

THESIS

SOIL NITROGEN CYCLING IN AGROECOSYSTEMS AS MODIFIED BY BIOCHAR
AMENDMENT AND PLANT PROCESSES

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ABSTRACT

SOIL NITROGEN CYCLING IN AGROECOSYSTEMS AS MODIFIED BY BIOCHAR AMENDMENT AND PLANT PROCESSES

Ecosystem productivity is dependent upon cycling of nutrients, such as nitrogen (N). In agricultural systems, humans have greatly altered N cycling through the application of synthetic fertilizers such that soil N in agroecosystems is lost at higher rates than N in unmanaged systems. A variety of strategies have been assessed to reduce losses of soil N through nitrous oxide (N₂O) emissions and leaching, which can negatively impact climate and water quality, respectively. The application of biochar, a carbon-rich soil amendment, has shown promise for increasing N retention in agricultural systems, but field and greenhouse studies often present less dramatic and often conflicting effects, suggesting the need for greater study in these environments. Further, the effects of biochar do not occur in isolation, but rather depend on plant processes that may affect soil N dynamics. This thesis explores these ideas through: (1) a greenhouse study considering the effects of different biochar types on N cycling with and without plants and (2) a field study looking at seasonal patterns of N cycling and fixation in alfalfa as altered by strategically-placed, low rates of biochar application. Study 1 sought to determine differential effects of biochar and plants, and raw and engineered biochar, on both fertilizer and innate soil N cycling using isotopically labelled fertilizer. While biochar effects on soil-derived N were minimal, we found that engineered biochar led to significantly higher leaching losses of fertilizer N. Plants, in

contrast, were found to reduce N loss and increase overall recovery of fertilizer N. Study 2 focused on the effects of low and economically feasible application rates of two different biochars on N fixation, N loss, and mineral N availability over a growing season. We found no biochar effects on any N cycling parameter and, rather, found significant temporal effects in all N pools. Seasonal dynamics suggest connections between SIN availability and N fixation and loss. Indications of increased N loss with engineered biochar in Study 1 urge the need for greater study of biochars in combination with a variety of fertilizer types in order to provide the best recommendations to farmers. Lack of effects with biochar in Study 2 indicate that low application rates of biochar may not be useful for increasing N retention, suggesting the need to find a balance between economic and effective biochar application rates. Since both studies suggest that plant processes have more substantial impacts on N cycling than biochar amendment, via reduced N loss (Study 1) or increased symbiotic N input (Study 2), it is important that plants are included in more biochar studies such that the strength of biochar effects can be more realistically evaluated.

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CHAPTER 1: INTRODUCTION

1. Background

This thesis seeks to explore the modification of nitrogen (N) cycling in temperate agricultural systems in response to the addition of biochar and plant processes. Nitrogen, like many nutrients in this anthropogenic period, occupies a dual role as a villain and hero in ecosystems. Nitrogen is a fundamental element for all living things and it is often the limiting nutrient for plant growth, but excess N can have negative consequences for the larger environment. This is especially true in agroecosystems, where plant growth is a commodity, and thus N is often overapplied to ensure it is not limiting. Increasing crop performance is essential for issues of global food security and farm profitability, while increasing the efficiency of N use by crops means we need fewer inputs to achieve the same outputs. Sustainable agriculture is a movement that seeks to strike a balance between providing food, enhancing producer livelihoods, and protecting the environment (Reganold et al., 1990). Biochar, organic matter burned with low oxygen to 350-1000°C and applied to soil (Lehmann and Joseph, 2015), is a relatively new tool for sustainable agriculture. Research on biochar sprung from the discovery of *terra preta de índio* or Amazonian Dark Earths, highly fertile and organic matter-rich soils that resulted from biomass burning by Amerindians 100s to 1000s of years ago (Lehmann, 2009). As of 2013, more than 300 primary research articles had been published on biochar, and this number only continues to grow with a dedicated biochar journal first being published in 2019 (Gurwick et al., 2013; *Biochar*, Springer). However, even with an increasingly large body of literature on biochar, we are still faced with many questions and uncertainties related to the potential agronomic and environmental benefits of biochar in agricultural

soil. Further, biochar effects do not occur in isolation, so evaluating the effects of plant processes in tandem with biochar effects will more realistically represent an agricultural system.

2. A brief review

Biochar effects on N have been broadly covered in reviews (Clough, et al., 2013; Clough & Condon, 2010; Gul & Whalen, 2016) and meta-analyses (Borchard et al., 2019; Cayuela et al., 2014; Nguyen et al., 2017) so this introduction focuses on broad-scale patterns and gaps of knowledge occurring in the literature, with special focus on plant processes such as biological N fixation and plant N uptake. It is important to note that biochar and its effects are highly variable because they depend on both biochar factors, such as feedstock type, application rate, and physio-chemical properties, as well as soil factors, such as pH and texture.

2.1 Biochar effects on soil N pools and internal soil cycling

2.1.1 Soil inorganic N

Biochar effects on soil inorganic N (SIN) have been the subject of intense study, as SIN is often considered a proxy for the plant available N pool and thus understanding its response to biochar is a crucial part of understanding the response of crop growth. Two meta-analyses have collated studies of SIN modification by biochar and found contrasting results. Nguyen et al. (2017) found that addition of biochar to soil, on average, reduced soil NO_3^- -N and NH_4^+ -N by $10 \pm 1.6\%$ and $11 \pm 2\%$, respectively, but that this was highly dependent on fertilizer type, crop type, biochar feedstock, and experiment length. Interactions between these drivers further muddled the analysis and prevented evaluation of the effects of experimental design (i.e. greenhouse, field, or lab study) (Nguyen et al.,

2017). A more recent analysis by Borchard et al. (2019) elaborates on the findings of (Nguyen et al., 2017) but focused only on NO_3^- . They, in contrast, found no effect of biochar, on average, on soil NO_3^- concentrations but, similarly, found this was highly dependent on the length of the experiment, with longer experiments leading to greater reductions in soil NO_3^- , and fertilizer type. Additionally, both studies show variation in SIN with biochar application rate - significant reductions in SIN were found for application rates greater than 2% by mass (Nguyen et al., 2017) and 20 Mg ha^{-1} (Borchard et al., 2019). This is not surprising as biochar effects on SIN are largely related to its physical properties. Biochar has a large negatively charged surface area and thus has been suggested to retain NH_4^+ via sorption (Zheng et al., 2010). Because NO_3^- is negatively charged, this pathway is less likely, rather, chemisorption is the suggested mechanism for retention of this species (Kammann et al., 2015). Biochar has also been shown to increase microbial biomass, potentially increasing microbial uptake of SIN, and reducing both forms of SIN in the soil (Gul & Whalen, 2016; Nguyen et al., 2017). Nitrification (i.e. the microbial oxidation of NH_4^+ to NO_3^-) can decrease with biochar amendment largely due to biochar-induced reduction of NH_4^+ (Gul & Whalen, 2016). However, when biochar is added in concert with N fertilizer, this limitation can be overcome and biochar-induced increases in nitrifier communities, O_2 availability, and pH, may lead to increased transformation to NO_3^- (Prommer et al., 2014). While the mechanisms and effects for NH_4^+ retention with biochar addition are clear, contrasting reports of biochar effects on NO_3^- suggest the need for further study of this N species, especially in response to different fertilizer, crop, and biochar types.

2.1.2 Soil organic N

The organic N pool in the soil is generally less studied than SIN pools and this holds true for biochar studies as well. Organic N makes up the majority of soil N and represents a comparatively stable pool (Kaye et al., 2002), which is thus important to study. Organic N is a balance between plant and microbial inputs and outputs via dissolved organic N (DON) leaching and mineralization. Increased plant yields and microbial biomass with biochar addition suggest increased N inputs leading to a buildup of organic N (Jeffery et al., 2017; Gul et al., 2015; Prommer et al., 2014). However, differential reports on the effect of biochar on mineralization (Bruun et al., 2012; Güereña et al., 2013; Prommer et al., 2014) and a lack of reports on the effect of DON sorption and loss (exceptions include Dempster et al., 2012; Güereña et al., 2013; Prommer et al., 2014), make it difficult to determine the overall impacts of biochar on the soil organic N pool. Further, stability of organic N is further determined by microbial substrate use efficiency and availability of mineral surfaces (Cotrufo et al., 2013), making biochar effects dependent on soil properties. Further research examining losses from the organic N pool and evaluating biochar interactions with soil properties will allow us to better understand how biochar might affect this more stable form of N.

2.1.3 Microbial biomass N

The amount of microbial biomass N (MBN) in the soil is determined by the biomass of the microbial community and the amount of its N uptake. As mentioned above, many studies find increased microbial activity with biochar addition (e.g. Gomez et al., 2014), but this effect appears to be stronger for mid-temperature (300-600°C) biochars that underwent slow pyrolysis (Gul et al., 2015). Nitrogen immobilization by microbes is a balance of the processes of uptake and mineralization. As reported above, the effects of

biochar on mineralization are fairly variable. There is less known about effects on microbial N uptake with biochar, which has been reported to increase (Güereña et al., 2013) and has often been cited as a mechanism for reduced soil N availability (Case et al., 2012; Clough et al., 2013), but is not often measured. Both of these processes depend on the C:N ratio of the substrate used by microbes, and because biochar generally has a high C:N, its addition may lead to dominance of immobilization over mineralization and increased mining of soil organic matter for N (Nelissen et al., 2012; Chan & Xu, 2015), leading to greater MBN. However, there is little empirical evidence of these claims so much more work is needed in this area.

2.2 Biochar effects on N fluxes to and from the soil

2.2.1 N fixation

The effect of biochar on N fixation has received less attention than other aspects of the N cycle but results are promising. The majority of studies show increased N fixation with biochar addition (e.g. Güereña et al., 2015; Mia et al., 2014; Rondon et al., 2007), but most studies have been carried out in the lab or greenhouse (exceptions are Mia et al., 2018; Van Zwieten et al., 2015) and most use high application rates (≥ 10 tons ha^{-1}). Suggested mechanisms for increased N fixation with biochar addition include reduced SIN (Rondon et al., 2007), introduction of micro- and macronutrients that are co-factors of the nitrogenase enzyme (namely P, B, K, and Mo; Rondon et al., 2007; Oram et al., 2014), and increased pH in acidic soils due to the commonly observed biochar-liming effect (Mia et al., 2014; Van Zwieten et al., 2015). However, it is important to evaluate more realistic (in field and lower application rates) scenarios of biochar amendment before concluding that biochar consistently has a positive effect on N fixation.

2.2.2 Plant uptake

Because biochar has been shown to reduce availability of SIN in the soil, there is concern that this could negatively affect plant growth and uptake of N. However, a meta-analysis indicated no effect of biochar on plant tissue N concentration (Biederman & Harpole, 2013) and individual studies find similar results (Dharmakeerthi et al., 2012; Schmidt et al., 2014). There are reports of increased N uptake with biochar amendment, but these are always in concert with fertilizer application (Dharmakeerthi et al., 2012; Saarnio et al., 2013), indicating the need for combining biochar with fertilizer addition to ensure positive plant effects with biochar amendment. While many biochar studies include fertilization, relatively few explicitly consider fertilizer-biochar interactions. Studies on biochar and fertilizer explicitly have focused on N dynamics, trace gas emission, and plant growth effects (Agegnehu et al., 2016; Nelissen et al., 2012; Zheng et al., 2012; Carter et al., 2013; Dharmakeerthi et al., 2012). Biochar alone was shown to increase barley yields relative to a control, although these yields increased with increasing fertilizer application rates (Agegnehu et al., 2016). In contrast to this, fertilization with biochar addition led to lower lettuce biomass than biochar alone in a pot study that used organic N fertilizers (Carter et al., 2013). Biochar-fertilizer interactions were observed by Zheng et al. (2012), where they found reduced N₂O emissions with biochar addition, but only when fertilizer was also added, and by Agegnehu et al. (2016) and Dharmakeerthi et al. (2012) who found increased N use efficiency and plant N uptake, respectively, with biochar application and fertilization. The aforementioned studies used only one fertilizer type (urea, NH₄NO₃, organic N), so a study by Nelissen et al. (2012) that evaluated different fertilizer types was important because fertilizer type has been found to

differentially affect SIN (Nguyen et al., 2017). While Nelissen et al. (2012) found reduced NO_3^- concentrations and NO with biochar addition regardless of fertilizer type, they found that reductions in N_2O emission only occurred when biochar was added with urea or KNO_3 and not NH_4Cl . Because different forms of N are differentially available to plants and have varied chemical properties, we expect them to interact differently with biochar application. Further evaluation of various biochar-fertilizer combinations will allow for more useful input to farmers about which management applications are best suited for their goals.

No studies to our knowledge have assessed how plant-biochar interactions impact soil N cycling, which is crucial for understanding how biochar will affect N dynamics in field settings and for determining whether findings from lab studies are relevant to the field.

2.2.3 Gaseous losses

Gaseous losses of N represent a variety of N compounds with differential environmental effects. Nitrous oxide (N_2O) is a greenhouse gas that is 298 times more efficient at trapping heat than carbon dioxide (IPCC, 2007) and thus its emission has serious implications for climate. A meta-analysis has shown that on average, application of biochar reduces N_2O emissions by 49% (Cayuela et al., 2015), likely through facilitation of complete denitrification to dinitrogen (N_2) due to shifts in soil redox conditions (Cayuela et al., 2013; Ramlow & Cotrufo, 2017), reductions in substrate availability (Taghizadeh-Toosi et al., 2011), or reductions in enzymatic activity due to increased soil pH (Mørkved et al., 2007). However, the effect of decreased N_2O emission is less apparent in field and greenhouse studies which generally have lower biochar application rates and soil moisture than lab studies (Cayuela et al., 2015). Additionally, no effect of biochar

(Ramlow et al., 2019) and increased N₂O emissions with biochar (Bruun et al., 2011; Verhoeven & Six, 2014) have also been reported, indicating the need for more studies under realistic agricultural conditions. Another form of gaseous N loss is nitric oxide (NO) emission. Biochar effects on NO are much less clear, as the two known studies that have measured NO found significant decreases, increases, and no effect with biochar addition, depending on application rates (Nelissen et al., 2014; Xiang et al., 2015). Only very high applications of biochar (20 t ha⁻¹) led to significant reductions in NO emission (Nelissen et al., 2014), indicating the need to evaluate more economical application rates. Volatilization of ammonia (NH₃), a pollutant that can be deposited from the atmosphere to adjacent ecosystems, had largely been thought to be reduced with biochar addition due to sorption of NH₃ to the biochar surface (Clough & Condon, 2010). However, more recent field studies of this phenomenon have found more variable effects with some studies reporting increases (Sun et al., 2017; Sun et al., 2014) and decreases (Subedi et al., 2015; Sun et al., 2014) in NH₃ volatilization with biochar amendment. Increases in NH₃ volatilization may be due to biochar-induced pH increases (Sun et al., 2017). Much like with NO, more research is needed to determine conditions that allow for reduction of NH₃ volatilization with biochar addition.

2.2.4 N leaching

The majority of studies demonstrate reduced N leaching following biochar addition and this has been confirmed by a meta-analysis that reported an average 13% decrease in NO₃⁻ leaching with biochar addition (Borchard et al., 2019). However, this effect has been posited to develop over time, since reduced NO₃⁻ leaching is likely related to the development of anion exchange capacity through formation of positively charged

functional groups on the biochar surface (Kammann et al., 2015). A few studies have reported increased NO_3^- leaching with biochar addition (Laird et al., 2010; Singh et al., 2010), but literature on NH_4^+ and total N leaching shows consistent decreases (e.g. Ding et al., 2010; Xu et al., 2016). In contrast, the little work that has examined organic N losses has found no effect of biochar (Dempster et al., 2012; Güereña et al., 2013). Research focusing on specific N species, especially NO_3^- and DON, will increase our understanding of biochar impacts on leaching.

3 Future directions

Biochar has been proven as a useful agronomic and ecological tool in lab studies, but these beneficial effects are less consistent when evaluated in the field (e.g. Cayuela et al., 2015). More research evaluating management practices and conditions that are more realistic for producers is needed before the feasibility of biochar as an agronomically effective amendment can be fully determined. A major aspect of realism that needs to be assessed is biochar-plant interactions, as effects of biochar do not occur in isolation from plant effects. Organic N, N fixation, and microbial pools all require more research before directionality of biochar effects can be established. There is great promise for biochar to reduce N losses through leaching and gaseous emissions and to increase N retention, but this needs to be evaluated with lower biochar application rates and varied fertilizer types. Perhaps the most pressing need for biochar research though, is determining whether it is economically feasible and socially acceptable for farmers, which this thesis will not delve into, but is sorely needed for this field to move forward. The extreme case dependency of any given soil-biochar-plant combination furthers this issue, as it is difficult to make general recommendations to land managers.

