# INTERACTIVE EFFECTS OF WARMING AND NITROGEN AVAILABILITY ON SOIL CARBON FLUXES: IMPLICATIONS FOR A CHANGING CLIMATE

By

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## ABSTRACT

The excess of greenhouse gasses like carbon dioxide in the atmosphere urgently calls for a deeper understanding of existing natural mechanisms for carbon sequestration. Soils act as a critical natural carbon sink, storing at least twice as much carbon as there is globally in the atmosphere and in plant biomass combined. However, climate change and associated increases in average temperatures can impact soil carbon storage processes, converting carbon sinks to carbon sources. The response of soil carbon to warming is complex and depends on many environmental factors. This study focuses on nitrogen (N) availability, which may mediate the effects of warming on soil carbon via its influence on microbial activity and decomposition. Strong support for this hypothesis is lacking, however, as these variables are difficult to isolate in the field. In this greenhouse experiment, I cross two warming treatments with four N treatments in order to disentangle some of these relationships. To get a picture of how carbon fluxes responded to my treatments, I measured soil organic matter (SOM) as a proxy for the amount of carbon stored in the soil, and soil respiration rate to see how much carbon the soil was releasing. I also measured nitrate and ammonium levels as well as nitrification and mineralization rates in the soil to get a fuller understanding of how my treatments actually affected both N availability and the soil microbial activity governing N availability. I found significant effects from both my warming treatments and my nitrogen treatments, along with significant interactions between the two. Because my pots had living microbes and plants, there were feedbacks between N and C above and belowground, creating complicated relationships between warming, N, SOM, and soil respiration (as expected). Further clarification of these dynamics will be critical for making accurate climate predictions and for strategically targeting restoration efforts to mitigate climate change.

# INTRODUCTION

The release of greenhouse gasses like carbon dioxide into the atmosphere contributes to global warming, which can, in turn, accelerate further release of gasses and create a self-reinforcing feedback cycle (Nyberg and Hovenden 2020). Carbon sequestration through natural ecosystem functions can help absorb and contain some of this excess carbon, and most of this sequestered carbon ends up stored in soil carbon pools (Morra et al. 2023). Previous research suggests that the amount of carbon naturally stored in soil globally is double or even triple the combined amount of carbon in the atmosphere and in plants (Davidson et al. 2000; Balser 2005; Jackson et al. 2017; Dignac et al. 2017; Guenet et al. 2018; Hou et al. 2019). This research implies that soils can be powerful carbon sinks; however, anthropogenic effects can convert soils to carbon sources by altering the processes that cycle carbon between plants, soils, water, and the atmosphere (Carney et al. 2007; Jackson et al. 2017; Reed et al. 2021). Such changes in cycling could place ecosystems with the highest capacity for carbon storage in danger of becoming huge carbon sources due to their large stocks of carbon (Reed et al. 2021). The release of greenhouse gasses from soils worldwide may be up to ten times greater than fossil fuel emissions (Ballantyne et al. 2015; Carey et al. 2016; Gerke 2022). Despite this massive impact on global climate and the large body of research in this field, the mechanisms behind these fluxes are still not very clearly understood, emphasizing the need for further research on the factors that impact soil carbon cycling dynamics.

*How carbon gets into and out of soils*

A main way that carbon is stored in soil is through decomposing plant matter. During the Calvin cycle in photosynthesis, plants fix inorganic carbon from the atmosphere (CO2) into organic carbon stored in their tissues (Ducat and Silver 2012). When these tissues die, they become plant residues and get incorporated into the soil by becoming part of the soil organic matter (SOM). SOM is then decomposed by heterotrophic microbes living in the soil, yielding soil organic carbon (SOC). This SOC does not exist as a permanent addition to the soil; rather, it exists in the different phases of SOM decomposition and is therefore constantly in flux (Prescott 2010). Decomposition simultaneously adds to and subtracts from SOC, adding to the dynamic nature of soil carbon fluxes. Decomposition adds to soil carbon stores by incorporating organic matter into the soil and transforming it into humus (this is called humification), which is a much more stable form of SOM than fresh biomass (Lal 2004; Prescott 2010). Humus can then become even more difficult to break down when it gets incorporated into mineral soil, which protects it from microbes chemically and/or physically (this is called carbon mineralization) (Prescott 2010). Stability of SOM is called recalcitrance; humus is highly recalcitrant because it is resistant to further decomposition by microbes (Balser 2005; Prescott 2010). However, microbes can also decrease SOC by decomposing SOM, respiring as they do and leading to carbon fluxes from the soil to the atmosphere (Ontl and Schulte 2010; Carey et al. 2016; Nyberg and Hovenden 2020). This makes up part of soil respiration– the other part is root respiration, where root cells release CO2 as they burn

glucose (Epron et al. 2010). This process of decomposition, respiration, and some mineralization means that, at any given time, around 5% of the carbon from dead plant biomass is stabilized into inorganic mineral soil (Hicks Pries et al. 2017), between 2% and 20% of it sits in soil organic carbon pools (Lal 2004), while the rest is lost to the atmosphere through soil respiration (Lal 2004).

The amount of SOM in the soil depends on the biomass inputs (Ontl and Schulte 2012). Because this study focuses on the fate of plant interactions with the soil, this literature review will focus on plant biomass and largely ignore biomass from animals. Aboveground plant biomass increases SOM by adding plant litter, and belowground biomass increases SOM by adding root litter (Ontl and Schulte 2012). Intuitively this implies that increases in overall plant biomass would lead to increased SOM and subsequently increased SOC when this biomass eventually dies and decomposes. However, increases in biomass may have no impact on soil carbon (Jackson et al. 2017), and biomass carbon inputs sometimes actually decrease soil carbon (Fontaine et al. 2004). This decrease can occur because soil microbes use the carbon in fresh SOM as an energy source to burn through both this fresh SOM and older SOM faster than before in a process called priming (Fontaine et al. 2004).

Priming is when fresh carbon inputs increase carbon turnover rates and stimulate the decomposition of older carbon, which decreases carbon retention in the soil (Fontaine et al. 2004; Luo et al. 2015; Li et al. 2017; Liu et al. 2020). The microbial mechanism behind this process is complex and not fully understood, but it likely happens because the addition of fresh carbon stimulates microbial

activity, which accelerates the decomposition process (Bond-Lamberty et al. 2004; Fontaine et al. 2004). Priming only happens some of the time, however, depending on the species of microbe that is stimulated (Fontaine et al. 2004). If a microbe species with an r life history strategy (shorter lifespan, more offspring) gets a population growth spurt from the fresh carbon input, then that population might chew through that fresh carbon pool quickly, peak, and then collapse back to pre-input population levels without having altered total SOC very much (Fontaine et al. 2004). On the other hand, if a K strategist microbe (longer lifespan, fewer offspring) gains momentum due to this carbon pulse, this slower life history strategy is less likely to burn through resources quickly and collapse and therefore may lead to longer-term impacts on SOC turnover (Fontaine et al. 2004). Many environmental factors (such as nitrogen) affect the competitive outcome between these two life history strategies (Fontaine et al. 2004). Further, priming effects may vary between aboveground and belowground biomass, with root biomass inputs potentially leading to less drastic priming effects than litter inputs (Luo et al. 2015). This could be because root litter tends to be more recalcitrant than leaf litter (Xia et al. 2015).

More generally, aboveground and belowground biomass have different effects on SOC (Campbell et al. 1991). I suspect that this is because aboveground biomass for leaves and forbs only alters SOC after it dies and falls down into the soil, while belowground biomass can influence SOC while it is still living. Roots, therefore, have more mechanisms through which they can alter SOM/SOC. In order to survive, roots have protection from easy decomposition in the soil, but

they can increase SOC through rhizodeposition, where carbon-rich root exudates, mucilage, and sloughed off cells dissipate into the soil throughout the root's life (McNear 2013; Dijkstra et al. 2020). Also, roots exchange resources with microbes in the soil through the rhizosphere, leading to C efflux from roots into the soil (McNear 2013). These processes create a flow of carbon into the soil from the plant roots (McNear 2013). Lastly, roots can stabilize soil C by creating aggregates which make SOC less available to microbes, although, paradoxically, roots can also destabilize soil C by destroying these same aggregates (Dijkstra et al. 2020). As mentioned above, roots also respire, leading to C losses (Hopkins 2013; Lambers and Oliveira 2019). Roots often play a more significant role in SOM dynamics than does aboveground biomass (Campbell et al. 1991; Kelly et al. 1996; Ontl and Schulte 2012), and I suspect this difference may be due to these different mechanisms, some of which avoid priming effects.

The carbon fluxes leading to carbon sequestration are clearly already quite complex on their own. These fluxes become exponentially more complex when placed in the context of a changing climate, where the environmental factors dictating these dynamics are experiencing dramatic shifts often caused or accelerated by humans. In this thesis I study how two variables associated with human-induced ecosystem changes, global warming and increased nitrogen availability, alter soil carbon storage both independently and when combined.

*Warming and carbon storage*

One factor associated with climate change that will likely impact soil carbon storage is warming temperatures. Warming may increase both aboveground and belowground biomass (Rustad et al. 2001; Lin et al. 2010; Chen et al. 2020). Because biomass makes up a large part of SOM (Kelly et al. 1996), warming could therefore lead to increases in C stocks (Figure 1). However, due to the priming effect described above, increases in biomass inputs may not always lead to increases in C stocks (Fontaine et al. 2004). Warming can also affect soil carbon through soil respiration, which is the release of carbon from the soil (Carey et al. 2016; Nyberg and Hovenden 2020). Many studies have shown that warming increases soil respiration rates (Rustad et al. 2001; Carey et al. 2016; Nyberg and Hovenden 2020), leading to decreased soil carbon storage (Tate 1992; Kirschbaum 1995; Reed et al. 2021). Because of all these interactions, these effects are complex (Figure 1).

As briefly mentioned above, soil respiration happens through two mechanisms: heterotrophic respiration done by free-living microbes during decomposition, and rhizosphere respiration done by roots (Epron et al. 2010; Figure 1). Warming can affect both of these differently, through direct effects on microbe/root metabolism and indirect effects via changes in biomass due to warming. Warming may affect heterotrophic respiration through its direct impact on the microbes doing the respiring; warming can increase microbial activity, which increases respiration (Sistla et al. 2013; Jackson et al. 2017; Walker et al. 2018). As it increases plant growth (Kelly et al. 1996; Sistla et al. 2013;

Ernakovich et al. 2014), warming can also indirectly increase heterotrophic respiration by giving the heterotrophs more fresh labile/available (the opposite of recalcitrant) food to eat (Fontaine et al. 2004). This is the basis of priming – more litter can lead to higher heterotrophic respiration (Fontaine et al. 2004).

The effect of warming on rhizosphere respiration depends on root thickness and depth (Jarvi and Burton 2020), but is generally positive. Warming can directly increase root respiration (Hartley et al. 2007), leading to C loss because increased activity in the rhizosphere leads to increased carbon losses (Körner and Arone 1992). Warming may more indirectly affect rhizosphere activity just by stimulating root growth, which also increases rhizosphere respiration (Kelly et al. 1996). Roots can be responsible for anywhere between 10 and 90 percent of soil respiration, depending on vegetation type and season (Hanson et al. 2000). The relative contributions of root respiration and heterotrophic microbial respiration to overall soil respiration are therefore constantly in flux, and warming affects these two respiration mechanisms differently (Epron et al. 2010; Wang et al. 2014). These constantly shifting forces could help explain some of the variability in warming's effect on soil respiration.

Given this variation, it is not surprising that soil respiration's effect on soil carbon turns out to be equally complex. Meta-analyses find no conclusive evidence about how warming-induced changes to rates of soil respiration and decomposition impact the actual amount of carbon in the soil (Bond-Lamberty and Thompson 2005; Bai et al. 2023), and field studies that have looked into this question present conflicting results. Mayer et al. (2017) and Ouyang et al. (2015)

found that temperature-induced increases in soil respiration were a major contributor to declines in soil carbon stocks. However, Chen et al. (2020) found in a meta-analysis of Tibetan grasslands that increased soil respiration due to warming did not affect total carbon stocks. This lack of effect was attributed to the study being too short and plant biomass temporarily making up for SOC losses (Chen et al. 2020). In the short term, warming-induced respiration may increase soil carbon turnover without net C losses because the carbon cycles more quickly due to exaggerated outputs and inputs (Jan van Groenigen et al. 2014). Over the long term this increased C cycling could be bad for climate warming because the respiring microbes may reach deeper into older carbon stores, destabilizing long-term belowground carbon pools. These contrasting experimental results could also be due to variation in overall temperature profiles, moisture, and rates of decomposition in these very different habitats. Lastly, conflicting findings could be because effects vary temporally; losses of soil carbon from respiration due to warming may become less and less dramatic over time as microbial communities shift (Knorr et al. 2005; Melillo et al. 2011).

The complexity in how warming affects respiration and how respiration affects soil carbon leads to conflicting results on how warming affects soil carbon. Sistla et al. (2013) found that warming in an Alaskan tundra caused many ecosystem shifts but did not affect soil carbon stocks. On the other hand, Tate (1992) found that warming decreased carbon storage in New Zealand tussock grasslands, and Kirschbaum (1995) found that warming by one degree Celsius decreased soil carbon stores by 3-10% in many different ecosystems depending

on mean annual temperatures. Hopkins et al. (2012) also found that warming seemed to negatively affect soil carbon storage. These contradictory results could be due to the processes described above, and they could also be partially due to the carbon itself; soil carbon's temperature sensitivity can depend on its recalcitrance, with more recalcitrant SOM being more sensitive to decomposition with temperature increases (Knorr et al. 2005; Hartley and Ineson 2008; Hopkins et al. 2012). All of these influences make it extremely difficult to predict the response of soil carbon fluxes to warming, as demonstrated in Figure 1. These responses seem to depend on the location (Carey et al. 2016), which could be because different ecosystems have different microbial communities and biogeochemical composition. More specifically, nitrogen availability in the soil can mediate warming-induced soil carbon losses (Lavoie et al. 2011, Bai et al. 2023).

