CONSEQUENCES AND RECOVERY AFTER NUTRIENT ENRICHMENT AND HERBIVORE REDUCTION IN THE BOREAL FOREST UNDERSTORY

by

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Abstract

Atmospheric nitrogen deposition poses a serious threat to plant communities globally. Furthermore, nitrogen-induced shifts in plant community composition may create positive feedbacks via litter decomposition by the soil microbial community. These feedbacks could prevent the recovery of plant communities, even in the absence of further nutrient addition, because nutrient availability and cycling remain high. We investigated the role of nutrients and herbivores in regulating plant and soil microbial communities in the boreal forest understory in northwestern Canada. We used an experiment that began in 1990 where plots were fertilized, exclosed (herbivore reduction), or both, in a 2 x 2 factorial design. In early 2000, plots were divided in half; treatments were continued on one half, and discontinued on the other half. In 2009, we sampled plant community composition, along with carbon/nitrogen ratios and total phenolics in the plant tissue. Using phospholipid fatty-acid analyses and extracellular enzyme activity assays, we estimated the soil microbial community composition and activity. Lastly, we measured soil pH and chemistry. Overall, fertilization had significant impacts on the variables we measured; herbivore exclosures mostly had no detectable impacts. In fertilized plots, species richness declined and the plant community became dominated by *Epilobium angustifolium* and *Mertensia paniculata*. Total phenolics and the carbon/nitrogen in the plant tissue declined. The total microbial biomass declined, as did the ratio of fungi to bacteria, indicating a more bacteria-dominated food web in the soil. Extracellular enzymes involved in the breakdown of cellulose increased in activity, but those involved in the acquisition of nitrogen and phosphorus were mostly unaffected, except urease, which declined in activity. Soil pH declined significantly, and fertilizer increased the availability of many nutrients. In recovery plots, the results do not fit the predictions of a plant-soil feedback hypothesis. Instead, the system follows a “cascade of responses”, where soil chemistry recovers first, then plant tissue chemistry, followed more slowly by plant community composition. After 10 years, the soil microbial community has yet to show significant signs of recovery. These results highlight the sensitivity of the boreal forest to nutrient enrichment and demonstrate that recovery of these ecosystems may take decades.
Preface

Chapter 2 was co-authored by Roy Turkington and will be submitted for publication. The experiment was designed and conducted by Roy since 1990 for 19 seasons. Roy determined sampling protocols for plant community composition; I decided on the sampling methods for the plant tissue chemistry. I also collected and analyzed the data, and wrote the drafts of the manuscript. Roy provided advice and feedback on the content and organization of the manuscript, and provided editorial assistance.

Chapter 3 was co-authored by Roy Turkington and will be submitted for publication. Again, the experiment used was designed and conducted by Roy since 1990 for 19 seasons. I decided on the sampling methods for all soil abiotic and biological properties and carried out laboratory analyses. I also analyzed the data and wrote drafts of the manuscripts. Roy provided advice and feedback on the content and organization of the manuscript, and provided editorial assistance.
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1 INTRODUCTION

By 2030, many regions of the world are predicted to receive elevated rates of nitrogen deposition (Bobbink et al. 2010, Dentener et al. 2006), caused mainly by human activities. Humans’ needs for energy, food and transportation have resulted in unprecedented increases of nitrogen oxide in the atmosphere, mainly through the burning of fossil fuels and agricultural activity (Galloway et al. 2004). While atmospheric nitrogen deposition historically has been a minor contributor to biogeochemical cycling, it is now becoming the dominant source of nitrogen in many ecosystems (Galloway et al. 2004). Atmospheric nitrogen is highly mobile and may travel over thousands of kilometers, therefore many ecosystems with little natural input of nitrogen are now receiving additional inputs of active nitrogen (Phoenix et al. 2006), threatening to cause major changes in ecosystem structure (Bobbink et al. 2010, Cleland and Harpole 2010, Gilliam 2006).

Humans are also altering global nutrient cycling by increasing the amount of carbon dioxide released into the atmosphere. This is an especial concern in northern ecosystems which are expected to be disproportionately affected by increases in carbon dioxide in the atmosphere (IPCC 2007). Temperature increases over the next century could lead to elevated nutrient availability via increases in decomposition rates, soil respiration and nutrient mineralization (Lükewille and Wright 1997, Rustad et al. 2001, Schuur et al. 2009). This increase in nutrient availability could exacerbate the impacts caused by atmospheric nitrogen deposition.

In addition, the redistribution and extinction of species has become ubiquitous in virtually all ecosystems. Despite international efforts, the loss of species and biodiversity is continuing at an alarming rate (Butchart et al. 2010) and there are major concerns about the effects of these losses on how ecosystems function (Cardinale et al. 2006). Although often overlooked, the introduction and redistribution of herbivores is also a major source of concern (Wardle and Bardgett 2004). Herbivores can consume from <1% to >50% of annual net primary production in an ecosystem (Cebrian 1999, Cebrian and Lartigue 2004, McNaughton et al. 1989), and therefore alterations in their abundance, either through
extinction or redistribution, may have large, and sometimes unforeseen, impacts on ecosystems (Turkington 2009).

Many studies seek to understand how plant communities and ecosystems will react to changes in nutrient availability and herbivore abundance in the context of top-down and bottom-up regulation. Most appear to be controlled by a combination of the two (Turkington 2009), but it is often difficult to predict, *a priori*, the relative strengths of top-down and bottom-up forces and their consequences on the community. The balance between bottom-up and top-down controls is highly context-dependent, complicating our ability to predict what will happen to ecosystems under global change scenarios. As global change phenomena threaten an increasing number of ecosystems, the relationship between top-down and bottom-up controls may be altered. For example, increased nutrient availability can change patterns of herbivory (Gough *et al.* 2008), or phenology (Fremlin *et al.* in press) in an ecosystem complicating predictions about how nitrogen deposition may impact a plant community.

One promising avenue of research focuses on interactions between the plant and soil microbial communities. The soil microbial community, including both fungi and bacteria, influences many key ecosystem processes (Van der Heijden *et al.* 2008), including decomposition, carbon storage, and the cycling of nutrients (O’Donnell *et al.* 2005, Wardle 2005, Zhu and Miller 2003). The soil microbial community directly and indirectly influences competitive interactions between plants (Van der Heijden *et al.* 2008), and can mediate the response of the plant community to global change phenomena, such as global climate change and nitrogen deposition (Bardgett and Wardle 2010). By studying both the plant and soil microbial community simultaneously, we may get a better understanding of the importance of plant-soil interactions in controlling the response of vegetation to anthropogenic pressures and their subsequent recovery (Wardle 2002, 2005, Wardle *et al.* 2004).

The study I describe here seeks to understand how fertilization and herbivory affect both the plant and soil microbial communities in an effort to more fully understand how they influence aspects of ecosystem and community structure and function, aboveground and belowground.
1.1 Plant-Soil Interactions

While often studied separately, the structure and function of plant and soil microbial communities are strongly interrelated. Here I will briefly introduce the major pathways by which plant and soil microbial communities interact, and how anthropogenic disturbances affect this relationship. First, I will highlight the mechanisms by which the plant community influences the soil microbial community. Second, I will focus on the important influence of the soil microbial community on aboveground interactions. Third, I will consider how fertilization and foliar herbivory alter plant-soil interactions. Last, I will present potential consequences of plant-soil feedbacks on the potential for recovery of ecosystem and community structure and function from fertilization and herbivory.

1.1.1 Influence of Plant Communities on the Soil

The plant community influences and structures the soil microbial community in many ways. Two important sources of resources for the soil microbial community are, first, root exudation and belowground carbon allocation, and second, decomposing plant litter. Patterns of root exudation, carbon allocation and the quality of decomposing litter differ between species, and can be altered in individual plants. Therefore, disturbances can have short-term consequences by altering individual plants, and long-term consequences if the functional composition of the plant community is altered (Bardgett and Wardle 2010).

Plants exude a considerable amount of carbon from their roots (Hogberg and Read 2006, Wardle and Bardgett 2010), stimulating the growth and activity of the microbial community in the rhizosphere (Turkington et al. 1988, Chanway et al. 1991, Hogberg and Read 2006). Changes in patterns of carbon allocation have been shown to alter the composition of the soil microbial community, particularly reducing the biomass and activity of plant symbionts (Janssens et al. 2010). For example, tree girdling (which terminates belowground carbon allocation) reduced the abundance of ectomycorrhizal fungi in the soil (Hogberg et al. 2007). Not only does the amount of carbon allocated belowground strongly influence soil microbial communities, but the type of compound entering the soil is important as well. Plants release many different compounds into the soil, including carbohydrates, amino acids, organic acids, fatty acids, enzymes (Grayston et al. 1997, Hogberg and Read 2006, Van Hees et al. 2005) and phenolics (Meier et al. 2008).
composition of the microbial community, as different compounds select for particular microbial communities better able to utilize the specific compound (Bardgett and Wardle 2010, Grayston et al. 1998).

Soil microbes also receive resources from the plant community via the decomposition of litter. The quality of litter affects its decomposability and the availability of resources for the soil community (Bardgett and Wardle 2010, McLaren and Turkington 2010, Wardle 2002). The amount of nitrogen, lignin and defensive compounds in plant tissue determine the decomposability of litter (Sinsabaugh et al. 2002, Wardle and Bardgett 2010), which differs greatly among functional groups of plants (Cornwell et al. 2008). The type of compound entering the soil can determine the competitive outcome amongst soil organisms, and determine the general structure of the soil microbial community (Turkington et al. 1988). For example, white rot fungi are the only members of the soil community able to completely degrade lignin (Prescott 2005), and therefore will be more competitive where highly recalcitrant litter predominates. Recalcitrant litter (with a high lignin content) is hypothesized to select a fungi-dominated food web slow at cycling nutrients, but when high-quality litter is more common, bacteria-dominated food webs are more common in the soil (Wardle 2005). The quality of litter also affects the size of the microbial community. For example, soils of coniferous forests generally have a lower microbial biomass than those of deciduous forests because they have lower quality litter (Wardle 2002).

Less is understood about how the quantity of litter affects the soil microbial community. The quantity of litter added to the soil can be an important determinant of the biomass of the microbial community (Hernandez and Hobbie 2010), but the relationship is not entirely clear. Decreases in net primary productivity (NPP) can have both positive and negative effects on soil microbial communities (Bardgett and Wardle 2010). This may be due to the relative strengths of top-down and bottom-up controls in soil food webs and changes in competitive interactions for nutrients (Wardle 2002).

1.1.2 Influence of Soil Biota on the Plant Community

Despite the difficulties in studying soil microbial communities (Leckie 2005), we know soil microbes control many ecosystem processes. Without microbes, there would be no biogeochemical cycling of carbon and nitrogen, no decomposition, no gas exchange, limited
possibilities for pollutant mitigation (O’Donnell et al. 2005) and a lowered ability for soils to store carbon (Zhu and Miller 2003). Microbes can either enhance the productivity of ecosystems by increasing rates of decomposition and nutrient cycling, or decrease it by competing with plants for key resources (Wardle 2005).

The soil community can have both positive and negative influences on the aboveground community, through both direct and indirect pathways. Directly, root herbivores (e.g. arthropods) and soil pathogens can remove nutrients and carbon from the plant, and remove plant tissue (Van der Heijden et al. 2008). Alternatively, mutualisms can be formed that aid in the plant’s ability to obtain nutrients, such as can occur with direct interactions with mycorrhizal fungi (Callaway et al. 2001, 2003). Indirectly, soil organisms exert control over the availability of nutrients. This can be beneficial to the plant community such as when soil microbes release nutrients bound up in organic debris through the activity of enzymes. Yet, the influence can also be negative, because soil microbes must also compete with plants for limiting resources, potentially immobilizing key nutrients in the soil (Van der Heijden et al. 2008).

The relative strength of these processes can determine the relative success of plants because these belowground interactions do not affect all plants equally. The differential impact among individuals has the potential to structure the plant community. For example, negative feedbacks can occur where plants of the same species grow less well in soils where conspecifics have grown, due to the accumulation of pathogens (Bever 1994). These negative feedbacks promote diversity in plant communities, because the growth of abundant species can be restrained. In contrast, positive feedbacks may also occur, reinforcing the success of dominant species, thereby promoting low-diversity systems (Kulmatiski et al. 2008).

1.1.3 The Effects of Fertilization and Herbivory on Plant-Soil Interactions

Plant-soil interactions and soil properties can be significantly altered by nutrient enrichment and changed herbivore abundance. Nitrogen deposition, or fertilization, can increase nutrient cycling and nitrogen mineralization (Clark et al. 2009), reduce soil pH (Bobbink et al. 1998), and alter soil microbial communities (Bardgett et al. 1999). Likewise, herbivores have been shown to influence many soil attributes, including nutrient availability (Holland
and Detling 1990), decomposition (Stark et al. 2000), and the soil microbial community (Guitian and Bardgett 2000), among others. The mechanistic basis for the impacts of fertilizer and herbivores on plant-soil interactions can be organized into effects on resource quality and resource quantity that enters the soil (Bardgett and Wardle 2003). This discussion is not exhaustive, but highlights a number of key mechanisms by which fertilization and herbivory can be expected to influence ecosystems.

Resource quality: Nutrient enrichment can impact the quality of resources entering the soil by altering the chemical composition of plant tissue, and in the long-term, by causing shifts in the functional composition of the plant community. Nutrient enrichment has been shown to increase nitrogen content and decrease levels of phenolics in plant tissue (Clark et al. 2009, Strengbom et al. 2003). Likewise, nutrient enrichment can cause shifts in plant community composition. Generally, nitrogen deposition reduces diversity while favouring species with large stores of aboveground tissue and species that are able to exploit resources quickly (Clark and Tilman 2008). Fast-growing species typically decompose quickly, speeding up nutrient cycling, thereby providing more nutrients to the plant community (Wardle 2002).

The quality of resources can be altered by herbivory through a number of pathways. First, herbivores can directly provide highly labile nutrients through the deposition of faeces and urine, bypassing the decomposition of litter composed of recalcitrant compounds (Bardgett and Wardle 2003). Second, herbivores can stimulate changes in plant tissue chemistry. Herbivory can increase the quality of plant tissue by increasing foliar nitrogen content (Green and Detling 2000) which may increase nutrient mineralization rates. For example, deer browsing causes decreased carbon/nitrogen (C/N) ratios in lupine leaf tissue, and increased nitrogen mineralization rates (McNeil and Cushman 2005). Alternatively, herbivory can induce the production of secondary defense compounds and favor the build-up of physical defenses (Strengbom et al. 2003) that decompose slowly (Grime et al. 1996).

At the community scale, herbivory can induce shifts in the functional composition of plant communities. Herbivores have been reported to decrease the abundance of nitrogen-rich plants (Sirotna and Huntly 2000), these are replaced by plants with higher levels of defense chemicals, and higher levels of physical defenses against herbivory (Wardle and Bardgett 2010). Woody species, and those high in phenolic content, can decrease nutrient cycling in ecosystems (Pastor et al. 1993, 1998, Persson et al. 2005).
**Resource quantity:** The quantity of resources entering the soil may be altered by nutrient enrichment, or foliar herbivory, in the short-term via changes in patterns of root exudation, and in the long-term, by changing NPP. Fertilization studies in forest communities have shown a reduction in the allocation of carbon belowground as plants divert resources to aboveground tissue production (Hogberg *et al.* 2007, Litton *et al.* 2007). This reduction in resources is particularly unfavourable to root symbionts, such as mycorrhizae (Janssens *et al.* 2010). Positively, nutrient enrichment often boosts NPP (Cleland and Harpole 2010), providing more resources through increases in litter quantity.

The impact foliar herbivory has on the quantity of resources entering the soil is more complicated. Foliar herbivory has been shown to increase root exudation, stimulating microbial activity in the rhizosphere (Hamilton *et al.* 2008). This has been shown to benefit individual plants, because nutrient availability for the plant increases, thereby stimulating growth (Hamilton and Frank 2001). Over the long-term, herbivores can alter the NPP of plant communities and the supply of root exudates. Whether the effect is positive or negative is likely highly dependent on site characteristics such as fertility and the plant species affected (Hawkes and Sullivan 2001, Bardgett and Wardle 2003). There is some evidence that increases in NPP occur in grassland systems (Belovsky and Slade 2000), but it may not be the case in other systems such as forest understories. For example, moose browsing in the boreal forest reduced NPP, which is suspected to have caused subsequent decreases in soil respiration (Persson *et al.* 2009).

### 1.1.4 Plant-Soil Interactions: Consequences for Ecosystem Recovery

In addition to initiating change in the structure and function of ecosystems, fertilization and herbivory can promote feedbacks between plants and soil that may prevent the recovery of ecosystems from anthropogenic disturbance\(^1\) (Wardle 2002). If the changes in vegetation promoted by fertilization or herbivory cause shifts in soil properties that further promote the

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\(^1\) Grime (1977) defines disturbance as causing the partial or total destruction of biomass. In a more general sense a disturbance is any event that opens new space for animal or plant recruitment. In this thesis I will use the word disturbance to refer to any anthropogenically imposed disruption to natural processes.
newly established vegetation, the return of the plant community to its previous state may be delayed, or may not occur at all.

