

Ecosystem Responses to Warming-Induced Plant Species Loss and Increased Nitrogen
Availability in a Rocky Mountain Subalpine Meadow

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Molly Elizabeth Smith

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Committee in charge:

Professor John Harte, Chair

Professor Todd E. Dawson

Professor Whendee L. Silver

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ABSTRACT

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Professor John Harte, Chair

Climate change is predicted to be an important driver of future biodiversity changes, especially in mountainous environments. Climate warming-induced plant species loss is likely to be non-random and based on species-specific susceptibility to rising temperatures. Experimental warming results from a subalpine meadow in Colorado suggest that warming adversely affects shallow-rooted forb species in this ecosystem. To examine the ecological consequences of losing this warming-sensitive species group, I experimentally removed all shallow-rooted forb species from otherwise intact subalpine meadow plots. Since experimental warming also resulted in increased soil nitrogen availability, I crossed the removal treatment with a nitrogen addition treatment to determine whether the loss of shallow-rooted forbs altered the community's response to a perturbation in nitrogen availability.

After three years of experimental species removal, tap-rooted forbs and grasses were able to fully compensate for the loss of shallow-rooted forbs with increased

biomass production. Moreover, the remaining plant community yielded a larger biomass response to nitrogen addition when shallow-rooted forbs were removed, possibly because removal led to increased soil moisture. The loss of shallow-rooted forbs and addition of nitrogen did not have strong effects on nitrogen cycling beyond increases in the amount of nitrate moving down through the soil profile. Uptake of nitrogen into plant tissue was also not affected by either the shallow-rooted forb removal or nitrogen addition treatments, suggesting that nitrogen may not have been the most limiting resource during the experiment. I found that spatial heterogeneity generally had a greater influence on soil microbial community composition than any of the experimental treatments.

I conclude that the warming-induced loss of shallow-rooted forbs did not affect biomass production, nitrogen cycling, or soil microbial community composition, but did increase the ecosystem's sensitivity to a perturbation in nitrogen availability. My results demonstrate that non-random scenarios of species loss do not necessarily follow the pattern of decreased productivity, decreased nitrogen uptake, increased nitrogen losses, and dampened response to nitrogen addition with species loss that is predicted by theory and experimental results from randomly-assembled plant communities. Therefore, caution should be used in applying results from randomly-assembled communities to natural communities undergoing non-random species loss.

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CHAPTER 1: INTRODUCTION

BACKGROUND

Human activities are increasingly stressing terrestrial ecosystems as a result of increased nitrogen (N) deposition (Vitousek *et al.* 1997), the loss of populations and species (Chapin *et al.* 2000), and a warmer climate (Walther *et al.* 2002). Climate change, in turn, is likely to further affect terrestrial ecosystems by altering plant species composition and biogeochemical cycles (Shaver *et al.* 2000). While the effects of warming on terrestrial ecosystems have been studied in several field manipulations (e.g., Chapin *et al.* 1995; Harte *et al.* 1995; Zavaleta *et al.* 2003b; Walker *et al.* 2006), it is often difficult to disentangle the direct effects of warming on temperature-dependent processes, for example net primary productivity (NPP), from indirect effects on NPP stemming from a warming-induced change in plant species composition or nutrient availability (Shaver *et al.* 2000) (Figure 1.1). However, these indirect pathways are likely to be as important as the direct effects of warming on ecosystem processes, particularly in ecosystems that are nitrogen (N) limited or where vegetation changes have a strong effect on ecosystem functioning (Shaver *et al.* 2000). Understanding these indirect effects of warming are crucial to forming a complete picture of how future changes in climate could alter ecosystem functioning.

Impact of climate warming on plant diversity and composition

In mountainous alpine environments, anthropogenic climate change is predicted to be an especially important driver of biodiversity change over the next 100 years (Sala

et al. 2000). Several experimental tests of the effect of warming on plant diversity support this prediction. A meta-analysis of 11 arctic and alpine warming experiments using passive open-top chambers found that overall plant species diversity significantly decreased with warming after only two growing seasons (Walker et al. 2006). One of those studies, in an alpine rangeland on the Tibetan Plateau, found that experimental warming resulted in a 26-36% decrease in plant species richness (Klein et al. 2004). However, a few of the studies found no significant change in plant diversity with warming (e.g., Hollister *et al.* 2005; Jonsdottir *et al.* 2005; Wahren *et al.* 2005), suggesting that warming impacts on plant diversity are likely to differ from site to site. Suspended infrared heat lamp warming experiments in a grassland site at Jasper Ridge, CA and a subalpine meadow at the Rocky Mountain Biological Station found no significant change in plant species diversity with warming, but both studies showed differential productivity responses of some species and/or functional groups to the warming treatments (Harte & Shaw 1995; de Valpine & Harte 2001; Zavaleta *et al.* 2003a; Zavaleta *et al.* 2003b). In addition to suggesting that warming could alter plant species richness, these studies all suggest that plant species composition and abundance is likely to change with future warming (Harte & Shaw 1995; de Valpine & Harte 2001; Zavaleta *et al.* 2003a; Zavaleta *et al.* 2003b; Walker *et al.* 2006).

Impact of climate warming on soil nitrogen availability

Soil nitrogen availability and mineralization rates are sensitive to changes in temperature (Nadelhoffer *et al.* 1991; Binkley *et al.* 1994; Robinson *et al.* 1995). Several experimental warming manipulations have found that warming increases soil

nitrogen availability by increasing microbial-mediated net nitrogen mineralization rates (Hobbie 1996; Rustad *et al.* 2001; Shaw & Harte 2001; Schmidt *et al.* 2002). While there is some indication that this effect of warming on N mineralization rates may dampen through time (e.g., Peterjohn *et al.* 1994), the rate of atmospheric deposition of nitrogen from anthropogenic activities is also expected to increase over the next several decades (Vitousek *et al.* 1997). Therefore, it is highly likely that soil nitrogen availability for plant growth will be significantly enhanced in the future, especially in mountainous areas where cold temperatures reduce microbial activity rates and nitrogen pollution is a relevant concern.

Impacts of plant species loss on ecosystem functioning

In light of impending changes in plant diversity and species composition, understanding the ecosystem consequences of declining diversity has become a focus of ecological research (Chapin *et al.* 2000; Kinzig *et al.* 2002; Loreau *et al.* 2002b; Hooper *et al.* 2005). Theoretical work has primarily focused on two mechanisms that could lead to a positive relationship between diversity and ecosystem function: niche complementarity and the selection effect. Niche complementarity (also called niche differentiation) assumes that plant species or functional groups access non-overlapping resource niches, and that this complementary use of niches results in a more efficient use of the total available resource space (Tilman *et al.* 1997b; Loreau 2000). Therefore, a plant system with a more diverse means of nutrient capture is more likely to exhibit complementary resource niche partitioning, use resources more efficiently, be more productive and retain more nutrients relative to a less diverse system (Tilman *et al.*

1997b; Loreau 2000). An alternative explanation of how increasing diversity could lead to increased productivity relies on the selection effect (also called the sampling effect), or the fact that a more diverse system has a greater probability of including a highly productive species or suite of species (Huston 1997). There has been an extensive debate over whether the selection effect is a mechanism by which diversity leads to increased productivity, or simply an artifact of the design of biodiversity experiments (Grime 1997; Huston 1997; Tilman 1999; Wardle 1999)

The first generation of experimental biodiversity studies used artificially seeded plant communities where species assemblages were chosen at random, and the results tended to show that decreasing diversity led to decreased productivity and nutrient retention (e.g., Tilman *et al.* 1996; Hector *et al.* 1999). However, this random combinatorial experimental approach was widely criticized for two main issues: 1) they did not allow for differentiation between niche complementarity and the selection effect; and 2) they did not distinguish between the effect of species number, species identity and the importance of functional groups (Grime 1997; Huston 1997). There was also a concern over how to apply results from artificially-created plant communities to more complex natural ecosystems (Wardle *et al.* 1997). Observational studies of naturally occurring gradients in plant diversity revealed more variability in the relationship between diversity and ecosystem functioning, with some studies showing a positive relationship between diversity and plant productivity (e.g., Troumbis & Memtsas 2000), others showing a negative relationship (e.g., Wardle *et al.* 1997), and at least one study showing that plant community composition was a better predictor of productivity than was diversity (Kahmen *et al.* 2005).

Subsequent research has focused on improving these issues by designing experiments that allow for a quantification of niche complementarity and the selection effect (Loreau & Hector 2001), and that separate out the effects of species and functional group number and identity (e.g., Hooper & Vitousek 1997; Tilman *et al.* 1997a; Reich *et al.* 2004; Spehn *et al.* 2005). Experimental removal manipulations in otherwise unmanipulated plant communities is a relatively new approach that allows for experimentation in more realistic, intact ecosystems (Diaz *et al.* 2003). Results from this second generation of biodiversity studies tend to emphasize that the identity of the species or functional type that is lost is more important than the number being removed (e.g., Hooper & Vitousek 1997; Symstad *et al.* 1998; Wardle *et al.* 1999; Symstad & Tilman 2001; Reich *et al.* 2004), and indicate that relationships between diversity and productivity can vary significantly across ecosystem types and scales (Wardle & Zackrisson 2005).

By focusing on either random species assemblages or complete factorial combinations of all species and/or functional groups, the majority of existing experimental studies have addressed questions related to the effect of diversity *per se* on ecosystem functioning. However, future changes in diversity, especially as a result of human activities, are not likely to be random – they are more likely to be targeted at species with traits that make them particularly vulnerable to change (Loreau *et al.* 2001; Grime 2002; Smith & Knapp 2003; Raffaelli 2004; Solan *et al.* 2004; Zavaleta & Hulvey 2004; Hooper *et al.* 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005). Several theoretical and empirical studies have shown that ecosystem functioning responds differently to random vs. non-random species loss (e.g., Ives & Cardinale 2004; Solan

et al. 2004; Zavaleta & Hulvey 2004; Gross & Cardinale 2005; Larsen et al. 2005; Schlapfer et al. 2005). Therefore, an understanding of how diversity *per se* affects ecosystem functioning may not provide sufficient insight into how specific, realistic scenarios of species loss could alter ecosystem functioning. Several articles have highlighted the need for additional investigations into how ecosystems may respond to non-random changes in biodiversity as a result of specific human activities in order to better predict the ecosystem consequences of future species loss caused by humans (Loreau et al. 2001; Grime 2002; Naeem & Wright 2003; Hooper et al. 2005). Some attempts to fill this knowledge gap in plant communities include several studies examining the effect of the loss of rare species (e.g., Lyons & Schwartz 2001; Smith & Knapp 2003; Zavaleta & Hulvey 2004), and a study that examines the effect of species loss in a grassland following high intensity fertilizer and cutting management (Schlapfer et al. 2005). Until now, no one has examined the ecosystem consequences of warming-induced plant species loss in a manipulation experiment.

Impacts of increased nitrogen availability on ecosystem functioning

Increases in soil nitrogen availability tend to increase plant productivity and uptake of nitrogen and carbon in temperate terrestrial ecosystems that are nitrogen-limited (Chapin 1980, Vitousek et al. 1997). Nitrogen additions over a short time period (1-2 years) in an alpine meadow ecosystem at Niwot Ridge, Colorado resulted in significant increases in aboveground production, foliar nitrogen concentrations, total soil nitrogen levels, and net nitrogen mineralization and nitrification rates (Bowman et al. 1993; Fisk & Schmidt 1996). The BioCON (Biodiversity, CO₂ and N) manipulation

experiment in a Minnesota grassland also demonstrated significant nitrogen limitations as aboveground biomass, total plant nitrogen content, net nitrogen mineralization and soil inorganic nitrogen pools all increased under enhanced nitrogen conditions (Reich *et al.* 2001a). Several nitrogen addition experiments in forest ecosystems have shown similar effects of increased foliar N concentrations and/or leaching losses, but some of those studies have seen no change or even declines in biomass production (e.g., McNulty *et al.* 1996; Kjonaas *et al.* 1998; Rainey *et al.* 1999; Magill *et al.* 2004). This is generally attributed to extremely high levels of experimental N addition meant to simulate N saturation in areas that receive (or are predicted to receive in the future) high rates of atmospheric N deposition. The response of a plant community to nitrogen additions will likely depend on the extent to which growth is nitrogen limited, and the amount of increase in N availability over background deposition rates.

Impacts of plant species loss and increased nitrogen availability on ecosystem functioning

Very few studies have directly manipulated both nitrogen availability and plant species diversity. Results from a few of these studies are mixed with both Reich *et al.* (2001a) and Fridley (2002) reporting that decreasing species diversity dampened the community's biomass response to N addition, and Bret-Harte *et al.* (2004) discovering that response to N addition was dependent on the identity of the plant group being removed. This research does not generally agree with some theoretical and empirical research which predicts a "diversity-begets-stability" relationship whereby a more diverse community is more resistant, or exhibits less change, in response to a

perturbation due to an increased likelihood of having positively-affected species which can compensate for declines in the growth of negatively-affected species (e.g., Tilman 1996; Naeem & Li 1997; Yachi & Loreau 1999; Tilman *et al.* 2006). Other research, however, presents evidence that less diverse plant systems can display greater resistance to a perturbation (Pfisterer & Schmid 2002). Most empirical research on the relationship between diversity and resistance to perturbations has focused on negative stresses such as drought and invasion by exotics, whereas very few experimental studies have looked at how diversity affects the magnitude of a community's response to potentially growth-enhancing perturbations such as increased nitrogen availability (Loreau *et al.* 2002a, although see Reich *et al.* 2001, Fridley 2002 and Bret-Harte *et al.* 2004). Loreau & Behra (1999) theorize that a more diverse plant system might exhibit lower resistance to nitrogen addition since a more diverse community has an increased probability of containing species that demonstrate a positive growth response to increased nitrogen availability. Additional studies that directly focused on the question of how changing plant diversity affects a system's response to nitrogen addition are clearly needed to further clarify these issues.

THIS DISSERTATION

The goal of this dissertation was to explore how future climate warming might indirectly affect ecosystem functioning in a subalpine meadow in the Colorado Rocky Mountains through the effects of warming on plant species diversity and nitrogen availability. To determine how warming could alter plant species diversity and soil nitrogen availability in our subalpine meadow study system, I examined results from an

on-going 15-year warming experiment. Long-term results from the warming experiment suggest that warming adversely affects shallow-rooted forb species and increases net nitrogen mineralization rates (Harte & Shaw 1995; de Valpine & Harte 2001; Shaw & Harte 2001). To explore the ecological consequences of these warming-induced changes in plant community composition and nitrogen availability, I conducted a field experiment where I removed shallow-rooted forb species and added inorganic nitrogen to otherwise intact subalpine meadow plots. By analyzing how this realistic scenario of warming-induced plant species loss and changes in soil nitrogen availability affected plant productivity, N cycling and soil microbial community structure, my research improves our ability to predict how anthropogenic activities could affect important ecosystem processes.

Research Questions

The primary research questions I addressed with my dissertation research were:

1. How does the loss of shallow-rooted forb species affect plant productivity, nitrogen cycling, and soil microbial community composition?
2. How does N addition affect plant productivity, nitrogen cycling, and soil microbial community composition?
3. How does the loss of shallow-rooted forb species affect ecosystem responses to N addition?

A conceptual model of my research strategy and how this dissertation complements the on-going warming experiment is summarized in Figure 1.1.

Experimental Approach

To address these research questions, I conducted an *in situ* species removal and nitrogen addition field experiment. The use of species removal experiments to alter plant species diversity and composition is one of many approaches to investigating the direct relationship between plant diversity/species composition and ecosystem processes. Previously, plant diversity manipulations mainly involved the creation of artificial plant communities by planting seed mixtures in either the field (e.g., Hooper & Vitousek 1997; Hector *et al.* 1999; Tilman *et al.* 2001) or the laboratory (e.g., Naeem *et al.* 1994). However, the applicability of results from these studies to naturally assembled communities is hotly debated (see Grime 1997). Observational examinations of natural variation in species diversity (e.g., Wardle *et al.* 1997; Troumbis & Memtsas 2000), on the other hand, may reveal diversity-related patterns in natural systems, but do not allow us to readily distinguish between correlation and cause-and-effect relationships.

In situ species removal experiments are a relatively new approach to exploring the relationship between altered species composition/diversity and ecosystem function (e.g., Wardle *et al.* 1999; Lyons & Schwartz 2001; Symstad & Tilman 2001; Diaz *et al.* 2003; Bret-Harte *et al.* 2004; Buonopane *et al.* 2005). By working in otherwise unmanipulated ecosystems, removal experiments can explore the role that species diversity/composition plays in complex natural systems, while maintaining the ability to look at cause-and-effect relationships by directly altering community composition (Diaz *et al.* 2003). Removal experiments also have trade-offs, mainly relating to the choice of

removal method and how to account for the loss of biomass in addition to the loss of species (Diaz *et al.* 2003). However, an international network of scientists conducting species removal experiments has developed a set of guidelines that I followed to minimize complicating factors associated with removal experiments (see <http://gcte.org/Prac-Guidelines-to-Removal-Experiments.pdf>). As a member of the Species Removal Network, my results are being included in a meta-analysis of data from removal studies across the globe. To my knowledge, this is the first species removal experiment to examine non-random plant species loss resulting from a specific anthropogenic activity.

Dissertation Outline

The remaining chapters of this dissertation report on how various ecosystem processes and parameters responded to the experimental removal of shallow-rooted forb species and addition of inorganic nitrogen. Chapter 2 discusses treatment effects on above and belowground plant biomass production, with a particular focus on whether the remaining plant community was able to compensate for the loss of the entire group of shallow-rooted forb species, and whether the classification of shallow-rooted forbs as a functional group is appropriate. Chapter 3 presents results on how species removal and N addition treatments affected aspects of the N cycle such as plant uptake of N, soil N pools, net N transformation rates, and movement of inorganic N down through the soil profile. Chapter 4 explores the response of the soil microbial community to experimental treatments, and discusses the importance of spatial heterogeneity of the microbial community and environmental variables.

Taken together, the chapters of this dissertation present a picture of how the loss of shallow-rooted forbs and increased inorganic nitrogen availability could potentially alter ecosystem functioning in this Rocky Mountain subalpine meadow. This research contributes to our understanding of how global changes such as climate warming, plant diversity loss and enhanced nitrogen availability could alter important ecosystem processes. It provides an important next step in furthering the field of biodiversity and ecosystem function research by considering a specific, plausible scenario of plant species loss. Additional experiments such as this one, that combine realistic scenarios of species loss and changing resource availability, over even longer timescales are needed to improve our ability to predict the ecosystem consequences of interacting anthropogenic environmental changes.

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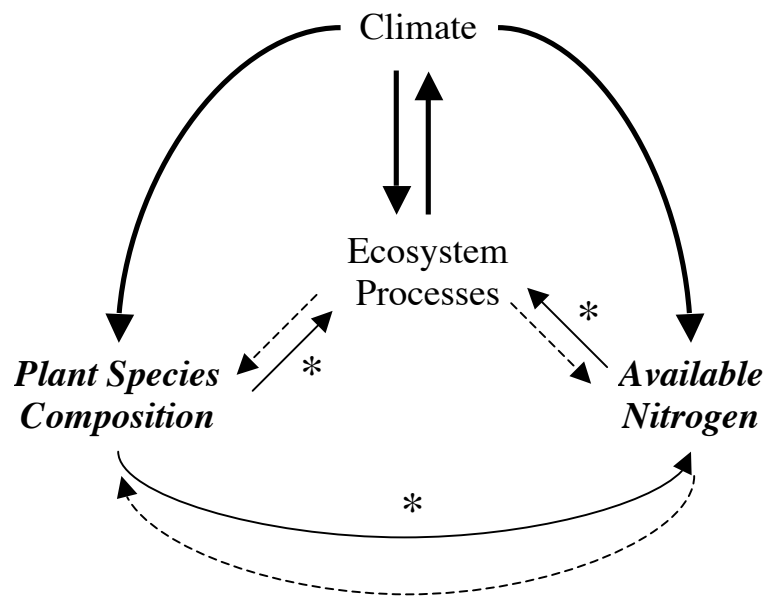
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FIGURES

Figure 1.1. Schematic diagram of possible direct and indirect effects of climate change on ecosystem processes. The diagram highlights connections between an on-going warming experiment (Harte *et al.* 1995) and the plant species removal/nitrogen-addition experiment described in this dissertation. The warming experiment examines the direct effects of warming on plant species composition, available soil nitrogen (N), and ecosystem processes; and feedbacks to the climate system from changing ecosystem processes (solid bold arrows). This dissertation was motivated by results from this warming experiment, and examined the direct effects of altered plant species composition and increased N availability on ecosystem processes (plant productivity, soil N cycling, and soil microbial community structure) (solid arrows with stars). The dashed arrow indicates connections that exist within the ecosystem, but were not directly investigated by this dissertation or the warming experiment.



CHAPTER 2: COMPENSATORY RESPONSES TO LOSS OF WARMING-SENSITIVE PLANT SPECIES

INTRODUCTION

Human-induced environmental changes such as land-use, climate change, nitrogen (N) deposition, atmospheric carbon dioxide (CO₂) enrichment and the introduction of exotic invasive species are predicted to have significant negative impacts on global biodiversity by the end of the next century (Sala *et al.* 2000). In light of these impending changes, understanding the ecosystem consequences of losing biodiversity has become a focus of ecological research (Chapin *et al.* 2000; Loreau *et al.* 2002b; Hooper *et al.* 2005). Most experimental studies of the ecosystem consequences of plant species loss have focused on randomly-assembled plant communities (e.g., Naeem *et al.* 1994; Tilman *et al.* 1997a; Hector *et al.* 1999; Reich *et al.* 2001a; Tilman *et al.* 2001). However, changes in diversity as a result of human activities are not likely to be random – they are more likely to be targeted at species with traits that make them particularly vulnerable to change (Loreau *et al.* 2001; Grime 2002; Smith & Knapp 2003; Raffaelli 2004; Solan *et al.* 2004; Zavaleta & Hulvey 2004; Hooper *et al.* 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005). Investigating how ecosystems may respond to non-random changes in biodiversity as a result of specific human activities is important to predicting ecosystem consequences of future species loss due to human activities (Loreau *et al.* 2001; Grime 2002; Naeem & Wright 2003; Hooper *et al.* 2005).

Species removal experiments have recently been proposed as an appropriate manipulation approach for examining the effects of non-random species loss in naturally-assembled communities (Diaz *et al.* 2003; Hooper *et al.* 2005). By working in otherwise unmanipulated ecosystems, removal experiments explore the consequences of non-random species loss in complex natural systems, while maintaining an ability to look at cause-and-effect relationships by directly altering community composition (Diaz *et al.* 2003). Previous removal studies of non-random plant species loss have focused on the loss of rare species (e.g., Lyons & Schwartz 2001; Smith & Knapp 2003), or on the loss of various combinations of functional groups and/or dominant species (e.g., Sala *et al.* 1989; Hobbie *et al.* 1999; Wardle *et al.* 1999; Symstad & Tilman 2001; Bret-Harte *et al.* 2004; Buonopane *et al.* 2005; Wardle & Zackrisson 2005). Since climate change is predicted to be an important driver of biodiversity change over the next 100 years, especially in mountainous alpine environments (Sala *et al.* 2000), we examined the consequence of removing those plant species most likely to be adversely affected by increasing temperatures, as indicated by a 15-year warming experiment at the same study site (Harte *et al.* 1995). To our knowledge, this is the first removal study that examines non-random plant species loss resulting from a specific anthropogenic activity.

Long-term experimental warming decreased the growth of forbs (Harte & Shaw 1995), but not all forb species responded in the same way to warming (de Valpine & Harte 2001). Warming decreased the aboveground biomass and flowering success of shallow-rooted forbs, and decreased or had no effect on the growth of tap-rooted forb species (de Valpine & Harte 2001; Saavedra *et al.* 2003). Although experimental

warming has not led to the complete disappearance of any plant species, spatial patterns across the experimental plots suggest that plant species richness is reduced under conditions of early snowmelt and enhanced nitrogen availability (J. Harte, unpublished data). The observation that manipulated climate warming reduces soil moisture in shallow soil layers, advances the timing of snowmelt, and increases nitrogen availability (Harte *et al.* 1995; Shaw & Harte 2001), provides further evidence that shallow-rooted forb species may be lost from this meadow ecosystem under future warming.

While these results suggest that shallow-rooted forb species share traits that determine their response to climate warming (*functional response traits*), it is not clear whether they also share traits that affect key ecosystem processes such as productivity and nutrient cycling (*functional effect traits*). To the extent that shallow-rooted forb species can be categorized as a “functional group” both in terms of their response to warming (functional response group) as well as their effect on ecosystem processes (functional effect group), their loss could have significant ecological consequences. Understanding whether a group of species which share functional response traits also share functional effect traits is important in determining how the loss of a group of species due to climate warming might effect ecosystem functioning (Lavorel & Garnier 2002; Naeem & Wright 2003; Hooper *et al.* 2005). Ecosystem function is generally preserved when species that share functional effects traits differ in their response to environmental change since unaffected species within that group can often compensate for the loss of susceptible species (Chapin *et al.* 1997; Hooper *et al.* 2005). However, when species that share functional effect traits all respond negatively to a particular environmental change, full compensation for the loss of that group may not be possible

if there are no remaining species to fill-in the available niche space (Diaz & Cabido 2001; Larsen *et al.* 2005).

We hypothesized that shallow-rooted forbs, which share a *response* to experimental warming, would also have a similar *effect* on productivity since they acquire their nutrients from a similar depth of soil, share a similar rooting morphology, and therefore occupy a specific resource niche that may not be as accessible to species with other belowground nutrient acquisition strategies. In this way, they might be categorized as both a functional response group and a functional effect group of species. Previous research has demonstrated that plant species can partition soil nutrient resources by soil depth, and that a greater diversity of rooting morphologies can result in more efficient use of soil resources and greater accumulation of biomass (Berendse 1982; McKane *et al.* 2002; Dimitrakopoulos & Schmid 2004; Fargione & Tilman 2005). We therefore expected that the loss of shallow-rooted forb species would decrease total biomass since the remaining plant community would not be able to exploit the available soil resource niche space as thoroughly as would a community with a greater diversity of rooting morphologies. We also expected that the loss of shallow-rooted forbs would dampen the community's biomass response to an increase in soil nitrogen availability. We chose to perturb the system by altering nitrogen availability because we observed increased rates of net nitrogen mineralization in response to experimental warming (Shaw & Harte 2001) and because atmospheric deposition of nitrogen from anthropogenic activities is expected to increase over the next several decades (Vitousek *et al.* 1997). We tested our hypotheses using an *in-situ* species removal experiment.