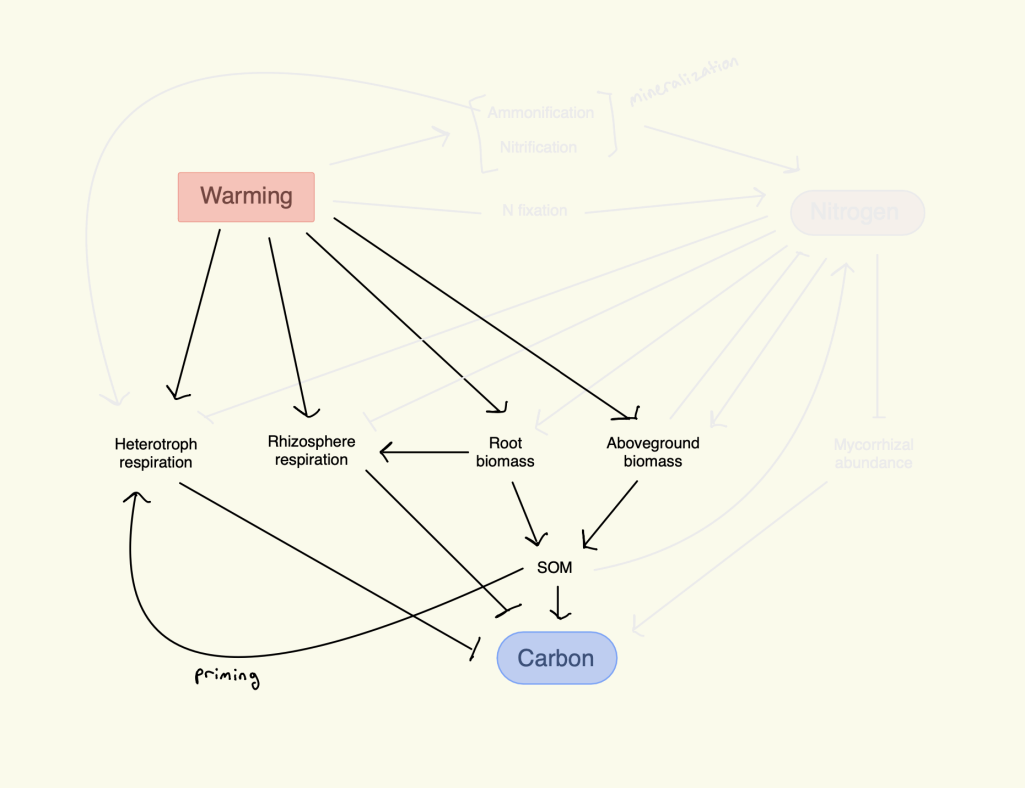


Figure 1: Summary of major mechanisms behind the effect of warming on soil carbon pools. Normal arrows (-->) indicate a positive influence while stopped arrows (--|) indicate a negative effect.

*Nitrogen and carbon storage*

In order to understand nitrogen's potentially mediating influence over soil carbon responses to warming, it is important to first understand how nitrogen affects soil carbon directly. Humans are dramatically increasing the availability of N in ecological systems, both through agricultural inputs and through fossil fuel combustion (Vitousek et al. 1997; Smil 1999). Similarly to warming, nitrogen availability in the soil has a strong influence over soil carbon through its direct effect on the decomposition of SOM through microbes, and its indirect effect on the decomposition of SOM through changing organic matter inputs (Binkley 2005).

N affects soil carbon by directly impacting microbial decomposition processes. Although some studies have shown that N increases microbial biomass and soil respiration by providing fuel (Jesmin et al. 2021), a large body of research has shown that N generally decreases soil microbial biomass (Bääth et al. 1981; Fog 1988 and cited within; Treseder 2008; Liu and Greaver 2010; Jian et al. 2016; Liu et al. 2018) and activity (Compton et al. 2004; Wallenstein et al. 2006; Lu et al. 2021). These decreases in microbial biomass and activity have been partially attributed to an N-induced decrease in pH, which acidifies the soil and makes living conditions less optimal for microbes (Treseder 2008; Liu and Greaver 2010; Lu et al. 2021). By decreasing soil microbial biomass and activity, N inputs can decrease decomposition (Keeler et al. 2009; Li et al. 2014), which decreases soil respiration (Bääth et al. 1981; Treseder 2008; Liu and Greaver 2010; Sun et al. 2014; Liu et al. 2018; Lu et al. 2021; Figure 2). The effect of N is not equal across all stages of decomposition, however.

Increased nitrogen availability can actually increase early stage decomposition rates while decreasing late stage decomposition rates (Hobbie et al. 2012; Jílková et al. 2020; Gill et al. 2021; Gill et al. 2022 and cited within; Wang et al. 2022). Early stage decomposition is the microbial processing of fresh, only partially decomposed organic input into slightly more mature forms in the soil. The microbes doing this processing are likely N-limited, so adding N increases C demand, leading to more decomposition (Gill et al. 2022). This shift in decomposition rates has been shown in other studies, in which fresh litter with high N is processed faster than litter with low N (Cornwell and Weedon 2014),

and in many other examples of N stimulation of early stage decomposition (Hobbie et al. 2012). Late stage decomposition is the processing of already partially-decomposed organic matter. Experimentally, N decreases this stage of decomposition (Hobbie et al. 2012; Jílková et al. 2020; Gill et al. 2022) and therefore decreases SOM degradation/release (Binkley 2005; Frey et al. 2014; Vandenenden 2021).

The mechanisms behind this pattern are slightly unclear, but it could be because N can have opposite effects on the enzymes that the microbes use to degrade different molecules in plant tissues (Keeler et al. 2009). For example, N can enhance cellulose-degrading enzymes (Keeler et al. 2009) while inhibiting lignin-degrading enzymes (Frey et al. 2004; Jian et al. 2016). Cellulose and lignin are important components of plant tissues, and they vary in their recalcitrance; cellulose is a polymer made up of glucose (among other things) and is therefore attractive food for microbes (Blagodatskaya et al. 2014), while lignin is highly recalcitrant and difficult for microbes to break down (Kögel-Knabner 2002; Ruiz-Dueñas and Martínez 2009). Cellulose decomposition therefore dominates early-stage decomposition (Berg and McClaugherty 2014), while lignin decomposition dominates late-stage decomposition (Hobbie et al. 2012). If N increases cellulose-decomposing enzyme activity while decreasing lignin-decomposing enzyme activity, then it makes sense that N would increase early stage decomposition while decreasing late stage decomposition. These mechanisms are not confirmed, however (Fioretto et al. 2005; Hobbie et al. 2012), and are complex because lignin itself

can affect cellulose decomposition, and because these dynamics depend on the type of litter, the environment, and seasonality (Yue et al. 2016).

Although some of the mechanisms behind it need clarification, the pattern of N increasing early stage decomposition while decreasing late stage decomposition is fairly well-demonstrated and drives the effects of N on C in the soil (Hobbie et al. 2012; Jílková et al. 2020; Gill et al. 2021; Gill et al. 2022 and cited within). Accelerating early stage decomposition while decelerating late stage decomposition leads to increased litter mean residence time in the soil, which in turn increases carbon accumulation in the soil (Gill et al. 2022). This stabilization of SOC in the soil by increasing residence time is not the only way N affects microbial activity; through other enzymatic pathways, N can also stimulate humification (Prescott 2010) and reduce microbial community priming effects (Liu et al. 2018).

N can also have more indirect effects on decomposition by changing plant litter inputs, similar to warming. Because nitrogen is essential for all proteins, increased access to N can increase plant growth and biomass production (LeBauer and Treseder 2008; Yue et al. 2021; Figure 2). This can have positive effects on SOM (Vitousek et al. 1997; Frey et al. 2014), but, as discussed in above sections, increased plant growth and contributions to soil organic biomass may actually decrease carbon retention in the soil by priming soil microbial communities toward species that then break down this fresh plant biomass (Fontaine et al. 2004; Figure 2). Further, N availability affects priming more directly by influencing the competitive outcomes between r and K microbe

strategists which determine the magnitude of priming effects (Fontaine et al. 2004). N effects on biomass can therefore affect soil C in different ways.

Adding another layer of complexity, the effect of N on biomass is itself also complex: aboveground and belowground biomass responses to N can differ, which is important because aboveground biomass and belowground biomass can affect soil C differently (described in section 1 of introduction). When a plant is stressed by mild N limitation, it tends to allocate more of its resources belowground in its root biomass in search of better N pools (Johnson and Thornley 1987; Griffith et al. 2000; Topa 2004; Jia and Wirén 2020). This fits into a general trend of nutrient limitation leading to an increased fraction of overall plant biomass going to root production to increase access to soil nutrients (Kramer-Walter and Laughlin 2017). Adding N can alleviate this stress (Johnson and Thornley 1987; Topa 2004), leading to decreased root to shoot ratio in plants in two large meta-analyses (Liu and Greaver 2010; Yue et al. 2021). Some studies have found the opposite effect, where N addition seemed to give roots more nutrients and so they grew more (Griffith et al. 2000; Topa 2004); differences in the form of inorganic N (nitrate or ammonium) added could help explain these discrepancies (Yan et al. 2019). The overall trend seems to be that N addition leads to a smaller proportion of biomass allocated to roots, which would likely decrease rhizosphere respiration and therefore help explain N's inhibiting effect on soil respiration (Figure 3).

In both field studies (Vitousek et al. 1997; Liu and Greaver 2010; Jian et al. 2016) and meta-analyses (Huang et al. 2020), N addition largely increased

carbon sequestration. For example, two decades of nitrogen enrichment enhanced soil carbon storage in a deciduous forest (Lovett et al. 2013). However, there is a limit to this effect: chronic nitrogen addition through fertilizer runoff and other anthropogenic inorganic nitrogen inputs can reduce soil's ability to hold onto carbon in some ecosystems (Marchetti et al. 2023). The effect of increasing nitrogen availability may depend on many different environmental factors such as microbial community structure (Fog 1988, Zhu et al. 2016). For example, increased nitrogen availability significantly decreased mycorrhizal abundance in a Swedish pine forest (Bääth et al. 1981) by as much as 15% (Treseder 2004). Losing these plant facilitators can decrease carbon storage (Erisman et al. 2013), although nitrogen's effect on mycorrhizae varies widely with the ecosystem (Treseder 2004). N addition may increase SOC in the top layers of the soil but not in mineral layers, making it difficult to make long-term predictions (Liu and Greaver 2010; Huang et al. 2020). Despite this complexity, it seems as though minor increases in nitrogen generally increase soil carbon storage.

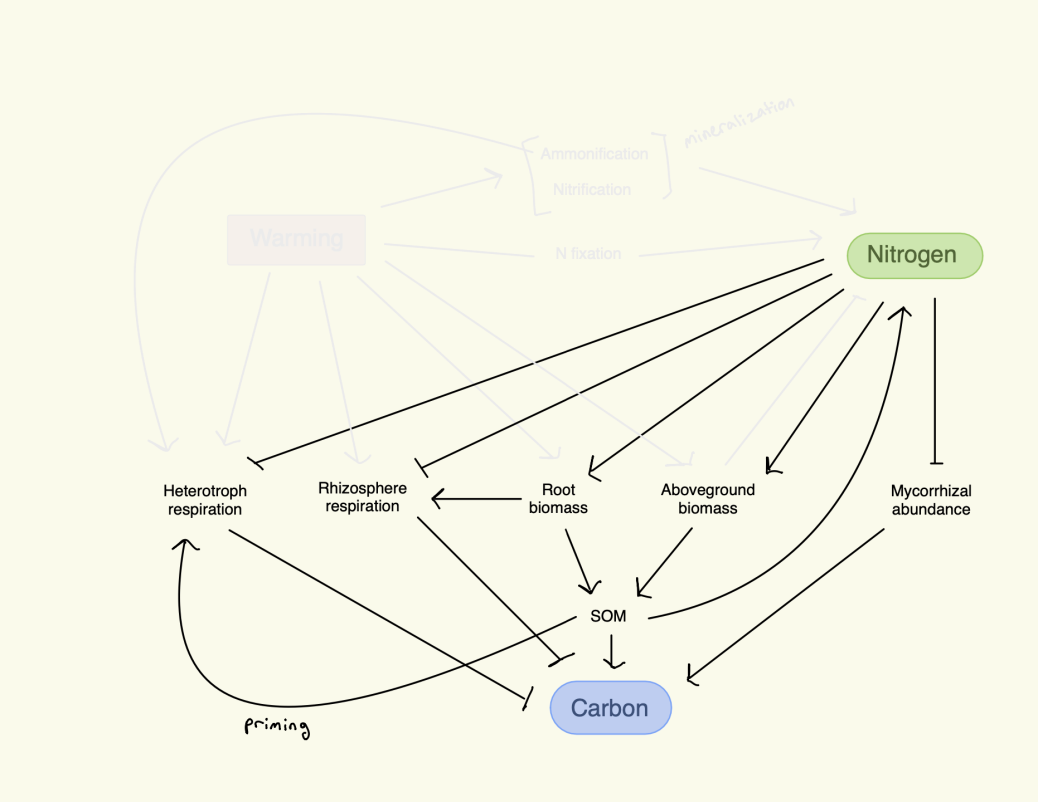


Figure 2: Summary of major mechanisms behind the effect of nitrogen on soil carbon pools. Normal arrows (-->) indicate a positive influence while stopped arrows (--|) indicate a negative effect.

*Warming and Nitrogen*

One last piece to consider before zooming out to understand how nitrogen may mediate effects of warming on soil carbon is how warming affects nitrogen availability. The effect of warming on nitrogen availability is also complex, as it can have varying impacts on the different stages of the nitrogen cycle. In the soil, N is present in four main forms: organic N, ammonium, nitrite, and nitrate (Dodds and Whiles 2017; Figure 3). First, nitrogen is stored in the tissues of primary producers (Strock 2008). When heterotrophic microbes break down these tissues during decomposition, they release ammonium ions in a process

called ammonification (Strock 2008; Figure 3). This ammonium is converted into nitrite and then nitrate by nitrifying autotrophs in a process called nitrification (Alexander 1965; Figure 3). These nitrifying bacteria are called nitrosomonas and nitrobacters, which get their energy from this oxidation of inorganic nitrogen compounds (Prosser 2007). The overarching process of converting organic N in plant tissues to inorganic N in ammonium and nitrate pools through ammonification and nitrification is often called net mineralization (Tietema and Wessel 1992; Schütt et al. 2014), although some studies use this term when just referring to ammonification (Butler et al. 2012), leading to some confusion when comparing studies. Both ammonium and nitrate are taken up by plants to build plant tissues, and then the cycle begins again with N in its organic form (Strock 2008). Nitrogen can also get into the soil through nitrogen fixation, which transforms $N_2$ gas into ammonium (Mylona et al. 1995; Figure 3; mechanism described below). Warming affects almost every stage of this cycle.