CHAPTER 2: PLANT PRESENCE REDUCES ENGINEERED BIOCHAR-INDUCED NITROGEN LOSS FROM AN ALKALINE TEMPERATE AGRICULTURAL SOIL

1. Introduction

Enhancing the efficiency of nitrogen (N) use in agricultural systems is crucial for reducing deleterious N losses and increasing profitability. Approximately 20% of the N added to agroecosystems for plant uptake is lost from the system, making cropping systems the largest source of reactive N in the biosphere (Fowler et al., 2013; Leach et al., 2012). The two major forms of N loss from agricultural systems are gaseous and leaching losses (Fowler et al., 2013). Gaseous N losses represent a wide array of compounds, including emissions of the greenhouse gas, nitrous oxide (N_2O), which is important due to its negative effects on climate (Ravishankara et al., 2009). Leaching losses are also important due to their harmful downstream effects on water quality and ecosystem functioning (Billen et al., 2013). N loss is largely controlled by the availability of substrate, soil inorganic N (SIN), as well as other soil biophysical properties (Quemada et al., 2013; Sgouridis & Ullah, 2015). Due to differences in chemistry and associated mobility of different forms of SIN, it is crucial to understand the effect of agricultural practices on both ammonium (NH_4^+) and nitrate (NO_3^-). It is important to note that nitrification, i.e. the microbial oxidation of NH_4^+ to NO_3^- , can be rapid and both NH_4^+ and NO_3^- are commonly applied in fertilizers.

While a number of potential technologies have been proposed to enhance crop N use efficiency, biochar exists as a highly promising soil amendment for improving N retention as well as providing other agronomic and environmental benefits (Atkinson et

al., 2010; Nguyen et al., 2017; Lehmann and Joseph, 2015). Biochar is defined as a carbon-rich soil amendment created by heating biomass to 350-1000°C in the absence of, or with limited, oxygen (pyrolysis) (Lehmann and Joseph, 2015). While consistent yield benefits with biochar addition have been shown for highly weathered soils in the tropics, there is less certainty for temperate areas that constitute most of the world's agricultural lands (Jeffery et al., 2017). Thus, further research is needed in temperate systems to determine where biochar might be the most effective, and potentially offer co-benefits (beyond yield), such as impacts on N use efficiency and retention in agricultural soils.

Biochar has been suggested to increase SIN retention through both abiotic and biotic pathways. These retention pathways are suggested to reduce N₂O emissions (Butterbach-bahl et al., 2013) and N leaching (Laird et al., 2010) by reducing SIN availability (Nguyen et al., 2017). Abiotic pathways include chemisorption to biochar surfaces and physisorption in biochar pores (Lehmann and Joseph, 2015; Nguyen et al., 2017). Because the majority of functional groups on biochar surfaces are negatively charged, chemisorption is a more important retention pathway for NH₄⁺ which can be retained via electrostatic attraction (Zheng et al., 2010). However, positively-charged functional groups, unconventional H-bonding, and cation bridging can serve as methods of chemisorption of NO₃⁻ to biochar surfaces (Kammann et al., 2015; Mukherjee et al., 2011; Amonette and Joseph, 2009). Biotic pathways of N retention with biochar are linked to its effects on soil biota. Generally, it has been found that biochar increases soil microbial biomass, through protection pathways or co-location with resources (Gomez et al., 2014; Gul & Whalen, 2016; Jiang et al., 2016; Pietikäinen et al., 2000; Saito,

1990). This may allow for more efficient microbial action which could provide more available N for plant use (Lehmann et al., 2011). Alternatively, higher microbial activity may lead to higher immobilization of inorganic N due to enhanced nutrient uptake by microbial communities (Nguyen et al., 2017). While abiotic and biotic pathways of N retention with biochar addition present great potential for mitigating N losses, there is a growing body of literature that shows no effect of biochar application on SIN retention or N losses (Foster et al., 2016; Ramlow et al., 2019; Verhoeven & Six, 2014) and a few studies have even reported greater losses of N through leaching (Singh et al., 2010) or N₂O emissions (Bruun et al., 2011; Verhoeven & Six, 2014) with biochar addition. Thus, it is crucial that we continue to investigate the conditions that allow for N retention or losses with biochar addition to soils. This is especially important in the eastern plains of Colorado, where our soil was collected and where nitrate (NO₃⁻) contamination of groundwater is highly problematic (Rupert, 2003).

Plants represent another biotic pathway of N retention that may interact with biochar and their effect is often neglected in biochar N retention studies. In sustainable agroecosystems, the goal is to support growth and nutrition of plants while mitigating environmental harm. Many studies have assessed biochar's effect on N cycling, but few explicitly consider the role of plants in N cycling, their interaction with biochar, and how their presence is affected by, and could contribute to, a sustainable agricultural system. Beyond Weng et al. (2015), who consider the effects of biochar-plant interactions on soil carbon priming effects, we know of no other study that has explicitly considered the role of plants by assessing biochar effects with and without their presence.

While biochar shows clear promise, its effects are highly dependent on biochar feedstock and production conditions as well as the properties of the soil in which it is applied (Crane-Droesch et al., 2013; Gul & Whalen, 2016; Nguyen et al., 2017). To combat some of this variability, investigators have begun to develop “designer” or “engineered” biochars which are meant to benefit specific aspects of a given system, although this work has mostly focused on pollution removal in wastewater (Mayer et al., 2014; Rajapaksha et al., 2016; Yao et al., 2013). Engineered biochar involves a post-treatment following the pyrolysis process, thus resulting in biochar with more physio-chemically consistent properties than that from pyrolysis alone. These post-treated biochars generally retain lower amounts of toxic substances on their surfaces, which can inhibit nitrification (Clough & Condon, 2010), and have more consistent porosity and surface area, potentially translating to more effective N use. Engineered biochars may lead to differential effects on soil N cycling and thus warrant greater study in soil systems.

To better understand how raw and engineered biochars interact with plants in regulating N dynamics in temperate agroecosystems, we looked at the effect of three different biochars on the fate of N fertilizer with and without lettuce plants in a greenhouse experiment using temperate agricultural soils from the eastern plains of Colorado, USA. To test the efficacy of engineered biochars, we compared practical application rates of one raw biochar and two engineered biochars. We used ^{15}N isotopically labeled NO_3^- fertilizer to trace the fate of N fertilizer in the soil-plant system in response to our treatments. We hypothesized that treatments with engineered biochars and plants present would have the highest N recovery in the soil and plant

pools, and consequently the lowest N losses through leaching and N₂O emissions, due to plant N uptake and biochar-induced soil N retention. Additionally, we expected fertilizer NO₃⁻ to be more mobile in the system and to account for a greater proportion of plant uptake and soil losses, as compared to soil-derived N, and that biochar addition would reduce this mobility.

2. Materials and Methods

2.1 Soil and biochars

To assess the effects of biochar and plant presence on N cycling in a temperate agricultural soil, we performed a greenhouse experiment in the Colorado State University (CSU) Plant Growth Facilities in Fort Collins, CO. For this experiment, we used a soil classified as a Fort Collins clay loam (Halvorson & Stewart, 2015) from the CSU Agricultural Research, Development, and Education Center (ARDEC) located 6.5 km north of Fort Collins, Colorado (40°39'10.3"N 104°59'46.6"W). Soil was collected in March 2018 to 20cm depth and passed through an 8mm sieve, air-dried, and homogenized before taking initial measurements. Initial soil total C and N concentrations, NH₄⁺, NO₃⁻, and pH were measured as described below. Before the start of the experiment, soil contained 18.3 g C kg⁻¹ (including inorganic C), 1.16 g N kg⁻¹, 2.03 mg NH₄⁺-N kg⁻¹, and 10.3 mg NO₃⁻ kg⁻¹ and had a pH of 9.1.

Biochars were created through slow pyrolysis and produced from coconut shell or pine feedstocks. The coconut shell feedstock was pyrolyzed at a maximum temperature of 600°C to produce an initial “raw” coconut biochar (RCB) and an “engineered” coconut biochar (ECB) that was further treated to provide more uniform physio-chemical properties. An “engineered” pine biochar (EPB) was also produced by

pyrolysis to a maximum temperature of 650°C and post-processed. Post-processing of biochars is proprietary information, so specific processes used are not reported here (Cool Planet, Inc., Greenwood Village, CO). Biochars were characterized by ultimate analyses (ASTM D3176-15, 2015) performed by Hazen Research, Inc. (Golden, CO) and other properties were reported by Cool Planet Energy Systems, Inc. (Camarillo, CA). Properties of each biochar are presented in Table 1.

Table 1: Selected properties for the three biochars used in the experiment: Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB). Electrical conductivity (ECe) represents the salinity of the biochars and hydrophobicity was measured using the Molarity of an Ethanol Drop (MED) method, where higher numbers are more hydrophobic materials.

Biochar	Organic C (%)	H:C _{org} (molar ratio)	C:N (mass ratio)	Ash (%)	Moisture (%)	pH	Surface Area (m ² g ⁻¹)	ECe (dS m ⁻¹)	Porosity (cc ml ⁻¹)	Hydrophobicity	Bulk Density (g cm ⁻³)
RCB	72.8	0.480	158	5.41	6.80	6.60	230	4.35	0.25	3	0.451
ECB	66.6	0.414	162	14.76	5.01	6.77	230	3.17	0.23	2	0.632
EPB	76.4	0.330	273	4.59	7.65	7.20	288	0.36	0.50	0	0.420

2.2 Experimental design

The greenhouse experiment consisted of four biochar application treatments: Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB) and a control with no biochar (C); and two plant treatments (with and without lettuce; *Lactuca sativa* var. Black Seeded Simpson) in a full-factorial design with four replicates (n=32). Lettuce was chosen as test plant for its fast growth, relatively small size, and because it is commonly grown in greenhouses (Corey et al., 1996; Dickerson, 1996).

The experiment was conducted using pots constructed out of 20 cm diameter PVC pipe cut to 20 cm lengths with a flat PVC base. A small hole (12 mm diameter) was drilled into the bottom of each pot to allow for drainage. A connector was fit into the hole and tubing was attached to ease collection of leachates as described below. This hole

was covered on the inside of the pot with double-layered window screen (1mm mesh size) to prevent soil loss (Fig. 1). A PVC chamber (7.5 cm tall x 20 cm dia.) was fit to the top of the PVC pot for headspace sampling for N₂O flux measurements as described below. A hole (0.64 cm) drilled into the side of each chamber fitted with a 10 cm-long stainless-steel tube was used for ventilation. A thermocouple and septa were installed in the top of the chamber for measurement of chamber air temperature and chamber air collection, respectively. Reflective tape was adhered to the top of the chamber to minimize temperature change in the chamber during the measurement period. Rubber weather seal attached to the bottom of the chamber and tire innertubes cut to 5 cm widths were used to ensure an airtight seal between the pot and chamber during gas measurement (Fig. 1).

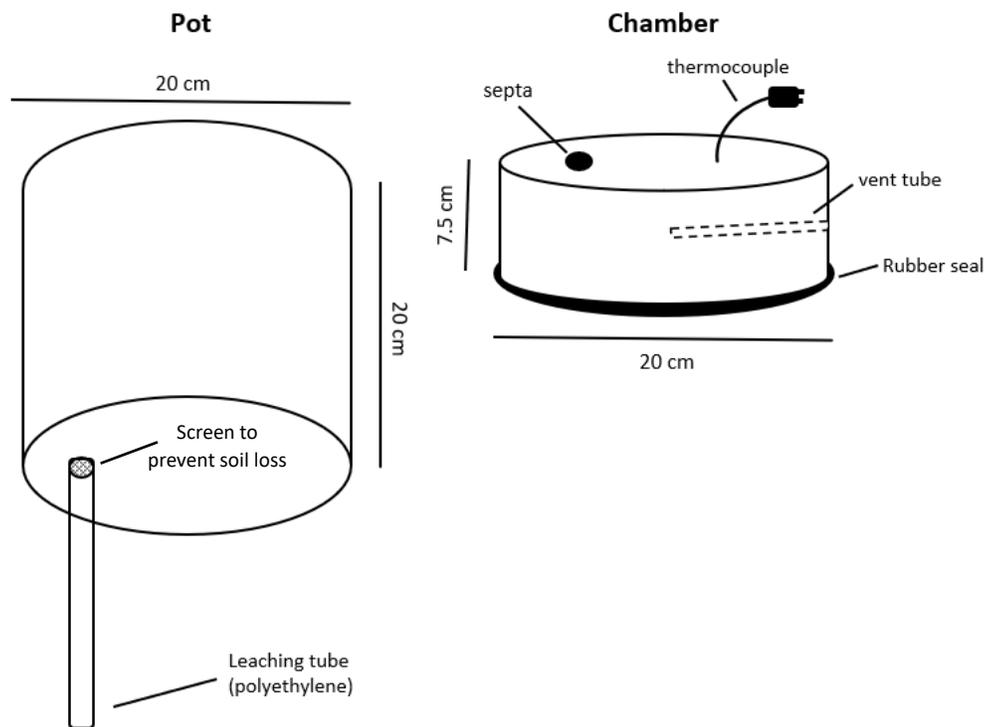


Figure 1: Pot and chamber created from 20cm diameter PVC pipe for the greenhouse experiment.

2.3 Greenhouse Experiment

The greenhouse experiment began on April 11th, 2018, when one lettuce seedling was planted in each pot, where applicable. Before planting, pots were filled with 8 mm-sieved, homogenized soil mixed with pure quartz sand at a 1.5:1 soil to sand ratio. Biochar was applied to respective pots before planting at a rate of 1% by volume (i.e., equivalent to a field application rate of 5-8 t ha⁻¹ depending on biochar bulk density; Table 1). The bottom of each pot was filled with 2.54 cm of sand to prevent soil loss and to facilitate leachate movement. Phosphorous (P) was added as Triple Super Phosphate (TSP) according to local recommendations at a rate of 224 kg P₂O₅ ha⁻¹ (150 mg P kg⁻¹ soil) directly before transplanting the lettuce seedlings from potting soil (Ells, Schwartz, & Cranshaw, 1990). To trace fertilizer-derived N through the system, 6.93 g L⁻¹ of isotopically labelled KNO₃ (98 at. % ¹⁵N, Sigma-Aldrich, Darmstadt, Germany) was mixed with 94.24 g L⁻¹ of unlabeled KNO₃ (0.37 at. % ¹⁵N, Fisher Scientific, Waltham, MA), to create the target label for the experiment of 6.5 at. %¹⁵N. To obtain the fertilizer endmember for the mixing model, a sub-sample of the fertilizer solution was freeze-dried and measured on an elemental analyzer (Costech Analytical Technologies, Inc., Valencia, CA) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA), referenced below as the EA-IRMS. The isotopically diluted KNO₃ (6.5 at. %¹⁵N) was added at a rate of 50 mg N kg⁻¹ soil (equivalent to 112 kg N ha⁻¹) split into two applications: (1) directly before the lettuce seedlings were transplanted and (2) 16 days after planting, to ensure that the lettuce plants had adequate N availability throughout the growing period (Ells, Schwartz, & Cranshaw, 1990). To simulate burial of fertilizer N in field plots, the ¹⁵N labeled KNO₃

was distributed vertically by drawing a syringe inserted to 5 cm depth out of the soil as the plunger was pushed down and was distributed horizontally by adding the fertilizer at 5 equally-spaced points in the pot. Six pots without labeled N treatment were also established to obtain the natural abundance ^{15}N values for subsequent calculations (see below).

All pots were watered bi-weekly to a pre-determined field capacity, based on weight. Field capacity was determined previously in the lab for each biochar-soil treatment separately to ensure uniform water potential across treatments. Briefly, field capacity was determined by taking the average difference in weight between three replicates of oven dry soil/sand or soil/sand/biochar mixtures and mixtures that were kept saturated in water for 24 hours. The target field capacities were 0.23, 0.27, 0.31, and 0.28 g water g⁻¹ dry media for C, RCB, ECB, and EPB, respectively.

The experiment was terminated when lettuce reached maturity, 36 days after initial transplanting to pots, on May 17th, 2018.

2.4 Evaluation of N fluxes

N lost through leaching and N₂O emissions was measured throughout the greenhouse experiment. Leachates were collected every two weeks during the growth period (days 6, 20, 33). The first leachate collection occurred before adding the fertilizer and plant to establish a baseline for each pot. Leachates were collected by adding water in excess of the calculated volume needed to reach field capacity and waiting until water flowed from the hole in the bottom of the pot (Fig. 1). Amount of water added to induce leaching in each pot (300-1340 mL) as well as amount of leachate collected per pot (53-290 mL) were recorded. Leachate samples were then immediately frozen until

analysis. After leaching, soil water was assumed to return to field capacity, as field capacity is defined as the soil water content after allowing drainage by gravity. For non-leaching weeks (days 1, 13, 27), watering was achieved by weighing pots and then adding water to return pots to their estimated field capacity. For leachate analysis, samples were thawed, diluted 1:10 sample: deionized water, and measured for total N on a total nitrogen (TN) analyzer (Shimadzu Corp., Kyoto, Japan) for each leachate sampling. Leachates were then refrozen for three weeks and then thawed and pooled by microcosm across sampling times for analysis of N isotopic composition using the EA-IRMS. To prepare the composite samples for isotopic analysis, 25 mg of K_2SO_4 was added to 4 mL of each pooled sample and freeze-dried.