MATERIALS AND METHODS

Study Site

Our experiment was conducted in an ungrazed subalpine meadow at the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado (38° 53' N, 107° 02' W; elevation 2920 m), approximately 150 m from an ongoing warming experiment (Harte & Shaw 1995; Harte *et al.* 1995). The meadow plant community in our experimental plots is composed of long-lived and largely perennial herbaceous forbs and grasses. (See Harte & Shaw (1995) and Harte *et al.* (1995) for additional soil and climate details).

Experimental Design

In summer 2001, plant species removal and nitrogen addition treatments were established in 48 2 x 2 m plots, arranged in 8 blocks of 6 contiguous plots. Blocks were spaced approximately 3 m apart within a section of hillside where slope, aspect, hydrologic regime and plant community type were relatively uniform. Before assigning treatments to each plot, we collected pre-manipulation data on peak aboveground biomass (AGB) of grasses, deep tap-rooted forbs and shallow-rooted forbs.

Plots within each block were randomly assigned to one of three plant species removal treatments – removal of all AGB of shallow-rooted forb species (Shallow Removal, SR treatment), removal of random forb AGB (Random Removal, RR treatment), or control plots with no removal of biomass (Control, CONT treatment) – for a total of two replicates of each species removal treatment in each 6-plot block. Forb

species were classified into shallow- and tap-rooted categories based on field excavations of root morphology (Appendix A). Shallow-rooted forbs were designated as those with fibrous rooting structures that were concentrated in the top 10cm of the soil profile, whereas tap-rooted forbs were characterized by a coarse taproot structure that can access deeper soil resources. The RR treatment was included to distinguish between the effect of species loss and the effect of biomass loss. We randomly chose one replicate of each species removal treatment within a block to be fertilized by inorganic nitrogen (+N treatment) while the other replicate remained at ambient nitrogen levels. Therefore, each block contained one replicate of each of 6 total treatment combinations: CONT, CONT+N, SR, SR+N, RR, RR+N.

At the time of peak standing AGB in late July 2001, we removed aboveground vegetation from the SR and RR plots by clipping plants at their base. Biomass in the RR plots was removed by clipping all of the forb AGB (regardless of rooting structure) within a set of randomly chosen 25 by 25 cm quadrats, until we had removed an equivalent amount of biomass to the amount removed from the other SR plots within that block. Removal treatments were applied to the entire 4m² area of each plot. Before applying the removal treatments there were on average 18 forb species (minimum = 13 and maximum = 24) in the central 1m² of the plots, 13 of which were shallow-rooted species (minimum = 8 and maximum = 17). Depending on pre-manipulation species composition within each plot, the SR treatments removed from 61.9% to 87.5% of all forb species. Therefore, the SR treatment manipulated both species richness and functional response group richness. Although grasses were present in the experimental plots, we chose not to target grasses for removal in any of the treatments since warming

has had no effect on grass AGB in the warming meadow experiment (Harte & Shaw 1995). In 2002 and 2003, we removed aboveground plant parts of all shallow-rooted forb species from the SR plots as soon as they emerged aboveground after snowmelt and could be identified to species. Plots were then continually weeded throughout the growing season to prevent aboveground growth of the target species. In the RR plots, the same 25 by 25 cm quadrats used to conduct the random removals in 2001 were kept free of forb biomass by weeding all forbs from those quadrats throughout the 2002 and 2003 growing seasons. In 2004, we discontinued weeding in both the SR and RR plots.

Inorganic nitrogen was applied to the soil surface of fertilized (+N) plots as ammonium-nitrate (NH_4NO_3) in solution. A total of 6 g N/m^2 was applied to each +N plot each year, divided into four equal doses of 1.5 L of NH_4NO_3 solution. This level of N addition is comparable to the approximate doubling of net N mineralization rates that has been observed in the heated plots relative to the controls in the warming meadow experiment (Shaw & Harte 2001). To account for the small amount of water addition that inevitably accompanies the application of fertilizer in solution, we added an equivalent amount of de-ionized water to non-fertilized plots, an amount that is negligible relative to incoming precipitation levels. The first nitrogen and water applications were applied just after snowmelt in spring 2002, with the remaining three doses applied roughly every 2-3 weeks thereafter. Nitrogen and water additions were continued in the same manner in 2003 and 2004.

We measured all response variables in the central 1 m^2 of each plot to reduce potential edge effects. We estimated aboveground plant productivity using peak standing AGB. Since all aboveground vegetation senesces at the end of each growing

season in this system, peak-standing AGB is a conservative estimate of annual aboveground production. We measured AGB non-destructively by calibrating estimates of aerial coverage (AC) to biomass via regressions for each plant group (tap- and shallow-rooted forbs, and grasses) with off-plot harvests. We used maximum AC over the course of the growing season to calculate peak standing AGB for each plant group. We measured fine (<2mm diameter) root biomass by taking three cores (3.5 cm diameter by 10 cm depth) per plot at the peak of the growing season in 2002 and 2003. Roots were hand-sorted into live and dead biomass based on visible physical criteria such as tensile strength, friability and color of all fragments ≥ 0.5 cm in length. We were unable to separate roots by species. We measured soil moisture at depths of 10 and 30 cm approximately once a week throughout the growing season in 2002 and 2003 using permanently installed gypsum blocks that were calibrated to % gravimetric soil moisture using soil samples taken directly from the plots.

Biomass data were initially analyzed by repeated measures MANOVA, but in almost all of the analyses there was a significant time effect and there was often a significant time by removal interaction. Therefore, we subsequently conducted separate ANOVA analyses for each year of the experiment with block, removal and N addition as main effects, and a removal by N addition interaction term ($n = 8$ blocks). We considered $p \leq 0.05$ to be significant, and $0.05 < p \leq 0.10$ to be marginally significant. When a main effect or interaction term was significant or marginally significant within our ANOVA model, we conducted planned post-hoc contrasts, or compared all means with the Tukey-Kramer HSD test. Data were analyzed using JMP statistical software (SAS Institute Inc., version 5.1). All reported p-values are from the ANOVA model

specified above and all data are reported as mean $\pm$ 1 standard error. When the effect of N addition was insignificant, we report means that are averaged across N addition treatments.

RESULTS

Pre-manipulation data from 2001 show that total AGB and the amount of grass and tap-rooted forb AGB were equal across all plots before the experimental treatments were applied, with only a small difference ($p = 0.05$) in the average amount of shallow-rooted AGB initially found in plots designated for CONT and RR treatments (Figure 2.1a). The removal treatments successfully shifted plant community composition (measured as the proportion of total biomass in each plant group), although it took nearly two years of weeding to remove almost all of the shallow-rooted forb AGB from the SR plots (Figure 2.1c, black bars). This shift in composition held even when weeding was discontinued in 2004 (Figure 2.1d). While the RR treatment reduced total AGB relative to CONT levels, the ratio of shallow-rooted forb AGB to tap-rooted forb AGB in RR and CONT treatments remained equal throughout the experiment ($p > 0.50$ in all years; Figure 2.1). The average amount of biomass removed from each of the removal treatments decreased over successive years of the experiment (Figure 2.2). Although there were slight differences in the amount of biomass removed from SR and RR plots in 2001 and 2003, there was no difference in the cumulative amount of biomass removed from any of the SR and RR treatments over the duration of the experiment (Figure 2.2).

In plots where shallow-rooted forbs or random biomass were removed but no nitrogen was added, total AGB of the remaining plants in 2003 was $19.3 \pm 6.8\%$ (SR) and $20.3 \pm 7.0\%$ (RR) below CONT AGB (Figure 2.1c). Total AGB in the ambient nitrogen SR and RR plots recovered to CONT levels in 2004 when weeding was discontinued (Figure 2.1d). Adding nitrogen to the RR+N plots did not change this pattern of reduced AGB in 2003 and recovery to CONT AGB levels in 2004 (Figure 2.1c-d). However, when nitrogen was added to the SR+N plots, total AGB remained indistinguishable from AGB in both the CONT and CONT+N plots in 2002 through 2003, and was significantly higher than in RR+N plots in 2003 and 2004 (Figure 2.1b-d). There was no significant effect of nitrogen addition on total AGB in either of the more diverse (in terms of both species and functional group richness) CONT or RR treatments in any year of the experiment, whereas nitrogen addition significantly increased total AGB in the less diverse SR plots in 2002, 2003 and 2004 ($p < 0.01$ in each year; Figure 2.1b-d).

Neither grasses nor shallow-rooted forbs showed a response to nitrogen addition in any of the removal or control treatments in any year of the experiment. Grasses did respond to the removal of shallow-rooted forbs with significantly increased AGB relative to the CONT treatment ($p < 0.003$ in each year), but the response was the same in SR and RR treatments ($p > 0.1$ in each year; Figure 2.1b-d). Tap-rooted forbs did respond positively to the addition of inorganic nitrogen, but only in plots where shallow-rooted forbs had been removed ($p < 0.01$ in each year; Figure 2.1b-d).

In 2002, species and biomass removal significantly decreased live root biomass, with live root biomass in the RR and SR plots $15.5 \pm 8.1\%$ and $17.7 \pm 5.6\%$ lower than

in the CONT plots, respectively ($p = 0.05$; Figure 2.3). Live root biomass declined by $58.1 \pm 3.1\%$ between 2002 and 2003 across all treatments, while dead root biomass increased by $44.9 \pm 9.7\%$ between those same years, leading to an overall decline in total root biomass of $25.9 \pm 3.6\%$ between 2002 and 2003 (Figure 2.3). In 2003, there was a marginal effect of removal treatment on live root biomass, with RR plots having $27.1 \pm 7.0\%$ less live root biomass than CONT plots, and SR plots being intermediate between the two ($p = 0.10$; Figure 2.3). There was no effect of nitrogen addition on any of the root biomass measures in either 2002 or 2003.

Gravimetric soil moisture varied throughout the growing season, between soil depths, and between years (Figure 2.4). There was no effect of removal treatment on average shallow (10 cm) soil moisture during the 2002 growing season (mid-May through mid-August) (Figure 2.4a); but average deep (30 cm) soil moisture in 2002 was significantly higher in SR plots ($19.6 \pm 0.5\%$) than in CONT plots ($18.2 \pm 0.2\%$), with RR soil moisture intermediate between the two ($19.2 \pm 0.5\%$) ($p = 0.04$; Figure 2.4c). In summer 2003, average shallow soil moisture in SR plots ($27.7 \pm 0.6\%$) was significantly higher than in both CONT ($25.7 \pm 0.5\%$) and RR plots ($26.1 \pm 0.6\%$) ($p = 0.02$; Figure 2.4b). Average deep soil moisture was also significantly affected by removal treatment in 2003, with SR and RR plots ($30.1 \pm 0.6\%$ and $29.8 \pm 0.6\%$, respectively) being significantly wetter than CONT plots ($28.1 \pm 0.3\%$) ($p = 0.01$; Figure 2.4d).

DISCUSSION

After 3 years of removal followed by 1 year without weeding, the subalpine meadow community had fully compensated for the loss of shallow-rooted forbs, an entire functional response group, in terms of total aboveground biomass (AGB) (Figure 2.1d). Both tap-rooted forbs and grasses contributed to the compensation in the shallow-rooted forb removal (SR) treatment. The more species- and functional group-rich control (CONT) and random removal (RR) treatments showed no response to N addition, while the less species- and functional group-rich SR treatment responded to N addition with significantly increased growth of tap-rooted forbs (Figure 2.1b-d). This response to N addition resulted in a faster rate of compensation for the loss of shallow-rooted forbs in the SR+N treatment. These AGB responses represented true changes in total plant biomass, rather than shifting allocation between roots and aboveground tissue, since root biomass showed very little response to the experimental treatments beyond decreased live root growth with the removal of both shallow-rooted forbs and random biomass in 2002 and 2003 (Figure 2.3).

Comparisons between the SR and RR treatments allowed us to determine the importance of the loss of a group of species versus the loss of biomass. The initial decline in total AGB in the SR and RR treatments in 2002 and 2003 are likely due to biomass removal rather than to the loss of shallow-rooted forbs in the SR treatment since total AGB dropped by equivalent amounts in both treatments. Furthermore, the recovery in total AGB that occurred in the SR treatment between 2003 and 2004 cannot be attributed to the loss of species because total AGB in the more diverse RR treatment recovered just as quickly. However, the fact that the SR plots were as productive as the

more diverse CONT and RR plots in 2004 is an indication that the tap-rooted forbs and grasses were able to fully compensate for the loss of the entire group of shallow-rooted forbs, in terms of peak standing AGB over the duration of this study. The observation that the less diverse shallow-rooted forb removal plots were more responsive to added N can be attributed to the loss of shallow-rooted forbs as opposed to a loss of biomass since AGB did not increase in response to N addition in the RR+N plots where random biomass was removed. We conclude that the warming-induced loss of shallow-rooted forbs did not have a strong effect on biomass production in this plant community over the duration of this study due to compensatory growth by other remaining plants. It also appears that the ability of tap-rooted forbs to compensate for the loss of shallow-rooted forb biomass will likely be enhanced if future warming is accompanied by increased soil nitrogen availability. However, the loss of shallow-rooted forbs could have had a significant effect on other important ecosystem processes, such as soil nitrogen and carbon cycling (which we will discuss in a forthcoming paper).

Our findings fit into the spectrum of results from non-random removal experiments which tend to show no systematic change in community-level biomass or its response to N addition with the removal of species or functional groups (e.g., Hobbie *et al.* 1999; Wardle *et al.* 1999; Symstad & Tilman 2001; Smith & Knapp 2003; Bret-Harte *et al.* 2004; Wardle & Zackrisson 2005). These removal studies tend to reveal that the identity of the removed species or functional group is more important than the number being removed (Hobbie *et al.* 1999; Wardle *et al.* 1999; Symstad & Tilman 2001; Bret-Harte *et al.* 2004), and indicate that relationships between diversity and productivity can vary significantly across ecosystem types and scales (Wardle &

Zackrisson 2005). Several removal studies show at least some level of biomass compensation, although not always complete compensation, by remaining species and functional groups (Sala *et al.* 1989; Hobbie *et al.* 1999; Wardle *et al.* 1999; Symstad & Tilman 2001; Smith & Knapp 2003; Buonopane *et al.* 2005). While our study does not encompass a full gradient of species and functional group richness, it is interesting to note that our results do not follow the pattern of decreased productivity and a dampened response to N addition with species loss that is observed in many randomly-assembled gradient experiments (e.g., Tilman *et al.* 1997a; Hector *et al.* 1999; Tilman *et al.* 2001; Reich *et al.* 2004). The findings from this and other removal studies appear to question whether conventional diversity-ecosystem functioning theory, which is generally based on random species loss, is applicable to non-random scenarios of plant species loss.

The fact that this subalpine meadow plant community was able to fully compensate for the removal of shallow-rooted forb biomass suggests that while shallow-rooted forbs might be classified as a functional response group because of their similar response to warming, they do not appear to constitute a functional effect group in terms of their effect on biomass production over the duration of this study. Understanding this differentiation between species that share functional response traits and species that share functional effects traits is critical to being able to predict how the loss of a group of species due to anthropogenic activities might effect ecosystem functioning (Lavorel & Garnier 2002; Naeem & Wright 2003; Hooper *et al.* 2005). Other studies have demonstrated the poor ability of *a priori* functional group classifications to predict effects on ecosystem functioning (Wright *et al.* 2006). Our study shows how functional response groups do not necessarily correspond to

functional effect groups of species, and that caution should be used when making predictions about how global change-induced species loss could affect community-level biomass production.

The loss of shallow-rooted forbs could have important consequences for ecosystem stability in terms of the system's "resistance", or ability to maintain function in response to a perturbation. Understanding how species loss affects ecosystem stability has been a long-standing ecological question (McCann 2000). Some research suggests a "diversity-begets-stability" relationship whereby a more diverse community is more resistant, or exhibits less change, in response to a perturbation due to an increased likelihood of having positively-affected species which can compensate for declines in the growth of negatively-affected species (e.g., Tilman 1996; Naeem & Li 1997; Yachi & Loreau 1999). Other research, however, presents theoretical and empirical evidence that less diverse plant systems can display greater resistance to a perturbation (Pfisterer & Schmid 2002). Most empirical research on the relationship between diversity and resistance to perturbations has focused on negative stresses such as drought and invasion by exotics, whereas very few experimental studies have looked at how diversity affects the magnitude of a community's response to potentially growth-enhancing perturbations such as increased nitrogen availability (Loreau et al. 2002a, although see Mellinger & McNaughton 1975, Fridley 2002, Reich et al. 2004, and Bret-Harte et al. 2004). Loreau & Behra (1999) theorize that a more diverse plant system might exhibit lower resistance to growth-enhancing perturbations such as nitrogen addition since a more diverse community would have an increased probability of containing species that demonstrate a positive growth response to increased nitrogen

availability. This would result in a larger magnitude of change in biomass relative to a system that is less diverse and therefore less likely to contain those nitrogen-responsive species.

We found that the loss of shallow-rooted forbs resulted in an *increased* magnitude of biomass change in response to N addition. This result seems to follow the pattern predicted by diversity-begets-stability theory, but does not appear to agree with the prediction made by Loreau & Behera (1999). Empirical data on how species loss affects community response to N addition is limited and the results mixed with Reich *et al.* (2004) and Fridley (2002) reporting that decreasing species diversity dampened the community's biomass response to N addition, Mellinger & McNaughton (1975) finding that decreased diversity increased the N addition response, and Bret-Harte *et al.* (2004) discovering that response to N addition was dependent on the identity of the plant group being removed. Since our experiment only looked at one specific scenario of species loss it is unclear how applicable our results are to theory and experimental results that are based on a randomly-assembled gradient in species and/or functional group richness. However, our results suggest that the loss of shallow-rooted forbs from this plant community made the system less resistant to a perturbation in N availability. Whether the system would also be less resistant to other perturbations remains to be seen, but could be a concern.

A potentially complicating factor in interpreting the observed responses to N addition may be that water is also likely to be limiting plant growth in our system (de Valpine & Harte 2001). Our assumption that this meadow community is N-limited was based on the observation that several species had responded to added nitrogen with

increased growth (de Valpine & Harte 2001). However, water is also considered to be an important resource (de Valpine & Harte 2001), and 2000-2003 was a period of severe drought throughout the southwestern U.S. (Breshears *et al.* 2005). If we assume that water was the primary limiting resource in our system during our experiment, then the observed increase in tap-rooted forb AGB in response to nitrogen addition in the SR+N plots could be explained at least in part by changes in soil moisture in response to the removal treatment. Soil moisture was significantly higher during the peak of the growing season in SR plots relative to CONT plots at 30 cm depth in 2002 and 2003, and at 10 cm depth in 2003 (Figure 2.4b-d). Changes in soil moisture of the magnitude we measured have been known to affect biogeochemical and plant processes in this system (Saleska *et al.* 2002), so the observed soil moisture difference may have sufficiently alleviated water limitations in the SR+N plots and allowed the tap-rooted forbs in those plots to respond positively to the nitrogen addition. However, this explanation does not account for the lack of response of tap-rooted forbs to N addition in the RR+N treatment, where soil moisture at all depths in 2002 and at 30 cm depth in 2003 was indistinguishable from that in the SR and SR+N treatment (Figure 2.4).

A more thorough exploration of how the species and functional groups within this plant community are limited by water and nitrogen is needed to further elucidate the mechanisms behind our observed responses since it appears that there could be a complicated interplay between water and nitrogen limitations and the loss of shallow-rooted forbs. Therefore, while our results suggest that the ability of tap-rooted forbs to compensate for the loss of shallow-rooted forbs will likely be enhanced if future warming is accompanied by increased nitrogen availability, this nitrogen addition effect

may be mediated by soil water availability. If future climate change results in decreased soil water availability via decreased precipitation, earlier snowmelt and/or increased evaporation, plants may not be able to respond to increased nitrogen availability.

The effect of anthropogenic species loss on ecosystem function and stability will likely depend on the identity of species being lost, the extent to which remaining species can compensate for the lost species, and the amount of overlap between functional response and effect traits within a community. Our results show that realistic non-random scenarios of species loss do not necessarily follow the pattern of decreased productivity and dampened response to N addition with species loss that is predicted by theory and experimental results from randomly-assembled plant communities. Additional experiments such as this one, that combine plausible species loss and perturbation scenarios, over even longer timescales are needed to improve our ability to predict the ecosystem consequences of human activity-driven declines in diversity.

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FIGURES

Figure 2.1. Plant aboveground biomass (AGB) by treatment in: A) 2001 (pre-manipulation); B) 2002 (with weeding and nitrogen (N) addition); C) 2003 (with weeding and N addition); and D) 2004 (no weeding, but N addition). White = grass AGB; Black = shallow-rooted forb AGB; Gray = tap-rooted forb AGB. Full height of bar is total AGB. Error bars are ± 1 SE of total AGB for each treatment. Treatment abbreviations are: CONT = no removal, ambient N; CONT+N = no removal, added N; RR = random forb removal, ambient N; RR+N = random forb removal, added N; SR = shallow-rooted forb removal, ambient N; SR+N = shallow-rooted forb removal, added N. Different letters at the top of each bar denote significant differences among treatments at $p \leq 0.05$ using an ANOVA model with a block effect and the Tukey-Kramer HSD test to compare all pairs. Bracketed significance levels (n.s. = not significant; * = $p < 0.01$) refer to *a priori* contrasts: CONT vs. CONT+N, RR vs. RR+N, and SR vs. SR+N.

Figure 2.1.

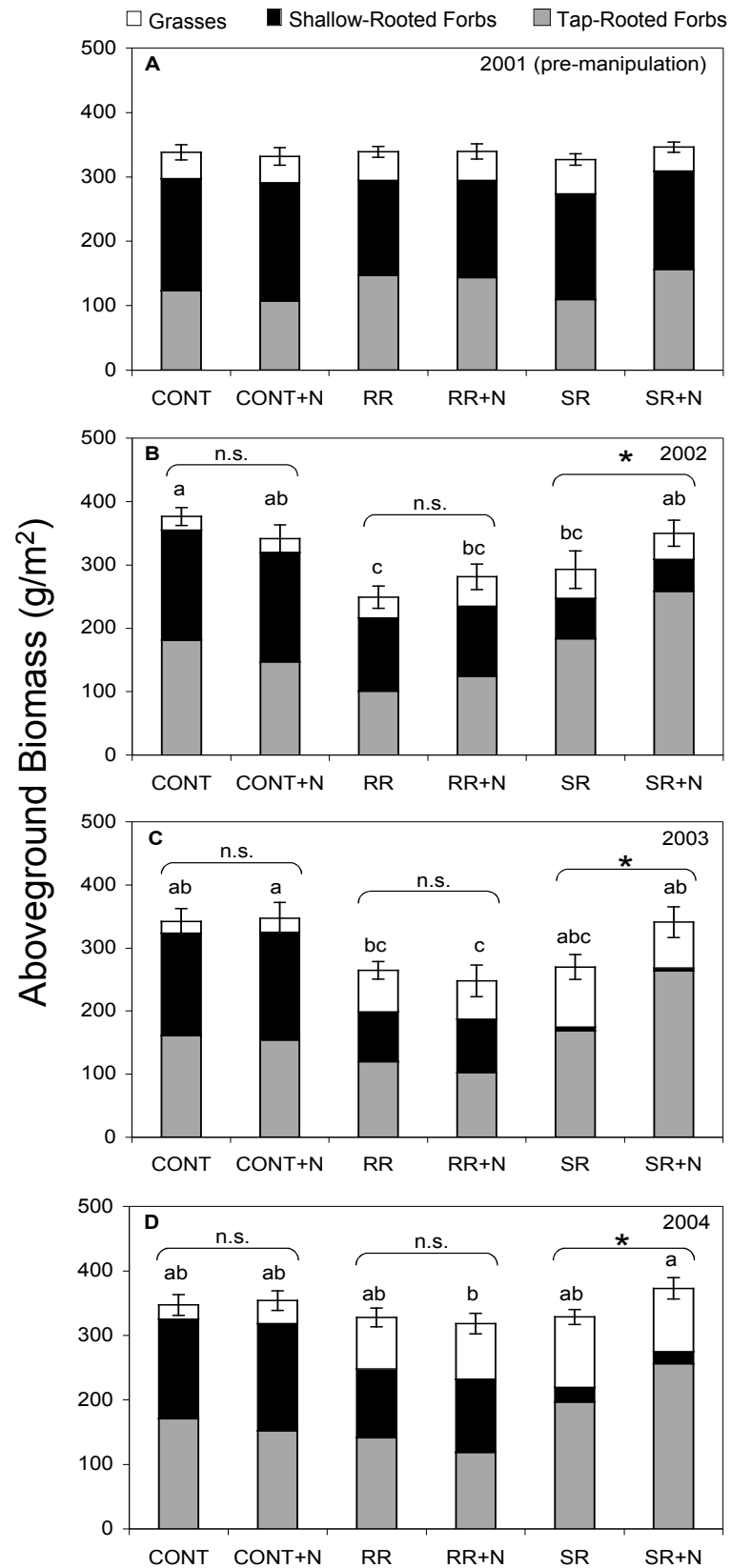


Figure 2.2. Average amount of biomass removed per plot (4m²) from the RR (black bars), RR+N (gray bars), SR (white bars) and SR+N (hatched gray bars) removal treatments in 2001 (when removal occurred at peak biomass, but before any N addition), 2002 (with weeding and N addition), 2003 (with weeding and N addition), and cumulatively over all three years. Different letters at the top of each bar denote significant differences among treatments within each year at $p \leq 0.05$ using an ANOVA model with a block effect and the Tukey-Kramer HSD test to compare all pairs. Error bars are ± 1 SE. See Figure 2.1 for treatment abbreviations.