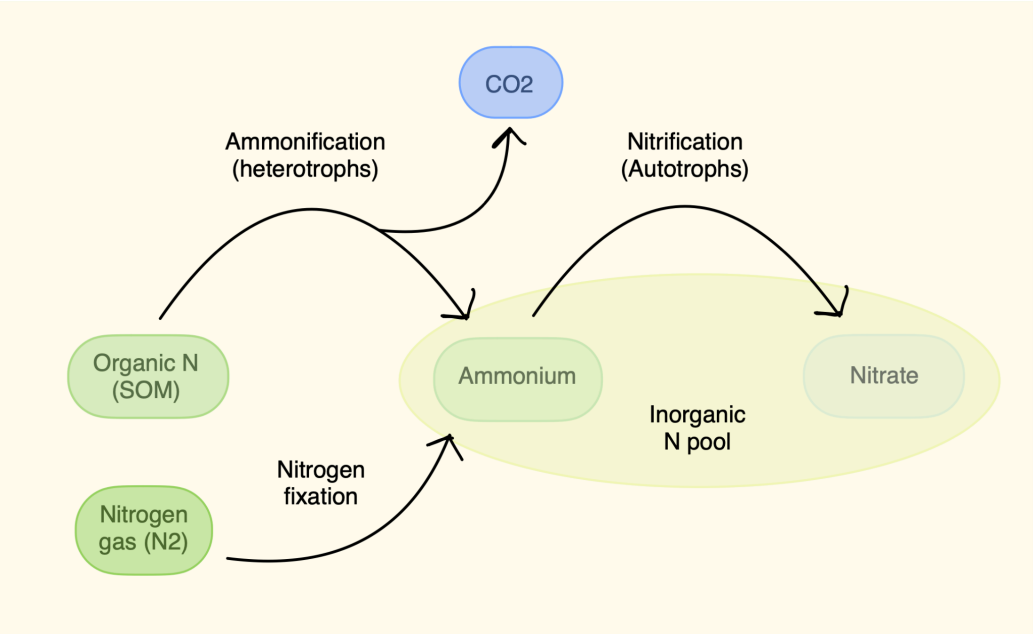


Figure 3: Diagram explaining the steps of N mineralization. Mineralization is defined here as total movement from soil organic N into soil inorganic N pools. N-fixation from $N_2$ gas into ammonium is also included.

First, by increasing the biomass of plants in the system, warming affects the organic plant inputs that can be decomposed (Sistla et al. 2013; Ernakovich et al. 2014). More organic N input via this biomass leads to higher nitrification and mineralization because the microbes have more to work with (Falkengren-Grerup and Schöttelndreier 2004). However, more biomass also means that plants might be pulling more N up out of the soil in order to build their tissues (Gastal and Lemaire 2002; Figure 4). Second, warming affects the rate at which the N in these dead plant tissues in the soil is physically broken down through mineralization during decomposition (Lett and Michalsen 2014; Zhang et al. 2016; Figure 4). Warming can accelerate total N mineralization (Rustad et al. 2001; Butler et al. 2012; Bai et al. 2013; Lett and Michalsen 2014; Zhang et al. 2016; Figure 4). This is because the microbes involved in ammonification

(Myers 1975) and nitrification (Butler et al. 2012; Bai et al. 2013) are temperature-sensitive.

So do these warming-induced accelerations increase nitrogen availability, or do they just lead to a faster cycling process where nitrogen spends less time in each stage but overall nitrogen levels stay about the same? The increases in microbial processes with warming can be drastic and often do lead to increases in overall nitrogen availability (Butler et al. 2012; Bai et al. 2013; Zhang et al. 2016). Warming also increased N pools in a large meta-analysis (Bai et al. 2013). However, this effect is not universal, with high global variance (Sun et al. 2022). In a semiarid grassland, warming increased nitrogen availability (Carrillo et al. 2012). Somewhat contrastingly, Chen et al. (2020) found that warming increased soil ammonium but not total nitrogen in a meta-analysis of Tibetan grasslands. This implies accelerated ammonification without associated accelerations in nitrification, which fits with the Bai et al. (2013) meta-analysis that warming can proportionally increase ammonification more than nitrification. Differences in warming effects on ammonification and nitrification may be due to microbial adaptations to different environments (Thamdrup and Fleischer 1998). Although these many interactions clearly make the process complex, it seems that a few degrees of warming generally increase nitrogen availability by increasing N mineralization.

As I alluded to above, N mineralization is not the only way through which N becomes available in the soil. Nitrogen fixation through mutualism between plants and N-fixing bacteria plays a huge role in mobilizing N (Mylona

et al. 1995; Vitousek et al. 1997; Figure 4). This mutualism happens between specially adapted plants (such as legumes) and several nitrogen-fixing bacteria. For most legumes, this nitrogen-fixing bacterium is in the genus *Rhizobium*, and researchers refer to multiple bacteria in this genus as *Rhizobia* (Mylona et al. 1995). The *Rhizobia* live in nodules that form on plant roots, converting $N_2$ gas into ammonium for the plant in exchange for carbohydrates (Mylona et al. 1995). Warming has a variable impact on nitrogen fixation (Lett and Michalsen 2014). Warming can depress nodulation and N-fixation in *Vigna radiata* (Hafeez et al. 1991). Some mosses in an Arctic heath decreased N-fixation in response to warming as well, although some stayed the same (Sorensen and Michelsen 2010; Rouse and Michalsen 2016), and some increased (Deslippe et al. 2005). Lett and Michalsen (2014) found that the effect of warming in a subarctic heath depended on the season, with warming increasing fixation by two to fivefold in the late spring but decreasing fixation in the long term. Some studies have shown that perhaps fixation by legumes actually may not rely on temperature, with moisture being a more important factor (Whittington et al. 2013; Rousk et al. 2018). Understanding how warming affects N in real-world ecosystems requires better knowledge of how warming affects N-fixation.

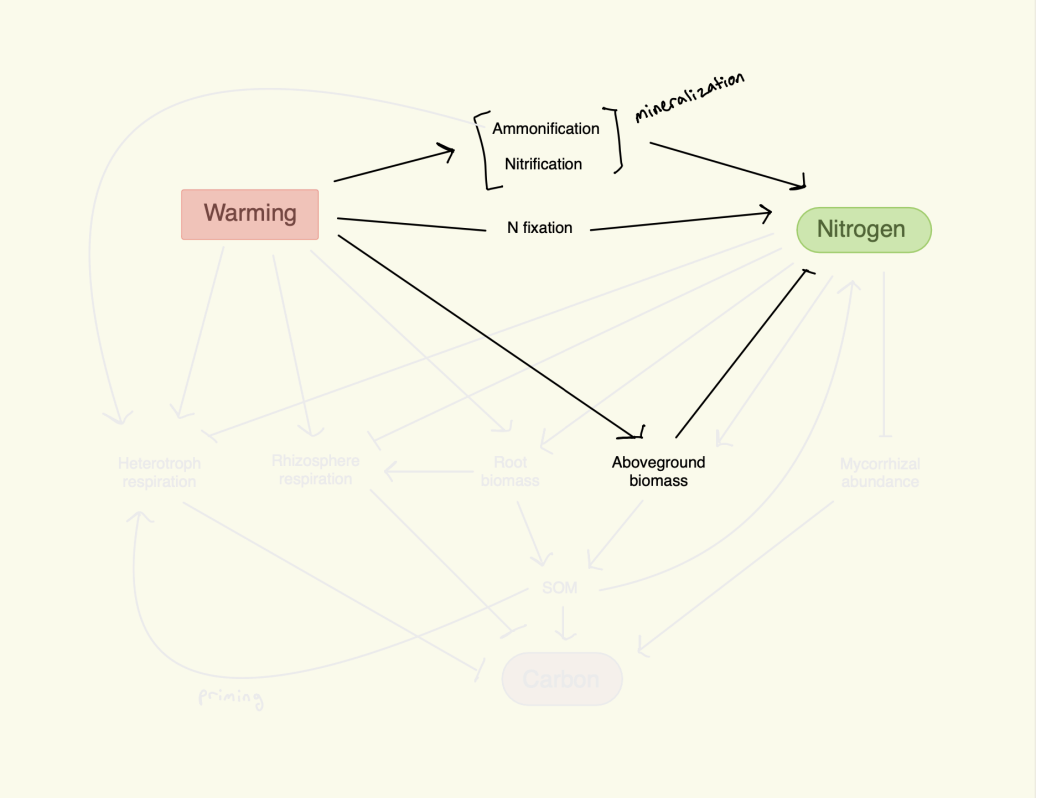


Figure 4: Summary of major mechanisms behind the effect of warming on soil nitrogen pools. Normal arrows (-->) indicate a positive influence while stopped arrows (--|) indicate a negative effect. No end to the arrow (N fixation) indicates a lack of clarity in the literature.

*Warming, nitrogen, and carbon*

These three pieces — warming effects on carbon, nitrogen effects on carbon, and warming effects on nitrogen — play out in interesting ways. Increasing nitrogen seemed to alleviate warming-induced carbon losses from the soil in a meta-analysis (Bai et al. 2023). The positive effect of increased nitrogen and the negative effect of increased warming on soil carbon storage are not additive, though (Vandenden et al. 2021). The direction and size of the effect of warming on carbon can vary based on the N content in the soil (Bai et al. 2023; Li et al. 2023). None of these factors are affecting carbon independently; as

shown in Figure 5, each factor is acting on many mechanisms, which can then create feedbacks affecting how all the other variables function in the system. Because nitrogen seems to respond in many different ways to warming, and because nitrogen has such a strong influence over decomposition, it makes sense that, when nitrogen and warming are combined, the effects on soil carbon are variable (Figure 5).

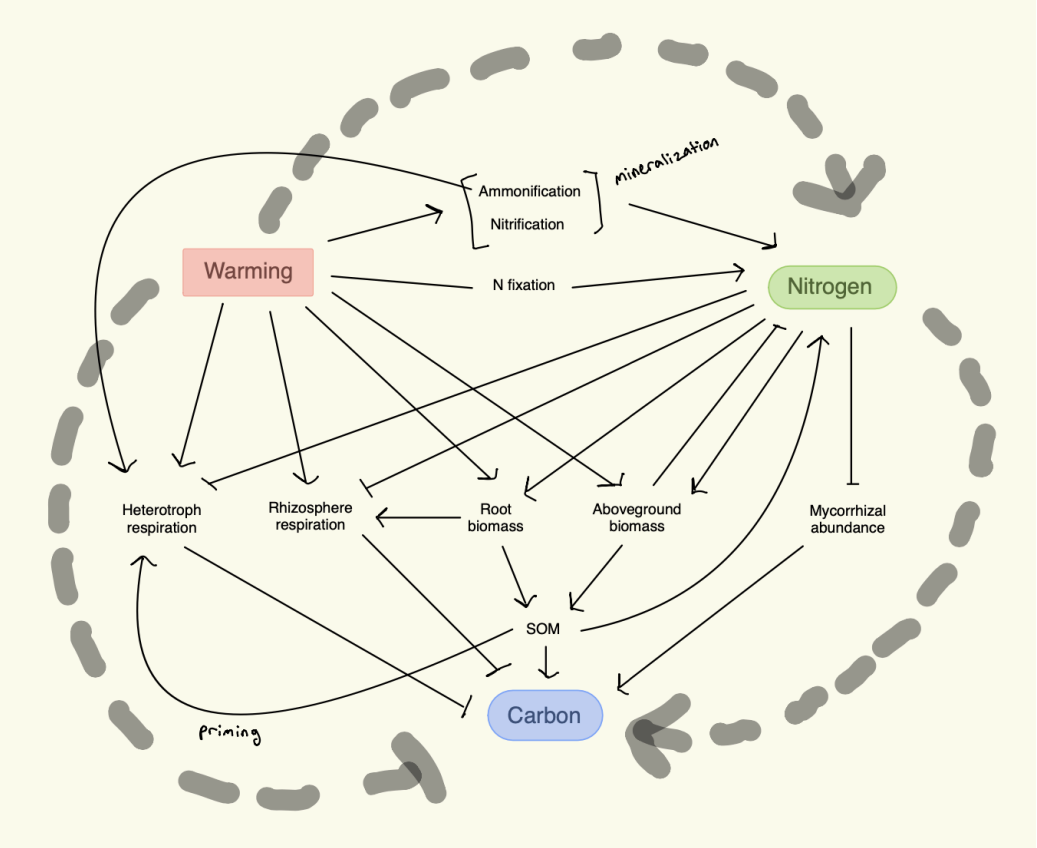


Figure 5: Visualizing the web of interactions between warming, nitrogen, and carbon. Normal arrows (-->) indicate a positive influence while stopped arrows (--|) indicate a negative effect. Thick dashed arrows indicate general patterns derived from the literature.

*Hypotheses*

The purpose of my thesis is to disentangle these interconnected cycles in order to clarify the roles of different factors when isolated and when in combination. I will do this by manipulating warming and nitrogen availability in a controlled greenhouse environment. Hopefully my data will be able to corroborate field studies and add another perspective to the growing body of knowledge in this area.

A second purpose of this study is to replicate a student project done last year, which looked at the effects of the non-native N-fixer, *Trifolium repens* (white clover), on native species and the role of nitrogen in this interaction. I am replicating their experiment and then testing the soil for nitrogen, which will hopefully provide some clarity for interpretation of their results. I am using their four *Trifolium* treatments to create a spectrum of N-levels with which I will test my hypotheses by crossing these four N manipulations with two warming treatments (Figure 6). This makes my project more interesting because I can look at not just N but the interplay between plant growth (with associated N uptake), N-fixation, and belowground N and C inputs.

I did this experiment in greenhouse pots with four *Trifolium* treatments to manipulate nitrogen availability and warming mats to raise the temperature for half of the pots. I measured soil respiration, nitrate, ammonium, nitrification and mineralization rates, and SOM as a proxy for SOC. My goal was to answer the following questions. How do the experimental treatments from last year's *Trifolium* study affect N availability in the soil? How does warming affect soil

carbon storage and soil respiration? How does nitrogen availability affect soil carbon storage and soil respiration? How does warming affect nitrogen availability? How does warming affect nitrification and mineralization rates? Lastly, how do nitrogen availability and warming interact to affect soil carbon storage and respiration?

Before I explain the expected effects of my *Trifolium* treatments on N, I will need to briefly explain what they were (see Figure 6 for a visual). My first treatment had live *Trifolium* growing in the pots throughout the entire 10-week growing period (I called this my live treatment). My second treatment also had live *Trifolium* growing in it, but I removed the aboveground biomass after 5 weeks (I called this my removed treatment). My third treatment was the same as the removed treatment except I took the aboveground biomass that I had removed, ground it up into a slurry, and poured it back onto the soil surface (I called this my slurry treatment). My last treatment was a control, which had no plants growing in it and no slurry added.

