CARBON, PLANT AND MICROBIAL COMMUNITY DYNAMICS IN LOW-ARCTIC TUNDRA

by

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Abstract

Anthropogenic climate change threatens the stability of Arctic C stores. Soil microbes are central to the C balance of ecosystems as decomposers of soil organic matter and as determinants of plant diversity. In four experiments in the tundra, I address critical gaps in our understanding of the role of soil microbial communities in the response of an Arctic ecosystem to climate change. My objectives were 1) to asses the role of mycorrhizal networks (MN) in plant-plant interactions; 2) to determine the effects of warming and fertilization on the ectomycorrhizal (ECM) community of *Betula nana*; 3) to determine the effect of warming on soil fungi and bacteria over time; 4) to assess the role of the mycorrhizal symbiosis in C-allocation to rhizosphere organisms. I show that MNs exist in tundra and facilitate transfer of C among *Betula nana* individuals, but not among the other plants examined. C-transfer among *Betula nana* pairs through MNs represented 5.5 ± 2.2% of photosynthesis, total belowground transfer of C was 10.7 ± 2.1%. My results suggest that C-transfer through MNs may alter plant interactions, increasing competition by *Betula nana,* and that this will be enhanced with warming. I show that warming leads to a significant increase of fungi with proteolytic capacity, particularly *Cortinarius* spp., and a reduction of fungi with high affinities for labile N, especially *Russula* spp. My findings suggest that warming will alter the ECM community and nutrient cycling, which may facilitate *Betula nana* in tundra. I show that warming leads to a 28% and 22% reduction in the richness of soil fungi and bacteria in tundra, respectively, as well as corresponding declines in diversity. My data agree with reductions in plant community richness with warming at this site, and suggest that warming will reduce total community diversity in tundra. I show that Gram-negative bacteria and a species-specific community of mycorrhizal fungi are the primary consumers of rhizodeposit C among tundra shrubs. Together, these results strongly suggest that soil microbes play a critical role in plant community dynamics and C-cycling in Arctic tundra, and that this role will become increasingly important as climate warms.

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Co-authorship statement

Chapter 2 was co-authored by Suzanne Simard who assisted with study design, data analysis, and preparation of the manuscript. I selected the field site, installed all experimental treatments and plots, and with the help of my field assistants Cathryn Hale, Hsin-fu Hou, Chris Mallon, Andrew Yamada, and, on occasion, several different colleagues at Toolik Lake Research Station, conducted ^{13}C-CO_2 labeling and biomass harvests. Cathryn Hale, Hsin-fu Hou, Andrew Yamada, and Asalatha Manda prepared plant materials for mass spectrometry at the Stable Isotope Laboratory at the University of California, Davis, USA. Gary Laursen (The University of Alaska, Fairbanks, USA) assisted me with sporocarp identifications. Gus Shaver and Jim Laundre (both Marine Biological Lab, Woods Hole, USA) of the Arctic LTER, and Christie Haupert (The University of Alaska, Fairbanks, USA) of the Toolik Lake Environmental Data Center for provided soil temperature and weather station data. In consultation with Suzanne Simard, and Francois Teste (The University of Alberta, Edmonton, Canada) I analyzed all data. In consultation with Suzanne Simard I wrote the manuscript, Sue Grayston provided a critical review of an earlier version.

Chapter 3 was co-authored by Bill Mohn and Suzanne Simard. Bill Mohn and Suzanne Simard assisted with study design and preparation of the manuscript. I designed the study and collected soil cores with the help of Hinrich Schaefer. I did all laboratory work, except DNA sequencing, which was done by the Genome Sciences Center, Vancouver Canada. Andrew Yamada assisted me by editing and assembling raw sequence files. Gus Shaver, Jim Laundre and the Arctic LTER provided soil temperature, and plant community data. I analyzed all data and wrote the manuscript. Suzanne Simard and Bill Mohn provided feedback on an earlier version of the manuscript.

Chapter 4 was co-authored by Martin Hartmann, Bill Mohn, and Suzanne Simard. Martin Hartmann developed the method to analyze ARISA profiles that I used in the study and provided guidance in technical aspects of the laboratory work, as well as providing feedback on an earlier version of the manuscript. Bill Mohn and Suzanne Simard aided in study design and in reviewing the manuscript. I designed the study and conducted all field work. Chris Mallon provided field assistance in 2007. I did all laboratory work except DNA extractions and sequencing, which were done by Gwenn Farrell, and the Genome Sciences Center, Vancouver Canada, respectively. Andrew Yamada assisted me by editing and assembling raw sequence

files. Gus Shaver, Jim Laundre and the Arctic LTER provided soil temperature, and plant community data. I analyzed all data and wrote the manuscript in consultation with Martin Hartmann, Bill Mohn, and Suzanne Simard. Sue Grayston also provided feedback on an earlier version of the manuscript.