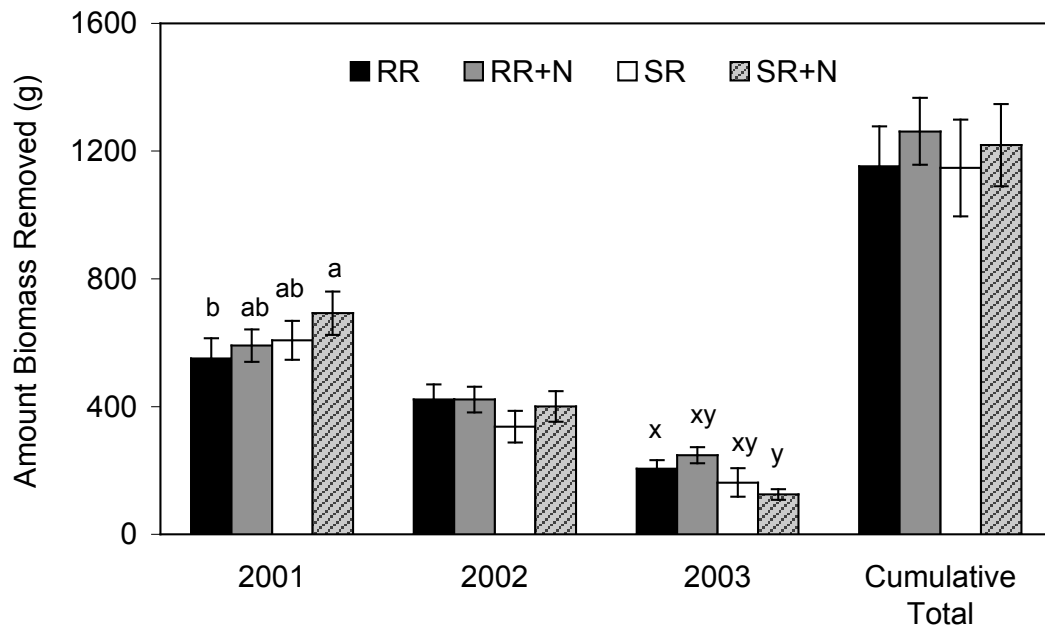


Figure 2.3. Live, dead and total fine root biomass in 2002 and 2003 for CONT (no removal = black bars), RR (random forb removal = gray bars) and SR (shallow-rooted forb removal = white bars). Data were pooled across nitrogen addition treatments. Different letters at the top of each bar denote significant differences among treatments at $p \leq 0.05$ using an ANOVA model with a block effect and *a priori* contrasts (CONT vs. SR, SR vs. RR). Error bars are ± 1 SE.

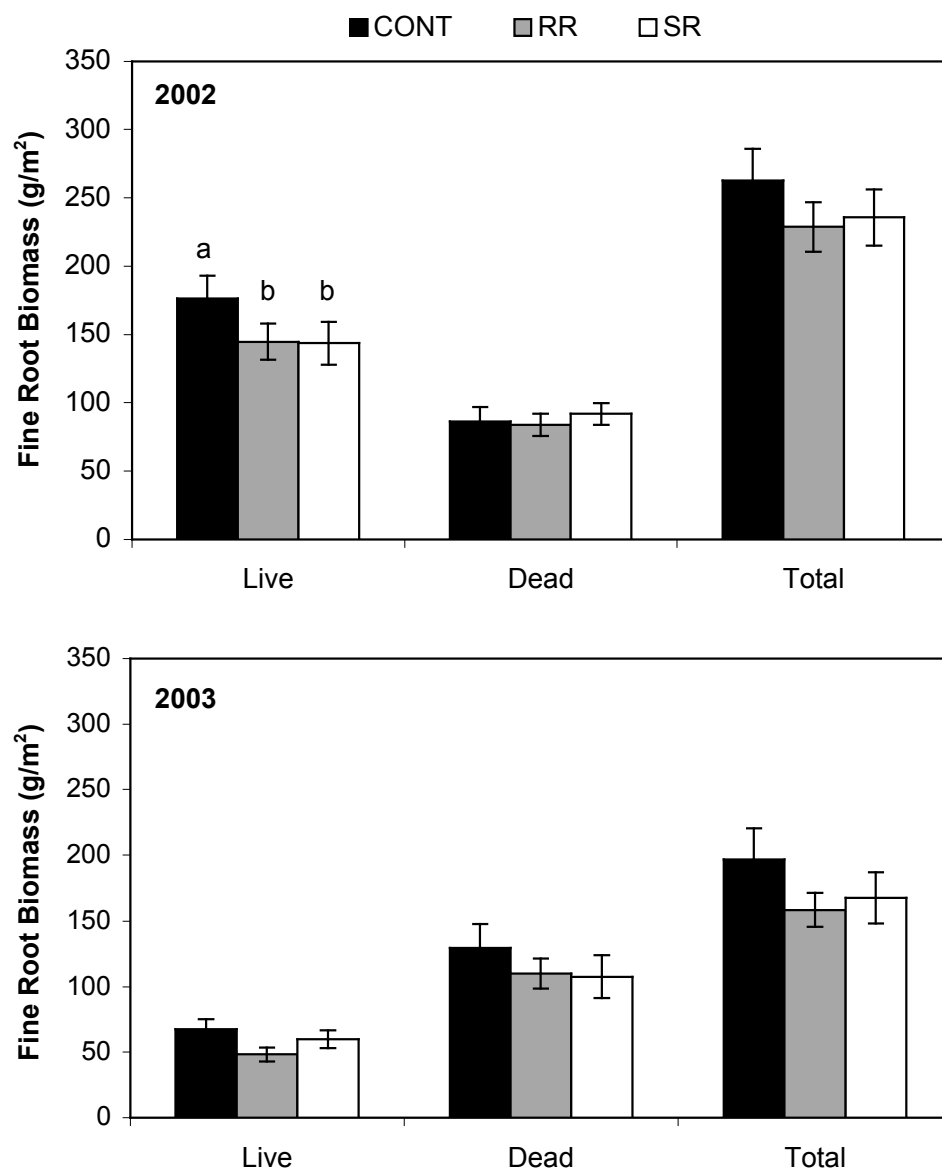
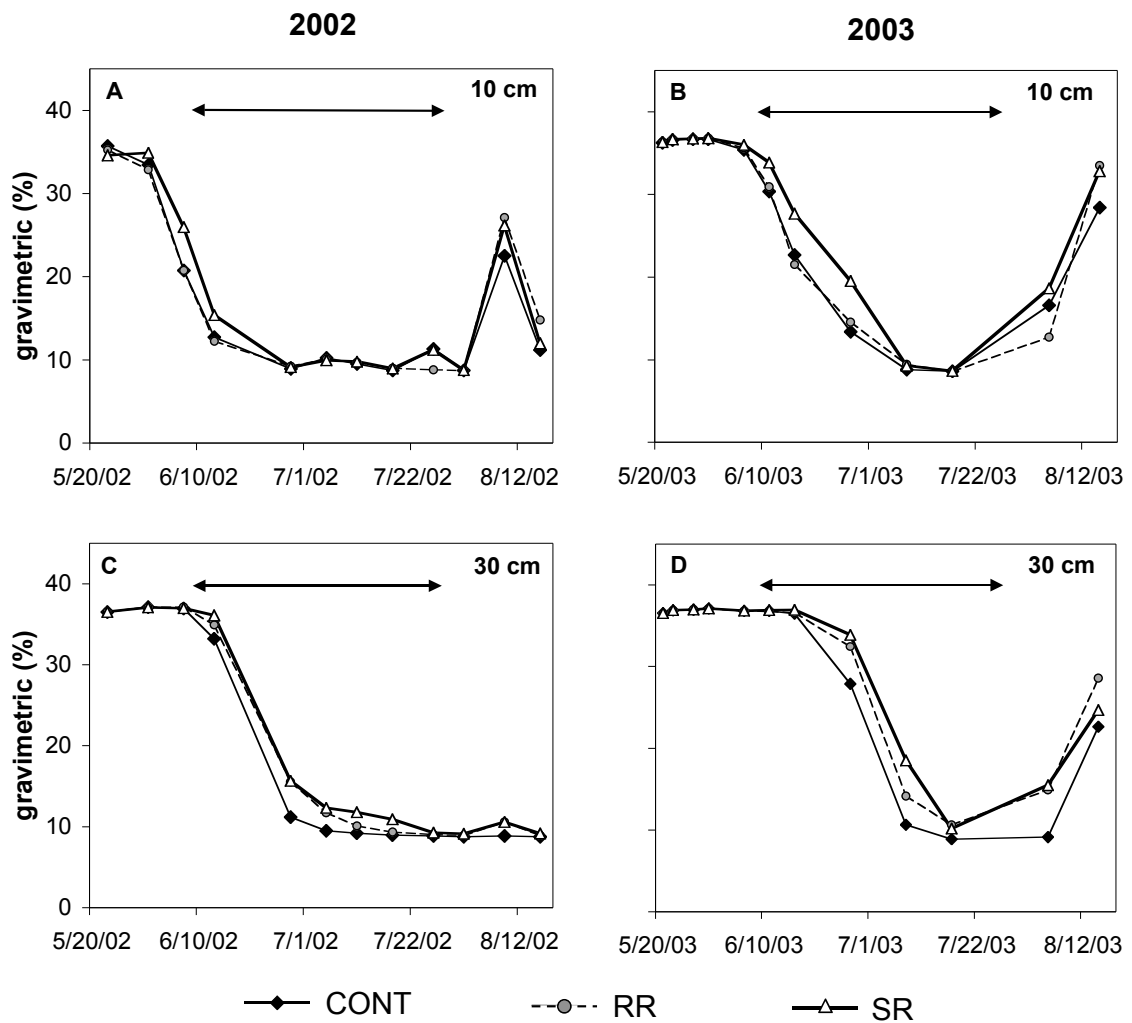


Figure 2.4. Gravimetric soil moisture at 10 cm and 30 cm in 2002 (A, C) and 2003 (B, D) for CONT (no removal = diamonds, solid line); RR (random forb removal = circles, dashed line); and SR (shallow-rooted forb removal = triangles, heavy line). Data were pooled across nitrogen addition treatments. Horizontal arrows approximately indicate the peak 6 weeks of the growing season.



CHAPTER 3: NITROGEN CYCLING RESPONSES TO WARMING-INDUCED PLANT SPECIES LOSS AND ENHANCED NITROGEN AVAILABILITY

INTRODUCTION

Human-induced environmental changes such as land-use, climate change, nitrogen (N) deposition, atmospheric carbon dioxide (CO₂) enrichment and the introduction of exotic invasive species are predicted to have significant negative impacts on global biodiversity by the end of the next century (Sala *et al.* 2000). In light of these impending changes, understanding the ecosystem consequences of losing biodiversity has become a focus of ecological research (Chapin *et al.* 2000; Loreau *et al.* 2002b; Hooper *et al.* 2005). Most experimental studies of the ecosystem consequences of plant species loss have focused on randomly-assembled plant communities (e.g., Naeem *et al.* 1994; Tilman *et al.* 1997a; Hector *et al.* 1999; Reich *et al.* 2001a; Tilman *et al.* 2001). However, changes in diversity as a result of human activities are not likely to be random – they are more likely to be targeted at species with traits that make them particularly vulnerable to change (Loreau *et al.* 2001; Grime 2002; Smith & Knapp 2003; Raffaelli 2004; Solan *et al.* 2004; Zavaleta & Hulvey 2004; Hooper *et al.* 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005). Several theoretical and empirical studies have shown that ecosystem functioning responds differently to random vs. non-random species loss (e.g., Ives & Cardinale 2004; Solan *et al.* 2004; Zavaleta & Hulvey 2004; Gross & Cardinale 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005). Therefore, investigating how ecosystems may respond to non-random changes in biodiversity as a result of specific human activities is important to predicting ecosystem

consequences of future species loss caused by humans (Loreau *et al.* 2001; Grime 2002; Naeem & Wright 2003; Hooper *et al.* 2005).

Anthropogenic climate change is predicted to be an important driver of biodiversity change over the next 100 years, especially in mountainous alpine environments (Sala *et al.* 2000). A meta-analysis of 11 arctic and alpine warming experiments found that plant species diversity significantly decreased with warming after only two growing seasons (Walker *et al.* 2006). One of those studies, in an alpine rangeland on the Tibetan Plateau, found that experimental warming resulted in a 26-36% decrease in plant species richness, and that the losses were non-random with respect to plant functional groups (Klein *et al.* 2004). Long-term results from an on-going 15-year warming experiment in a subalpine meadow ecosystem in the Colorado Rocky Mountains also suggests that some plant species in that system may be more adversely affected by increasing temperatures than other species (Harte *et al.* 1995). We found that warming decreased the growth of forbs (Harte & Shaw 1995), but not all forb species responded in the same way to warming (de Valpine & Harte 2001). Warming decreased the aboveground biomass and flowering success of shallow-rooted forbs, and decreased or had no effect on the growth of tap-rooted forb species (de Valpine & Harte 2001; Saavedra *et al.* 2003). Although warming has not led to the complete disappearance of any plant species from this experimental system, spatial patterns across the experimental plots suggest that plant species richness is reduced under conditions of early snowmelt and enhanced nitrogen availability (J. Harte, unpublished data). The observation that manipulated climate warming reduces soil moisture in shallow soil layers, advances the timing of snowmelt, and increases nitrogen availability

(Harte *et al.* 1995; Shaw & Harte 2001) provides further evidence that shallow-rooted forb species may eventually be lost from this meadow ecosystem under future warming.

To examine the impact that the non-random, warming-induced loss of shallow-rooted forb species may have on ecosystem functioning, we conducted an *in-situ* species removal experiment. Species removal experiments have recently been proposed as an appropriate manipulation approach for examining the effects of non-random species loss in naturally-assembled communities (Diaz *et al.* 2003; Hooper *et al.* 2005). By working in otherwise unmanipulated ecosystems, removal experiments explore the consequences of non-random species loss in complex natural systems, while maintaining an ability to look at cause-and-effect relationships by directly altering community composition (Diaz *et al.* 2003). Previous removal studies of non-random plant species loss have focused on the loss of rare species (e.g., Lyons & Schwartz 2001; Smith & Knapp 2003), or on the loss of various combinations of functional groups and/or dominant species (e.g., Sala *et al.* 1989; Hobbie *et al.* 1999; Wardle *et al.* 1999; Symstad & Tilman 2001; Bret-Harte *et al.* 2004; Buonopane *et al.* 2005; Wardle & Zackrisson 2005). To our knowledge, ours is the first removal study that examines non-random plant species loss resulting from a specific anthropogenic activity.

To quantify how ecosystem processes respond to the loss of shallow-rooted forbs, we measured plant biomass production (above- and below-ground), ecosystem nitrogen cycling, and soil microbial community composition. We also investigated whether the loss of shallow-rooted forb species altered the system's response to a perturbation. Since experimental warming significantly increased rates of net nitrogen mineralization (Shaw & Harte 2001), and because atmospheric deposition of nitrogen

from anthropogenic activities is expected to increase over the next several decades (Vitousek *et al.* 1997), we chose to perturb the system by increasing soil nitrogen availability. While Chapters 2 and 4 of this dissertation discuss the plant biomass and soil microbial community responses to our experimental species removal and nitrogen addition treatments, this Chapter examines nitrogen cycling responses.

Nitrogen availability is often a primary resource limiting ecosystem productivity in temperate terrestrial ecosystems (Vitousek & Howarth 1991). Understanding how nitrogen supply rates, plant uptake and leaching losses are affected by removal of shallow-rooted forbs is crucial to understanding how warming-induced plant species loss could affect the longer-term sustainability of nitrogen availability for plant growth (Niklaus *et al.* 2001). Plant communities that retain more nitrogen are likely to have sufficient nitrogen resources for future plant generations, whereas “leaky” systems with high nitrogen leaching rates (relative to supply rates via mineralization and nitrogen-fixation) could experience increased nitrogen limitation in the future. There are several ways that plant species removal could affect nitrogen retention and loss. Shifts in plant species composition can alter the quantity and quality of litter entering the soil organic pool, and therefore the amount of inorganic nitrogen mineralized by the soil microbial community and made available for plant growth (e.g., Hobbie 1992; Eviner & Chapin III 2003). Plant species also vary in their ability to compete for nitrogen resources, and/or their ability to access nitrogen resource niches that are less heavily used by other species (i.e., use complementary resource niches) (e.g., Tilman *et al.* 1997b). Changes in nitrogen uptake into plants and the supply of labile nitrogen via mineralization and nitrification can also affect leaching losses (e.g., Scherer-Lorenzen *et al.* 2003).

Several studies examining relationships between plant diversity and nitrogen cycling have found that reducing plant species and functional group richness can decrease nitrogen capture by the plant community, increase soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations, and increase NO_3^- leaching losses (Tilman *et al.* 1996; Tilman *et al.* 1997a; Hooper & Vitousek 1998; Knops *et al.* 2001; Niklaus *et al.* 2001; Symstad & Tilman 2001; Scherer-Lorenzen *et al.* 2003; Zak *et al.* 2003; Bret-Harte *et al.* 2004; Spehn *et al.* 2005). Some studies attribute that pattern to a decrease in complementary use of resource niches and resource use efficiency when diversity is lowered (e.g., Tilman *et al.* 1996; Knops *et al.* 2001; Spehn *et al.* 2005), while others highlight the importance of species identity and the presence of particular plant species or functional groups in determining nitrogen cycling (e.g., Hooper & Vitousek 1998; Symstad *et al.* 1998; Wardle *et al.* 1999; Symstad & Tilman 2001; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Bret-Harte *et al.* 2004). The presence and abundance of nitrogen-fixing legumes have been shown to be particularly important to nitrogen cycling, with increased legumes often corresponding to higher nitrogen concentrations in plant tissue (both in the legumes themselves as well as in neighboring non- N_2 -fixing plants) and increased NO_3^- leaching losses (Hooper & Vitousek 1998; Reich *et al.* 2001b; Mulder *et al.* 2002; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Spehn *et al.* 2005). Evidence of how plant species loss might affect soil nitrogen transformation rates is limited, and existing results range from increased mineralization (e.g., Zak *et al.* 2003), decreased mineralization (e.g., Dijkstra *et al.* 2005), and the direction of response depending on the specific species being removed (e.g., Hooper & Vitousek 1998; Symstad & Tilman 2001).

Inorganic nitrogen additions have also been shown to affect nitrogen cycling. In nitrogen-limited ecosystems, the addition of nitrogen generally leads to increased nitrogen uptake into plant biomass through an increase in tissue nitrogen concentrations and/or an increase in biomass accumulation (e.g., Bowman *et al.* 1993; Chapin *et al.* 1995; Fahey *et al.* 1998; Reich *et al.* 2001a; Magill *et al.* 2004; Reich *et al.* 2004). The effect of inorganic nitrogen addition on soil nitrogen dynamics is more difficult to predict since microbially-mediated nitrogen mineralization processes are determined by the balance between both plant tissue quantity and quality, and the extent to which microbial activity in the system is nitrogen vs. carbon limited (Fisk & Fahey 2001; Dijkstra *et al.* 2004). Soil nitrogen processes in some systems are driven by the effect of the amount and nitrogen concentration of litter on gross mineralization, while other systems are driven more by microbial demand for nitrogen (Fisk & Fahey 2001). In the first case, nitrogen additions will likely increase nitrogen mineralization, whereas there may be decreases in mineralization when microbial immobilization of nitrogen dominates net rates in the second case. For this reason, results from studies examining the effect of nitrogen fertilization on soil nitrogen cycling are mixed with some studies showing increases in net nitrogen transformation rates (e.g., McNulty & Aber 1993; Fisk & Schmidt 1996; Kjonaas *et al.* 1998; Dijkstra *et al.* 2005) and others showing decreases (e.g., Fisk & Fahey 2001; Magill *et al.* 2004).

Understanding how the loss of an entire group of plant species will alter the ecosystem's response to nitrogen addition is similarly difficult. Niche complementarity theory predicts that the loss of species could decrease the efficiency with which the remaining plant community can take up the added nitrogen resource (Tilman *et al.*

1997b). Assuming that mineralization is dominated by substrate quantity and quality, that decrease in nitrogen uptake could reduce the amount of organic nitrogen available for mineralization by soil microbes. Only a few studies have directly examined the effect of manipulated plant species loss and increased nitrogen availability on nitrogen cycling (Reich *et al.* 2001a; Bret-Harte *et al.* 2004), and results from those studies are mixed with Reich *et al.* (2001a) reporting that species loss dampened the community's uptake of nitrogen in response to N addition, and Bret-Harte *et al.* (2004) discovering that response to N addition was dependent on the identity of the plant group being removed. The effect of plant species loss and increased nitrogen availability on soil nitrogen cycling depends on exactly how those treatments alter plant tissue quantity and quality of each plant species and for the community as a whole.

Our study examines how a specific scenario of non-random plant species loss – the removal of species that were negatively affected by manipulated warming – affects the response of the plant community to nitrogen additions. Our experiment was designed to test several specific hypotheses:

- H1: The loss of all shallow shallow-rooted forb species will decrease the diversity of rooting morphologies and lead to decreased resource use efficiency.
- H2: Addition of inorganic nitrogen will alleviate nitrogen-limitations and lead to increased plant uptake and growth.
- H3: The loss of shallow-rooted forb species will dampen any increase in nitrogen uptake by plants with nitrogen addition since the decrease in the diversity of rooting morphologies is expected to result in less efficient use of the added nitrogen resource.

H4: Biomass of lupine, a common nitrogen-fixing species, will be correlated with higher nitrogen tissue concentrations and increased NO_3^- leaching losses.

If the loss of rooting morphological diversity does lead to decreased resource use efficiency as predicted in H1, then we would expect to see a decrease in the remaining plant community's uptake of nitrogen for plant growth when shallow-rooted forbs are removed. Decreased uptake of nitrogen into plant tissue would result in more inorganic nitrogen being left in the soil and an increase in soil NO_3^- and NH_4^+ pools (at least over the short-term). Since NO_3^- is generally very mobile in soils and leaches easily, we would also expect to see an increase in the amount of NO_3^- in deeper soil layers. Although initially we expect soil NO_3^- and NH_4^+ pools to increase in response to decreased plant uptake of nitrogen, we ultimately expect that decreased uptake of nitrogen will decrease plant litter %N and therefore reduce net nitrogen mineralization, nitrification and ammonification rates. Changes in plant species composition through the removal of shallow-rooted forbs may also alter the C/N ratio, or quality, of plant and soil organic matter in a way that alters nitrogen transformation rates.

The response of the plant community to nitrogen addition will strongly depend on the degree to which plant growth is limited by nitrogen availability (H2). If this subalpine meadow community is strongly nitrogen limited, then adding nitrogen will increase the uptake of nitrogen into plant tissue and we will see a change in the concentration of nitrogen or total amount of nitrogen in plant biomass. However, if plant growth is more tightly regulated by a resource other than nitrogen, then the addition of nitrogen will not increase the uptake of nitrogen into plant tissue, and

instead we would expect to see the added nitrogen leach down through the soil profile. If the plant community is strongly nitrogen-limited and nitrogen addition does lead to increase uptake of nitrogen into plant tissue, then we would expect to see higher rates of net nitrogen mineralization, nitrification and ammonification due to increased organic nitrogen inputs to the soil via plant litter.

Hypothesis H3 combines hypotheses H1 and H2 and assumes that the loss of an entire rooting morphological group will result in less efficient use of the added nitrogen resource. Therefore we would expect to see decreases in nitrogen uptake into plant tissue and increases in NO_3^- leached to deeper soil layers when shallow-rooted forbs are removed and inorganic nitrogen is added, relative to treatments with a higher diversity of rooting morphologies.

Since legumes provide their own nitrogen via N_2 -fixation and are poor competitors for soil NO_3^- , they tend to be associated with higher nitrogen tissue concentrations and increased NO_3^- leaching losses (H4). We therefore expect that the biomass of lupine, a common nitrogen-fixing species, will be correlated with increased NO_3^- moving down through the soil profile and increased plant tissue nitrogen concentration.

MATERIALS AND METHODS

Study Site

Our experiment was conducted in an ungrazed subalpine meadow at the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado (38° 53' N, 107° 02' W; elevation 2920 m), approximately 150 m away from our ongoing warming

experiment (Harte & Shaw 1995; Harte *et al.* 1995). The meadow plant community at this site is characterized by long-lived and largely perennial herbaceous forbs and grasses. Additional site details can be found in Harte and Shaw (1995) and Harte *et al.* (1995).

Experimental Design

In summer 2001, plant species removal and nitrogen addition treatments were established in 48 2 x 2 m plots, arranged in 8 blocks of 6 contiguous plots. Blocks were spaced approximately 3 m apart within a section of hillside where slope, aspect, hydrologic regime and plant community type were relatively uniform. Before assigning treatments to each plot, we collected pre-manipulation data on peak aboveground biomass (AGB) of grasses, deep tap-rooted forbs and shallow-rooted forbs.

Plots within each block were randomly assigned to one of three plant species removal treatments – removal of all AGB of shallow-rooted forb species (Shallow Removal, SR treatment), removal of random forb AGB (Random Removal, RR treatment), or control plots with no removal of biomass (Control, CONT treatment) – for a total of two replicates of each species removal treatment in each 6-plot block. Forb species were classified into shallow- and tap-rooted categories based on field excavations of root morphology (Appendix A). Shallow-rooted forbs were designated as those with fibrous rooting structures that were concentrated in the top 10cm of the soil profile, whereas tap-rooted forbs were characterized by a coarse taproot structure that can access deeper soil resources. The RR treatment was included to distinguish between the effect of species loss and the effect of biomass loss. We randomly chose

one replicate of each species removal treatment within a block to be fertilized by inorganic nitrogen (+N treatment) while the other replicate remained at ambient nitrogen levels. Therefore, each block contained one replicate of each of 6 total treatment combinations: CONT, CONT+N, SR, SR+N, RR, RR+N.

At the time of peak standing AGB in late July 2001, we removed aboveground vegetation from the SR and RR plots by clipping plants at their base. Biomass in the RR plots was removed by clipping all of the forb AGB (regardless of rooting structure) within a set of randomly chosen 25 by 25 cm quadrats, until we had removed an equivalent amount of biomass to the amount removed from the other SR plots within that block. Removal treatments were applied to the entire 4m² area of each plot. Before applying the removal treatments there were on average 18 forb species (minimum = 13 and maximum = 24) in the central 1m² of the plots, 13 of which were shallow-rooted species (minimum = 8 and maximum = 17). Depending on pre-manipulation species composition within each plot, the SR treatments removed from 61.9% to 87.5% of all forb species. Therefore, the SR treatment manipulated both species richness and functional response group richness. Although grasses were present in the experimental plots, we chose not to target grasses for removal in any of the treatments since warming has had no effect on grass AGB in the warming meadow experiment (Harte & Shaw 1995). In 2002 and 2003, we removed aboveground plant parts of all shallow-rooted forb species from the SR plots as soon as they emerged aboveground after snowmelt and could be identified to species. Plots were then continually weeded throughout the growing season to prevent aboveground growth of the target species. In the RR plots, the same 25 by 25 cm quadrats used to conduct the random removals in 2001 were kept

free of forb biomass by weeding all forbs from those quadrats throughout the 2002 and 2003 growing seasons. In 2004, we discontinued weeding in both the SR and RR plots.

Inorganic nitrogen was applied to the soil surface of fertilized (+N) plots as ammonium-nitrate (NH_4NO_3) in solution. A total of 6 g N/m² was applied to each +N plot each year, divided into four equal doses of 1.5 L of NH_4NO_3 solution. This level of N addition represents an approximate doubling of net N mineralization rates in the CONT plots, a magnitude of change roughly comparable to that observed in the heated plots relative to the controls in the warming meadow experiment (Shaw & Harte 2001). To account for the small amount of water addition that inevitably accompanies the application of fertilizer in solution, we added an equivalent amount of de-ionized water to non-fertilized plots, an amount that is negligible relative to incoming precipitation levels. The first nitrogen and water applications were applied just after snowmelt in spring 2002, with the remaining three doses applied roughly every 2-3 weeks thereafter. Nitrogen and water additions were continued in the same manner in 2003 and 2004.

