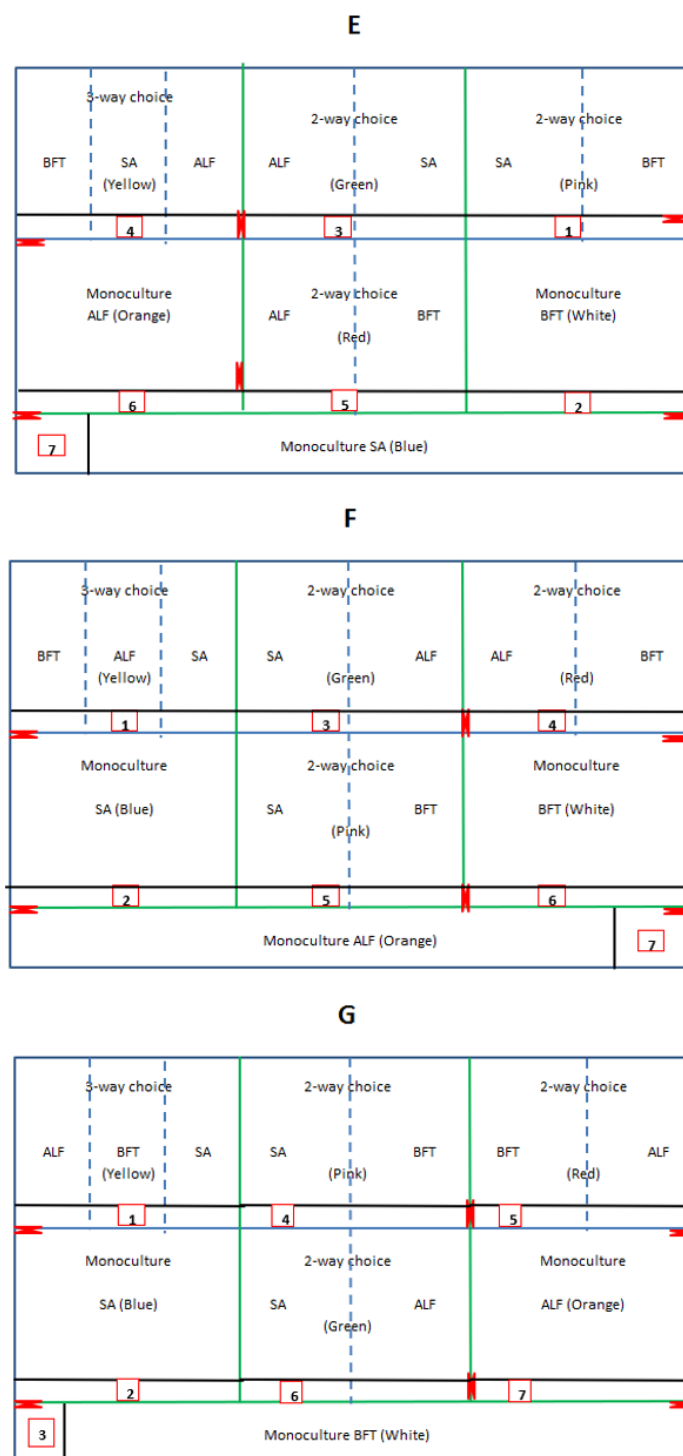
#### 4.1. Conclusions

This study observed C:N ratio driven soil C and N cycling dynamics, with evidence of CT complexation and protection of mineral N in pasture soils as well as when incubated with cattle feces. These results are consistent with the literature, and support the idea that other secondary compounds such as saponins may produce a similar, but shorter-lived effect on soil N cycling as condensed tannins. However, my research is limited in that I only have one year of data for experiment one to monitor the effects of CTs in the field over time. My incubation experiment was also limited because I did not include urine with my fecal treatment so I was not able to fully observe how shifts in N from urine to feces affects soil N and C mineralization patterns. I recommend that future research monitors the effects of CTs and specific microbial communities involved in C

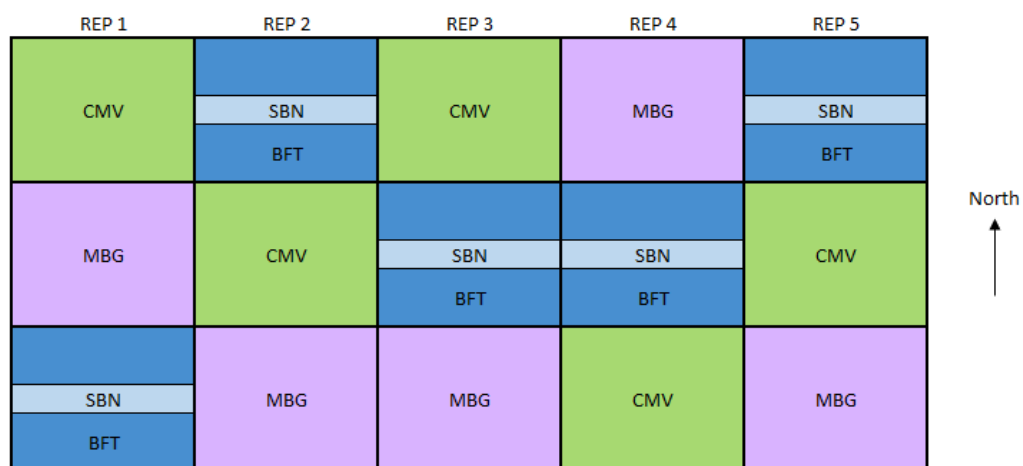


and N mineralization to investigate possible shifts in community structure as well as the effect of shifts in urinary and fecal N over more than one field season. I also recommend researching the effect of purified tannins and saponins isolated from forage species on soil C and N cycling to investigate whether saponins and tannins produce similar effects under controlled settings.

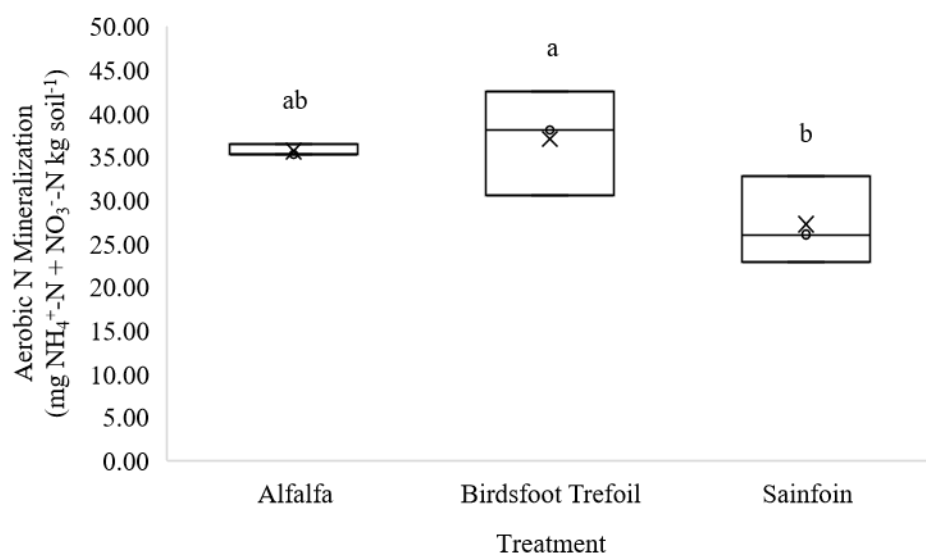
## 5. Figures and tables



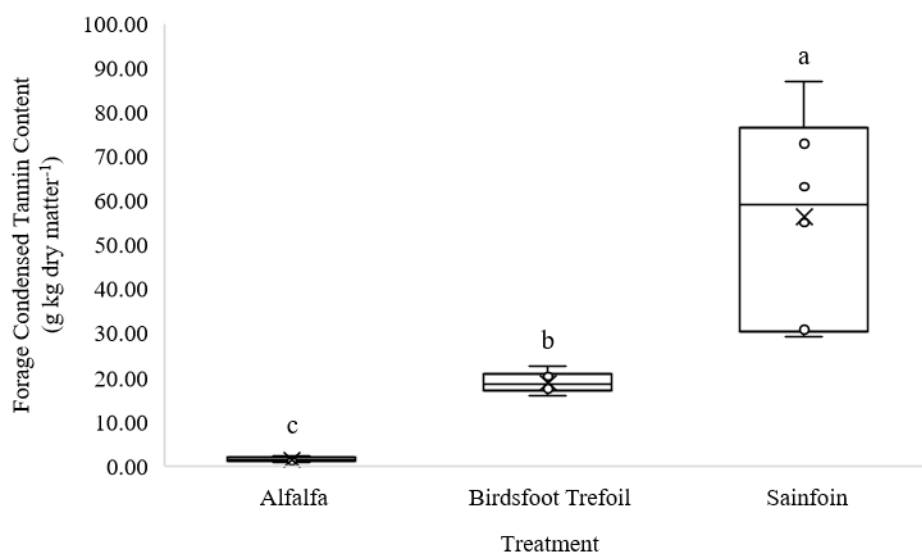
**Fig. 1.** Plot layout for field experiment one at Utah State Agricultural Experiment Station in Lewiston, Utah. The experiment is a randomized complete block design with three blocks and three treatments (alfalfa, birdsfoot trefoil, and sainfoin) per block.



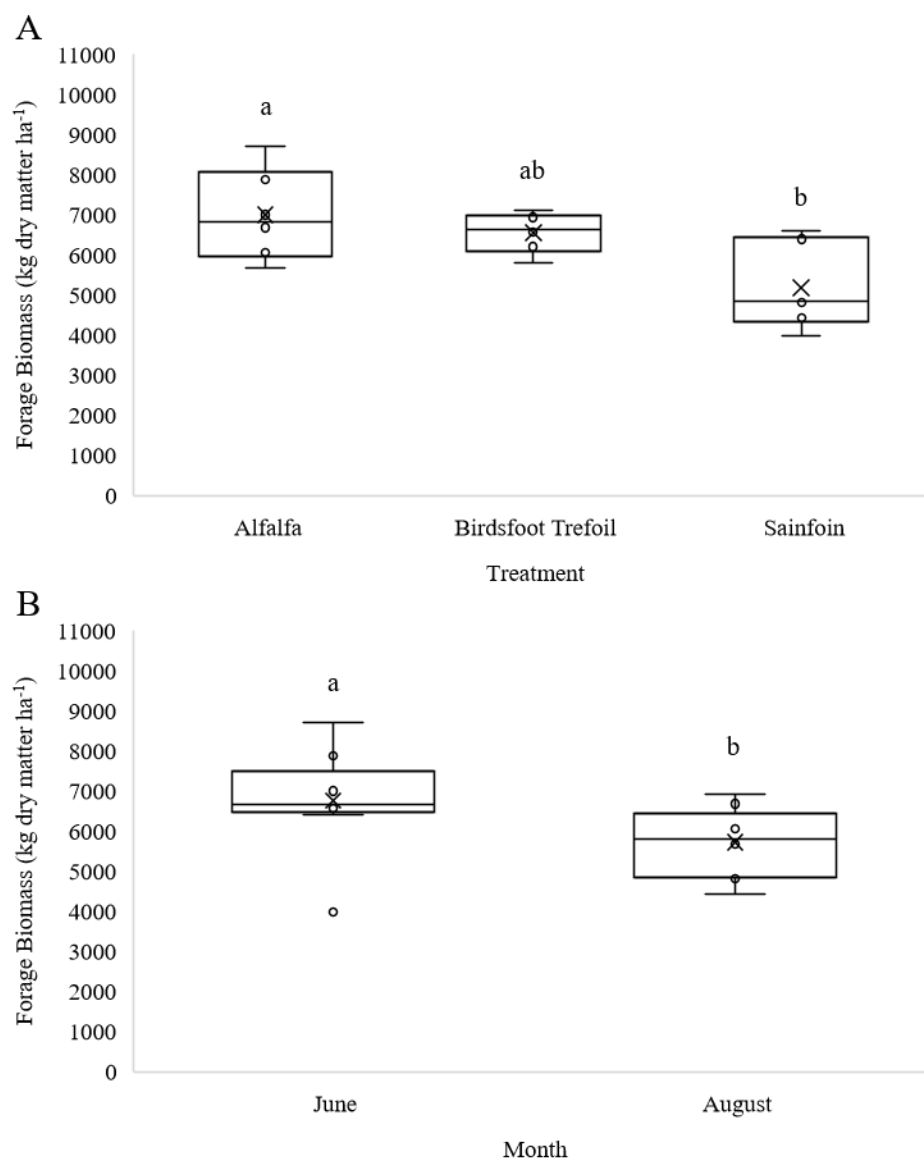
**Fig. 2.** Plot layout for field experiment two at Utah State Agricultural Experiment Station in Lewiston, Utah. The experiment is a randomized complete block design with five blocks and four treatments (birdsfoot trefoil (BFT), cicer milkvetch (CMV), meadow brome grass (MBG), and small burnet (SBN) per block.



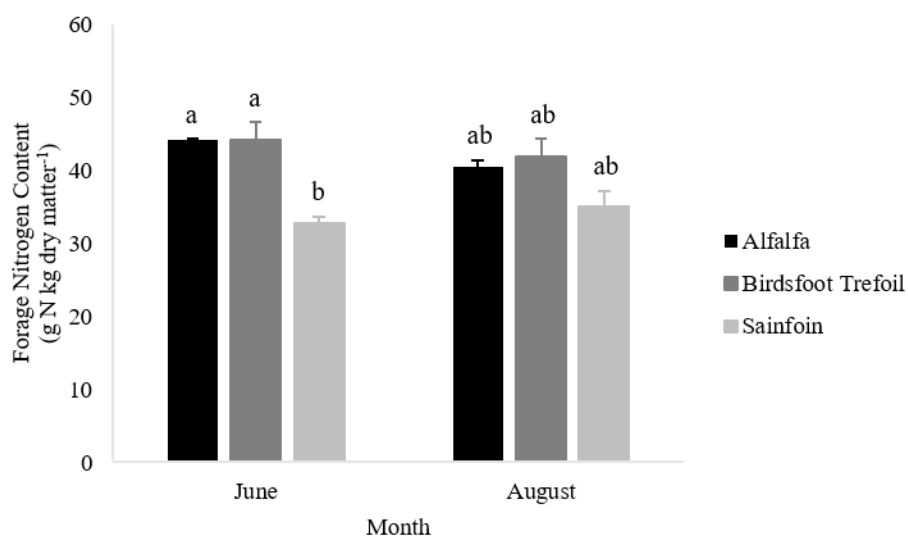
**Fig. 3.** Average potential aerobic soil nitrogen mineralization over a 21-day incubation from soils under grazed alfalfa, birdsfoot trefoil, and sainfoin pastures in field experiment one. Letters denote a significant difference at  $p < 0.1$  for the main effect of treatment ( $n=3$ ,  $p=0.0765$ ). Birdsfoot trefoil had higher potential aerobic nitrogen mineralization than sainfoin.



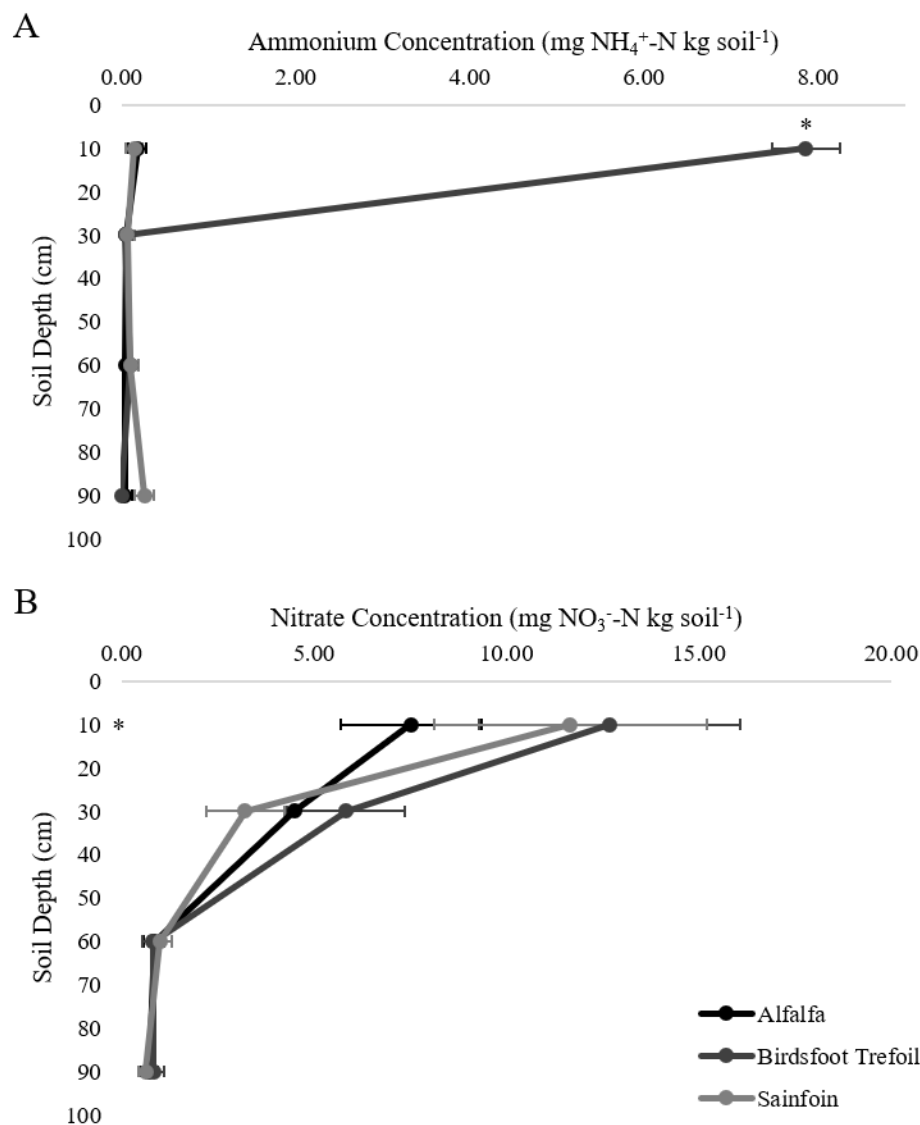
**Fig. 4.** Average forage condensed tannin content for alfalfa, birdsfoot trefoil, and sainfoin forages in field experiment one. Letters denote significant differences in condensed tannin content at  $p < 0.05$  for the main effects of treatment ( $p < 0.0001$ ,  $n = 3$ ). Sainfoin had a significantly higher condensed tannin content than birdsfoot trefoil and alfalfa, and birdsfoot trefoil had a significantly higher condensed tannin content than alfalfa. There was no significant effect of month ( $p = 0.1714$ ) or the interaction between treatment and month ( $p = 0.2003$ ).