It was suggested by Chapin (1991) that in sites of high fertility, herbivory may cause increases in nutrient cycling, while at sites of low fertility, herbivory may actually retard nutrient cycling. The mechanism for this is an herbivore-induced shift in the plant community composition. At high fertility, herbivores can promote the growth fast-growing, high-quality plants that decompose quickly. This could enhance nutrient cycling, making more nutrients available for the plant community. This would further promote the growth of fast-growing, high quality plants, inhibiting the return of more slow-growing, nitrogen-conservative plants. By promoting fast-growing plants, fertilization of nitrogen-poor ecosystems could cause similar responses, even in the absence of herbivory.

Alternatively, in sites of low fertility, herbivory is expected to further impede nutrient cycling. The mechanism is similar to that described for sites of high fertility. High levels of herbivory would select for slow-growing, well-defended plant species with low quality plant tissue. This would retard nutrient cycling, promoting a fungal-dominated microbial community, and further lessen the availability of nutrients for the plant community (Wardle 2002). Again, this would inhibit fast-growing species but enhance the competitive ability of slow-growing species.
1.2 Conclusions

A primary objective of this study is to determine the long-term influences of fertilization and herbivory on both the plant community and the soil microbial community, and to monitor the recovery of these communities once fertilization ceases. Taking advantage of a long-term study that began in 1990 (Turkington et al. 2002) in the understory of the boreal forest, we were able to get a snapshot of the effects of fertilization and herbivory on the plant and soil microbial communities. This research will investigate the plant community and soil microbial community separately, searching for patterns that will allow us to speculate about how the plant and soil microbial communities are interacting. Chapter 2 focuses on the plant community, investigating plant community composition and tissue chemistry. Chapter 3 focuses belowground, including the soil microbial community composition and activity, as well as the pH and nutrient availability of the soil. In Chapter 4, I speculate about some of the possible interactions between plant species and the soil microbial community that may be influencing the recovery of the boreal forest understory ecosystem.
2 EFFECTS OF FERTILIZATION AND HERBIVORE REDUCTION ON PLANTS

2.1 Introduction

Humans have significantly altered rates of global nutrient cycling (Vitousek et al. 1997), so an understanding of the widespread impacts of nutrient enrichment on terrestrial plant communities has become an important area of ecological research. Recent reviews and meta-analyses have shown that plant communities are responding to nitrogen deposition in many ecosystems (Bobbink et al. 2010, Cleland and Harpole 2010, Gilliam 2006). Typically there are declines in species diversity and large changes in plant community composition towards fast-growing, nitrophilous species, causing concern about global declines in biodiversity and altered ecosystem functions (Clark et al. 2008, Cleland and Harpole 2010).

The degree to which a community responds to elevated levels of nutrients is largely determined by the relative strengths of top-down and bottom-up structuring forces acting on plant communities. While once a dichotomous area of debate, where research focused on whether one force or the other determined plant composition, it has become clear that most ecosystems exhibit some form of interactive control (Gruner et al. 2008, Hillebrand et al. 2007). There is considerable experimental evidence on the effects of nutrient enrichment and herbivore removal (or reduction) on plant communities but surprisingly, few field experiments have been conducted investigating their interactive effects (Turkington et al. 2002, Turkington 2009). It is possible that herbivores can either mitigate or exacerbate shifts in plant community composition through selective feeding, or through functional and numerical shifts in their behaviour and abundance. Fertilization can increase the palatability of plants to herbivores, making them more vulnerable to herbivory, and favouring species with higher levels of physical and chemical defenses. Alternatively, herbivory can intensify a fertilizer-induced shift in the plant community by favouring fast-growing species such as graminoids that often are more tolerant of herbivory, or are more capable of re-growth (Altesor et al. 2005).
Most studies testing top-down and bottom-up effects have typically focused on community-level phenomenon, but top-down and bottom-up forces also interact at the level of the individual plant. The quality of plant tissue, including secondary metabolite concentrations, may depend on both resource levels in the soil and levels of herbivory (e.g. Bryant et al. 1983, Coley et al. 1985, Herms and Mattson 1992). Fertilization has been shown to increase the nutrient content of plant tissue (Clark et al. 2009, Gilliam 2006, Power et al. 2006) and to reduce levels of carbon-based defensive chemicals such phenolics in woody species (Koricheva et al. 1998). Herbivory can have positive effects on plant tissue quality by stimulating the production of nutrient-rich tissue during re-growth (Green and Detling 2000), or negative effects through stimulating the production of defensive chemicals (Sharam and Turkington 2009). Changes in the chemical content of plant tissue can have important consequences for the composition of the plant community, and also has important implications for the structure of the soil microbial community and rates of nutrient cycling (Wardle 2002).

While many studies have investigated plant community and plant tissue responses to nutrient enrichment or herbivory (Bakker et al. 2004, 2009, Bobbink et al. 2010, Cleland and Harpole 2010, Gilliam 2006, Olff and Ritchie 1998, Ritchie et al. 1998, Turkington 2009), fewer have investigated how plant communities recover from these disturbances (but see Clark and Tilman 2008, Clark et al. 2009, Strengbom et al. 2001, Strengbom and Nordin 2008). It is not known how long it takes plant communities to recover from fertilization or herbivore eradication, or if the effects are fully reversible. Studies that have investigated recovery from fertilization indicate the recovery process may be extremely slow, with changes in the plant community composition not being detectable until decades after fertilization is stopped (Strengbom et al. 2001, Strengbom and Nordin 2008). This recovery process can also be impacted by the biotic context of the plant community. Herbivores have the potential to either accelerate or slow down changes in plant community composition by selective feeding of plant species and by slowing or speeding up nutrient cycling (Gilliam 2006, Wardle and Bardgett 2004). For example, it is common in forests for herbivores to accelerate succession, as more palatable species are eaten. This promotes the dominance of slow-growing species with recalcitrant litter, slowing the cycling of nutrients and further reinforcing the competitive advantage of slow-growing species (Pastor et al. 1988, 1993). Alternatively, in more fertile systems, herbivores maintain the vigour of fast-growing species that have a high capacity for re-growth. This retards succession, as these fast-growing
species have easily decomposable litter, promoting nutrient cycling, and preventing the dominance of slow-growing, late-successional species (Augustine and McNaughton 1998). It is therefore important to understand the role herbivores play in controlling plant community dynamics under fertilization.

Given the pivotal importance of nutrients and herbivores in structuring plant communities and determining the chemical content of individual plants, it is important to conduct long-term studies researching their individual and interactive effects. This research may provide general insights into how top-down and bottom-up forces affect plants, as well as provide insights into how plant communities will respond to human-induced changes in the environment, such as increased nitrogen deposition, climate change, and species extinctions. Furthermore, as fewer and fewer ecosystems remain free from human-induced change, it is important to understand the extent to which the change is permanent or reversible.

This analysis comes in the 20th year of a long-term fertilization and herbivore reduction study in the understory of the boreal forest in southwest Yukon, Canada. The understory of the boreal forest has to cope with harsh conditions, including low-light levels, long winters with cold temperatures, brief summers and soils having low fertility. Also, the understory plant community is subject to herbivory by snowshoe hares that go through 8-10-year population cycles that can reach peak densities of up to 300 km$^{-2}$. The first 10 years of this study (Turkington et al. 2002) showed the plant community to be controlled predominately from the bottom-up. Annual fertilization caused reductions in both the richness and evenness of the community, as accompanied by major changes in composition from prostrate woody-species to herbaceous dicots and grasses. Herbivores had little effect on any measurements made on the plant community such as percent cover (Turkington et al. 2002) or phenology (Fremlin et al. in press). A small number of herbaceous species increased in abundance inside exclosures during the hare peak of 1990-91, when hare numbers were extremely high. When fertilized, a few species may have been fed upon preferentially.

In this study we use the 20th year of measurements on the plant community to address 3 questions:
1. What is the relative strength of top-down and bottom-up forces in this boreal forest understory after 20 years of treatments? Bottom-up forces dominated the system after 10 years of treatments, but short-term responses of plant communities to disturbances are not necessarily indicative of the longer-term response (Milchunas and Laurenroth 1995, Tilman et al. 1994).

2. What effects do fertilization and herbivory have on the chemical composition of plant tissues? Given that there was almost no response to herbivore removal by the plant community after 10 years, we extended the study to ask how fertilization and herbivory may affect individual plants.

3. Do plant communities recover from long-term fertilization and herbivore removal? After the first 10 years of this study were complete, treatments were discontinued in half of each plot, providing the opportunity for plots to recover. This gave us the opportunity to determine the long-term impact of fertilization, and the role of herbivores, in the recovery of this community.
2.2 Methods

2.2.1 Research Site

The study site is located in the understory of the boreal forest near Kluane Lake, in southwestern Yukon in northern Canada, and is described in detail in Turkington et al. (2002). The site is in the Shakwak Trench, a glacial valley in the rain shadow of the St. Elias Mountains. The area receives an annual mean precipitation of c. 280 mm, which falls mostly as rain during the summer. Snowfall during winter months averages about 100 cm at Burwash Landing, 40 km north of the research sites (Environment Canada 2010). The forest is a closed to open forest dominated by white spruce (*Picea glauca* (Moench) Voss) with stands of trembling aspen (*Populus tremuloides* Michx.) and balsam poplar (*Populus balsamifera* L.). The shrub community is dominated by shrub willows (*Salix glauca* (L.) and other *Salix* spp.), and also includes dwarf birch (*Betula glandulosa* Michx.) and soapberry (*Shepherdia canadensis* (L.) Nutt.). The ground layer consists chiefly of arctic lupine (*Lupinus arcticus* S. Wats.), northern rough fescue (*Festuca altaica* Torr.), twin-flower (*Linnaea borealis* L.), bearberry (*Arctostaphylos uva-ursi* (L.) Spreng.), bluebell (*Mertensia paniculata* (Aiton) G. Don), and yarrow (*Achillea millefolium* L. var. *borealis* (Bong) Farwell). In 1995, an outbreak of spruce bark beetle caused a massive die-off of adult spruce, which allowed more light to penetrate to the understory than in the past.

The principal herbivore in the study area is the snowshoe hare (*Lepus americanus* Erxleben), which undergoes 8-10 year population cycles (Krebs et al. 1986). During this experiment, hare population densities peaked on 3 occasions. In 1990 at the beginning of the experiment, hare numbers reached 148 km\(^{-2}\) and declined to 8 km\(^{-2}\) in 1994 (Boutin et al. 1995; Krebs et al. 1995). The second peak was in 1998 when hare densities reached 198 km\(^{-2}\) (Hodges et al. 2001). The most recent peak occurred in 2006 when hare densities reached 92 km\(^{-2}\), much lower than the previous two peaks, and declined to 29.5 km\(^{-2}\) by 2009 (Krebs 2011).
2.2.2 Experimental Design

The experimental site is 2 km south of Boutelier Summit, (61° 02' N, 138° 22' W; km 1695 on the Alaska Highway). The site is in a moderately open spruce forest, with a canopy cover of 45-65% and 160-220 stem/ha. The last time the sites were burned was probably 1872 (Dale et al. 2001, Francis 1996). The understory is well developed with approximately 90% vegetation cover.

In 1990, 16 5 x 5 m plots were set up in small meadows in the forest that had few rooted trees and minimal shrub cover. The plots were divided evenly into 4 treatments: no treatment (control), fertilizer only, exclosure only, fertilizer + exclosure. Exclosures are 1 m high, galvanized chicken wire with 2.5 cm mesh. Fertilizer (N:P:K 35:10:5) was applied in granular form -- N was applied as NH$_4$NO$_3$; P was applied as H$_3$PO$_4$; K was applied as K$_2$O. After snow melt in late May – mid June, 1.25 kg of fertilizer were added to 5 x 5 m plots, which amounts to an application rate of 17.5 g N/m$^2$/yr, 5 g P/m$^2$/yr, and 2.5 g K/m$^2$/yr. To prevent clonal species from connecting outside the plots, a 20 cm deep trench was dug around each plot.

In 2000, after 10 years of annual treatments, each of the 16 plots was divided in half. At random, one half of the plot ceased receiving treatments, while the other half continued to receive treatments for the next 10 years until 2009, resulting in 16 plots, in which half of each plot was continuously treated with fertilizer, exclosures, or both for 20 years, and the other half was free of treatment during the past 10 years. For ease of explanation, the treatments will be referred to as: C (+), C (-), F (+), F (-), E (+), E(-), FE (+) and FE (-), where C (Control), F (Fertilized), E (Exclosure), ‘+’ are the 20 years continuous treatment, and ‘-’ are those half plots that have been free of treatments from 2000-2009.

2.2.3 Measurement of Vegetation Composition

Percent cover of each plant species less than 1m tall was estimated in each half (sub)plot using point samples every 10 cm along five 2 m transects. This was done each year from 1990-2009, but only the data from 2009 were analyzed here.
2.2.4 Analysis of Leaf Tissue Carbon/Nitrogen (C/N), Phenolics, and Condensed Tannins

Plant leaves for the analysis of leaf tissue C/N, total phenolics, and condensed tannins were collected from subplots in mid-July, 2009, placed on ice, and brought to the nearby Arctic Institute of North America Base camp. Four species (*A. millefolium*, *M. paniculata*, *E. angustifolium*, and *F. altaica*) were selected because they were present in all treatments. *Mertensia paniculata* was absent from five of 32 subplots, and *E. angustifolium* was absent from two subplots. Leaf tissue was air-dried for one week in the shade, then finely ground for chemical analysis. Total phenolic content was determined by the Folin-Ciocalteu method (Waterton and Mole 1994), with tannic acid as a standard curve. Condensed tannins were analyzed by the butanol-HCl tannin assay modified from Porter et al. (1986), using an aspen tannin standard curve. Condensed tannins were not detectable in any plant tissue and will not be further discussed. The material was also analyzed for percent carbon and nitrogen using a CE-440 CHN/O/S Elemental Analyzer (Exceter Analytical).

2.2.5 Statistical Analyses

Treatment and interaction effects were analyzed using linear mixed effects models with fertilizer, exclosure and treatment cessation (Trt Cess: whether treated for 20 years or 10 years) as fixed effects and plot as the random effect using the “nlme” package (Pinheiro et al. 2010) in R 2.12.1 (R Development Core Team 2010). Linear mixed-effects models were used instead of regular split-plot ANOVAs due to some missing values in the plant tissue analyses (Crawley 2007). For consistency, linear mixed-effects models were used for all analyses. Data were transformed if necessary to satisfy the assumptions of independence of data, normality, and equality of variance. Despite transformations, there was still heterogeneity of variance in a number of models. To correct this, variance structures were assigned that corrected for different variances among experimental treatments using varIdent in the “nlme” package in R, as suggested by Zuur et al. (2009). Diagnostic plots and AIC values were used to select the best variance structure for each model. Species that had been analyzed by Turkington et al. (2002) were analyzed separately, while other species were lumped together as infrequent species. Wald F-tests with Type III Sums of Squares were used to test significance of terms in the models.
2.3 Results

2.3.1 Plant Community Composition

Fertilization for 20 years significantly affected the composition of the plant community, whereas herbivore removal had relatively little impact. Species richness declined from 13.75 in control plots to approximately 7.25 in fertilized plots, whether fenced or not (Figure 2.1 a). In fertilized plots, percent cover was significantly increased only in *E. angustifolium* (16 to 83.75 % cover), and *M. paniculata* (2.25 to 29.75% cover) (Figure 2.1 b and c). Five species declined in abundance; two prostrate woody species, *L. borealis* and *A. uva-ursi*, and *L. arcticus* and mosses all but disappeared from fertilized plots, though the effect is not statistically significant for *A. uva-ursi* and mosses; *F. altaica* declined from 55.25 to 3.75 % cover (Figure 2.1 d,e,f,g,h). The combination of infrequent species, as well as *A. millefolium*, and *Salix* spp. were unaffected by fertilization (Figure 2.1 i, j, k). The only species that were affected by herbivore exclosures were the mosses, which increased from 2.5 to 27.5 % cover in fenced areas (Figure 2.1 g).