N_2O sampling occurred weekly, two days after watering (on days 2, 8, 15, 22, 29) and a baseline measurement was taken before plant transplanting. Headspace air was sampled using the static chamber method (Parkin & Venterea, 2010). After placing the chamber on top of a pot, three headspace air samples were drawn (at 0, 10 and 20 min after closure). N_2O was measured for six pots at a time using six separate chambers such that the time 0, 10, and 20 samples were all collected at similar times (~1.5 min apart) for those six pots. For each sample, chamber air was mixed by drawing 35 mL of chamber air in and out of the syringe twice and a 25 mL sample was taken and transferred to a previously evacuated Exetainer® 12 mL glass vial. N_2O concentrations in each vial were measured within a week from collection on a fully-automated Gas Chromatograph (Varian 3800, Varian Inc., Palo Alto, CA) at the USDA Agricultural Research Service in Fort Collins, CO. Nitrous oxide flux was calculated using linear regression over time or, when the data curvi-linearity index was greater than 1.2 (Parkin

& Venterea, 2010), by using the Hutchinson-Mosier method (Hutchinson & Mosier, 1981) to account for decreased rate of N₂O efflux over chamber deployment time. Approximately 1/3 of the data was calculated using the Hutchinson-Mosier method, with the rest calculated using linear regression. Fluxes that fell below the detection limit were given a value of zero (~40% of the data). Cumulative N₂O emissions over the growing period were calculated using linear integration of the weekly flux measurements (Parkin & Venterea, 2010).

2.5 Plant and bulk soil measurements

At harvest, each lettuce plant was cut at the base for determination of total aboveground biomass. Fresh samples were weighed, and a subsample was collected for determination of dry weight and for use in subsequent analyses. Each PVC pot was then emptied on top of an 8 mm sieve and roots were carefully collected from soil passing through the sieve for further analyses. A subsample of the soil was collected and refrigerated until analysis described below. A subsample of the 8mm sieved soil was sieved to 2mm. All plant root and aboveground biomass as well as a subsample of the 2 mm-sieved soil were oven-dried at 60°C, finely ground, and sub-samples were measured for total and isotopic N concentration on the EA-IRMS. Soil pH was measured on a mixture of air-dried, 2 mm-sieved soil and water (2:1 ratio by mass) using a pH electrode (Expandable IonAnalyzer EA 940, Orion Research, Jacksonville, FL).

2.6 Soil N pools

Soil NH₄⁺ and NO₃⁻ concentrations were determined by extracting a subsample (10 g) of the 8 mm-sieved bulk soil using 2M KCl (5:1 KCl/soil ratio by mass), shaking

for 1 hour, filtering (Whatman #40 ashless filter paper), and analyzing the extraction colorimetrically (Alpkem Flow Solution IV Automated wet chemistry system; O.I. Analytical, College Station, TX) (McTaggart & Smith, 1993) within one week from harvest. Soil inorganic N extractions were freeze-dried, and isotopic compositions were determined on the EA-IRMS. As a proxy for microbial biomass N (MBN), we used the chloroform-fumigation extraction method (Brooks et al., 1985) on 8 mm-sieved bulk soil, modified from a 24 hour to a 5-day incubation to ensure adequate time for chloroform to enter biochar pore spaces (Foster et al., 2016), with extractable N measured on the TN analyzer, within a week from harvest. Following Foster et al. (2016), we assessed biochar effects on N extraction efficiency by adding each biochar at the same rate as for the greenhouse experiment to 4 replicates of control soils immediately before fumigation. Nitrogen extracted from control soils which did not receive the biochar was not significantly different from those with biochar added, so uncorrected values were used for analysis. We used extractable N from the non-fumigated soils as the total dissolved N (DN) concentrations in the soil.

2.7 Fertilizer-N recovery

To determine the contribution of fertilizer-derived and soil-derived N to each N pool, we used the isotopic mixing model (Hauck & Bremner, 1976). The fraction of fertilizer-derived N (f_F) in the pools of interest was determined using the equation:

$$f_F = ({}^{15}\text{N}_s - {}^{15}\text{N}_N) / ({}^{15}\text{N}_F - {}^{15}\text{N}_N)$$

where ${}^{15}\text{N}_s$, ${}^{15}\text{N}_F$, and ${}^{15}\text{N}_N$ are the atom % ${}^{15}\text{N}$ of the sample, fertilizer (6.5 atom % ${}^{15}\text{N}$), and the natural abundance control sample, respectively. The model was applied to individual treatment reps and those values were used to calculate fractional contribution

of different sources to each measured pool by replicate. The natural abundance control values represent averages across controls with biochar (n=3) and those without (n=3). Averages of the biochar/soil and soil controls were used in models for biochar and control treatments, respectively. Fraction of N derived from the soil was determined using the equation $f_N = 1 - f_F$. Nitrogen concentrations in natural abundance controls for SIN were too low to obtain isotopic values. Thus, total soil N natural abundance values were used for the soil-derived endmember in the mixing models for SIN. While fractionations can be as high as -35‰ for nitrification (Hogberg, 1997), natural abundance fractionations can be ignored in heavy isotope enrichment experiments, such as this, because this fractionation has a negligible effect on the final distribution of the heavy isotope (Fry, 2006).

2.7 Statistical analyses

Statistical analyses were carried out using R statistical software (R Core Team, 2017). Two-way ANOVA was used to examine the influence of biochar, plants, and their interaction on soil properties and N recovery in soil pools and one-way ANOVA was used to assess biochar effects on plant growth and N uptake (car; Fox and Weisberg, 2011). Pairwise comparisons (emmeans; Lenth, 2018) were used to determine differences within treatments and among fractional contributions from fertilizer-derived or soil-derived N. Repeated measures analysis was used to assess patterns of N₂O emission and N leachate over time. When response variables did not fit the assumptions of the linear model, natural log and Box-Cox power transformations were assessed and applied for data analysis (Box & Cox, 1964). Total recovery data was analyzed using a reciprocal transformation to meet the assumptions of a normal

distribution. Pot water content varied between treatments during the growing period as a result of the imposed leaching events and our watering approach, and ECB treatment pots ended up producing a greater volume of leachate than the control pots (Table 2). Given that differences in pot water content (a proxy for water-filled pore space) may have affected plant growth and N dynamics, average pot weight following leaching and watering over the entire growth period (a proxy for water content) was added as a covariate to the model (Table 2). For models where adding the covariate changed results, statistics from that model are presented. Differences between treatments were considered significant at $p < 0.05$.

Table 2: Differences in cumulative leachate volume and average pot weight over the growing season and between treatments presented as average values \pm standard errors ($n=4$). Letters indicate significance differences between treatments, where biochar type is represented as Control (C), Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB).

	<i>Experimental Treatments</i>							
	<i>C</i>		<i>RCB</i>		<i>ECB</i>		<i>EPB</i>	
	<i>Plant</i>	<i>No Plant</i>	<i>Plant</i>	<i>No Plant</i>	<i>Plant</i>	<i>No Plant</i>	<i>Plant</i>	<i>No Plant</i>
<i>Total</i>								
<i>Leachate</i>	689.50	673.75	688.75	711.50	1069.50	1301.00	801.50	901.25 \pm
<i>Volume</i>	$\pm 55.22^A$	$\pm 70.93^A$	$\pm 50.52^A$	$\pm 29.43^A$	$\pm 26.96^A$	$\pm 245.84^B$	$\pm 90.87^A$	80.96^{AB}
<i>(mL pot⁻¹)</i>								
<i>Average</i>								
<i>Pot Weight</i>	8463.16	8523.10	8525.77	8578.06	8502.18	8666.64	8491.65	8543.19
<i>(g)</i>	$\pm 64.48^A$	$\pm 14.29^A$	$\pm 33.77^A$	$\pm 31.61^A$	$\pm 7.13^A$	$\pm 19.23^A$	$\pm 46.67^A$	$\pm 28.11^A$

3. Results

3.1 Plant growth

There was a tendency towards higher aboveground biomass and higher total biomass for plants under ECB (Table 3). However, there were no significant effects of biochar treatment on any plant growth parameter (i.e., aboveground, belowground, and total biomass, and shoot:root ratio). Overall, plant growth and survival were good,

however, one control plant had to be replaced at day 16. Excluding this plant in analysis of biomass and N uptake had no effect on statistical results.

3.2 Treatment effects on N pools and pH

3.2.1 Plant N

Plant N uptake mirrored plant growth in that there were not significant biochar effects on shoot or root N content (Table 3). There was a tendency for higher aboveground N uptake in the ECB treatment, but this was not significant for total N, fertilizer-derived N, or soil-derived N (Table 3). Shoots in the ECB treatment had approximately double the amount of mean total, fertilizer-derived, and soil-derived N as compared to the other biochar treatments. This pattern was largely driven by plant biomass differences, as plant N concentrations were very similar across biochar treatments (data not shown). Fertilizer-derived N made up 73-75% of shoot N and 72-75% of root N, which was significantly higher than plant N derived from the soil ($p < 0.001$).

Table 3: Plant growth and uptake data presented as mean \pm 1 standard error (n=4) for each biochar type: Control (C), Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB). Biochar was not a significant predictor for any plant growth factor.

	Biochar Type				
	C	RCB	ECB	EPB	
Aboveground biomass (g)	2.32 \pm 0.71	2.30 \pm 0.58	3.83 \pm 1.36	2.37 \pm 0.54	
Belowground Biomass (g)	0.41 \pm 0.08	0.39 \pm 0.08	0.52 \pm 0.08	0.42 \pm 0.06	
Total Biomass (g)	2.73 \pm 0.78	2.69 \pm 0.58	4.35 \pm 1.35	2.79 \pm 0.56	
Shoot: root ratio	5.14 \pm 1.20	6.42 \pm 1.90	7.91 \pm 3.35	5.99 \pm 1.37	
Shoot N Uptake (mg N)	Total	104.30 \pm 28.68	97.76 \pm 7.94	182.73 \pm 70.02	112.05 \pm 26.67
	Fertilizer	76.78 \pm 21.26	73.35 \pm 5.86	133.73 \pm 49.23	81.95 \pm 18.63
	Soil	27.51 \pm 7.86	24.42 \pm 2.17	49.00 \pm 20.97	30.09 \pm 8.11
Root N Uptake (mg N)	Total	13.31 \pm 2.51	7.19 \pm 1.10	13.98 \pm 1.12	10.87 \pm 2.55
	Fertilizer	9.68 \pm 1.63	5.40 \pm 0.97	10.48 \pm 0.78	8.15 \pm 1.97
	Soil	3.63 \pm 0.98	1.80 \pm 0.17	3.50 \pm 0.37	2.72 \pm 0.59

3.2.2 Soil N

In contrast to its effects on plant growth, biochar had significant effects on soil N pools, especially when considering N derived from fertilizer (Table 4). Biochar treatment was a significant predictor of total, soil-derived, and fertilizer-derived soil N.

Unsurprisingly, soil-derived N made up a significantly higher portion of the total soil N pool than fertilizer-derived N ($p < 0.01$) and pairwise comparisons show the control as having significantly higher soil-derived N concentrations than EPB ($p < 0.01$) (Table 4). Similar effects were observed for fertilizer-derived N concentrations, but both the control and RCB were significantly higher than EPB and ECB ($p < 0.05$) (Table 4). Plant presence generally reduced the amount of N in soil pools, especially for fertilizer-derived N – pots without plants had 1.5 times higher fertilizer-derived soil N ($p < 0.001$) (Table 4).

Dissolved, mineral, and microbial biomass soil N pools were differentially affected by biochar application and plant presence (Fig. 2). Biochar had significant effects on total soil NH_4^+ concentrations and total MBN, but biochar was not a significant predictor for DN, dissolved organic N (DON), or total, soil-derived, or fertilizer-derived NO_3^- concentrations (Table 4). Pairwise comparisons indicate significantly higher soil NH_4^+ concentrations in the control as compared to EPB ($p = 0.003$) and significantly higher MBN in RCB as compared to ECB ($p < 0.02$). Pots without plants had significantly higher total, fertilizer-derived, and soil-derived NO_3^- ($p < 0.05$; Fig. 2). In contrast, pairwise comparisons indicate higher soil NH_4^+ in plant treatments rather than no plant treatments ($p < 0.001$) and plant presence had no effect on MBN (Fig. 2).

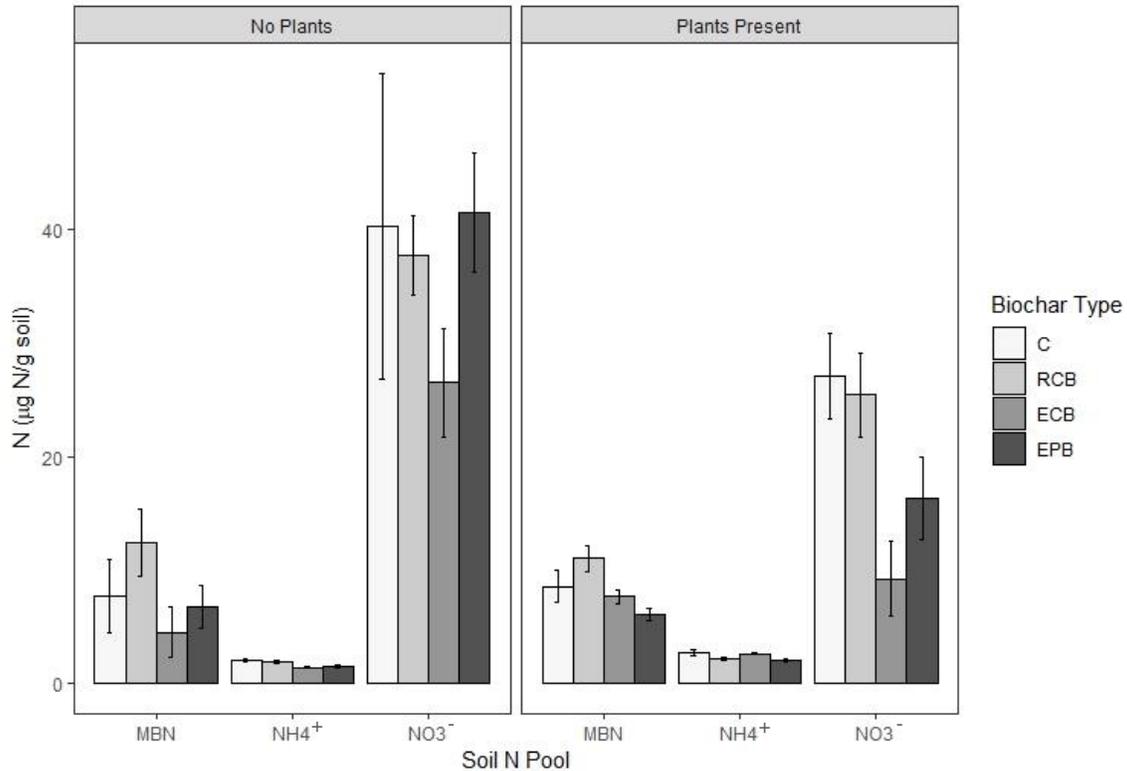


Figure 2: Soil N concentrations for each biochar type with and without plants. Error bars represent 1 standard error (n=4). The main effect of biochar was significant for MBN and NH₄⁺ ($p < 0.025$) and the main effect of plant was significant for NH₄⁺ and NO₃⁻ ($p < 0.005$). The interaction term was not significant so differences between plant and no plant within a given biochar type were not analyzed. Biochar types are represented as Control (C), Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB).

Biochar was not a significant predictor of soil pH, although there was a tendency for higher pH in ECB and EPB, with mean values ~0.1 U higher than those with C and RCB, respectively (Table 4). Plant presence had no effect on soil pH.

3.2.3 N losses through leaching and N₂O

Engineered biochars had higher N losses through leaching than the control or RCB treatments. Biochar effects on total leachate N losses were driven by differences in fertilizer N loss among treatments, but fertilizer N did not make up a significantly higher proportion of leachate N losses across treatments ($p = 0.1$; data not shown). ECB had significantly higher cumulative leachate total and fertilizer-derived N losses

than C or RCB ($p < 0.006$). This pattern is driven by significantly higher leachate N losses in ECB as compared to RCB and the control for the first and second leaching events ($p < 0.04$; data not shown), significantly higher fertilizer N concentrations in leachate from ECB as compared to RCB ($p < 0.05$; Fig. 3), and significantly higher volume of leachate loss in ECB compared to all other biochar treatments ($p < 0.01$; Table 1). In models of leachate N concentration, pot weight (a proxy for water content), as well as biochar type, were significant predictors of soil-derived leachate N concentration (Fig. 3). Plant presence generally reduced the magnitude of leachate N losses, especially for N derived from fertilizer. Plant presence reduced leaching losses by 21% for total N ($p < 0.01$) and 27% for fertilizer N ($p < 0.01$). This pattern was largely driven by significantly higher leachate N losses from treatments without plants for the third leaching event ($p < 0.001$; data not shown). We did not assess pot water content as a covariate for total leachate N loss because this metric inherently includes differential volumetric loss between treatments. Plant was not a significant predictor of total, soil-derived, or fertilizer-derived leachate N concentrations (Fig. 3). There were not significant differences between biochar types or plant presence for N_2O emissions (Table 4). Interactions between biochar and plant presence were not significant for leaching or N_2O emissions.