**Fig. 5.** Average forage biomass for alfalfa, birdsfoot trefoil, and sainfoin forages in field experiment one. Letters denote significant differences in biomass at  $p < 0.05$  for the main effects of treatment (panel A,  $p = 0.0181$ ,  $n = 3$ ) and month (panel B,  $p = 0.0242$ ,  $n = 2$ ). Alfalfa had greater average biomass than sainfoin, and forage biomass was greater in June than August. There was no significant interaction between treatment and month ( $p = 0.3960$ ).

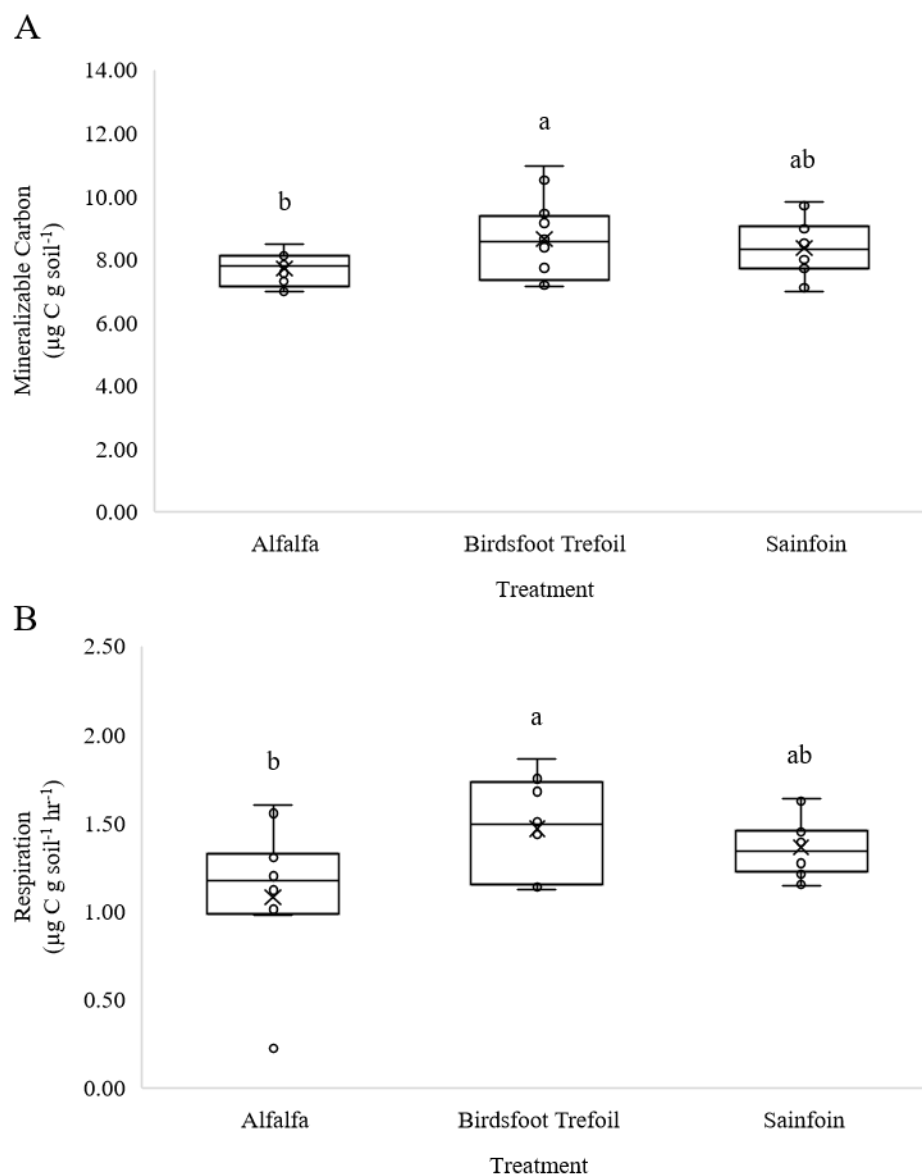


**Fig. 6.** Average nitrogen content in alfalfa, birdsfoot trefoil, and sainfoin forages. Letters denote significant differences in nitrogen content among treatments and months at  $p < 0.05$  for the interaction of treatment  $\times$  month in field experiment one ( $p = 0.0420$ ,  $n = 3$ ). Error bars represent standard error. Alfalfa and birdsfoot trefoil had a higher N content than sainfoin in June, but not in August.

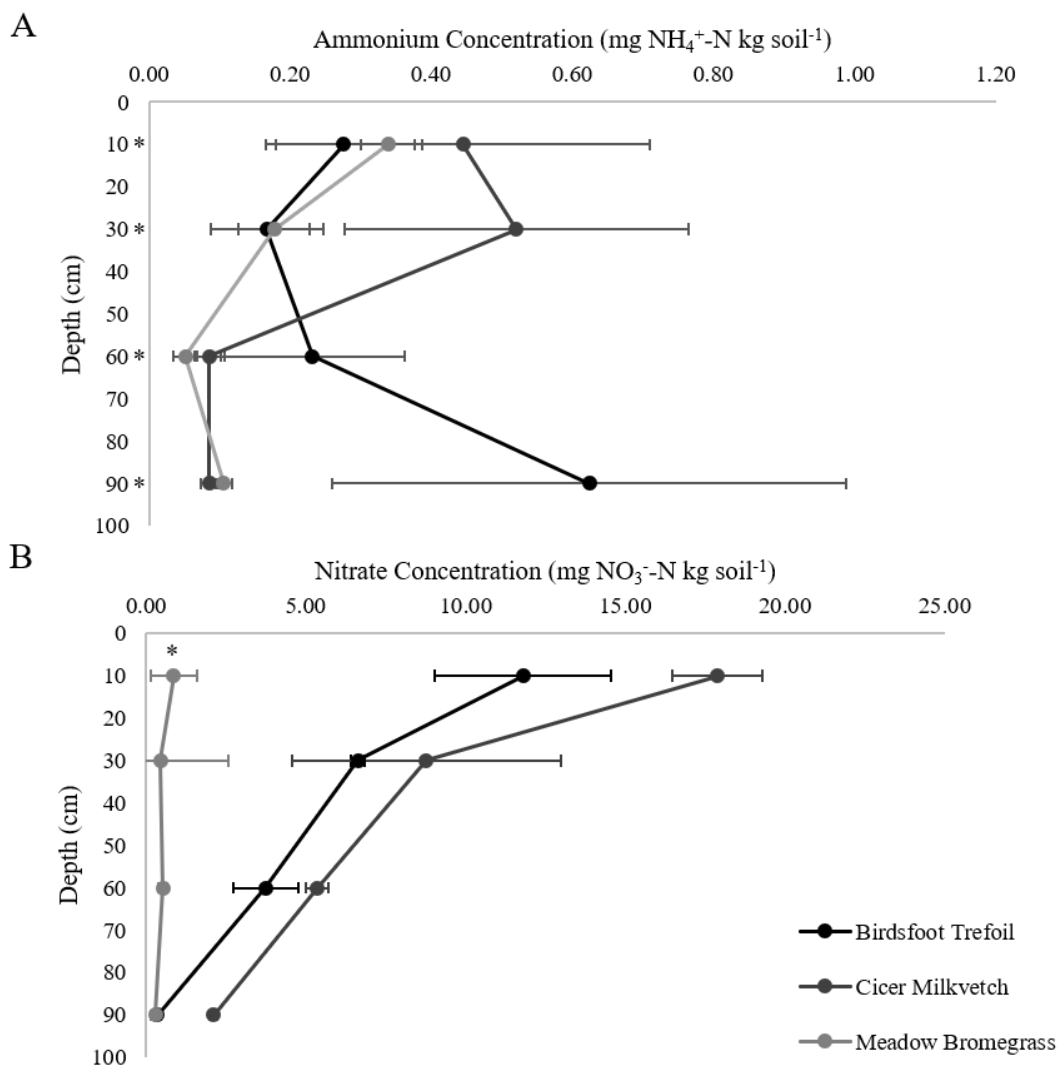


**Fig. 7.** Average soil ammonium and nitrate from soils under grazed alfalfa, birdsfoot trefoil, and sainfoin pastures in field experiment one. Asterisks (\*) denote significant differences for the interaction of treatment  $\times$  day (panel A,  $p=0.0061$ ,  $n=3$ ) and the main effect of depth (panel B,  $p<0.0001$ ,  $n=3$ ) at  $p<0.05$ . Error bars represent standard error. Soils under birdsfoot trefoil had significantly higher ammonium concentrations from 0-10 cm than alfalfa. Nitrate concentrations were higher from 0-10 cm than all other depths.

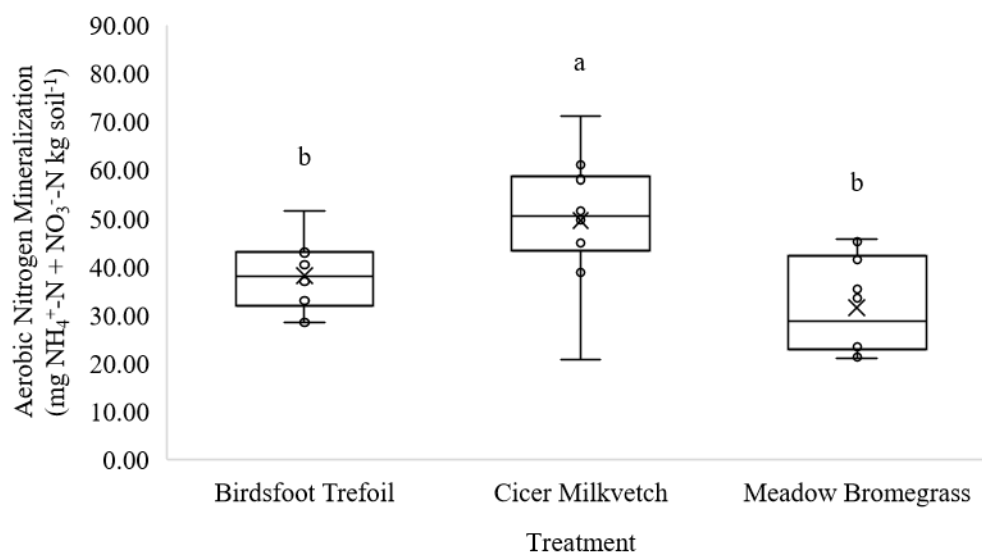




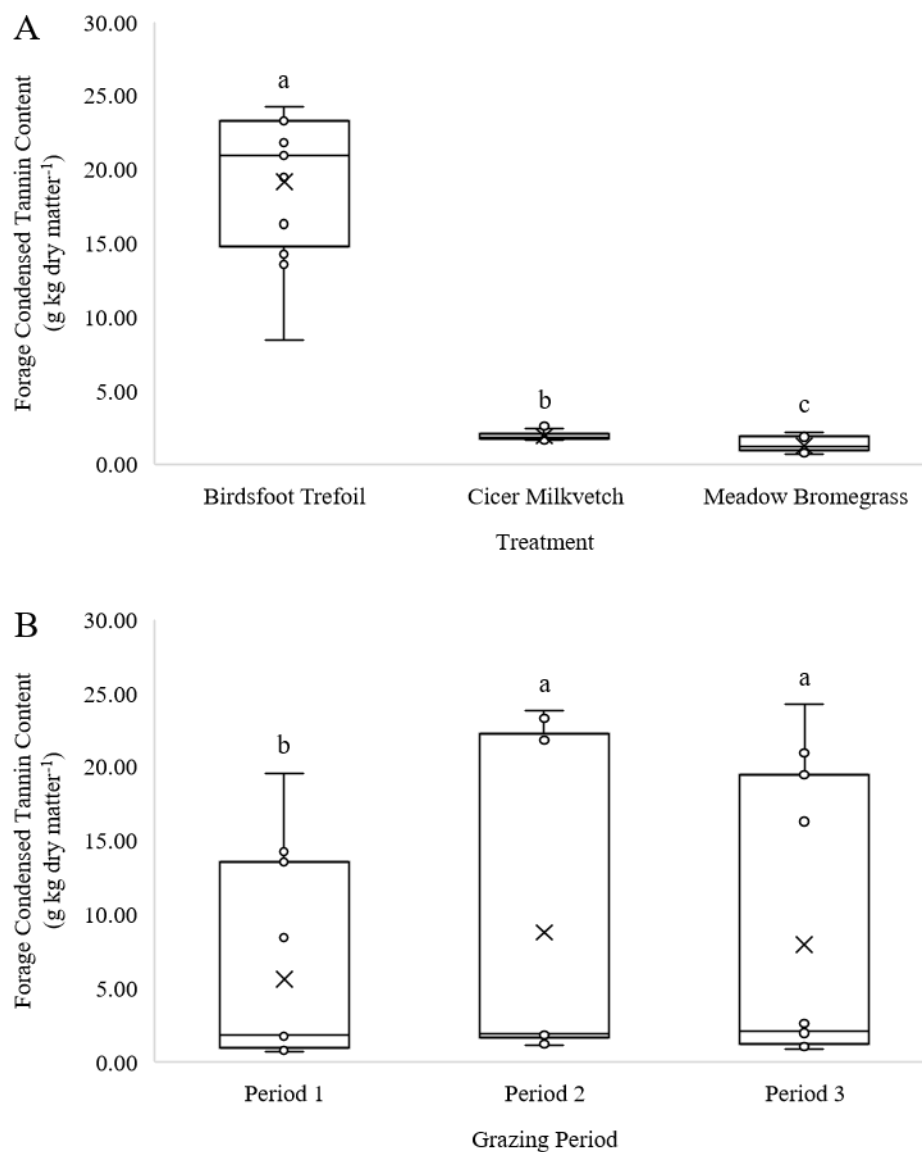
**Fig. 8.** Average soil mineralizable carbon and soil respiration rate from soils under grazed alfalfa, birdsfoot trefoil, and sainfoin pastures in field experiment one. Letters denote a significant difference for the main effect of treatment at  $p < 0.05$  in panel A ( $p = 0.0337$ ,  $n = 3$ ) and panel B ( $p = 0.0282$ ,  $n = 3$ ). Soil under birdsfoot trefoil had significantly higher mineralizable carbon and respiration rates than alfalfa, while sainfoin did not differ from either treatment.



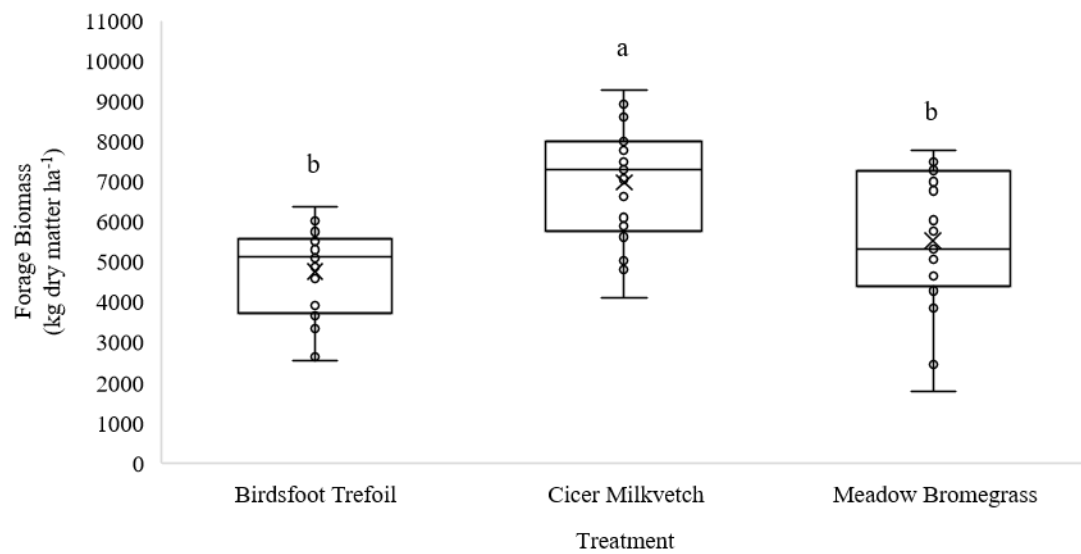
**Fig. 9.** Average soil ammonium and nitrate concentrations from soils under grazed birdsfoot trefoil, cicer milkvetch, and meadow brome grass pastures in field experiment two. Asterisks (\*) denote a significant difference for the main effect of depth in panel A ( $p=0.0030$ ,  $n=5$ ) and the interaction of treatment  $\times$  depth in panel B ( $p=0.0013$ ,  $n=5$ ) at  $p<0.05$ . Error bars represent standard error. Soil ammonium concentrations were higher from 0-10 cm than 30-60 cm. Soil nitrate concentrations were lower under meadow brome grass than birdsfoot trefoil or cicer milkvetch from 0-10, 0-30, and 30-60 cm.