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1 Introduction

Climate change in Arctic regions

Despite steady orbitally driven reductions in summer insolation to Arctic regions over the past 2000 years, recent meta-analysis of temperature proxy data for 23 Arctic sites reveals that cooling in Arctic regions has reversed during the present century, with four of the five warmest decades occurring between 1950 and 2000 (Kaufman *et al.* 2009). Arctic warming has exceeded global mean warming by a factor of 2-3 over the past 150 years (Trenberth *et al.* 2007), and is currently greater than the rate of warming during the Pleistocene-Holocene transition, which coincided with widespread vegetation shifts and faunal extinctions in the circumpolar Arctic (Post *et al.* 2009 and references therein). Arctic warming is spatially non-uniform, with the western North American Arctic generally warming the fastest, at rates of up to 0.1^{o}C per year in the past 35 years (Anisimov *et al.* 2007). Despite the buffering effect of landscape heterogeneity, the terrestrial, aquatic, and marine Arctic ecosystems, and the trophic relationships that structure them, have been severely perturbed (Post *et al.* 2009).

Many ecosystem responses consistent with the predicted effects of climate warming have already been observed in Arctic regions and these responses feedback to effect greater ecosystem change. For example, warming has led to the earlier onset of plant growth in Greenland but not to changes in the timing of caribou calving. This is leading to an increasing trophic mismatch between the food demand of reproductive females and seasonal peak resource availability, and is associated with reduced production and survival of caribou calves (Post *et al.* 2008, 2009). Likewise, differences in the relative productivity of tundra plant species with warming have led to ecosystem-scale changes in plant community composition characterized by the expansion of shrubs (Hobbie 1996, Hobbie and Chapin 1998, Sturm *et al.* 2001, Strum *et al.* 2005) and the consequent decline of mosses and evergreen ericaceous species, leading to reductions in plant community diversity (Chapin *et al.*1995, Hobbie 1996, Hobbie and Chapin 1998, Bret-Harte *et al.* 2001). Shrub growth has positive feedbacks to microclimatic conditions that enhance ecosystem change and lead to greater climate forcing (Sturm *et al.* 2001). Beneath shrub thickets, increased local snow-trapping in winter, increased soil insulation, higher winter and spring-time soil temperatures, and increased rates of nutrient

mineralization lead to local conditions that further favour shrub growth and expansion onto tussock tundra (Sturm *et al.* 2001, Strum *et al.* 2005, Weintraub and Schimel 2005).

Arctic C storage will be mediated by soil microbes

Of particular concern in Arctic regions are changes in the globally significant C and nutrient stores that have accumulated in tundra soils since the last glacial maximum because cold and short growing seasons and the presence of permafrost, which restricts drainage, limit decomposition more than plant net primary productivity. These processes and cryoturbation, by which soil organic C is buried and incorporated into permafrost, have led to almost twice as much C stored in soils of Arctic and Boreal regions as is currently present in the atmosphere (Schuur *et al.* 2009 and references therein). Arctic warming is associated with increased microbial activity, increased plant N availability (Chapin 1983, Nadelhoffer *et al.*1992, Chapin *et al.*1995, Aerts 2006), and faster C turnover in Arctic soils (Hobbie and Chapin 1998, Mack *et al.* 2004, Shaver *et al.* 2006, Nowinski *et al.* 2008, Schuur *et al.* 2009). These findings have led to a growing concern that warming threatens the stability of Arctic C stores which, upon release, are likely to result in significant additional positive climate forcing (Cox *et al.* 2000, Friedlingstein *et al.* 2006, Schuur *et al.* 2009).

The stability of Arctic soil C stores may not only be sensitive to changes in the activity of soil microorganisms but also to changes in the composition of microbial communities. For example, temperature-induced changes in the structure of soil bacterial communities in an American hardwood forest soil led to a non-linear relationship between soil temperature and microbial respiration and to the hypothesis that increased temperature leads to shifts in microbial community composition, wherein dominant populations at higher temperatures have access to substrates that are not utilized by members of the community at lower temperatures (MacDonald *et al.* 1995, Zogg *et al.* 1997). However, our current understanding of the relationships between temperature and microbial community composition and function in Arctic soils is very limited (Nemergut *et al.* 2005).