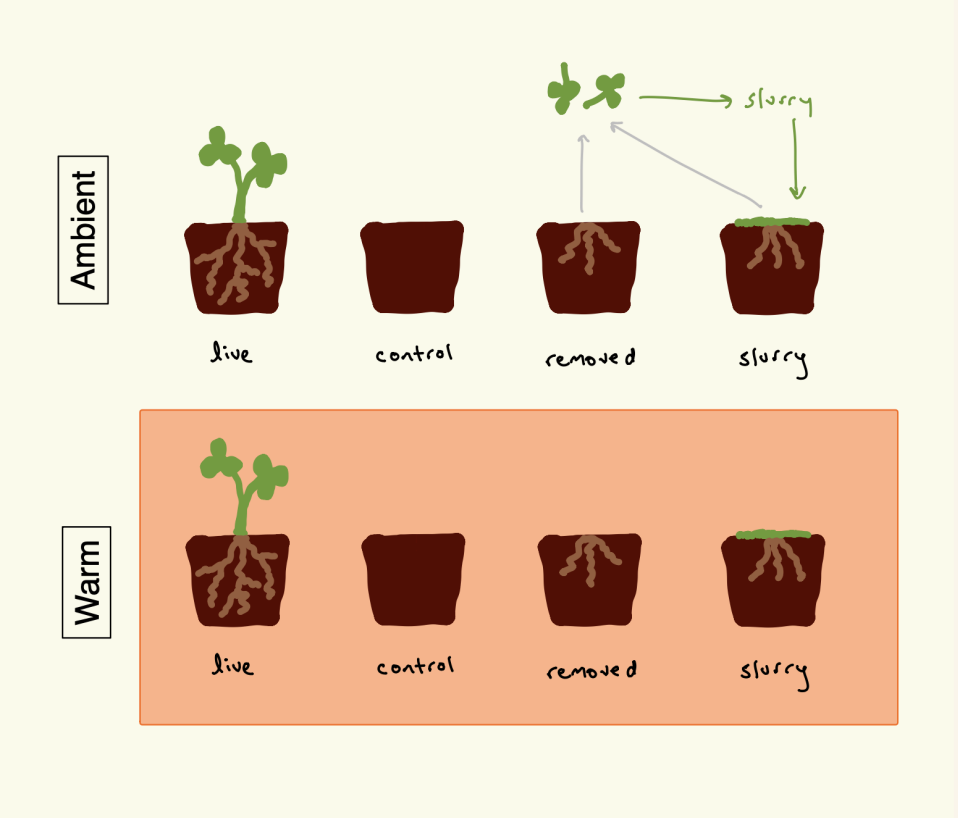


Figure 6: Diagram of my experimental design.

In terms of nitrogen, I predicted that these *Trifolium* treatments would affect N availability in different ways. In all of my treatments that had ever had plants growing in them (live, removed, slurry), I predicted that N levels would be reduced compared to controls because, in order to build their tissues, plants need to uptake N. I thought that the slurry treatment would replenish some of this N because I manually broke down the organic nitrogen in the plant tissues and poured it directly back onto the soil, making it highly available for ammonifiers and nitrifiers to convert it into inorganic N. I thought that my live treatment might also replenish some of the N taken up by the plants by facilitating N-fixation; on the other hand, the N uptake in these live *Trifolium* pots would

also be greater because the plants were growing for longer. These processes moving in both directions meant I was not exactly sure what to expect. I guessed that the live treatment might have more N because it had a root system to hold onto N during watering while the N in controls and pots with less-developed root systems might just be getting washed out. Lastly, I thought that the *Trifolium* removal treatment would have the lowest N availability because the plants had enough time to draw some N out of the soil but not enough to replace that withdrawn N after N-fixation began. The lack of clarity around some of these hypotheses and predictions illustrates why this study is useful for informing the effects of the *Trifolium* treatments on last year's experiment.

Further, I predicted that warming would magnify all of these effects (both on N and on C) by accelerating both plant and microbial growth. Overall, I predicted that warming would increase nitrification and mineralization, and that this increased rate of transformation into inorganic N would increase nitrogen availability across all treatments. I hypothesized that this increased nitrogen availability would slightly increase soil carbon storage by decreasing soil respiration and accelerating early stage decomposition while slowing late stage decomposition. On the other hand, I hypothesized that warming would increase soil respiration due to increased microbial activity, and that this microbial activity would lead to decreased carbon storage; however, I predicted that, when nitrogen and warming treatments were mixed, these warming-induced losses in soil carbon would be lower under higher N-availability.

To add one last complication, I predicted that my *Trifolium* treatments

would also vary in terms of their effects on belowground C dynamics. Two treatments (removed and slurry) had dead (and therefore decomposing) roots left in the soil. One treatment (live) had live roots in the soil. It is probable that these roots were a carbon input, which intuitively would lead to higher soil carbon. I predicted that this effect would be stronger in warming pots compared to ambient pots, because the plant carbon would be broken down faster and integrated into the soil more quickly. This increased carbon could potentially cancel out carbon losses from accelerated decomposition due to warming. However, it is actually not clear that increased carbon inputs always lead to increased soil carbon, as described in the above sections on priming and shifting microbial community composition. Priming effects could then cancel out the idea of warming enhancing carbon storage by accelerating carbon inputs from roots, so I predicted that there might still be a negative effect of warming on soil carbon in pots with roots. When interpreting my results and looking for relationships between warming, N, and C, I knew that I would have to account for *Trifolium* effects on both N and C.

## METHODS

*Greenhouse setup*

I used a potted greenhouse experiment with four nitrogen treatments and two warming treatments with ten replicates (for a total of eighty pots) to explore these questions. For my nitrogen manipulations, I used three experimental nitrogen treatments with one control. I manipulated nitrogen by following the protocols set by the thesis project done last year, which involve the growth of *Trifolium repens*, a nitrogen-fixing legume species. I summarized these manipulations briefly in the introduction, but to review: the first experimental treatment was planting live *Trifolium* that grew throughout the entire experiment, the second experimental treatment was removal of the aboveground biomass of the live *Trifolium* after 5 weeks of growing (I did this by pinching off the tops by hand), and the third experimental treatment was the same as the second treatment except I added the removed *Trifolium* back into the soil as a slurry (see below for more details). The control treatment had no *Trifolium*.

I planted the *Trifolium* seeds in 3.5'' x 3.5'' pots on September 30, 2023. Before planting the seeds I inoculated them using N-Dure seed inoculant for clover plants from Verdesian Life Sciences to promote nodulation. The active ingredients were *Sinorhizobium meliloti* 2 x $10^8$ CFU/g and *Rhizobium leguminosarum* biovar *trifolii* (Clover) 2 x $10^8$ CFU/g. However, after around five weeks there was no nodulation in the roots of this first round of plants, which meant that the *Rhizobia* had not formed the nitrogen-fixing mutualism in the way that we were hoping. Although we used the lowest nutrient potting soil

available at the MHC greenhouse, it did have some added nutrients and therefore plenty of available nitrogen, making the N-fixing mutualism unnecessary. I removed all of the *Trifolium* from each pot, including the roots, on November 7, 2023, mixed all of the soil from the pots with *Trifolium* together in a bucket (keeping the warming treatment separate from the ambient treatment), removed any remaining root material, and added around 6 potfuls of fresh potting soil to the combined mixture for each warming treatment to add volume. I then planted a second round of *Trifolium* in this combined soil, keeping warmed and ambient treatments separate. My hope was that the first round of plants had drawn up a lot of the available soil nitrogen, leaving N-depleted soil that would encourage the *Trifolium* and nitrogen-fixing *Rhizobia* to create nodules and fix nitrogen. I also added more *Rhizobia* inoculum to the seeds and planted more seeds per pot (around 80, using a marked circle in a small spoon) because the plants were looking a bit sparse in the first round (I did not count the seeds in the first round, but had been using a slightly smaller circle on the same spoon). Lastly, I used the newly inoculated seeds to plant four extra trays of *Trifolium* in this same soil from the buckets, so that we would have enough *Trifolium* tissue to make the slurry. Two trays of extra *Trifolium* were warmed and two trays were left in the ambient conditions of the greenhouse.

The second round of plants nodulated, and, five weeks after the second planting, with the help of other members of the Hoopes lab, I removed all the aboveground *Trifolium* plant biomass from the removed and slurry treatment pots and poured the slurry back onto the slurry treatment pots. I made this slurry, with

the help of Martha Hoopes, by blending removed aboveground tissues with 600 mL of water for a couple minutes until we saw no change in consistency. We made a separate slurry with the *Trifolium* tissues from the warming treatment to pour on the warmed pots and from the ambient treatment tissues for the ambient pots. There was not enough aboveground biomass to make a sufficient amount of slurry to pour over all the pots requiring slurry, so we added to the blend the extra *Trifolium* that we had grown in separate trays for each warming treatment, keeping warm with warm and ambient with ambient. We then poured 50 mL of this mixture evenly into the slurry pots and 50 mL of water into all the other pots. I deconstructed all the pots after 10 weeks to collect data.

For my warming manipulation, I used a warming mat on which I put half of the pots. This warming mat consistently warmed the pots to around 23.3$^{o}$C, which was around 3.3$^{o}$C warmer than the approximate average greenhouse temperature of 20$^{o}$C. I randomly placed the pots in trays using the Excel randomizing function, and twice a week I rotated each tray of pots 180 degrees and switched the location of the warmed and ambient trays. The greenhouse staff checked on the plants nearly every day and watered them depending on drying conditions.

*Biomass*

When I deconstructed all the pots at the end of the 10-week growing period, I removed all of the remaining aboveground biomass from my pots (the live pots were the only ones with aboveground biomass left) and weighed each

individual pot's worth of biomass to get a picture of how warming might have affected plant growth.

*Soil respiration*

I measured soil respiration a couple of days after removing the biomass to minimize the effect of this disturbance on the respiration data. I measured soil respiration using a Labquest 3 by Vernier with a CO2 sensor attachment that fit snugly into the top of a 250 mL square Nalgene bottle. Kris Camp, Jason Andras, and Kate Ballantine helped me saw off the bottom of the bottle so that I could place the bottle over each pot. In order to ensure a tight seal over the soil, I had to push the bottle about an inch into the soil. The sensor then recorded how quickly CO2 built up in the small container with a graph of CO2 ppm over time. The monitor recorded CO2 ppm once every two seconds and plotted it as a line. The line was usually a bit unreliable for the first 150 seconds, so I calculated the slope from 150-300 seconds to find my respiration rate for each pot. I took two measurements per pot, fanning and airing out the bottle between measurements but not removing it because I did not want to disturb the soil. Between measurements, the sensor needed to be aired out for 2-5 minutes or until the CO2 ppm number stabilized. It was important to take these measurements in the shade because the CO2 sensor was very unreliable in direct sunlight. Sometimes, the Vernier suggested that the slope reversed direction between runs so I discarded these data and was able to re-do these runs. It was important to keep the soil in the pot relatively intact and undisturbed for these soil respiration measurements,

but once I finished I was able to take samples of soil out of my pots to run other tests.

*SOM*

I measured SOM to get a picture of carbon storage. I used a muffle furnace to burn off the SOM and then calculated the mass lost on ignition (LOI method). SOM data collected this way is tightly correlated with SOC (Saleska et al. 2002). I collected around 10-15 grams of soil from each pot in aluminum tins and put them all into a drying oven at 60$^{o}$C for four days to dry (I did not want water to be included in the mass lost on ignition). I then put them into a 105$^{o}$C drying oven for one more night to make sure all of the moisture was removed. Once the samples were dried, I took one sample out of the drying oven at a time to process. I spent one minute for each sample picking out the chunks of wood and peat using tweezers, sterilizing the tweezers between each sample. I took these big pieces out firstly because I wanted to measure the SOM in the soil itself and not in these big blocks of carbon, and secondly because they would have led to huge variability in the proportional amount of carbon between samples. After picking out the larger pieces from the soil, I measured the mass of a labeled crucible, put the soil into that crucible, and measured the mass again. Only 40 crucibles fit into the muffle furnace at a time, so I did two rounds of combustion at 550$^{o}$C for six and a half hours each. I then took the samples out and put them into a desiccation chamber so that they could cool without gaining mass by absorbing moisture from the air. I then took the mass of each sample again,

including the crucible but subtracting it from the measurement later during calculations. To calculate percent SOM, I divided the change in soil mass (mass before muffle furnace minus after muffle furnace) by the original soil mass (before the muffle furnace) and multiplied by 100.

*Ammonium and nitrate*

I measured both ammonium and nitrate. I did a KCl extraction to get these forms of N in solution and then ran reactions (described in detail below) on those extracts in Allison Gill's lab at Williams College. For each sample's KCl extraction, I measured out around 10 grams of wet soil and exactly 50mL of a 2M KCl solution (a mixture of solid KCl and distilled water) into a baby food jar and shook it on a shaker table at 180 rpm for 60 minutes. I could fit around 20 jars on the shaker table at a time. Before I put the soil into the jar, I first dumped out the entire pot into a tray and homogenized it so that I was getting a picture of the N in the entire pot and not just in one pocket of soil. This was important because moisture and temperature varied by depth and distance from the center, and there may have been mini pools of N. Also, roots may have caused some heterogenization of N and moisture. I washed out the tray between samples. While I was weighing out this homogenized soil into the baby food jars, I was also weighing out around 10 g of it into aluminum tins which I put into the drying oven and weighed again after a few days to get the dry soil fraction for each sample. I was able to multiply this dry soil fraction by the mass of soil that I had added to the jar so that I knew exactly how much dry soil I had added to each

jar. I later used this information to calibrate my N calculations.

After the jars were finished shaking, I filtered each jar's mixture through a Whatman #1 filter into a vial which I froze and transported to Williams to test for nitrate and ammonium (described below). I also ran two blanks through this shaking and filtering process, so that I had two jars with just the KCl solution and no soil. At Williams to test for nitrate and ammonium concentrations, I ran my KCl extracts through a set of reactions (one for nitrate and one for ammonium) in a 96-well microplate with Allison Gill in order to translate concentrations of these different types of nitrogen to color intensities that are readable using spectrophotometry. For both ammonium and nitrate, I subtracted the ammonium/nitrate concentration in the KCl blanks described above from my samples' ammonium/nitrate concentrations to account for any contamination in the KCl solution.