In plots that were not fertilized for years11 - 20, species richness began to recover to control levels, reaching 10.75 species, nearing the 13.75 species in control plots (Fig. 2.1 a). Of the species affected by fertilization, only *F. altaica* and *M. paniculata* had significant returns towards control levels (Figure 2.1 h and c). With the exception of *L. arcticus*, there was a trend of all other species returning towards control abundances, but these changes in percent cover were not significant (p > 0.05).
a) *All species*
Fert: $F_{1,12} = 55.97$, $p < 0.001$
Fert x Trt Cess: $F_{1,12} = 9.89$, $p = 0.009$

b) *Epilobium angustifolium*
Fert: $F_{1,12} = 14.37$, $p = 0.003$
c) *Mertensia paniculata*

Fert: $F_{1,12} = 20.19$, $p = 0.001$

Fert * Trt Cess: $F_{1,12} = 3.33$, $p = 0.093$

d) *Linnaea borealis*

Fert: $F_{1,12} = 11.97$, $p = 0.005$
e) *Arctostaphylos uva-ursi*
Fert: $F_{1,12} = 4.01, p = 0.068$
Fert * Trt Cess: $F_{1,12} = 3.38, p = 0.091$

\[\text{Percent Cover}\]
\[\begin{array}{c|c|c|c|c}
 & C & E & F & FE \\
\hline
\text{Percent Cover} & 0 & 5 & 10 & 15 \\
\end{array}\]

f) *Lupinus arcticus*
Fert: $F_{1,12} = 21.18, p = 0.001$
g) *Moss species*

Exc: $F_{1,12} = 14.37, p = 0.003$

Fert x Exc: $F_{1,12} = 9.34, p = 0.010$

h) *Festuca altaica*

Fert: $F_{1,12} = 44.02, p < 0.001$

Fert * Trt Cess: $F_{1,12} = 9.66, p = 0.009$
i) *Infrequent species*

![Graph showing percent cover for Infrequent species across different treatments (C, E, F, FE).]

j) *Achillea millefolium*

![Graph showing percent cover for Achillea millefolium across different treatments (C, E, F, FE).]
Figure 2.1 Species richness (±1 SE) and % cover (±1 SE) in different experimental treatments for different species. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. Gray bars are plots treated for 20 years, black bars indicate plots that were treated for 10 years and then treatments were discontinued for years 11-20. Significant F statistics and p-values (p = 0.05) are shown based on Wald tests done on linear mixed-effects models.
2.3.2 Foliar Tissue Chemistry

C/N ratios in the plant leaf tissue of all four species (*A. millefolium*, *E. angustifolium*, *M. paniculata*, *F. altaica*) significantly decreased when fertilized (Figure 2.2 a,b,c,d), but began to recover towards control levels when treatments were stopped during years 11-20; the effect was not significant for *E. angustifolium* (Fert x Trt Cess: $F_{1,11} = 1.25$, $p = 0.288$). Herbivore exclusion did not significantly alter C/N ratios in any species.

When fertilized, total phenolics in leaf tissue significantly decreased in all species except *F. altaica* (Fert: $F_{1,12} = 1.60$, $p = 0.230$, Figure 2.3 a, b, c, d). Even though phenolic levels began returning to control levels in all species, especially when combined with the removal of exclosures (Fig. 2.3 a, b, c, d), the only significant interaction indicating recovery to control phenolic levels was for *A. millefolium* (Fert x Trt Cess: $F_{1,12} = 6.95$, $p = 0.022$). Exclosures alone did not alter the level of total phenolics in any species.
a) *Achillea millefolium*

Fert: $F_{1,12} = 325.66$, $p < 0.001$

Fert x Trt Cess: $F_{1,12} = 55.57$, $p < 0.001$

b) *Epilobium angustifolium*

Fert: $F_{1,11} = 61.06$, $p < 0.0001$
Figure 2.2 Carbon/nitrogen ratios (±1 SE) in foliar tissue of four species in different experimental treatments. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. Gray bars are plots treated for 20 years, black bars indicate plots that were treated for 10 years and then treatments were discontinued for years 11 – 20. Significant F statistics and p-values (p = 0.05) are shown based on Wald tests done on linear mixed-effects models.
a) *Achillea millefolium*
Fert: $F_{1,12} = 41.36, \ p < .0001$
Fert x Trt Cess: $F_{1,12} = 6.95, \ p = 0.022$

b) *Epilobium angustifolium*
Fert: $F_{1,11} = 6.36, \ p = 0.028$
Figure 2.3 Total phenolics (±1 SE) in foliar tissue of four species in different experimental treatments. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. Gray bars are plots treated for 20 years, black bars indicate plots that were treated for 10 years and then treatments were discontinued for years 11 – 20. Significant F statistics and p-values (p = 0.05) are shown based on Wald tests done on linear mixed-effects models.
2.4 Discussion

2.4.1 Top-Down and Bottom-Up Controls on the Plant Community

After 20 years of treatments on the understory of the boreal forest, results support the hypothesis of bottom-up control of the plant community, and reject the top-down hypothesis. The plant community responded strongly to fertilization, while almost no impact of herbivore removal was detected. As would be predicted by the bottom-up hypothesis, fertilization caused reductions in the richness and evenness of the community, shifted plant community composition towards herbaceous dicots, and increased the quality of plant tissue by reducing C/N ratios and total phenolics. Herbivore exclusion, on the other hand, only significantly increased the percent cover of mosses, and had no effect on the overall abundance of vegetation or the richness of the plant community. The community also showed signs of resilience to effects of nutrient enrichment, as plant community composition changed, albeit slowly, towards the species composition of control communities when the fertilizer treatment was stopped from years 11 - 20.

Results after 20 years reinforce the findings of the first decade. After 10 years, the vegetation community was largely controlled from the bottom-up. The large effect of fertilizer in boreal forests is not surprising. Boreal forests are adapted to low nutrient conditions, and many studies have shown the sensitivity of the boreal forest understory to changes in nutrient levels. When fertilized, boreal forests typically have increases in grasses, and decreases in mosses and lichens, as well as woody species, such as shrubs (Strengbom et al. 2001, Strengbom and Nordin 2008). Only four species appeared to have the capacity to react positively to nutrient enrichment in our study: F. altaica, E. angustifolium, M. paniculata, and A. borealis (Turkington et al. 2002). Yet, since 1999, E. angustifolium and M. paniculata have competitively excluded the other two. It is also not surprising that few other species were able to increase in abundance with fertilization. Boreal systems are dominated by slow-growing, N-conservative species adapted to the short-growing seasons and nutrient-poor conditions. These species often lack the flexibility to respond to changes in resource levels (Graham and Turkington 2000).

A number of plausible mechanisms may account for the large impacts fertilization had on the plant community. Most obviously, fertilization increases the pool of available nutrients,
favouring faster-growing species such as some of the herbaceous dicots and grasses. Fertilization also generally increases the biomass of the community, consequently, tall plants cause more light interception leading to increasing light competition among smaller plants. Light limitation can be as important as nutrient levels in determining community structure in boreal forests (Strengbom et al. 2004). In this study, two tall herbaceous species, *E. angustifolium* and *M. paniculata*, dominated the fertilized plots, and the ratio of ground-level to above-canopy photosynthetic active radiation decreased from 0.62 in control plots to only 0.15 in fertilized plots (Chapter 3). This may explain the large reductions in the percent cover of *F. altaica* and *A. millefolium* during years 11 – 20, both of which initially showed positive responses to fertilization during the first 10 years of the study.

Fertilization with $\text{NO}_3^-$ and $\text{NH}_4^+$ has other consequences. Typically, due to low mineralization rates, boreal forest soils have low levels of $\text{NO}_3^-$, and most of the nitrogen available to plants is in the form of $\text{NH}_4^+$ or amino acids. Our fertilization experiment sharply increased levels of $\text{NO}_3^-$, relative to increases in $\text{NH}_4^+$, making $\text{NO}_3^-$ the dominant form of nitrogen in the soil (Chapter 3). Many tree, shrub and herbaceous plant species in the boreal forest have a limited capacity to use $\text{NO}_3^-$ (Kronzucker *et al.* 1997, Nordin *et al.* 2001, 2004, 2006). In nitrogen-rich habitats, plant species are well adapted to use $\text{NO}_3^-$, but have less capacity to take up organic forms of nitrogen and $\text{NH}_4^+$ (Nordin *et al.* 2001, 2006). This is a likely scenario in our study plots, where the slow-growing woody species declined greatly in fertilized plots.

Two other important consequences of nitrogen addition are soil acidification and changes in cation availability. The addition of $\text{NH}_4^+$ can cause the acidification of soil as $\text{NH}_4^+$ is nitrified in the soil. This acts as a double filter on the community, as acid-tolerant and nitrophilous species are favoured (Brunet *et al.* 1998). The pH of the soil in our study decreased from 6.05 to 4.97 in fertilized plots (Chapter 3). Finally, fertilization can reduce the availability of essential cations in the soil, such as $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$, which can limit the growth of plants (Gilliam *et al.* 1994, Gilliam 2006). $\text{Mg}^{2+}$ levels significantly decreased in fertilized plots, while $\text{Ca}^{2+}$ levels showed increases with fertilization, but declined drastically in the recovery plots (Chapter 3).

Although herbivory had little impact on the vegetation or on individual species, in this study, we know that it is important in the larger system (Sinclair *et al.* 2001). Herbivory can have
large impacts on plant communities of boreal and other northern ecosystems. In the Kluane region, snowshoe hares are known to have large effects on winter vegetation, especially during peak population densities. Shrubs such as Betula spp. and Salix spp., and the dominant tree, Picea glauca, often experience intense herbivory during the winter months, altering the growth and chemical composition of these species. Yet, our study showed an extremely limited response of the plant community to herbivory, with mosses being the only plants to increase in abundance in exclosures. This could be due to a number of reasons.

First, the density of hares may not have reached levels where significant damage to summer vegetation occurs. Hares may be limited by winter food shortages and predation, keeping hare numbers lower than would be necessary for populations to have a measurable impact on summer vegetation. Snowshoe hares go through 10-year population cycles, where in peak years, the abundance or hares can reach 300 km$^2$, but be as low as 8 km$^2$ in non-peak years. Evidence for a herbivore effect on a number of species occurred in the first peak during the study (1990-91), where F. altaica, A. millefolium, L. arcticus and M. paniculata showed some response to exclosures, but a herbivore effect was not detected in the 1998 peak. The third peak occurred in 2006, but hare densities were considerably lower (92 km$^2$) than the previous two peaks (198 km$^2$ in 1998 and 148km$^2$ in 1990). It is unlikely, then, that the abundance of hares reached high enough levels to significantly impact summer vegetation in recent years.

Second, hares may not be feeding frequently enough on the typical understory species in this study. This could be due to a high reliance on woody species, even in the summer months, or could be due to general unavailability of herbaceous vegetation due to chemical defense. Snowshoe hares are highly selective in their summer diet, feeding preferentially on a number of shrub species (Salix spp., B. glandulosa and S. canadensis) and a limited number of herbaceous species (F. altaica and L. arcticus)(Seccombe-Hett and Turkington 2008). During the first hare peak in 1990, both of the preferred understory species, F. altaica and L. arcticus, showed some response to fertilization and herbivory (Turkington et al. 2002), yet this interaction is less likely to occur now because both F. altaica and L. arcticus had declined to quite low abundances in 2009 in the fertilized plots.

There is some indication, though, that the exclusion of herbivores had a minor impact on plants in fertilized plots, despite the lack of statistically significant exclosure effects.
Epilobium angustifolium and M. paniculata had lower abundances in FE(+) plots than in F(+) plots, and F. altaica and infrequent species were slightly more abundant in FE(+) plots. This indicates herbivores may be further reducing F. altaica abundance in unfenced plots that have been fertilized, allowing for greater dominance by other species.

2.4.2 Nutrient and Herbivore Control of Foliar Tissue Chemistry

As with the composition of the plant community, fertilization had a greater impact on the foliar tissue chemistry of all four species than did herbivory. In general, foliar nitrogen concentrations increased, thereby decreasing C/N ratios, and decreased levels of total phenolics. These finding are consistent with previous studies investigating the impact of nutrient additions on foliar chemistry, and support hypotheses on the role of soil fertility in influencing concentrations of secondary metabolites in plants.

Many studies have shown that fertilization increases the nutrient content of foliar tissue and can have important community- and ecosystem-level consequences. Typically, nitrogen fertilization increases the concentration of nitrogen in plant tissue quite rapidly and this has been shown to increase susceptibility of plants to herbivory and infection by pathogens (Nordin et al. 2009, Strengbom et al. 2002, Throop and Lerdau 2004, Wiedermann et al. 2007), which could have further consequences for plant community composition. Furthermore, increased nutrient content in plant tissue can increase rates of decomposition (Cornwell et al. 2008) and change the soil microbial community composition (Wardle et al. 2004), potentially altering rates of nutrient cycling and eventually nutrient availability for the plants. The combination of changes in plant community composition to herbaceous dicots, which generally have higher nutrient contents than grasses or woody species, and increased levels of nutrients in the leaf tissue, makes increased rates of nutrient cycling a distinct possibility.

The reduction of total phenolics in leaf tissue is consistent with predictions of both the growth-differentiation balance (GDB) hypothesis (Herms and Mattson 1992) and the carbon-nutrient balance (CNB) hypothesis (Bryant et al. 1983) The GDB predicts that the concentration of secondary metabolites in plant tissue depends on the environmental context of the plant and the subsequent trade-offs a plant must make between allocating resources to growth (i.e. the production of new plant tissue) and processes of differentiation.
(e.g. the production of secondary metabolites). If an environmental factor slows growth more than photosynthesis, the plant will have more resources available for differentiation processes such as the production of defense chemicals, at little cost (Stamp 2003). Growth, typically, is more restricted by nutrient and water availability than is photosynthesis (Herms and Mattson 1992). Therefore, fertilization should allow plants to allocate more resources to growth, thereby decreasing levels of secondary metabolites. Herbivory, on the other hand, reduces the nutrients available for growth, allowing an excess of carbohydrates for producing secondary metabolites at little cost. The CNB makes similar predictions, though with different justifications (Stamp 2003). Although our experiment was not designed to test these hypotheses and is not a perfect test of their predictions (see Stamp 2003), our results are consistent with them; fertilization resulted in increased growth, and a decline in total phenolics.

The lack of response by plant tissue to herbivore removal was likely affected by the summer diet selection of the snowshoe hare. Of the four plant species studied, only _F. altaica_ is an important food source for snowshoe hares in this study area (Seccombe-Hett and Turkington 2008). Phenolic compounds are less important as defense compounds in grasses than silica and other physical defenses (Massey _et al._ 2007), so it is not surprising herbivore removal caused little change in the concentration of phenolics in _F. altaica_. Both _E. angustifolium_ and _A. millefolium_ are part of the snowshoe hare diet, but less so than would be expected based on their abundance. _Mertensia paniculata_ is generally not eaten by snowshoe hares. Therefore, the only way these species might be affected by herbivory is indirect, through snowshoe hares’ feeding on other species and altering nutrient cycling and availability for the plants, but hares were probably not present at a high enough density to cause these kinds of changes in ecosystem processes.

The use of total phenolics as a proxy for defensive chemicals in plant tissue is a very simplified measure, though it is commonly used (see Appel _et al._ 2001, Heil _et al._ 2002). Phenolics produced by plants are a diverse group of chemicals with many different functions, both defensive and structural (Appel 1993, Appel _et al._ 2001, Levin 1971). Therefore, interpreting these results in terms of plant defense must be done carefully and conservatively. For example, we cannot make any statements about differences between species. Despite the limitations in using total phenolics as a measure of investment in plant defense (Appel _et al._ 2001), phenolic compounds are still considered to be important in
deterring herbivory (Bryant et al. 1983, Coley et al. 1985, Herms and Mattson 1992),
controlling soil microbial community structure (Eskelinen et al. 2009) and can alter
ecosystem processes such as nutrient cycling (Meier et al. 2008, Northup et al. 1998,
Wardle et al. 2004))

2.4.3 Recovery After Cessation of Long-Term Treatments

Ten years after the cessation of treatments, the plant community is showing signs of
recovery, albeit slowly. The slow recovery of plant communities following nutrient
enrichment seems to be typical. Other studies have shown that decades after a fertilization
treatment in forests, effects on the herb-layer composition can be detectable in forests
(Strengbom et al. 2001, Strengbom and Nordin 2008). In the first decade after fertilization
has ceased, little recovery can be expected. At times, the richness of the community may
return to pre-fertilization levels, yet the composition of the community can be significantly
altered (Clark and Tilman 2008). So, while it appears that the effects of fertilization may be
somewhat reversible, the effects are long lasting, and the original composition of the
community may take decades to return.

In contrast to the slow recovery of the composition of the plant community, plant foliar tissue
is recovering quickly, as reported in other studies (Boxman et al. 1998, Clark et al. 2009,
Power et al. 2006). C/N ratios are approaching control levels in the previously fertilized
plots, and total phenolics in the leaf tissue are recovering, although they have not yet
reached control levels. It is likely that nitrogen concentrations in the litter are still elevated,
as there is likely a lag between living and dead plant tissue that can accumulate over many
years (Clark et al. 2009).

The removal of herbivores appears to have little impact on the recovery of the plant
community. Other studies have shown that herbivores can either accelerate or slow down
succession in ecosystems by selective feeding. In this system, if herbivores selectively fed
on E. angustifolium and M. paniculata, then we might expect the plant community to recover
more quickly, as the removal of these dominants would allow other, slower-growing species,
to return and increase abundance. However, if hares continue to feed on the more
infrequent species in fertilized plots, they might slow down the recovery of the plant
community. Although we did not explicitly test the effect of herbivores on recovery,
herbivory likely did not impact recovery rates much, given the minor impact of herbivores throughout the rest of the study.

2.4.4 The Importance of Long-Term Studies: Changes Since 1999

Long-term studies provide researchers with the ability to pick up long-term trends in the data, as well as unique events in the history of plant community. This study documented a noticeable shift in the composition of the plant community in the control plots since 1999. These shifts are not related to the experimental treatments, but show how ambient conditions may be changing in the boreal forest understory. A number of species have increased in abundance, including *E. angustifolium* (1.5 % cover in 1999, >15% cover in 2009), and *A. millefolium* (3.6 % cover in 1999, 6% cover in 2009). The most notable declines in abundance occurred in *A. uva-ursi* (28.3 % cover in 1999, 4 % cover in 2009) and *L. arcticus* (40.8% cover in 1999, 8 % cover in 2009). One plausible explanation is changing light availability. In 1995, a major outbreak of spruce bark beetle occurred that caused a mass die-off of mature white spruce. Since then, the canopy has opened allowing more light penetration to the understory, potentially favouring faster-growing species such as *E. angustifolium*, whereas prostrate woody species such as *A. uva-ursi* would be out-competed.