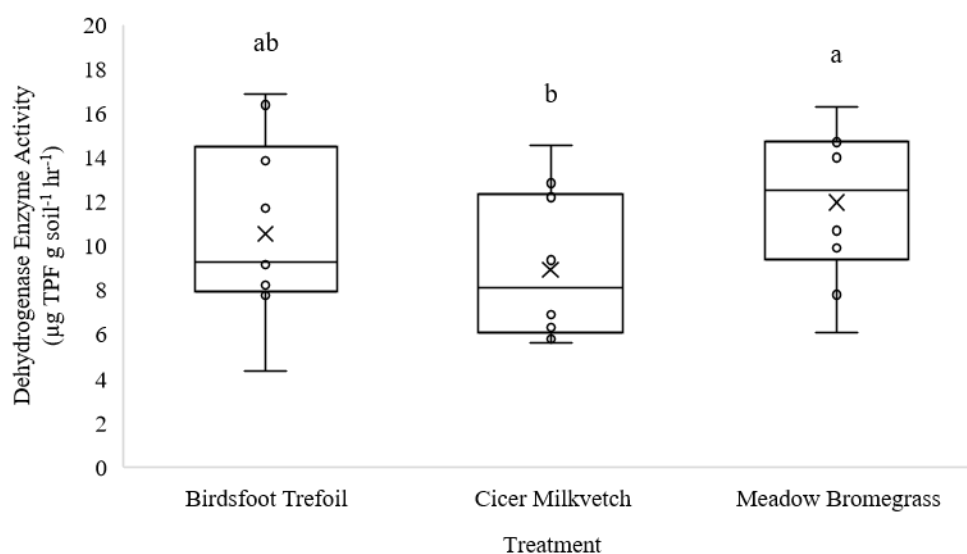
**Fig. 10.** Average aerobic soil nitrogen mineralization over a 21-day incubation from soils under grazed birdsfoot trefoil, cicer milkvetch, and meadow bromegrass pastures in field experiment two. Letters denote a significant difference at  $p < 0.05$  for the main effect of treatment ( $p = 0.0003$ ,  $n = 5$ ). Cicer milkvetch had higher average aerobic soil nitrogen mineralization than birdsfoot trefoil or meadow bromegrass.



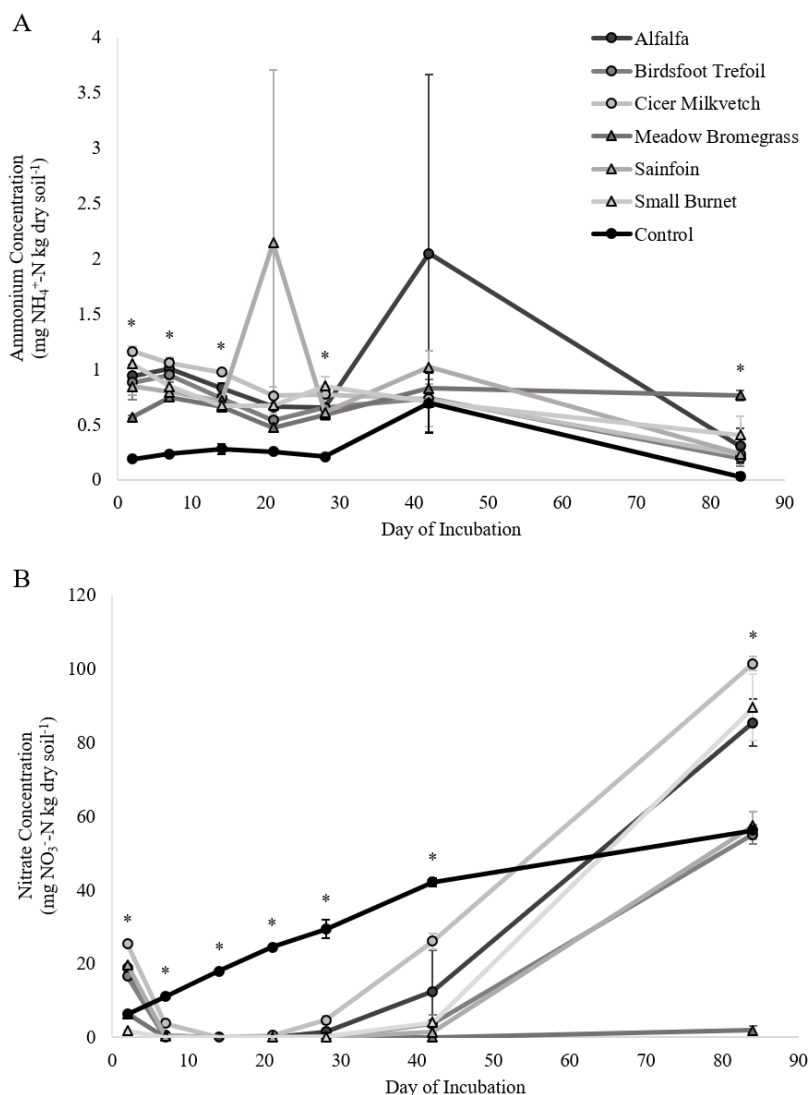
**Fig. 11.** Average forage condensed tannin content for birdsfoot trefoil, cicer milkvetch, and meadow bromegrass forages in field experiment two. Letters denote significant differences in condensed tannin content at  $p < 0.05$  for the main effects of treatment (panel A,  $p < 0.0001$ ,  $n = 3$ ) and grazing period (panel B,  $p = 0.0006$ ,  $n = 3$ ). Birdsfoot trefoil had a greater average condensed tannin content than cicer milkvetch or meadow bromegrass, and cicer milkvetch had a greater average condensed tannin content than meadow bromegrass. Average forage condensed tannin content was greater in grazing periods 2 and 3 than period 1.



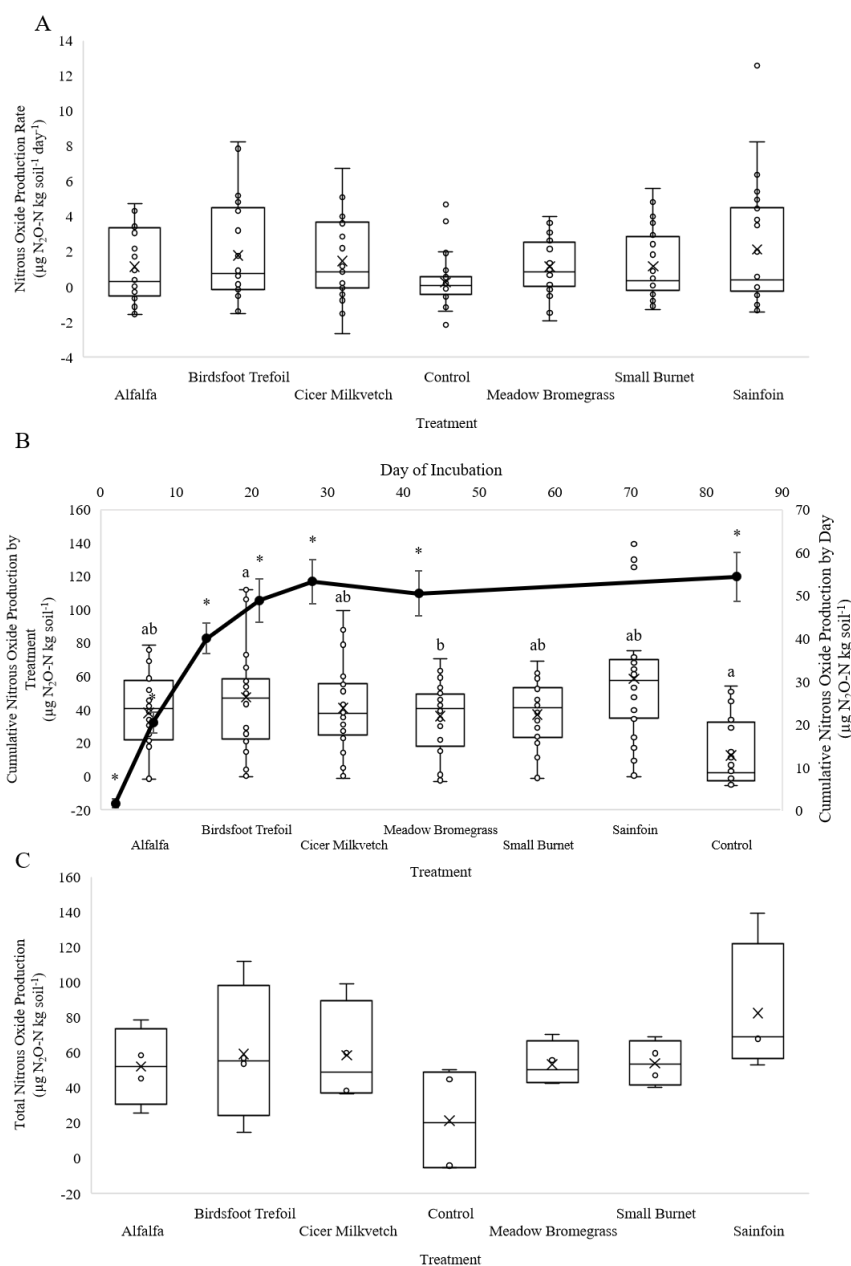
**Fig. 12.** Average biomass of birdsfoot trefoil, cicer milkvetch, and meadow bromegrass in field experiment two. Letters denote a significant difference at  $p < 0.05$  for the main effect of treatment ( $p = 0.0002$ ,  $n = 5$ ). Cicer milkvetch had greater biomass than birdsfoot trefoil or meadow bromegrass.



**Fig. 13.** Average dehydrogenase enzyme activity from soils under grazed birdsfoot trefoil, cicer milkvetch, and meadow bromegrass pastures in field experiment two. Letters denote a significant difference at  $p < 0.05$  for the main effect of treatment ( $p = 0.0058$ ,  $n = 5$ ). Meadow bromegrass had higher dehydrogenase enzyme activity than cicer milkvetch.

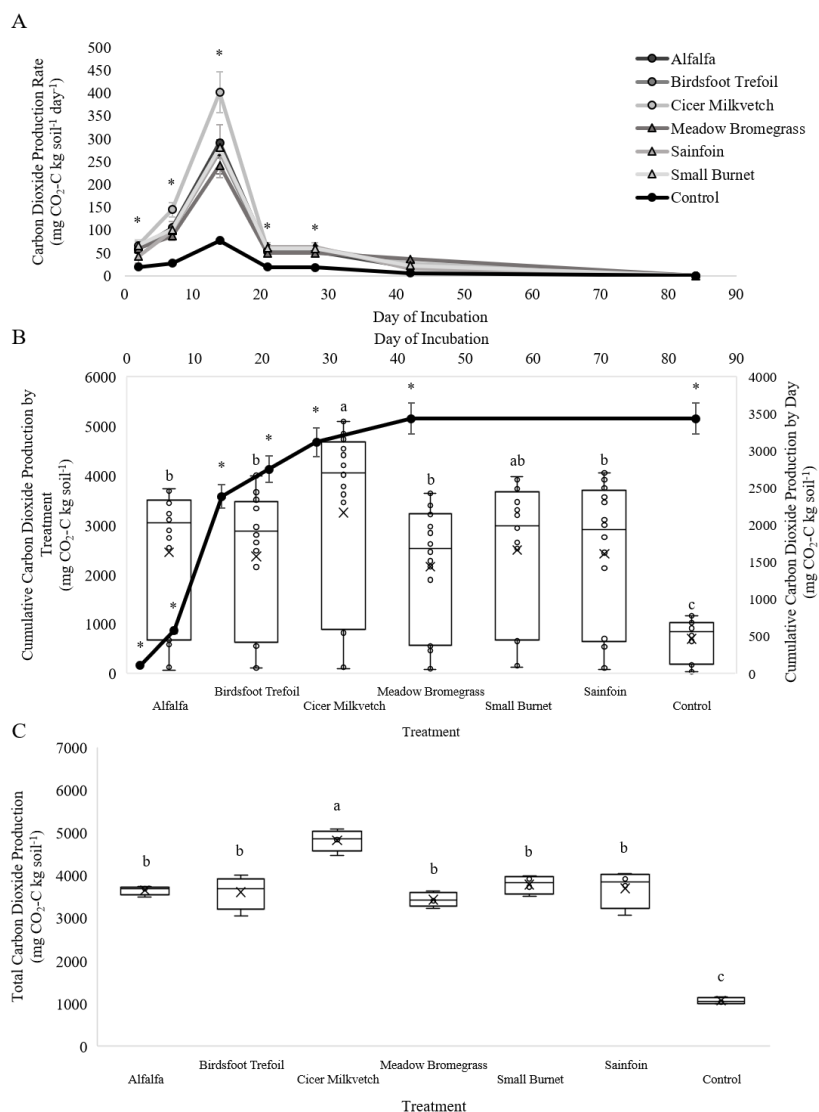


**Fig. 14.** Average soil ammonium and nitrate concentrations of soils incubated with alfalfa-, birdsfoot trefoil-, cicer milkvetch-, meadow bromegrass-, small burnet-, or sainfoin-containing feces, or an unamended soil control. Asterisks (\*) denote significant differences for the interaction of treatment × day in panels A ( $p < 0.0001$ ,  $n = 3$ ) and B ( $p < 0.0001$ ,  $n = 3$ ) at  $p < 0.05$ . Error bars represent standard error. Values for ammonium and nitrate concentration for day 0 can be found in Table 6 and have been removed to show increased detail. The soil control initially had higher ammonium concentrations than the rest of the treatments on day 0 (not shown), but the differences disappeared between days 7 and 28. The meadow bromegrass treatment had higher ammonium concentrations than the soil control by the end of the incubation. Nitrate concentration followed a pattern of immobilization and mineralization for fecal treatments, while nitrate in the soil control steadily increased. By the end of the incubation, the meadow bromegrass treatment had lower nitrate concentrations than the rest of the treatments.

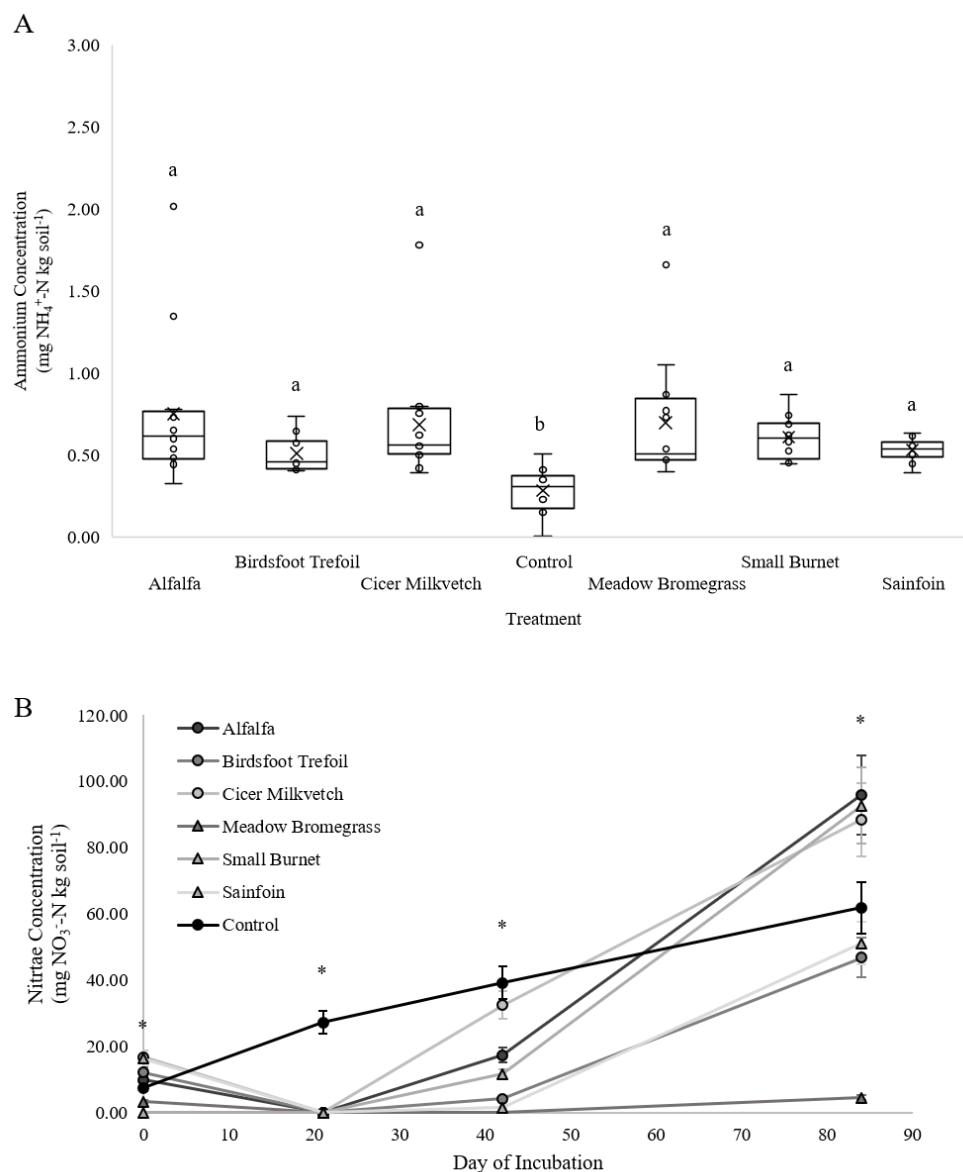


**Fig. 15.** Average nitrous oxide production rate, cumulative nitrous oxide production, and total nitrous oxide production of soils incubated with alfalfa-, birdsfoot trefoil-, cicer milkvetch-, meadow bromegrass-, small burnet-, or sainfoin-containing feces, or an unamended soil control. Letters denote a significant difference for the main effect of treatment for nitrous oxide production rate in panel A ( $p=0.9836$ ,  $n=4$ ), cumulative nitrous oxide production in panel B ( $p=0.0153$ ,  $n=4$ ), and total nitrous oxide production in panel C ( $p=0.2017$ ,  $n=4$ ), with cumulative nitrous oxide production by day overlaid in panel B ( $p<0.0001$ ). The soil control had lower cumulative nitrous oxide production than the birdsfoot trefoil and sainfoin treatments. Cumulative nitrous oxide production was highest on day 84 (equal to days 28, 21, and 14), followed by day 42 (equal to days 28, 21, and 14), day 7, and day 2.