The ammonium ($NH_4$+) reactions involved a few steps. I put three 50 µL replicates of my samples from the KCl extraction into the 96-well plate along with two 50 µL replicates of the eight standards described below. I then added 25µL of a citrate reagent to all of my wells and allowed it to react for one minute, which chelated any divalent metal ions that would have potentially interfered with the following reactions. I then added 50µL of salicylate-nitroprusside reagent, 25µL of hypochlorite reagent, and 125 µL of nanopure to all wells. When hypochlorite reacts with ammonium it forms monochloramine, which reacts with the salicylate to form benzoquinone monoimine. This benzoquinone monoimine when coupled with the salicylate yields colored indophenol dye. The

intensity of the color of this dye is related to the concentration of ammonium in the beginning of the reaction process, and I measured color intensity using a microplate spectrophotometer at 667 nm after leaving the microplate in the dark at room temperature for 30 minutes to allow the color to develop. In these plates alongside my samples, I had eight $NH_4$+ standards, in which I knew the concentration of ammonium. From this $NH_4$+ standard data I made a standard curve with known ppm of ammonium on the x axis and absorbance values on the y axis. This allowed me to get an accurate measurement of ppm ammonium from my samples' absorbances. Allison Gill made the standards by dissolving 1.90944 g of $NH_4Cl$ in enough nanopure water to make 500 mL total. This was the standard stock solution of 1000 ppm $NH_4$+, which she diluted to make the eight different standards ranging from 0-10 ppm ammonium.

The nitrate reactions were slightly more complex because in order to measure nitrate I needed to first reduce it to nitrite using Vanadium (III) chloride ($VCl_3$). In order to capture the full range of nitrite concentrations, I needed to do a run to measure the low concentrations and a run to measure the high concentrations. First, I did a run to capture the low concentrations of nitrite, using the high concentration $VCl_3$ solution. I did this by adding 100µL of each KCl-extracted sample to each well and 80µL of the concentrated $VCl_3$ solution. To make the concentrated $VCl_3$ solution, Allison Gill dissolved around 200 mg of sulfanilamide and around 10mg of N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) in 100mL of 1M HCl using a spin bar. In another beaker on a scale, she put 50mL of a 1.0M HCl solution and then quickly added

around 400mg $VCl_3$. It was okay not to have exact amounts because $VCl_3$ is a slam reagent. She did all of this under the fume hood and quickly because $VCl_3$ reacts with air. She then added the $VCl_3$ mixture to the sulfanilamide and NEDD solution, yielding the final concentrated $VCl_3$ solution. I then did a run to capture high concentrations of nitrite, using the low-concentration $VCl_3$ solution. To do this, I loaded the microplate with three 9µL replicates of my extracted samples and 200µL of diluted $VCl_3$ solution. To make the diluted $VCl_3$ solution, Allison Gill followed the same protocol as the concentrated vanadium solution except instead of dissolving the sulfanilamide and NEDD in 100mL 1M HCl, she dissolved it in 400mL nanopure water.

The way both of these nitrate reactions (concentrated and diluted) worked was that, when I mixed the $VCl_3$ solutions with my samples, first the $VCl_3$ reduced nitrate to nitrite, then the nitrite diazotized the NEDD so that it could couple with the sulfanilamide (this happens under acidic conditions, which is why we needed to add the HCl) to form chromophores that the spectrophotometer could detect. I then read the absorbance in each well at 540 nm, which gave me data about the intensity of the color. The color intensity is related to the amount of nitrite, and I calculated exact concentrations of nitrite in my samples using a standard curve, as I did for ammonium. To make this standard curve, I ran two sets of nine standards in the plates alongside my samples. I added the same volume of standard solution to the plate as I was adding sample solution (100µL for low concentration nitrite and 9µL for high concentration nitrite). Allison Gill made the $NO_3$- standards by adding 0.3609 g

potassium nitrate ($KNO_3$) to 500 mL ultrafiltered deionized water to create a 100 ppm stock solution, diluted this stock to 10 ppm, and then diluted from there to make the nine standards ranging from 0-10 ppm $NO_3$-. I took the data from the high nitrate concentration run, and when a result had a number that was too low for this run to have been able to capture it accurately (under 15µg per g dry soil) then I took the data from the low nitrate concentration run.

*Ammonification and nitrification*

To get a picture of the microbial processes going on in the soil that were dictating N dynamics, I took these ammonium and nitrate measurements twice, with a two week incubation at 20$^{o}$C in the dark in between. This gave me information about how my *Trifolium* and warming treatments changed the rate of microbial transformations between the different forms of N even after these treatments were removed. I did the first round of KCl extraction a few days after pot deconstruction, and then froze the extracts until I was able to go to Williams a week or so later to run the reactions. I then put my soil into the incubator, waited two weeks, and did the second round of KCl extraction. I was not able to make it to Williams to run the reactions for this second round of data collection, so Allison Gill did them for me. To calculate ammonification, I subtracted initial ammonium from final ammonium. To calculate nitrification, I subtracted initial nitrate from final nitrate.

*Data analysis*

I analyzed my data in R version 4.3.1.To look for effects of *Trifolium* treatment on available nitrogen, I ran two ANOVAs (using aov in R), using nitrate as the response variable for one and ammonium as the response variable for the other. Each test was a two-way ANOVA with warming (two levels) and nitrogen treatment (four levels) as fixed factors and looked for main effects as well as interactions. For each ANOVA, I identified significant differences between treatments with a Tukey post-hoc test and used an alpha value of 0.05 to determine significance. I did the same thing to look for effects of *Trifolium* treatment on net ammonification, net nitrification, soil respiration, and SOM. To look for relationships between variables, I ran regressions using the lm function in R. I looked at the response of soil respiration and SOM to both ammonium and nitrate, and I looked at nitrate and ammonium response to biomass. I also looked at the response of percent SOM to biomass. For each regression, I used an alpha value of 0.05 to determine significance. To look at the effect of warming on biomass, I used a two-way ANOVA using aov in R with two levels of warming and looked for main effects. I used an alpha value of 0.05 to determine significance for this test as well.

# RESULTS

ANOVA analyses

*Nitrogen levels immediately after harvesting of live* Trifolium

There was a significant effect of my *Trifolium* treatments on ammonium ($F_{3,72}$ =3.901, p = 0.012) and nitrate ($F_{3,72}$ =19.981, p = 1.59 x $10^{-9}$) levels in my pots (Figure 7). Ammonium on average was marginally significantly higher in live compared to removed (p=0.0664), and significantly higher in slurry compared to removed (p=0.0462; Figure 7). Nitrate on average was significantly higher in control pots compared to live pots (p<0.0001), removed pots (p<0.0001), and slurry pots (p<0.0001; Figure 7). There were no other significant effects of *Trifolium* treatments on N.

There was a significant effect of my warming treatments on ammonium ($F_{1,72}$ = 49.487, p = 9.54 x $10^{-10}$) and nitrate ($F_{1,72}$ = 46.781, p = 2.18 x $10^{-10}$) levels in my pots, with warming increasing both (Figure 7).

There was a significant interaction between warming and *Trifolium* treatments on ammonium ($F_{3,72}$ = 2.870, p = 0.0422), and a marginally significant interaction between warming and *Trifolium* treatments on nitrate ($F_{3,72}$ = 2.683, p = 0.053; Figure 7).

*Ammonification and nitrification during incubation*

There was no significant effect of my *Trifolium* treatments on ammonification ($F_{3,72}$ =0.423, p = 0.7368), but there was a significant effect of *Trifolium* treatment on nitrification ($F_{3,72}$ =4.61, p = 0.0052; Figure 8).

Nitrification on average was significantly higher in controls compared to live (p=0.0075) and removed (p=0.0186) pots, although it was not significantly higher than slurry pots (p=0.3801; Figure 8). There were no other significant effects of *Trifolium* treatments on nitrification or mineralization.

There was a significant effect of my warming treatments on ammonification ($F_{1,72}$ = 9.295, p = 0.00321) and nitrification ($F_{1,72}$ = 15.62, p = 0.0002) levels in my pots, with warming increasing both (Figure 8).

There was no significant interaction between warming and *Trifolium* treatments on ammonification ($F_{3,72}$ = 0.730, p = 0.5376), and there was a significant interaction between warming and *Trifolium* treatments on nitrification ($F_{3,72}$ = 6.21, p = 0.0008; Figure 8).

*Carbon fluxes*

There was no significant overall effect of my warming treatment on soil respiration ($F_{1,72}$ = 1.25, p = 0.267; Figure 9). There was a marginally significant effect of my warming treatment on SOM ($F_{1,71}$ = 3.936, p = 0.0511), with warming slightly increasing SOM (Figure 9).

There was a significant effect of my *Trifolium* treatments on soil respiration ($F_{3,72}$ = 10.74, p = 6.49 x $10^{-6}$; Figure 9). Soil respiration was higher in live pots compared to control pots (p=0.0001), removed pots (p=0.0001), and slurry pots (p=0.0019; Figure 9). There was no significant effect of my *Trifolium* treatments on SOM ($F_{3,71}$ = 0.127, p = 0.9438; Figure 9).

There was a significant interaction between my *Trifolium* treatments and

my warming treatment on soil respiration ($F_{3,72} = 11.59$, $p = 2.78 \times 10^{-6}$; Figure 9). There was no significant interaction between my *Trifolium* treatments and my warming treatment on SOM ($F_{3,71} = 0.313$, $p = 0.8160$; Figure 9).

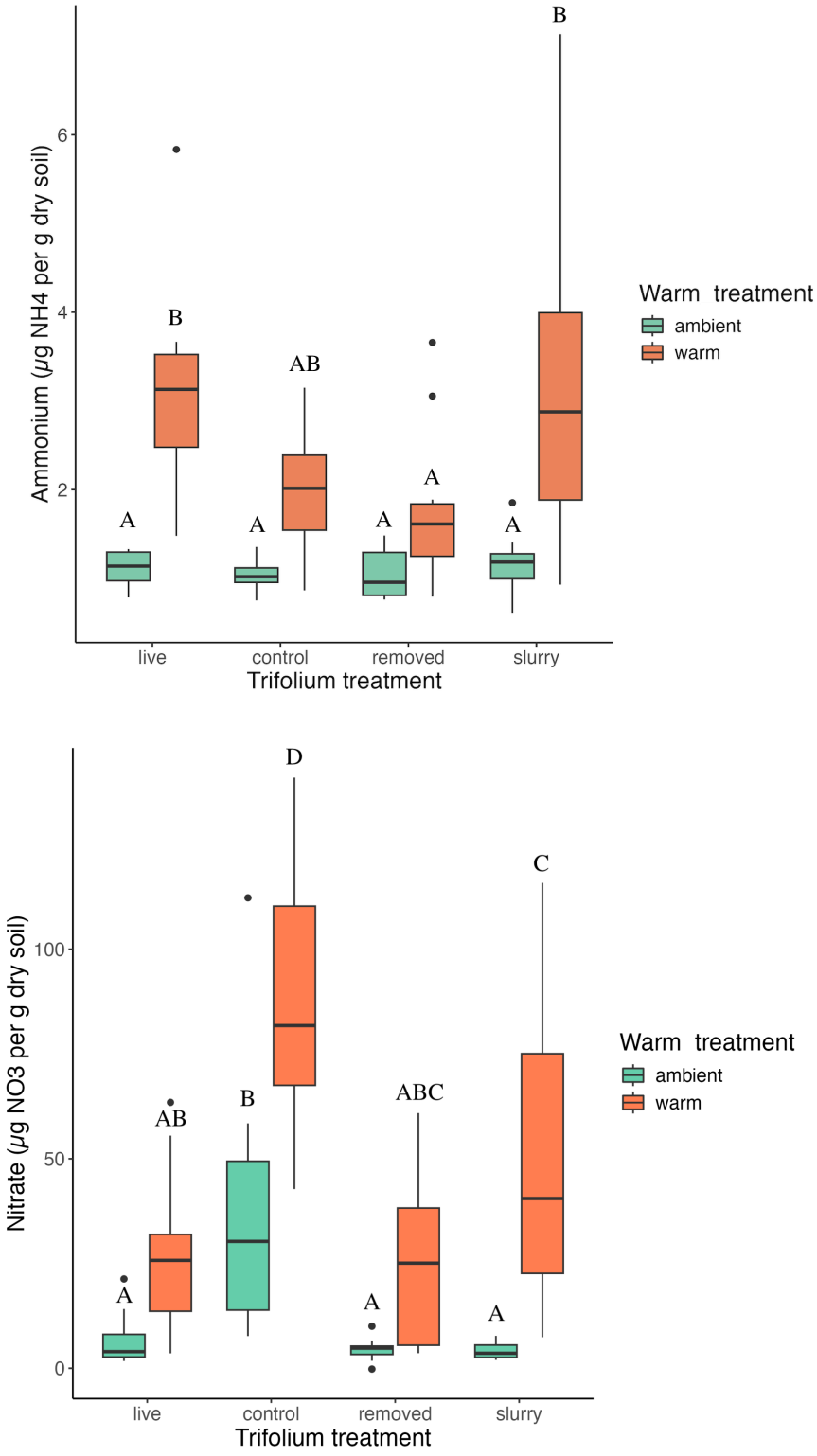


Figure 7: Ammonium (top) and nitrate (bottom) levels vs *Trifolium* treatment. Orange bars represent warmed pots, and green bars represent ambient pots. Bars with different letters are significantly different from one another using an alpha value of 0.05.