2.4.5 Conclusions

It is evident from this study and previous studies in this system (Turkington *et al.* 2002) that the primary control of plant community composition and dynamics in the boreal forest understory is the availability of nutrients. Fertilization caused significant changes in richness and composition of the plant community, as well as significantly altering the chemical composition of plant tissue. This study also indicates that recovery from fertilization may take a long time, but is possible. After a decade of no treatments, plots that had been fertilized are showing signs of recovery, both in plant community composition and in plant tissue quality, but the signs of recovery are small. This study highlights the importance of long-term studies, as the composition of the plant community in 2009 is noticeably different in both control and treatment plots from that of 1999.
3 THE EFFECTS OF FERTILIZATION AND HERBIVORE REDUCTION ON SOIL CHEMISTRY AND MICROBIAL COMMUNITY

3.1 Introduction

The soil microbial community (SMC) is an influential and relatively understudied component of many terrestrial systems. The soil microbial community, consisting of both fungi and bacteria, controls or influences many key ecosystem processes, such as decomposition, nutrient cycling, and carbon sequestration (O’Donnell et al. 2005, Wardle 2005, Zhu and Miller 2003). Furthermore, the soil microbial community can influence the outcome of plant-plant interactions, and alter rates of succession and ecosystem recovery (Wardle 2002). Just as the soil microbial community influences plant communities, the plant community exerts strong influences belowground by providing key resources to soil microbes (Wardle et al. 2004). Understanding how the soil microbial community responds to and recovers from anthropogenic disturbances such as fertilization and herbivore reduction is an important step in understanding how plant and soil communities respond to the same disturbances, and how they might influence each other.

In most global change models, soil communities are included as a ‘black box’, which implicitly assumes that the soil microbial community is either resistant or resilient to disturbance, although there is considerable evidence opposing this view (Allison and Martiny 2008). Indeed, the soil microbial community composition can be highly sensitive to fertilization and nitrogen deposition (Allison and Martiny 2008, Compton et al. 2004). A number of studies have shown decreases in the total microbial biomass with fertilization, though this is not always the case (Allison et al. 2008). In agricultural fields, grasslands and forests, fertilization typically decreases the ratio of fungi to bacteria, indicating a shift in the food web structure of soils (Bardgett et al. 1996, de Vries et al. 2006, Wallenstein et al. 2006). Large shifts in the make-up of fungal communities are common (Allison et al. 2008, 2009, 2010, Demoling et al. 2008), with mycorrhizal fungi being particularly susceptible (Hogberg et al. 2003, 2007, Lilleskov et al. 2001, 2002, Lucas and Casper 2008). Other...
rhizosphere-associated organisms such as gram-negative bacteria and nitrifying bacteria also are commonly negatively impacted by increases in mineral nutrients. Frequently, the ratio of gram-positive to gram-negative bacteria increases (Rinnan et al. 2007). These shifts in the soil microbial community may be directly related to altered nutrient conditions, but, at times, may also be the results of decreases in soil pH or changes in the vegetation community. Soil organisms have differing tolerances to low or high pH (Baath and Anderson 2003, Rousk et al. 2010), and are sensitive to the differing resources supplied by different plant species.

Despite the sensitivity of the soil microbial community composition to changes in nutrient availability, the effects on soil microbial community activity appear to be more variable and less clear (Allison et al. 2008, 2009, 2010, Bell et al. 2010, Carreiro et al. 2000, Chung et al. 2007). In general, it has been shown that nitrogen cycling and mineralization rates increase, and over time, there are increased rates of nitrogen loss through leaching and the volatilization of gaseous nitrogen (see Clark et al. 2009) – all processes influenced by the soil microbial community. Yet, the links between the soil microbial community and these ecosystem processes are not clear. Studies have shown that changes in the composition of soil microbial communities may or may not affect the activity and function of the soil community (Allison et al. 2008, Waldrop et al. 2004). One measure of the functionality of a soil microbial community is the measurement of extracellular enzyme activity (EEA). Extracellular enzymes are released by soil microbes and aid in the breakdown of organic substrates that enter the soil, making resources available for other soil organisms and the plant community. Extracellular enzyme activity is strongly correlated to decomposition rates in soils and different enzymes are produced to obtain nutrients from organic substrates. For example, phenol oxidase is a critical enzyme necessary for the breakdown of lignin, whereas cellobiohydrolase aids in obtaining carbon from cellulose. Other enzymes free key nutrients, such as nitrogen and phosphorus. It is hypothesized that soil microbes will increase the production of an enzyme if the nutrient needed is in limited supply (Sinsabaugh et al. 1994), and therefore offers a sensitive measurement of the resource requirements of the soil microbial community.

Extracellular enzyme activity can be impacted strongly by nutrient addition, though the results are often variable and ecosystem specific. Nitrogen deposition often slows the decomposition of recalcitrant litter, whereas more labile litter decomposes more quickly (Fog
The activity of enzymes involved in the breakdown of recalcitrant litter, such as phenol oxidase, is often suppressed by the addition of nitrogen (Sinsabaugh et al. 2002). Likewise, the activity of protein and chitin-degrading enzymes has been reported to decrease in response to high levels of nitrogen (Allison et al. 2008). Alternatively, enzymes involved in carbon acquisition such as those involved in cellulose breakdown increase (Allison et al. 2010). These results indicate that the soil microbial community's ability to function is also strongly impacted by changing nutrient levels, which could result in altered rates of decomposition and nutrient cycling.

Herbivores also have the potential to influence soil microbial community composition and function (Wardle 2002). Herbivores can alter the quantity and quality of resources entering the soil by causing changes in plant community composition, changing tissue chemistry and root exudation patterns in plants, and by direct inputs of feces and urine (Wardle and Bardgett 2004). For example, grazing by sheep has been shown to increase bacteria relative to fungi in the soil by causing changes in root carbon allocation (Bardgett et al. 1996, 1998). Also, by reducing aboveground biomass, herbivores reduce the amount of resources entering the soil, and decrease soil microbial biomass (Northup et al. 1999). Other studies have shown that herbivores can influence ecosystem processes such as nitrogen mineralization rates positively (Frank andGroffman 1998, Tracy and Frank 1998), negatively (Pastor et al. 1988, Van Wijnen et al. 1999), or not at all (Stark and Grellman 2002). The direction and magnitude of the effect of herbivores on soil microbes depends on many factors (e.g. site fertility, plant species, intensity of herbivory, etc.) and it is therefore difficult to make specific predictions as to their effects.

Not only is there considerable evidence that soil microbial community composition and function are sensitive to disturbance, there is also evidence that soil microbes and the processes they influence are not as resilient as commonly assumed (Allison and Martiny 2008). The recovery of soil microbial communities may lag behind plant communities after disturbances, thereby also inhibiting the plant community’s recovery (Holtkamp et al. 2008). van der Wal (2006) showed that fungal biomass was still low three decades after agricultural land use ceased, although there are indications of more rapid recovery in other studies (Bardgett et al. 1996). Spore counts of fungi in boreal forest soils were still depressed 9 and 47 years after fertilization ceased (Strengbom et al. 2001). In a nitrogen-reduction experiment designed to simulate recovery after nitrogen deposition, the microbial and fungal
biomass had only risen slightly after 16 years (Enowashu et al. 2009). Changes in biogeochemical cycling can persist for a long time; studies have shown nitrogen mineralization and cycling can remain elevated for decades after fertilization or nitrogen deposition ceases (Boxman et al. 1998, Chen and Hogberg 2006). Therefore, given the intricate and complex relationship between the soil microbial community, the plant community, and key ecosystem processes, it is important to further explore how the soil microbial community responds to fertilization and herbivore removal, and its subsequent recovery when these treatments are stopped. This study seeks to investigate the relationship between soil microbial community structure and function, and the role of nutrients and herbivores in the regulation of soil microbial community composition and function in a boreal forest understory. Furthermore, understanding both plant (Chapter 2) and soil microbial responses to fertilization and herbivore removal may allow us to better predict how boreal forests will respond to widespread anthropogenic change.

This study was done in the 20th year of a long-term fertilization and herbivore removal experiment in the understory of the boreal forest in southwest Yukon, Canada. The plant community has been intensively studied, and bottom-up forces predominately control vegetation community composition and plant tissue chemistry (Chapter 2, Dlott and Turkington 2000, John and Turkington 1995, 1997, Turkington et al. 1998, 2002). Typically, the boreal forest understory vegetation in our region consists of a well-mixed community of low-growing woody shrubs (Arctostaphylos uva-ursi, Linnaea borealis), grasses (primarily Festuca altaica) and a variety of herbaceous dicots (Lupinus arcticus, Achillea borealis, Mertensia paniculata). After long-term fertilization, the community became dominated by two species of herbaceous dicots (Epilobium angustifolium and Mertensia paniculata). Once fertilization treatments were stopped, plant species began returning to pre-treatment abundances, but the recovery has been slow.

The objective of this chapter is to explore changes in the soil microbial community and soil abiotic properties that may be driving the change and recovery of the plant community. We seek to address three primary questions:

1) How does fertilization and herbivore removal influence soil microbial community composition and function, and the abiotic properties of the soil?

The addition of nutrients caused significant changes in the plant community (Chapter
2, Turkington et al. 2002). This fertilization potentially altered many variables in the soil besides nutrient availability, and this study gives us the opportunity to investigate changes in the soil that might be affecting the plant community.

2) **How do the soil microbial community and abiotic properties of the soil recover from fertilization and herbivore removal?** After the first 10 years of the experiment (1990-1999), treatments were stopped in half of each plot. This allows us to explore the long-term consequences of fertilization and herbivore removal on the soil, and its ability to recover.

3) **Are the abiotic properties of the soil, soil microbial community composition, and soil microbial community function related?** By measuring key components of the soil (abiotic properties, soil microbial community and activity), we can attempt to determine how these variables relate to each other.
3.2 Methods

3.2.1 Study Site and Experimental Design

The experimental site is located near Kluane Lake in the south-western Yukon, 2 km south of Boutelier Summit, (61° 02' N, 138° 22' W; km 1695 on the Alaska Highway). The site is in a moderately open spruce forest, with a canopy cover of 45-65% and 160-220 stem/ha. The last time the sites were burned was probably 1872 (Dale et al. 2001, Francis 1996). The understory is well developed with approximately 90% vegetation cover. The soils of this forest are brunisolic, with a thin organic layer approximately 2-10 cm deep. More detailed accounts of the vegetation and climate are in Chapter 2.

The principal herbivore in the study area is the snowshoe hare (Lepus americanus Erxleben). It undergoes 8-10 year population cycles (Krebs et al. 1986), reaching densities of up to 148 km\(^{-2}\) and lows to 8 km\(^{-2}\) (Boutin et al. 1995; Krebs et al. 1995). The hare cycle peaked three times during the course of this study, in 1990, 1998, and 2006.

In 1990, 16 5 x 5 m plots were set up in small meadows in the forest that had few rooted trees and minimal shrub cover. The plots were divided into 4 treatments: no treatment (control), fertilizer only, exclosure only, fertilizer + exclosure. Exclosures are 1 m high galvanized chicken wire with 2.5 cm mesh. Fertilizer (N:P:K 35:10:5) was applied in granular form -- N was applied as NH\(_4\)NO\(_3\); P was applied as H\(_3\)PO\(_4\); K was applied as K\(_2\)O. After snow melt in late May – mid June, 1.25 kg of fertilizer were added to 5 x 5 m plots, which amounts to an application rate of 17.5 g N/m\(^2\)/yr, 5 g P/m\(^2\)/yr, and 2.5 g K/km\(^2\)/yr. To prevent clonal species from connecting outside the plots, a 20 cm deep trench was dug around each plot.

In early 2000, before the 11\(^{th}\) year of annual treatments, each of the 16 plots was divided in half. At random, one half of the plot ceased receiving treatments, while the other half continued to receive treatments for the next 10 years until 2009, resulting in 16 plots, in which half of each plot was continuously treated with fertilizer, exclosures, or both for 20 years, and the other half was free of treatment during the past 10 years. For ease of explanation, the treatments will be referred to as: C (+), C (-), F (+), F (-), E (+), E(-), FE (+)
and FE (-), where C (Control), F (Fertilized), E (Exclosure), ‘+’ are the 20 years continuous treatment, and ‘-’ are those half plots that have been free of treatments from 2000-2009.

3.2.2 Photosynthetic Active Radiation

Photosynthetic active radiation (PAR) was measured in early July 2009 using a Spectrum 6 Sensor Quantum bar (Apogee Instruments Inc., Logan, UT, USA). Ten measurements were taken on each half-plot, five taken at ground level (~5 cm) and five above the canopy of understory plants (~1.05m). PAR was calculated as the ratio of ground-level to above-canopy measurements, indicating the difference in light availability due to treatments. Measurements were all taken on an overcast day (Parent and Messier 1996).

3.2.3 Abiotic Soil Response Measurements

Ten cores, each 2 cm in diameter and 20 cm deep, were collected to assess rooting depth. Greater than 90% of roots were found in the organic layer. Five additional soil cores, 2 cm diameter and consisting of the entire organic layer (2 – 8 cm deep), from each subplot were collected in mid-July, 2009, and pooled together. Samples were air-dried for one week. To test soil pH, approximately 10 g of organic layer soil were mixed with deionized water in a 1:2 ratio of soil to water. Measurements were made using a Waterproof pH Tester 20 (Eutech Instruments, Illinois) after stirring the solution periodically for 30 minutes, followed by no stirring for 30 minutes. The remaining soil was ground and analyzed for % carbon and nitrogen using a CE-440 CHN/O/S Elemental Analyzer (Exceter Analytical, Coventry, UK).

Soil moisture levels were determined using a Hydrosense Water content measurement system (Campbell Scientific, Thuringowa Central, QLD, Australia) at ten locations in each subplot on July 9, 2009 to get an average for the subplot.

Ion exchange membranes (Plant Root Simulator (PRS)™ probes; Western Ag Innovations Inc., Saskatoon, SK) were used to measure in situ soil nutrient supply rates. Eight probes (4 anion, 4 cation) were placed randomly in each subplot at the beginning of the growing season in early June. Probes were removed in mid-August and analyzed by Western Ag Innovations Inc., (Saskatoon, SK), for NO₃, NH₄, P, K, S, Ca, Mg, Mn, Fe, Cu, Zn, B, Al, and Pb.
3.2.4 Soil Microbial Community Measurements

In late July 2009, ten cores were obtained from each subplot, and were composited to form a single sample. Each core consisted of the entire organic layer, and was 2 cm in diameter. Soils were placed on ice, shipped to Vancouver, BC the following day and placed in a -20°C freezer within 24 hours of collection. After about 2 months storage, samples were briefly thawed and were sieved using a 2mm sieve, allowing for homogenization. A subset of each sample designated for phospholipid fatty acid analysis was freeze-dried in batches of 11-12 for 48 hours. All samples were returned to the -20°C freezer.

3.2.4.1 Phospholipid Fatty Acid (PLFA\(^2\)) Analysis

The structure of the living soil microbial community structure was determined by phospholipid fatty acid (PLFA) analysis, modified from Bligh and Dyer (1959) and Frostegard et al. (1993). Different taxonomic groups of soil microorganisms can be identified by unique membrane lipids associated with them. Approximately 0.75-1.0 g of freeze-dried soil was extracted using citrate buffer, methanol and chloroform. Membrane lipids were extracted from approximately 0.75-1.0 g of freeze-dried soil with citrate buffer, methanol and chloroform. Chloroform was then used to split the extract into two phases. The lipid phase was collected and stored in the dark at -20°C. The lipid phase was then passed through a solid phase extraction column. Neutral lipids and waxes were eluted with acetone and were discarded. Phospholipids were eluted with methanol, collected, and converted to fatty acid methyl esters (FAMEs) via methylation with methanol, potassium hydroxide, toluene and hexane. These were analyzed using gas chromatography. Methyl nonadecanoate fatty acid (19:0) was used as the internal standard.