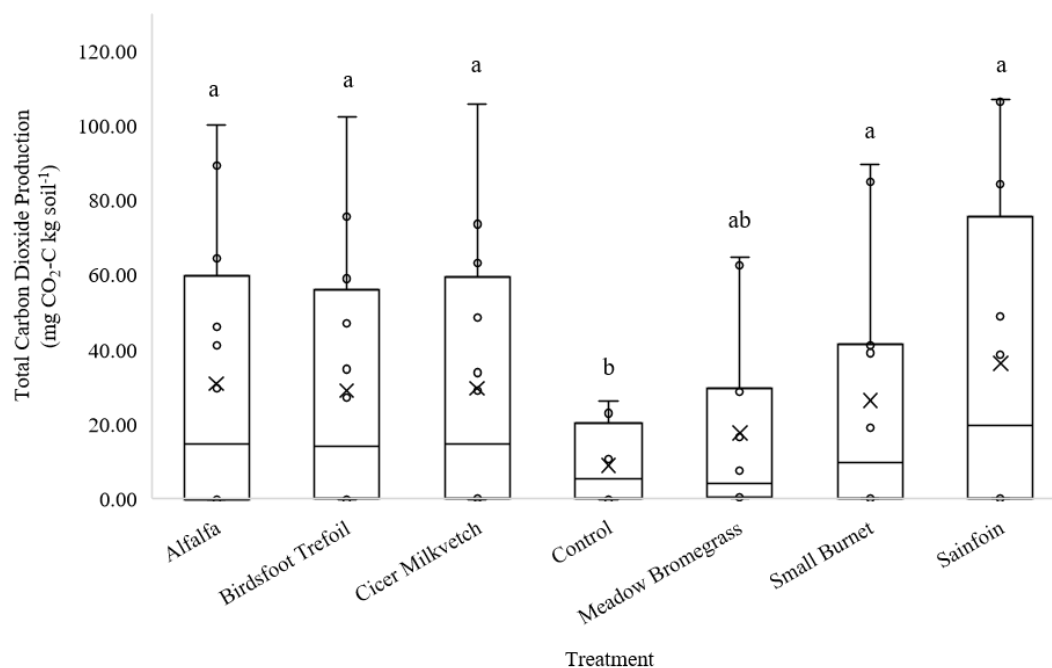




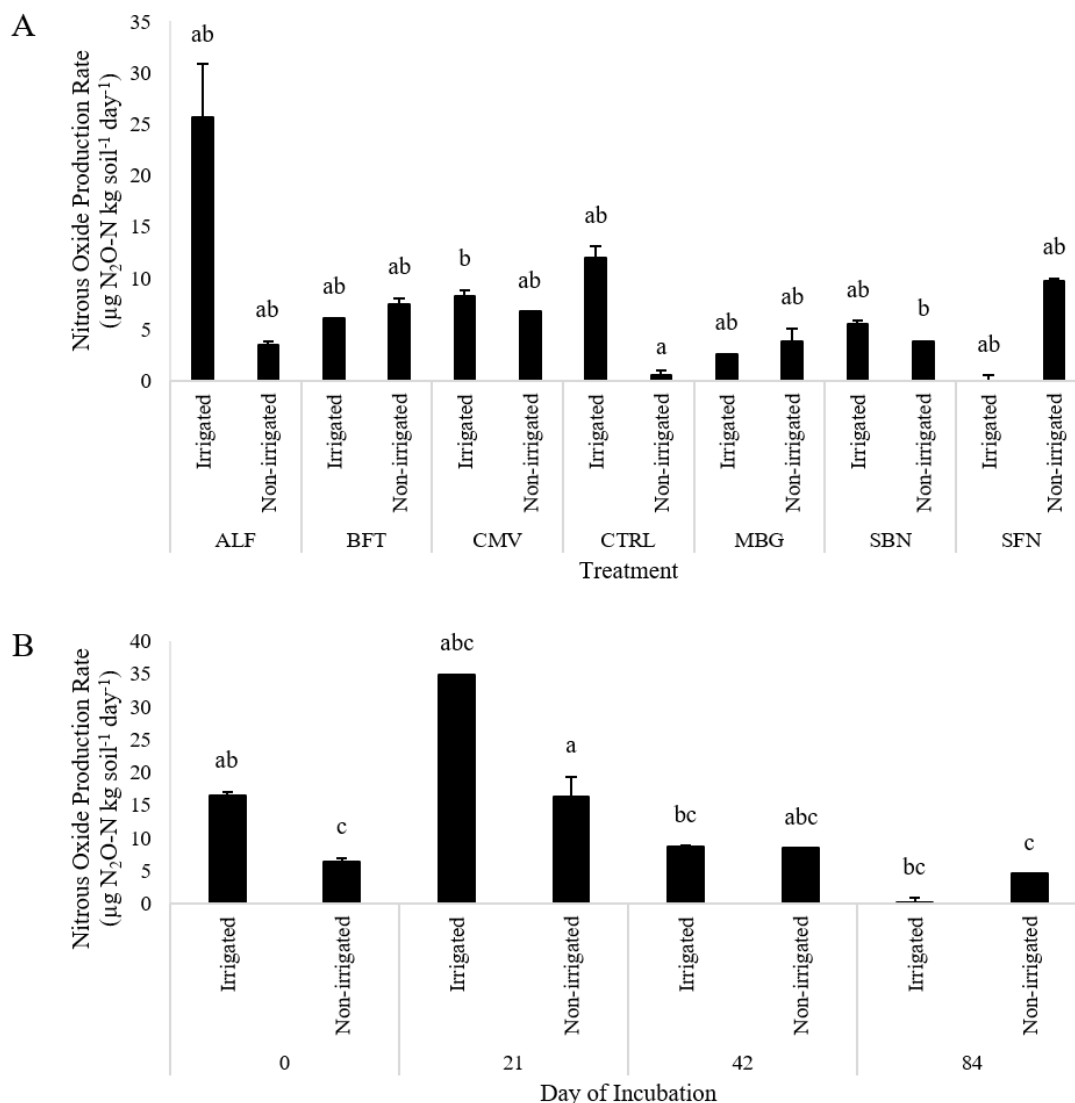
**Fig. 16.** Average carbon dioxide production rate, cumulative carbon dioxide production, and total carbon dioxide production of soils incubated with alfalfa-, birdsfoot trefoil-, cicer milkvetch-, meadow bromegrass-, small burnet-, or sainfoin-containing feces, or an unamended soil control. Letters or asterisks (\*) denote a significant difference for the main effect of treatment for cumulative carbon dioxide production in panel B ( $p < 0.0001$ ,  $n = 4$ ) and total carbon dioxide production in panel C ( $p < 0.0001$ ,  $n = 4$ ) with cumulative carbon dioxide production by day overlaid in panel B, and the interaction of treatment  $\times$  day for carbon dioxide production rate in panel A ( $p < 0.0001$ ,  $n = 4$ ) at  $p < 0.05$ . Error bars in panel A represent standard error. The soil control had lower carbon dioxide production rates than the fecal treatments until day 28 of the incubation, and lower cumulative carbon dioxide production than the fecal treatments. Cumulative production was higher on each consecutive day until days 42 and 84 when production did not differ. Cicer milkvetch had higher total carbon dioxide production than all other treatments, and higher cumulative carbon dioxide production than all treatments besides small burnet.



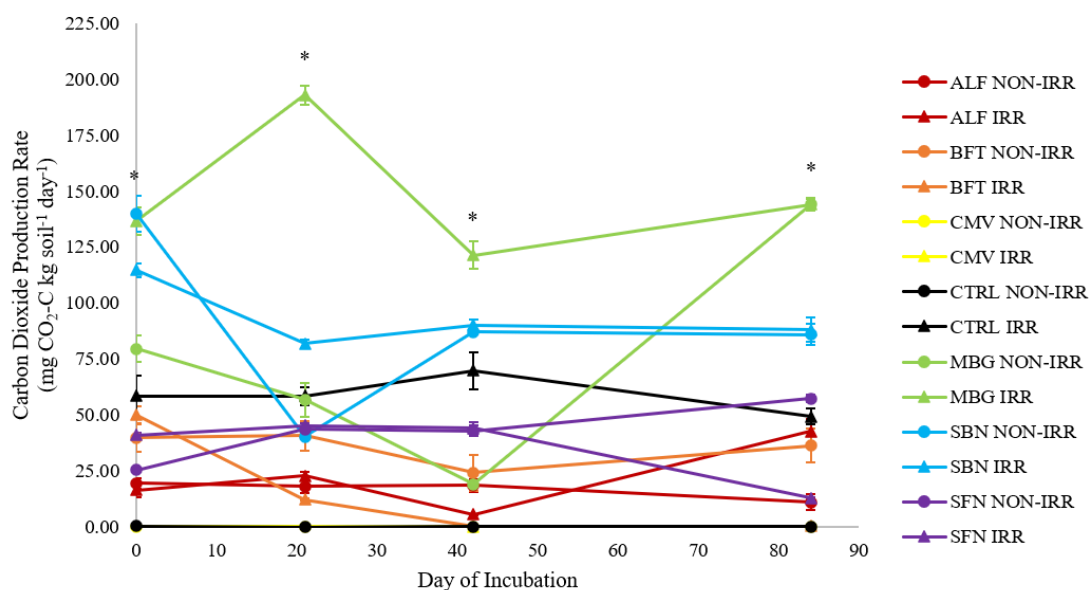
**Fig. 17.** Average soil ammonium concentrations over 84 days and average soil nitrate concentrations by day in irrigated soils incubated with alfalfa-, birdsfoot trefoil-, cicer milkvetch-, meadow bromegrass-, small burnet-, or sainfoin-containing feces, or a soil control. Letters or asterisks (\*) denote a significant difference for the main effect of treatment for soil ammonium concentration (panel A,  $p < 0.0001$ ,  $n = 3$ ) or differences among treatments on each day for the treatment  $\times$  day interaction for soil nitrate concentration (panel B,  $p < 0.0001$ ,  $n = 3$ ). Error bars in panel B represent standard error. The soil control had lower ammonium concentrations than the fecal treatments. Soil nitrate concentrations followed patterns of N immobilization and mineralization. Substrate C:N ratio appeared to be the primary influence soil N cycling, with evidence of increased N immobilization from secondary compounds.



**Fig. 18.** Total carbon dioxide production from irrigated soils incubated with alfalfa-, birdsfoot trefoil-, cicer milkvetch-, meadow brome grass-, small burnet-, or sainfoin-containing feces, or an unamended soil control. Letters denote significant differences among treatments for the main effect of treatment over 84 days ( $p < 0.0001$ ,  $n = 3$ ). The soil control had lower total carbon dioxide production than all treatments except for meadow brome grass.



**Fig. 19.** Nitrous oxide production rates from irrigated and non-irrigated soils incubated with alfalfa (ALF)-, birdsfoot trefoil (BFT)-, cicer milkvetch (CMV)-, meadow brome grass (MBG)-, small burnet (SBN)-, or sainfoin (SFN)-containing feces, or an unamended soil control (CTRL). Letters denote a significant difference among samples for the treatment  $\times$  irrigation interaction in panel A ( $p=0.0033$ ) and the day  $\times$  irrigation interaction in panel B ( $p<0.0001$ ). ( $n=3$  for irrigated samples,  $n=4$  for non-irrigated samples.) Error bars represent standard error.



**Fig. 20.** Carbon dioxide production rates from irrigated and non-irrigated soils incubated with alfalfa (ALF)-, birdsfoot trefoil (BFT)-, cicer milkvetch (CMV)-, meadow brome grass (MBG)-, small burnet (SBN)-, or sainfoin (SFN)-containing feces, or an unamended soil control (CTRL). Asterisks (\*) denote a significant difference on each day for the treatment × irrigation × day interaction ( $p=0.0309$ ,  $n=3$  for irrigated samples,  $n=4$  for non-irrigated samples). Error bars represent standard error. Non-irrigated fecal treatments generally had higher CO<sub>2</sub> production rates than the non-irrigated control while any clear trends were masked in irrigated samples.

**Table 1**

Field experiment one Pearson correlations. Asterisks (\*) denote a significant difference at  $p < 0.05$ .

Parameters		Pearson Correlation Coefficient	p-values
Potential Aerobic N Mineralization	Forage Biomass	0.56248	0.1149
Potential Aerobic N Mineralization	Forage Condensed Tannin Content	-0.73165	0.0251*

**Table 2**

Field experiment one total soil N treatment  $\times$  depth interaction. Averages are calculated after the removal of outliers and assuming that negative values are equal to zero with back-transformed standard errors. Letters denote a significant difference at  $p < 0.05$  across all depths ( $n=3$ ).

Parameter	Depth (cm)								
	0-30			30-60			60-90		
	ALF	BFT	SFN	ALF	BFT	SFN	ALF	BFT	SFN
Total N (mg N cm <sup>3</sup> soil <sup>-1</sup> )	0.867 $\pm$ 0.146a	0.325 $\pm$ 0.111abc	0.641 $\pm$ 0.088ab	0.214 $\pm$ 0.105bc	0.424 $\pm$ 0.138abc	0.501 $\pm$ 0.090abc	0.121 $\pm$ 0.129bc	0.141 $\pm$ 0.118bc	0.012 $\pm$ 0.080c
<b>p-value</b>									
Total N	0.0314*								

**Table 3**

Field experiment one soil characteristics by treatment. Averages are calculated with standard errors after the removal of outliers and assuming that negative values are equal to zero. Letters denote a significant difference at  $p < 0.05$  ( $n=3$ ).

Parameter	Treatment		
	ALF	BFT	SFN
Dehydrogenase Enzyme Activity ( $\mu\text{g TPF g soil}^{-1} \text{ hr}^{-1}$ )	$8.35 \pm 0.53$	$9.48 \pm 0.62$	$9.53 \pm 0.63$
Microbial Biomass ( $\mu\text{g C}_{\text{mic}} \text{ g soil}^{-1}$ )	$132.32 \pm 8.36$	$147.88 \pm 5.82$	$133.62 \pm 4.29$
Metabolic Quotient ( $\mu\text{g CO}_2\text{-C } \mu\text{g C}_{\text{mic}}^* \text{ hr}^{-1}$ )	$0.008 \pm 0.002$	$0.010 \pm 0.001$	$0.010 \pm 0.000$
pH	$8.22 \pm 0.04$	$8.06 \pm 0.04$	$8.20 \pm 0.08$
EC ( $\mu\text{S cm}^{-1}$ )	$225.5 \pm 39.3$	$267.0 \pm 28.3$	$239.5 \pm 37.4$
CEC ( $\text{cmol kg}^{-1}$ )	$11.02 \pm 0.68$	$11.54 \pm 0.39$	$11.92 \pm 0.62$
Zn ( $\text{mg kg}^{-1}$ )	$1.06 \pm 0.08$	$1.34 \pm 0.29$	$1.04 \pm 0.08$
Fe ( $\text{mg kg}^{-1}$ )	$8.52 \pm 0.84$	$8.48 \pm 0.60$	$9.09 \pm 0.39$
Cu ( $\text{mg kg}^{-1}$ )	$1.14 \pm 0.07$	$1.05 \pm 0.02$	$1.08 \pm 0.02$
Mn ( $\text{mg kg}^{-1}$ )	$16.21 \pm 0.76$	$15.91 \pm 0.67$	$15.84 \pm 0.90$
Olsen P ( $\text{mg kg}^{-1}$ )	$6.28 \pm 2.80$	$4.78 \pm 1.58$	$7.75 \pm 1.65$
Olsen K ( $\text{mg kg}^{-1}$ )	$163 \pm 12$	$203 \pm 72$	$226 \pm 63$
<b>p-values</b>			
Dehydrogenase Enzyme Activity	0.2569		
Microbial Biomass C	0.2042		
Metabolic Quotient	0.1023		
pH	0.072		
EC	0.6342		
CEC	0.1146		
Zn	0.4606		
Fe	0.7568		
Cu	0.4337		
Mn	0.9306		
Olsen P	0.1041		
Olsen K	0.4984		



**Table 4**

Field experiment one soil characteristics by treatment and depth with standard errors. Letters denote a significant difference at  $p < 0.05$  ( $n=3$ ).