Plant Uptake of Nitrogen

In 2003, we monitored treatment effects on plant uptake of nitrogen by looking at the concentration as well as the total amount of nitrogen in above- and below-ground plant tissue of grasses, tap- and shallow-rooted forbs. We also separated out the nitrogen-fixing *Lupinus sp.* (lupine) from the other tap-rooted forb species. It was not the only nitrogen fixing plant species in this community, but since on average it represented 30% of tap-rooted forb biomass (range 0-100%), we thought it might be an important player in nitrogen cycling. To quantify plant tissue nitrogen content, we

randomly sampled lupine, non-lupine tap-rooted forb, shallow-rooted forb and grass AGB from the central 1 m² of each plots at the peak of the growing season in the third week of July. We collected fine root (<2mm diameter) biomass by taking three cores (3.5 cm diameter by 10 cm depth) per plot at the peak of the growing season in July 2003. Roots were hand-sorted into live and dead biomass based on visible physical criteria such as tensile strength, friability and color of all fragments ≥ 0.5 cm in length (Vogt & Persson 1991), but only live roots were analyzed for nitrogen uptake.

All above and belowground plant samples were oven-dried for 72 hours at 65°C, ground to a homogeneous powder and analyzed on a Carlo-Erba C:N autoanalyzer for total organic nitrogen concentration (%N) for each plant group and all live fine roots. We calculated the average %N for the entire plant community by weighting each plant group's %N by its relative abundance in the community. Aboveground plant tissue %N was multiplied by AGB to calculate the total amount of N taken up into each plant type. Fine root biomass %N was multiplied by total live root biomass to calculate total nitrogen uptake into live fine roots. Since roots could not be identified to growth-form group, only total plot-level uptake of N into roots was measured. Total uptake of N by the entire plant community was calculated as the sum of N in AGB and fine roots. Plant tissue quality was calculated as the ratio of carbon to nitrogen (C/N) in lupine, grass, shallow-rooted forb, non-lupine tap-rooted forb and fine root biomass; and we calculated average aboveground plant tissue C/N for the entire plant community by weighting each plant group's C/N by its relative abundance in the community.

Soil Nitrogen Transformations

In 2002 and 2003, net nitrogen mineralization, nitrification and ammonification rates were measured using an *in situ* ion exchange resin core method with intact soil cores (DiStefano & Gholz 1986; Binkley & Hart 1989; Hart & Firestone 1989; Shaw & Harte 2001). Three soil cores (2.5 cm diameter by 10 cm length) were extracted from each plot with clear plastic tubes. We attached a mesh bag made from nylon stockings and filled with approximately 1g of mixed-bed ion exchange resins (50-60% moisture, JT Baker #4631) to both the top and bottom of each tube using perforated plastic caps. The core was then placed back in its original hole and the surface litter layer was replaced. To determine initial nitrate (NO_3^-) and ammonium (NH_4^+) concentrations, we aggregated three smaller soil samples (1.7 cm diameter by 10 cm length) collected adjacent to each of the intact cores. The intact cores were placed in the ground approximately 5-6 weeks after snowmelt and were retrieved after a 6-week incubation period to determine nitrogen transformation rates during the main part of the growing season. After incubating, we analyzed the soil inside each core for final inorganic nitrogen concentrations.

We extracted a ~20 g subsample of both the initial and final soils in 100 ml of 2M KCl. The top and bottom resin bags were rinsed in de-ionized water and extracted in 20 ml of 2M KCl (only 10 ml in 2002). Soil KCl extracts were shaken on a mechanical shaker for two hours, filtered through Whatman #42 filter paper, and frozen for storage. Resin bag KCl extracts were shaken for one hour and frozen without filtering. Thawed extracts were analyzed using a Lachat flow-injection autoanalyzer at the University of California, Berkeley or the Division of Agriculture and Natural

Resources (DANR) laboratory at the University of California, Davis. We calculated net mineralization by adding the amount of NO_3^- -N and NH_4^+ -N that leached out of the incubating soil and onto the resin bags at the bottom of each core to the difference between initial and final $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ in the soil. Net nitrification was similarly calculated by adding the amount of NO_3^- -N on the bottom resin bags to the change in soil $[\text{NO}_3^-]$ pool during incubation; and net ammonification was calculated as the change in soil $[\text{NH}_4^+]$ during incubation plus the amount of NH_4^+ -N on the bottom resin bags. The three replicates from each plot were averaged to obtain one rate for each experimental plot.

Soil Nitrogen Pools

Soil inorganic nitrogen pools at 0-10 cm was measured in 2002 and 2003 at ~5-6 weeks after snowmelt as the initial $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ concentrations for the net nitrogen transformation rates using the method described above. We measured total soil organic nitrogen at 0-10 cm in 2003 by aggregating three soil cores (2.5 cm diameter) from each plot, sieving out particles > 2mm in diameter, removing the majority of the visible root material, grinding the sample to a homogeneous powder, and analyzing a subsample on a Carlo-Erba C:N autoanalyzer. The ratio of soil organic carbon to nitrogen (C/N) was calculated as a measure of soil quality.

To measure the amount of inorganic nitrogen moving down through the soil profile, we inserted two mesh bags filled with $\sim 15 \pm 0.75$ g mixed-bed ion exchange resin (IER) (50-60% moisture, JT Baker #4631) at 30 cm depth in each plot (Binkley 1984; Giblin *et al.* 1994). In 2003, we left the IER bags in place for an entire year

(October 2002 through October 2003). In 2004, the bags were left in place for 8 months since we removed the bags one month after snowmelt (October 2003 through June 2004). After removing the bags from the ground, we rinsed them with de-ionized water, extracted them in 80 mL of 2M KCl, put them on a shaker for 1 hour, and filtered them through Whatman #42 filter paper. Extracts were analyzed using a Lachat flow-injection autoanalyzer as described above for net N transformation rates. Although not considered a true measure of inorganic nitrogen leaching, the amount of NO_3^- and NH_4^+ extracted from the 30cm IER bags is an indication of the amount of nitrogen moving down through the soil profile since the IER bags are largely collecting nitrogen ions that are transported via convective flow in soil water (Binkley 1984).

Soil Moisture

We measured soil moisture at 10 and 30 cm approximately once a week throughout the growing season in 2002 and 2003 using permanently installed gypsum blocks that were calibrated to % gravimetric soil moisture using soil samples taken directly from the plots.

Statistical Analyses

Treatment effects on nitrogen cycling were analyzed using multi-factorial analysis of variance (ANOVA) with the following model: block, removal and N addition as main effects, and a removal by N addition interaction term (with $n = 8$ blocks). Since soil moisture, fine root biomass, biomass of nitrogen-fixing *Lupinus spp.*, and inorganic nitrogen pools can also affect nitrogen cycling, we included these

variables (when appropriate) as covariates in an analysis of covariance (ANCOVA). We considered $p \leq 0.05$ to be significant, and $0.05 < p \leq 0.10$ to be marginally significant. When a main effect or interaction term was significant or marginally significant within our ANOVA or ANCOVA model, we conducted planned *a priori* contrasts or compared all means using the Tukey-Kramer HSD test. Regressions were conducted using general linear models. Deep ion-exchange resin bag data was transformed to $y = \ln(x+1)$ to improve normality of the data. Unless stated otherwise, all reported p-values are from the ANOVA model specified above and all data are reported as mean $\pm$ 1 standard error. Data were analyzed using JMP statistical software (SAS Institute Inc., version 5.1).

RESULTS

Effect of species removal on nitrogen uptake and tissue quality

Removal treatments had no main effect on the concentration of nitrogen (%N) in aboveground biomass (AGB) for any of the plant types (non-lupine tap-rooted forbs, shallow-rooted forbs, grasses, lupine) (Figure 3.1). The loss of shallow-rooted forbs led to a significant decrease in fine root %N relative to CONT (*a priori* contrast, $p = 0.03$), with fine root %N in RR indistinguishable between SR and CONT (Figure 3.1). Average aboveground tissue %N for each plant type differed significantly with grass having the lowest %N ($1.73 \pm 0.05\%$) and lupine having the highest %N ($2.58 \pm 0.09\%$). Shallow-rooted forb %N ($2.12 \pm 0.06\%$) and non-lupine tap-rooted forb %N ($2.19 \pm 0.08\%$) were significantly higher than grass %N and lower than lupine %N (Tukey HSD, $p = 0.05$), but indistinguishable from one another (Tukey HSD, $p = 0.05$).

Although the removal of shallow-rooted forbs significantly altered plant community composition (Chapter 2), average %N of AGB for the entire plant community (weighted by the relative abundances of each plant group type) was not altered by removal treatments ($p = 0.72$; data not shown).

The removal of both shallow-rooted forbs and random forb biomass resulted in a significant decrease in the total amount of nitrogen taken up into plant biomass (aboveground tissue plus fine roots) relative to CONT levels ($p = 0.0002$; Figure 3.2). Since plant tissue nitrogen concentrations were not strongly affected by removal treatments, the effect of removal on the total amount of nitrogen taken up into plant tissue of each different plant type tended to follow the same patterns as removal treatment effects on AGB of each of those plant types (Chapter 2; Figure 2.1). Removal of shallow-rooted forb species and random forb biomass led to a significant increase in the amount of nitrogen taken up into grass AGB relative to CONT plots ($p = 0.001$; Figure 3.2). There was a significant effect of removal treatment on the amount of nitrogen taken up in to non-lupine tap-rooted forbs ($p < 0.0001$), but that effect was driven by a significant removal by nitrogen interaction (discussed below in *The effect of species loss on response to nitrogen addition* results section). The effect of removal treatment on the amount of nitrogen taken up into fine roots also followed treatment effects on live fine root biomass: CONT had slightly more N in fine roots than RR ($p = 0.02$), with SR indistinguishable between RR and CONT (Figure 3.2).

Plant tissue quality, measured as the ratio of C/N in live plant tissue, differed significantly among plant groups (Figure 3.3). Grass AGB and fine root tissue for all species had significantly higher C/N ratios than lupine, shallow-rooted forbs, and non-

lupine tap-rooted forbs (Tukey HSD, $p = 0.05$). However, average C/N of plant tissue for the entire plant community was not altered by the removal of either shallow-rooted forbs or random forb biomass ($p = 0.99$; data not shown). There was a marginal increase in live fine root C/N with the removal of shallow-rooted forbs relative to CONT ($p = 0.08$), with fine root C/N in RR plots indistinguishable between SR and CONT (Figure 3.3).

Effect of species removal on soil nitrogen

Removal treatments had no effect on inorganic soil $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ at 0-10 cm depth in either 2002 or 2003 (Figure 3.4). The concentration of shallow (0-10 cm) soil organic nitrogen (%N) in SR treatments was significantly higher than in RR treatments ($p = 0.05$), but was indistinguishable from shallow soil %N in CONT treatments (Figure 3.5). There was no main effect of removal treatment on shallow soil C/N ratios (data not shown).

The amount of NH_4^+ on ion exchange resin (IER) bags at 30 cm was unaffected by removal treatment (data not shown), but there was an effect of removal treatment on the amount of NO_3^- on IER bags at 30 cm in both 2003 (Figure 3.6). In 2003, the removal of both random forb biomass and shallow-rooted forb species led to a marginal increase in the amount of NO_3^- at 30 cm, relative to CONT levels ($p = 0.07$). In 2004, when the deep IER bags were left in place for only one month following snowmelt, there was no effect of removal treatment on the amount of NO_3^- at 30 cm ($p > 0.10$). The amount of NO_3^- at 30 cm in 2003 was negatively correlated with total fine root biomass ($R^2 = 0.28$, $p = 0.0001$), and marginally positively correlated to deep (0-10 cm)

soil moisture ($R^2 = 0.07$, $p = 0.07$) (Figure 3.7). As discussed in Chapter 2, the removal of both random and shallow-rooted forb AGB reduced total fine root biomass (although not significantly; Figure 2.3) and increased deep soil moisture ($p = 0.01$; Figure 2.4). Therefore, the observed increase in NO_3^- at 30 cm in RR and SR treatments is likely related to a decrease in root uptake of NO_3^- in the 0-10 cm zone and an increase in the amount of soil water at 10-30 cm depth under those treatments (see Chapter 2 for additional details on fine root and soil moisture responses to experimental treatments).

Effect of species removal on soil nitrogen transformation rates

In 2002, net nitrogen mineralization (N-MIN) and nitrification (N-NITR) rates were significantly greater in SR relative to CONT plots, but were indistinguishable from rates in RR plots ($p = 0.05$; Figure 3.8a-b). 2002 shallow (0-10 cm) soil moisture was significantly positively correlated with 2002 N-MIN ($R^2 = 0.18$, $p = 0.01$) and 2002 N-NITR ($R^2 = 0.16$, $p = 0.01$). When 2002 shallow soil moisture was included as a covariate, the effect of shallow soil moisture was significant (ANCOVA: $p = 0.01$) and its inclusion in the model eliminated the significant effect of removal treatment on both 2002 N-MIN (from $p = 0.05$ [ANOVA] to $p = 0.19$ [ANCOVA]) and 2002 N-NITR (from $p = 0.06$ [ANOVA] to $p = 0.24$ [ANCOVA]). As discussed in Chapter 2, there was a non-significant trend towards higher 2002 shallow soil moisture in SR plots relative to RR and CONT plots (Chapter 2, Figure 2.4). There was no effect of removal treatment on net nitrogen ammonification (N-AMM) in 2002, and no removal treatment effect on any of the net nitrogen transformation rates in 2003 (Figure 3.8a-c).

2003 plant tissue and soil quality (measured as average AGB C/N and shallow (0-10 cm) soil C/N) showed no correlation with 2003 N-MIN, N-NITR or N-AMM rates (data not shown). 2003 fine root tissue quality (measured as the ratio of C/N) was a significant covariate on 2003 N-MIN and N-AMM (ANCOVA: $p = 0.05$ and $p = 0.01$, respectively) with higher root C/N correlating to lower N-MIN and N-AMM rates, but having no effect on the significance of any of the treatment effects on N-MIN or N-AMM.

Effect of nitrogen addition on soil nitrogen, plant uptake and tissue quality

Experimental nitrogen addition resulted in a significant increase in soil inorganic nitrogen pools in both 2002 and 2003 ($p \leq 0.001$ both years; Figure 3.4). Mean soil $[\text{NO}_3^-]$ was almost 2.4 times greater in fertilized +N treatments than ambient N treatments in 2002, and more than 3.5 times greater in 2003. Mean soil $[\text{NH}_4^+]$ was 1.6 times greater in fertilized +N treatments than ambient N treatments in 2002, and just over 3 times greater in 2003. Total fine root biomass was negatively correlated with soil $[\text{NO}_3^-]$ in both 2002 ($R^2 = 0.10$, $p = 0.02$) and 2003 ($R^2 = 0.19$, $p = 0.002$), but the increase in soil $[\text{NO}_3^-]$ with nitrogen addition remained significant even when we accounted for variation in fine root biomass (Figure 3.9). There was a marginal decrease in shallow (0-10 cm) soil organic %N with nitrogen addition ($p = 0.08$), but no main effect of nitrogen addition on shallow soil C/N ratios (data not shown).

Nitrogen addition had no main effects on %N or C/N ratio of AGB for any of the plant types (non-lupine tap-rooted forbs, shallow-rooted forbs, grasses, lupine) (Figures 3.1 and 3.3). However, nitrogen addition did marginally increase average %N

of AGB for the entire plant community from $2.06 \pm 0.06\%$ to $2.18 \pm 0.07\%$ ($p = 0.10$). There was no change in total plant nitrogen uptake (Figure 3.2) or average C/N of plant tissue for the entire plant community (data not shown) with nitrogen addition.

The amount of NH_4^+ on IER bags at 30 cm was unaffected by nitrogen addition in 2003 and 2004 (data not shown). In 2003, when the IER bags were left in place for the entire growing season, nitrogen addition resulted in a significant increase in the amount of NO_3^- at 30 cm ($p = 0.03$; Figure 3.6), even when we accounted for variation in fine root biomass and deep soil moisture (Figure 3.7). This response seems to be due to the observed increase in soil $[\text{NO}_3^-]$ with nitrogen addition (Figure 3.4) since 2003 soil $[\text{NO}_3^-]$ was positively correlated with NO_3^- at 30 cm ($R^2 = 0.25$, $p = 0.0003$). In 2004, when the deep IER bags were left in place for only one month following snowmelt, there was still an increase in NO_3^- at 30 cm with nitrogen addition, but the effect was only marginally significant ($p = 0.07$; Figure 3.6).

Effect of nitrogen addition on soil nitrogen transformation rates

There was no effect of N addition on N-MIN and N-NITR rates in 2002 or 2003 (Figure 3.8a-b). Nitrogen addition had no effect on N-AMM rates in 2002, but it led to a significant decrease in N-AMM from net production to net immobilization of NH_4^+ in 2003 ($p = 0.03$; Figure 3.8c). Since N-AMM rates were negatively correlated with soil $[\text{NH}_4^+]$ in both 2002 ($R^2 = 0.27$, $p = 0.0002$) and 2003 ($R^2 = 0.35$, $p < 0.0001$), it appears that the increase in soil $[\text{NH}_4^+]$ with N addition is what drives the correlation between N addition and N-AMM rates. When 2003 soil $[\text{NH}_4^+]$ was added as a covariate in the regular ANOVA model, the effect of N addition on 2003 N-AMM rates

was no longer significant and 2003 soil $[\text{NH}_4^+]$ was the only significant factor affecting 2003 N-AMM (ANCOVA: $p = 0.007$). For 2003 N-NITR, adding 2003 soil $[\text{NO}_3^-]$ as a covariate made the N addition effect marginally significant (from $p = 0.43$ [ANOVA] to $p = 0.06$ [ANCOVA]) with nitrogen addition leading to an increase in N-NITR rates. 2002 soil $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ showed no correlation with 2002 N-MIN, N-NITR, or A-AMM rates (data not shown).

The effect of species loss on response to nitrogen addition

There was a significant removal by nitrogen addition interaction on N uptake into non-lupine tap-rooted forb AGB ($p = 0.01$), whereby non-lupine tap-rooted forbs responded to nitrogen addition with increased nitrogen uptake in SR plots, but not in RR and CONT plots (Figure 3.2). None of the other plant groups (lupine, grasses, shallow-rooted forbs) responded to nitrogen addition with increased uptake in any of the SR, RR, or CONT removal treatments. There was a marginally significant removal by nitrogen addition interaction on grass %N ($p = 0.07$) and with an increase in %N in grass AGB with N addition in SR and RR plots, while grasses in CONT plots trended towards decreased %N with N addition (Figure 3.1). Those changes in grass %N also led to a marginal removal by nitrogen addition interaction effect on the C/N ratio of grass AGB tissue with the addition of nitrogen leading to decreased grass C/N in SR and RR plots, but no change in CONT plots ($p = 0.08$; Figure 3.3).

The effect of lupine on nitrogen cycling

The amount of nitrogen-fixing lupine AGB was positively correlated with the amount of NO_3^- found at 30 cm in the IER bags ($R^2 = 0.12$; $p = 0.02$; Figure 3.10). When lupine AGB was included as a covariate on NO_3^- at 30 cm (ANCOVA: $p = 0.07$), the significance of the removal effect increased (from $p = 0.07$ [ANOVA] to $p = 0.03$ [ANCOVA]), and the effect of nitrogen addition remained significant (from $p = 0.03$ [ANOVA] to $p = 0.04$ [ANCOVA]). So even after accounting for variation in lupine AGB, SR and RR treatments had increased amounts of NO_3^- on deep IER bags relative to CONT treatments, and nitrogen addition increased the amount of NO_3^- at 30 cm. We did not find any relationship between lupine AGB and soil $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ at 0-10 cm, N-MIN, N-NITR or N-AMM rates. However, since the %N of lupine AGB was significantly higher than in tissue from the other plant groups (Figure 3.1), increasing lupine AGB did increase the average %N of AGB for the entire plant community (data not shown). Although increasing lupine AGB was not correlated with lupine %N or the %N of grasses or shallow-rooted forbs, it was positively correlated with non-lupine tap-rooted forb %N ($R^2 = 0.19$, $p = 0.002$) (Figure 3.11).

DISCUSSION

Several theoretical and experimental studies of random declines in plant richness suggest that decreases in species diversity will on average decrease complementary resource niche use and therefore have negative consequences for nitrogen cycling in terms of decreased capture by vegetation, increased leaching losses, and a dampened response to nitrogen addition (e.g., Tilman *et al.* 1996; Tilman *et al.* 1997b; Loreau

1998; Knops *et al.* 2001; Reich *et al.* 2001b; Mulder *et al.* 2002; Spehn *et al.* 2005). However, there also exists a large body of evidence that species and functional group identity matters as much to nitrogen cycling, if not more, than the number of species present in an ecosystem (e.g., Hooper & Vitousek 1998; Symstad *et al.* 1998; Wardle *et al.* 1999; Symstad & Tilman 2001; Mulder *et al.* 2002; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Bret-Harte *et al.* 2004; Reich *et al.* 2004). Since plant species loss driven by anthropogenic activities is not likely to be random, it is important that we investigate how specific scenarios of plant loss could affect nitrogen cycling processes. To our knowledge, ours is the first removal study that examines non-random plant species loss resulting from a specific anthropogenic activity by examining the ecological consequences of losing shallow-rooted forbs, a group of species we predict to be adversely affected by climate change based on their response to experimental warming. Although some nitrogen cycling processes were affected by the removal of shallow-rooted forbs and the addition of inorganic soil nitrogen, the responses often did not agree with our predictions based on theory and experimental results from randomly-assembled gradients in plant diversity.

Effects of shallow-rooted forb removal

We expected the loss of shallow-rooted forbs to decrease the amount of nitrogen taken up into above and belowground plant tissue (H1). However, we found no change in the nitrogen concentration (%N) of aboveground plant tissue in lupine, grasses, shallow-rooted forbs and non-lupine tap-rooted forbs, and only a slight decrease in %N of fine roots in response to the loss of shallow-rooted forbs under ambient nitrogen

conditions (Figure 3.1). There was also no effect of shallow-rooted forb removal on average %N and C/N of AGB for the entire plant community. Although the concentration of nitrogen in plant tissue did not change with the removal of shallow-rooted forbs, there was a significant decrease in the total amount of nitrogen taken up into plant tissue when shallow-rooted forbs were removed relative to controls (Figure 3.2). However, there was a similar decrease in nitrogen uptake with the removal of random forb biomass (Figure 3.2). So while we did see a drop in total nitrogen uptake into above and belowground plant tissue with the loss of shallow-rooted forbs as we predicted following hypothesis H1, it appears that drop was mostly due to the removal of biomass rather than the loss of species.

Although we expected the loss of an entire group of species to increase inorganic soil nitrogen pools, we did not see a change in soil $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ when shallow-rooted forbs were removed under ambient nitrogen conditions (Figure 3.4). We also did not see any consistent changes in the supply of NO_3^- and NH_4^+ for plant growth through N-MIN, N-NITR and N-AMM rates with the loss of shallow-rooted forbs as we expected. Despite the fact that the removal of shallow-rooted forbs significantly increased N-MIN and N-NITR rates in 2002 by increasing soil moisture %, there was no effect of shallow-rooted forb removal on 2003 N-MIN, N-NITR and N-AMM rates (Figure 3.8). We expected the decrease in total nitrogen uptake into plant tissue under ambient nitrogen SR and RR treatments to reduce the amount of organic nitrogen available for microbial mineralization, but perhaps the decrease was not sufficient to alter microbial activity. Another reason why microbial nitrogen transformation rates were not affected by shallow-rooted forb removal could be that shallow-rooted forbs

and non-lupine tap-rooted forbs do not significantly differ in their tissue %N or C/N (Figures 3.1 and 3.3). Therefore, average plant tissue quality (measured as %N or C/N) was not changed by the loss of shallow-rooted forbs even though shallow-rooted forb biomass was replaced in part by non-lupine tap-rooted forb biomass. This suggests that microbially-mediated nitrogen transformation rates in this ecosystem may be more strongly controlled by the quality of organic nitrogen inputs than the quantity.

We did measure an increase in the amount of NO_3^- extracted from IER bags at 30 cm when shallow-rooted forbs were removed (Figure 3.6). Since the removal of shallow-rooted forbs also decreased fine root biomass at 0-10 cm (Figure 2.3) and increased soil moisture % at 10-30 cm (Chapter 2; Figure 2.4), the increase in NO_3^- at 30 cm in SR plots may have been due to a decrease in root uptake of NO_3^- near the soil surface and an increase in soil water transport of NO_3^- ions down through the soil profile. While this suggests that the loss of shallow-rooted forbs could make the ecosystem more “leaky” in terms of the amount of NO_3^- leaching down through the soil profile, we recorded similar responses to the loss of random forb biomass so it appears that the increase in NO_3^- at 30 cm is as much due to the loss of plant biomass as it is due to the loss of shallow-rooted forb species. Caution should also be used in directly relating NO_3^- at 30 cm on IER bags to leaching losses since we cannot determine if and/or how much nitrogen transformation by microbes contributed to the amount of NO_3^- at 30 cm. It is also not clear whether the NO_3^- at 30 cm remains at that depth (where it can be taken up by plants or microbes), or leaches out of the soil into ground- and surface-water.

Our findings suggest that this non-random scenario of plant species loss with future warming does not lead to decreased nitrogen uptake and increased nitrate leaching losses that is predicted by theory and some experimental results which consider more thorough gradients in species and functional group richness (e.g., Tilman *et al.* 1996; Tilman *et al.* 1997b; Loreau 1998; Knops *et al.* 2001; Niklaus *et al.* 2001; Spehn *et al.* 2005). While those studies demonstrate that, on average, random increases in richness will also increase nitrogen capture and retention, there are often some less diverse replicates that are as efficient at taking up nitrogen as some of the higher diversity replicates (e.g., see Figure 6 in Spehn *et al.* 2005). Therefore, while there may be an underlying effect of diversity *per se* on nitrogen uptake and retention, these studies do not necessarily reveal what may happen under specific, plausible scenarios of plant species loss. Several studies have determined that plant species identity and community composition are more important than species richness to understanding the effect of species loss on nitrogen cycling (Hooper & Vitousek 1998; Symstad *et al.* 1998; Symstad & Tilman 2001; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Bret-Harte *et al.* 2004). This argues in favor of conducting additional experiments such as the one presented here in order to better understand how non-random species loss can affect nitrogen cycling processes.