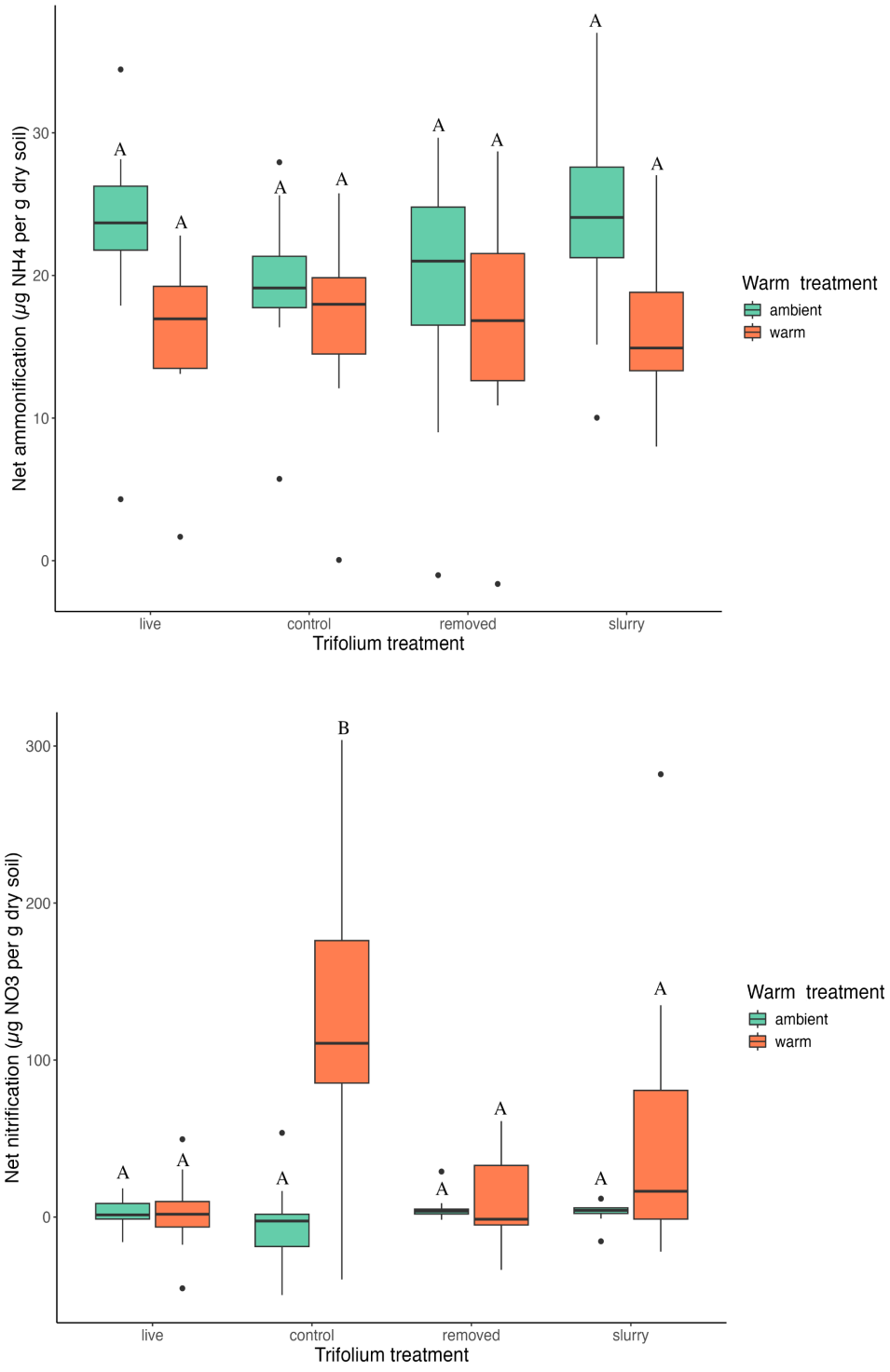


Figure 8: Net ammonification (top) and net nitrification (bottom) vs *Trifolium* treatment. Calculated as the increase in ammonium and nitrate, respectively, over a 2 week incubation in the dark at 20°C. Orange bars represent warmed pots, and green bars represent ambient pots. Bars with different letters are significantly different from one another using an alpha value of 0.05.

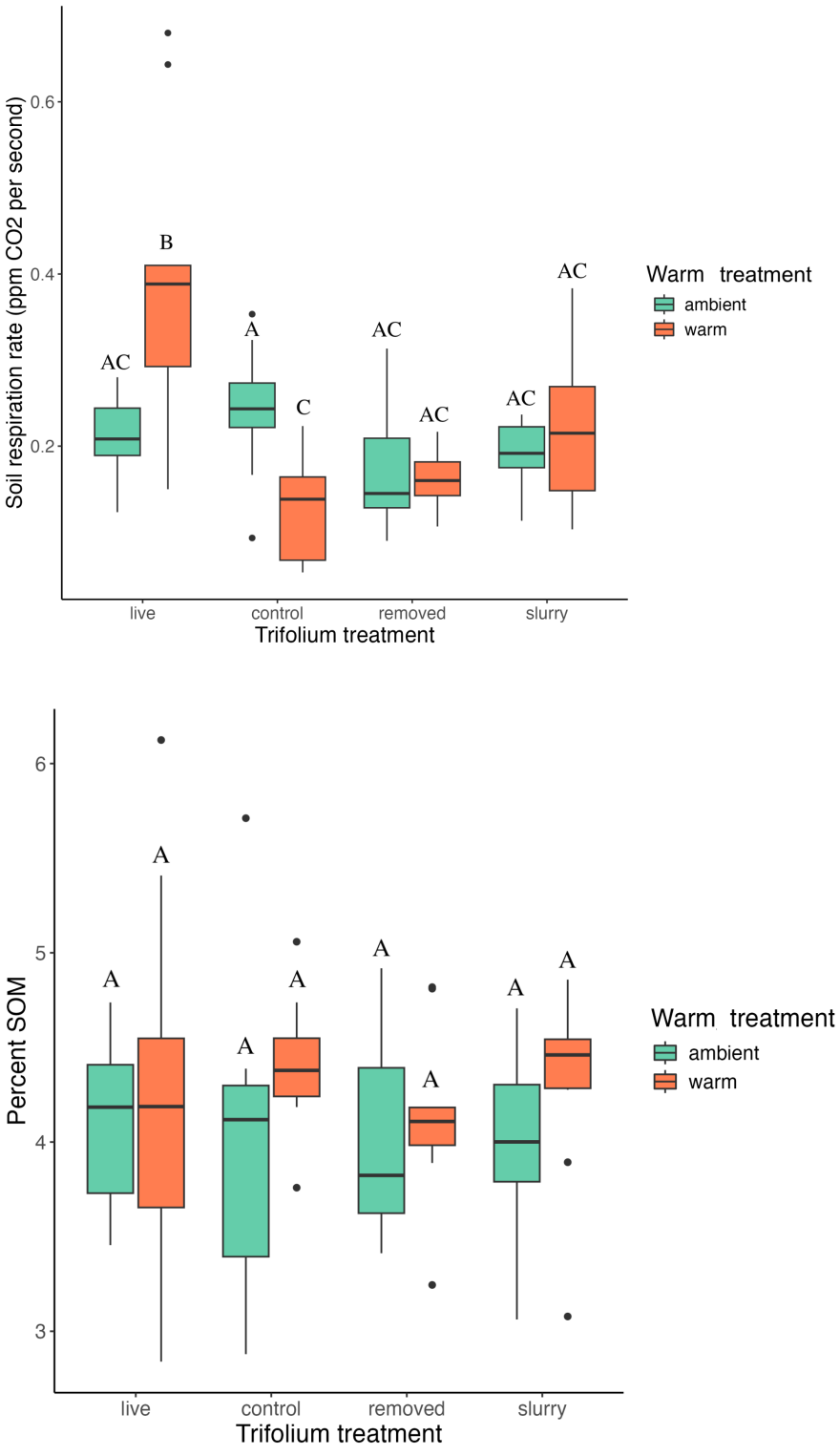


Figure 9: Soil respiration (top) and percent SOM (bottom) vs *Trifolium* treatment. Orange bars represent warmed pots, and green bars represent ambient pots. Bars with different letters are significantly different from one another using an alpha value of 0.05.

Regression analyses

*Soil respiration vs N*

Soil respiration increased significantly with higher ammonium in warm pots ($F_{1,34}$ = 5.802, slope = 3.7093, $R^2$ = 0.1458, p = 0.02157; Figure 10). There was no significant relationship between soil respiration and ammonium in the ambient pots ($F_{1,34}$ = 0.309, slope = -0.4066, $R^2$ = 0.0090, p = 0.5819; Figure 10).

There was no significant relationship between soil respiration and nitrate in warm pots ($F_{1,34}$ = 2.998, slope = -70.73, $R^2$ = 0.08103, p = 0.09244; Figure 10) or ambient pots ($F_{1,34}$ = 0.8016, slope = 54.920, $R^2$ = 0.02303, p = 0.3769; Figure 10).

*SOM vs N*

There was no significant relationship between ammonium and SOM in warmed pots ($F_{1,35}$ = 2.657, slope = 0.5856, $R^2$ = 0.07056, p = 0.1121; Figure 11) or ambient pots ($F_{1,36}$ = 0.6806, slope = -0.06128, $R^2$ = 0.01855, p = 0.4148; Figure 11).

Nitrate and SOM did not have a significant relationship in warmed ($F_{1,35}$ = 1.66, slope = 12.076, $R^2$ = 0.04527, p = 0.2061; Figure 11) or ambient ($F_{1,36}$ = $7.857 \times 10^{-5}$, slope = 0.05577, $R^2$ = $2.182 \times 10^{-6}$, p = 0.993; Figure 11) pots.

*Ammonium vs biomass*

Ammonium had no significant relationship with aboveground biomass from the live treatment in either the warmed ($F_{1,8}$ = 2.237, slope = -1.3944, $R^2$ =

0.2185, p = 0.1731; Figure 12) or the ambient pots (($F_{1,8}$ = 4.027, slope = 1.8385, $R^2$ = 0.3348, p = 0.0797; Figure 12).

Nitrate had a significant negative relationship with biomass in warmed pots ($F_{1,8}$ = 8.437, slope = -36.05, $R^2$ = 0.5133, p = 0.0198; Figure 12) but no significant relationship in ambient pots ($F_{1,8}$ = 0.0618, slope = -6.719, $R^2$ = 0.0077, p = 0.8099; Figure 12).

*SOM vs soil respiration*

There was no significant relationship between percent SOM and soil respiration rate in warmed pots ($F_{1.34}$ = 1.256, slope = 0.8318, $R^2$ = 0.03562, p = 0.2703; Figure 13) or ambient pots ($F_{1,34}$ = 0.0171, slope = 0.2165, $R^2$ = 0.0005, p = 0.8968; Figure 13).

T-test analysis

*Biomass differences with warming treatment*

Warmed pots had significantly higher aboveground biomass in the live *Trifolium* treatment by the end of the growing period than in ambient pots (t = 5.174, df = 1 in 8, p = 0.0354; Figure 14).

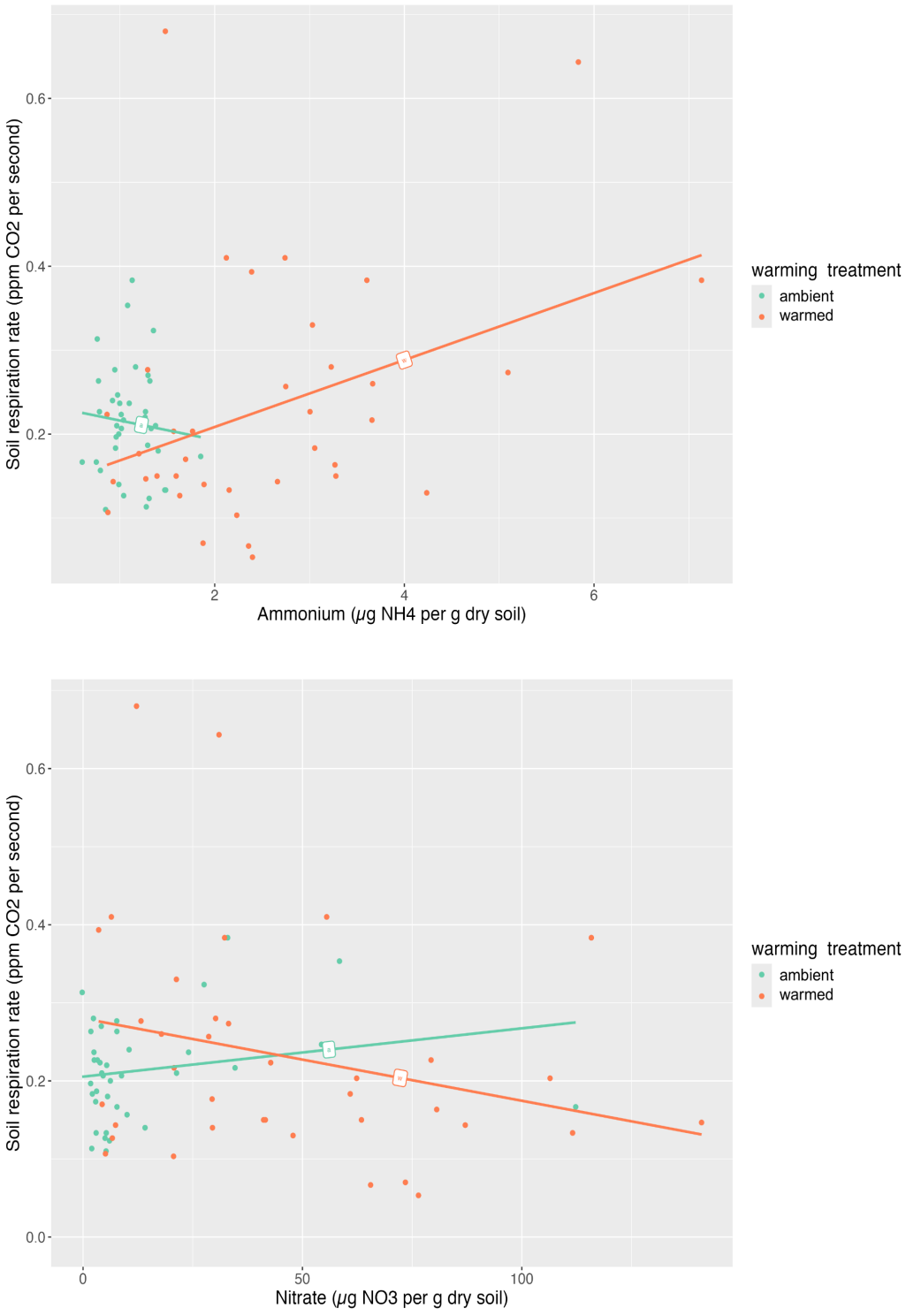


Figure 10: Soil respiration vs ammonium (top) and nitrate (bottom). Warmed pot data shown in orange, and ambient pot data shown in green.