Parameter	Treatment			Depth (cm)			
	ALF	BFT	SFN	0-10	0-30	30-60	60-90
Soil Water Content (%)	8.43 $\pm$ 0.90	8.66 $\pm$ 0.96	9.40 $\pm$ 1.71	6.57 $\pm$ 0.25a	7.20 $\pm$ 0.07ab	9.90 $\pm$ 0.59bc	11.65 $\pm$ 0.87c
Bulk Density (g cm <sup>3</sup> <sup>-1</sup> )	1.27 $\pm$ 0.01	1.26 $\pm$ 0.01	1.26 $\pm$ 0.01	N/A	1.25 $\pm$ 0.01	1.27 $\pm$ 0.01	1.27 $\pm$ 0.02
Soil Porosity (%)	52.13 $\pm$ 0.45	52.53 $\pm$ 0.52	52.28 $\pm$ 0.46	N/A	52.86 $\pm$ 0.43	52.02 $\pm$ 0.37	52.06 $\pm$ 0.59
Sand (%)	71.86 $\pm$ 1.49	74.35 $\pm$ 0.98	73.01 $\pm$ 1.47	N/A	71.96 $\pm$ 0.67	73.54 $\pm$ 1.94	73.73 $\pm$ 1.14
Silt (%)	12.70 $\pm$ 1.02	12.17 $\pm$ 0.92	12.21 $\pm$ 1.32	N/A	16.00 $\pm$ 0.38a	11.39 $\pm$ 0.60b	9.70 $\pm$ 0.71c
Clay (%)	15.44 $\pm$ 1.35	13.47 $\pm$ 0.83	14.78 $\pm$ 1.07	N/A	12.05 $\pm$ 0.36b	15.08 $\pm$ 1.43a	16.57 $\pm$ 0.62a
Total Organic C (mg organic C cm <sup>3</sup> <sup>-1</sup> )	4.71 $\pm$ 1.39	5.80 $\pm$ 2.31	4.85 $\pm$ 1.73	N/A	11.78 $\pm$ 0.78a	2.03 $\pm$ 0.44b	0.091 $\pm$ 0.56b
<b>p-values</b>	<b>Treatment</b>			<b>Depth</b>			
Soil Water Content	0.5324			0.0001*			
Bulk Density	0.7607			0.4062			
Soil Porosity	0.8085			0.3977			
Sand	0.1483			0.2981			
Silt	0.529			<0.0001*			
Clay	0.1854			0.0016*			
Total Organic C	0.711			<0.0001*			

**Table 5**

Field experiment two Pearson correlations. Asterisks (\*) denote a significant difference at  $p < 0.05$ .

Parameters		Pearson Correlation Coefficient	p-values
Potential Aerobic N Mineralization	Forage Biomass	0.25818	0.1683
Potential Aerobic N Mineralization	Forage Condensed Tannin Content	0.08555	0.7618

**Table 6**

Field experiment two soil characteristics by treatment and depth. Averages are calculated with back-transformed standard errors after the removal of outliers. Letters denote a significant difference at  $p < 0.05$  ( $n=5$ ).

Parameter	Treatment			Depth (cm)			
	BFT	CMV	MBG	0-10	0-30	30-60	60-90
Soil Water Content (%)	14.73 $\pm$ 0.41b	16.17 $\pm$ 0.35a	15.86 $\pm$ 0.43ab	14.51 $\pm$ 0.37b	14.90 $\pm$ 0.44b	16.43 $\pm$ 0.40a	17.42 $\pm$ 0.52a
Bulk Density (g cm <sup>3</sup> <sup>-1</sup> )	1.55 $\pm$ 0.01	1.53 $\pm$ 0.01	1.54 $\pm$ 0.01	N/A	1.49 $\pm$ 0.01b	1.58 $\pm$ 0.01a	1.56 $\pm$ 0.01a
Soil Porosity (%)	41.69 $\pm$ 0.39	42.11 $\pm$ 0.51	41.82 $\pm$ 0.52	N/A	43.81 $\pm$ 0.42a	40.53 $\pm$ 0.40b	41.29 $\pm$ 0.37b
Sand (%)	66.6 $\pm$ 1.7	65.7 $\pm$ 1.0	64.1 $\pm$ 1.9	N/A	65.1 $\pm$ 1.1	65.8 $\pm$ 1.5	N/A
Silt (%)	18.2 $\pm$ 1.3	18.0 $\pm$ 1.2	21.2 $\pm$ 2.1	N/A	20.5 $\pm$ 0.8	17.8 $\pm$ 1.6	N/A
Clay (%)	15.2 $\pm$ 1.2	16.4 $\pm$ 1.3	14.7 $\pm$ 0.5	N/A	14.4 $\pm$ 0.3	16.4 $\pm$ 1.1	N/A
Total Organic C (mg organic C cm <sup>3</sup> <sup>-1</sup> )	8.34 $\pm$ 1.13	8.83 $\pm$ 1.16	9.24 $\pm$ 1.24	N/A	14.97 $\pm$ 0.48a	5.47 $\pm$ 0.64b	3.54 $\pm$ 0.28b
Total N (mg N cm <sup>3</sup> <sup>-1</sup> )	0.76 $\pm$ 0.11	0.74 $\pm$ 0.10	0.60 $\pm$ 0.12	N/A	0.96 $\pm$ 0.09a	0.53 $\pm$ 0.07b	0.53 $\pm$ 0.13b
<b>p-values</b>	Treatment			Depth			
Soil Water Content	0.0261*			<0.001*			
Bulk Density	0.6577			0.0008*			
Soil Porosity	0.7119			0.0007*			
Sand	0.2901			0.6212			
Silt	0.2051			0.1068			
Clay	0.4031			0.2328			
Total Organic C	0.7726			<0.0001*			
Total N	0.1006			<0.0001*			

**Table 7**

Field experiment two soil characteristics by treatment. Averages are calculated with back-transformed standard errors. Letters denote a significant difference at  $p < 0.05$  ( $n=5$ ).

Parameter	Treatment		
	BFT	CMV	MBG
Mineralizable Carbon ( $\mu\text{g C g soil}^{-1}$ )	$16.15 \pm 2.06$	$15.02 \pm 1.21$	$16.61 \pm 0.69$
Respiration ( $\mu\text{g C g soil}^{-1} \text{ hr}^{-1}$ )	$7.31 \pm 0.39$	$7.22 \pm 0.50$	$7.21 \pm 0.83$
Microbial Biomass ( $\mu\text{g C mic g soil}^{-1}$ )	$883.91 \pm 76.19$	$872.97 \pm 44.96$	$812.42 \pm 44.42$
Metabolic Quotient ( $\mu\text{g CO}_2\text{-C } \mu\text{g C}_{\text{mic}}^{-1} \text{ hr}^{-1}$ )	$0.008 \pm 0.000$	$0.008 \pm 0.000$	$0.009 \pm 0.001$
pH	$8.31 \pm 0.05\text{b}$	$8.40 \pm 0.03\text{b}$	$8.53 \pm 0.03\text{a}$
EC ( $\mu\text{S cm}^{-1}$ )	$200.48 \pm 14.76$	$209.80 \pm 13.08$	$184.16 \pm 11.34$
CEC ( $\text{cmol kg}^{-1}$ )	$12.28 \pm 0.28$	$12.62 \pm 0.30$	$12.76 \pm 0.29$
Zn ( $\text{mg kg}^{-1}$ )	$1.34 \pm 0.14\text{b}$	$1.46 \pm 0.20\text{ab}$	$1.79 \pm 0.14\text{a}$
Fe ( $\text{mg kg}^{-1}$ )	$7.42 \pm 0.66$	$8.00 \pm 0.57$	$7.73 \pm 0.53$
Cu ( $\text{mg kg}^{-1}$ )	$1.77 \pm 0.41$	$1.73 \pm 0.33$	$1.97 \pm 0.47$
Mn ( $\text{mg kg}^{-1}$ )	$12.58 \pm 1.21$	$12.52 \pm 1.28$	$12.33 \pm 1.37$
P ( $\text{mg kg}^{-1}$ )	$9.66 \pm 1.25$	$9.71 \pm 1.22$	$8.68 \pm 1.07$
K ( $\text{mg kg}^{-1}$ )	$192.20 \pm 24.75$	$198.30 \pm 25.68$	$209.90 \pm 27.21$
<b>p-values</b>			
Mineralizable Carbon	0.2827		
Respiration	0.9936		
Microbial Biomass	0.4808		
Metabolic Quotient	0.7783		
pH	0.0040*		
EC	0.4086		
CEC	0.2893		
Zn	0.0270*		
Fe	0.6157		
Cu	0.9771		
Mn	0.4516		
P	0.3522		
K	0.7290		

**Table 8**

Incubation study average initial soil characteristics with standard errors.

Parameter	Average
Texture	Sandy Loam
Sand Content (%)	$57 \pm 0.33$
Silt Content (%)	$27 \pm 0.33$
Clay Content (%)	$16 \pm 0.58$
pH	$7.8 \pm 0.00$
ECe (ds m <sup>-1</sup> )	$0.52 \pm 0.01$
Olsen P (mg kg <sup>-1</sup> )	$8.4 \pm 0.12$
Olsen K (mg kg <sup>-1</sup> )	$251 \pm 5.04$
Soil Organic Matter (%)	$2.3 \pm 0.09$
Sodium Adsorption Ratio	$0.58 \pm 0.05$
Total C (g kg <sup>-1</sup> )	$15.77 \pm 0.22$
Total N (g kg <sup>-1</sup> )	$1.10 \pm 0.00$

**Table 9**

Incubation study non-irrigated sample nitrogen cycling characteristics. Values are expressed as averages and as average percentages of initial feces nitrogen content combined over 84 days. Most of the measured nitrogen was in the form of mineral nitrate and ammonium, with much lower amounts in the form of nitrous oxide.

Parameter	Treatment						
	ALF	BFT	CMV	MBG	SBN	SFN	Control
Average ammonium (mg $\text{NH}_4^+$ -N)	$0.04 \pm 0.01$	$0.04 \pm 0.02$	$0.06 \pm 0.03$	$0.01 \pm 0.00$	$0.04 \pm 0.02$	$0.05 \pm 0.02$	$0.00 \pm 0.00$
Average nitrate (mg $\text{NO}_3^-$ -N)	$0.23 \pm 0.09$	$0.15 \pm 0.06$	$0.30 \pm 0.10$	$0.03 \pm 0.01$	$0.19 \pm 0.09$	$0.16 \pm 0.06$	$0.36 \pm 0.05$
Total nitrous oxide production (mg $\text{N}_2\text{O}$ -N)	$0.00078 \pm 0.00017$	$0.00089 \pm 0.00030$	$0.00088 \pm 0.00022$	$0.00080 \pm 0.00010$	$0.00081 \pm 0.00010$	$0.00124 \pm 0.00029$	$0.00032 \pm 0.00023$
Average ammonium (% Feces N)	$0.57 \pm 0.21$	$0.66 \pm 0.28$	$0.70 \pm 0.30$	$0.28 \pm 0.05$	$0.44 \pm 0.18$	$0.70 \pm 0.29$	N/A
Average nitrate (% Feces N)	$3.36 \pm 1.26$	$2.28 \pm 0.85$	$3.68 \pm 1.21$	$0.65 \pm 0.21$	$1.97 \pm 0.97$	$2.16 \pm 0.80$	N/A
Total Nitrous Oxide Production (% Feces N)	$0.011 \pm 0.002$	$0.013 \pm 0.005$	$0.011 \pm 0.003$	$0.016 \pm 0.002$	$0.009 \pm 0.001$	$0.017 \pm 0.004$	N/A
Average ammonium (% Soil+Feces N)	$0.17 \pm 0.06$	$0.19 \pm 0.08$	$0.24 \pm 0.10$	$0.07 \pm 0.01$	$0.16 \pm 0.07$	$0.22 \pm 0.09$	$0.03 \pm 0.00$
Average nitrate (% Soil+Feces N)	$1.00 \pm 0.37$	$0.66 \pm 0.25$	$1.25 \pm 0.41$	$0.15 \pm 0.05$	$0.73 \pm 0.36$	$0.68 \pm 0.25$	$2.18 \pm 0.32$
Total Nitrous Oxide Production (% Soil+Feces N)	$0.00340 \pm 0.00072$	$0.00389 \pm 0.00131$	$0.00359 \pm 0.00089$	$0.00378 \pm 0.00045$	$0.00316 \pm 0.00038$	$0.00527 \pm 0.00123$	$0.00195 \pm 0.00138$

**Table 10**

Incubation study initial soil, fecal, and combined soil and fecal treatment characteristics.

		Treatment						
		ALF	BFT	CMV	MBG	SBN	SFN	Control
Soil	<b>Weight (g)</b>	600	600	600	600	600	600	600
	<b>Total C (g)</b>	9.48	9.48	9.48	9.48	9.48	9.48	9.48
	<b>Total C (g kg<sup>-1</sup>)</b>	15.80	15.80	15.80	15.80	15.80	15.80	15.80
	<b>Total N (g)</b>	0.66	0.66	0.66	0.66	0.66	0.66	0.66
	<b>Total N (g kg<sup>-1</sup>)</b>	1.10	1.10	1.10	1.10	1.10	1.10	1.10
	<b>C:N Ratio</b>	14.36	14.36	14.36	14.36	14.36	14.36	14.36
Feces	<b>Weight (g)</b>	11.72	10.34	11.98	11.9	11.94	13.91	0
	<b>Total C (g)</b>	5.40	5.40	5.40	5.40	5.40	5.40	N/A
	<b>Total C (g kg<sup>-1</sup>)</b>	460.75	522.24	450.75	453.78	452.26	388.21	N/A
	<b>Total N (g)</b>	0.28	0.27	0.34	0.20	0.39	0.30	N/A
	<b>Total N (g kg<sup>-1</sup>)</b>	23.89	26.11	28.38	16.81	32.66	21.57	N/A
	<b>C:N Ratio</b>	19.4	20.1	16.0	26.5	14.0	17.9	N/A
	<b>Condensed Tannin (g kg<sup>-1</sup>)</b>	4.06	8.41	7.41	3.22	5.92	15.81	N/A
Soil + Feces Combined	<b>Total C (g)</b>	14.86	14.89	14.87	14.87	14.87	14.86	9.48
	<b>Total C (g kg<sup>-1</sup>)</b>	24.29	24.40	24.30	24.30	24.30	24.21	15.80
	<b>Total N (g)</b>	0.9	0.9	1.0	0.9	1.0	1.0	0.7
	<b>Total N (g kg<sup>-1</sup>)</b>	1.54	1.52	1.63	1.41	1.72	1.56	1.10
	<b>C:N Ratio</b>	16.2 : 1	16.3 : 1	15.2 : 1	17.4 : 1	14.3 : 1	15.1 : 1	14.4 : 1
	<b>Condensed Tannin (g kg<sup>-1</sup>)</b>	0.078	0.142	0.145	0.063	0.116	0.358	0.000
	<b>Day 0 NH<sub>4</sub><sup>+</sup> (mg NH<sub>4</sub><sup>+</sup>-N kg soil<sup>-1</sup>)</b>	14.79 ± 0.32	18.56 ± 0.04	25.06 ± 0.64	2.84 ± 0.11	17.19 ± 0.97	21.26 ± 0.89	0.35 ± 0.07
	<b>Day 0 NO<sub>3</sub><sup>-</sup> (mg NO<sub>3</sub><sup>-</sup>-N kg soil<sup>-1</sup>)</b>	7.36 ± 0.14	6.18 ± 0.03	7.88 ± 0.25	8.88 ± 0.16	5.57 ± 0.46	6.38 ± 0.35	4.79 ± 1.06

**Table 11**

Significant incubation experiment non-irrigated greenhouse gas Pearson correlations.  
Correlations were significant at  $p < 0.05$ .

Parameter 1	Parameter 2	Pearson Correlation Coefficient	p-values
Nitrous Oxide Production Rate	Day	-0.47545	0.0006
	Nitrate Concentration	-0.34084	0.0166
	Carbon Dioxide Production Rate	0.61012	<.0001
	Cumulative Carbon Dioxide Production	-0.30896	0.0308
	Total Nitrous Oxide Production	0.31729	0.0263
Cumulative Nitrous Oxide Production	Day	0.88491	<.0001
	Cumulative Carbon Dioxide Production	0.54718	<.0001
	Total Carbon Dioxide Production	0.41449	0.0031
	Total Nitrous Oxide Production	0.54946	<.0001
	Total Soil & Feces C	0.46029	0.0009
	Total Soil & Feces N	0.45344	0.0011
	Soil & Feces Tannin Content	0.36719	0.0095
Total Nitrous Oxide Production	Cumulative Carbon Dioxide Production	0.34393	0.0155
	Total Carbon Dioxide Production	0.74573	<.0001
	Total Soil & Feces C	0.81199	<.0001
	Total Soil & Feces N	0.85042	<.0001
	Soil & Feces Tannin Content	0.70725	<.0001
Carbon Dioxide Production Rate	Day	-0.45492	0.001
	Nitrate Concentration	-0.41443	0.0031
Cumulative Carbon Dioxide Production	Day	0.61629	<.0001
	Total Carbon Dioxide Production	0.47321	0.0006
	Total Soil & Feces C	0.42441	0.0024
	Total Soil & Feces N	0.43244	0.0019
Total Carbon Dioxide Production	Total Soil & Feces C	0.91733	<.0001
	Total Soil & Feces N	0.92095	<.0001
	Soil & Feces Tannin Content	0.47057	0.0003



**Table 12**

Incubation experiment irrigated greenhouse gas parameters by treatment and day. Averages are calculated with back-transformed standard errors (n=3).