Effects of nitrogen addition

As we expected (H2), the addition of nitrogen significantly increased soil $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ (Figure 3.4). In response to increased nitrogen availability, there was a marginal increase in the average nitrogen concentration (%N) of aboveground plant

tissue for the entire plant community, but no change in the total amount of nitrogen taken up into plant biomass (aboveground tissue plus fine roots) (Figure 3.2). Nitrogen addition also did not alter average plant tissue quality (measured as C/N). The addition of nitrogen did increase the amount of NO_3^- at 30 cm, even after accounting for variation in fine root biomass at 0-10 cm and soil moisture at 10-30 cm (Figures 3.6 and 3.7).

Despite the marginal increase in average %N in plant AGB with nitrogen addition, our findings generally do not support hypothesis H2 and suggest that plant growth in this system is not strongly limited by nitrogen. The increase in NO_3^- at 30 cm with nitrogen addition provides additional evidence that the plant community was unable to take up the added nitrogen which may have resulted in NO_3^- moving down through the soil profile. Therefore, we conclude that this plant community was likely limited by other non-nitrogen resources that prevented the plants from more fully capitalizing on the added nitrogen. This agrees with our findings for the response of plant productivity to nitrogen addition, where nitrogen addition had no over-all effect on total aboveground biomass (Chapter 2). Our initial assumption that this meadow community might be nitrogen limited was based on the observation that several plant species had responded to added nitrogen with increased growth (de Valpine & Harte 2001). However, water is also considered to be an important resource (de Valpine & Harte 2001), and 2000-2003 was a period of severe drought throughout the southwestern U.S. (Breshears *et al.* 2005). If water was more limiting to plant growth during the years of our experiment, it could explain why we did not see a larger plant response to nitrogen addition.

Nitrogen addition significantly decreased N-AMM in 2003, but did not have any effect on N-MIN or N-NITR (Figure 3.8). Although we expected nitrogen addition to increase net nitrogen transformation rates, that hypothesis was dependent on nitrogen addition leading to increased nitrogen uptake by plants. However, we did not see any change in total plant nitrogen uptake or average plant tissue quality (measured as %N or C/N) with the addition of nitrogen. Therefore, our observation that nitrogen addition did not consistently affect nitrogen transformation rates is not surprising. The increase in microbial immobilization of NH_4^+ with nitrogen addition (through decreased N-AMM rates) suggests that the microbes were able to take up the added NH_4^+ directly rather than through ammonification of organic matter, however this does not appear to have been a major factor affecting N-MIN or N-NITR since initial soil $[\text{NO}_3^-]$ and $[\text{NH}_4^+]$ did not affect those rates.

Although these results do not agree with several other nitrogen fertilization studies that measured increased plant uptake of nitrogen with increasing inorganic nitrogen availability (Bowman *et al.* 1993; Chapin *et al.* 1995; Fahey *et al.* 1998; Reich *et al.* 2001a; Magill *et al.* 2004; Reich *et al.* 2004), the difference is likely explained by differences in the strength of nitrogen limitation on plant growth versus limitations by other resources (e.g., soil water). Since results from other studies looking at the effect of nitrogen addition on net nitrogen transformation rates are so variable, it is hard to put our finding of no consistent change in N-MIN and N-NITR and a slight decrease in N-AMM in 2003 into context. Our results suggest that the lack of a nitrogen addition effect on net nitrogen transformation rates is due to the fact that nitrogen addition also did not change average plant tissue quantity and quality, and therefore did not alter

organic nitrogen inputs to the soil. However, since we did not separately measure gross nitrogen transformation rates and microbial immobilization, we cannot pinpoint the exact mechanism behind this pattern. Our finding that nitrogen addition led to increased NO_3^- at 30 cm agrees with evidence of a positive relationship between nitrogen addition and increased NO_3^- leaching that mainly comes from forested ecosystems (e.g., Magill *et al.* 2004; Micks *et al.* 2004; Pregitzer *et al.* 2004).

Effects of shallow-rooted forb removal on response to nitrogen addition

We expected that the loss of shallow-rooted forbs would dampen the increased uptake of nitrogen by the plant community in response to nitrogen addition, and that this reduced uptake would result in an even greater amount of NO_3^- leaching down the soil profile (H3). Instead, we found that the loss of shallow-rooted forbs increased the total uptake of nitrogen into plant tissue with nitrogen addition, relative to the more species- and functional group-rich CONT and RR treatments which did not take up any additional nitrogen when nitrogen was added (Figure 3.2). We also did not see any additional increase in the amount of NO_3^- at 30 cm depth when we removed shallow-rooted forbs and added nitrogen in either 2003 or 2004 (Figure 3.6). The increased uptake of nitrogen in plots where shallow-rooted forbs had been removed and nitrogen added resulted in the SR+N plots taking up an equivalent amount of nitrogen as the more diverse CONT and CONT+N plots (Figure 3.2). This was mainly due to a significant increase in the uptake of nitrogen by non-lupine tap-rooted forbs in the plots where shallow-rooted forbs had been removed and nitrogen added (Figure 3.2). Grasses also took up more nitrogen when shallow-rooted forbs were removed, but did not

increase their uptake when nitrogen was added (Figure 3.2). These results suggest that the loss of the entire group of shallow-rooted forbs did not decrease the ability of the plant community to take up the additional nitrogen resources, as we initially predicted might occur (H3), since non-lupine tap-rooted forbs and grasses were able to fully compensate for the loss by taking up additional nitrogen and increasing biomass production.

Our results provide an example where the loss of an entire group of plant species did not reduce the ability of the plant community to take up additional nitrogen resources. This finding does not conform to general niche complementarity theory that predicts resource capture will be greater under higher diversity since a more diverse system is more likely to include a combination of species that utilize complementary resource niches by differing in their spatial and temporal use of resources, and the exact form of the resource used. For example, plant species can differ in the depth to which they extract nitrogen from the soil, the timing when they depend on nitrogen resources (e.g., early-, mid-, or late-season), and the form of nitrogen used (e.g., NO_3^- , NH_4^+ , organic nitrogen compounds, or N_2 via fixation) (Berendse 1979; Nadelhoffer *et al.* 1996; Hooper & Vitousek 1998; Nordin *et al.* 2001; McKane *et al.* 2002; Fargione & Tilman 2005). Previous research has demonstrated that a greater diversity of rooting morphologies and depths can result in more efficient use of soil resources and greater accumulation of biomass (Berendse 1982; McKane *et al.* 2002; Dimitrakopoulos & Schmid 2004). Although we do not know the exact nitrogen resource niche occupied by each of the plant species in our system, we expected that the loss of all shallow-rooted forbs species would represent a significant loss of rooting morphological diversity in

our plant community and therefore reduce the community's ability to take up nitrogen under both ambient and enhanced nitrogen conditions.

We did find that the loss of shallow-rooted forbs resulted in decreased nitrogen uptake under ambient nitrogen conditions, but that response was no different than the response to the removal of random forb biomass where a diversity of rooting morphologies was maintained (Figure 3.2). Under enhanced nitrogen availability, we found that only the less diverse SR treatment responded to added nitrogen with increased uptake, which does not agree with our initial prediction based on niche complementarity theory (H3). There are several reasons why our results might not have conformed to our initial predictions. One is that there is less resource niche partitioning in this plant community than we assumed. When niche overlap, and therefore competition for resources, is high we might expect that the removal of a species can lead to competitive release of remaining species and a relatively large amount of compensation for the lost species (Hooper & Vitousek 1997, 1998; Wardle *et al.* 1999). The increased uptake of nitrogen by grasses and non-lupine tap-rooted forbs when shallow-rooted forbs were removed suggests that some amount of competitive release likely occurred in our experiment (Figure 3.2). However, those plants were not able to fully compensate for the loss under ambient nitrogen conditions – we only saw complete compensation for the loss of shallow-rooted forb nitrogen uptake when nitrogen was added and non-lupine tap-rooted forbs increased their uptake even more. A better understanding of exactly how each plant group's resource niches overlap or are differentiated in this ecosystem would allow us to better understand these patterns of compensation.

Another possible reason why we only saw increased nitrogen uptake in the SR+N treatment could be that the species removal treatment altered other abiotic conditions in a way that enabled the plant community to respond to the added nitrogen. As discussed in Chapter 2, the removal of shallow-rooted forbs significantly increased average soil moisture relative to CONT plots at 30 cm in 2002 and 2003, and at 10 cm in 2003 (Chapter 2, Figure 4b-d). If we assume that water, rather than nitrogen, was the primary limiting resource in our system during our experiment, then the observed increase in nitrogen uptake by non-lupine tap-rooted forbs in response to nitrogen addition in the SR+N plots could be explained at least in part by changes in soil moisture in response to the removal treatment. The observed soil moisture difference may have sufficiently alleviated water limitations in the SR+N treatment and allowed the non-lupine tap-rooted forbs in those plots to respond positively to nitrogen addition with increased uptake. Although complex interactions between water and nitrogen limitations are certainly important in this system, this explanation does not account for the lack of response of non-lupine tap-rooted forbs to N addition in the RR+N treatment (Figure 3.2), where soil moisture at all depths in 2002 and at 30 cm in 2003 was indistinguishable from that in the SR and SR+N treatment (Chapter 2, Figure 2.4).

A third possible explanation is that the loss of shallow-rooted forbs could have affected the ecosystem's stability in terms of its "resistance", or ability to maintain function in response to a perturbation – in this case nitrogen addition. As discussed in Chapter 2, some research suggests a "diversity-begets-stability" relationship whereby a more diverse community is more resistant, or exhibits less change, in response to a perturbation due to an increased likelihood of having positively-affected species which

can compensate for declines in the growth of negatively-affected species (e.g., Tilman 1996; Naeem & Li 1997; Yachi & Loreau 1999). Other research, however, presents theoretical and empirical evidence that less diverse plant systems can display greater resistance to a perturbation (Pfisterer & Schmid 2002). While most of this research has focused on perturbations that represent a negative stress such as drought and invasion by exotics, a few studies have looked at how manipulated plant diversity affects ecosystem responses to nitrogen addition – a generally growth-enhancing perturbation (Reich *et al.* 2001a; Bret-Harte *et al.* 2004). Results from those studies are mixed with Reich *et al.* (2004) reporting that decreasing species diversity dampened the community's biomass response to N addition, and Bret-Harte *et al.* (2004) discovering that response to N addition was dependent on the identity of the plant group being removed. Although it is unclear what the mechanism is behind our observation that the less diverse SR+N treatment was more responsive to nitrogen addition, our findings agree with the diversity-begets-stability theory. However, a more detailed understanding of how the loss of shallow-rooted forbs affects species-level responses to nitrogen addition over an even longer timeframe is needed before we can make a definitive link to ecosystem stability.

Importance of lupine

Many studies have demonstrated that plant species or functional group identity is more important to nitrogen cycling than is the number of species or functional groups (Hooper & Vitousek 1998; Symstad & Tilman 2001; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Spehn *et al.* 2005). In particular, several studies have shown the

presence and abundance of legumes to be an important factor in the relationship between plant diversity and nitrogen cycling (Hooper & Vitousek 1998; Reich *et al.* 2001b; Mulder *et al.* 2002; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Spehn *et al.* 2005). Research from plant diversity manipulation experiments has shown that the presence and abundance of legumes leads to increased NO_3^- leaching due to reduced uptake of soil NO_3^- (Scherer-Lorenzen *et al.* 2003) and increased nitrogen in aboveground plant biomass through fixation of N_2 (Mulder *et al.* 2002; Spehn *et al.* 2002). Mulder *et al.* 2002 and Spehn *et al.* 2002 also found evidence that legumes not only increased average plant community nitrogen uptake, but also the uptake of nitrogen by non-fixing plant species.

We expected to find similarly positive relationships between lupine biomass and NO_3^- at 30 cm depth and plant nitrogen content (H4). We did find a positive relationship between lupine AGB and NO_3^- at 30 cm depth (Figure 3.10); and since lupine plant tissue had the highest nitrogen concentration of all the plant groups (Figure 3.1), increasing lupine AGB also increased the average nitrogen concentration for the entire plant community (data not shown). We also found a positive correlation between lupine AGB and the %N in AGB of non-lupine tap-rooted forbs (Figure 3.11), suggesting that the presence of lupine may possibly increase nitrogen availability for other plants. However, a more thorough analysis of nitrogen sources for each plant group and/or species is needed before we can conclusively say whether the presence of lupine directly affects the nitrogen concentration of non-fixing plants.

These results suggest that legumes play an important role in nitrogen cycling in this subalpine meadow, similar to findings from other temperate grassland communities

(Mulder *et al.* 2002; Spehn *et al.* 2002; Scherer-Lorenzen *et al.* 2003; Spehn *et al.* 2005). However, caution should be used in interpreting these results since we only examined the effect of one particular nitrogen-fixing species that represented a significant proportion of the tap-rooted forb biomass. There were several other potentially nitrogen-fixing legumes in our plant community, including *Lathyrus laetivirens* and *Vicia americana* (both shallow-rooted forbs) that we did not track at the species-level. We also did not verify that lupines (or any of the other legumes) were actively fixing nitrogen in our experimental system. Despite these limitations, our results suggest that future nitrogen cycling research in this plant community should explicitly take into account the role of legumes.

Consequences of warming-induced plant species loss for nitrogen cycling

Our results suggest that the loss of shallow-rooted forbs due to future warming may not have a large effect on nitrogen cycling in this subalpine meadow ecosystem, over the relatively short duration of our experiment. Non-lupine tap-rooted forbs, and to some extent grasses, seem capable of compensating for the loss of shallow-rooted forbs. Non-lupine tap-rooted forbs not only compensated by taking up additional nitrogen into aboveground plant tissue, but since they have the same %N tissue concentration as shallow-rooted forbs they most likely had a similar effect on soil nitrogen transformation rates and the subsequent supply of NO_3^- and NH_4^+ for plant growth. The loss of shallow-rooted forbs did alter the ecosystem's response to nitrogen addition by increasing nitrogen uptake in plots where shallow-rooted forbs had been removed compared to more diverse treatments. Although we do not fully understand the

mechanisms behind this result, our findings suggest that complex interactions between water and nitrogen availability, the extent to which resource niches overlap, and the effect that losing an entire group of species has on ecosystem stability could all play a role.

Our findings show that realistic non-random scenarios of species loss do not necessarily follow the pattern of decreased nitrogen uptake, increased nitrogen losses, and dampened response to nitrogen addition with species loss that is predicted by theory and experimental results that examine the role of diversity *per se*. Most likely, the effect of species loss on nitrogen cycling will depend on the identity of the species being removed and the extent to which there is resource niche partitioning between the species being removed and those that remain. We suggest that an increased understanding how and when the resource niches of plant species and/or groups overlap or are partitioned is necessary to better understand the impacts of species loss on nitrogen cycling. Since anthropogenic plant species loss is not likely to be random, additional experiments such as this one that combine plausible species loss and perturbation scenarios are needed to improve our ability to predict the consequences of human-driven declines in diversity on nitrogen cycling.

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FIGURES

Figure 3.1. Plant tissue %N by treatment for each plant type and fine roots. Treatment abbreviations are: CONT = no removal, ambient N [black bars]; CONT+N = no removal, added N [hatched black bars]; RR = random forb removal, ambient N [gray bars]; RR+N = random forb removal [hatched gray bars], added N; SR = shallow-rooted forb removal, ambient N [white bars]; SR+N = shallow-rooted forb removal, added N [hatched white bars]. Error bars are ± 1 SE. Shallow-rooted forb AGB in SR and SR+N treatments was removed from the %N analysis since the only shallow-rooted forb AGB in the SR plots were persistently re-sprouting young leaves that were especially high in nitrogen.

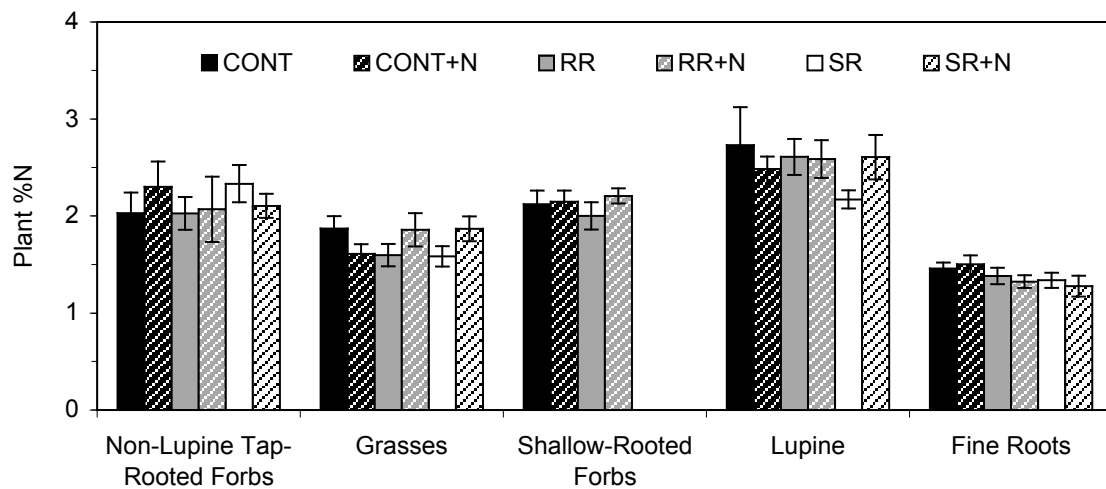


Figure 3.2. Total amount of N in plant biomass (aboveground tissue and fine roots) by treatment. Treatment abbreviations are same as in Figure 3.1. Hatched = fine roots (0-10 cm); dark gray = non-lupine tap-rooted forbs; light gray = lupine; black = shallow-rooted forbs; white = grasses. Full height of bar is total nitrogen in plant biomass. Error bars are ± 1 SE of total nitrogen for each treatment. Different letters at the top of each bar denote significant differences in total plant nitrogen among treatments at $p \leq 0.05$ using an ANOVA model with a block effect and the Tukey-Kramer HSD test to compare all pairs.

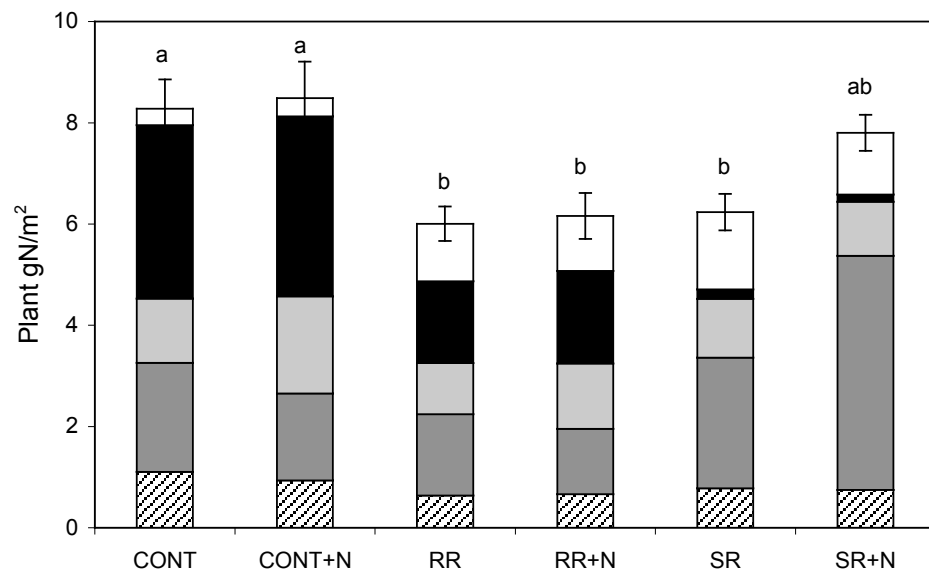


Figure 3.3. Above and belowground plant tissue C/N ratio by treatment. Treatment abbreviations are same as in Figure 3.1. Shallow-rooted forb AGB in SR and SR+N treatments was removed from the %N analysis since the only shallow-rooted forb AGB in the SR plots were persistently re-sprouting young leaves that were especially high in nitrogen. Error bars are ± 1 SE.

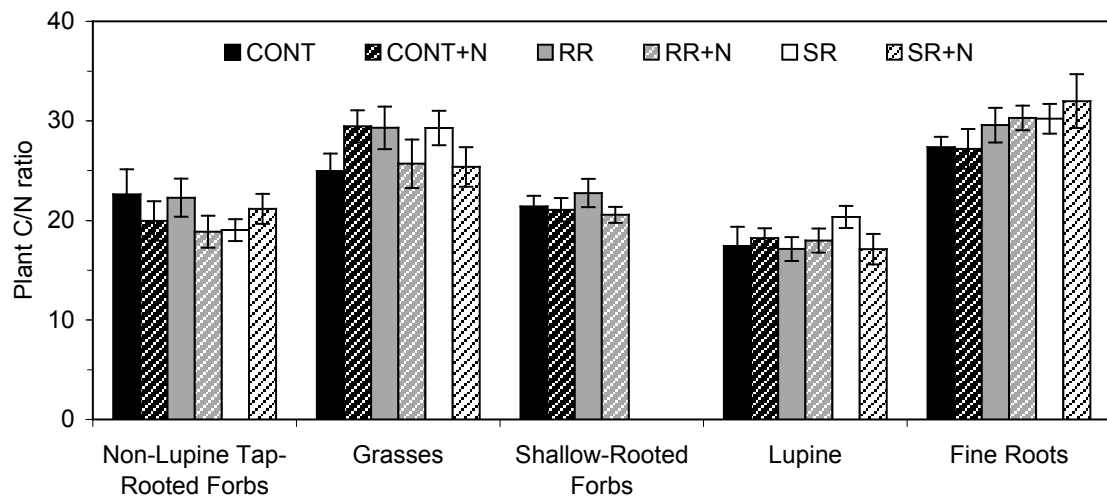


Figure 3.4. Concentration of ammonium (NH_4^+) and nitrate (NO_3^-) extracted from soil samples in late June of 2002 (A) and 2003 (B) by treatment. Treatment abbreviations are same as in Figure 3.1. Error bars are ± 1 SE.

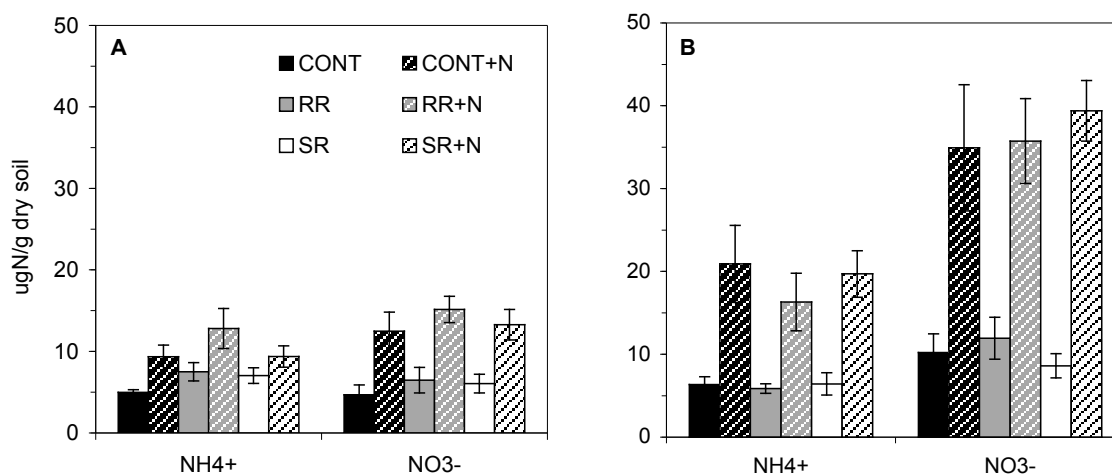


Figure 3.5. Soil nitrogen concentration (%N) by treatment at 0-10 cm depth. Treatment abbreviations are same as in Figure 3.1. Error bars are ± 1 SE. Different letters at the top of each bar denote significant differences among treatments at $p \leq 0.05$ using an ANOVA model with a block effect and the Tukey-Kramer HSD test to compare all pairs.

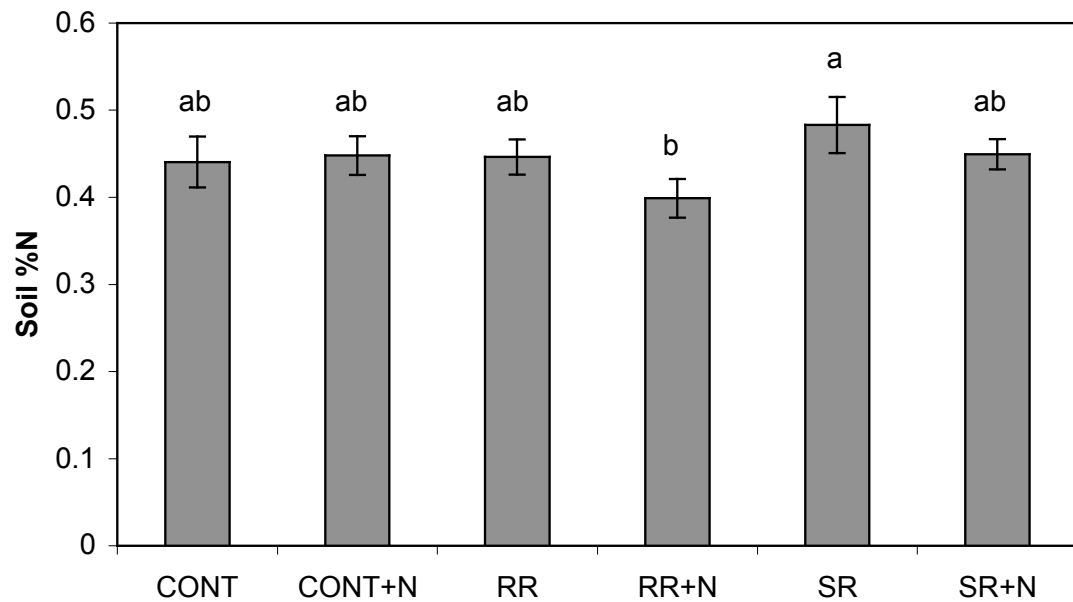


Figure 3.6. Amount of nitrate (NO_3^-) extracted from ion exchange resin bags buried at 30 cm depth in 2003 and 2004 by treatment. Treatment abbreviations are same as in Figure 3.1. In 2003, bags were buried for 12 months (Oct. 2002 until Oct. 2003). In 2004, bags were buried for 8 months (Oct. 2003 until June 2004). Error bars are ± 1 SE.