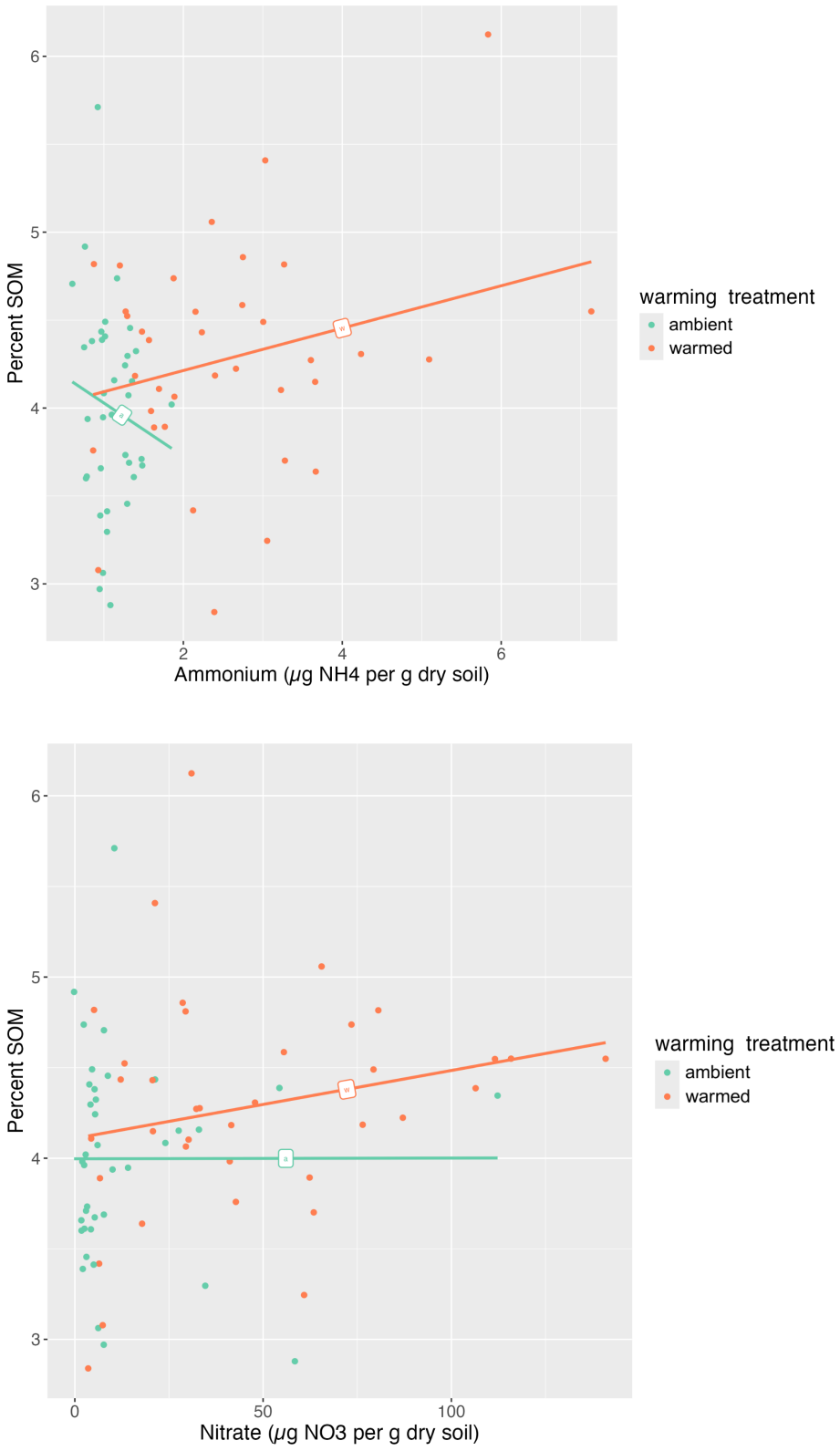


Figure 11: Percent SOM vs ammonium (top) and nitrate (bottom). Warmed pot data shown in orange, and ambient pot data shown in green.

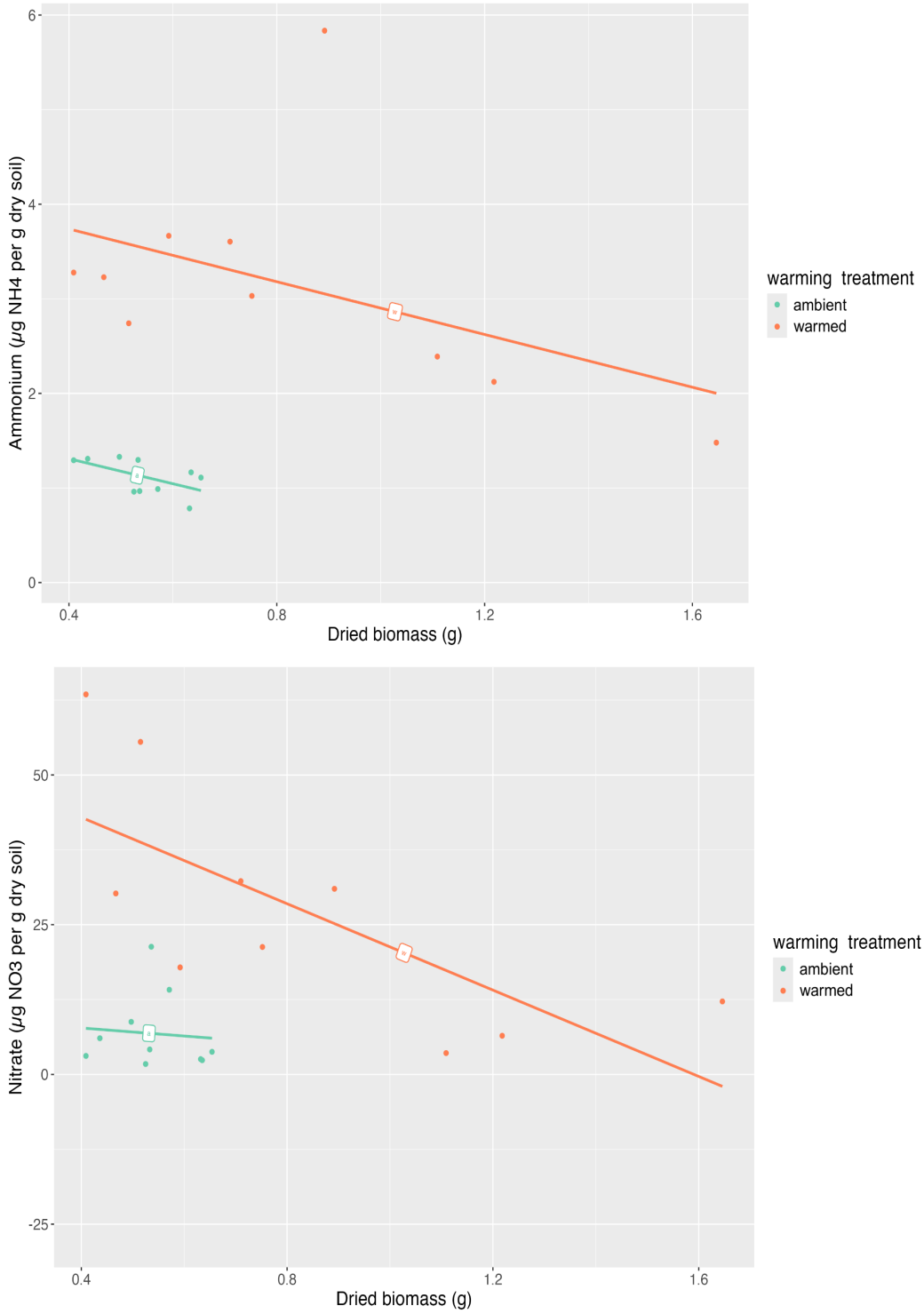


Figure 12: Biomass vs ammonium (top) and nitrate (bottom). Data is from live pots only. Warmed pot data shown in orange, and ambient pot data shown in green.

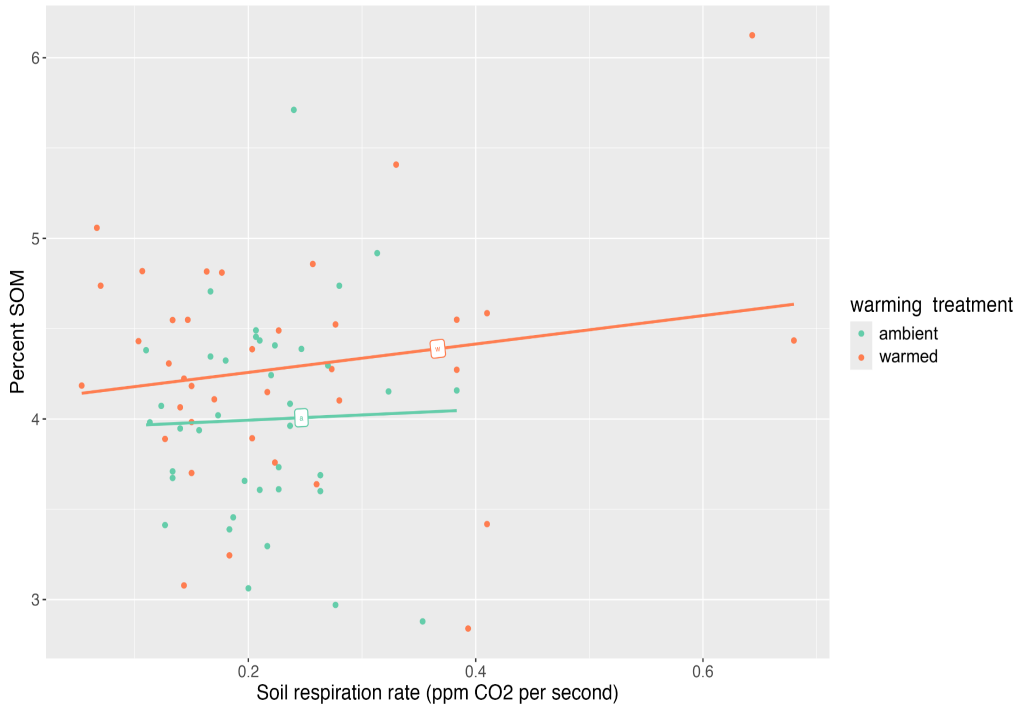


Figure 13: SOM vs soil respiration. Warmed pot data shown in orange, and ambient pot data shown in green.

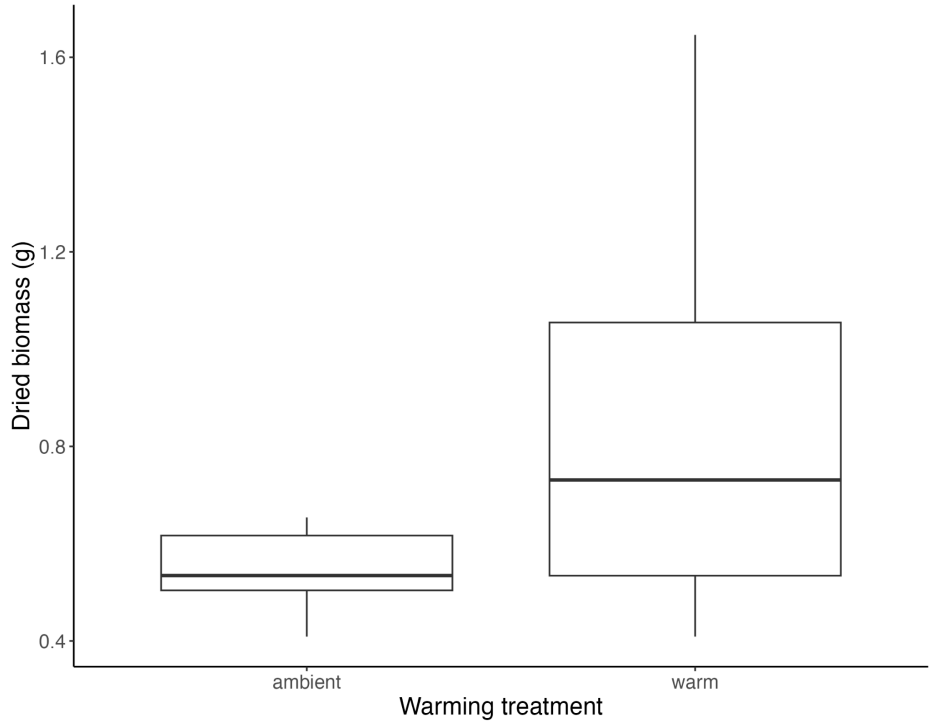


Figure 14: Biomass vs warming treatment. Data from live pots only.

# DISCUSSION

My primary goal in this experiment was to explore how warming and nitrogen affected stored soil carbon. Unexpectedly, I saw almost no effect of my *Trifolium* treatments on SOM (Figure 9) and no relationship between inorganic N and SOM (Figure 11). Warming had only a marginally significant effect on stored soil carbon (SOM), and that effect was positive (Figure 9), which was the opposite of what I predicted. There was no significant interaction between warming and *Trifolium* treatments on SOM (Figure 9). There also was no consistent effect of warming on soil respiration, but there were very significant effects of both my *Trifolium* treatment and the interaction between warming and *Trifolium* treatment on soil respiration (Figure 9). Warming increased measured inorganic N levels significantly in all of my treatments and seemed to magnify the inorganic N differences between my *Trifolium* treatments (Figure 7). These effects, however, showed up differently in soil ammonium ($NH_4^+$) and nitrate ($NO_3^-$), pointing to domination of different microbial communities with my treatments.

## *Effect of* Trifolium *on inorganic N pools*

The *Trifolium* treatments affected both nitrogen inputs and carbon inputs, leading to complex yet interesting effects on inorganic N. Many of these effects were only visible with warming (Figure 7), demonstrating the magnifying effect of warming on these processes. To review, I had a live treatment, which had *Trifolium* growing in it throughout the 10-week-long experiment, a removed

treatment, which had the aboveground biomass clipped off after 5 weeks, a slurry treatment, which had aboveground biomass clipped after 5 weeks and then had those tissues immediately added back into the system in a ground up liquid, and a control which never had *Trifolium* growing in it.

These treatments directly affected inorganic N pools through two different pathways of N addition (N fixation by *Rhizobia* and protein addition by adding a slurry) and by removing existing inorganic soil N to allow plant growth. My removed treatment did end up having the lowest ammonium and nitrate (Figure 7), as expected, likely because the *Trifolium* had time to pull up N but did not have enough time to replace it through N-fixation. My slurry treatment had slightly higher ammonium and nitrate (Figure 7), which shows that the N in the added plant proteins was transformed into inorganic N by microbes in the soil, as predicted. My live treatment marginally increased ammonium compared to the removed treatment (Figure 7), which likely means that it was able to contribute to inorganic N pools by fixing nitrogen because N-fixation yields ammonium (Mylona et al. 1995; Figure 3). The live treatment did not then increase nitrate; there are two potential explanations for this. Both of these lines of reasoning allow me to explain why my live treatment increased ammonium but not nitrate while my control treatment had the opposite result of increasing nitrate but not ammonium (Figure 7).