Parameter	Treatment							Day			
	Control	ALF	BFT	CMV	MBG	SBN	SFN	0	21	42	84
CO <sub>2</sub> Production Rate (mg CO <sub>2</sub> -C kg soil <sup>-1</sup> day <sup>-1</sup> )	72.23 ± 4.02	172.16 ± 6.42	217.59 ± 7.58	215.74 ± 6.83	69.47 ± 4.90	215.31 ± 7.10	215.04 ± 7.58	49.90 ± 6.25b	572.05 ± 31.33a	77.30 ± 2.85c	0.87 ± 0.86d
N <sub>2</sub> O Production Rate (mg N <sub>2</sub> O-N kg soil <sup>-1</sup> day <sup>-1</sup> )	-403.85 ± 449.89	112.68 ± 48.84	-335.84 ± 446.66	-269.25 ± 351.83	-400.33 ± 463.40	121.78 ± 51.93	-313.03 ± 480.76	24.61 ± 4.24a	124.50 ± 42.24ab	1.30 ± 263.53a	-964.40 ± 475.53b
Total N <sub>2</sub> O Production (mg N <sub>2</sub> O-N kg soil <sup>-1</sup> )	6.00 ± 2.79	12.87 ± 2.62	3.08 ± 2.37	4.15 ± 2.28	1.30 ± 1.74	2.78 ± 2.30	-0.09 ± 1.82	4.74 ± 2.08a	9.98 ± 2.15a	2.45 ± 1.63ab	0.02 ± 1.14b
<b>p-values</b>	Treatment							Day			
CO <sub>2</sub> Production Rate	0.2514							<0.0001*			
N <sub>2</sub> O Production Rate	0.7442							0.0011*			
Total N <sub>2</sub> O Production	0.4552							0.0058*			

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CHAPTER III.  
EFFECT OF POLYPHENOLIC TYPE AND DOSE ON SOIL N-CYCLING  
DYNAMICS

### **1. Introduction**

Tannin-containing legumes have recently been proposed as an economically and environmentally viable alternative forage for grass-fed beef production systems. Replacing grass with legumes may increase cattle average daily gains (ADGs) by increasing forage quality and soil N retention while decreasing greenhouse gas (GHG) emissions. Tannins and other secondary plant compounds have been documented to alter soil organic matter (SOM) accumulation, decomposition, and carbon (C) and nitrogen (N) mineralization processes in the soil by complexing with organic materials and inhibiting microbial function (Halvorson et al., 2012; Kraus et al., 2003; Smolander et al., 2012). In recent field studies comparing the effect of tannin- and non-tannin-containing forages on pasture soil C and N cycling, forages containing saponins produced similar results to tannin-containing treatments (Clemensen, 2018). This suggests that saponins may be able to alter soil C and N cycling in similar ways to tannins naturally occurring in legume forages. If saponins are able to reduce C and N mineralization in the soil, it will have significant implications for managing pasture systems and reducing N losses and GHG emissions.

Tannins are a class of heterogeneous polyphenolic compounds produced by some plant species. Tannins are classified as being either condensed (comprised of flavan-3-ols with C-C bonds), or hydrolyzable (comprised of sugars and gallic or ellagic acids) (Kraus



et al., 2003; Nierop et al., 2006a). Tannins enter the soil in plant residue, leachate, or feces, where they can be converted to humic substances, chelated, leached as dissolved organic C (DOC), or adsorbed to soil particles (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003). In the soil, tannins are capable of altering C and N cycling. Tannins may form insoluble complexes with or chemically react with SOM. This chemical protection may inhibit SOM decomposition and C mineralization (Halvorson et al., 2012; Kraus et al., 2003; Smolander et al., 2012). However, tannins contain C and can also act as a substrate for microorganisms which may stimulate temporary N immobilization (Kraus et al., 2003; Smolander et al., 2012). Independent of changes in C cycling, tannins can form protein complexes and reduce the mineral N pool. This complexation has been linked to an increase in the ratio of DON to mineral N, diverting N away from the mineralization process (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003; Smolander et al., 2012). While the literature generally agrees that phenolics tend to decrease N mineralization, results regarding their effect on nitrification is mixed (Kraus et al., 2003; Nierop et al., 2006a; Smolander et al., 2012). It is unclear if decreases in nitrification rates are due to indirect effects of reduced mineral N pools, or the direct inhibition of nitrifying bacteria (Adamczyk et al., 2013; McCarty and Bremner, 1986). These processes are likely affected by other factors such as soil type, litter quality, organic matter composition, tannin concentration, and tannin structure (Adamczyk et al., 2012, 2013; Halvorson et al., 2016, 2012, 2009; Nierop et al., 2006a, 2006b; Smolander et al., 2012). Tannins may directly influence microbial function via cell membrane interference, enzyme complexation, and limiting metal availability (Adamczyk et al., 2013; Alberto et al., 2001; Kraus et al., 2003; McDonald et al., 1996; Mila et al., 1996;

Smolander et al., 2012; Ultee et al., 2002).

Saponins are a heterogeneous class of secondary plant metabolites defined by glycosylated steroids, steroidal alkaloids, and triterpenes (Haralampidis et al., 2002). Alfalfa specifically contains triterpene glycoside saponins (Lu and Jorgensen, 1987). When saponins enter the soil, they may be mineralized to CO<sub>2</sub>, adsorb to humic acids, or be assimilated into microbial biomass (Okumura et al., 1999). Certain compounds found in alfalfa saponins such as medicagenic acid have been observed to inhibit enzyme activity and cause cell death in certain rhizosphere bacteria strains (Hoagland et al., 2001). Saponins have also been documented to affect soil N cycling. In peat, alfalfa saponins have stimulated N immobilization and denitrification processes, and inhibited proteolysis, ammonification, and N mineralization (Levanon et al., 1982). While the first four processes have been attributed to the sugar structure found in saponins, changes in N mineralization have been attributed to fungal community inhibition.

While tannins have been studied extensively in forest systems, few studies have examined the influence of tannins found in forage species. In a study that examined the effects of tannin- and non-tannin-containing dung, Crush (1993) found that dung from cattle fed big trefoil (*Lotus pedunculatus*) vs. white clover (*Trifolium repens*) forage had decreased nitrification rates in a pasture soil. However, a subsequent study found that this effect may disappear over time as the soil microbial community adapts to the tannin-containing species (Crush and Keogh, 1998). Few studies have focused on the effects of tannins and saponins on soil nutrient cycling in pastures. In order to understand the effects of tannins and saponins on pasture nutrient cycling, it is necessary to understand how the type and dose of purified phenolics from forage species of interest affect soil C

and N cycling in a controlled setting. Tannins and saponins occur in forages in much lower doses than in forest species. Furthermore, the structure, and therefore the effect, of secondary compounds varies among species. As a consequence, it is crucial to use compounds from my species of interest to understand their effects on a pasture soil. If tannins can inhibit N mineralization in pasture soil, it could increase soil N retention and decrease GHG emissions.

In order to address this knowledge gap, I performed an 84-day soil incubation with purified condensed tannins from birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*), and saponins from alfalfa (*Medicago sativa*). The objective of this study was to quantify the effect of phenolic type and concentration against an unamended control on measures of C and N cycling in a uniform soil in a controlled incubation setting. I hypothesized that all phenolic treatments would decrease inorganic N concentrations, N<sub>2</sub>O production, and autoclaved citrate extractable protein (ACEP) compared to the control, with high concentrations having the greatest effect.

## **2. Materials and methods**

### **2.1. Soil and amendment preparation**

Condensed tannins isolated from birdsfoot trefoil and sainfoin leaves using the LH-20 Sephadex method of Hagerman (2011) and saponins isolated from alfalfa leaves according the method of Lee et al. (2001) were incubated with a uniform soil for 84 days. Soil samples were collected from 0-15 cm from grass alleyways at the Utah Agricultural Experiment Field Station in Lewiston, Utah (41°57'4" N, 111°52'26" W) in June of 2018 and consisted of Lewiston fine sandy loam (coarse-loamy, mixed, superactive, mesic

Aquic Calcixeroll). Samples were homogenized and sieved to 2 mm.

## 2.2. Incubation setup

Polyphenolic compounds were added to the soil on a  $\text{mg g}^{-1}$  dry soil basis.

Tannins from BFT and SFN were added in two concentrations: low (3  $\text{mg/g}$  soil or 0.3% by dry weight) and high (15  $\text{mg/g}$  soil or 1.5% by dry weight), while saponins from ALF were only added at a low (3  $\text{mg/g}$  soil or 0.3% by dry weight) dose as they only naturally occur in low concentrations. A soil control treatment was included with no added compounds. These concentrations were based on the concentrations of tannins used in similar soil incubation studies, which have ranged from 0-20  $\text{mg tannin g soil}^{-1}$  (Adamczyk et al., 2013; Halvorson et al., 2016, 2012, 2009; Halvorson and Gonzalez, 2008). The final CT percentages of the amended soils in this study were lower than what is typically found in forage biomass (1-4% in BFT and 3-8% in SFN). However, the final CT percentages of these amended soils were approximately equal to the percentage of CTs found in the feces of cows fed BFT (0.841%) or SFN hays (1.581%). The final CT percentages of these amended soils were higher than the final %CT of the feces amended soils (0.014% for feces with BFT and 0.036% for feces with SFN) used in chapter II.

Amendment total C and N were analyzed using an Elementar Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to an Isoprime VisION isotope-ratio mass spectrometer (IRMS) (Elementar UK Ltd, Cheadle, UK). Samples were combusted at  $1000^{\circ}\text{C}$  in a reactor packed with chromium oxide and silvered copper oxide. Samples were then reduced in a reduction reactor (reduced copper at  $650^{\circ}\text{C}$ ). The helium carrier then flowed through a water trap

(magnesium perchlorate and phosphorous pentoxide) Carbon dioxide was retained on an adsorption trap until the N<sub>2</sub> peak was analyzed. The adsorption trap was then heated to release the CO<sub>2</sub> to the IRMS (Table 13).

Five grams of oven dry equivalent soil was weighed into 40 mL borosilicate glass vials with rubber septa caps. Each polyphenolic treatment was dissolved in distilled de-ionized (DDI) water such that each sample received the correct phenolic treatment dose and was adjusted to 22% moisture. The control samples received DDI water only. Each treatment included 39 individual samples, plus three blanks with no soil or phenolics for analysis in triplicate at each time point to be preserved throughout the experiment. An additional six replicates of each treatment were included during the first and last day of the incubation: three for soluble C and N, and three for autoclaved citrate extractable protein (ACEP).

### 2.3. Soil and headspace analysis

Samples were analyzed on days 0, 2, 7, 14, 28, 39, 56, 70, and 84 of the study to determine rates of N mineralization, immobilization, and CO<sub>2</sub> and N<sub>2</sub>O production. Concentrations of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> were analyzed using a 2M potassium chloride (KCl) extraction method described in Gavlak et al. (2005) (Method S-3.50) and analyzed in duplicate using a Lachat Quikchem 8500 Flow Injector analyzer (Lachat Instruments, Loveland, CO, USA) according to Harbridge (2007a) for NH<sub>4</sub><sup>+</sup> and Harbridge (2007b) for NO<sub>3</sub><sup>-</sup>. Headspace samples collected using a syringe and vials were subsequently flushed to ambient conditions between sampling time points. Headspace samples were analyzed for CO<sub>2</sub> using a HP 6890 Series Gas Chromatograph System with thermal conductivity detection (Hewlett-Packard, Palo Alto, CA, USA) at 50°C with a 80/100

Chromosorb 12 6 ft x 1/8 in (2.1 mm) SS column. Nitrous oxide was analyzed using an Agilent Technologies 6850 Series II Network GC System (Agilent Technologies, Santa Clara, CA, USA) with electron capture detector at 55°C with an 80/100 Chromosorb 102 6 ft x 1/8 in (2.1 mm) SS column.

Soluble total C, inorganic C, total N, and total organic C (by difference of total and inorganic C) were extracted using one cold and three subsequent hot water extractions as described in Halvorson et al. (2009) and analyzed on a Shimadzu TOC-L analyzer with an ASI-L autosampler (Shimadzu Corp., Kyoto, Japan). Samples were treated with cool water for one hour. Samples were then treated with hot water for 16 and 21 hours on days 0 and 84 respectively for the first hot water extraction, and 24 hours for each of the second and third hot water extractions. Autoclaved citrate extractable protein was analyzed using the bicinchoninic acid (BCA) assay as described in Hurisso et al. (2018).

#### 2.4. Statistical analysis

Statistical analysis was performed using a mixed linear model and a randomized complete block design for analysis of variance. Parameters were analyzed for the main effects of treatment by dose and day, and their interaction where day was accounted for as a repeated measure. Carbon dioxide production rate, cumulative CO<sub>2</sub> production, ACEP, soluble TC, soluble TOC, and soluble TN were log-transformed to attain normality. Nitrous oxide production rate, cumulative N<sub>2</sub>O production, NH<sub>4</sub><sup>+</sup> concentration, and NO<sub>3</sub><sup>-</sup> concentration were analyzed using a non-parametric ranking procedure. Outliers were removed by assessing residuals. Single outliers were removed if their removal was critical in creating a normally distributed dataset. Outliers were kept in

boxplots and graphs. Statistical analysis was performed using the MIXED procedure in SAS Studio University Edition (version 9.4, SAS Institute, Cary, NC, USA).

### 3. Results

Differences in soluble C and N among the CT and saponin treatments suggested that both types of compounds can increase N retention, but through different mechanisms. There were significant treatment  $\times$  day interactions for total extracted soluble C (soluble TC, organic C, TN,  $p < 0.0001$ ), although the interactions appeared to be random and there were no major crosses in the data. There were significant treatment (TC, organic C, TN,  $p < 0.0001$ ) and day (TC, organic C, TN,  $p < 0.0001$ ) effects for all three parameters (Fig. 21a-c). Significantly more soluble TC ( $p < 0.0001$ ), organic C ( $p < 0.0001$ ), and N ( $p < 0.0001$ ) were extracted from samples on day 84 than day 0, suggesting that microbially-derived C and N was released throughout the incubation. The 15 mg/g SFN (15 mg/g BFT, 3 mg/g BFT, 3 mg/g SFN, control  $p < 0.0001$ ) and 3 mg/g SAP (3 mg/g BFT, 3 mg/g SFN, control  $p < 0.0001$ ; 15 mg/g BFT  $p = 0.0010$ ) treatments yielded significantly more soluble TC than the rest of the treatments, followed by the 15 mg/g BFT treatment which yielded significantly more soluble TC than the remaining control ( $p < 0.0001$ ), 3 mg/g BFT ( $p < 0.0001$ ) and SFN treatments ( $p < 0.0001$ ). The control yielded significantly more soluble TC than the 3 mg/g SFN treatment ( $p = 0.0424$ ), and the 3 mg/g BFT treatment did not differ from the control ( $p = 0.1822$ ) or 3 mg/g SFN treatment ( $p = 0.9766$ ). Similar results were seen for total soluble organic C yields, as the 15 mg/g SFN and 3 mg/g SAP treatments, followed by the 15 mg/g BFT treatment, yielded significantly greater soluble organic C than the control (15 mg/g SFN, 3 mg/g SAP, 15 mg/g BFT  $p < 0.0001$ ) or 3 mg/g BFT (15 mg/g SFN, 3 mg/g SAP, 15 mg/g BFT

$p < 0.0001$ ) or SFN (15 mg/g SFN, 3 mg/g SAP, 15 mg/g BFT  $p < 0.0001$ ) treatments. The 3 mg/g BFT and SFN treatments yielded significantly less soluble organic C than the control (3 mg/g BFT  $p = 0.0438$ , 3 mg/g SFN  $p = 0.0019$ ). This suggests that low concentrations of condensed tannins may be binding soil organic matter because they did not yield significantly more soluble total C or organic C than the control, despite adding C to the soil samples. The main effect of treatment was also significant for soluble N ( $p < 0.0001$ ) (Fig. 21c). The control yielded significantly more N than any of the BFT or SFN treatments (15 mg/g BFT  $p < 0.0001$ , 15 mg/g SFN  $p = 0.0002$ , 3 mg/g BFT  $p < 0.0001$ , 3 mg/g SFN  $p < 0.0001$ ) and yielded equal amounts of N as the 3 mg/g SAP treatment ( $p = 0.0817$ ). The 3 mg/g SAP treatment was also equal to the 15 mg/g SFN treatment ( $p = 0.1661$ ). Both the 3 mg/g SAP (15 mg/g BFT, 3 mg/g BFT, 3 mg/g SFN  $p < 0.0001$ ) and 15 mg/g SFN (15 mg/g BFT, 3 mg/g BFT, 3 mg/g SFN  $p < 0.0001$ ) treatments were significantly higher than the remaining BFT and 3 mg/g SFN treatments. This suggests that all CT treatments are complexing with organic N and increasing N retained in the soil.

Nitrogen mineralization patterns provided some evidence for increased N retention, as well as phenolic degradation and subsequent immobilization. There were significant treatment  $\times$  day interactions for  $\text{NH}_4^+$  ( $p < 0.0001$ ) and  $\text{NO}_3^-$  concentrations ( $p < 0.0001$ ) (Fig. 22a-b). When  $\text{NH}_4^+$  concentrations were compared among treatments within day, the only significant differences were on days 0 and 14. At the start of the incubation, the 15 mg/g SFN treatment had significantly higher  $\text{NH}_4^+$  concentrations than the control ( $p < 0.0001$ ), and the control treatment had significantly lower  $\text{NH}_4^+$  concentrations than the remaining treatments (15 mg/g BFT, 3 mg/g SAP  $p < 0.0001$ ; 3



mg/g SFN  $p=0.0002$ , 3 mg/g BFT,  $p=0.0007$ ). On day 14, the 3 mg/g SAP and 15 mg/g SFN treatments had significantly higher  $\text{NH}_4^+$  concentrations than the 15 (3 mg/g SAP  $p=0.0100$ , 15 mg/g SFN  $p=0.0478$ ) or 3 mg/g BFT (3 mg/g SAP  $p=0.0015$ , 15 mg/g SFN  $p=0.0089$ ) treatments which did not contain any detectable  $\text{NH}_4^+$ . By the end of the incubation there were no significant differences in  $\text{NH}_4^+$  concentration among treatments.