\(^2\) PLFA nomenclature follows Steer and Harris (2000). The number of carbon atoms is followed by a colon, then the number of double bonds. The suffix ‘\(\omega\)’ following the number of the first carbon atom is used to indicate the position of the double bond from the methyl end of the molecule. The ‘c’ after the double-bond configuration refers to a cis configuration. Branched fatty acids are indicated by the prefix ‘i’ for iso-branching, and ‘a’ for an anteiso-branching pattern. 10Me indicates a methyl-group on the tenth carbon from the carboxyl end of the molecule. The prefix ‘cy’ refers to cyclopropane fatty acids.
Different taxonomic groups can be identified by membrane lipids unique to the group. Gram-positive bacteria are represented by i15:0, a15:0, i16:0, 10Me16:0, 10Me17:0, a17:0, i17:0, 10Me18:0, and Me19:0. PLFAs indicative of gram-negative bacteria were considered to be 16:1ω7c, i16:1ω7c, i17:1ω8c, cy17:0, 18:1ω7c, 18:1ω5c, cy19:0 (Zogg et al. 1997), and 16:1ω9c (Fritze et al. 2000). Actinobacteria are represented by 10Me16:0, 10Me17:0, 10Me18:0, 10Me19:0 (Kroppenstedt 1992). Two specific PLFAs are representative of all bacteria: 15:0 (Federle 1986, Frostegard et al. 1993, Tunlid et al. 1989) and 17:0 (Tunlid et al. 1989). A ratio of gram-positive to gram-negative bacteria was calculated according to the above designation. The sum of all bacterial PLFAs listed was also used as a measure of total bacteria. Fungal PLFAs include 18:2ω6,9 and 18:1ω9c for saprophytic fungi (Bardgett et al. 1996, Stahl and Klug 1996), and were used to calculate total fungal biomass. PLFA 16:1ω5c is indicative of arbuscular mycorrhizae (Balser et al. 2005, Olson 1999), although PLFA 16:1ω5c is also found to a lesser degree in prokaryotic organisms (mostly gram-negative bacteria) and must be interpreted carefully. The sum of all the indicated PLFAs was used as a measure of total biomass, and also included two unclassified PLFAs: 16:0 and 18:0 (Mitchell et al. 2010). For consistency in comparing results from different studies, the fungal to bacterial biomass ratio was calculated using the bacterial PLFAs i15:0, a15:0, i16:0, a17:0, cy17:0, 18:1ω7c, and cy19:0, while the PLFA 18:2ω6,9 was used to represent fungi (Bardgett et al. 1996). Two indicators of stress in the microbial community were also calculated. When under stress (e.g. changes in pH, temperature, heavy metals, fertilizer), bacterial membrane lipids change, including an increase in cyclopropyl fatty acids to their monoenic precursors (Iyyemperumal and Shi 2007, Kieft et al. 1997). Two stress ratios were calculated: cy19:0 to its precursor 18:1ω7c, and cy17:0 to its precursor 16:1ω7c. All values, except total microbial biomass, total bacteria, and total fungi, are expressed as a proportion of the total PLFA (mol%). Units for the total microbial biomass are nmol of PLFA/g of soil.

3.2.4.2 Extracellular Enzyme Activity (EEA)

When the soils were first removed from -20°C freezer and thawed, subsets of each sample were weighed for enzyme assays. Freezing of samples does not affect comparisons of treatments, and is common in other studies (e.g. Keeler et al. 2009). Assays for the activity rates of six enzymes were adapted from the procedure of Sinsabaugh et al. (2003) for fluorometric and spectrophotometric assays, and Sinsabaugh et al. (2000) for urease.
Fluorometric measurements were conducted for β-1,4-N-acetylglucosaminidase (NAG), cellobiohydrolase (CBH), β-1,4-glucosidase (BG), and acid phosphatase (aP). CBH and BG are involved in the breakdown of cellulose: CBH depolymerises cellulose into cellobiose, and BG hydrolyzes cellobiose to glucose (Keeler et al. 2009). NAG aids in the breakdown of chitin, thereby assisting in the acquisition of organic N. aP releases PO₄³⁻ from organic phosphorus sources, and therefore indicates the investment of microbes in phosphorus acquisition. 0.1 g of sieved soil was homogenized in 100 mL of 50 mM acetate buffer (pH 5.0). Soil suspensions, substrate (NAG = 4-MUB-N-acetyl-β-glucosaminide, CBH = 4-MUB-β-D-cellobioside, BG = 4-MUB-β-D-glucoside, AP = 4-MUB-phosphate), acetate buffer, and MUB standard (10 µM 4-methylumbelliferone) were added to 96-well plates. For the analysis, 16 replicate sample wells (200 µl soil suspension + 50 µl substrate), 8 replicate blank wells (200 µl soil suspension + 50 µl buffer), 8 negative controls wells (50 µl substrate + 200 µl buffer), and 8 standard quench wells (50 µl MUB standard + 200 µl soil suspension) were done per assay. Plates were incubated at 20°C for 2-7 hours, depending on the substrate. At the end of the incubation, 20 µl aliquots of 0.5 N NaOH were added to each well to stop the reactions. Measurements of activity were made fluorimetrically, and are calculated as nmol of substrate converted per hour per gram of soil (nmol/h/g).

For phenol oxidase, an enzyme involved in the breakdown of recalcitrant litter and lignin, 0.5 g of homogenized soil was added to 100 mL of 50 mM acetate buffer (pH 5.0). Assays were done with 25 mM L-3,4-dihydroxyphenylalanine (DOPA) as a substrate. In 96-well plates, 16 replicate samples wells (200 µl soil suspension + 50 µl substrate), 8 replicate negative control wells (50 µl substrate + 200 µl buffer), 8 replicate blank wells (200 µl soil suspension + 50 µl buffer), and 8 buffer control wells (250 µl buffer) were used per assay. Plates were incubated at 20°C for 18hr. Measurements of absorbance were made spectrophotometrically and are expressed as µmol of substrate converted per hour per g of soil (µmol/h/g).

The activity rates of amidohydrolase (urease), which converts urea to ammonium and carbon dioxide and is important for nitrogen mineralization, were measured spectrophotometrically with urea as a substrate. 2.0 g of soil were added to 100 mL of 50 mM acetate buffer (pH 5.0) to create a soil suspension. 96-well plates consisted of 16 replicate sample wells (10 µl substrate + 200 µl soil suspension), 8 replicate negative control wells (10 µl dH2O + 200 µl soil suspension), 8 replicate substrate control wells (10 µl
substrate + 200 µl buffer), 8 replicate positive control wells (10 µl dH20 + 200 µl 1 µM ammonium chloride) and 8 replicate buffer control wells (10 µl dH20 + 200 µl buffer). Plates were incubated at 20°C for 18 hr. Salicylate and cyanurate reagents were added to the wells and allowed to sit for 20 min to quantify ammonium concentrations before readings were made with the spectrophotometric plate reader. Units are expressed as µmol of ammonium released per hour per g of soil (µmol NH₄⁺/h/g).

3.2.5 Data and Statistical Analyses

The experiment was designed as a split-plot experiment, with plots being treated with fertilizer, exclosures, or both, and subplots being either treated continuously for 20 years, or treated for 10 years and allowed to recover for 10 years. To analyze the data, a combination of principal components analyses (PCA) and linear mixed effects models were used. PCAs were conducted separately for nutrient and other chemical species, PLFAs, and the activity of extracellular enzymes. The PCA for nutrients and chemical species in the soil consisted of all those detected with the PRS probes. PCA for PLFA data included the 24 PLFA’s mentioned in the methods, and was conducted after PLFA abundances were converted to mol %. PCA for EEA data included five of six enzymes, excluding only phenol oxidase because it was undetectable in all samples. All PCAs were scaled using the correlation matrix, to deal with large differences in variance. Pearson’s correlation coefficients were calculated between the first three PCA axes of the nutrient, enzyme and PLFA data, as well as a soil pH and soil C/N ratio. All PCAs were done in R 2.12.1 (R Development Core Team 2010), using the “prcomp” function.

Linear mixed effects models were used to determine the effect of treatments and detect recovery in plots. Main effects included the experimental treatments of fertilizer (Fert) and exclosures (Exc) and whether or not the treatments were discontinued (Trt Cess). Plot was included as a random effect. All analyses were done using the “nlme” package (Pinheiro et al. 2010) in R 2.12.1 (R Development Core Team 2010). Linear mixed-effects models were used instead of regular split-plot ANOVAs due to missing values in the plant tissue analyses (Crawley 2007). We continued to use linear mixed-effects models to keep the statistical analyses consistent. Data were transformed as necessary to satisfy assumptions of normality and homogeneity of variance. Despite these efforts, there was still some heterogeneity of variance in a number of models. To correct this, variance structures were
assigned that corrected for differing variances among experimental treatments using varIdent in the “nlme” package in R, as suggested by Zuur et al. (2009). Diagnostic plots and AIC values were used to select the best variance structure for each model. Wald F-tests with Type III Sums of Squares were used to test the significance of terms in the models.
3.3 Results

3.3.1 Soil Chemistry

Fertilization for 20 years significantly altered the concentration of various nutrients in the soil, while herbivore removal had very little impact. Principal components analysis of the nutrient content of the soil showed that those plots that were fertilized for 20 years were strongly differentiated from all other plots along PC1 (Fig. 3.1 a), and to a much lesser extent along PC2. PC1 accounted for 48.7 % of the total variation, and PC2 accounted for only 12.0 % of the total variation. Those nutrients that generally increased with fertilization were positively aligned along PC1 (Fig. 3.1 b). The differentiation along PC2 is less clear.
Figure 3.1 Summary results of a PCA for soil chemistry, a) PCA scores from different treatments ± 1 SE, b) PCA loadings for specific chemical species. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. (+) symbols are plots treated for 20 years, (-) symbols are plots that were treated for 10 years and then treatments were discontinued for years 11-20.
When analyzed separately, many nutrients increased in concentration, most notably $\text{NH}_4^+$ and $\text{NO}_3^-$; the levels of P, K, S, Al, Zn and Mn also increased (Table 3.1). Mg was the only nutrient that decreased in concentration with fertilization, and concentrations of Ca, Cu, and B were not significantly altered. With the exception of K and Mg, the availability of all nutrients returned to control levels after fertilization ceased. Mn showed a significant effect of treatment cessation in the control plots ($F_{1,12} = 6.76, p = 0.0232$), although this result is probably not biologically relevant as availability only increased 1.65 $\mu$g/10cm$^2$/61 days above control levels, whereas fertilization caused an increase of 45.6 $\mu$g/10cm$^2$/61 days above control levels. Levels of Pb and Cd were not detectable in the soils.
Table 3.1 Mean, standard error (S.E.) and Wald test statistics (F-ratio and p-values) from linear mixed-effects models for the effects of fertilization, exclosures (herbivore removal) and treatment cessation on fertilized plots on soil and ecosystem properties. Values in bold are significant (p<0.05). Levels of Pb and Cd were not detectable in any samples. PAR equals the ratio of ground-level to canopy level measurement of photosynthetic active radiation. All measurements except for PAR and soil moisture are in µg/10cm²/61 days. Mean and standard error calculations based on half plots (control, exclosure and fertilizer = treated 20 years continuous, fertilizer x trt cess = fertilized for 10 years then allowed to recover).

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 4)</th>
<th>Exclosure (n = 4)</th>
<th>Fertilizer (n = 4)</th>
<th>Fertilizer x Trt Cess (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.E.</td>
<td>Mean</td>
<td>S.E.</td>
</tr>
<tr>
<td>PAR</td>
<td>0.62</td>
<td>0.08</td>
<td>0.58</td>
<td>0.09</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>17.15</td>
<td>2.10</td>
<td>20.65</td>
<td>4.23</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>1.55</td>
<td>0.66</td>
<td>2.40</td>
<td>0.54</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>6.60</td>
<td>1.43</td>
<td>7.15</td>
<td>1.53</td>
</tr>
<tr>
<td>P</td>
<td>13.10</td>
<td>2.79</td>
<td>11.75</td>
<td>4.87</td>
</tr>
<tr>
<td>K</td>
<td>122.60</td>
<td>31.33</td>
<td>126.90</td>
<td>27.57</td>
</tr>
<tr>
<td>S</td>
<td>2.85</td>
<td>0.86</td>
<td>1.90</td>
<td>1.10</td>
</tr>
<tr>
<td>Ca</td>
<td>1703.15</td>
<td>115.60</td>
<td>1583.65</td>
<td>276.87</td>
</tr>
<tr>
<td>Al</td>
<td>27.35</td>
<td>4.44</td>
<td>21.25</td>
<td>0.82</td>
</tr>
<tr>
<td>Mn</td>
<td>0.95</td>
<td>0.28</td>
<td>1.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Mg</td>
<td>293.50</td>
<td>28.90</td>
<td>243.85</td>
<td>27.40</td>
</tr>
<tr>
<td>Cu</td>
<td>0.25</td>
<td>0.05</td>
<td>0.25</td>
<td>0.05</td>
</tr>
<tr>
<td>Zn</td>
<td>0.45</td>
<td>0.10</td>
<td>0.60</td>
<td>0.14</td>
</tr>
<tr>
<td>Pb</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cd</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>1.40</td>
<td>0.54</td>
<td>1.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Fertilization significantly altered other measured ecosystem properties. Soil pH declined from 6.05 to 4.98 (Figure 3.2 a). In plots where fertilization was stopped, pH is returning towards control levels (Figure 3.2 a). Soil C/N declined from 25.7 to 19.5 (Figure 3.2 b) with fertilization, and has not returned to control levels with the cessation of treatment. The ratio of ground-level to above-plant photosynthetic active radiation (PAR) also decreased from 0.62 to only 0.15 with fertilization and is returning to control levels, though the effect is not significant (Table 3.1). Soil moisture was not significantly altered by any of the treatments (Table 3.1).
Figure 3.2 a) pH (±1 SE) and b) % C/N ratio (±1 SE) of the soil organic layer in different experimental treatments for different species. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. Gray bars are plots treated for 20 years, black bars are plots that were treated for 10 years and then treatments were discontinued for years 11-20. Significant F statistics and p-values (p < 0.05) are shown based on Wald tests done on linear mixed-effects models.

a) Fert: $F_{1,12} = 75.68$, $p < .0001$,
Fert x Trt Cess: $F_{1,12} = 54.31$, $p < 0.001$

b) Fert: $F_{1,12} = 11.33$, $p = 0.006$
3.3.2 Soil Microbial Community Composition

Fertilization caused differentiated PLFA profiles for the SMC, whereas plots where herbivores were excluded did not differ from controls (Fig. 3.3a). The first three principal components accounted for 63% of the total variation in PLFAs. PC1 accounted for 33.1% of the total variation; PC2 and PC3 accounted for 17.4% and 13.3% of the total variation, respectively. Individual PLFA’s had different loadings along PC1 and PC2 (Fig. 3.3b). In particular, the PLFAs representing gram-negative bacteria cy19:0, cy17:0, 16:1ω7c, the gram-positive bacterial PLFA a15:0, as well as the more general PLFAs 16:0, and 18:0, had positive loadings along PC1. The gram-negative bacterial PLFAs 18:1ω5c, 18:1ω7c, 16:1ω9c, 16:1ω5c, and the fungal PLFA 18:2ω6,9 had negative loadings on PC1. On PC2, PLFAs 18:1ω9c, 16:1ω7c and 16:0 had negative loadings, while a large number of PLFAs had positive loadings, including PLFAs representative of actinobacteria (10Me18:0 and 10Me19:0), gram-positive bacteria (i17:0, a17:0, i16:0, i15:0) and gram-negative bacteria (i16:1ω7c).
Figure 3.3 Summary results of a PCA for PLFA data (soil microbial community composition), a) PCA scores from different treatments ± 1 SE, b) PCA loadings for specific PLFAs. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. (+) symbols are plots treated for 20 years, (-) symbols are plots that were treated for 10 years and then treatments were discontinued for years 11-20.
The total PLFA declined by 25% with fertilization (Fig. 3.4 a), and the cessation of fertilization did not indicate a return to control levels. The ratio of fungi to bacteria also significantly decreased from 0.26 to 0.18 (Fig. 3.4 b). Reduction in the total microbial biomass was driven by decreases in both total bacteria (Fig. 3.4 c) and fungi (Fig. 3.4). The fungi were disproportionately affected, decreasing in abundance by 37%, whereas the total bacteria only decreased by 25%, causing the decrease in the fungi to bacteria ratio. The ratio of gram-positive to gram-negative bacteria remained unchanged with fertilization (Fig. 3.4 e). Fertilization significantly altered the two stress ratios, cy17:0 to 16:1o7c (Fig. 3.4 f) and cy19:0 to 18:1o7c (Fig. 3.4 g), and both recovered with the cessation of fertilization treatments.
Total PLFA nmol/g

a) Fert: $F_{1,12} = 8.76$, $p = 0.012$

Fungi : Bacteria

b) Fert: $F_{1,12} = 11.37$, $p = 0.006$
c) Fert: $F_{1,12} = 7.28$, $p = 0.019$

d) Fert: $F_{1,12} = 20.15$, $p = 0.001$
Gram-positive / Gram-negative bacteria

e) Fert: $F_{1,12} = 4.56$, $p = 0.054$

f) Fert: $F_{1,12} = 7.90$, $p = 0.016$
Fert x Trt Cess: $F_{1,12} = 14.97$, $p = 0.002$
Figure 3.4 Results from PLFA analysis of the soil organic layer, a) total PLFA (microbial biomass) (±1 SE), b) ratio of fungi to bacteria (±1 SE), c) total bacteria (±1 SE), d) total fungi (±1 SE), e) ratio of gram negative to gram positive bacteria (±1 SE), and f,g) stress ratios (±1 SE). C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosures. Gray bars are plots treated for 20 years, black bars are plots treated for 10 years and then treatments were discontinued for years 11-20. Key F statistics and p-values (p = 0.05) are shown based on Wald tests done on linear mixed-effects models.
3.3.3 Extracellular Enzyme Activity

Extracellular enzymes showed minimal responses to fertilization and herbivore removal, as detected by PCA. A PCA of five enzymes showed little differentiation in soils due to treatments (Fig. 3.5 a), although loadings of individual enzymes showed more distinctive responses of enzymes along PC1 and PC2 (Fig. 3.5 b). PC1 accounted for 43.6 % of the total variation and PC2 accounted for 27.1 %. Urease was the only enzyme with a positive loading along PC1. The enzymes involved in carbon acquisition (cellobiohydrolase, β-1,4-glucosidase) and urease were positively loaded on PC2, while the nitrogen acquiring enzyme, N-acetyl-glucosamidase, and the phosphorus-acquiring enzyme acid phosphatase were negatively loaded on PC2.
Figure 3.5 Summary results of a PCA for extracellular enzyme activity assay data (soil microbial community activity), a) PCA scores from different treatments ± 1 SE, b) PCA loadings for extracellular enzymes. C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosure. (+) symbols are plots treated for 20 years, (-) symbols are plots that were treated for 10 years and then treatments were discontinued for years 11-20.
When analyzed separately, fertilization significantly increased the activity level $\beta$-1,4-glucosidase (Fig 3.6 a) and cellobiohydrolase (Fig. 3.6 b), and decreased activity rates of urease (Fig. 3.6 c). Fertilization did not significantly alter activity rates of N-acetyl-glucosamidase or acid phosphatase (Fig. 3.6 d, e). Herbivore exclosures did not significantly alter activity rates of any enzymes under study, though activity rates of urease decreased slightly. Cellobiohydrolase and $\beta$-1,4-glucosidase activity rates showed signs of returning to pre-treatment levels, though the interaction is not significant. Activity rates of phenol oxidase were not detectable in any samples.
a) Fert: $F_{1,12} = 4.34, p = 0.059$

b) Fert: $F_{1,12} = 4.96, p = 0.046$
c) Fert: $F_{1,12} = 6.20, p = 0.028$
Figure 3.6 Results from extracellular enzyme activity assays of the soil organic layer, a) β-1,4-glucosidase (±1 SE), b) cellobiohydrolase (±1 SE), c) urease (±1 SE), d) N-acetyl-glucosamidase (±1 SE), and e) acid phosphatase (±1 SE). C = control, E = herbivore exclosures, F = fertilizer, FE = fertilizer + herbivore exclosures. Gray bars are plots treated for 20 years, black bars are plots treated for 10 years and then treatments were discontinued for years 11-20. Key F statistics and p-values (p = 0.05) are shown based on Wald tests done on linear mixed-effects models.
3.3.4 Correlations Between SMC Composition, Function, and Soil Abiotic Variables

SMC composition and function were not strongly correlated overall (Table 3.2). PC1 from the PLFAs and enzymes were negatively correlated, while PC1 of the enzymes and PC3 of the PLFAs were significantly positively correlated.