Explanation 1: *Trifolium* preference for nitrate

The first explanation is that the increased ammonium in the live treatment was converted into nitrate and then was immediately sucked up by the plant because *Trifolium* likely prefers nitrate to ammonium as a nitrogen source. Many factors influence a plant's ammonium vs nitrate preference, including ratios of available ammonium to nitrate (Daryanto et al. 2018), temperature (Marschner 2011), pre-existing N levels (Marschner 2011), and the type of plant (Marschner 2011; Sheppard et al. 2013). Usually, species preferring less acidic soils prefer nitrate to ammonium (Falkengren-Grerup and Lakkenborg-Kristensen 1994; Yan et al. 2019), and *Trifolium* does prefer less acidic soils (Simpson et al. 1987), probably because it is a nitrogen fixer and acidic soils inhibit root nodulation (Ferguson et al. 2012). My biomass data showed modest support for *Trifolium* preference for nitrate, at least under warmed conditions, because, as biomass increased, nitrate significantly decreased while ammonium did not (Figure 12). If *Trifolium* indeed prefers nitrate to ammonium, it makes sense that *Trifolium* increased ammonium but not nitrate; the plant facilitated fixation of $N_2$ gas into ammonium and, although some of this ammonium was converted into nitrate, that nitrate was drawn up almost immediately by the growing plants. Comparing live pots to control pots reinforces this idea: the live pots had plants growing in them for the longest amount of time out of any treatment, while the control pots had plants growing in them for zero time, which led to controls having the highest nitrate. Controls did not have very different ammonium from the treatments with plants growing in them because those plants were not taking up

very much ammonium. This first explanation for the nitrate/ammonium difference between live and control pots is compelling; however it may not be the sole explanation because it does not help explain the different respiration results (Figure 9) or the different nitrification results (Figure 8) between live and control treatments.

Explanation 2: *Trifolium* affects N by influencing carbon availability

The second explanation for the live treatment increasing ammonium but not nitrate can help integrate some of these other results. The ammonium that the live plant had fixed may not have been able to be converted to nitrate due to microbial shifts in the soil. These microbial shifts may have been triggered by differing carbon availability in my pots due to my *Trifolium* treatments. Although there was no difference in SOM between my treatments (Figure 2), the system with live *Trifolium* likely had high C availability because of the high belowground input of biomass from roots. My control pots, on the other hand, contained no fresh or decomposing organic material, only the stable and recalcitrant carbon in the peat material used to make the potting mix (Lal 2004; Prescott 2010). Therefore I hypothesize that all treatments except for controls had a carbon pool that was quite available to microbes, with live *Trifolium* pots having the highest available carbon. The differences between live and control pots can therefore be the most elucidating comparison when discussing carbon availability.

Differences in carbon availability are highly relevant to the microbes

involved in N transformation. A microbial community dominated by ammonifiers would lead to higher ammonification and therefore a bigger ammonium pool, while a microbial community dominated by nitrifiers would lead to higher nitrification and therefore a bigger nitrate pool. The microbes that produce ammonium through ammonification are almost all heterotrophic, meaning that they depend on a carbon source in the soil to survive, and use the citric acid cycle to be very efficient under high-C-available conditions (Sepers 1981). The microbes that produce nitrate through nitrification are almost all autotrophic (Montrás et al. 2008; Papp et al. 2016; Isobe 2019; Figure 3), meaning that their main source of carbon is from CO2, not organic matter in the soil (Matin 1978). Based on these different resource acquisition strategies, it makes sense that the amount of each type of microbe might shift differently based on environment (Isobe 2019), and carbon availability might be a factor that impacts competitive outcomes between heterotrophs and autotrophs (Degrange et al. 1997). Very little research has been done specifically on the effect of carbon on nitrifier/ammonifier competitive outcomes, but nitrifiers and ammonifiers do sometimes compete for resources (Verhagen et al. 1992), so one explanation for the increase in ammonium in live *Trifolium* (carbon-available) pots and the dramatic increase in nitrate in control (carbon-limited) pots (Figure 1) in my study could be that carbon availability affected competitive outcomes between nitrifying and ammonifying microbes in the soil by giving ammonifiers the edge. Whether or not this result was due to competition or just independent shifts in relative abundances of each type of microbe, the hypothesis of a microbial shift

is strongly supported by the dramatic increase in nitrification in controls (Figure 8).

My soil respiration data also support this hypothesis; soil respiration was very low in control pots yet quite high in live pots (Figure 9). Although this result could be because there was more carbon in live pots for respiring microbes to eat, the change in C availability between *Trifolium* treatments and warming treatments was not huge, (otherwise I would have seen it more significantly in my SOM data in Figure 9), while the change in N was enormous (with controls nearly doubling total N compared to live– see Figure 7). It is hard to imagine that this drastic change in N had nothing to do with the different responses of microbial respiration in live and control treatments to warming. Ammonifying heterotrophs respire and release CO2 as they process matter in the soil, while nitrifying autotrophs do not respire (Matin 1978). Both ammonifier respiration and the absence of nitrifier respiration were visible in Figure 10: I found a significant positive association between ammonium and soil respiration rates in warmed pots and no significant associations between nitrate and soil respiration rate. I think this difference in microbial respiration can help explain Figure 9; the live pots likely had the highest available carbon because they had the most roots, therefore they probably had the strongest swing towards ammonifier domination, which would lead the soil in those pots to respire more. The roots themselves were also likely respiring. The control pots had the least available carbon, which could have led to domination of autotrophic nitrifiers which do not respire, which would explain why those pots had the lowest soil respiration. This pattern

supports my second explanation for Figure 7, which is that *Trifolium* affected ammonium and nitrate differently in live and control treatments because of the plants' effect on C availability.

*Effect of warming on SOM: microbial biomass*

Warming accelerated many processes in this system such as plant growth and microbial activity; this acceleration seemed to drastically exacerbate most treatment effects just described. Not only did warming increase microbial activity, as shown in the dramatically increased rate of N transformations (Figure 7), but it also likely increased microbial biomass. This potential increase in microbial biomass is supported by my SOM data, which showed marginally significantly higher SOM in warmed pots compared to ambient pots (Figure 2). This increase at first glance could have been because warming increased plant biomass, at least in live pots (Figure 14). However, live pots actually seemed to be the only treatment that did not increase SOM (Figure 9). The biomass explanation also does not quite work because warming increased SOM even when there were no roots present (in control treatment); if warming was increasing SOM only by accelerating root carbon inputs, then warming would not have increased SOM in controls. By process of elimination, this sequence of results supports the microbial biomass explanation for increases in SOM. Microbial biomass counts as a proportion of SOM because it gets burned off when doing LOI testing (the method I used), and it has recently been recognized as a significant contributor to SOM (Miltner et al. 2011). The inclusion of

microbial biomass in SOM helps explain why my results did not support my original hypothesis that warming would diminish SOM by diminishing SOC pools; perhaps warming-induced SOM losses were outweighed by dramatic increases in microbial populations in my experiment. It is also possible that the short-term nature of my experiment did not allow enough time for soil respiration to begin to affect SOM; I saw no relationship between these two (Figure 13).

My hypothesis that warming increased microbial biomass is further supported by the lasting effect that I measured of warming on microbial processing of N during incubation (Figure 8). During incubation, all the pots were in the same dark space at the same temperature, and I had removed all aboveground *Trifolium* tissues from the pots. Nonetheless, I still saw dramatically increased nitrification in warmed controls compared to the rest of the treatments during the incubation period (Figure 8). The continued effect of my warming treatment during that incubation period suggests a "legacy effect," meaning that the treatments had altered the microbial community composition enough that the communities in different pots and different treatments could not revert back to some sort of neutral or central community. If each individual microbe's activity was more or less equal (with a set amount of resources) during this incubation period (because they were now at the same temperature), then Figure 8 demonstrates that warming had a long-term effect on the size of the microbial community and not just the activity. Warming clearly also exacerbated the long-term effect on microbial community composition (heterotroph vs autotroph domination) in controls. This increase in size of the microbial

community with warming helps explain the increased SOM in warmed pots.

*Connections to other studies*

My data run parallel to the result in Li et al. (2023) that warming stimulates the priming effect (where inputs of more available carbon"prime" microbial communities towards increased activity, leading to more carbon loss through respiration) in conditions with high C:N ratios but inhibits the priming effect in conditions with low C:N ratios. In my experiment, I think that my live pots likely had a high C:N ratio due to high root carbon inputs and relatively low total N compared to controls, while controls had huge amounts of total N and low amounts of available C and therefore had a low C:N ratio (see Figure 7 for N data). In live pots (likely high C:N), I saw more respiration with warming, while in control pots (likely low C:N) I saw less respiration with warming. Because respiration through microbial activity is the mechanism behind priming (Fontaine et al. 2004), my results align with the important result of Li et al. (2023) that warming-induced priming effects depend on C:N ratios. My result that respiration may depend on C:N ratios also provides a partial answer to my original questions about how N might mediate C response to warming, although because both N and C were shifting, this effect was uncertain. (To see how I would suggest finding a fuller answer to these questions, see the "Future directions" section below.)

A last and slightly unexpected result is that my experiment also points to an understudied potential interaction between warming and carbon availability on

ratios of different forms of nitrogen in soils. The ratio of nitrate to ammonium in a soil has a strong influence on plants, affecting plant growth, photosynthetic rate, and many other important metabolic processes (Liu et al. 2017; Collado-González et al. 2022). Moreover, temperature stress can affect a plant's preferred ratio of nitrate to ammonium, hinting at an interesting potential interaction between warming and this ratio of different forms of N (Liu et al. 2017). My study potentially provides insight into this interaction by demonstrating how temperature can affect this ratio under different carbon availability levels. For example, differences in carbon availability could help explain contradictory findings on how warming affects ammonium/ammonification compared to nitrate/nitrification (Butler et al. 2012; Chen et al. 2020; Ren et al. 2020). A lot of research on the effects of nitrate to ammonium levels has been done on plants for agricultural purposes (Liu et al. 2017; Collado-González et al. 2022), and while this research is important, more studies should be done from the perspective of understanding larger ecological impacts. Results derived from this experiment clearly have value and carry weight in real-world ecosystems; however, if I wanted to answer my original questions, I might design a future experiment slightly differently.

*Future directions*

To more clearly isolate the effects of N addition in future experiments, my nitrogen addition treatments would use inorganic sources of nitrogen like urea or fertilizers instead of live plants. The way I set up my experiment partially

obscured the effects of N on C firstly because I had no idea how much nitrogen I was adding with each *Trifolium* treatment; it was difficult if not impossible to predict how plant N uptake and N addition would balance each other. Secondly, the live *Trifolium* plants were simultaneously changing C, confounding the effect of N on C by affecting them both at the same time and adding to the entanglement because adding more available C affected fluxes of N. Of course, in real ecosystems, all three variables (N, C, and warming) are dependent on many of the same environmental processes and factors, and N and C do affect each other. However, the original goal of my greenhouse experiment was to disentangle these effects, and an experiment simplified by excluding live plants would likely be helpful in doing that. A neater incubation experiment with fewer confounding variables would be helpful in answering these questions, and many have been done (Thiessen et al. 2013; Eberwein et al. 2015; Bai et al. 2023; Lavoie et al. 2011). My experiment, on the other hand, has value in that it demonstrates how plants can tip the scales in these ecological balances. It also exposes largely unexplored areas of research, pointing to new and interesting lines of study.

For example, this experiment brought up questions surrounding N shifts driven by interactions between carbon and warming in the soil microbiome. To pursue this slightly different line of questioning, I would design a slightly different experiment. Instead of warming and N being my independent variables with carbon as a response, I would make warming and carbon availability my independent variables with N, microbial activity, microbial biomass, and

microbial community composition as the responses (there would still be feedbacks between C and N, but without live plants these would hopefully be more predictable). I could do this in an experiment with around five different levels of carbon addition into the soil using straw and sugar, which are more recalcitrant and more available C additions, respectively (Corbin and D'Antonio 2004). I would make sure to do this experiment with a baseline soil that has a fairly recalcitrant SOM pool to make sure that I could see the effect of C limitation on the ammonifier vs nitrifier balance within microbial community composition. I would cross these treatments with warming treatments because, based on my results, warming may influence these specific microbial dynamics. Based on the data in this paper and some suggestions about heterotrophic/autotrophic competition (Degrange et al. 1997), I would expect that more carbon availability would lead to more ammonification compared to nitrification (paired with a higher ratio of ammonifiers to nitrifiers) and vice versa with lower carbon availability. I would also expect to see warming exacerbate these effects, and for effects to last some time after treatments are removed. If I found evidence for microbial shifts, I would be interested in doing a follow-up experiment to determine whether competition between nitrifiers and ammonifiers was a plausible mechanism behind these shifts or if microbial ratios were being altered by independent changes in relative nitrifier/ammonifier abundances. This seems to be a relatively unexplored area in the literature; very few studies have looked at C limitation effects on nitrifier vs ammonifier ratios and/or competition, making this an exciting area of research.

These experimental designs would help answer both my original questions and some new questions sparked by my research. In addition to different study designs, I would also suggest a slightly different set of measurements to be taken in these studies. For example, measuring C:N ratio would provide more clear insights from the data, as this ratio is quite a significant player in determining responses to warming (Li et al. 2023). Next, using soil microbe molecular analysis techniques as in Montrás et al. (2008), Jones et al. (2018), and Friedman et al. (2018) to confirm shifts in nitrifier/ammonifier balance would strengthen any dataset in this area. Measuring ammonium to nitrate ratio within N pools may also provide new and important understandings about implications for the broader ecosystem of these C, N, and warming interactions. If I were doing another experiment with live plants, relative contributions of root and microbe respiration to overall soil respiration and how that fits into the web of interactions would be another interesting set of data to have. Lastly, and perhaps most importantly, I would recommend taking at least a few sets of measurements throughout the experiment instead of just one set of measurements at the end. My data were limited in that they allowed me to see a snapshot of what was happening only at one moment in time. Because everything in the system was constantly shifting, measurements taken throughout the experiment would have revealed a much clearer picture of the actual fluxes and relationships that I was interested in. Multiple measurements were not feasible for my project due to the time-consuming nature of my measurements and because many of the measurements require destructive sampling. I would need a

much larger sample size in order to sacrifice a portion of the experiment at stages.

The gaps in my experiment point to many new questions and new ways of asking these questions, while the results of my experiment support research on current questions in the literature about carbon and nitrogen fluxes. More broadly, this experiment highlights the need to update our climate modeling; when models include complexities such as priming (where carbon inputs can actually lead to net carbon losses by altering microbial activity), they can change drastically (Guenet et al. 2018). It is hard to include these complexities when we do not fully understand them, however. Including as many of these interacting variables as possible in climate models, firstly by verifying the relationships between them with studies like this one, will allow us to 1) better understand the factors influencing current global climate shifts, 2) predict future shifts, and 3) respond accordingly with targeted and informed restoration efforts.

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