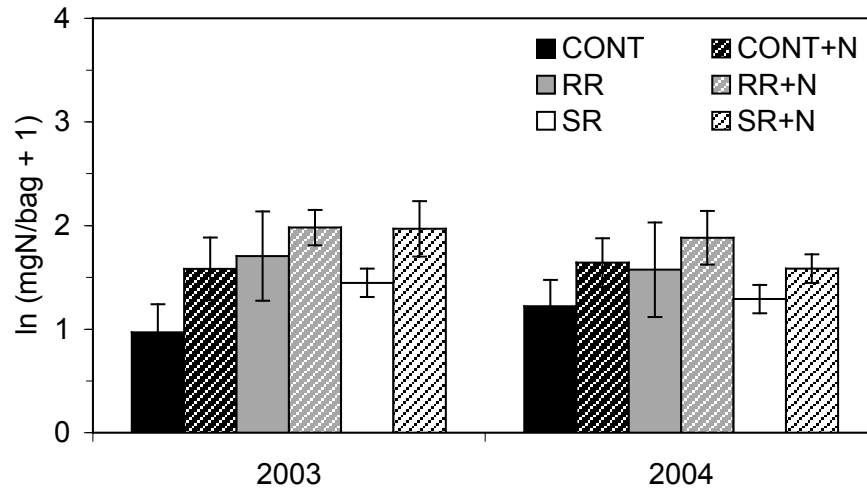


Figure 3.7. Relationship between the amount of nitrate (NO_3^-) on deep (30 cm) ion exchange resin (IER) bags and A) total fine root biomass (live plus dead) or B) deep (10-30 cm) soil moisture for ambient nitrogen (open squares, dashed line) and nitrogen addition (closed squares, solid line) treatments in 2003. The slopes of the lines were not significantly different between ambient and added nitrogen treatments, but the elevations were significantly different between the nitrogen treatments for soil moisture ($p = 0.05$) and marginally different for fine root biomass ($p = 0.06$).

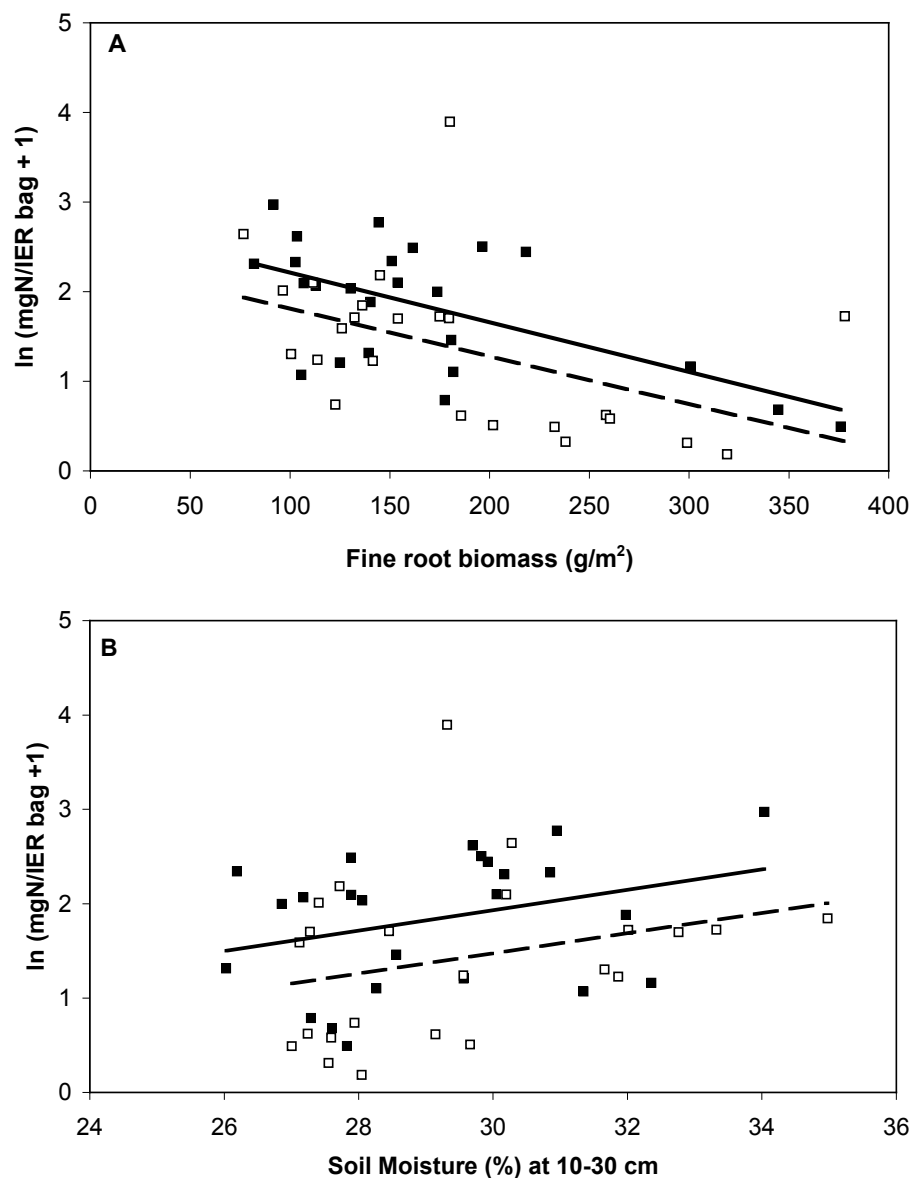


Figure 3.8. Net nitrogen mineralization (A), net nitrification (B) and net ammonification (C) during the peak 6 weeks of the growing season by treatment in 2002 and 2003.

Treatment abbreviations are same as in Figure 3.1. Error bars are ± 1 SE.

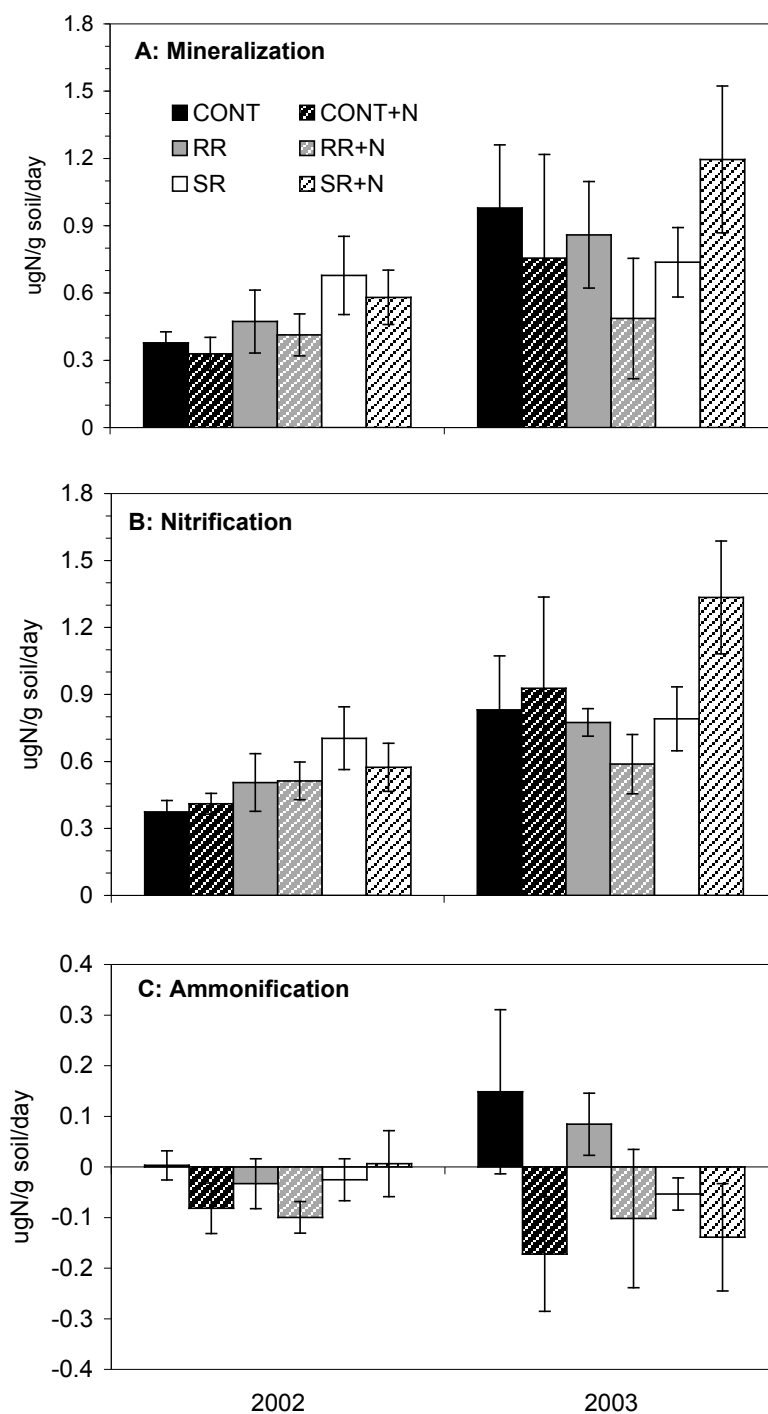


Figure 3.9. Relationship between total fine root biomass (live plus dead) and soil nitrate (NO_3^-) in ambient nitrogen (open squares, dashed line) and nitrogen addition (closed squares, solid line) treatments in 2002 (A) and 2003 (B). In 2002, the slopes were not significantly different among treatments, but the elevation of the lines did differ between ambient and added nitrogen ($p < 0.0001$). In 2003, both slopes and elevations of the lines were significantly different between nitrogen treatments (both $p < 0.01$).

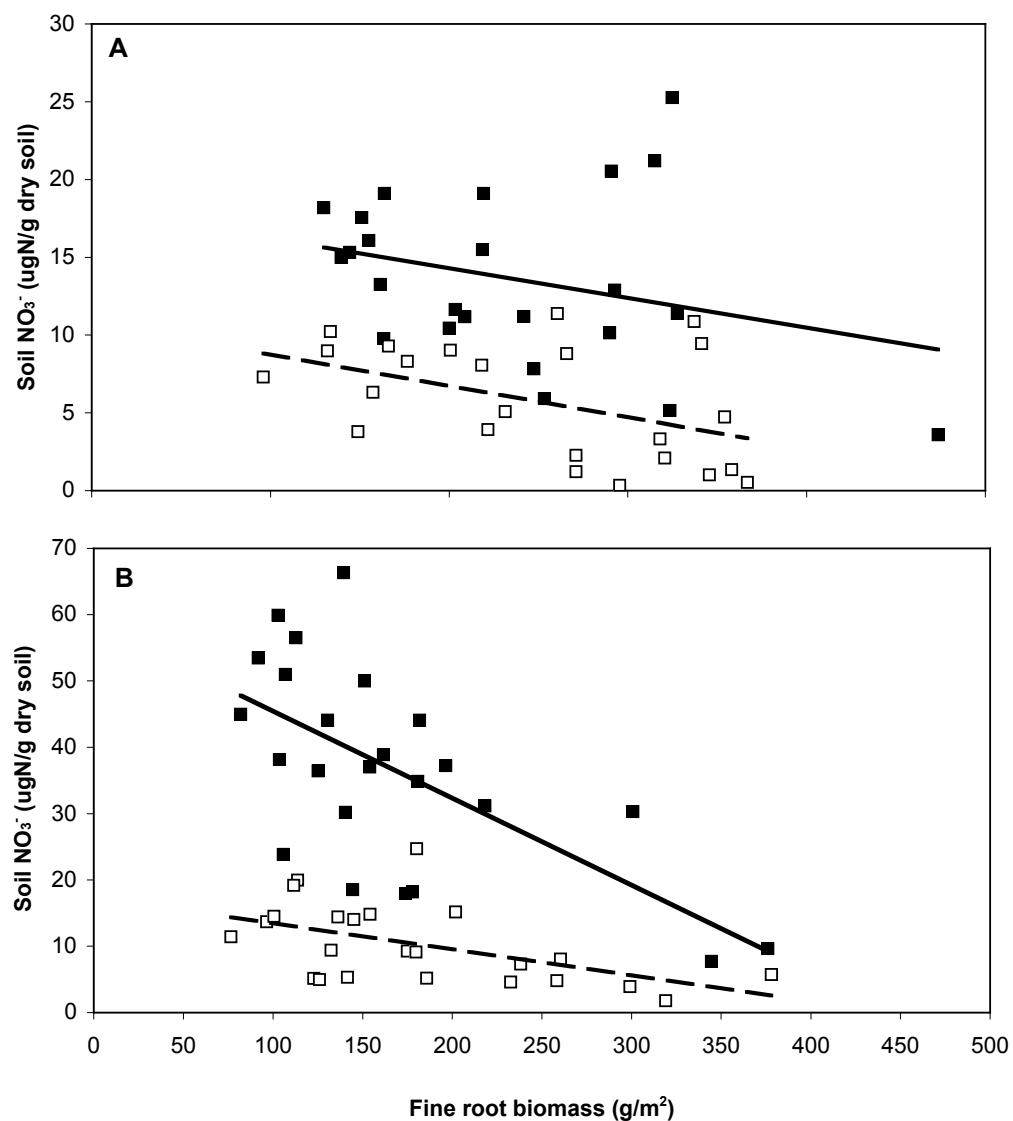


Figure 3.10. Relationship between lupine aboveground biomass (AGB) and the amount of nitrate (NO_3^-) on ion exchange resin (IER) bags at 30 cm.

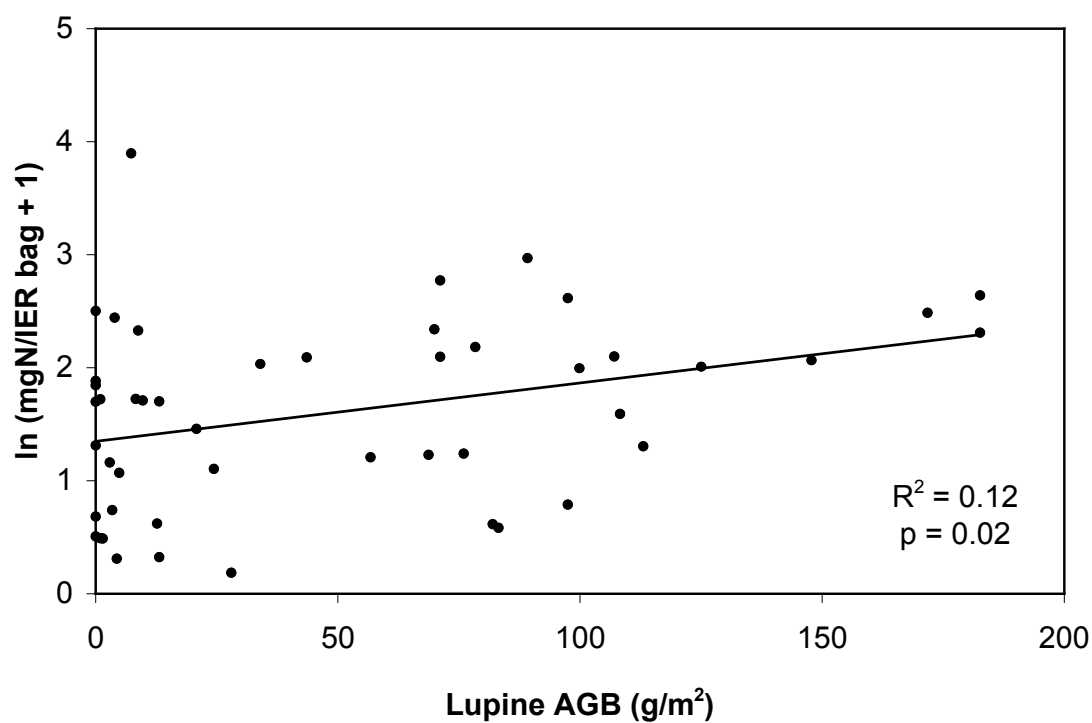
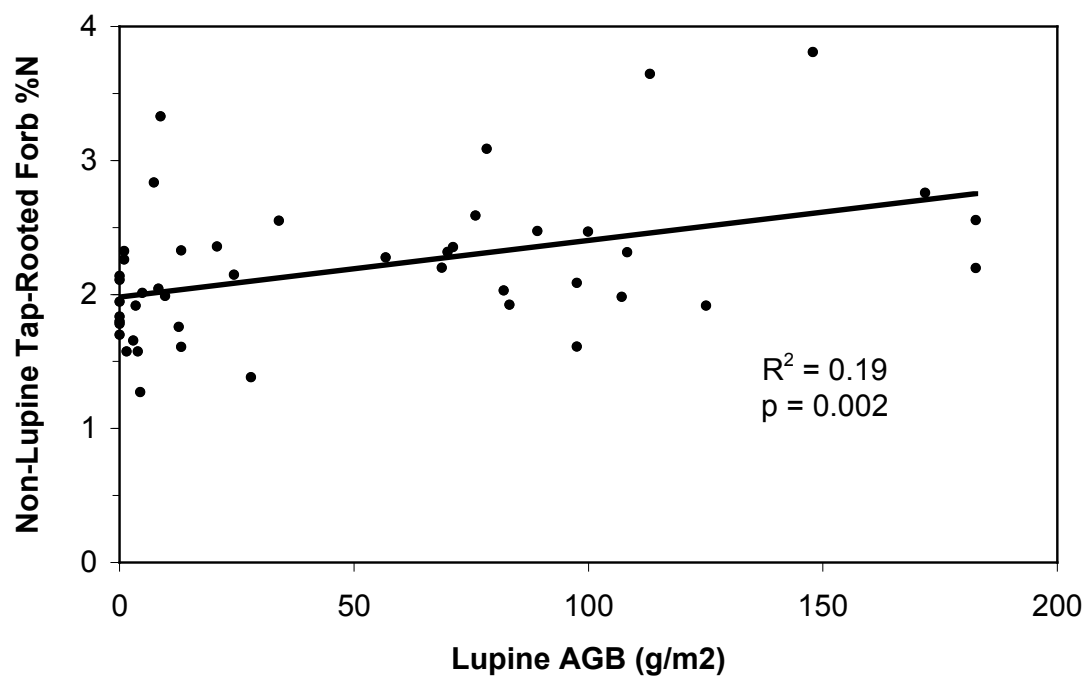


Figure 3.11. Relationship between lupine aboveground biomass (AGB) and the concentration of nitrogen (%N) in non-lupine tap-rooted forb AGB.



CHAPTER 4: EFFECTS OF WARMING-INDUCED PLANT SPECIES LOSS AND ENHANCED NITROGEN AVAILABILITY ON SOIL MICROBIAL COMMUNITY COMPOSITION

INTRODUCTION

Human-induced environmental changes such as land-use, climate change, nitrogen (N) deposition, atmospheric carbon dioxide (CO₂) enrichment and the introduction of exotic invasive species are predicted to have significant negative impacts on global biodiversity by the end of the next century (Sala *et al.* 2000). In light of these impending changes, understanding the ecosystem consequences of losing biodiversity has become a focus of ecological research (Chapin *et al.* 2000; Loreau *et al.* 2002b; Hooper *et al.* 2005). Most experimental studies of the ecosystem consequences of plant species loss have focused on randomly-assembled plant communities (e.g., Naeem *et al.* 1994; Tilman *et al.* 1997a; Hector *et al.* 1999; Reich *et al.* 2001a; Tilman *et al.* 2001). However, changes in diversity as a result of human activities are not likely to be random – they are more likely to be targeted at species with traits that make them particularly vulnerable to change (Loreau *et al.* 2001; Grime 2002; Smith & Knapp 2003; Raffaelli 2004; Solan *et al.* 2004; Zavaleta & Hulvey 2004; Hooper *et al.* 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005). Several theoretical and empirical studies have shown that ecosystem functioning responds differently to random vs. non-random species loss (e.g., Ives & Cardinale 2004; Solan *et al.* 2004; Zavaleta & Hulvey 2004; Gross & Cardinale 2005; Larsen *et al.* 2005; Schlapfer *et al.* 2005). Therefore, investigating how ecosystems may respond to non-random changes in

biodiversity as a result of specific human activities is important to predicting ecosystem consequences of future species loss caused by humans (Loreau *et al.* 2001; Grime 2002; Naeem & Wright 2003; Hooper *et al.* 2005).

Anthropogenic climate change is predicted to be an important driver of biodiversity change over the next 100 years, especially in mountainous alpine environments (Sala *et al.* 2000). A meta-analysis of 11 arctic and alpine warming experiments found that plant species diversity significantly decreased with warming after only 2 growing seasons (Walker *et al.* 2006). One of those studies, in an alpine rangeland on the Tibetan Plateau, found that experimental warming resulted in a 26-36% decrease in plant species richness, and that the losses were non-random with respect to plant functional groups (Klein *et al.* 2004). Long-term results from an ongoing 15-year warming experiment in a subalpine meadow ecosystem in the Colorado Rocky Mountains also suggests that some plant species in that system may be more adversely affected by increasing temperatures than other species (Harte *et al.* 1995). We found that warming decreased the growth of forbs (Harte & Shaw 1995), but not all forb species responded in the same way to warming (de Valpine & Harte 2001). Warming decreased the aboveground biomass and flowering success of shallow-rooted forbs, and decreased or had no effect on the growth of tap-rooted forb species (de Valpine & Harte 2001; Saavedra *et al.* 2003). Although warming has not led to the complete disappearance of any plant species from this experimental system, spatial patterns across the experimental plots suggest that plant species richness is reduced under conditions of early snowmelt and enhanced nitrogen availability (J. Harte, unpublished data). The observation that manipulated climate warming reduces soil moisture in

shallow soil layers, advances the timing of snowmelt, and increases nitrogen availability (Harte *et al.* 1995; Shaw & Harte 2001) provides further evidence that shallow-rooted forb species may eventually be lost from this meadow ecosystem under future warming.

To examine the impact that the non-random, warming-induced loss of shallow-rooted forb species may have on ecosystem functioning in this subalpine meadow ecosystem, we conducted an *in-situ* species removal experiment. Species removal experiments have recently been proposed as an appropriate manipulation approach for examining the effects of non-random species loss in naturally-assembled communities (Diaz *et al.* 2003; Hooper *et al.* 2005). By working in otherwise unmanipulated ecosystems, removal experiments explore the consequences of non-random species loss in complex natural systems, while maintaining an ability to look at cause-and-effect relationships by directly altering community composition (Diaz *et al.* 2003). Previous removal studies of non-random plant species loss have focused on the loss of rare species (e.g., Lyons & Schwartz 2001; Smith & Knapp 2003), or on the loss of various combinations of functional groups and/or dominant species (e.g., Sala *et al.* 1989; Hobbie *et al.* 1999; Wardle *et al.* 1999; Symstad & Tilman 2001; Bret-Harte *et al.* 2004; Buonopane *et al.* 2005; Wardle & Zackrisson 2005). To our knowledge, ours is the first removal study that examines non-random plant species loss resulting from a specific anthropogenic activity.

To quantify how our subalpine meadow ecosystem responded to the loss of shallow-rooted forbs, we measured plant biomass production (above- and below-ground), ecosystem nitrogen cycling, and soil microbial community composition. We also investigated whether the loss of shallow-rooted forb species altered the system's

response to a perturbation. Since experimental warming significantly increased rates of net nitrogen mineralization (Shaw & Harte 2001), and because atmospheric deposition of nitrogen from anthropogenic activities is expected to increase over the next several decades (Vitousek *et al.* 1997), we chose to perturb the system by altering soil nitrogen availability. While Chapters 2 and 3 of this dissertation discuss the vegetation, nitrogen cycling and carbon storage responses to our experimental species loss and nitrogen addition treatments, this Chapter examines the response of the soil microbial community.

The size and structure of the soil microbial community is important to many ecosystem processes, including soil organic matter dynamics and nutrient mineralization (Six *et al.* 2006). Understanding how the microbial community responds to species loss and increased nitrogen availability are crucial to understanding the overall impact that those environmental changes may have on ecosystem functioning in general, and soil carbon sequestration and nutrient availability in particular. The ratio of decomposer fungal to bacterial biomass can be used to indicate the likelihood of soil carbon accrual since fungi are assumed to: 1) metabolize substrates more efficiently (thereby maintaining more of the consumed carbon as biomass rather than respired as CO₂) (Holland & Coleman 1987, although see Six *et al.* 2006 for discussion of how evidence is limited); 2) produce detritus that is more recalcitrant due to the presence of more resistant chitin and melanin compounds in their cell walls (Holland & Coleman 1987; Guggenberger *et al.* 1999); and 3) produce extracellular polysaccharides and hyphae that form soil macroaggregates that physically protect soil organic matter (Jastrow & Miller 1998; Zhu & Miller 2003; Simpson *et al.* 2004). Therefore, soils with

higher fungi to bacteria ratios are expected to sequester more carbon than would a soil that is more dominated by bacteria (Bailey *et al.* 2002).

Climate warming, changing plant species diversity, and nitrogen addition have all been shown to have the ability to alter microbial community size and structure (e.g., Bardgett *et al.* 1999; Clegg *et al.* 2003; Staddon *et al.* 2003; Zak *et al.* 2003). However, many of the observed changes in the microbial community have been attributed to the effect that warming, plant diversity or nitrogen addition has had on the quantity and quality of plant inputs to the soil. Since the tissue of different plant species exhibit varying biochemical properties, and the quantity and type of biochemical compounds in plant-derived detritus can control the size and function of the microbial community, it is expected that changes in plant productivity and species composition can have significant effects on microbial community dynamics (Zak *et al.* 2003; Leckie 2005). Plant species also vary in their association with fungal symbionts and changes in plant species composition towards plants that have strong AMF associations, for example, can significantly affect the overall presence of fungal biomass in the microbial community (Staddon *et al.* 2003). Distinguishing between the relative contributions of the direct effects of warming/plant diversity/nitrogen from the indirect effects via changing plant species composition and productivity is therefore important to understanding the role that future environmental change may have on ecosystem functioning.

We examined how microbial community composition responded to shallow-rooted forb removal and nitrogen addition treatments, both separately and in combination. Since the loss of species in a removal experiment is necessarily

confounded with the loss of biomass, and because the microbial community is likely to be sensitive to a decrease in plant biomass, we included a random biomass removal treatment that reduced biomass but not species diversity. We expected to see a significant effect of both shallow-rooted forb removal and nitrogen addition on microbial community composition and the ratio of fungi to bacteria. We expected that the loss of shallow-rooted forbs would have an additional effect on the microbial community beyond just the effect of a reduction in plant biomass since the shift in plant species composition could alter the quality of litter inputs to the microbes and change the strength of AMF associations. We measured changes in microbial community composition using the phospholipid fatty acid (PLFA) technique. PLFAs are an important component of cellular membranes that decompose rapidly on cell death. Therefore, PLFAs extracted from soil samples represent living microbial biomass (Frostegard & Baath 1996; Leckie *et al.* 2004). Since certain related groups of soil microorganisms exhibit signature PLFA structures, it is possible to use marker PLFA signatures to differentiate between types of soil microbes (Frostegard *et al.* 1993; Zak *et al.* 1996; Olsson 1999; Zelles 1999). Changes in the relative proportions of each PLFA signature can then be used to represent changes in soil microbial community structure.