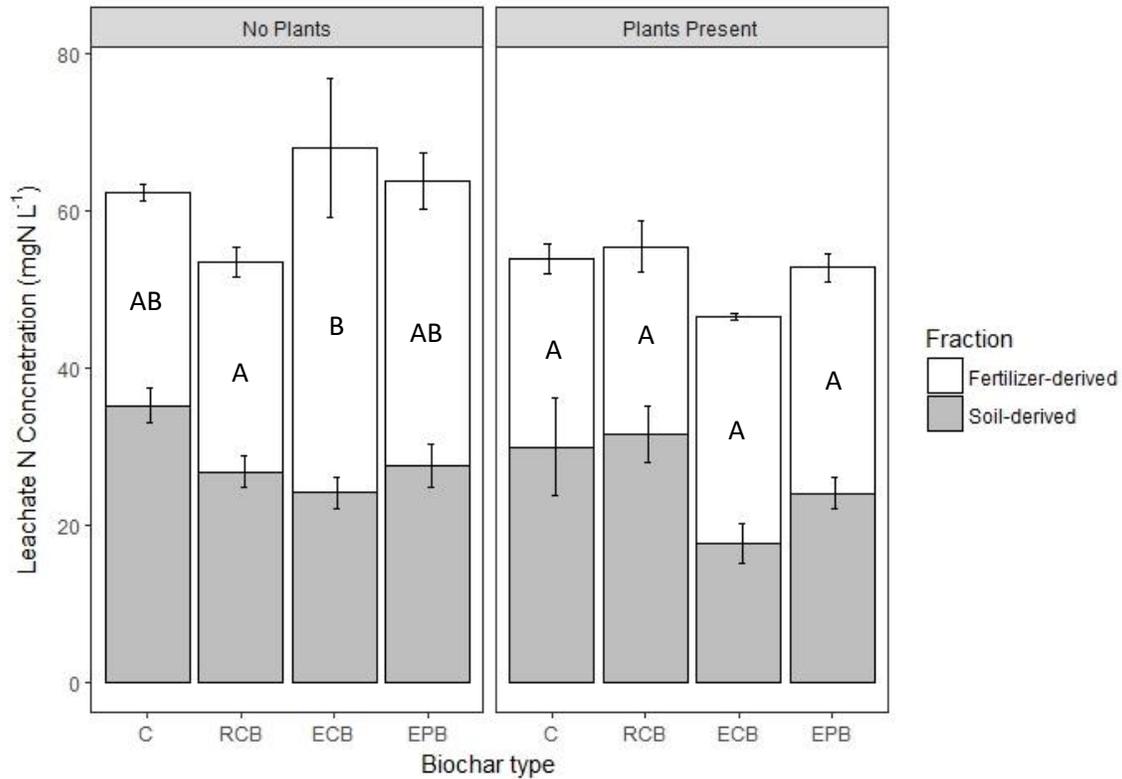


Figure 3: Average leachate N concentration for each biochar type, with and without plants, broken into fertilizer- (white) and soil-derived (grey). Error bars represent 1 standard error (n=4). Letters denote significant difference in fertilizer-derived leachate N concentration from other biochar types within a plant group when average pot weight is included as a covariate. There were not significant differences between plant and no plant for total, soil-derived, or fertilizer-derived leachate N concentrations. The interaction term was not significant so differences between plant and no plant within a given biochar type were not analyzed. Average pot weight was a significant predictor of soil-derived leachate N concentration. Biochar types are represented as Control (C), Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB).

Table 4: Chemical analyses presented as mean \pm standard error and their associated p-values for each biochar type: Control (C), Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB), with and without plants. Blank spaces indicate that the p-value was above $\alpha = 0.05$. The interaction term and the covariate were either not significant or not applicable for all pools displayed below with the exception of total soil N (covariate, $p = 0.002$).

Pool	Fraction	Experimental Treatment								Biochar Significance	Plant Significance		
		C		RCB		ECB		EPB					
		Plant	No Plant	Plant	No Plant	Plant	No Plant	Plant	No Plant				
Soil N (mg N g ⁻¹ soil)	Total	82.02 \pm 5.22	89.75 \pm 2.08	76.78 \pm 3.78	80.91 \pm 1.69	78.54 \pm 3.34	75.31 \pm 1.20	70.73 \pm 7.77	73.08 \pm 0.77	<0.001			
		3.09 \pm 0.32	4.56 \pm 0.05	3.09 \pm 0.39	4.51 \pm 0.32	1.47 \pm 0.41	2.64 \pm 0.38	2.14 \pm 0.43	3.43 \pm 0.15				
	Fertilizer	78.93 \pm 5.32	85.19 \pm 2.10	73.69 \pm 3.49	76.40 \pm 1.38	77.07 \pm 3.24	72.68 \pm 1.42	68.60 \pm 7.37	69.65 \pm 0.62			0.033	
		Soil	34.70 \pm 5.20	42.71 \pm 3.86	36.93 \pm 3.86	38.08 \pm 3.41	50.62 \pm 3.05	85.23 \pm 9.61	42.97 \pm 4.31			58.83 \pm 6.41	0.002
	Cumulative Leachate N Loss (mg N)		15.45 \pm 1.95	18.76 \pm 2.33	15.65 \pm 1.42	19.12 \pm 2.28	31.36 \pm 0.292	54.51 \pm 12.69	23.54 \pm 2.88			33.74 \pm 4.85	<0.001
		Soil NO ₃ ⁻ (μ g N g ⁻¹ soil)	19.25 \pm 3.75	23.95 \pm 1.71	21.28 \pm 2.81	18.96 \pm 1.42	19.25 \pm 2.83	30.71 \pm 6.52	19.43 \pm 1.56			25.09 \pm 2.38	
Soil NO ₃ ⁻ (μ g N g ⁻¹ soil)	10.92 \pm 1.73		22.46 \pm 7.60	12.02 \pm 1.78	17.56 \pm 2.91	2.49 \pm 1.38	13.82 \pm 3.45	3.69 \pm 1.15	20.26 \pm 3.69		0.001		
	Dissolved Total N (μ g N g ⁻¹ soil)	16.17 \pm 2.29	17.81 \pm 6.13	13.40 \pm 2.22	20.14 \pm 0.87	6.73 \pm 1.93	12.70 \pm 1.9	12.67 \pm 2.74	21.22 \pm 2.34		0.042		
N ₂ O Flux (mg N ₂ O m ⁻²)		409.88 \pm 36.94	494.20 \pm 76.81	389.88 \pm 11.29	448.49 \pm 30.97	306.74 \pm 23.26	434.75 \pm 20.61	347.79 \pm 19.20	511.01 \pm 40.66		<0.001		
	pH	19.77 \pm 3.47	13.35 \pm 4.17	29.65 \pm 5.58	32.08 \pm 5.05	43.77 \pm 5.82	32.86 \pm 9.19	41.77 \pm 10.23	35.33 \pm 16.48				
		8.57 \pm 0.03	8.52 \pm 0.04	8.56 \pm 0.03	8.56 \pm 0.03	8.65 \pm 0.43	8.68 \pm 0.03	8.76 \pm 0.07	8.57 \pm 0.06				

3.2.4 Recovery of fertilizer N

Total recovery of fertilizer N was significantly affected by biochar type ($p = 0.01$; Fig. 4), with pairwise comparisons showing non-significantly higher recovery in the control as compared to ECB and EPB ($p < 0.08$). Treatments with plants present had higher average recoveries (77-89%) than treatments without plants (64-83%) for a given biochar type but main effects of plant and the interaction were not significant (Fig. 4). Partitioning the recovery by aboveground biomass, belowground biomass, soil, and leachate pools mirrors patterns of N uptake and N leaching presented above, which indicated lower soil N in ECB, higher leaching losses with the engineered biochars, reduced leaching losses with plants present, and minimal differences between the control and RCB.

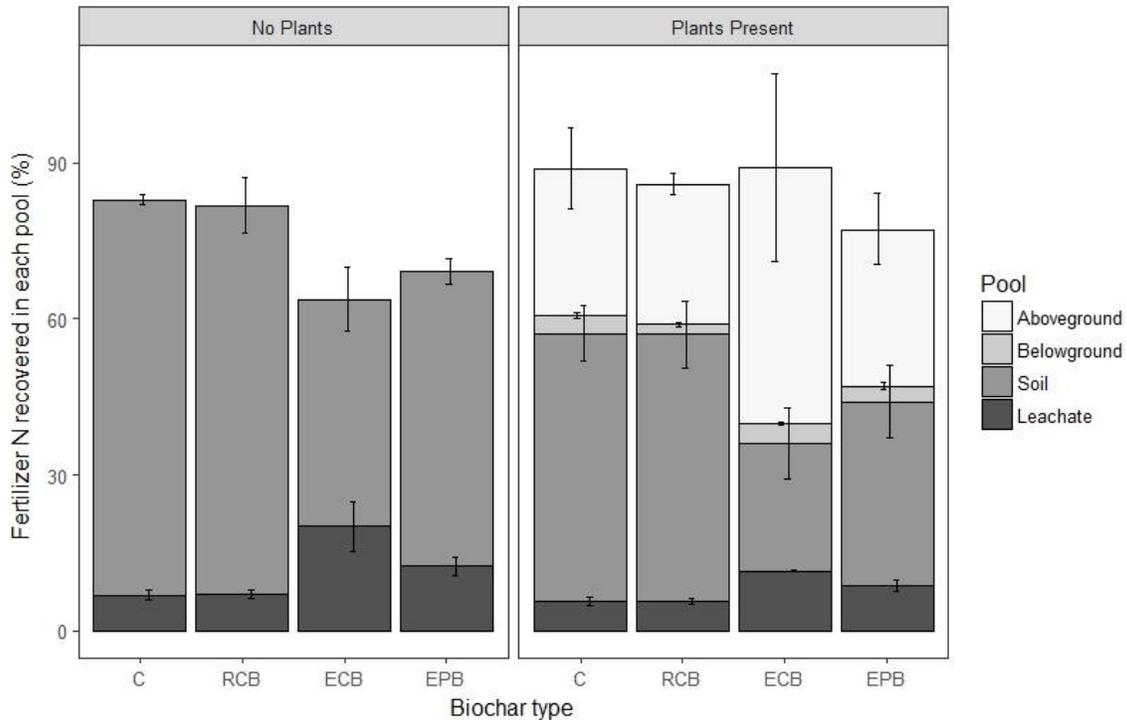


Figure 4: Recovery of fertilizer N in each pool with and without plants present. Error bars represent standard error ($n=4$). Biochar types are represented as Control (C), Raw coconut shell biochar (RCB), Engineered coconut shell biochar (ECB), and Engineered pine biochar (EPB).

4. Discussion

4.1 *Biochar-plant interactions*

While biochar has been shown as a promising tool for reducing SIN and N losses through leaching and N₂O emissions (Cayuela et al., 2014; Laird et al., 2010; Nguyen et al., 2017), our results largely contradicted this, falling in line with other recent studies reporting no effects on or increased N losses with biochar addition to the system (Foster et al., 2016; Ramlow et al., 2019; Singh et al., 2010; Verhoeven & Six, 2014; Bruun et al., 2011). Additionally, we saw the largest biochar effects with our engineered (post-processed) biochar treatments, which have rarely been considered in agronomic contexts (although the soil contaminant literature is rich: reviewed in (Rajapaksha et al., 2016)), and thus are difficult to directly compare to data on biochar N interactions reported in previous research.

In contrast, we found that the presence of plants is useful for reducing losses of N, which is not surprising, as plants assimilate N for their growth. In our study, biochar and plants had no interacting effects, even for pools and fluxes for which they both, individually, had significant effects. This was surprising since there seemed to be differential effects of biochar type when plants were or were not present. We know of only one other study that has assessed biochar effects with and without plants and they found important interactions between these (Weng et al., 2015) but this discrepancy could be due to differences in response variables, biochar properties, or study systems. Further studies considering biochar-plant interactions will elucidate the validity of lab experiments without plants present for understanding effects of biochar application in cropping systems. These studies would be especially important in areas where N loss

can be very high and plant presence could have significant benefits, such as in sandy soils or areas with high fertilizer N application.

4.2 Effect of N origin on N cycling

Few studies have considered biochar effects on NO_3^- fertilizer, which would be expected to behave differently than NH_4^+ or organic N fertilizers. As we predicted, fertilizer N was much more mobile in the system as compared to soil-derived N and was more significantly affected by plant presence and biochar addition. Fertilizer N made up a higher proportion of plant N uptake, drove patterns of N loss through leaching, and was generally more affected by plant presence than soil-derived N. We observed significantly higher fertilizer N concentrations in leachates from ECB treatments, indicating an N mobilization effect for ECB amended soils that goes beyond higher volumetric losses in this treatment. We know of only one other study that has explicitly studied the effects of biochar on N cycling with NO_3^- fertilizer application but they did not measure N leaching (Nelissen et al., 2014), which is especially important to study when applying a highly mobile fertilizer like KNO_3 . Additionally, they did not apply an isotopically labelled fertilizer, so they were unable to distinguish between losses of soil- and fertilizer-derived N, for which we saw the largest leaching losses. However, Nelissen et al. (2014) did study other forms of N loss and found reduced emission of NO and N_2O with application of NO_3^- fertilizer and biochar, which they attributed to reduced soil NO_3^- . In contrast, we found no effect of biochar on N_2O emission or soil NO_3^- , but this may be attributed to our use of engineered biochar or our comparatively low application rate ($5\text{-}8 \text{ t ha}^{-1}$ as compared to 20 t ha^{-1}), which would reduce the likelihood

of the abiotic immobilization pathway that was suggested to reduce soil NO_3^- and consequently N_2O emission (Nelissen et al., 2012).

The general reduction in fertilizer N pools and losses with plant presence found here are to be expected. While NH_4^+ is more energetically favorable for plant uptake, availability of N is more important than assimilation cost (Andersen et al., 2017). Since NO_3^- was 3-20 times higher in our soils compared to NH_4^+ , the expected greater plant uptake of NO_3^- likely led to reduced fertilizer-derived soil N and N loss in our study.

4.3 Biochar and plant effects on soil N cycling

We expected all biochar types to have somewhat similar effects on N cycling in our system, with respect to the control, with differences in the strength of effects between engineered and raw biochar. However, responses grouped similarly in engineered biochars vs. the raw biochar and control. The raw biochar and the control had higher soil-derived and fertilizer-derived soil N concentrations relative to the engineered biochars. These results contrast findings from a meta-analysis of increased total soil N with biochar addition, which were attributed to N input directly from the biochar (Biederman & Harpole, 2013). Our biochars were applied at low application rates (1% by volume; 0.39-0.58% by weight, depending on bulk density), to assess the validity of cost-effective biochar applications, and had low N contents (<0.5%), so addition of N directly from biochar amendment was limited. It is possible that high losses of fertilizer-derived soil N from engineered biochar treatments in the forms of leaching and plant uptake led to reduced soil N in these treatments.

Both organic and mineral N can represent important substrates for agricultural plant uptake and biochar may affect the availability of these (Clough et al., 2013;

Nasholm et al., 2000). Because we saw no differences in plant uptake of N between biochar treatments, it is unlikely that biochar limited N bioavailability. Biochar did not have effects on dissolved N pools in the soil. This is somewhat surprising as the few other studies who have measured these have found increased DON concentrations and sorption with biochar amendment, which were attributed to biochars high surface area and physical entrapment of DON in biochar pores (Güereña et al., 2013; Prommer et al., 2014). Since DON accounted for 90-99% of DN at the end of the experiment, we can expect similar mechanisms to explain the effect of biochar on both pools. The discrepancy in our results is likely due to our considerably lower application rates (5-8 t ha⁻¹ as compared to 12-72 t ha⁻¹) which would reduce the effects of biochar physio-chemical properties.

A recent meta-analysis by Nguyen et al. (2017) indicated that biochar leads to general reduction in both NH₄⁺ and NO₃⁻ availability and our data showing reduced NH₄⁺ concentrations in EPB-amended soils as compared to the control agree with this. Reduced NH₄⁺ concentrations are generally attributed to sorption to negatively charged biochar surfaces or increased microbial immobilization (Deenik et al., 2010; Yao et al., 2012). Microbial immobilization did not seem to play a role, as MBN concentrations were very similar for the control and EPB. However, sorption could be the driving process for this result, as EPB had the highest surface area of the biochars applied (288 m² g⁻¹ vs. 230 m² g⁻¹ for RCB and ECB) and was thus likely to have the highest sorption potential. Higher NH₄⁺ sorption supports our hypothesis of increased N retention in engineered biochars. However, we did not find any difference in total, soil-derived, and fertilizer-derived NO₃⁻ in any of our treatments. This lack of effect has been reported by

a recent meta-analysis (Borchard et al., 2019) and found for a field experiment using the same soil type as ours (Ramlow et al., 2019), indicating that this soil type may not be ideal for biochar-induced NO_3^- retention, because its relatively high clay content may already provide sorption effects also provided by biochar. However, a study with a similar soil amended with fertilizer and two different sized biochars found reduced NO_3^- with biochar addition, albeit with a higher biochar application rate, which may explain this discrepancy (Zheng et al., 2012). Plant presence reduced NO_3^- and DN availability but was associated with increased NH_4^+ availability. This indicates that plants were not using NH_4^+ as a substrate, which is not surprising given the much higher availability of NO_3^- (see Section 4.2).