Table 3.2 Pearson’s correlation coefficients between the first three principal components of extracellular enzyme activity (EEA) and phospholipid fatty acid (PLFA) data. Values in bold are significant at p = 0.05.

<table>
<thead>
<tr>
<th></th>
<th>PLFA</th>
<th>EEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC1</td>
<td>PC2</td>
</tr>
<tr>
<td>PC1</td>
<td>-0.36</td>
<td>-0.11</td>
</tr>
<tr>
<td>PC2</td>
<td>0.31</td>
<td>0.12</td>
</tr>
<tr>
<td>PC3</td>
<td>-0.33</td>
<td>0.07</td>
</tr>
</tbody>
</table>

SMC composition was significantly correlated with a number of abiotic properties of the soil, including soil nutrients (PC1), pH, and C/N ratio (Table 3.3). PC1 of EEA was significantly correlated to soil C/N ratio, and PC3 was significantly correlated with soil pH (Table 3.3).

Table 3.3 Pearson’s correlation coefficients between the first three principal components of phospholipid fatty acid (PLFA) and extracellular enzyme activity (EEA) data, and some abiotic soil properties.

<table>
<thead>
<tr>
<th></th>
<th>PLFA</th>
<th>EEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC1</td>
<td>PC2</td>
</tr>
<tr>
<td>Soil C/N</td>
<td>0.72***</td>
<td>-0.08</td>
</tr>
<tr>
<td>pH</td>
<td>0.87***</td>
<td>-0.04</td>
</tr>
<tr>
<td>moisture</td>
<td>0.011</td>
<td>-0.27</td>
</tr>
<tr>
<td>PC1 nutrients</td>
<td>0.89***</td>
<td>-0.18</td>
</tr>
<tr>
<td>PC2 nutrients</td>
<td>0.11</td>
<td>0.22</td>
</tr>
<tr>
<td>PC3 nutrients</td>
<td>-0.07</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Significant at p = 0.05  
** Significant at p = 0.01  
*** Significant at p = 0.001
3.4 Discussion

Theoretically, both fertilization and herbivores have the potential to strongly influence both the soil microbial community and ecosystem properties. In this study, fertilization had significant and long-lasting impacts on both abiotic and biotic components of soil, whereas herbivore removal had almost no measurable impact, similar to responses reported for the plant community (Chapter 2). This contributes to a growing body of evidence that soil microbial communities are not as resistant or resilient to disturbance as is commonly thought (Allison and Martiny 2008). Furthermore, these finding suggest that more effort is required to identify how plant and soil microbial communities interact and influence each other, in order to better predict how ecosystems will respond to anthropogenic disturbances. Because herbivore removal had virtually no influence on the vegetation or on the soil microbial community, I will focus most of the discussion on the effects of fertilization that produced large responses in the soil chemistry and microbial communities.

3.4.1 Soil Microbial Community Composition

The composition of the soil community was altered significantly by fertilization, and total microbial biomass (fungi and bacteria) decreased by 25%. This is consistent with other studies investigating total microbial biomass under N deposition or fertilization (e.g. Wallenstein et al. 2006), including a recent meta-analysis which showed microbial biomass declined on average by 15% across ecosystems, while in boreal forests the decline was 22% (Treseder 2008). In the same study, fungi decreased significantly across ecosystems (though not in boreal systems), and bacteria did not consistently respond to N fertilization. In contrast, in this study, in fertilized plots there was a decline in the ratio of fungal to bacterial PLFAs indicating a more bacteria-dominated soil food web (Wardle 2002).

Although, reasons for declines in total microbial biomass, fungi, and gram-negative bacteria are not definitively known, there are a number of plausible hypotheses. One common suggestion focuses on the C/N ratio of the soil, due to the differences between fungi and bacteria in C/N ratios (Strickland and Rousk 2010). Bacteria have much lower
C/N ratio than fungi do, generally between ~3-6. The C/N ratio of fungi is much higher, at ~ 5 – 15 on average. This amount can vary considerably and there may be considerable overlap between the C/N ratios of bacteria and fungi. Microbes in the soil, generally, are carbon limited, even in soils where there is a high C/N ratio (Demoling et al. 2008). The C/N ratio of soils in the study site declined from ~ 25 to ~19 when fertilized and this may have been sufficient to cause a shift in dominance between fungi and bacteria. Bacteria thrive in higher quality substrates, where the C/N ratio is lower. In this study, because the C/N ratio remained high, it is unlikely the shift in C/N of the soil favoured bacteria strongly. Furthermore, this rationale does not explain decreases in microbial biomass and gram (−) bacteria. However, bacteria are declining less than fungi — i.e., the bacteria respond more favourably to this environment than do fungi.

A second hypothesis focuses on the belowground allocation of carbon by plants. Plants contribute a substantial amount of soluble organic compounds to the soil microbial community, providing energy and resources for saprophytic organisms as they decompose organic material. This phenomenon is known to occur both through the trees of forest communities (Hogberg et al. 2007, Yarwood et al. 2009), and the understory plants (Manninen et al. 2009). As the level of resources available to plants increases, they allocate less carbon to fine roots system and more to aboveground tissue (Litton et al. 2007). This is also known to have a direct negative effect on plant symbionts, such as mycorrhizal fungi. Given that fine roots and mycorrhizal fungi provide the majority of the resources to the soil microbial community, there is a large decrease in the amount of resources plants release into the soil.

This decrease in carbon allocation belowground and the reduction in root exudation could have a strong negative effect on a number of organismal groups in the soil. Mycorrhizae and other fungal symbionts are most susceptible to a reduction in carbon supply because they directly rely on plant hosts for carbon. This may explain the substantial decrease in the PLFA marker 18:2ω6,9, which is considered to be a good indicator of ectomycorrhizal fungi in soil. It is also a reasonable explanation for the decrease in the ratio of fungi to bacteria, as fungi decreased more than bacteria in this experiment. Reduction of below-ground carbon supply, however, does not only affect fungi. There was also a decrease in the gram-negative bacterial PLFAs. Gram-negative bacteria thrive in the rhizosphere, presumably because of the resources available near
plants roots via root exudation (Turkington et al. 1988). Therefore, a reduction in belowground carbon supply and root production with fertilization may reduce the growth and activity of rhizosphere dwellers such as gram-negative bacteria (Philips and Fahey 2007).

Third, alterations in the composition of the soil microbial community may be due to changes in the pH caused by the addition of nitrogen. The pH of fertilized plots in our study declined to 4.99, compared to 6.05 in control soils (p< 0.05). The pH of the soil plays an important role in structuring microbial communities, as groups of organisms are favoured differentially along gradients in pH (Baath and Anderson 2003, Hogberg et al. 2007, Rousk et al. 2009, 2010, Strickland and Rousk 2010). For example, Baath and Anderson (2003) found that even a difference of 0.5 pH negatively affects certain bacterial groups, such as gram-negative bacteria. In general, acidic soils contain lower microbial biomass that is dominated more by fungi (Rousk et al. 2010, Strickland and Rousk 2010), whereas soils with a higher pH are more dominated by bacteria. Similar patterns to those reported by Baath and Anderson (2003) and Rousk et al. (2010) were identified in this study, indicating the drop in pH in the soil is having some effect on microbial community composition. This included decreases in key PLFAs associated with gram-negative bacteria. But, low soil pH generally favours fungi (or at least doesn’t strongly affect), and we detected large declines in fungi biomarkers. This indicates soil pH may be a contributing factor to the changes we found in the microbial community, but doesn’t provide a complete explanation. It is likely that fertilization is driving changes in the soil microbial community through a variety of mechanisms. Changes in soil C/N ratios, carbon allocation patterns by plants, and soil pH are likely all contributing to the patterns we detected.

### 3.4.2 Extracellular Enzyme Activity

Despite the large shifts in soil microbial community composition, fertilization and herbivore removal failed to cause large changes in the activity of the extracellular enzymes we measured. These results contribute to a growing body of research on soil enzymes showing how complex enzyme-substrate-environment interactions are, and how difficult it is to accurately predict the direction and magnitude of enzyme responses to disturbance.
Consistent with many other studies (Ajwa et al. 1999, Olander and Vitousek 2000, Saiya-Cork et al. 2002, Stursova et al. 2006), we have shown an increase in cellulose-degrading enzymes with fertilization. One explanation for this predicts that enzyme production is likely constrained by the availability of nitrogen in most ecosystems, as it is an essential component of enzymes (Keeler et al. 2009). Adding nitrogen may increase the demand for carbon, thereby increasing the production of enzymes involved in acquiring it (Sinsabaugh et al. 2005). Furthermore, as nitrogen is a critical component of enzyme structure, adding nitrogen increases the potential for microbes to produce carbon-acquiring enzymes. This is evident in the high correlation between the activity rates of β-1,4-glucosidase and cellobiohydrolase and the C/N ratio of the soil.

While many studies have reported decreases in phenol oxidase activity with fertilization (Carreiro et al. 2000, DeForest et al. 2004, Frey et al. 2004), we did not detect any phenol oxidase activity in any samples. Although this is somewhat surprising, it is not entirely unusual (see Keeler et al. 2009, Sinsabaugh et al. 2005, Sinsabaugh 2010). Phenol oxidase is an important enzyme involved in humification, the production of secondary compounds, decomposition (particularly of lignin) and defense in microbes, although determining its particular function is problematic (Sinsabaugh 2010). Studies have linked the activity of phenol oxidase to mass loss in decomposing organic matter, as phenol oxidase is one of the few enzymes capable of breaking down lignin (Allison and Vitousek 2004, Sinsabaugh et al. 1992), often a rate-limiting step in decomposition. Due to its importance in mediating key ecosystem processes, such as carbon mineralization, humification and lignin decomposition, one would expect it to be abundant year-round, especially in forests with an abundance of recalcitrant litter. Yet, activity rates often decline in the summer, possibly due to moisture limitation (Sinsabaugh 2010), although reasons for this decline remain elusive (Keeler et al. 2009).

The inability to detect any phenol oxidase activity may also be the result of the assay technique used. The substrate DOPA (L-3,4-dihydroxyphenylalanine), used in this study, has been criticized for being either too sensitive or not sensitive enough (Sinsabaugh 2010). These assays are susceptible to chemical oxidation by Mn and Fe species, as well as competitive reactions that can consume the oxidation products. This limits color development in the assay, complicating its interpretation (Sinsabaugh 2010).
Furthermore, DOPA has a slightly basic pH optimum (Sinsabaugh 2010), whereas our assays were conducted under acidic conditions more closely resembling the pH of boreal forest soils.

In addition to predicting an increase in cellulase activity, and a decrease in phenol oxidase, we predicted that enzymes involved in nitrogen and phosphorus acquisition would have decreased activity with fertilization. According to resource allocation theory (Allison and Vitousek 2005), if nitrogen is a limiting nutrient, then nitrogen-acquiring enzymes will be produced in order to obtain it. Alternatively, if nitrogen is plentiful in the environment, microbes will invest energy into the production of enzymes that will help obtain other nutrients, like the increased production of carbon-acquiring enzymes we see in this study. The same applies to acid phosphatase, a phosphorus-acquiring enzyme. Therefore, N-P-K fertilization should be expected to decrease investment in enzymes used to acquire those nutrients. Our results suggest that this hypothesis may be too simplistic. We detected no change in the activity of acid phosphatase, N-acetyl-glucosamidase, and only minor changes in urease. Yet, these results are similar to those reported by others who have shown that N-acetyl-glucosamidase activity increases with fertilization (Saiya-Cork et al. 2002, Michel and Matnzer 2003), or does not change (Waldrop and Zak 2006). Sinsabaugh et al. (2005) suggest the production of N-acetyl-glucosamidase is associated with the fungal community, and therefore reflects changes in the community composition of fungi. This is unlikely in this study because the fungal community showed dramatic declines with fertilization with no change in N-acetyl-glucosamidase.

The lack of significant changes in extracellular enzyme activity in the soil suggests there may be considerable functional redundancy for particular processes carried out by the soil microbial community. Furthermore, the non-significant correlation between the primary principal components of the PLFA and EEA data suggest a decoupling of the structure and function of soil microbial communities. “Narrow processes”, such as methanogenesis and nitrification, may be carried out by a relatively small group of microorganisms (Schimel et al. 2005). For these processes, small shifts in soil microbial community composition may considerably affect the process. But, it is likely that many other processes could be considered “broad”. The acquisition of carbon, nitrogen, and phosphorus may all be broad processes, accomplished by a diverse set of organisms.
Therefore, even large shifts in the soil microbial composition are not having significant impacts on the activity of most of the extracellular enzymes in this study.

### 3.4.3 Insensitivity to Herbivore Reduction Treatments

Herbivore reduction had virtually no detectable impact on any of the soil measurements; neither soil microbial community composition or activity, nor soil chemistry changed in plots with exclosure treatments. In order for snowshoe hares to have a measureable impact on soil microbial communities, there needs to be a significant direct input of feces or urine, or herbivores must cause a significant change in litter chemistry, root exudation patterns, or plant community composition (Bardgett and Wardle 2010). This means that there must be a change in either the quantity or quality of resources entering the soil. Snowshoe hares had very few impacts on the plant community, causing no obvious changes in plant tissue chemistry or community composition (Chapter 2). Therefore, litter quality was not altered by herbivores, and likely did not cause any changes in resource inputs to the soil microbial community. Furthermore, there was very little evidence of snowshoe activity in any experimental plots (pers. obs.).

One likely explanation for this is that herbivores never reached high enough densities to impact soil properties. In the first 10 years of this study, there was evidence for slight impacts of snowshoe hares on plant community composition (Turkington et al. 2002). During this time, hare numbers peaked at 148 and 198 km$^{-2}$ in 1990 and 1998, respectively (Krebs et al. 1995, Hodges et al. 2001). Recent estimates of hare densities are much lower. In 2006, hare densities peaked at 92 km$^{-2}$ (Krebs 2011).

### 3.4.4 Recovery from Fertilization

While there have been many studies investigating the effects of nutrient enrichment on both plants and soil, we have less understanding of their reversibility. Within a few years after the cessation of nitrogen application, the chemical parameters of ecosystems show strong recovery, leading researchers to predict complete reversibility (Boxman et al. 1998). But, other studies have shown that the biological components of ecosystems take considerably longer, and may not be fully reversible (Strengbom et al. 2001). For
example, the nitrogen content of plant tissue, may recover quickly (Boxman et al. 1998), while nitrogen mineralization rates or fungal sporocarp production, may take decades to return to normal (Clark et al. 2009, Strengbom et al. 2001). The results from this study highlight how long-term studies are necessary to determine the time required for ecosystems to recover, how different measured variables give different interpretations about whether recovery is possible, and how long it may take.