Our ability to predict the response of soil microbial communities to climate change is partly hampered by the many interacting factors that influence the structure of soil microbial

communities. For instance, compositional shifts in Arctic microbial communities may be a direct result of increased temperature, or may be mediated by changes in plant communities or other soil organisms, and the timescales of these interactions may differ, complicating observed trends. For example, a significant increase in the density of fungal-feeding nematodes was observed after 6 years of warming a Swedish tundra heath (Ruess *et al.* 1999), but a significant decline in the abundance of fungal biomarkers was only observed after 15 years of warming in this experiment (Rinnan *et al.* 2007). Likewise, 7 years of warming at a High-Arctic site resulted in no change in the composition of the root-associated fungal community of *Salix arctica* but there was an increase in root-associated fungal biomass (Fujimura *et al.* 2008). Similarly, 13 years of warming an Alaskan tussock tundra site led to a significant increase in all fungal biomarkers, and a significant increase in the mycelial production of mycorrhizal fungi. However, the increase in mycorrhizal biomass was associated with a strong increase in the biomass of the host shrub *Betula nana* and was interpreted to represent an overall reduction of mycorrhizal fungal biomass relative to plant root biomass (Clemmensen *et al.* 2006).

The role of mycorrhizal fungi in the response of Arctic ecosystems to climate change warrants more attention than it has received. Shrubs, which are the tundra plants that show the strongest positive biomass response to climate change (Brete-Hart *et al.* 2001, Sturm *et al*. 2001), form obligatory symbioses with ectomycorrhizal (ECM) fungi. Symbiotic ECM are the main pathway for plant access to inorganic and organic soil nutrients (Leake & Read, 1997; Smith & Read, 1997). A model based on the natural abundance of ^{13}C and ^{15}N in plant and fungal tissues from Alaskan tundra estimated that 61-86% of the nitrogen in plants is filtered through mycorrhizal fungi (Hobbie and Hobbie 2006). Congruent with above ground shrub biomass, ECM biomass appears to increase in response to climate change factors (Clemmensen *et al.* 2006).This change may enhance C-storage in surface layers of tundra soils because ECM fungi are a significant sink for plant photosynthate (Simard *et al.* 2002, Hobbie and Hobbie 2006) and fungal hyphae are comprised of complex C compounds that are recycled slowly and degraded only by a subset of the microbial community (de Boer *et al.* 2006). On the other hand, high substrate affinities, unique enzymatic capabilities, and a tendency to grow prolifically in organic substrates (Bending 2003, Leake *et al.* 2004, Read *et al.* 2004) enable mycorrhizal fungi to significantly alter soil C stores as they act to release nutrients in soil organic matter (Langley and Hungate 2003). We currently have a limited understanding of climate

change effects on mycorrhizal fungi in Arctic ecosystems and we know even less about how these potential effects will influence C and nutrient cycling in Arctic tundra.

Arctic plant community dynamics will be mediated by soil microbes

A concerted experimental effort to assess the impacts of climate change on terrestrial ecosystems in the circumpolar Arctic has led to the development of a good understanding of the strong direct responses of tundra plant communities to climate change factors. However, there is growing recognition that ecosystem response to climate change will be a complex response modulated by trophic interactions, including those among plants, their mycorrhizae, and decomposer microbial communities (Read *et al.* 2004, Brooker 2006, Bargett *et al.* 2008, Post *et al.* 2009). Soil microbes are central to the C and nutrient balance of tundra ecosystems not only in their role as decomposers of soil organic matter, but also as determinants of plant community diversity (van der Heijden *et al.* 2008), which in turn controls the quality and quantity of C inputs to soils (De Deyn *et al.* 2008, Bardgett *et al.* 2008). Soil microbial communities may alter plant-plant interactions through positive or negative effects on plant growth, leading to the competitive exclusion or coexistence of competitors in ecosystems (Bever 2003). Presently, we know little of the role of species interactions in determining ecosystems response to climate change in Arctic regions.

One important mechanism by which soil microorganisms may influence plant communities is through the formation of mycorrhizal networks (MNs). MNs occur because most mycorrhizal associations are non-specific, and the mycelium of one or more mycorrhizal fungal species can colonize two or more nearby plants of the same or different species (Molina *et al.* 1992). Mycorrhizal networks have been shown to link plants within ECM, ericoid (ERM) and arbuscular mycorrhizal (AM) plant communities (Fitter *et al.* 1998, Vrålstad *et al.* 2002, He *et al.* 2003, Larat *et al.* 2002, Beiler *et al.* 2009). Arctic tundra plants are likely to be interconnected in a MN because of their low mycorrhizal specificity and dense, intermingling root architecture (Read *et al.* 2004). MNs are increasingly shown to influence plant community structure through nutrient transfers or through mycorrhizal colonization effects on plant establishment, interspecific competition, and community diversity (Perry *et al.* 1989, Fitter *et al.* 1998, van der Heijden *et al.* 1998, Klironomos 2002, Simard and Durall 2004; Teste *et al.* 2009). Thus, growing theoretical and empirical evidence suggests that MNs are drivers of change in plant communities and consequent storage of C in ecosystems (see Read *et al.* 2004, Brooker 2006).