4.4 Soil N losses

Losses in the form of leaching were more affected by biochar application and plant presence than losses as N_2O emissions. We found higher losses through leaching in engineered biochar treatments as compared to the control and RCB. These high losses from engineered biochar-amended soils may explain reduced total and dissolved soil N in these treatments. While differences in watering are inherently included in cumulative leachate losses, we also saw significantly higher fertilizer-derived N concentrations in leachate under ECB as compared to RCB, indicating very high mobile N concentrations in ECB, where we would expect a diluted signal due to higher volumetric leachate loss. This was contrary to our expectations, as we expected the lowest losses in our engineered biochar treatments. Additionally, greater N loss through leaching with biochar addition is rarely seen in the literature. The majority of biochar studies report reduced leaching of NO_3^- (Haider et al., 2017), NH_4^+ (Ding et al., 2010; Singh et al.,

2010), or both (Laird et al., 2010; Xu et al., 2016) and a recent meta-analysis found a 13% average reduction in NO_3^- leaching with biochar addition (Borchard et al., 2019). We found no effect of biochar on leachate N concentration in RCB and EPB as compared to the control. Anion exchange capacity (AEC) and, consequently, the ability of biochar to reduce NO_3^- leaching, has been posited to develop over time (Borchard et al., 2019; Kammann et al., 2015). We performed a relatively short (36 day) experiment, which could explain the lack of biochar effect for EPB and RCB. However, we only know of two other studies that have reported increased NO_3^- leaching with biochar addition and they attribute their findings to low AEC of the biochar and biochar-induced increases in nitrification (Laird et al., 2010; Singh et al., 2010). The suggested nitrification mechanism does not align with our data, as we see the lowest soil-derived NO_3^- for ECB. The AEC mechanism suggested by Singh et al. (2010) is plausible for our study as NO_3^- sorption has been shown to decrease with greater pH (Fidel et al., 2018) and our ECB treatment had higher mean pH than the control or RCB, albeit not significantly higher and by only 0.1 units. Additionally NO_3^- leaching has been shown to increase with biochar addition, on average, for soils with pH greater than 7 and for low biochar application rates ($<10 \text{ Mg ha}^{-1}$) (Borchard et al., 2019). Other potential mechanisms for high N loss from ECB include the high ash content of ECB reducing the surface area available for sorption and 1.9 times higher microbial uptake of N in RCB compared to ECB, although low MBN values are unable to quantitatively account for differences in N loss. None of these explanations have robust support in the data so further work should be directed at the mechanisms underlying changes in N leaching with biochar and NO_3^- fertilizer application.

Effects of biochar on losses of total dissolved and dissolved soil organic N are less often studied than losses of SIN through leaching. Of the few studies who have pursued this work, two have reported no effect of biochar on DON losses (Dempster et al., 2012; Güereña et al., 2013). The low DN and DON concentrations in soils amended with ECB suggest higher losses of DN and DON from this treatment, indicating the need for further study of these forms of N loss with biochar addition.

It is clear from this data that plant presence is key for reducing leaching losses, regardless of biochar application. This is not surprising, as we expect plants to take up N, but it is important to consider when we extrapolate findings in the lab without plants to the field. Considering the presence of plants may reduce the strength of biochar effects, a greater number of field experiments evaluating the effect of biochar on N leaching is needed.

Unlike for leaching, plant presence did not affect cumulative N₂O emissions and neither did biochar application. These results are somewhat surprising because plant presence reduced soil N and thus we would expect reduced N₂O emission. However, pots without plants had consistently higher water contents than pots with plants (Table 2), potentially facilitating complete denitrification to N₂, and thus offsetting benefits of plant uptake of N. Further, a large amount of our N₂O data was below detection limit (~40%) so we were limited in our ability to assess these patterns. A meta-analysis has shown that biochar is able to reduce N₂O emission across many soil and biochar types but this effect is reduced in greenhouse studies and for low application rates, due to reduced physical impacts on soil, which may help explain our results (Cayuela et al., 2014; Cayuela et al., 2015). Other studies have reported a lack of effect on N₂O

emission with biochar application, particularly in field studies, which generally use lower biochar application rates and have lower soil moisture than studies in the lab (Ramlow et al., 2019; Schimmelpfennig et al., 2014; Cayuela et al., 2015). Thus, applying low rates of biochar in field and greenhouse settings may not be an effective tool for reducing N₂O emissions. Other forms of gaseous N loss, such as nitric oxide and N₂, were not evaluated in this study, but losses as N₂ were likely to be high, as N₂ emission can account for up to 85% of denitrification products in high pH (~8) soils such as ours (Wagena et al., 2017), which may explain our incomplete recovery of fertilizer N.

5. Conclusions

Contrary to our expectations, our greenhouse experiment assessing biochar-plant interactions on N cycling revealed that biochar addition to a temperate agricultural soil did not increase N retention. In fact, amendment with engineered biochars, especially the coconut feedstock biochar, enhanced leaching N losses and suggested higher plant growth (though not significant), indicating a mobilization effect with NO₃⁻ fertilizer-derived N. This effect requires further study on post-processed biochars and NO₃⁻ fertilizer to ensure sustainable combinations of biochar and fertilizer types in agricultural systems. In contrast, addition of raw biochar had no effects on the system relative to the control, indicating that relatively low application rates of traditional biochar that is not post-processed may not significantly affect temperate agricultural alkaline soil N cycling. Additionally, plant presence played a crucial role for reducing N loss in our study, indicating that effects of biochar on N loss found in lab studies without plants may not be as strong when evaluated in cropping systems. This study suggests that biochar is

not effective for N retention in this system but underscores the value of plants for reducing N losses.

CHAPTER 3: AGROECOSYSTEM NITROGEN DYNAMICS DEPEND MORE ON SEASONAL PATTERNS THAN LOW APPLICATION OF BIOCHAR IN A SANDY SOIL

1. Introduction

A major facet of the soil health and sustainable agriculture movements is supporting the ecosystem services soils provide (Kibblewhite et al., 2008). One key ecosystem service that is greatly altered by cultivation is the ability of soils to retain and cycle nutrients, especially nitrogen (N) (Galloway et al., 2003). Nitrogen is crucial for crop growth but over-application of fertilizers and adverse environmental conditions can lead to large losses of N from agroecosystems (Fowler et al., 2013). While there are many proposed solutions for enhancing N retention and cycling in agricultural systems, the use of N-fixing legumes and the application of biochar have both shown promising results (Nguyen et al., 2017; Tonitto et al., 2006).

1.1 Biochar effects on N cycling

Biochar is a soil amendment created by pyrolyzing biomass at high temperatures (350-1000°C), resulting in a carbon-rich, recalcitrant material that can persist in the soil for centuries (Lehmann and Joseph, 2015; Wang et al., 2016). Biochar has been proposed as a potential win-win-win solution for climate change mitigation, soil health, and agricultural production, due to its ability to provide carbon (C) sequestration while increasing microbial activity and crop yield (Biederman & Harpole, 2013). With respect to N, biochar has been posited to mitigate climate change and reduce environmental impacts of agricultural production by decreasing N₂O emissions (Butterbach-bahl et al., 2013) and N leaching (Laird et al., 2010), through reduction in soil inorganic N (SIN)

availability. The pathways for lower concentrations of SIN with biochar addition include both biotic and abiotic mechanisms. Biochar-induced increases in microbial activity may lead to higher immobilization of inorganic N (Nguyen et al., 2017). Additionally, the high surface area and porous nature of biochar allows for chemi- and physi-sorption of N compounds, respectively (Nguyen et al., 2017; Lehmann & Joseph, 2015). Further, biochar may also limit mineralization of organic matter through suppression of enzymatic activity via direct sorption of enzymes to the biochar surface (Foster et al., 2018). Biochar-induced reductions of L-leucine aminopeptidase (LAP) and β -1,4-N-acetylglucosaminidase (NAG) activities, which catalyze protein and chitin degradation, respectively, could thus also contribute to reduced mineral N availability through reduced breakdown of these nitrogenous compounds. Biochar is highly variable though, so assessing biochar with differential properties will allow for finer parsing of the controlling physio-chemical mechanisms for potential SIN reductions. Notably, biochar-induced SIN reductions have been shown to reduce crop yield, and thus biochar applications may require a co-amended N source to prevent growth limitation (Nelissen et al., 2014).

1.2 Legume effects on N cycling

Nitrogen limitation, which is prevalent in most terrestrial systems, and often exacerbated by biochar addition, may be overcome in systems with legumes, which are able to fix atmospheric dinitrogen (N₂) into a bioavailable form. Additionally, diversifying cropping systems by adding a legume has been shown to enhance microbial biomass and bioavailable N, while reducing greenhouse gas emissions and external inputs (King & Hofmockel, 2017; Stagnari et al., 2017). However, our knowledge on N fixation lags

behind that of other forms of N cycling due to measurement difficulties, so it is important to employ multiple lines of evidence to measure changes in N fixation (Vitousek et al., 2013).

Extent of biological N fixation (BNF) in N-fixing plants can be determined using stable N isotopes, when plant-available soil N has a significantly different ^{15}N signal than the atmosphere (Hogberg, 1997). Proportion of N fixed by the plant can be determined by using the natural abundance isotope dilution method (Shearer & Kohl, 1986). Alternatively, when the reference plant and N-fixing plants $\delta^{15}\text{N}$ values are not sufficiently separated to calculate N derived from the atmosphere, difference in $\delta^{15}\text{N}$ values between the reference plant and N-fixer can indicate relative differences in N fixation between treatments and over time, as well as transfer of fixed N from the N-fixing plant to the reference plant (Chalk, 1998; Malik et al., 1991). Additionally, because the abundance of the nitrogenase reductase gene (*nifH*), which catalyzes reduction of N_2 to ammonia (NH_3), is correlated with N fixation in soil (Terakado-Tonooka et al., 2013), it represents another proxy for relative amount of N fixation.

1.3 Combined biochar-legume effects on N cycling

High rates of BNF are generally associated with low SIN, high phosphorus availability, and adequate availability of the micronutrients potassium, boron, molybdenum, iron, and sulfur, which are co-factors of the nitrogenase enzyme that is responsible for catalyzing fixation (Rubio & Ludden, 2008). Since reduced SIN forces a legume to rely more on BNF for N requirements, biochar application may lead to increased input of biologically-fixed N combined with reduced losses of SIN to the environment. Previous studies assessing the effect of biochar application on N fixation

have shown promising results (Güereña et al., 2015; Liu et al., 2017; Mia et al., 2014; Oram et al., 2014; Quilliam, DeLuca, & Jones, 2013; Rondon et al., 2007; Van Zwieten et al., 2015), although the number of field studies is limited (Mia et al., 2018; Van Zwieten et al., 2015) and the majority of studies have evaluated relatively high application rates (≥ 10 tons ha^{-1}). However, these high application rates are not economically feasible for farmers in temperate regions (Galinato et al., 2011), urging the need to test the effects of low biochar application rates for the overall assessment of biochar use as a sustainable agricultural practice. Low rate biochar applications, added in-row, in close proximity to the seed, have shown promise in an irrigated maize system (Foster et al., submitted). Further, Rajkovich et al. (2012) found significantly increased tissue N and N uptake with decreasing biochar application rates, such that there may be multiple agronomic benefits of low biochar application rates. Additionally, the effect of reduced SIN with biochar application is especially strong in coarse textured soils, making low rates of biochar application in sandy soils an intriguing agricultural practice to evaluate (Nguyen et al., 2017).

However, biochar amendment and N fixation do not occur in isolation, making it relevant to understand their effects on broader N cycling. Soil N cycling involves a variety of functional genes that, when quantified, can give insights into N transformations within the soil-plant-atmosphere system (Wallenstein & Vitgalys, 2005). The genes encoding nitrite reductase (*nirK*) and nitrous-oxide reductase (*nosZ*) are part of the process of denitrification. Because *nirK* catalyzes the reduction of nitrite to nitric oxide, it represents an environmentally-harmful gaseous loss pathway of N, whereas *nosZ*, which catalyzes the transformation of N_2O to N_2 , represents a loss pathway that

does not have harmful climate effects. Thus, relative abundances of nirK and nosZ can be representative of potential for greater N loss as NO/N₂O or N₂, respectively. While addition of biochar generally reduces N loss as N₂O (Cayuela et al., 2014), this effect is reduced in field studies (e.g. Ramlow et al., 2019), and legumes have been shown to increase emissions relative to non-N-fixers (Rochette & Janzen, 2005). Thus, understanding the effects of these combined management practices on N loss is important for a holistic view of N cycling.

To better understand how low biochar application rates could affect N cycling in alfalfa in semiarid agroecosystems, we looked at the effect of two different biochars on N cycling over the growing season in a commercial alfalfa field with sandy soil. We used natural abundance ¹⁵N of alfalfa and dandelions, our reference plant, in combination with N gene abundance, enzymatic activity, and SIN availability, to assess N fixation, soil-plant N dynamics, and potential N loss. We hypothesized that biochar would increase N fixation and nifH abundance and reduce gene abundance associated with N loss through decreased SIN availability. We expected this effect to be stronger for biochar with higher surface area and C:N due to increased sorption of SIN and enzymes and immobilization of SIN, respectively. We also hypothesized that biochar would reduce SIN early in the growing season (Nelissen et al., 2014), leading to increased BNF in biochar treatments for the rest of the growing season.

2. Materials & Methods

2.1 Study site

The experiment was conducted on a commercial alfalfa (*Medicago sativa* var. *Pioneer 5010*) field at Lost Creek Land & Cattle Co (40°13'17.75"N, 104°19'28.35"W) in

Roggen, Colorado, USA from May to September 2019. At the time of sampling, the field had been in alfalfa for 5 years and has been maintained with organic amendments of compost and chicken litter, although precise timing and rates of these applications is not known. Previously, dating back to 1990, this field was in a rotation with corn, sugar beets, and beans. Soils are part of the Valent sand series and are classified as sandy using particle size distribution determined by the hydrometer method (Soil Science Division Staff, 1993). Before the start of the experiment, soils contained 5.8 g kg⁻¹ C, 0.66 g kg⁻¹ N, and had an average pH of 7.9. In 2018, compost sourced from feed lot manure (C:N ≈ 5) was applied at a rate of 7.4 tons ha⁻¹ in early March before the growing season began and harrowing was done in the last week of March and in early April. The field was pivot-irrigated continuously over the growing season for a total application of 28.17 inches of water between April and September 2018.

2.2 Biochars

Two biochars were assessed in this study: a pelletized pine biochar product (PBC) and an engineered coconut biochar (CBC). Biochars were created through continuous pyrolysis at maximum temperatures below 650°C and produced from pine and coconut shell feedstocks, respectively. Both biochars were post-processed to provide more uniform physio-chemical properties (proprietary information; Cool Planet, Inc., Greenwood Village, CO). PBC is an agglomerate product that is 38-40% biochar (proprietary information; Cool Planet, Inc., Greenwood Village, CO). Biochars were characterized by ultimate analyses (C, H, N, ash; ASTM D3176-15, 2015) performed by Wyoming Analytical Labs, Inc. (Laramie, WY) and all other properties were determined by Cool Planet Energy Systems, Inc. (pH, surface area, Ece; Camarillo, CA; Table 5),

with the exception of moisture, which was determined by mass difference of biochar as applied and biochar oven-dried for 48 hours at 105°C.

Table 5: Selected properties for the two biochars used in the experiment: Pine biochar product (PBC) and Coconut shell biochar (CBC). Asterisks indicate values for the PBC biochar base material. All other values were measured for the agglomerate product. Electrical conductivity (Ece) is an indicator of salinity.

Biochar	Organic C (%)	H:C _{org} (molar ratio)	C:N (mass ratio)	Ash (%)	pH	Surface Area (m ² /g)	Moisture (%)	Ece (mmhos/cm)
PBC	72.07*	0.659*	30*	1.02*	6.13	97-110	2.25	1.88
CBC	62.25	0.418	148	1.88	7.61	200-300	4.29	1.92

2.3 Experimental design

The experiment consisted of three biochar treatments: application of the PBC, application of the CBC, and no biochar application, as a control (C), each replicated six times in six blocks (n = 18).

The six blocks, each with three 4 m² treatment plots, were established approximately two weeks after harrowing, on April 18th, 2018 (Fig. 5). Biochar was incorporated by hand at plot establishment, at a rate of 112 kg ha⁻¹ (45 g plot⁻¹) by replicating a no-till drill with minimal soil disturbance to ensure proximity to alfalfa roots. Application rate and method of application were determined based on recommended broadcast rates provided by Cool Planet for row crops (Cool Planet, 2019) and on recommended application methods provided by Cool Planet for perennial crops (Cool Planet, 2019), respectively. Biochar was applied in rows spaced approximately 22 cm apart to 3 cm depth and soil was quickly covered to prevent biochar loss. Initial soil samples were taken to a depth of 20 cm with an auger to assess any differences between blocks. Blocks were located to ensure dandelions (our reference plant; *Taraxacum officinale*) were within the block area.