MATERIALS AND METHODS

Study Site

Our experiment was conducted in an ungrazed subalpine meadow at the Rocky Mountain Biological Laboratory (RMBL), Gunnison County, Colorado (38° 53' N, 107° 02' W; elevation 2920 m), approximately 150 m away from an ongoing warming

experiment (Harte & Shaw 1995; Harte *et al.* 1995). The meadow plant community at this site is characterized by long-lived and largely perennial herbaceous forbs and grasses. Additional site details can be found in Chapter 2 of this dissertation, Harte and Shaw (1995) and Harte *et al.* (1995).

Experimental Design

In summer 2001, plant species removal and nitrogen addition treatments were established in 48 2 x 2 m plots, arranged in 8 blocks of 6 contiguous plots. Blocks were spaced approximately 3 m apart within a section of hillside where slope, aspect, hydrologic regime and plant community type are relatively uniform. Before assigning treatments to each plot, we collected pre-manipulation data on peak aboveground biomass (AGB) using the following growth form categories: grasses, deep tap-rooted forbs and shallow-rooted forbs.

Plots within each block were randomly assigned to one of three plant species removal treatments – removal of all aboveground biomass of shallow-rooted forb species (Shallow Removal, SR treatment), removal of random forb aboveground biomass (Random Removal, RR treatment), or control plots with no removal of biomass (Control, CONT treatment) – for a total of two replicates of each species removal treatment in each 6-plot block. Forb species were classified into shallow- and tap-rooted categories based on field excavations of root morphology (Appendix A). Shallow-rooted forbs were designated as those with fibrous rooting structures that were concentrated in the top 10cm of the soil profile, whereas tap-rooted forbs were characterized by a coarse taproot structure that can access deeper soil resources. The RR

treatment was included to distinguish between the effect of the loss of species, and the effect of the loss of biomass. We randomly selected one replicate of each species removal treatment within each block to be fertilized by inorganic nitrogen (+N treatment) while the other replicate remained at ambient nitrogen levels. Therefore, each block contained one replicate of each of 6 total treatment combinations: CONT, CONT+N, SR, SR+N, RR, RR+N.

At the time of peak standing AGB in late July 2001, we removed aboveground vegetation from the SR and RR plots by clipping plants at their base. Biomass in the RR plots was removed by clipping all of the forb AGB (regardless of rooting structure) within a set of randomly chosen 25 by 25 cm quadrats, until we had removed an equivalent amount of biomass to the amount removed from the other SR plots within that block. Removal treatments were applied to the entire 4m² area of each plot. Before applying the removal treatments there were on average 18 forb species (minimum = 13 and maximum = 24) in the central 1m² of the plots, 13 of which were shallow-rooted species (minimum = 8 and maximum = 17). Depending on pre-manipulation species composition within each plot, the SR treatments removed from 61.9% to 87.5% of all forb species. Therefore, the SR treatment manipulated both species richness and functional response group richness. Although grasses were present in the experimental plots, we chose not to target grasses for removal in any of the treatments since warming has had no effect on grass AGB in the warming meadow experiment (Harte & Shaw 1995). In 2002 and 2003, we removed aboveground plant parts of all shallow-rooted forb species from the SR plots as soon as they emerged aboveground after snowmelt and could be identified to species. Plots were then continually weeded throughout the

growing season to prevent aboveground growth of the target species. In the RR plots, the same 25 by 25 cm quadrats used to conduct the random removals in 2001 were kept free of forb biomass by weeding all forbs from those quadrats throughout the 2002 and 2003 growing seasons. In 2004, we discontinued weeding in both the SR and RR plots.

Inorganic nitrogen was applied to the soil surface of fertilized (+N) plots as ammonium-nitrate (NH_4NO_3) in solution. A total of 6 g N/m^2 was applied to each +N plot each year, divided into four equal doses of 1.5 L of NH_4NO_3 solution. This level of N addition is comparable to the approximate doubling of net N mineralization rates that has been observed in the heated plots relative to the controls in the warming meadow experiment (Shaw & Harte 2001). To account for the small amount of water addition that inevitably accompanies the application of fertilizer in solution, we added an equivalent amount of de-ionized water to non-fertilized plots, an amount that is negligible relative to incoming precipitation levels. The first nitrogen and water applications were applied just after snowmelt in spring 2002, with the remaining three doses applied roughly every 2-3 weeks thereafter. Nitrogen and water additions were continued in the same manner in 2003 and 2004.

Microbial Community Sampling

Changes in soil microbial community composition were analyzed using the abundances of specific phospholipid fatty acid (PLFA) signatures extracted from the soil. In August 2004, we aggregated three soil samples (2.5 cm diameter and 10 cm depth) from each experimental plot for PLFA analysis. Samples were frozen until they could be processed in the laboratory. Thawed samples were passed through a 2mm

sieve, freeze-dried, and all visible root particles were hand-picked from the remaining soil. Lipids were extracted using an adaptation of the technique described by (Bligh & Dyer 1959). Following extraction, the PLFAs were separated from other lipids using silicic acid column chromatography (Vestal & White 1989; Zak *et al.* 1996). PLFAs were then methylated and the resulting fatty acid methyl esters (FAMES) were identified using a gas chromatograph. See Allison *et al.* (2005) for additional details on the PLFA extraction process. Fatty acids were described using the A:BωC nomenclature, where “A” is the total number of carbon atoms in the chain; “B” indicates the number of double bonds; and “C” represents the position of the double bond from the methyl end of the molecule. The suffixes “c” and “t” indicate *cis* and *trans* geometries; and prefixes “i”, “a”, and “Me” refer to iso, anteiso, and midchain methyl branching, respectively. The “cy” prefix denotes a cyclopropyl ring structure.

In addition to considering each PLFA signature separately, we also classified the specific marker signatures into soil microbial functional groups (Table 4.1); and calculated the ratios of all fungi to all bacteria (F/B), and saprophytic fungi to all bacteria (SAP/B). The total amount of PLFA extracted is considered a measure of active microbial biomass (Petersen *et al.* 1991; Zelles *et al.* 1997), while the F/B ratio is an additional way to measure a community composition shift in terms of the relative proportion of fungi vs. bacteria. The SAP/B ratio is potentially relevant to soil carbon sequestration since fungi are assumed to be more efficient decomposers and to contribute more recalcitrant detritus to the soil organic pool. We also calculated the ratio of cyclopropyl PLFAs (cy17:0 and cy19:0) to their precursors (16:1ω7c and 18:1ω7c) as an index of the physiological status of the gram-negative bacterial

community (Bacterial Stress Index; BSI). When gram-negative bacteria experience stress, such as nutrient (i.e., carbon, oxygen) limitations, they shift from logarithmic to stationary-phase growth and convert precursors into cyclopropyl fatty acids (Guckert *et al.* 1986; Bossio & Scow 1998; Jackson *et al.* 2003). Therefore, a higher BSI indicates a higher level of physiological stress within the bacterial community.

Environmental Variables

We measured several environmental variables that we expected could influence soil microbes: total soil organic carbon (SOC, %C) and nitrogen (TN, %N), the ratio of soil C/N, aboveground plant biomass (AGB), and soil moisture. We measured all environmental variables in the central 1m² area of each plot in order to reduce potential edge effects. Soil moisture and AGB were measured in 2004, the same year that the microbial community was sampled. Since all AGB senesces at the end of each growing season, we also included measurements of plant AGB in 2003 as an indication of the relative quantity of plant tissue that entered the soil pool in each plot at the end of the 2003 growing season. SOC and TN were measured in 2003. Since SOC and TN do not rapidly change between years, 2003 SOC and TN are relevant to determining relationships between soil organic C and N on microbial community measures in 2004.

We measured SOC and TN at 0-10 cm and 10-30 cm depths by aggregating three soil cores (2.5 cm diameter) from each plot, sieving out particles > 2mm in diameter, removing the majority of the visible root and litter material, grinding the sample to a homogeneous powder, and analyzing a subsample on a C:N autoanalyzer (Carlo-Erba, Italy). We estimated aboveground plant productivity using peak standing

AGB. Since all aboveground vegetation senesces at the end of each growing season in this system, peak-standing AGB is a conservative estimate of annual aboveground production. We measured AGB non-destructively by calibrating estimates of aerial coverage (AC) to biomass via regressions for each plant group (tap- and shallow-rooted forbs, and grasses) with off-plot harvests. We used maximum AC over the course of the growing season to calculate peak standing AGB for each plant group (grass, tap- and shallow-rooted forbs). We summed peak AGB for each group to get peak total AGB for each plot. We measured soil moisture at depths of 10 and 30 cm on June 14, July 6, July 14 and July 19, 2004 using permanently installed gypsum blocks that were calibrated to % gravimetric soil moisture using soil samples taken directly from the plots. We then averaged the soil moisture values for those four dates to get mean soil moisture for the peak of the growing season in 2004.

Statistical Analyses

The composition of the soil microbial community was represented visually using a multivariate reciprocal averaging (RA) ordination technique using PLFA proportional abundances. RA reduces the dimensionality of multivariate data to allow for easier interpretation of differences between samples (i.e., plots). The ordination graphs that RA produces show the relative positions of samples to one another, and the average position of each PLFA signature along each axis. Samples that are closer together on a RA ordination graph have more similar PLFA compositions than do samples that are further apart. Comparison of the location of samples and PLFA signatures along the RA ordination axes allow for interpretation of which PLFA signatures are responsible

for differences in community composition among plots. The effects of removal and N addition treatments on microbial community composition were statistically analyzed using a distance-based multivariate analysis (DISTLM v.5) (Anderson 2001; McArdle & Anderson 2001). We used the Bray-Curtis distance measure and 9999 permutations to obtain pseudo F-values for each of the main effects (block, removal, N addition), and the removal by N addition interaction.

We used Pearson correlation coefficients to determine relationships between RA axis values, environmental variables, and other microbial community measures. Analysis of variance (ANOVA) and covariance (ANCOVA) were used to determine treatment effects on total PLFA, SAP/B and F/B ratios, and bacterial stress index (BSI) using block, removal and N addition as main effects, and a removal by N addition interaction term. Environmental variables that we found to be significantly correlated with RA axis locations in the Pearson correlation analysis were included in the ANCOVA model as covariates to see if they altered the significance of treatment effects on the individual microbial community measures. ANOVA and ANCOVA analyses were performed using JMP statistical software (SAS Institute Inc., version 5.1); RA was performed using PC-ORD (MJM Software Design, version 4.0).

RESULTS

We extracted a total of 22 PLFA signatures from our experimental soils, all of which were used in the reciprocal averaging (RA) ordination analysis. Axis 1 from the RA explained 55.1% of the variance in microbial community composition, while Axis 2

explained 18.3% of the variance. Axis 3 explained 15.9% of the variance, but since RA axes become less reliable after the first few axes we do not discuss Axis 3 any further.

Neither removal nor nitrogen addition treatments resulted in a visual shift in microbial community composition along either RA Axis 1 or 2 (Figure 4.1a-b). However, there was a visual shift in microbial community composition between experimental blocks with Blocks 1, 2 and 3 having more negative values along Axis 1 relative to Blocks 6 and 8 (Figure 4.1c). Along Axis 2, Blocks 3 and 7 were more positive than Block 10, with the other blocks indistinguishable and intermediate between those two groups (Figure 4.1c). The distance based multivariate analysis also revealed that there were no main effects of removal and N addition on microbial community composition (removal: pseudo $F_{2,35} = 0.14$, $p = 0.92$; N addition: pseudo $F_{1,35} = 0.09$, $p = 0.88$), but that there was a significant effect of block (pseudo $F_{7,35} = 3.08$, $p = 0.009$). There was also a significant removal by nitrogen interaction (pseudo $F_{2,35} = 3.31$, $p = 0.04$).

More negative Axis 1 values were most strongly correlated with high amounts of bacterial PLFA, (especially Gram (-) bacteria), low fungal to bacterial PLFA ratios (F/B), high bacterial stress indices (BSI), and high total PLFA (a measure of active microbial biomass) (Table 4.2; Figure 4.2). There were also significant changes in the relative abundance of fungi and bacteria along Axis 1, with a decline in bacteria relative abundance and an increase in fungi relative abundance with increasing values on Axis 1 (Figure 4.3). Most of the increase in fungal abundance with increasing Axis 1 values was due to an increase in the abundance of saprophytic fungi, with AMF showing a significant but weaker relationship with Axis 1 values (Figure 4.4). More positive Axis

2 values were significantly but not very strongly correlated with a high F/B ratios and lower amounts of desulfobacter PLFA (Table 4.2; Figure 4.2).

To identify the environmental characteristics that are most likely to be driving changes in microbial community composition along RA Axes 1 and 2, we calculated Pearson correlation coefficients between the ordination values and several environmental variables for each sample plot (Table 4.2). Position along RA Axis 1 was most strongly correlated (negatively) with soil organic carbon (SOC, %C) and nitrogen (TN, %N) at 10-30 cm. Axis 1 was also negatively, although less strongly, correlated with 2004 aboveground biomass (AGB), TN at 0-10 cm, SOC at 0-10 cm, and 2003 AGB (Table 4.3). Axis 2 was weakly although significantly positively correlated with soil C/N ratios at 0-10 cm and 10-30 cm, and was only marginally correlated with 2004 AGB and soil M at 0-10 cm (Table 4.3). The SAP/B and F/B ratios were also correlated with environmental conditions. The SAP/B and F/B ratios were most strongly (negatively) correlated with SOC and TN at 10-30 cm, but also showed weaker correlations with soil TN and C/N ratio at 0-10 cm, and 2004 AGB (Table 4.3). Bacterial stress index (BSI) was not correlated with any of the environmental variables measured for this study; and 2004 shallow (0-10 cm) and deep (10-30 cm) gravimetric soil moisture were not significantly correlated with any of the microbial community measures (Table 4.3).

Analysis of variance revealed that there were no significant main effects of removal or nitrogen addition treatments on total PLFA, SAP/B and F/B ratios (Table 4.4). However, there was a significant removal by nitrogen addition interaction on total PLFA ($p = 0.02$) where the addition of nitrogen lead to a significant decrease in total

PLFA in the random removal (RR) plots, and no change in total PLFA in the shallow-forb removal (SR) and control (CONT) plots (Table 4.4; Figure 4.5). The bacterial stress index (BSI) was marginally affected by the removal of both random forb biomass and shallow-rooted forbs, with the bacterial community showing signs of slightly higher stress RR and SR relative to CONT plots (Table 4.4).

Experimental removal and nitrogen addition treatments had significant effects on several environmental variables (Table 4.5). There was a significant effect of removal treatment on 2003 and 2004 total AGB with SR and CONT plots producing significantly greater aboveground biomass than RR plots, mostly due to a marginal removal by nitrogen interaction where only the SR plots responded to added nitrogen with increased growth (Table 4.5) (see Chapter 2 for a more detailed discussion of AGB responses to treatments). Shallow (0-10 cm) SOC and TN were significantly greater in SR plots relative to RR plots with CONT plots intermediate between the two, and shallow TN marginally decreased in response to nitrogen addition (Table 4.5). There was a marginally significant interaction between removal and nitrogen addition on deep (10-30 cm) SOC and TN, with RR plots showing a slight decrease in deep SOC and TN with nitrogen addition without any change in either SR or CONT plots (Table 4.5). There were no significant treatment effects on deep or shallow soil C/N or soil moisture (Table 4.5).

Overall, the block effect seems to be the most important driver of changes in both microbial community measures and environmental variables. Block explained the greatest variation in total PLFA, SAP/B, F/B, BSI, and microbial community composition; in addition to accounting for the greatest variation in deep and shallow

SOC and TN, 2004 AGB and soil C/N at 10-30 cm (Table 4.5). This finding suggests that there is substantial spatial heterogeneity in microbial, soil and vegetation properties in this ecosystem.

Since both block and deep (10-30 cm) SOC showed significant correlation with several microbial community measures and there was a significant effect of block on deep SOC, we included deep SOC as a covariate in our ANOVA model to see if it altered our ability to discern any treatment effects on the microbial community and/or accounted for any of the variation that was attributable to block (Table 4.6). Deep SOC was a significant factor in determining total PLFA, SAP/B, and F/B; and was marginally correlated with BSI (Table 4.6). Inclusion of deep SOC in our ANCOVA model tended to reduce the effect of block, although it only eliminated the significance of block completely for total PLFA (Table 4.6). Changes in deep SOC seemed to fully account for the apparent removal by nitrogen addition interaction effect on total PLFA, which was no longer significant when deep SOC was added as a covariate (Table 4.6). Inclusion of deep SOC in the ANCOVA model strengthened the effect of removal and nitrogen addition on the BSI, with the removal of random and shallow-rooted forbs leading to a significant increase in BSI and the addition of nitrogen leading to a marginal increase in BSI (Table 4.6). However, the absolute amount of change in BSI due to either removal of nitrogen addition treatments was small – an increase of only 0.002 ± 0.014 (mean $\pm$ s.e.) for nitrogen addition, an increase of 0.004 ± 0.013 for the removal of shallow-rooted forbs (SR), and an increase of 0.02 ± 0.01 for the removal of random forb biomass (RR). ANCOVA results were qualitatively similar when we used

deep TN as the covariate since deep SOC and TN are highly correlated ($R^2 = 0.95$, $p < 0.0001$).

DISCUSSION

Although we did not know *a priori* how plant tissue quality and fungal associations differ between shallow- and tap-rooted forbs and grasses, we expected that the removal of the entire group of shallow-rooted forb species and the addition of inorganic soil nitrogen would have significant impacts on microbial community measures. Instead, we found that spatial heterogeneity outweighed any impacts that our experimental treatments may have had on the microbial community measures we investigated. Reciprocal averaging (RA) ordination demonstrated that microbial community composition, measured as relative proportions of PLFA signatures, shifted more between experimental blocks than it did in response to either removal or nitrogen addition treatments (Figure 4.1a-c). Block was also a significant factor in determining individual microbial community measures, such as total PLFA, the ratios of saprophytic and all fungi to bacteria (SAP/B and F/B), and the bacterial stress index (BSI) (Table 4.4). While removal treatments did not alter microbial community composition or the ratios of saprophytic and all fungi to bacteria, the removal of random biomass and shallow-rooted forbs did increase the BSI (Table 4.4).

Our results differ from previous observational and experimental studies looking at the effect of nitrogen addition on the composition of PLFA signatures and fungal to bacterial ratios. A common finding in temperate grassland studies is that microbial community composition shifts along soil fertility gradients from less-managed sites to

those that are intensively fertilized by nitrogen, with bacterial decomposition being associated with increased nitrogen availability and fungal decomposition being associated with lower fertility soils (Bardgett *et al.* 1996; Yeates *et al.* 1997; Bardgett & McAlister 1999). Experimental addition of inorganic nitrogen fertilizer has also been shown to alter microbial PLFA composition (e.g., Bardgett *et al.* 1999; Clegg *et al.* 2003; Clegg 2006). However, Bardgett *et al.* (1999) found that nitrogen addition led to an increase in the ratio of fungal to bacterial PLFA, rather than a decrease as would have been predicted by observational gradient analyses. Bardgett *et al.* (1999) suggest that this difference might be mediated by changes in plant species composition and root exudation properties. In our experiment, we saw no response in either microbial community composition or fungal to bacterial ratios to nitrogen addition.

We did find that nitrogen addition marginally increased the BSI, after accounting for variation in SOC at 10-30 cm (Table 4.6). Although we cannot determine the exact mechanism for this response, it is possible that since bacteria need both nitrogen and carbon for growth, the addition of nitrogen alone could have stressed the bacterial community by making carbon more limiting to microbial growth. Other studies have suggested that unexpected responses to nitrogen addition could be linked to how N fertilization altered carbon availability (e.g., Fisk & Fahey 2001). The removal of random and shallow-rooted forbs also led to slight, but significant, increases in the BSI (Table 4.6). However, the absolute amount of change in BSI due to either removal of nitrogen addition treatments was small and therefore not likely to be biologically relevant.

The observation that the loss of shallow-rooted forbs species did not alter the microbial community is similar to results from Wardle *et al.* (1999) who found that separate removals of C₄ grasses, C₃ grasses, annual C₃ grasses, and dicotyledonous weeds from a perennial grassland site in New Zealand had no effect on total microbial PLFAs or the fungal to bacterial ratio. Instead, they suggested that the effects of spatial variability in soil properties were more influential than changes in plant species composition in determining soil microbial community dynamics (Wardle et al. 1999). We also found that microbial community measures in our system were strongly related to soil properties. Specifically, we found that soil organic carbon (SOC) and total soil nitrogen (TN) seem to be the most important factors predicting microbial community composition (measured as RA Axes 1 and 2) (Tables 4.3). Plant aboveground biomass (AGB) in both 2003 and 2004 were also significantly correlated with RA Axes 1 and 2 (Table 4.3), suggesting that plant biomass production is another critical factor in addition to soil C and N. All of these soil and vegetation parameters differed significantly between blocks (Table 4.5), suggesting that spatial variability in SOC, TN and AGB are likely to be important in determining soil microbial community structure. However, since removal and nitrogen addition treatments also affected these variables, we expected those treatment-induced changes to impact the microbial community. For example, 2003 and 2004 AGB, and shallow (0-10 cm) SOC and TN were all significantly affected by removal treatment (Table 4.5) and yet there were no significant effects of removal treatment on any of the microbial community measures (Table 4.4). Removal and nitrogen addition treatment effects on deep (10-30 cm) SOC did seem to account for a removal by nitrogen addition interaction effect on total PLFA (Tables 4.4

- 4.6), perhaps suggesting that deep SOC is a critical factor correlating with microbial biomass in this system.

While shallow SOC and TN could be directly related to the microbial community variables we measured at 0-10 cm, there is unlikely to be a direct connection between deep (10-30 cm) SOC and TN and shallow soil microbial measures. Instead, the strong correlation between deep SOC and microbial community measures could be due to an indirect relationship, such as between fine root biomass and soil C and N at 10-30 cm. Total fine root biomass at 0-10 cm was positively correlated with SOC at 0-10 cm in 2003 ($R^2 = 0.35$, $p < 0.0001$; data not shown). Although we did not measure fine root biomass at any depth in 2004, if the relationship between fine root biomass and SOC is consistently positive in both deep and shallow soil layers, it is possible that the observed positive relationship between deep SOC and microbial measures is actually due to a positive correlation between fine root biomass and SOC. Since fine roots provide microbes with C and N substrates through both root exudates and dead root biomass, increases in fine root biomass often increases microbial biomass and/or alters microbial community composition (e.g., Newman 1985; Bottner *et al.* 1988; Grayston *et al.* 1998; Fisk & Fahey 2001; Grayston *et al.* 2001; Lu *et al.* 2002; Allison *et al.* 2005; Brant *et al.* 2006).

Several possible explanations for why the observed effects of removal and/or nitrogen addition treatments on AGB, SOC and TN did not necessarily result in changes within the microbial community include: 1) the magnitude of treatment effects on vegetation and soil parameters were not sufficient to alter microbial community structure; 2) deep SOC and TN are more important predictors of microbial community

composition than the other variables; or 3) the experimental removal of shallow-rooted forb species and addition of nitrogen over a 3-year period was not long enough to alter the soil chemical properties sufficiently to have had an effect in microbial populations. Although AGB responses to the experimental treatments were roughly of the same magnitude as differences in AGB across blocks, there were some differences in the magnitude of changes in soil properties in response to experimental treatments that could contribute to the explanation. Differences in deep SOC and TN between the highest and lowest blocks were roughly 10 times larger than the average differences in deep SOC and TN between removal treatments; and differences in shallow SOC and TN between the highest and lowest blocks were roughly 3 times the average difference due to removal treatments (data not shown). Our level of nitrogen addition was also ~2-4 times lower than the quantity added in some other studies which examined microbial responses to nitrogen addition (e.g., Bardgett *et al.* 1999; Clegg *et al.* 2003; Clegg 2006). Since deep SOC and TN showed the strongest correlations with microbial community measures (Table 4.3), it is not surprising that spatial variation in those variables seemed to have a stronger effect than other environmental variables on the microbial community. Wardle *et al.* (1999) also hypothesized that vegetation changes might take longer than 3 years to alter soil properties that seem to drive microbial community dynamics so the relatively short duration of our experimental removals and nitrogen additions might account for the observation that between-block variation had a larger effect on microbial community measures than did treatments.

Overall, it appears that the magnitude of treatment effects on vegetation and soil properties, a strong relationship with deep SOC and TN (possibly an indirect measure of

fine root biomass), and a time-lag between changes in the vegetation and changes in soil properties are all potential explanations for the absence of removal and nitrogen addition treatment effects on microbial community composition. We are also unable to rule out the possibility that there may be other important environmental variables that we did not directly measure – such as soil temperature, root biomass, and phosphorus availability – or that we may not have measured the microbial community at the correct spatial scale. Microbial community structure is known to vary non-randomly across space with the scale of patchiness varying over several orders of magnitude from centimeters to several meters (e.g., Ettema & Wardle 2002; Franklin & Mills 2003). Our soil sampling scheme allowed us to distinguish between variation occurring at the ~2-6 m scale (within a block) and at the ~10-15 m scale (between blocks) since the significant block effect on microbial community composition (Table 4.4) indicated that microbial community structure is more similar within a block than between blocks; but it did not permit us to explore variation at finer scales. Soil microbial processes and community structure have been shown to vary within the extremely fine-scale (i.e., several millimeters) rhizosphere zone around plant roots (e.g., Sorensen 1997; Belnap *et al.* 2003). If our experimental treatments altered growth conditions for microbes within the rhizosphere, our soil sampling scheme would not have been able to capture that fine-scale variation.

Another reason we may not have seen any treatment effects on microbial community composition could be that there were composition shifts that could not be detected using PLFA analysis. Many PLFA signatures are non-specific and even the more specific PLFA signatures do not directly represent microbial “species”, but rather

related groups of microorganisms. There exist other molecular techniques for quantifying microbial community composition, such as DNA fingerprinting, which are able to distinguish between microbes at a finer “species”-like resolution than what we can distinguish using PLFAs (e.g., Clegg *et al.* 1998; van Elsas *et al.* 2000; Ranjard *et al.* 2001; Green *et al.* 2004; Leckie 2005). It is possible that those techniques might have revealed shifts in microbial community structure that we could not detect using PLFAs. Despite our inability to see treatment effects on microbial community structure as measured by PLFA, we also cannot rule out the possibility that microbial activity (as opposed to biomass or community structure) could have been affected by removal and nitrogen addition treatments. In Chapter 3 of this dissertation, we present results on how the loss of shallow-rooted forbs and nitrogen addition affected microbially mediated net nitrogen mineralization, nitrification and ammonification rates in 2002 and 2003. Since these processes were not measured in 2004 when the microbial community was sampled, we cannot directly compare microbial community changes with changing nutrient cycling processes.