The nutrient content of the soil recovers quite well post-treatment. After 10 years of no treatment, levels of NO$_3^-$ and NH$_4^+$ had almost returned to control levels, though both remain somewhat elevated. The same applies to all the other measured nutrients, with the exception of Mg$^{2+}$, which remained low, and K$^+$, which remained slightly elevated. These results are consistent with numerous other studies that show a quick return towards pre-treatment concentrations once nitrogen deposition ceases (Boxman et al. 1995, 1998). Although the initial recovery is quick, however, nutrient stores and cycling may take many years to completely recover. Both Power et al. (2006) and Clark et al. (2009) reported that total soil nitrogen was still elevated 6 and 12 years after treatments ceased, although the results were not statistically significant.

Other abiotic parameters of the soil showed differing degrees of recovery. Fertilization caused significant increases in soil acidity, but since the cessation of fertilization, it is returning to control levels. While it has not fully recovered, pH is expected to return to pre-treatment conditions, as other studies have shown (Power et al. 2006). This is important as soil pH strongly influences the structure of both plant and soil communities (Gilliam 2006, Rousk et al. 2010). Soil C/N ratios, in contrast, showed little sign of recovery. This is likely an indication of increased nitrogen in litter that has accumulated over many years. Due to the long cold winters and short growing seasons, we expect these litter stores to take a long time to decompose.

While there are significant signs of recovery in many of the abiotic parameters of the soil, the effects of fertilization on the soil microbial community are highly persistent. This has been suspected in many studies because nitrogen mineralization, a process strongly influenced by the soil microbial community, can remain elevated for over a decade after fertilization (Chen et al. 2006, Clark et al. 2009). The fungal biomass, and therefore the ratio of fungi to bacteria, and the total microbial biomass in this study showed little sign
of recovery in the 10 years since fertilization was stopped. Other studies have shown reductions in fungal biomass and abundance three decades after nitrogen additions stopped (van der Wal et al. 2006). The bacterial component of the soil microbial community is more resilient than the fungi, and is showing signs of recovery, probably due to the high number, dispersal ability and fast growth rate of bacteria (Fenchel and Finlay 2004).

3.4.5 Conclusions

This study shows the sensitivity of soil microbial communities to increased nutrient availability, and their inability to recover after a decade of natural levels of nutrient input. This contrasts with soil chemistry that responded strongly to fertilization, but recovered relatively quickly. So, while many readily measured chemical variables indicate good ecosystem recovery, the effects of fertilization on soil microbes are long lasting. Furthermore, this study highlights the relative unimportance of above-ground herbivores in influencing processes that happen belowground in the boreal forest.

While the long-term nature of this study has provided insight into how fertilization and herbivores affect the belowground component of ecosystems, we still have much to investigate. What controls the activity rates of enzymes involved in nitrogen and phosphorus cycling? How are composition and function of soil microbial communities related? Is it possible for soil microbial communities to completely recover without active restoration? These questions, and others, remain unanswered, and further research is needed.
4 CONCLUSIONS AND FUTURE DIRECTIONS

As the pressures of human activities increase on natural ecosystems, such as nitrogen deposition and climate change, there is a more urgent need to understand the basic biology of ecosystems. Understanding what controls and regulates plant and soil microbial communities will give us an improved ability to predict future changes, as well as provide us with better information for the management and restoration of ecosystems. This study has provided a long-term perspective of plant understory and soil microbial communities in the boreal forest, giving insight into the sensitivity of these communities to nutrient enrichment, the relative unimportance of herbivores to these plant and soil communities, and the length of time required for recovery after disturbance, if complete recovery is possible at all. In the two previous chapters, the plant and soil microbial communities were discussed separately, yet, we know they have important influences on each other. Here I discuss them together in the context of plant-soil feedbacks and the potential recovery of the ecosystem after a disturbance.
4.1 Plant-soil Feedbacks and Recovery from Fertilization

Whether or not ecosystems can fully recover from nutrient enrichment is a question open for debate. Evidence from this study and others indicate that at the very least the process can take an extremely long time – extending for decades (Strengbom et al. 2001, Strengbom and Nordin 2008). Theoretically, plant-soil feedbacks are one process capable of slowing recovery (Bargrett and Wardle 2010). Plant-soil feedbacks develop due to the strong interactions between plants and soil communities. Plants strongly influence belowground dynamics, selecting for specific microbial communities (Bardgett and Wardle 2010, Grayston et al. 1998), and the soil community influences aboveground dynamics, altering competitive interactions between plants (Bardgett and Wardle 2010). It is in this context that plant-soil feedbacks can develop.

Plant-soil feedbacks may function both through direct pathways and indirect pathways (Bargrett and Wardle 2010). Direct pathways generally work through the plant having contact with soil organisms, such as root symbionts (e.g. nitrogen-fixing bacteria and mycorrhizae), root herbivores, and pathogens, and lead to both positive and negative feedbacks. Negative feedbacks are suggested to be important processes maintaining plant diversity in grasslands in North America (Reynolds et al. 2003). Indirect pathways work through the decomposer organisms in the soil, whereby plants provide resources to decomposers via litter, and in turn the decomposers release nutrients back to the plants, leading to positive feedbacks. Wardle (2002) hypothesized that plants may select for decomposer communities that preferentially mineralize their own litter, thereby giving them a competitive advantage over other plant species. For example, fast-growing plants with high-quality litter may select for bacteria-dominated food webs that cycle nutrients quickly, allowing the fast-growing plants to further exploit the high availability of nutrients. If fertilization causes a shift in the plant community towards fast-growing nutrient-exploiting plants, then, although intuitively unreasonable, it is possible that even in the absence of continued nutrient inputs, a positive plant-soil feedback may develop allowing fast-growing plants to persist (see Manning et al. 2008). This could slow down the recovery of the plant community considerably (Bargrett and Wardle 2010). The possibility of this indirect feedback is the focus of this section.

While many studies suggest that plant-soil feedbacks may be important structuring forces, we have little empirical evidence from natural plant communities (see Bardgett and Wardle
Kulmatiski et al. (2008) found that plant-soil feedbacks are common, with negative feedbacks being more common than positive feedbacks, but the data used in their meta-analysis were predominantly from greenhouse studies. It’s possible that positive feedbacks are more important in natural systems due to the short-term nature of most greenhouse studies (favouring the proliferation of short-lived antagonists), and the use of early-successional herbaceous plants in those studies (Bardgett and Wardle 2010). Long-term studies in natural systems are critical to determine the importance of indirect plant-soil feedbacks, where the long-term processes of species turnover in the plant community, litter decomposition and nutrient mineralization are given enough time to impose an impact, if any, on structuring the plant community. It was a goal of our study to determine if there was evidence for plant-soil feedbacks that may have changed with fertilization, and that may prevent, or delay, the recovery of the plant community once fertilization was stopped.

While our experimental design does not allow for definitive conclusions about the presence or absence of plant-soil feedbacks, we can accumulate evidence that supports, or rejects, the idea that plant-soil feedbacks exist in our system. Essentially, we are attempting to detect significant changes in plant community composition and plant tissue quality, soil microbial community composition and activity, and nutrient availability in the soil (Bardgett and Wardle 2010). First, we predict that fertilization will cause a change in the plant community towards fast-growing herbaceous species. Second, the change in plant community composition will also include an increase in the quality of plant tissue (i.e. lower C/N ratio, lower concentration of defense chemicals). Third, the change in the plant community and tissue quality will select for a bacteria-based decomposer community that will result in more rapid decomposition and mineralization rates, and thereby increase the rate of nutrient cycling. Fourth, this will result in an increased nutrient availability in the soil that will favour fast-growing species that will be able to further exploit the resources and remain dominant.

I will now briefly review the evidence from our study pertaining to these predictions; the results discussed here are reported in more detail in Chapters 2 and 3, and focus on the plots in which the treatments were stopped 10 years ago in 2000. We will begin this plant-soil feedback scenario with the plant community, where we predicted an altered community dominated by fast-growing opportunistic species. First, the plant community did change significantly with fertilization and once fertilization was stopped, it only appears to be
recovering slowly towards control conditions. Two herbaceous species, *Epilobium angustifolium* and *Mertensia paniculata*, continued to dominate the previously fertilized plots after 10 years. These species are fast-growing with large amount of above-ground biomass (pers. observation). The second prediction is that plant tissue quality would increase with fertilization. Initially this was true – fertilization did increase plant quality by decreasing total phenolics and the C/N ratio of foliar tissue. But, importantly, this was a short-term effect. After 10 years recovery, tissue quality was showing strong signs of recovery. In fact, plant quality is reverting toward control conditions much more rapidly than the plant community is returning to the control composition.

The overall quality of resources entering the soil depends on the combination of plant tissue quality within a species and the differences between species in plant tissue quality making up the plant community (Bardgett and Wardle 2010). We only measured the differences within a species for C/N ratios and secondary defense chemicals, and only measured a small subset of the plants in the community. Therefore, it is still highly likely, that despite the high degree of recovery of plant tissue quality within a species, the change in plant community composition to fast-growing herbaceous dicots has also increased the overall quality of resources entering the soil, because fast-growing species generally have higher quality tissue (Bardgett and Wardle 2010). We have some evidence for this in our fertilized and formerly fertilized plots, as grasses and woody plants (typically high C/N ratios) have been replaced by fast-growing forbs (low C/N ratios). This indicates that the plant community, overall, has followed the expectations of a plant-soil feedback model.

The third expectation focuses on the composition of the soil microbial community. High-quality litter from fast-growing plants is expected to select for microbial communities dominated by bacteria that decompose litter quickly and increase the rate at which nutrients are cycled back to the plant community (Bardgett and Wardle 2010). As a note of caution, we did not directly measure litter quality. Plants reabsorb nutrients as they senesce, causing incongruity between the quality of living and dead tissue. Yet, although the nutrient content of litter is not directly comparable to living foliar tissue, in general plants with high-quality living tissue have higher quality litter (Bardgett and Wardle 2010). Here again we have evidence that there was a change in microbial composition. The fungi:bacteria ratio in the soil declined with fertilization and showed little sign of recovery. This indicates a shift towards a bacteria-dominated food web that is persistent. But, this must be interpreted with
caution because fertilization was not necessarily beneficial for bacteria. The added nutrients appear to have negatively affected bacteria as well as fungi, but fungi were affected more strongly. So, although we have detected a change towards bacterial dominance in the soil microbial community, overall there is a negative affect indicated by declines in both fungi and bacteria. Therefore, the expectation of a bacteria-based food web was met, even though bacteria were detrimentally affected by fertilization, and could greatly weaken any plant-soil feedback.

The last expectation focused on the cycling and availability of nutrients. The first part of this expectation, that nutrient cycling will increase and remain elevated post-fertilization, was indirectly measured via extracellular enzyme activity assays. Here, there is not much evidence for increased nutrient cycling. Urease activity, involved in the mineralization of nitrogen, declined, a finding indicating potentially slower nitrogen mineralization rates. In contrast, there were increases in the activity of both cellulases measured, which could indicate increased rates of decomposition. Studies in other systems have shown that high-quality labile litter decomposes more quickly when cellulase activity increases (Carreiro et al. 2000, Sinsabaugh et al. 2002). We did not measure decomposition directly, and therefore it is unknown if the increased enzyme activity actually resulted in increased decomposition rates. The final part of this expectation is an increase in nutrient availability due to higher nutrient cycling rates. We did not detect any evidence for this in our study. Ten years after fertilization ceased, the availability of nitrate and ammonium returned to close to control levels. This makes it highly unlikely that fast-growing plants are able to exploit the resources in the soil in order to remain competitively dominant.

In conclusion, we see little evidence supporting a plant-soil feedback through this indirect pathway. The changes we detected belowground in the soil microbial community composition and activity, and most importantly, the availability of nutrients, makes it highly unlikely this is a significant process slowing down the recovery of the plant community. Instead, we see what is more appropriately termed a ‘cascade of response’ beginning with soil chemistry (Boxman et al. 1998, Tietema et al. 1995). First there is a recovery by the soil chemistry, followed by a recovery of plant tissue chemistry, which can take three to four years post-fertilization (Boxman et al. 1998, Clark et al. 2009). After this, the plant community shows signs of recovery. Only when the plant community recovers will one see recovery of the soil microbial community. This ‘cascade of response’ is evident in our study,
because soil and tissue chemistry have recovered well, while the plant community is showing a much slower recovery. We have little indication, yet, that the soil microbial community is recovering at all.
4.2 The Use of Long-Term Experiments

This study took advantage of a long-term experiment originally set-up to determine the relative strengths of top-down and bottom-up control on understory plant communities of the boreal forest. Since the beginning of the experiment, the experiment has become relevant for studying questions pertinent to global change. Yet, whenever we use an older experiment to address new questions, there are drawbacks, some of which I will discuss here.

First, this study only observes the ecosystem in the 20\textsuperscript{th} year of the study, providing a snapshot of the ecosystem at one point in time, rather than measuring rates of change and the dynamics of interactions over time. This is the case with many long-term studies where new questions are asked of old experiments and studies (Silvertown \textit{et al.} 2006). As a consequence, we are sometimes not able to answer questions completely. For example, we did not have the ability to monitor how the soil system recovered over time, or how quickly plant tissue chemistry returned to normal post-fertilization levels. Other studies have shown soil chemistry, plant tissue, and plant community composition all recover at different rates, but we can only make inferences about the order and rates of recovery in this system (e.g. Boxman \textit{et al.} 1998, Clark \textit{et al.} 2009, Power \textit{et al.} 2006). Furthermore, we are unable to make definitive statements about whether the recovery of the plant community is driving the recovery of different soil variables, or vice versa.

Second, the experimental protocol applied high levels of nitrogen, phosphorus and potassium to the experimental plots. This has two drawbacks. First, the rate of application far exceeds the amount of nitrogen that would be added to a system via atmospheric nitrogen deposition (Dentener \textit{et al} 2006). Even though chronic low-level additions of nitrogen often have similar impacts (Clark and Tilman 2008), we must be cautious in our interpretations. Second, the addition of N-P-K fertilizer does not directly mirror atmospheric nitrogen deposition. The alleviation of multiple nutrient limitations simultaneously may have different consequences. It is often found that once nitrogen limitation is alleviated, phosphorus limitation becomes increasingly important in ecosystems (Allgeier \textit{et al.} 2011).

Third, fertilization caused many changes in the plant community, and belowground, making it difficult, if not impossible, to untangle the direct and indirect effects of fertilization. For
example, we cannot know for certain if the shift towards a bacteria-based soil food web is
due to direct effects of fertilization, or indirect effects via changes in plant community
composition or changes in soil pH. Some mechanisms are more plausible than others, but
without experimental manipulations, it is difficult to quantify the relative contribution of the
direct and indirect effects of fertilization, though direct effects are more common (e.g.
Manning et al. 2006).
4.3 Future Directions

Despite these limitations, this work has shed light on new avenues of research that can be explored in the future. First, to address some of the shortcomings listed above, we could use levels of fertilizer application that are a more realistic reflection of atmospheric nitrogen deposition rates or potential increases in nutrient availability due to global warming in northern systems. Second, we could add fertilizer consisting of single nutrients, instead of the combination N-P-K, to determine a more realistic effect of different nutrients on the ecosystem. Third, more work is needed to understand the length of time it takes for the ecosystem to recover (if at all), and the order of recovery. Future sampling of the experimental plots will allow better understanding about the rate and trajectory of recovery of this ecosystem post-fertilization.

This study also took a more exploratory perspective on the composition and function of the soil microbial community. Now that significant changes have been detected, more in-depth analyses of the soil microbial community would provide information that could better link the composition and function of the soil microbial community. More specifically, more intensive sampling of the soil microbial community and processes involved in nutrient cycling are needed. We assayed six extracellular enzymes produced by the soil microbial community at a single point in time. A more comprehensive suite of assays, sampled over a longer time period, within and between years, would provide a more complete picture of how the functioning of the soil microbial community changes with fertilization (Nannipieri et al. 2002).

It would also be useful to use molecular techniques to understand the composition of the soil microbial community. Phospholipid fatty acid analysis provides information about broad-scale patterns in the soil microbial community. Newly available molecular and genetic techniques would more readily provide finer-scale resolution allowing us to detect changes within these broad taxonomic groupings, such as the fungi (Leckie et al. 2005).
4.4 Conclusions

The long-term nature of this study has provided uniquely long-term insights into the regulation and control of understory plant and soil microbial communities in this part of the boreal forest in the context of global change. Using an experimental approach has allowed us to isolate the role the nutrients and herbivores in this system. Furthermore, we were able to address the question of recovery of the system over the long-term, a necessity if we want to understand the rate of recovery of “slow” systems such as the boreal forest. In summary, we found:

i) The plant community is regulated primarily from the bottom-up by resources. Increasingly the availability of nutrients created a plant community dominated by two species primarily, *Epilobium angustifolium* and *Mertensia paniculata*. Even species that appeared to respond positively in the short-term to increased nutrient availability, such as *Festuca altaica*, were out-competed in the long-term.

ii) Plant tissue chemistry of understory herbs is largely controlled by nutrient levels in the soil. The C/N ratios and levels of total phenolics in foliar tissue decrease significantly with fertilization.

iii) The composition of the soil microbial community is regulated by soil resource levels. Fertilization decreased the soil microbial biomass and changed the community to a more bacteria-based food web.

iv) The activity of the soil microbial community was relatively resistant to changing resource levels and herbivore absence.

v) The recovery of the system followed a ‘cascade of recovery’, beginning with soil chemistry, followed by foliar tissue chemistry, the plant community, and finally the soil microbial community.