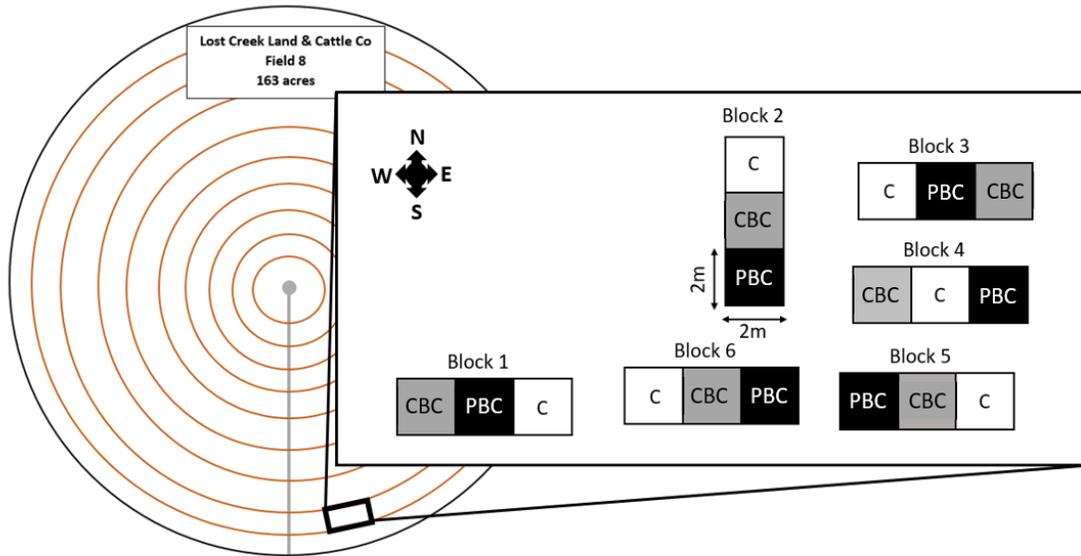


Figure 5: Plot layout for the experiment. All blocks were between the second and third wheel tracks of the pivot.

2.2 Plant and soil samples collection

Alfalfa was harvested four times during the growing season, on May 30th, July 4th, August 6th, and September 21st, which will be further referred to as H1 (Harvest 1), H2, H3, and H4, as the harvests were relatively regularly spaced and represent similar growth stages of alfalfa (flowering). Harvests were conducted a few days before the harvest of the whole field for hay production which was determined by the farm managers. At each harvest, biomass was cut by hand to height of approximately 4 cm and was collected from only the inner 1 m² of each plot to minimize potential edge effects. At each cutting, fresh alfalfa biomass was weighed in the field and a sub-sample was taken for determination of dry weight and further analyses in the lab. Additionally, for H2-H4, dandelions located within 2 m of each block were collected for ¹⁵N analysis. At each harvest, five soil cores were taken with an auger to 20 cm depth within each 1 m² subplot and pooled for further analyses. Plant and soil samples were kept in a cooler

until return to Colorado State University where soil samples were stored at 4°C until analysis and plants were oven-dried at 60°C. Subsamples of H4 soils were sieved to 2 mm and stored at -80°C for measurement of extracellular enzyme activity (5 g) and gene abundance (5 g).

2.3 Plant and soil analyses

Oven-dried alfalfa and dandelion aboveground biomass from each treatment plot at each harvest (only H2-H4 for dandelions) were ground to powder, and sub-samples were measured for N concentration and $\delta^{15}\text{N}$ isotopic composition on an elemental analyzer (Costech Analytical Technologies, Inc., Valencia, CA) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Alfalfa aboveground N stocks for each plot at each harvest were estimated by multiplying N concentrations by aboveground dry weights.

The differential isotopic values of an N-fixer (alfalfa) and a reference plant (dandelion) can indicate relative amounts of N fixation using the assumption that all dandelion N is derived from the soil which has a specific $\delta^{15}\text{N}$ value, whereas alfalfa will have N from both the soil and atmosphere. To use a reference plant, you must assume that it has access to the same pool of N as the N-fixing plant. Because dandelions are perennials and can have very deep tap roots (generally ~1 m but up to 4.6 m; Longyear, 1916; Rodriguez, 2019), comparable to root depths of 2.1-3.7 m seen in mature alfalfa (Weaver, 1926), they may be considered an appropriate reference plants for alfalfa and their N isotopic values can be used to represent plant-available soil N. Thus, greater difference between alfalfa and dandelion $\delta^{15}\text{N}$ will indicate greater contribution of atmospheric N to alfalfa N stocks.

Within a week from sampling, soil NH_4^+ and NO_3^- concentrations were determined by extracting a subsample of field-wet bulk soil using 2M KCl (5:1 soil/KCl ratio by mass), shaking for 1 hour, filtering, (Whatman #40 ashless filter paper), and analyzing the extraction colorimetrically (Alpkem Flow Solution IV Automated wet chemistry system; O.I. Analytical, College Station, TX) (McTaggart & Smith, 1993). Because of the sandy and homogenous nature of the soil, sieving was not necessary, but all roots were removed before performing these analyses.

2.4 Enzyme and DNA analyses

We assessed activities of two common extracellular enzymes that are associated with the degradation of nitrogenous compounds (LAP & NAG), to assess biochar effects on N mineralization. These were analyzed using a high throughput fluorometric assay, described by Bell et al. (2013). Bailey et al. (2011) found that fluorometric assays account for potential biochar sorption of enzymes better than colorimetric assays. Briefly, standard plates were prepared by creating a series of 16 dilutions (0.4-100 μM) of 4-Methylumbelliferone and 7-Amino-4-methylcoumarium stock solutions. To assess the quenching of fluorescence due to floating soil or organic particles, these stock solutions were combined with 800 μL of sample and read as described for the substrate-sample mixtures, below. To assess enzyme activity, 1 g of soil was combined with 30mL of Tris buffer and shaken for 20 minutes. A combination of 800 μL of the soil solution and 200 μL of the substrates was shaken for 3 hours. Standard-sample and substrate-sample solutions were transferred to black plates and read at 365nm excitation and 450nm emission on an Infinite M200 Microplate Reader (Tecan Trading AG, Switzerland).

Abundances of N cycling genes (nifH, nirK, and nosZ) and microbial markers (bacteria and fungi) were determined using qPCR, to estimate potential for N cycling and microbial processes, respectively. Extraction and qPCR followed the methods of Hallin et al. (2009). Briefly, DNA was extracted using a Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) as per the manufacturer's instructions. Standard curves with 8 ten-fold dilutions were prepared using plasmids for each gene and extracted DNA was combined with respective primers for measurement of DNA copy numbers on a Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers and conditions used for each measured gene are reported in Table 6.

Table 6: Primers and PCR conditions used for gene analysis (adapted from Trivedi et al., 2011).

<i>Primers</i>	<i>Sequence (5'-3')</i>	<i>Thermal Conditions</i>	<i>Reference</i>
Total Bacteria			
Eub338	ACT CCT ACG GGA GGC AGC AG	95°C, 15 min, 1 cycle	Fierer et al., 2005
Eub518	ATT ACC GCG GCT GCT GG	95°C for 1 min, 53°C for 30 s, 72°C for 1 min, 40 cycles	
Total Fungi			
ITS1f	TCC GTA GGT GAA CCT GCG G	Same as for bacteria	Fierer et al., 2005
5.8s	CGC TGC GTT CTT CAT CG		
nirK			
nirK876	ATY GGC GGV CAY GGC GA	95 C, 15 min, 1 cycle 95 C for 15 s, 63 to 58 C for 30 s (-1 C by cycle), 72 C for 30 s, 80 C for 15 s, 6 cycles	Hallin et al., 2009
nirK1040	GCC TCG ATC AGR TTR TGG TT		
nifH			
po1F	TGC GAT CCS AAT GCB GAC TC	Same as for nirK	Hallin et al., 2009
po1R	ATS GCC ATC CTY TCR CCG GA		
nosZ			
nosZ2F	CGC RAC GGC AAS AAG GTS MSS GT	95°C for 15 s, 1 cycle 95 C, 15 min, 1 cycle 95 C for 15 s, 65 to 60 C for 30 s (-1 C by cycle), 72 C for 30 s, 80 C for 15 s, 6 cycles 95 C for 15 s, 60 C for 30 s, 72 C for 30	Hallin et al., 2009
nosZ2R	CAK RTG CAK SGC RTG GCA GAA		

s, 80 C for 15 s, 40 cycles
95°C for 15 s, 60 to 95°C, 1
cycle

2.5 Data analyses

Statistical analyses were carried out using R statistical software (R Core Team, 2017). Repeated measures analysis was used to assess patterns of alfalfa aboveground biomass production, N uptake, alfalfa and dandelion $\delta^{15}\text{N}$, and SIN between harvests and in response to different biochar types with block as a random effect (lme4; Bates et al., 2015). One-way ANOVA with block as a random effect was used to examine the influence of biochar on enzyme activity and gene abundance. Pairwise comparisons (emmeans; Lenth, 2018) were used to determine differences between individual biochar types. When response variables did not fit the assumptions of the linear model, natural log and Box-Cox power transformations were assessed and applied for data analysis (Box & Cox, 1964). Correlations between alfalfa $\delta^{15}\text{N}$ and SIN parameters used data from the entire growing season. Correlations between gene abundance and SIN parameters used data only from H4. For correlations between alfalfa $\delta^{15}\text{N}$ and percent of SIN as NO_3^- , the latter was log transformed to meet the assumptions of normality for the residuals. All other data fit the assumptions of the normal model. Significant differences between treatments and correlation significance were determined where $P < 0.05$.

3. Results

3.1 Plant and soil N dynamics

Seasonal trends were much more pronounced than biochar effects for all measures of plant and soil N dynamics. Biochar type was not a significant predictor for alfalfa

biomass, alfalfa N concentration (Table 7), alfalfa or dandelion $\delta^{15}\text{N}$, or SIN. However, date was significant for all of these pools with the exception of alfalfa N concentration. The largest alfalfa harvest was at the beginning of the season, as alfalfa biomass for H1 was around 600 g m^{-2} for all treatments, 1.5-2 times higher than all other harvests ($p < 0.001$; Fig. 6). Similarly, soil NO_3^- was relatively high for H1 as compared to the other harvests ($p < 0.001$) as well as for H2 compared to H4 ($p = 0.004$; Fig. 7). In contrast, NH_4^+ was relatively low at the beginning of the growing season and increased through H3, such that H1 was significantly lower than all other dates ($p \leq 0.001$) and H2 and H3 were significantly higher than H4 ($p < 0.021$; Fig. 7). Additionally, NH_4^+ dominated the total SIN pattern at the end of the growing season as H2 and H3 had significantly higher SIN than H4 ($p < 0.002$; Fig. 7). These patterns in mineral N led to a significant decrease in percent of SIN as NO_3^- over time ($p < 0.04$). Additionally, the interaction between harvest date and biochar type was significant for percent of SIN as NO_3^- – the control had significantly greater SIN as NO_3^- as compared to the treatments with biochar for H1 ($p < 0.006$; Fig. 7).

Alfalfa $\delta^{15}\text{N}$ values ranged from -0.93 to 1.15 ‰ and followed a similar pattern as NH_4^+ values over time, such that H1 was significantly lower than all other harvests ($p < 0.001$) and H2 was significantly lower than H3 and H4 ($p < 0.001$; Fig. 8). Dandelion $\delta^{15}\text{N}$ values also increased over time ($p < 0.04$) and by H4 were not significantly different from alfalfa $\delta^{15}\text{N}$ values (Fig. 8).

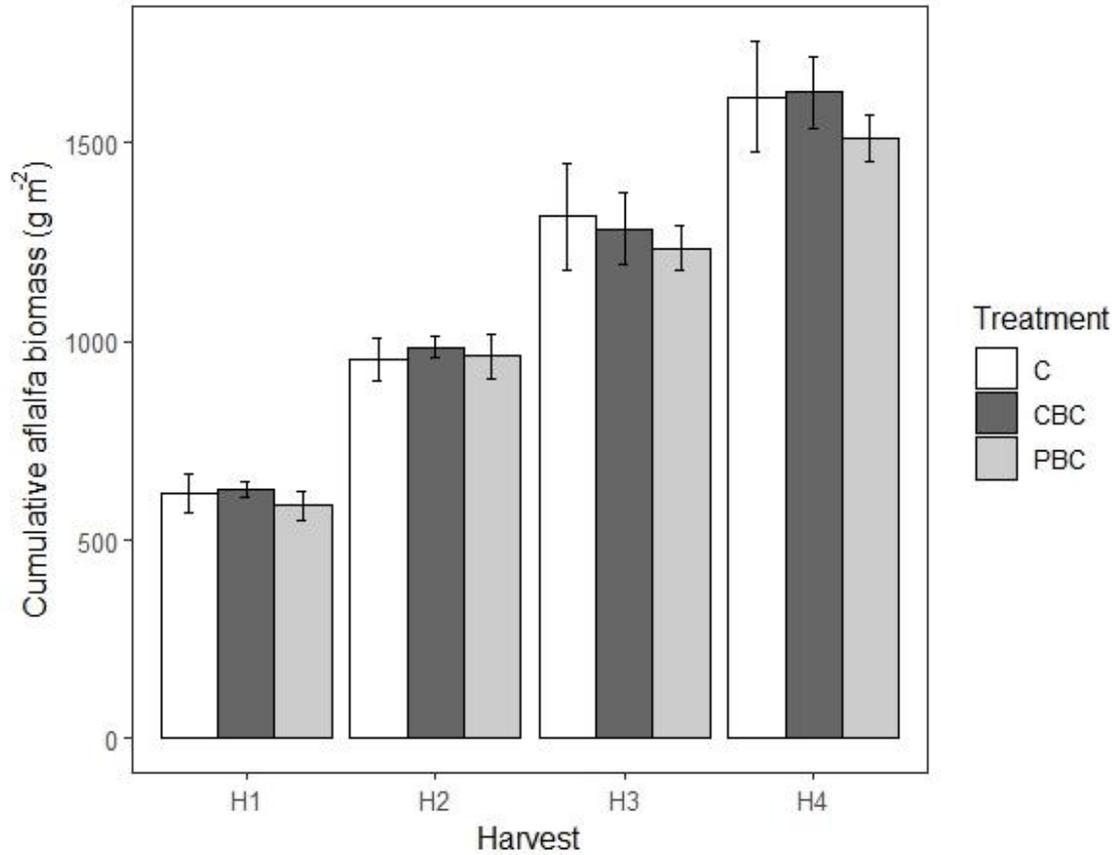


Figure 6: Cumulative biomass harvested over the growing season by biochar type. Error bars represent standard error (n=6). Biochar types are represented as Control (C; white), Coconut shell biochar (CBC; dark grey), Pine biochar (PBC; light grey). Date was a significant predictor for alfalfa biomass ($p < 0.001$), which was not predicted by biochar type nor the biochar by date interaction.

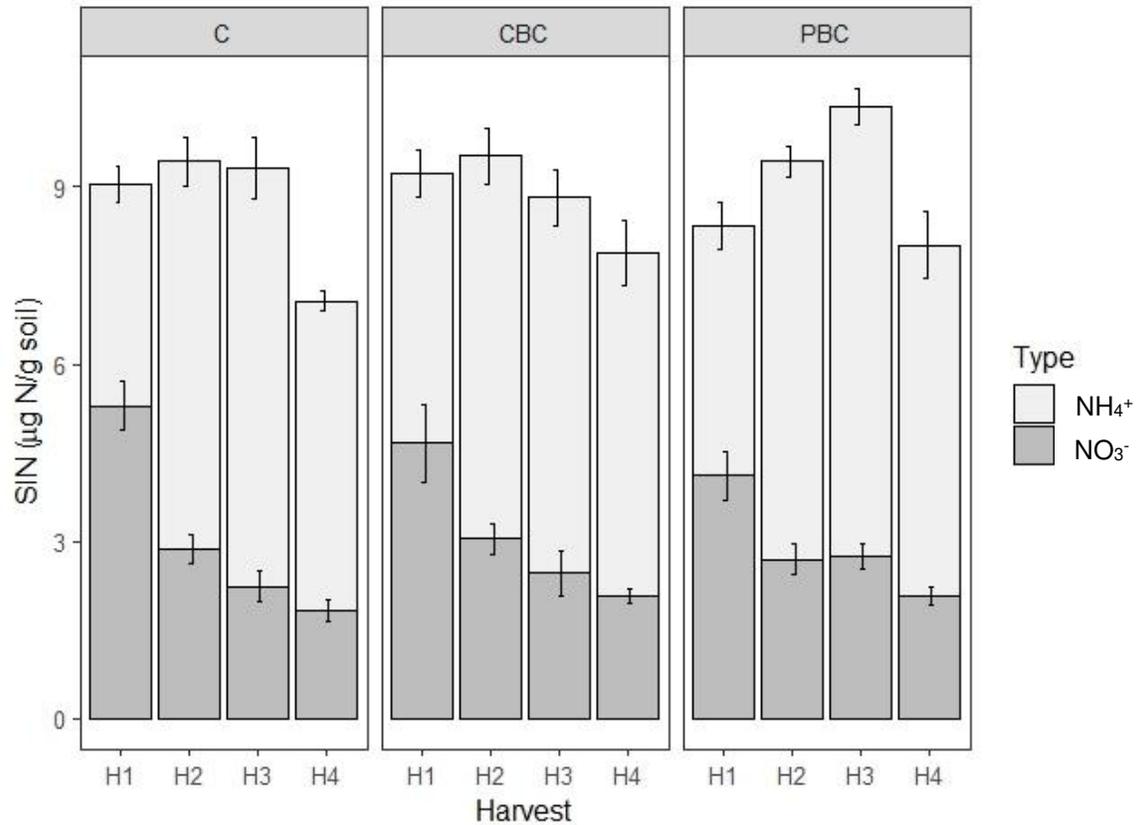


Figure 7: Average SIN concentration over time for each biochar type, broken into NH₄⁺ (light gray) and NO₃⁻ (dark gray). Error bars represent standard error (n=6). There were not significant differences between biochar types nor significant interactions for total SIN or individual inorganic N species. Date was a significant predictor for total SIN ($p < 0.001$) and individual inorganic N species ($p < 0.001$). The interaction was significant for percent of SIN as NO₃⁻, which was significantly higher in the control for H1 ($p < 0.006$). Biochar types are represented as Control (C), Coconut shell biochar (CBC), and Pine biochar (PBC).