In conclusion, it does not appear that the loss of shallow-rooted forbs and an increase in inorganic soil nitrogen availability has drastically altered the microbial community composition and ratio of fungi to bacteria over a roughly 3 year period. Instead, spatial heterogeneity of vegetation and soil properties seems to be more important in determining microbial community composition and structure. However, we do not know whether the loss of shallow-rooted forbs and/or nitrogen addition could have longer-term impacts on the microbial community. We also do not know if warming-induced plant species loss and enhanced nitrogen availability could alter

aspects of the microbial community that we could not detect using PLFA analysis. A better understanding of long-term impacts of plant removals and nitrogen addition on soil properties, and causal relationships between soil carbon and nitrogen concentrations and microbial PLFA relative abundances are needed before we can fully understand the relative roles that spatial heterogeneity, plant species loss and nitrogen availability may play in determining microbial community structure.

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TABLES AND FIGURES

Table 4.1. Assignment of signature phospholipid fatty acids (PLFAs) to soil microbial functional groups.

Group	Signature PLFAs				
Gram (-) Bacteria	cy17:0†	18:1ω7c§	16:1ω7c‡		
Gram (+) Bacteria	i15:0†	i16:0†			
Actinomycetes	10Me18:0†				
Desulfobacter	10Me16:0#				
Other Bacteria	14:0‡	a15:0†§	i17:0†§	a17:0†§	cy19:0a†§
Saprophytic Fungi	18:2ω6,9c†§	18:1ω9c‡			
Arbuscular Mycorrhizal Fungi	16:1ω5c¶				

† Zelles (1999); § Frostegård *et al.* (1993); ‡ Zak *et al.* (1996); # Coleman *et al.* (1993); ¶ Olsson (1999).

Table 4.2. Pearson correlation coefficients between RA Axis 1 and 2 values and microbial community measures.

	RA Axis 1 ¹	RA Axis 2
Total PLFA ² , n mol/g	-0.49***	n.s.
Total Bacteria PLFA, n mol/g	-0.52***	n.s.
Gram (-) Bacteria PLFA, n mol/g	-0.52***	n.s.
Gram (+) Bacteria PLFA, n mol/g	-0.47***	n.s.
Actinomycete PLFA, n mol/g	-0.36**	n.s.
Desulfobacter PLFA, n mol/g	-0.32*	-0.35**
Saprophytic Fungi PLFA, n mol/g	-0.27†	n.s.
Arbuscular Mycorrhizal Fungi (AMF) PLFA, n mol/g	-0.31*	n.s.
Total Fungi PLFA/Total Bacteria PLFA Ratio	0.73***	0.46***
Bacterial Stress Index (BSI: cyclopropyl/precursors ratio)	-0.52***	n.s.

¹ Correlation coefficients are significant at: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$;

† $p \leq 0.10$; n.s. = not significant.

² PLFA = phospholipid fatty acid.

Table 4.3. Pearson correlation coefficients between environmental variables and microbial community measures.

	Total PLFA ^{1,2}	RA Axis 1	RA Axis 2	SAP/B	F/B	BSI
2003 AGB, g/m ²	0.32*	-0.42**	n.s.	-0.26†	n.s.	n.s.
2004 AGB, g/m ²	n.s.	-0.52***	0.28†	-0.29*	-0.29*	n.s.
TN, %N (0-10 cm)	0.57***	-0.51***	n.s.	-0.32*	-0.29*	n.s.
SOC, %C (0-10 cm)	0.52***	-0.46***	n.s.	-0.25†	n.s.	n.s.
Soil C/N (0-10 cm)	-0.25†	n.s.	0.40**	0.33*	0.35*	n.s.
TN, %N (10-30 cm)	0.68***	-0.65***	n.s.	-0.42**	-0.42**	n.s.
SOC, %C (10-30 cm)	0.69***	-0.68***	n.s.	-0.42**	-0.42**	n.s.
Soil C/N (10-30 cm)	n.s.	n.s.	0.38**	n.s.	n.s.	n.s.
Soil M % (0-10 cm)	n.s.	n.s.	0.25†	n.s.	n.s.	n.s.
Soil M % (10-30 cm)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

¹ PLFA = phospholipid fatty acid; SAP/B = saprophytic fungal to bacteria ratio; F/B = total fungal to bacteria ratio; BSI = bacterial stress index (cyclopropyl/precursors ratio); AGB = aboveground biomass; TN = total soil nitrogen; SOC = soil organic carbon; Soil M = gravimetric soil moisture.

² Correlation coefficients are significant at: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; † $p \leq 0.10$; n.s. = not significant.

Table 4.4. ANOVA F-ratios for treatment effects on microbial community measures.

	d.f.	Total PLFA ^{1,2}	SAP/B	F/B	BSI
Removal	2	0.01	0.41	0.77	2.71†
N-Addition	1	0.13	0.37	0.01	1.86
Removal x N-Addition	2	4.15*	0.08	0.13	0.33
Block	7	3.25**	2.54*	3.90**	3.03**

¹ See Table 4.3 for abbreviations.

² F-ratios are significant at: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; † $p \leq 0.10$.

Table 4.5. ANOVA F-ratios for treatment effects on soil and vegetation properties.

	d.f.	2003 AGB ^{1,2}	2004 AGB	SOC 0-10cm	TN 0-10cm	Soil C/N 0-10cm
Removal	2	10.84***	3.54*	3.61*	3.37*	1.35
N-Addition	1	1.62	2.00	1.86	3.22†	1.13
Removal x N-Addition	2	2.92†	2.65†	0.86	1.45	0.70
Block	7	2.91*	4.53***	6.14***	7.72***	1.01

¹ See Table 4.3 for abbreviations.

² F-ratios are significant at: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; † $p \leq 0.10$.

Table 4.5 (continued).

	SOC 10-30cm	TN 10-30cm	Soil C/N 10-30cm	Soil M % 0-10 cm	Soil M % 10-30 cm
Removal	0.45	0.35	0.42	0.47	1.23
N-Addition	1.83	0.85	0.93	0.99	0.27
Removal x N-Addition	2.50†	2.72†	0.03	0.53	3.27*
Block	13.67***	12.69***	2.30*	1.14	1.52

¹ See Table 4.3 for abbreviations.

² F-ratios are significant at: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; † $p \leq 0.10$.

Table 4.6. ANCOVA F-ratios for treatment effects on microbial community measures with deep (10-30 cm) soil organic carbon (SOC, %C) as a covariate.

	d.f.	Total PLFA ^{1,2}	SAP/B	F/B	BSI
Removal	2	0.09	0.87	1.78	3.36*
N-Addition	1	1.02	0.01	0.66	3.03†
Removal x N-Addition	2	2.20	0.25	0.39	0.86
Block	7	0.28	2.21†	4.22**	3.15**
SOC, %C (10-30 cm)	1	7.82**	6.28*	10.54**	2.85†

¹ See Table 4.3 for abbreviations.

² F-ratios are significant at: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; † $p \leq 0.10$.

Figure 4.1. Microbial community composition assessed by relative abundance of signature PLFAs using reciprocal averaging (RA). RA Axis 1 accounted for 55.1% and RA Axis 2 accounted for 18.3% of the variance explained. Samples (plots) are grouped according to removal treatment (A), nitrogen addition treatment (B), or Block (C). For removal treatments, CONT = no removal control; RR = random forb removal; SR = shallow-rooted forb removal. Points represent the mean value for each grouping category, and error bars represent ± 2 SE along both Axes 1 and 2.

Figure 4.1.

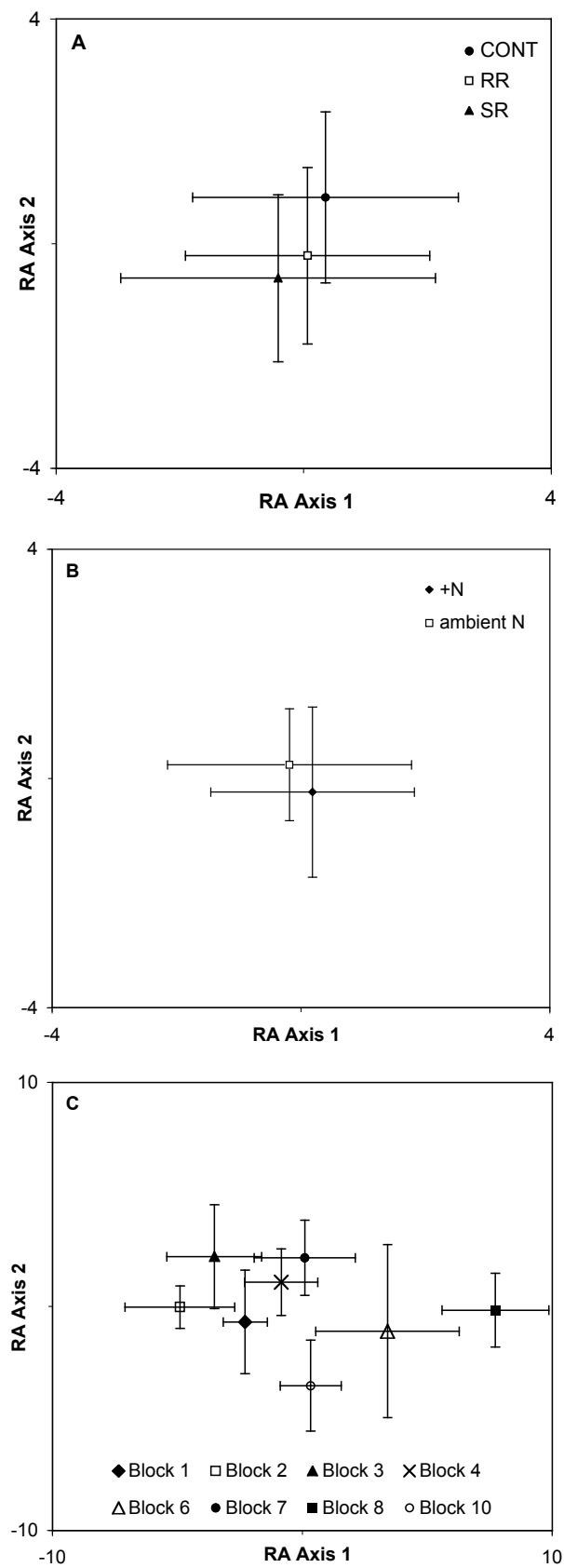


Figure 4.2. Distribution of signature PLFAs along RA Axes 1 and 2. RA Axis 1 accounted for 55.1% and RA Axis 2 accounted for 18.3% of the variance explained. Location of PLFA points indicate the relative contribution of each PLFA signature to the loading on RA Axes 1 and 2.

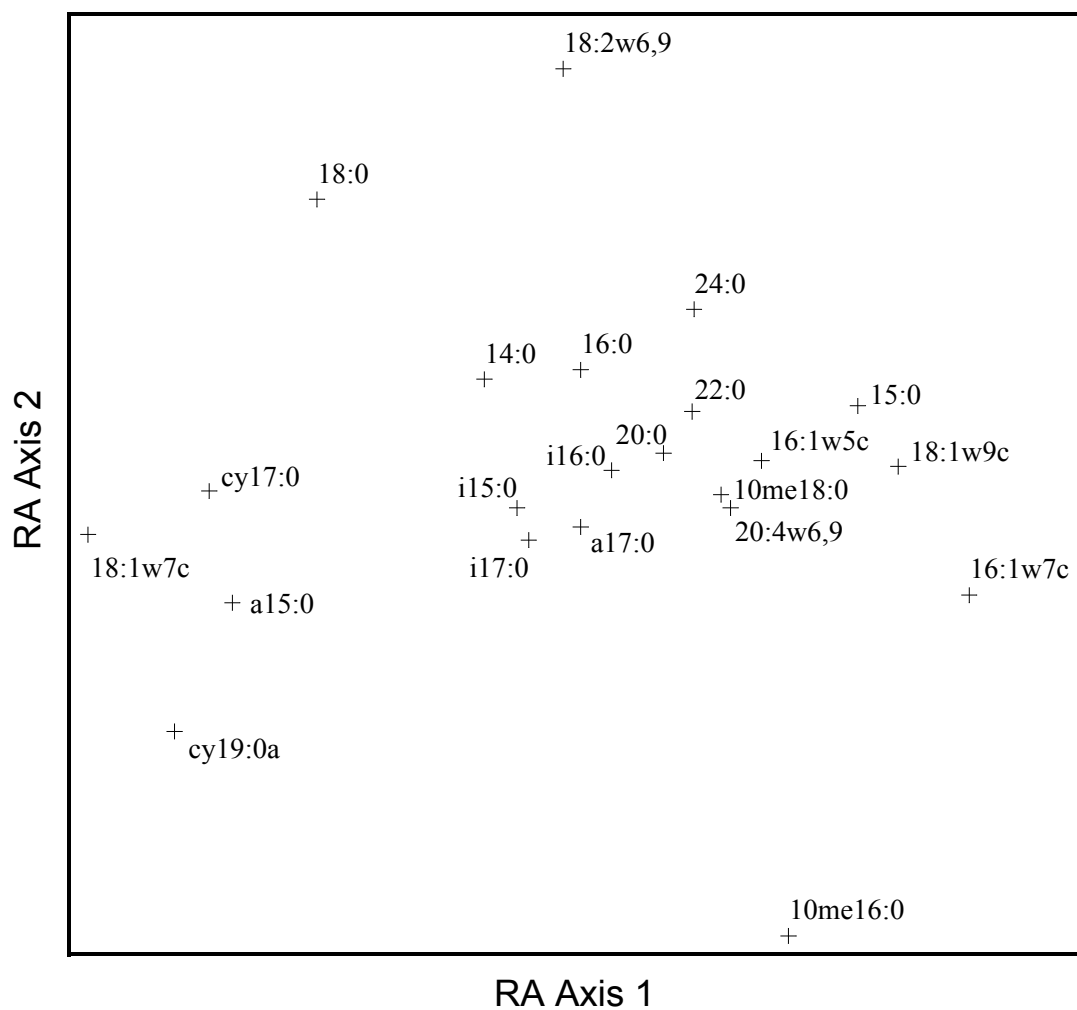


Figure 4.3. Change in relative abundance of all fungi (open squares, dashed line) and all bacteria (closed circles, solid line) PLFA along RA Axis 1. Note the different scales on the Y-axes for fungal and bacterial relative abundances.

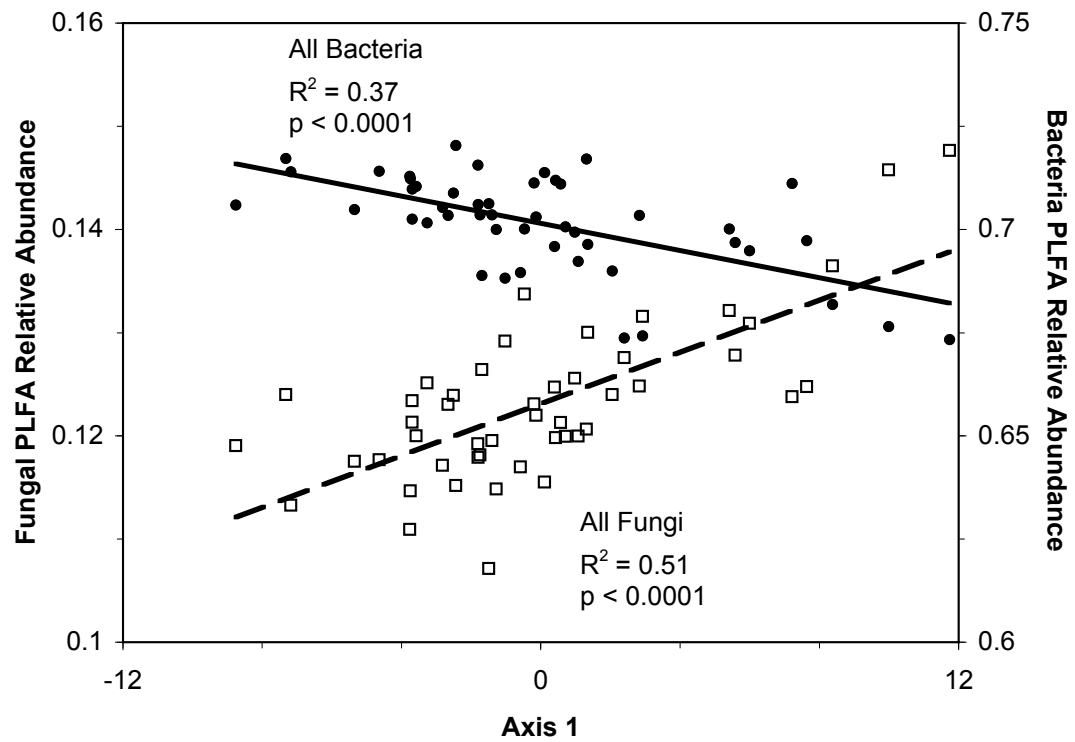


Figure 4.4. Change in relative abundance of saprophytic fungi (closed squares, solid line) and arbuscular mycorrhizal fungi (AMF) (open squares, dashed line) PLFA along RA Axis 1.

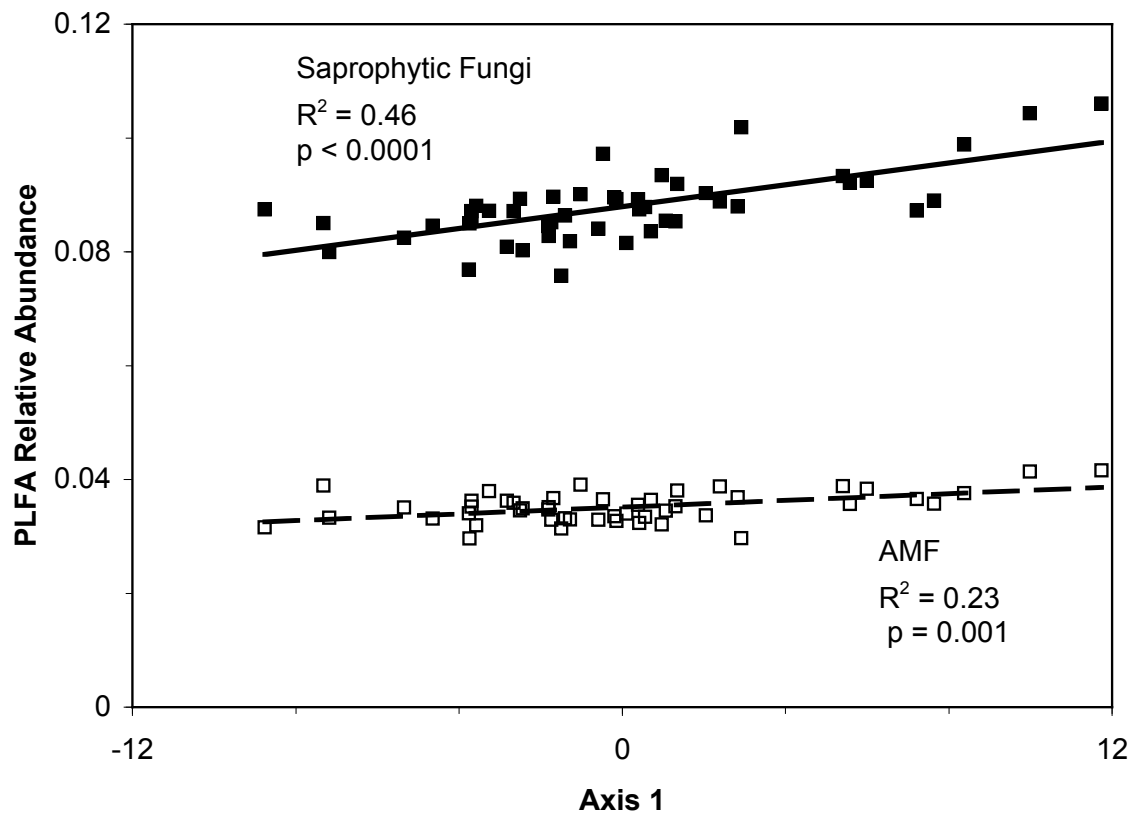
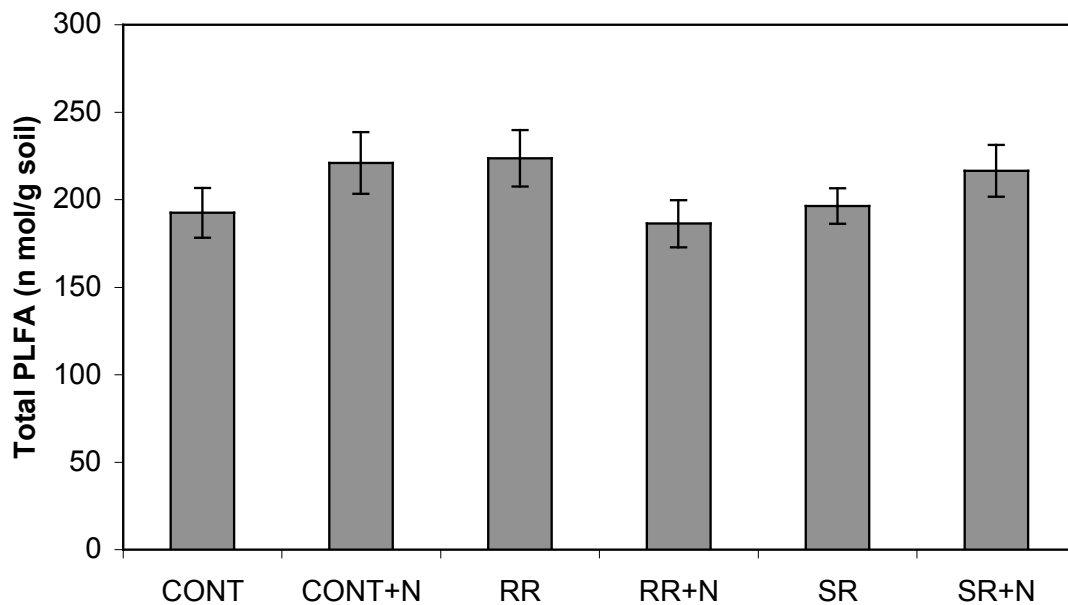


Figure 4.5. Total PLFA (a measure of total microbial biomass) by treatment. Treatment abbreviations are: CONT = no removal, ambient N; CONT+N = no removal, added N; RR = random forb removal, ambient N; RR+N = random forb removal, added N; SR = shallow-rooted forb removal, ambient N; SR+N = shallow-rooted forb removal, added N. Error bars are ± 1 SE. The interaction between removal and nitrogen addition treatments is significant (ANOVA: $p = 0.02$) with nitrogen addition leading to decreased total PLFA in RR, and having no effect on total PLFA in SR or CONT treatments.



APPENDIX A: PLANT SPECIES LIST

A list of all plant species in the experimental plots and their classification as grass, shallow- or tap-rooted forb. Forb species were classified into shallow- and tap-rooted categories based on field excavations of root morphology. Shallow-rooted forb species have fibrous rooting structures, mostly concentrated in the 0-10 cm soil layer. Tap-rooted forb species showed evidence of a thickened taproot structure that is assumed to penetrate deeper into the soil profile.

Species Name*	Plant Group
<i>Achillea millefolium</i>	shallow-rooted forb
<i>Agoseris</i> sp.	shallow-rooted forb
<i>Androsace septentrionalis</i>	shallow-rooted forb
<i>Aquilegia caerulea</i>	tap-rooted forb
<i>Arabis drummondii</i>	shallow-rooted forb
<i>Bromus carinatus</i>	grass
<i>Bromus porteri</i>	grass
<i>Calochortus gunnisonii</i>	shallow-rooted forb
<i>Chenopodium album</i>	shallow-rooted forb
<i>Cirsium</i> sp.	Tap-rooted forb †
<i>Claytonia lanceolata</i>	shallow-rooted forb
<i>Collomia linearis</i>	shallow-rooted forb
<i>Delphinium barbeyi</i>	tap-rooted forb
<i>Descurainia</i> sp.	shallow-rooted forb
<i>Draba spectabilis</i>	shallow-rooted forb
<i>Elymus trachycaulus</i>	grass
<i>Erigeron elatior</i>	shallow-rooted forb

<i>Erigeron speciosus</i>	shallow-rooted forb
<i>Erysimum capitatum</i>	shallow-rooted forb
<i>Erythronium grandiflorum</i>	shallow-rooted forb
<i>Festuca thurberi</i>	grass
<i>Fragaria virginiana</i>	shallow-rooted forb
<i>Frasera speciosa</i>	tap-rooted forb
<i>Galium boreale</i>	shallow-rooted forb
<i>Gentiana parryi</i>	tap-rooted forb †
<i>Geum triflorum</i>	shallow-rooted forb
<i>Helianthella quinquenervis</i>	tap-rooted forb
<i>Heliomeris multiflora</i>	shallow-rooted forb
<i>Hymenoxys hoopesii</i>	shallow-rooted forb
<i>Ipomopsis aggregata</i>	shallow-rooted forb
<i>Lathyrus laetivirens</i>	shallow-rooted forb
<i>Linum lewisii</i>	shallow-rooted forb
<i>Lupinus sp.</i>	tap-rooted forb
<i>Melica spectabilis</i>	grass
<i>Mertensia oblongifolia</i>	shallow-rooted forb
<i>Pedicularis procera</i>	tap-rooted forb
<i>Poa sp.</i>	grass
<i>Polygonum douglasii</i>	shallow-rooted forb
<i>Potentilla gracilis</i>	shallow-rooted forb
<i>Pseudocymopterus montanus</i>	shallow-rooted forb
<i>Ranunculus inamoenus</i>	shallow-rooted forb
<i>Senecio bigelovii</i>	tap-rooted forb †
<i>Solidago multiradiata</i>	shallow-rooted forb
<i>Stipa sp.</i>	grass
<i>Symphotrichum foliaceum</i>	shallow-rooted forb
<i>Taraxacum officinale</i>	tap-rooted forb

<i>Thalictrum fendleri</i>	shallow-rooted forb
<i>Tragopogon dubius</i>	shallow-rooted forb ‡
<i>Trisetum spicatum</i>	grass
<i>Valeriana edulis</i>	tap-rooted forb
<i>Valeriana occidentalis</i>	shallow-rooted forb
<i>Vicia americana</i>	shallow-rooted forb
<i>Viola sp.</i>	shallow-rooted forb

Notes:

* All species names are considered standard acceptable nomenclature by the Integrated Taxonomic Information System (<http://www.ITIS.usda.gov>) as of January 25, 2006.

“sp.” indicates that we could not identify the plant to species, only genus.

† These species were either rare, or it difficult to determine rooting structure. We treated them as if they were tap-rooted since in many instances they showed a somewhat thickened root structure just below the soil surface. None of these species were common in terms of frequency or biomass in any of the experimental plots.

‡ This species was found in only one experimental plot and was unidentified for the first few years of the experiment. Since we did not know its rooting structure, we treated it as if it were a tap-rooted forb.