When  $\text{NO}_3^-$  concentrations were compared among treatments by day, the 15 mg/g SFN treatment had significantly higher  $\text{NO}_3^-$  concentrations than all treatments except for the 3 mg/g SFN treatment on day 0 (15 mg/g BFT, 3 mg/g BFT, 3 mg/g SAP, control  $p<0.0001$ ). As the incubation progressed,  $\text{NO}_3^-$  concentrations indicated a pattern of N immobilization and subsequent mineralization. By day 2, the 15 mg/g SFN treatment still had significantly higher  $\text{NO}_3^-$  concentrations than all treatments except for the 3 mg/g SFN treatment (15 mg/g BFT, 3 mg/g BFT, control  $p<0.0001$ ; 3 mg/g SAP  $p=0.0006$ ), and the 3 mg/g SFN treatment had significantly higher  $\text{NO}_3^-$  concentrations than the 15 and 3 mg/g BFT ( $p=0.0016$ ) treatments and the control ( $p=0.0393$ ) as N began to be immobilized. By days 7 and 14, the 15 mg/g SFN treatment had significantly higher  $\text{NO}_3^-$  concentrations than all other treatments as  $\text{NO}_3^-$  concentrations remained low (15 mg/g BFT  $p=0.0018$ ,  $<0.0001$ ; 3 mg/g BFT  $p<0.0001$ ; 3 mg/g SFN  $p=0.0300$ ,  $<0.0001$ ; 3 mg/g SAP  $p<0.0001$ ; control  $p<0.0001$ ). Subsequent N mineralization began on day 28 as  $\text{NO}_3^-$  concentrations in the 15 mg/g SFN treatment were significantly higher than the 15 mg/g BFT ( $p=0.0070$ ) and 3 mg/g SAP ( $p=0.0070$ ) treatments, and the control and 3 mg/g SFN treatments began to increase significantly above the 15 mg/g BFT (control  $p<0.0001$ , 3 mg/g SFN  $p=0.0002$ ) and 3 mg/g SAP (control  $p<0.0001$ , 3 mg/g SFN  $p=0.0002$ ) treatments. These differences lasted through day 39, with the addition of the 3 mg/g BFT

treatment also rising above the 15 mg/g BFT ( $p=0.0227$ ) and 3 mg/g SAP ( $p=0.0002$ ) treatments. By days 56 and 70,  $\text{NO}_3^-$  concentrations reached a maximum with the 15 mg/g SFN treatment remaining significantly higher than 15 mg/g BFT (day 56  $p<0.0001$ , day 70  $p=0.0008$ ) and 3 mg/g SAP (day 56,70  $p<0.0001$ ) treatments. The  $\text{NO}_3^-$  concentrations in the 3 mg/g SAP treatment remained significantly lower than all treatments (15 mg/g BFT  $p<0.0001$ ; 3 mg/g BFT  $p=0.0171$ ,  $<0.0001$ ; 15 mg/g SFN  $p<0.0001$ ; 3 mg/g SFN  $=<0.0001$ ; control  $p<0.0001$ ) besides the 15 mg/g BFT treatment ( $p=0.8383$ ,  $0.4281$ ), and the 15 mg/g BFT treatment was significantly lower than the control ( $p=0.0227$ ,  $0.0116$ ). By the end of the incubation on day 84, the 3 mg/g SAP and the 15 mg/g BFT treatments had significantly lower  $\text{NO}_3^-$  concentrations than the 3 mg/g BFT (3 mg/g SAP  $p=0.0078$ , 15 mg/g BFT  $p=0.0018$ ) and SFN (3 mg/g SAP  $p=0.0010$ , 15 mg/g BFT  $p=0.0002$ ) treatments and the control (3 mg/g SAP  $p=0.0001$ , 15 mg/g BFT  $p<0.0001$ ), and the 15 mg/g SFN treatment continued to be significantly higher than the 15 mg/g BFT ( $p<0.0001$ ) and 3 mg/g SAP ( $p<0.0001$ ) treatments. However, none of the treatments had  $\text{NO}_3^-$  concentrations that significantly exceeded the control. These results confirm that the phenolic treatments did not add significant amounts of N to the samples and suggest that low concentrations of saponins and high concentrations of CTs may decrease N mineralization.

The addition of the phenolic treatments generally did not increase total  $\text{N}_2\text{O}$  emissions over the course of the incubation. There was a significant treatment  $\times$  day interaction for  $\text{N}_2\text{O}$  production rate ( $p=0.0064$ ) and cumulative  $\text{N}_2\text{O}$  production ( $p<0.0001$ ) (Fig. 23a-b). On day 84, the 3 mg/g BFT treatment had significantly higher  $\text{N}_2\text{O}$  production rates than the 3 mg/g SFN treatment ( $p=0.0010$ ), and the 15 mg/g SFN

treatment had significantly higher N<sub>2</sub>O production rates than the 3 mg/g SFN treatment (p=0.0042). The 15 mg/g SFN treatment had significantly higher cumulative N<sub>2</sub>O production than the control treatment on days 2 (p=0.0004), 7 (p=0.0025), and 14 (p=0.0464), and the 3 mg/g SAP and SFN Low treatments on days 2 (p=0.0004 and p=0.0042, respectively) and 7 (p=0.0028 and p=0.0168, respectively). On day 2, the 15 mg/g BFT treatment had significantly higher cumulative N<sub>2</sub>O production than the control (p=0.0283) or 3 mg/g SAP (p=0.0253) treatments. There was a significant treatment effect for total N<sub>2</sub>O production (p<0.0001) (Fig. 23c). The 15 mg/g SFN treatment had significantly higher total N<sub>2</sub>O production than all other treatments (15 mg/g BFT, 3 mg/g BFT, 3 mg/g SFN, 3 mg/g SAP, control p<0.0001). This suggested that none of the phenolic additions except for the high dose SFN treatment stimulated significant N<sub>2</sub>O production over a prolonged period of time.

While there was evidence that the phenolic treatments increased soil N retention, they also appeared to provide a C source and increase C mineralization. There were significant treatment × day interactions for CO<sub>2</sub> production rate (p<0.0001) and cumulative CO<sub>2</sub> production (p<0.0001), but the interactions appeared to be random and there were no obvious crosses. There was no treatment effect for CO<sub>2</sub> production rate, but there were significant treatment effects for cumulative (p<0.0001) and total CO<sub>2</sub> production (p<0.0001) (Fig. 24a-c, Table 14). The 15 mg/g SFN (3 mg/g BFT, 3 mg/g SFN, control p<0.0001; 3 mg/g SAP p=0.0001) and BFT (3 mg/g BFT p=0.0003, 3 mg/g SFN p=0.0011, 3 mg/g SAP p=0.0025, control p<0.0001) treatments had significantly higher cumulative CO<sub>2</sub> production than all other treatments indicating a stimulatory effect from high doses of CTs, the 3 mg/g SAP (p=0.0093) and SFN (p=0.0232)

treatments had cumulative production that was significantly greater than the control indicating a lesser stimulatory effect. The 3 mg/g BFT treatment's cumulative production was intermediate to the control, 3 mg/g SAP, and 3 mg/g SFN treatments. By the end of the incubation the only significant differences in total CO<sub>2</sub> production were observed for the 15 mg/g SFN (3 mg/g BFT  $p=0.0005$ , 3 mg/g SFN  $p=0.0006$ , 3 mg/g SAP  $p=0.0057$ , control  $p=0.0003$ ) and BFT (3 mg/g BFT  $p=0.0033$ , 3 mg/g SFN  $p=0.0044$ , 3 mg/g SAP  $p=0.0479$ , control  $p=0.0019$ ) treatments which had significantly higher total CO<sub>2</sub> production than the rest of the treatments, suggesting a stimulatory effect of CTs on C mineralization at higher doses. There was a significant treatment  $\times$  day interaction ( $p<0.0001$ ) for ACEP (Fig. 21d). On day 0, the 15 mg/g SFN and BFT treatments yielded significantly more ACEP than all treatments ( $p<0.0001$ ). By day 84, the 15 mg/g BFT treatment only yielded more ACEP than the 3 mg/g SAP ( $p=0.0480$ ) and control ( $p=0.0031$ ) treatments. This indicates that protein generally decreased through time, but decreased significantly more for the 15 mg/g BFT and SFN treatments.

Soil N cycling results including those for ammonium, nitrate, and N<sub>2</sub>O production were unexpectedly high for the 15 mg/g SFN treatment and have caused concerns of possible N contamination in that treatment. Upon further analysis, the 15 mg/g SFN treatment was the only treatment with detectable amounts of total N (Table 13). Because the basic catechin and epicatechin building blocks of condensed tannins do not contain N, it is suspicious that the SFN, but not the BFT treatments, would add such a considerable amount of N. Statistical analysis was run with and without the 15 mg/g SFN treatment included. When these analyses were compared, the removal of the 15 mg/g SFN treatment did not generally alter the results of the experiment or my conclusions. Despite

the unexpected results of the 15 mg/g SFN treatment, low doses of saponins and high doses of BFT-derived condensed tannins did appear to increase soil N retention without increasing N<sub>2</sub>O emissions.

#### **4. Discussion**

I determined the effect of phenolic type and concentration on soil C and N cycling processes in a controlled incubation setting over the course of 84 days. Previous research has concluded that tannins generally decrease net soil N mineralization and denitrification rates due to complexation with organic N and microbial inhibition. I hypothesized that all phenolic treatments would decrease inorganic N concentrations and ACEP in the soil, as well as decrease N<sub>2</sub>O production compared to the control. Consistent with my hypothesis, all condensed tannin treatments decreased soluble total N yields compared to the control, and low doses of CTs decreased soluble total and organic C yields. Additionally, the 15 mg/g BFT and 3 mg/g SAP treatments had significantly lower NO<sub>3</sub><sup>-</sup> concentrations than the control for the last 8 weeks of the incubation with none of the phenolic treatments exceeding the control by day 84. Contrary to my hypothesis, none of the phenolic treatments had significantly lower NH<sub>4</sub><sup>+</sup> concentrations compared to the control at any point during the incubation. Also contrary to my hypothesis, none of the phenolic treatments had significantly lower N<sub>2</sub>O production rates, cumulative production, or total N<sub>2</sub>O production than the control.

Reductions in soluble TN are consistent with past studies. Like the results of Halvorson et al. (2016), reductions in soluble N by the CT treatments were dose dependent when the 15 mg/g SFN treatment was excluded. This is consistent with the idea that CTs may increase N retention through complexation reactions with organic

and/or mineral N. Reductions in soluble C and organic C provided evidence of CT sorption to SOM at low concentrations, but not in the dose dependent manner described in prior literature (Adamczyk et al., 2012; Halvorson et al., 2016; Kraus et al., 2003; Northup et al., 1995). It is possible that the high doses of CTs added more C than could be sorbed, or that the higher doses of added C stimulated a greater microbial biomass which in turn contributed to the soluble C pools. Also contrary to my hypothesis, high concentrations of CTs appeared to increase extraction of ACEP compared to the control. Past studies have used now outdated measures of soil protein such as Bradford reactive soil protein which makes direct comparison difficult. Autoclaved citrate extractable protein has just recently been proposed as an indicator of available organic N, and my data will need to be compared against future studies. The apparent dose-dependent increase in ACEP would suggest that the assay is either extracting proteins contained in the treatments, or that high doses of CTs make soil protein more available because reductions in soluble C were not dose dependent.

Although the ammonium data did not directly support the idea of phenolic-driven N complexation, the nitrate data did. The lower nitrate concentrations in the 15 mg/g BFT and 3 mg/g SAP treatments did confirm that phenolics, including saponins, can inhibit N mineralization over a prolonged period (Crush, 1993; Crush and Keogh, 1998; Kraus et al., 2003; Nierop et al., 2006a, 2006b; Northup et al., 1995; Schimel et al., 1998; Smolander et al., 2012). This may also occur in a dose-dependent manner, although it is difficult to conclude this with certainty because the 15 mg/g SFN treatment was questionable and had high nitrate concentrations throughout the incubation. Total  $N_2O$  production was significantly higher in the 15 mg/g SFN treatment, but was likely due to

the high  $\text{NO}_3^-$  concentrations found in that treatment. While I believe that the high values of ammonium, nitrate, and  $\text{N}_2\text{O}$  production in the 15 mg/g SFN treatment are likely due to treatment contamination, it is worth noting that CT complexation with proteins is well-documented to depend on other factors such as tannin structure, pH, and chain length, among others (Adamczyk et al., 2012, 2013; Smolander et al., 2012). Despite this shortcoming, it was evident that the 15 mg/g BFT and 3 mg/g SAP treatments were able to reduce mineral N pools without stimulating  $\text{N}_2\text{O}$  production at low doses, suggesting a complexation reaction with soil N.

The effect of tannins on  $\text{CO}_2$  production in the literature is mixed. A study by Nierop et al. (2006a) found that CTs from different species incubated with pine litter had greater, equal, or lesser cumulative  $\text{CO}_2$  production compared to the control, and confirms that different CTs have different effects on C mineralization. The dose-dependent effect on C mineralization observed in my study would suggest that C from the CT treatments was used as a labile C source, as past studies have indicated (Nierop et al., 2006a, 2006b; Schimel et al., 1998). While  $\text{CO}_2$  production appears to be substrate driven, the subtle differences in soluble C patterns may suggest that saponins and condensed tannins work in different ways to increase soil N retention. Because the SAP treatment was able to reduce mineral N pools and  $\text{N}_2\text{O}$  production at low doses while not increasing C mineralization, this would point towards a complexation reaction. However, the yields of soluble total and organic C, and total N did not support this like the CT treatments. Therefore, it is more likely that saponins inhibited N cycling by inhibiting microbial communities. Microbial inhibition would decrease  $\text{N}_2\text{O}$  production and microbially-mediated nitrification, but the lack of complexation would allow N to be

extracted as soluble TN. This would be consistent with the literature, as medicagenic acid (a component of alfalfa saponins) has inhibited enzyme activity and led to cell death in some strains of rhizosphere bacteria (Hoagland et al., 2001). Levanon et al. (1982) observed alfalfa saponins inhibiting ammonification and N mineralization in peat and was attributed to fungal community inhibition. Unlike my results, the authors also observed increased denitrification. However, a recent study by Clemensen (2018) at the same location where my soils were collected, found reduced soil respiration and dehydrogenase enzyme activity in field soils under both alfalfa and sainfoin. This again suggests that both saponins and CTs can inhibit microbial activity in field soils. This was also confirmed in a soil-feces incubation, where high concentrations of feces from both alfalfa and sainfoin decreased N mineralization. This is further confirmed by the results of chapter II of this thesis, which found decreased field soil respiration under alfalfa compared to CT-containing birdsfoot trefoil.

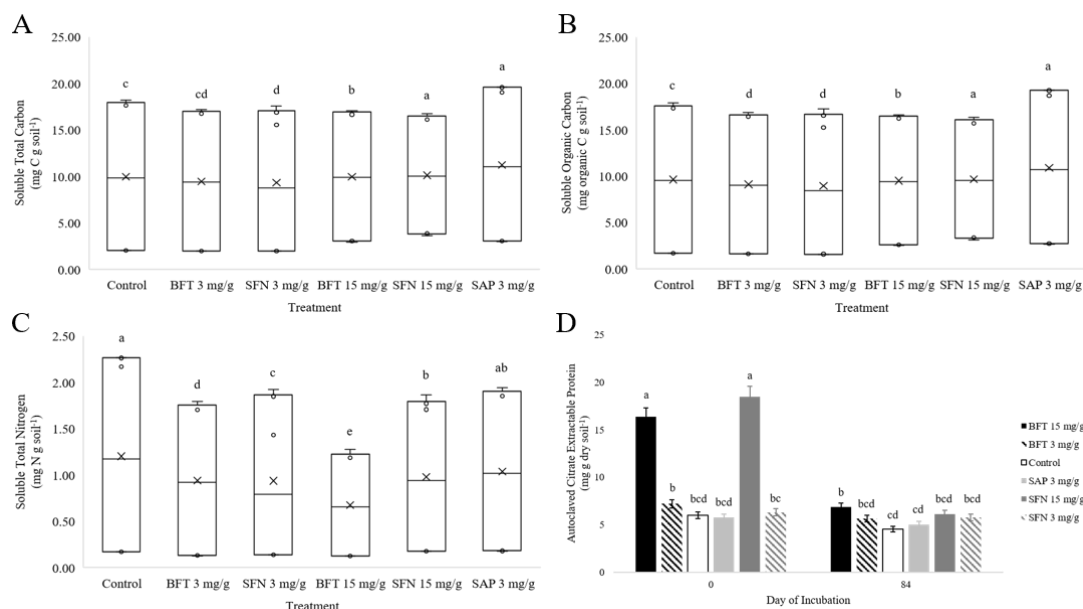
#### 4.1. Conclusions

This study investigated the effects of purified CTs and saponins isolated from BFT, SFN, and ALF forages on soil C and N cycling processes in a controlled setting. Both CTs and saponins inhibited N cycling processes, but in different ways. Condensed tannins likely elicited a combination of immobilization and complexation mechanisms, while saponins likely reduced N mineralization and denitrification through microbial inhibition. These effects have significant implications for increasing N retention in N loaded grazed pasture systems as increased immobilization and complexation coupled with reduced N mineralization may work to reduce N loss through leaching and denitrification to greenhouse gases.