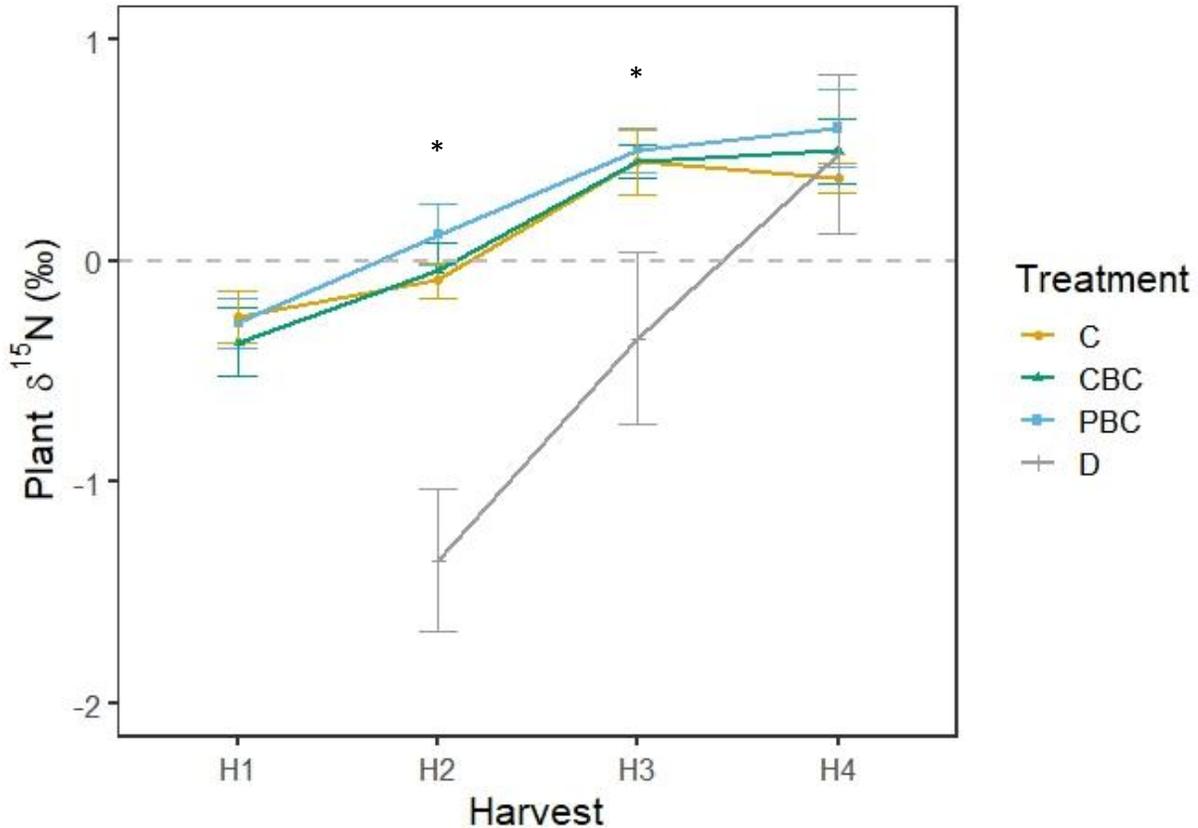


Figure 8: Average plant $\delta^{15}\text{N}$ over time for alfalfa under biochar treatments and for dandelions. Error bars represent 1 standard error ($n=6$). Asterisks denote significant difference between alfalfa and dandelion values. There were not significant differences between biochar types for alfalfa $\delta^{15}\text{N}$. Date was a significant predictor for alfalfa ($p < 0.001$) and dandelion ($p = 0.042$) $\delta^{15}\text{N}$. Treatments are represented as alfalfa under Control (C), Coconut shell biochar (CBC), and Pine biochar (PBC), and Dandelions averaged across treatments (D).

Table 7: Aboveground N concentration of alfalfa presented as mean \pm standard error ($n=6$) for each biochar type: Control (C), Coconut shell biochar (CBC), Pine biochar (PBC). The date effect, biochar effect, and interaction term were not significant for this metric.

Pool	Sampling Date	Units	Biochar Type		
			C	CBC	PBC
Aboveground N concentration	May 13th (H1)	mg N g^{-1} biomass	3.41 ± 0.24	3.03 ± 0.12	3.55 ± 0.16
	July 4th (H2)		2.82 ± 0.26	3.04 ± 0.04	3.31 ± 0.20
	August 6th (H3)		3.04 ± 0.16	3.16 ± 0.26	3.68 ± 0.30
	September 21st (H4)		3.31 ± 0.21	3.21 ± 0.25	3.18 ± 0.12

3.2 Microbial measurements

There was a trend towards higher nirK and nosZ abundance in PBC treatments, but biochar type was not a significant predictor of these values. This relationship was

strongest for nirK, where biochar type was marginally significant and PBC had twice the average DNA copy numbers as compared to CBC ($p = 0.064$; Fig. 9). In contrast, nifH was not significantly different between any treatments, but mean nifH copy numbers were ~1.5 times higher in the control relative to the biochar treatments (Fig. 9). Biochar type was not a significant predictor for fungal and bacterial abundance, nor enzyme activity (Table 8).

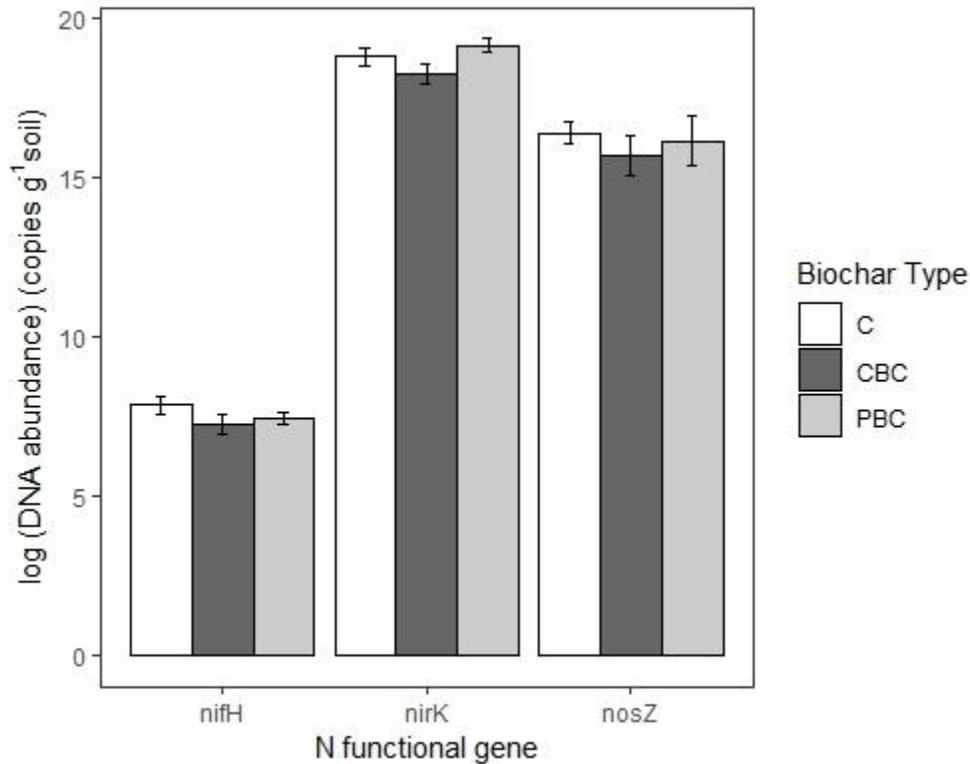


Figure 9: Average gene abundance for each N functional gene by biochar type. Error bars represent standard error (n=6). There were not significant differences between biochar types for any functional genes. Biochar types are represented as Control (C; white), Coconut shell biochar (CBC; dark grey), Pine biochar (PBC; light grey).

Table 8: Microbial analyses presented as mean \pm standard error (n = 6) for each biochar type: Control (C), Coconut shell biochar (CBC), Pine biochar (PBC). All microbial analyses were done for the H4 (September 21st) sampling date. Biochar type was not a significant predictor for any of these measurements. Note that the high error for the control for NAG is being driven by a single value and when it is removed there are still no significant differences between biochar treatments.

Measurement	Units	Biochar Type		
		C	CBC	PBC
Bacterial DNA abundance	copies g ⁻¹ soil	7.95e10 ⁹ \pm 3.91e10 ⁹	1.49e10 ⁹ \pm 3.24e10 ⁸	3.57e10 ⁹ \pm 7.92e10 ⁸

Fungal DNA abundance	copies g ⁻¹ soil	2.33e10 ¹¹ ± 1.03e10 ¹¹	3.52e10 ¹¹ ± 1.62e10 ¹¹	1.78e10 ¹¹ ± 9.77e10 ¹⁰
LAP	mmol g ⁻¹ soil	4.70 ± 0.81	2.87 ± 1.07	5.54 ± 1.50
NAG	mmol g ⁻¹ soil	7.93 ± 4.28	3.65 ± 0.83	3.55 ± 1.17

3.3 Correlations between measurements

To assess relationships between substrates and soil N processes, we examined strength of the relationships between SIN and isotopic values, microbial abundance, and enzyme activity (Fig. 10). Correlations used data for all treatments and times, since they were used to identify overarching drivers for change. Soil NO₃⁻ and NH₄⁺ both correlated with alfalfa δ¹⁵N (NO₃⁻: $r = -0.544$; $p < 0.001$, NH₄⁺: $r = 0.420$; $p < 0.001$), but total SIN did not. The strongest correlation with alfalfa δ¹⁵N was for percent of SIN as NO₃⁻ ($r = -0.606$; $p < 0.001$), which, like with NO₃⁻, was negatively related to alfalfa δ¹⁵N. In contrast, nosZ gene abundance was only related to total SIN ($r = 0.487$; $p = 0.041$) and not NO₃⁻, NH₄⁺, nor percent of SIN as NO₃⁻. Similarly, nirK correlated with NH₄⁺ ($r = 0.582$; $p = 0.011$) and total SIN ($r = 0.562$; $p = 0.015$), but not NO₃⁻ nor percent of SIN as NO₃⁻. The abundances of nifH, bacteria, and fungi, and activities of LAP and NAG were not significantly correlated with any measure of SIN.

3. Discussion

We found no effects of biochar on N cycling, microbial activity, nor plant growth in our system and rather found that time over the growing season was a more important predictor of N dynamics in this alfalfa field. Additionally, we found that proxies for N fixation and denitrification were differentially related to measures of SIN, which may have important implications for agricultural N management.

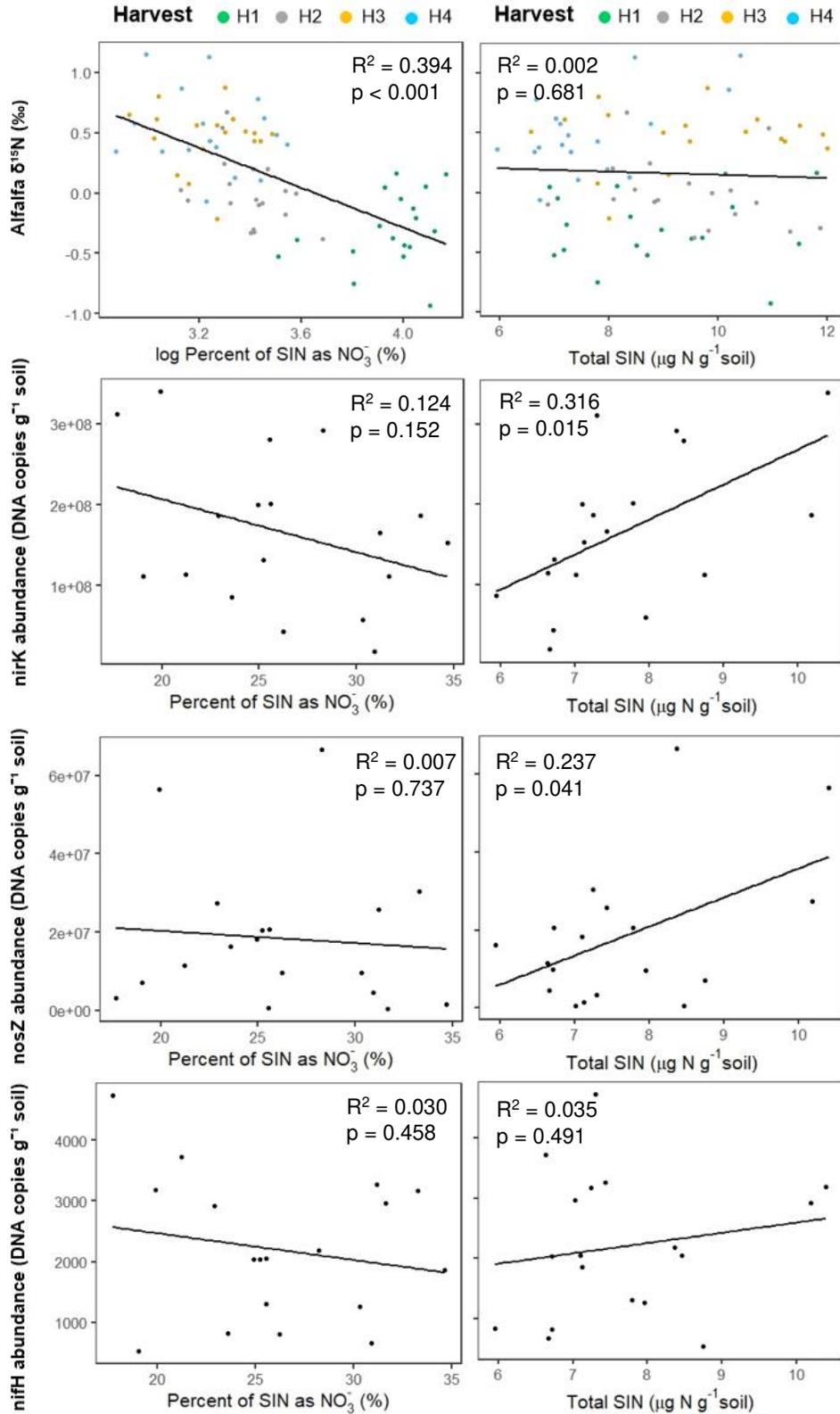


Figure 10: Correlations between total SIN or relative abundance of a given N species and metrics explicitly associated with N cycling (alfalfa $\delta^{15}\text{N}$, nirK, nosZ, and nifH). Points in the $\delta^{15}\text{N}$ plots (top panels) are colored by the harvest date because this was a significant predictor of these metrics. R^2 and p-values for each correlation are provided in the corner of each plot.

3.1 *Seasonal trends in N cycling*

While we expected a decrease in SIN over the growing season, changes in individual SIN species led to a more complex pattern in mineral N availability. There was a shift from approximately 60% of SIN as NO_3^- to less than 20% of SIN as NO_3^- over the growing season that was correlated with alfalfa $\delta^{15}\text{N}$. This correlation could be explained by a few scenarios:

1. We could assume higher (more enriched) $\delta^{15}\text{N}$ values are associated with greater BNF because dandelion $\delta^{15}\text{N}$ values began negative early in the season. Thus, negative values likely represent bioavailable soil N from previously fixed N or remnant litter from past growing seasons. The significant negative correlation between percent of SIN as NO_3^- and $\delta^{15}\text{N}$ could suggest that early in the growing season plants derived their N from relatively high NO_3^- in the soil that had built up over the drier winter and early spring when growth and potential soil losses are minimal (Ledgard & Steele, 1992). This NO_3^- would be expected to be especially high in this system after 5 successive years of alfalfa cropping during which N content would be expected to increase in the soil (Peoples et al., 2009), although the sandy nature of this soil may negate N build up to some extent (Silver et al., 2000). As plants reduced soil NO_3^- via uptake, BNF would no longer be inhibited (Peoples et al., 2009) and would be stimulated to provide additional N for the plant. Although NO_3^- levels in our soil were relatively low, BNF has been shown to increase linearly with decreasing NO_3^- (Voisin et al., 2002), indicating that even small decreases in NO_3^- should lead to increases in fixation. N fixed by BNF can enter the soil as NH_4^+ through rhizodeposition via (1) decomposition and decay of

root nodules and cells and (2) root exudation of soluble compounds (Joëlle et al., 2010). Furthermore, rhizodeposition is expected to increase as plants mature (Joëlle et al., 2010), which could explain the shift to NH_4^+ dominance in the late growing season, as our plants reached the end of their sixth cropping cycle.