The long-term plots will continue to be valuable. Future monitoring will allow us to re-evaluate the conclusions of this study after 20 years. Furthermore, the continued use of this experiment may detect shifts in the plant community due to climate change or unique events in the history of the area, such as the spruce bark beetle outbreak in 1996.
REFERENCES


# APPENDIX: RESULTS FROM LINEAR MIXED-EFFECTS MODELS

Table A.1 Wald test statistics (F-ratio and p-values) from linear mixed-effects models for the effects of fertilization, exclosures (herbivore removal) and treatment cessation on fertilized plots on percent cover values for plant community composition. Values in bold are significant (p<0.05). Fert = fertilizer, Exc = exclosure, Trt Cess = treated for 20 years continuously or treated for 10 years and allowed to recover 10 years.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fertilizer</th>
<th>Exclosure</th>
<th>Trt Cess</th>
<th>Fert x Exc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td><em>Achillea millefolium</em></td>
<td>0.14</td>
<td>0.711</td>
<td>0.40</td>
<td>0.540</td>
</tr>
<tr>
<td><em>Arctostaphylos uva-ursi</em></td>
<td>4.01</td>
<td>0.068</td>
<td>1.32</td>
<td>0.272</td>
</tr>
<tr>
<td><em>Epilobium angustifolium</em></td>
<td>25.52</td>
<td>&lt;0.001</td>
<td>0.05</td>
<td>0.834</td>
</tr>
<tr>
<td><em>Festuca altaica</em></td>
<td>44.02</td>
<td>&lt;0.001</td>
<td>0.11</td>
<td>0.743</td>
</tr>
<tr>
<td><em>Linnaea borealis</em></td>
<td>11.97</td>
<td>0.005</td>
<td>0.00</td>
<td>0.947</td>
</tr>
<tr>
<td><em>Lupinus arcticus</em></td>
<td>21.18</td>
<td>0.001</td>
<td>0.00</td>
<td>0.963</td>
</tr>
<tr>
<td><em>Mertensia paniculata</em></td>
<td>20.19</td>
<td>0.001</td>
<td>0.03</td>
<td>0.869</td>
</tr>
<tr>
<td><em>Moss spp.</em></td>
<td>2.33</td>
<td>0.153</td>
<td>14.39</td>
<td>0.003</td>
</tr>
<tr>
<td><em>Salix spp.</em></td>
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<td>0.319</td>
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<td>0.096</td>
</tr>
<tr>
<td>Rare species</td>
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<td>0.159</td>
<td>0.06</td>
<td>0.818</td>
</tr>
<tr>
<td>Species</td>
<td>Fert x Trt Cess</td>
<td></td>
<td>Exc x Trt Cess</td>
<td></td>
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<tr>
<td>-------------------------</td>
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<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td><em>Achillea millefolium</em></td>
<td>0.07</td>
<td>0.800</td>
<td>0.46</td>
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<tr>
<td><em>Arctostaphylos uva-ursi</em></td>
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<td>0.710</td>
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<td><em>Festuca altaica</em></td>
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<td><strong>0.009</strong></td>
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<td>0.378</td>
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<td><em>Lupinus arcticus</em></td>
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<td>0.476</td>
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<td>0.16</td>
<td>0.695</td>
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<tr>
<td><em>Moss spp.</em></td>
<td>0.26</td>
<td>0.617</td>
<td>0.57</td>
<td>0.466</td>
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<tr>
<td><em>Salix spp.</em></td>
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<td>0.451</td>
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<td>Rare spp.</td>
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<tr>
<td>Species richness</td>
<td>9.89</td>
<td><strong>0.009</strong></td>
<td>3.95</td>
<td>0.070</td>
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Table A.2 Wald test statistics (F-ratio and p-values) from linear mixed-effects models for the effects of fertilization, exclosures (herbivore removal) and treatment cessation on fertilized plots on plant tissue chemistry. Values in bold are significant (p<0.05). Fert = fertilizer, Exc = exclosure, Trt Cess = treated for 20 years continuously or treated for 10 years and allowed to recover 10 years.

<table>
<thead>
<tr>
<th>Species</th>
<th>Fertilizer</th>
<th>Exclosure</th>
<th>Trt Cess</th>
<th>Fert x Exc</th>
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<td></td>
<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td><strong>C/N ratios</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Achillea millefolium</em></td>
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<td>0.25</td>
<td>0.626</td>
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<tr>
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<td>0.360</td>
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<td>36.69</td>
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<tr>
<td><em>Mertensia paniculata</em>*</td>
<td>8.44</td>
<td>0.016</td>
<td>0.02</td>
<td>0.904</td>
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</table>

* Numerator and denominator degrees of freedom for F-statistic are 1 and 11, respectively.
** Numerator and denominator degrees of freedom for F-statistic are 1 and 10, respectively, for fertilizer, exclosure and fertilizer x exclosure effects. Numerator and denominator degrees of freedom for F-statistic are 1 and 9, respectively, for effect of treatment.
<table>
<thead>
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<th>Species</th>
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<th>Exc x Trt Cess</th>
<th>Fert x Exc x Trt Cess</th>
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<td>prob</td>
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<tr>
<td><strong>C/N ratios</strong></td>
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<td></td>
<td></td>
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<tr>
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<td>0.95</td>
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<td><strong>0.019</strong></td>
<td>0.76</td>
</tr>
<tr>
<td><strong>Total Phenolics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Achillea millefolium</em></td>
<td>6.95</td>
<td><strong>0.022</strong></td>
<td>0.01</td>
</tr>
<tr>
<td><em>Epilobium angustifolium</em></td>
<td>0.39</td>
<td>0.548</td>
<td>0.15</td>
</tr>
<tr>
<td><em>Festuca altaica</em></td>
<td>0.53</td>
<td>0.481</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Mertensia paniculata</strong></td>
<td>0.25</td>
<td>0.626</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* Numerator and denominator degrees of freedom for F-statistic are 1 and 11, respectively.

** Numerator and denominator degrees of freedom for F-statistic are 1 and 9, respectively.
Table A.3 Mean, standard error (S.E.) and Wald test statistics (F-ratio and p-values) from linear mixed-effects models for the effects of fertilization, exclosures (herbivore removal) and treatment cessation on fertilized plots on soil and ecosystem properties. Values in bold are significant (p<0.05). Levels of Pb and Cd were not detectable in any samples. PAR equals the ratio of ground-level to canopy level measurement of photosynthetic active radiation. All measurements except for PAR and soil moisture are in µg/10cm²/61 days. Fert = fertilizer, Exc = exclosure, Trt Cess = treated for 20 years continuously or treated for 10 years and allowed to recover 10 years.

<table>
<thead>
<tr>
<th></th>
<th>Fertilizer</th>
<th>Exclosure</th>
<th>Trt Cess</th>
<th>Fert x Exc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;1,12&lt;/sub&gt;</td>
<td>prob</td>
<td>F&lt;sub&gt;1,12&lt;/sub&gt;</td>
<td>prob</td>
</tr>
<tr>
<td>PAR</td>
<td>13.42</td>
<td>0.003</td>
<td>0.08</td>
<td>0.777</td>
</tr>
<tr>
<td>Organic layer pH</td>
<td>75.68</td>
<td>&lt;0.001</td>
<td>0.03</td>
<td>0.858</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>11.33</td>
<td>0.006</td>
<td>2.03</td>
<td>0.180</td>
</tr>
<tr>
<td>Soil Moisture %</td>
<td>1.16</td>
<td>0.302</td>
<td>0.85</td>
<td>0.375</td>
</tr>
<tr>
<td>NO3-</td>
<td>255.43</td>
<td>&lt;0.001</td>
<td>0.35</td>
<td>0.565</td>
</tr>
<tr>
<td>NH4+</td>
<td>18.12</td>
<td>0.001</td>
<td>0.06</td>
<td>0.814</td>
</tr>
<tr>
<td>P</td>
<td>7.43</td>
<td>0.018</td>
<td>0.57</td>
<td>0.464</td>
</tr>
<tr>
<td>K</td>
<td>5.05</td>
<td>0.044</td>
<td>0.01</td>
<td>0.934</td>
</tr>
<tr>
<td>S</td>
<td>14.70</td>
<td>0.002</td>
<td>0.86</td>
<td>0.373</td>
</tr>
<tr>
<td>Ca</td>
<td>1.02</td>
<td>0.332</td>
<td>0.27</td>
<td>0.613</td>
</tr>
<tr>
<td>Al</td>
<td>10.94</td>
<td>0.006</td>
<td>1.71</td>
<td>0.215</td>
</tr>
<tr>
<td>Mn</td>
<td>9.02</td>
<td>0.011</td>
<td>0.41</td>
<td>0.536</td>
</tr>
<tr>
<td>Mg</td>
<td>5.47</td>
<td>0.038</td>
<td>1.55</td>
<td>0.236</td>
</tr>
<tr>
<td>Cu</td>
<td>3.86</td>
<td>0.073</td>
<td>0.00</td>
<td>1.000</td>
</tr>
<tr>
<td>Zn</td>
<td>7.28</td>
<td>0.019</td>
<td>0.78</td>
<td>0.396</td>
</tr>
<tr>
<td>Pb</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cd</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>B</td>
<td>2.52</td>
<td>0.138</td>
<td>0.96</td>
<td>0.346</td>
</tr>
<tr>
<td></td>
<td>Fert x Trt Cess</td>
<td>Exc x Trt Cess</td>
<td>Fert x Exc x Trt Cess</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td>PAR</td>
<td>2.05</td>
<td>0.178</td>
<td>0.00</td>
<td>0.999</td>
</tr>
<tr>
<td>Organic layer pH</td>
<td>54.31</td>
<td>$&lt;0.001$</td>
<td>0.42</td>
<td>0.530</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>2.24</td>
<td>0.160</td>
<td>2.01</td>
<td>0.182</td>
</tr>
<tr>
<td>Soil Moisture %</td>
<td>0.07</td>
<td>0.790</td>
<td>0.22</td>
<td>0.646</td>
</tr>
<tr>
<td>NO3-</td>
<td>124.5</td>
<td>$&lt;0.001$</td>
<td>0.00</td>
<td>0.949</td>
</tr>
<tr>
<td>NH4+</td>
<td>26.85</td>
<td>$&lt;0.001$</td>
<td>0.00</td>
<td>0.984</td>
</tr>
<tr>
<td>P</td>
<td>11.00</td>
<td>0.006</td>
<td>1.53</td>
<td>0.239</td>
</tr>
<tr>
<td>K</td>
<td>0.75</td>
<td>0.404</td>
<td>0.01</td>
<td>0.910</td>
</tr>
<tr>
<td>S</td>
<td>49.85</td>
<td>$&lt;0.001$</td>
<td>0.17</td>
<td>0.690</td>
</tr>
<tr>
<td>Ca</td>
<td>4.12</td>
<td>0.065</td>
<td>0.00</td>
<td>0.947</td>
</tr>
<tr>
<td>Al</td>
<td>15.20</td>
<td>0.002</td>
<td>0.00</td>
<td>1.000</td>
</tr>
<tr>
<td>Mn</td>
<td>9.13</td>
<td>0.011</td>
<td>2.54</td>
<td>0.137</td>
</tr>
<tr>
<td>Mg</td>
<td>2.38</td>
<td>0.149</td>
<td>2.51</td>
<td>0.139</td>
</tr>
<tr>
<td>Cu</td>
<td>8.33</td>
<td>0.014</td>
<td>1.33</td>
<td>0.271</td>
</tr>
<tr>
<td>Zn</td>
<td>9.87</td>
<td>0.009</td>
<td>1.48</td>
<td>0.247</td>
</tr>
<tr>
<td>Pb</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cd</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>B</td>
<td>1.07</td>
<td>0.320</td>
<td>0.07</td>
<td>0.799</td>
</tr>
</tbody>
</table>
Table A.4 Wald test statistics (F-ratio and p-values) from linear mixed-effects models for the effects of fertilization, exclosures (herbivore removal) and treatment cessation on fertilized plots on soil microbial community composition. Values in bold are significant (p<0.05). Fert = fertilizer, Exc = exclosure, Trt Cess = treated for 20 years continuously or treated for 10 years and allowed to recover 10 years.

<table>
<thead>
<tr>
<th></th>
<th>Fertilizer</th>
<th>Exclosure</th>
<th>Trt Cess</th>
<th>Fert x Exc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;1,12&lt;/sub&gt;</td>
<td>prob</td>
<td>F&lt;sub&gt;1,12&lt;/sub&gt;</td>
<td>prob</td>
</tr>
<tr>
<td>Total microbial biomass</td>
<td>8.76</td>
<td>0.012</td>
<td>0.45</td>
<td>0.514</td>
</tr>
<tr>
<td>Fungi : Bacteria</td>
<td>11.37</td>
<td>0.006</td>
<td>0.29</td>
<td>0.601</td>
</tr>
<tr>
<td>Gram (+) : Gram (-)</td>
<td>4.56</td>
<td>0.054</td>
<td>0.32</td>
<td>0.581</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>7.28</td>
<td>0.019</td>
<td>0.38</td>
<td>0.551</td>
</tr>
<tr>
<td>Total fungi</td>
<td>20.15</td>
<td>0.001</td>
<td>1.31</td>
<td>0.274</td>
</tr>
<tr>
<td>Stress Ratios</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cy19:0 to 18:1ω7c</td>
<td>34.91</td>
<td>&lt;0.001</td>
<td>0.33</td>
<td>0.574</td>
</tr>
<tr>
<td>cy17:0 to 16:1ω7c</td>
<td>7.90</td>
<td>0.016</td>
<td>1.46</td>
<td>0.250</td>
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<tr>
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<td>Fert x Trt Cess</td>
<td>Exc x Trt Cess</td>
<td>Fert x Exc x Trt Cess</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------</td>
<td>----------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td>Total microbial biomass</td>
<td>0.38</td>
<td>0.551</td>
<td>3.58</td>
<td>0.083</td>
</tr>
<tr>
<td>Fungi:Bacteria</td>
<td>0.36</td>
<td>0.559</td>
<td>0.00</td>
<td>0.957</td>
</tr>
<tr>
<td>Gram (+) : Gram (-)</td>
<td>0.00</td>
<td>0.994</td>
<td>0.05</td>
<td>0.835</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>0.70</td>
<td>0.421</td>
<td>2.98</td>
<td>0.110</td>
</tr>
<tr>
<td>Total fungi</td>
<td>0.00</td>
<td>0.980</td>
<td>4.11</td>
<td>0.066</td>
</tr>
<tr>
<td>Stress Ratios</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$cy_{19:0}$ to $18:1w7c$</td>
<td>26.07</td>
<td>$&lt;0.001$</td>
<td>0.44</td>
<td>0.519</td>
</tr>
<tr>
<td>$cy_{17:0}$ to $16:1w7c$</td>
<td>14.97</td>
<td><strong>0.002</strong></td>
<td>0.08</td>
<td>0.788</td>
</tr>
</tbody>
</table>
Table A.5 Wald test statistics (F-ratio and p-values) from linear mixed-effects models for the effects of fertilization, exclosures (herbivore removal) and treatment cessation on fertilized plots on soil microbial activity. Values in bold are significant (p<0.05). Fert = fertilizer, Exc = exclosure, Trt Cess = treated for 20 years continuously or treated for 10 years and allowed to recover 10 years.

<table>
<thead>
<tr>
<th></th>
<th>Fertilizer</th>
<th>Exclosure</th>
<th>Trt Cess</th>
<th>Fert x Exc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td>β-1,4-glucosidase</td>
<td>4.34</td>
<td>0.059</td>
<td>0.00</td>
<td>0.970</td>
</tr>
<tr>
<td>N-acetyl-glucosamidase</td>
<td>0.06</td>
<td>0.813</td>
<td>0.57</td>
<td>0.463</td>
</tr>
<tr>
<td>Celllobiohydrodrolase</td>
<td>4.96</td>
<td><strong>0.046</strong></td>
<td>0.01</td>
<td>0.937</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>0.51</td>
<td>0.488</td>
<td>0.69</td>
<td>0.422</td>
</tr>
<tr>
<td>Urease</td>
<td>6.20</td>
<td><strong>0.028</strong></td>
<td>2.58</td>
<td>0.134</td>
</tr>
<tr>
<td>Enzyme Type</td>
<td>Fert x Trt Cess</td>
<td>Exc x Trt Cess</td>
<td>Fert x Exc x Trt Cess</td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----------------</td>
<td>----------------</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$F_{1,12}$</td>
<td>prob</td>
<td>$F_{1,12}$</td>
<td>prob</td>
</tr>
<tr>
<td>β-1,4-glucosidase</td>
<td>0.31</td>
<td>0.589</td>
<td>0.05</td>
<td>0.825</td>
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<td>N-acetyl-glucosamidase</td>
<td>0.11</td>
<td>0.748</td>
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<td>0.336</td>
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<tr>
<td>Cellobiohydrolase</td>
<td>1.56</td>
<td>0.235</td>
<td>0.09</td>
<td>0.774</td>
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<tr>
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<td>0.05</td>
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<tr>
<td>Urease</td>
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<td>0.873</td>
<td>0.03</td>
<td>0.871</td>
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</table>