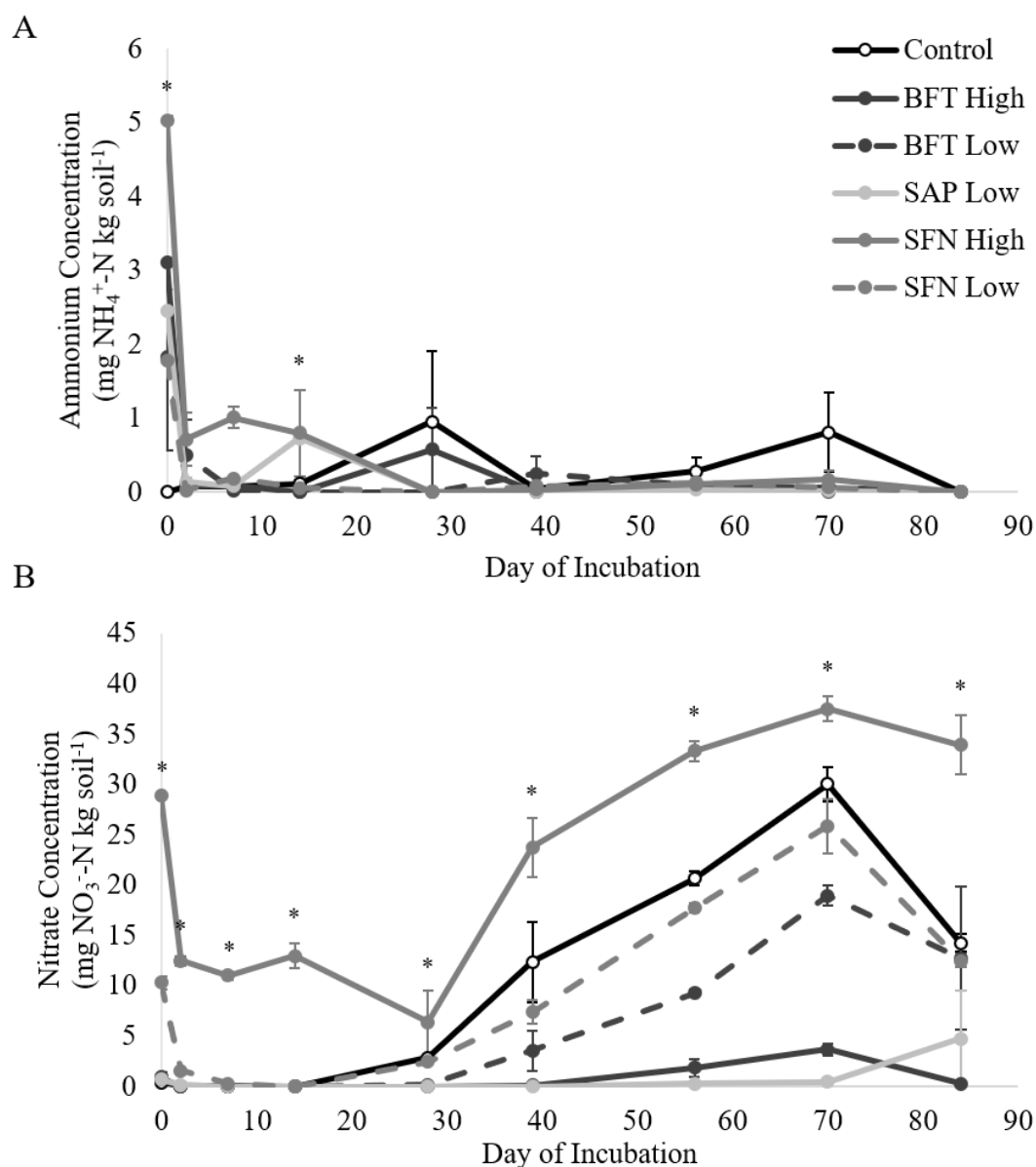


This study is limited in that potential contamination of the 15 mg/g SFN treatment reduced my ability to draw conclusions about the effects of CTs, particularly the effect of dose, on soil C and N cycling. It is also limited by use of a single dose of the saponin treatment. Future studies should continue to assess the effects of saponins in addition to CTs on field soil C and N cycling processes, as well as the effect of saponin dose.

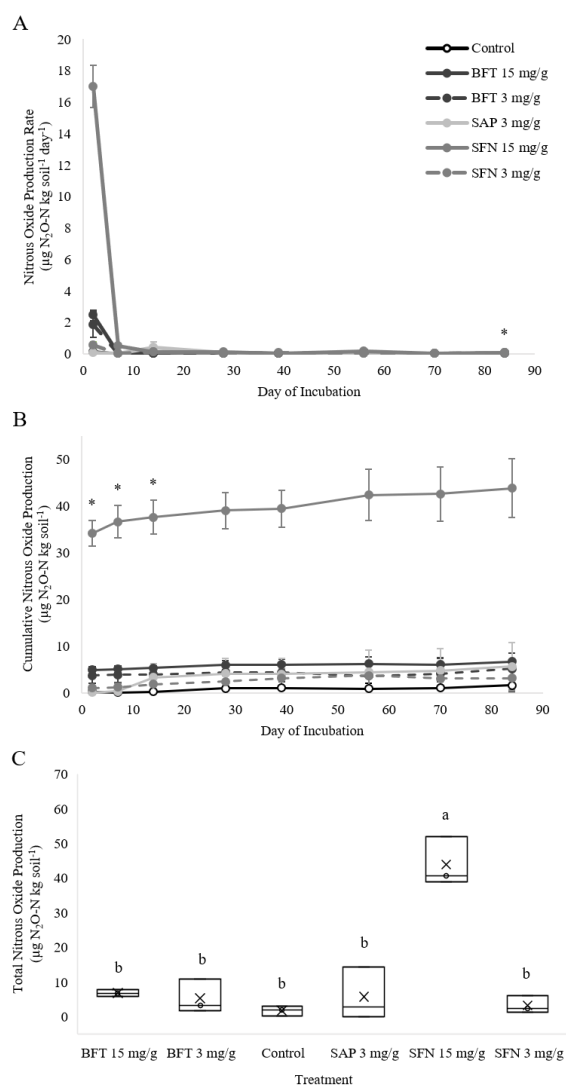
## 5. Figures and tables



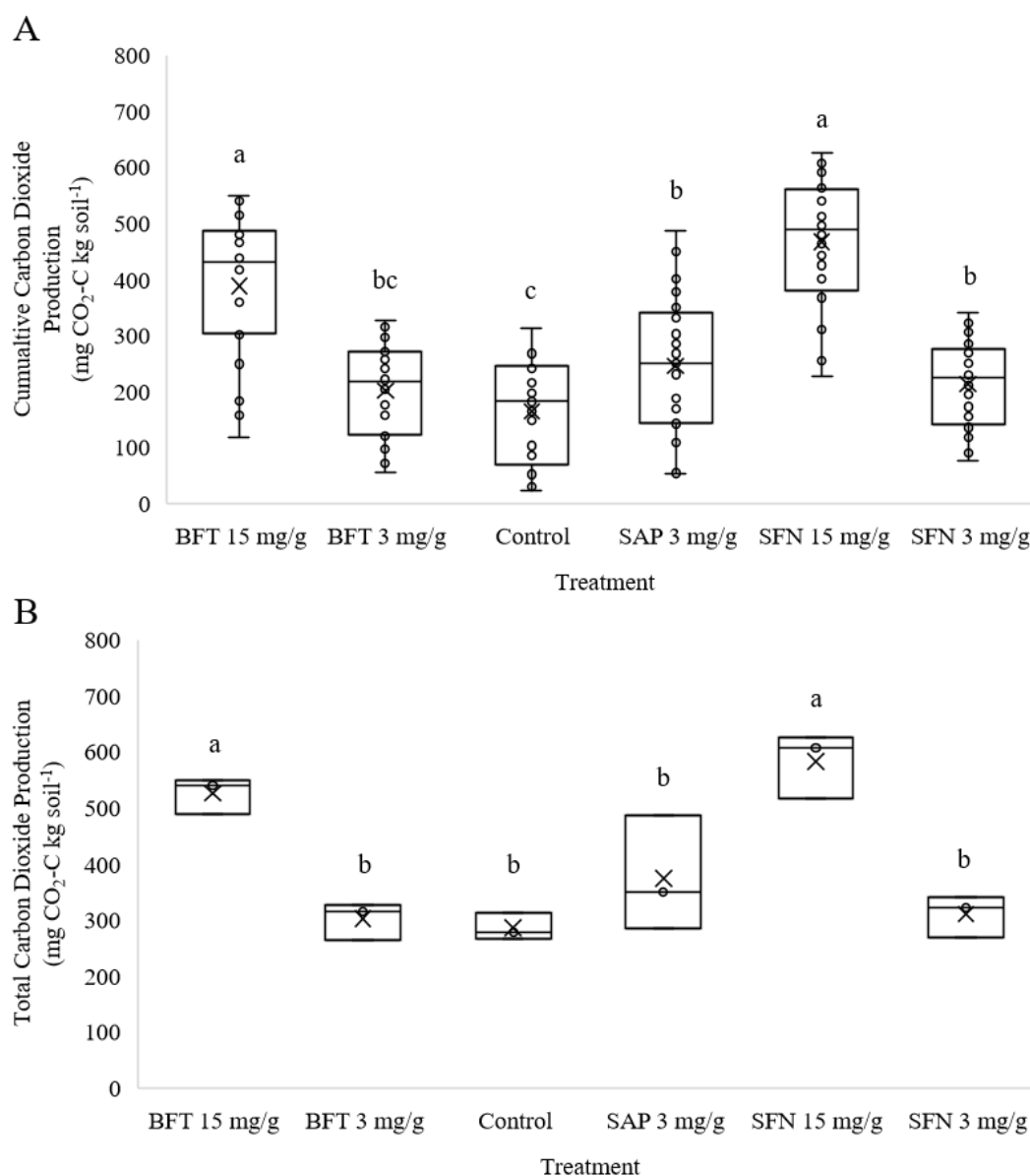
**Fig. 21.** Average soluble total carbon, soluble organic carbon, soluble total nitrogen, and autoclaved citrate extractable protein for days 0 and 84 from soils incubated with 3 mg/g or 15 mg/g doses of condensed tannins isolated from birdsfoot trefoil and sainfoin, saponins isolated from alfalfa, or an unamended soil control. Different letters denote a significant difference for the main effect of treatment for soluble total carbon in panel A ( $n=3$ ,  $p<0.0001$ ), soluble organic carbon in panel B ( $n=3$ ,  $p<0.0001$ ), soluble total nitrogen in panel C ( $n=3$ ,  $p<0.0001$ ) and the interaction of treatment and day for autoclaved citrate extractable protein in panel D ( $n=3$ ,  $p<0.0001$ ). Soil amendments had a lesser effect on soluble carbon and ACEP, but condensed tannin treatments decreased soluble total N in a dose dependent manner. BFT=birdsfoot trefoil, SFN=sainfoin, SAP=saponin, ACEP=autoclaved citrate extractable protein.



**Fig. 22.** Average soil ammonium and nitrate concentration in soils incubated with 3 mg/g or 15 mg/g doses of condensed tannins isolated from birdsfoot trefoil and sainfoin, saponins isolated from alfalfa, or an unamended soil control. Asterisks (\*) denote a significant difference for the interaction of treatment and day for soil ammonium in panel A ( $n=3$ ,  $p<0.0001$ ) and soil nitrate in panel B ( $n=3$ ,  $p<0.0001$ ). Error bars represent standard error. While treatment C:N ratio affected both ammonium and nitrate concentrations, secondary compounds increased mineralization and decreased nitrification processes. BFT=birdsfoot trefoil, SFN=sainfoin, SAP=saponin.



**Fig. 23.** Average nitrous oxide production rate, cumulative nitrous oxide production, and total nitrous oxide production from soils incubated with 3 mg/g or 15 mg/g doses of condensed tannins isolated from birdsfoot trefoil and sainfoin, saponins isolated from alfalfa, or an unamended soil control. Different letters or asterisks (\*) denote a significant difference for the main effect of treatment for total nitrous oxide production in panel C ( $n=3$ ,  $p<0.0001$ ), or the interaction of treatment and day for nitrous oxide production rate in panels A ( $n=3$ ,  $p=0.0064$ ) and cumulative nitrous oxide production in panel B ( $n=3$ ,  $p<0.0001$ ). Error bars in panels A and B represent standard error. The 15 mg/g SFN treatment had elevated nitrous oxide production rates as well as cumulative and total nitrous oxide production compared to the remaining treatments and control. BFT=birdsfoot trefoil, SFN=sainfoin, SAP=saponin.



**Fig. 24.** Average cumulative carbon dioxide production and total carbon dioxide production from soils incubated with 3 mg/g or 15 mg/g doses of condensed tannins isolated from birdsfoot trefoil and sainfoin, saponins isolated from alfalfa, or an unamended soil control. Different letters denote a significant difference for the main effect of treatment for cumulative carbon dioxide production in panel A ( $n=3$ ,  $p<0.0001$ ) and total carbon dioxide production in panel B ( $n=3$ ,  $p<0.0001$ ). Condensed tannin treatments appeared to increase measures of carbon dioxide production in a dose dependent manner. BFT=birdsfoot trefoil, SFN=sainfoin, SAP=saponin.

**Table 13**

Initial condensed tannin and saponin amendment total carbon and nitrogen characteristics.

	Treatment					
	Control	BFT 15 mg/g	BFT 3 mg/g	SFN 15 mg/g	SFN 3 mg/g	SAP 3 mg/g
<b>Amendment</b>						
Weight (mg)	N/A	15.00	3.00	15.00	3.00	3.00
Total C (mg)	N/A	6.32	0.65	4.59	0.30	0.50
%C	N/A	42.16	21.64	30.61	9.92	16.82
Total N (mg)	N/A	0	0	0.03	0	0
%N	N/A	Below detection	Below detection	0.22	Below detection	Below detection
C:N Ratio	N/A	N/A	N/A	139.9 : 1	N/A	N/A

**Table 14**

Carbon dioxide production rates by treatment and day. Averages are calculated with back-transformed standard errors. Letters denote a significant difference at  $p < 0.05$  ( $n=3$ ). The effect of treatment was significant for carbon dioxide production rate, but there were no significant differences among treatments using the Tukey method of means separation.

Parameter		
	CO <sub>2</sub> Production Rate (mg CO <sub>2</sub> -C kg soil <sup>-1</sup> day <sup>-1</sup> )	p-values
Treatment		0.0194
Control	4.96 ± 0.36	
BFT 15 mg/g	15.32 ± 0.54	
BFT 3 mg/g	7.42 ± 0.36	
SFN 15 mg/g	21.53 ± 0.51	
SFN 3 mg/g	8.45 ± 0.35	
SAP 3 mg/g	8.06 ± 0.42	
Day		<0.0001*
2	54.33 ± 2.45a	
7	13.69 ± 0.72b	
14	6.69 ± 0.37c	
28	4.66 ± 0.26d	
39	3.45 ± 0.20e	
56	1.01 ± 0.06h	
70	2.21 ± 0.12f	
84	1.62 ± 0.09g	

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## CHAPTER IV.

## CONCLUSIONS

This thesis aimed to assess the ecosystem services provided by soil under condensed tannin-containing legume grazed pasture systems. To address this goal, I compared the effects of various condensed tannin and non-condensed tannin-containing forage species on soil C and N mineralization processes and measures of microbial activity under a grazed pasture, compared the effects of CT- and non-CT-containing fecal additions to a pasture soil on C and N mineralization and denitrification processes, and compared the effects of purified CTs and saponins isolated from forages species of interest on pasture soil C and N cycling processes. I observed evidence for secondary compound complexation with N in field soils through significantly reduced aerobic N mineralization rates. I also observed evidence for increased N retention by both CTs and saponins through reductions in N mineralization, reduced soluble total N, and a lack of differences in N<sub>2</sub>O production in the incubation experiments. While both CTs and saponins provided evidence for reduced N mineralization, CTs appeared to work through a combination of immobilization and complexation processes while saponins appeared to work through microbial inhibition. Reductions in soil N cycling processes were observed both in controlled laboratory experiments and in the field, although other factors such as the C:N ratio of biomass and feces in more complex systems were also important in determining a system's nutrient cycling dynamics.

These results suggest that CT- and saponin-containing legume forages may successfully increase soil N retention in grazed pasture systems by limiting rates of soil N

mineralization. This may help producers raising pasture-finished beef to compete economically with feedlot finished beef, as well as reduce the environmental impact of pasture-based beef production. Changes in soil nutrient cycling coupled with reduced N fertilizer needs may substantially decrease the environmental footprint of beef production both on a per-kg beef and per-area of land basis. However, further life-cycle based analysis is necessary to fully understand how these changes to the C and N cycle affect the final GHG footprint of pasture-finished beef at the farm gate. Future research should also continue to study the effect of saponins on soil C and N cycling in addition to CTs and incorporate field GHG fluxes into field studies. Research should also investigate changes in specific soil microbial communities over time once these forages are established. While the most effective way to reduce the environmental impact of beef production is to reduce personal meat intake, the use of CT legumes may be also be an effective method of increasing the sustainability of beef production and supporting pasture ecosystem services.