These patterns are further supported by the lack of correlation between total SIN and $\delta^{15}\text{N}$, which indicates that extent of N fixation is not dependent on the total amount of mineral N in the system but rather specific species of inorganic N. The lack of significant correlations between nifH abundance and SIN measurements do not seem to support this idea, but nifH was only measured for H4 where the pattern between $\delta^{15}\text{N}$ and percent of SIN as NO_3^- was not as strong as for the whole growing season (Fig. 6), indicating that alfalfa may have been reducing N uptake and BNF as the growing season ended. Hooper & Vitousek (1998) found soil inorganic N pools in September that were twice as high compared to those in May in a leguminous plant community, supporting the idea that legumes reduce their uptake later in the growing season.

2. Correlations between plant $\delta^{15}\text{N}$ values of alfalfa and percent of SIN as NO_3^- could also be attributed to changes in N source over the growing season. The compost application rate was approximately two orders of magnitude higher than our biochar application rate and degradation of this N source could also lead to temporal patterns in plant $\delta^{15}\text{N}$. Generally 15-20% of compost N becomes plant available in the first year of application (Amlinger et al., 2003) and low C:N organic amendments, such as our compost, show peaks in soil available NO_3^- within two months of application (Cooperband, Bollero, & Coale, 2001). Further, nitrification

can exhibit fractionations as high as -35‰ (Hogberg, 1997), such that the compost, which began with $\delta^{15}\text{N} = 14.8 \pm 0.05$, may exhibit a more depleted $\delta^{15}\text{N}$ when transformed to NO_3^- and taken up by plants. This more depleted compost $\delta^{15}\text{N}$ could represent the plant available N and lead to the more negative alfalfa $\delta^{15}\text{N}$ values early in the growing season. Later in the season, as alfalfa depletes the compost available N, it may begin to perform N fixation and rhizodeposition, as presented above, or it may live off NH_4^+ that has built up over several years of alfalfa cropping, leading to NH_4^+ with a similar $\delta^{15}\text{N}$ as fixed N. Although, this second explanation is harder to support given the expected transformation from NH_4^+ to NO_3^- in this well-aerated, sandy soil (Barnard et al., 2005).

3. Change in acquisition depth of N may also explain this strong correlation between SIN availability and plant $\delta^{15}\text{N}$. Alfalfa has been shown to increase its N uptake from the subsoil over the growing season (Huang et al., 1996), so in our system, alfalfa may have shifted from nitrified compost readily available at the surface, with a more negative $\delta^{15}\text{N}$, to previously fixed N leached downward in the soil profile, with a more positive $\delta^{15}\text{N}$. However, we would not expect NH_4^+ to be leached to the subsoil due to its low mobility, so this scenario has somewhat less support, based on the strong correlation between plant $\delta^{15}\text{N}$ and SIN as NO_3^- .

Understanding the N dynamics that lead to enhanced N fixation over the growing season may allow land managers to better target fertilizer applications to encourage BNF.

3.2 Alfalfa-dandelion interactions

If scenario one is taken as true, the convergence of dandelion $\delta^{15}\text{N}$ values on alfalfa $\delta^{15}\text{N}$ values over the growing season may indicate uptake of biologically fixed N by dandelions. While this prevented us from using dandelions as a reference plant for calculating BNF, it may have important implications for N transfer to plants growing in concert with legumes. A review of legume N transfer found that 7-57% of tissue N of a non-legume growing in association with a legume could be derived from legume rhizodeposition (Joëlle et al., 2010). Additionally, increased N transfer over the growing season and after successive years of cropping has been reported in multiple studies evaluating alfalfa (Burity et al., 1989; Tomm, 1994; Frankow-Lindberg & Dahlin, 2013; Louarn et al., 2015), and because our alfalfa was in its sixth year of growth, it may have transferred particularly high amounts of N to perennial dandelions towards the end of the growing season. Alternatively, changes in $\delta^{15}\text{N}$ of plants may be attributed to changes in soil N source that are differentially preferentially taken up by dandelions and alfalfa (scenario 2) or differences in access to N due to differences in root structures and distribution in the soil profile (Shearer & Kohl, 1986; scenario 3), rather than solely differences in N fixation. Alfalfa has been shown to be an effective competitor for recycled N (Tomm et al., 1995), such that alfalfa may rely more on soil N built up from previous growing seasons, whereas dandelions may take up more of the readily accessible compost N, which may have a more negative $\delta^{15}\text{N}$ signature due to nitrification. Further, since alfalfa may have access to deeper soil N than dandelions, it may be taking up more fixed N that has leached downward in the soil profile over successive growing seasons (relatively enriched $\delta^{15}\text{N}$; Thorup-Kristensen, 2001), which would be expected in this sandy soil, whereas more shallowly rooted dandelions may use

compost N which is closer to the surface (relatively depleted $\delta^{15}\text{N}$). Additionally, while we assume N is transferred from the legume to the non-fixing plant, the opposite has also been shown to occur (Tomm, 1994), although this is unlikely to be an important mechanism for changes in alfalfa $\delta^{15}\text{N}$ in our system, as dandelions made up a very small proportion of biomass in the study field. Similar $\delta^{15}\text{N}$ values of alfalfa and dandelions at the end of the growing season indicate either that dandelions are deriving their N solely from fixed N, which is unlikely (Walley et al., 1996), or more likely, that alfalfa and dandelions are both deriving N from a mixture of soil available N and biologically fixed N. This may have implications for weed persistence in agricultural fields of N-fixing plants. Reduced alfalfa growth later in the season in this study could be attributed to (1) enhanced dandelion uptake of N (Vitousek et al., 2002), although this would be minimal in our alfalfa-dominated field, (2) resource allocation to N fixation (Gutschick, 1987), or (3) a combination of these. Alternatively, weed growth in concert with alfalfa growth could have environmental benefits, as deep-rooted perennials, such as dandelions, have promise as N catch crops, which could reduce N loss through leaching (Thorup-Kristensen & Rasmussen, 2015).

3.3 Microbial linkages to substrate availability

Substrate availability was important predictor for genes representative of N loss but not for *nifH*, N-associated enzyme activity, nor microbial abundance. Total SIN, rather than a specific SIN species, was a better predictor for *nirK* and *nosZ* gene abundance. This is contrast to the findings of Ducey et al. (2013) who found stronger correlations between $\text{NO}_3\text{-N}$ and *nirK* and *nosZ* than between soil %N and *nirK* and *nosZ*, although correlations with both N pools were significant. They attributed significant correlations of

soil %N and N genes to increased water holding capacity with biochar addition, which would allow for greater N transport. This was unlikely to occur in our study due to low application rates reducing physical retention of water (Basso et al., 2013). In contrast, our results likely indicate the requirement of N, regardless of form, for microbial metabolism (Clark et al., 2012; Kaiser et al., 1992). However, this is in opposition to our findings for enzyme activity, microbial abundance, and *nifH*, none of which were correlated with any measure of SIN. The lack of correlation was especially surprising for *nifH* given significant associations between $\delta^{15}\text{N}$ and the other N genes, but this could be due to the time of measurement (see section 3.1). Alternatively, the abundance of *nifH* has been found to be primarily positively associated with microbial biomass carbon across a large number of environmentally variable sites (Hayden et al., 2010), and our low organic matter soil is expected to have relatively low MBC, perhaps explaining the low *nifH* abundance relative to other genes measured. Lack of correlation between measurements of SIN availability and LAP and NAG are also surprising, considering activity of these enzymes should be associated with greater production of SIN (Sinsabaugh et al., 1993), although enzymatic activities can be interpreted in multiple ways. Further, a global scale analysis found that NAG and LAP correlated the most strongly with soil pH (Sinsabaugh et al., 2008) whereas SIN was not correlated with pH across ecosystems (Booth et al., 2005), indicating that these pools may be largely controlled by different environmental variables. The considerably higher abundances of fungal and bacterial DNA as compared to the abundances of N genes indicate that there was a large amount of functional diversity in our soils relative to diversity of N cycling microbes, likely leading to non-significant correlations between microbial

abundance and SIN measurements. Taken all together, findings related to N dynamics imply that eliminating fertilizer addition may stimulate N fixation, while initially reducing total SIN, which, via reduced abundance of denitrifying microbes, may reduce N losses as N₂O or N₂.

3.4 Lack of biochar effects

We see no biochar effects on N cycling, plant growth, and microbial measurements and also no differences between the two types of biochar applied. This was contrary to our expectations, as we expected CBC to be associated with greater reductions in SIN due to its higher C:N, leading to greater immobilization (Borchard et al., 2019) and higher surface area, allowing for greater sorption (Zheng et al., 2010). However, it is possible that biochar differences were masked by general lack of effects with biochar addition, which were likely due to our very low application rate, that was an order of magnitude lower than most biochar studies (112 kg ha⁻¹ vs. rates in tons ha⁻¹), and relatively shallow incorporation depth as compared to rooting depth. The literature supports weaker effects at low application rates – reductions in N₂O emissions and NO₃⁻ leaching are minimized to non-significance at low application rates (Borchard et al., 2019; Cayuela et al., 2014), likely due to reduced chemico-physical effects of biochar. Additionally, lack of effects of biochar on soil biology were also supported by our data as there were no biochar effects on bacterial and fungal abundance, as well as on enzymatic activity, thus negating SIN reduction mechanisms of immobilization and physical sorption of N mineralizing enzymes, respectively. However, some meta-analyses report increased yields or soil NO₃⁻ concentrations with low rates of biochar addition, but these are grouped as <10 tons/ha or Mg/ha (Borchard et al., 2019; Liu et

al., 2013), which could include application rates much higher than what was investigated in this study. In contrast, several biochar studies have found lack of changes in SIN concentration, N₂O, and MBN (Foster et al., 2016; Ramlow et al., 2019; Verhoeven & Six, 2014), even with higher application rates (15-30 Mg ha⁻¹), indicating that biochar may not affect these pools in certain systems, regardless of application rate. Increased N input from legumes could negate increased abiotic and biotic immobilization of SIN with biochar addition, potentially explaining our non-significant effects (Verhoeven & Six 2014). Although, the lack of biochar effect is especially surprising given many reports of stronger increases in water-holding capacity and reductions of SIN with biochar application in sandy soils, like ours, as compared to more clay soils (Atkinson et al., 2010; Nguyen et al., 2017; Spokas et al., 2012). However, a recent meta-analysis assessing crop yields has contested this (Crane-Droesch et al., 2013) and reported the strongest yield increases with biochar amendment to weathered and degraded soils. Because the soil in this study is used for agricultural production, it has received organic amendments that may have increased soil quality, such that we do not see beneficial biochar effects we would expect with more degraded soils.

Our low application rate could also explain the non-effect of biochar on our proxy for N fixation ($\delta^{15}\text{N}$) – Mia et al. (2014) found increased biomass, percent N derived from the atmosphere, and N fixed per pot at 10 tons ha⁻¹ but not at 1 ton ha⁻¹, which is still an order of magnitude higher than the rate used in our study. Since biochar application did not lead to reduced SIN, which was our expected mechanism for increased N fixation, it is not surprising to see non-significant effects of biochar on $\delta^{15}\text{N}$. Non-significant differences in nifH gene abundance between the biochar types may have also been due

to low biochar application rate or, alternatively, due to when genes were measured. Other studies have found increased nifH gene abundance with biochar addition (Ducey et al., 2013; Harter et al., 2014), but this effect diminished after 22 days (Harter et al., 2014), and a longer term study found no significant differences between biochar-amended and control soils after 6 and 12 months (Bai et al., 2015). Since gene abundance was measured on soils collected ~5 months after biochar application, any positive effects of biochar on nifH abundance may have already been reduced to comparable levels with the control. Several studies attribute increased N fixation with biochar to increased availability of micronutrients (e.g. Mia et al., 2014; Oram et al., 2014; Rondon et al., 2007), which our low application rate also may not allow for. However, another study with a low application rate found significantly ($p < 0.1$) increased potassium early in the season (Foster et al., submitted), but even this relatively low application rate was eight times higher than ours (0.8 vs. 0.1 Mg ha^{-1}), potentially representing a threshold effect for benefits of low application rates of biochar. Additionally, the post-processing procedure used on our biochar has been shown to reduce the amount of biochar potassium, boron, and phosphorus, further reducing any benefits of nutrient addition with biochar amendment (data not shown). Our findings support the idea that low application rates of biochar are likely not sufficient to induce increased N fixation.

4. Conclusions

While previous studies have found that biochar is a promising tool for increasing N fixation, our field study found no effects of low application rates of biochar to a sandy soil on N cycling. However, we did find seasonal patterns in N cycling that may have

indicated a shift from plant dependence on soil NO_3^- to biologically fixed N towards the end of the growing season. Additionally, total SIN, rather than a specific inorganic N species, was a better predictor of nosZ and nirK gene abundance. Taken together, these results could have management implications for soil N availability – reducing SIN may both increase N fixation and decrease gaseous N losses, especially through reduced NO_3^- availability. Further study on application rates of biochar that are agronomically, environmentally, and economically effective are needed for biochar to be widely applicable in agriculture.

CHAPTER 4: CONCLUSION

Biochar undoubtedly offers promise for sustainable agriculture. While it is logical to be excited about the prospect of a soil amendment that can do it all – sequester carbon, boost yields, and increase N retention – we cannot allow this excitement to cloud our judgement as scientists. A large number of studies have provided evidence that amending soil with biochar leads to reduced N loss through leaching and N₂O emission, and further, that biochar can increase N input via increased biological N fixation. However, our findings in large part do not support these previous reports.

The second chapter of this thesis explored the fate of fertilizer N in response to both traditional and post-processed biochars, in the presence and absence of plants, in a greenhouse study. Addition of traditional biochar had no effects on N cycling relative to the control in our temperate alkaline soil. These findings agree with others that find minimal biochar effects in temperate regions, where the majority of the world's agriculture is found. In contrast, addition of engineered biochar was associated with increased losses of N, suggesting negative effects of biochar addition. These findings, while potentially associated with specific methods used in our study, provide contrary evidence to previous findings of reduced N loss with biochar amendment. The third chapter of this thesis focused on the effects of low rates of biochar addition on seasonal N cycling in an alfalfa field with a sandy soil. Findings indicated that low application rates of biochar are ineffective for increasing N retention or fixation, even in a sandy soil, where benefits of biochar addition are expected to be highest. These findings

indicate a need to find thresholds of application rates where biochar can have beneficial effects that outweigh the cost of applying that biochar.

My findings suggest that biochar amendments have minimal agronomic benefits, but this is not true of all situations. Positive effects of biochar on N cycling and yield in temperate systems have been shown, albeit generally with high application rates. Farmer incentive is needed for biochar to be effective in temperate agroecosystems. Thus, further study is needed to determine which application rates are economically feasible for farmers, especially given that there is not currently a carbon market in the US that credits for biochar application. These rates will need to be evaluated to determine if they can provide beneficial impacts on plant growth and N retention that can increase profit and/or decrease fertilizer cost for growers.

Studies in this thesis find that plant processes, in contrast to biochar additions, are crucial for increasing N retention and availability. The second chapter of this thesis demonstrated the importance of plant presence for reducing N loss. While this is not entirely surprising, it has implications for agricultural management and suggests the need for careful interpretation of laboratory experiments. While highly controlled laboratory studies may be valuable for elucidating mechanisms, they may overestimate the strength of biochar-induced reductions in loss. There is a need for more studies evaluating biochar effects with and without plants to determine how applicable lab experiments without plants are to agricultural field settings. Findings presented in the third chapter of this thesis suggest leguminous plants leave large amounts of N in the soil following successive years of cropping that can support new growth at the beginning of the growing season. As available N gets used by plants and microbes,

biological N fixation is likely stimulated, allowing for continued growth of the legume and nearby weeds. Understanding plant dynamics over the growing season may allow for more targeted fertilizer application that would reduce N loss and increase biological N fixation by legumes.

Identifying methods to increase N retention in agroecosystems is a crucial hurdle to overcome in reaching a sustainable agricultural system. The growing demand for food coupled with the increased need to reduce human pressure on the environment requires agricultural solutions that can reduce N loss while maintaining crop yields. While biochar did not benefit N retention nor increased crop growth in the systems evaluated in this thesis, plant processes were important for both reducing N losses and increasing N inputs, further confirming the important role of plants in nutrient cycling in terrestrial ecosystems.

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