To date there has been no quantitative work done on the occurrence or role of MNs in tundra ecosystems.

Research objectives and outline of thesis

In this thesis I sought to address critical gaps in our understanding of the role of soil microbial communities in the response of an Arctic ecosystem to climate change. Recognizing that direct effects of climate change factors on soil communities are likely to feed back to plant communities through symbiotic and trophic interactions, my approach was two-fold: firstly, I sought to characterize the relationships among plants and their associated soil microbial communities by elucidating the fate of newly photosynthesized C in a tundra ecosystem; and secondly, I sought to characterize changes in the structure and composition of soil microbial communities that result from climate change factors.

My research addressed three central questions:
1) What is the ecological role of soil microbes in belowground C transfer within a tundra plant community?
2) Which microbial groups are important in the cycling of newly photosynthesized C in a Low-Arctic ecosystem?
3) What impact do climate change factors have on the composition of symbiotic and free-living soil microbes, and what is the temporal component to their response?

I answered these questions in a series of 4 integrated experiments, each designed to test specific hypotheses regarding the key factors that regulate C-dynamics in a Low-Arctic ecosystem.

In Chapter 2, I applied $^{13}CO_2$ to tundra plants in the field in order to test the hypothesis that mycorrhizal networks (MNs) are involved in the interplant transfer of C in a Low-Arctic tundra plant community adjacent to Toolik Lake, Alaska, USA. I predicted that belowground C-transfer would be greater through MNs than through soil pathways, and I characterized the role of MNs by testing specific hypotheses regarding their function. These hypotheses included increasing C-transfer with increasing mycorrhizal connectivity among donor and receiver plants, increasing C-transfer with increasing sink strength of receivers, and increasing total C-transfer over time. Finally, I tested the hypothesis that the amount of C transferred among plants would be of sufficient magnitude to potentially facilitate shrub expansion in Low-Arctic tundra.

Betula nana responds strongly to experimental simulations of climate change, and it is rapidly expanding in Alaskan tussock tundra as climate warms. In Chapter 3, I examine the response of the ECM community of *Betula nana* to long-term experimental warming and fertilizer additions. I sampled *Betula nana* roots in control plots and in plots that had received either fertilizer, warming by greenhouses, or both, since 1989 in Arctic Alaska. I constructed clone libraries of the ITS region of the fungal rRNA gene for each treatment to test the hypothesis that long-term fertilizer addition and warming by greenhouses would alter the composition of the mycorrhizal community of *Betula nana* in Low-Arctic tussock tundra. Despite the observation that warming and fertilizer amendment are known to have additive effects on gains in above ground biomass by *Betula nana* (Chapin *et al.*1995), I hypothesized that the two treatments would led to different rather than additive responses of the ECM community. I predicted that fertilization will result in the increase of ECM taxa characteristic of sites with high N availability while warming would lead to an ECM community adapted to N limitation.

In Chapter 4, I test the hypothesis that long-term warming will alter the composition of soil bacterial and fungal communities in Low-Arctic tundra. I predict that changes in microbial community structure due to warming would exceed those due to normal seasonal succession. I sampled soils from control plots and those treated with warming by greenhouses repeatedly during the summers of 2006 and 2007 and used Ribosomal Intergenic Spacer Analysis of the Internal Transcribed Spacer region of 16S/18S rRNA to profile the bacterial and fungal communities in each sample. To characterize the microbial community composition, I constructed clone libraries for bacteria and fungi in two soil horizons from the warming and control treatments.

In Chapter 5, I used stable-isotope probing of phospholipid fatty acids (PLFA-SIP) and DNA (DNA-SIP) to examine the fate of newly photosynthesized plant C in a Low-Arctic tundra ecosystem. I hypothesized that carbon acquisition patterns of rhizosphere organisms would be a function of plant mycorrhizal type, and I selected plant species based on their mycorrhizal strategies: *Betula nana* (ectomycorrhizal), *Salix pulchra* (ecto- and arbuscular mycorrhizal), and *Ledum palustre* (ericoid mycorrhizal). The combination of these methods provided unique insight to the key microbial players in the carbon cycle of this Low Arctic tundra ecosystem.

In Chapter 6, I integrate data from the four primary chapters and discuss general findings that arise from this work. I relate this research to the current body of literature on the role of microbial communities in ecosystem response to climate change in Arctic regions. I further discuss strengths and limitations of my contribution. I identify gaps that remain in our understanding of Arctic plant-microbial interactions and suggest priority areas for future research that will be most valuable for predicting biological response to climate change.

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2 Carbon transfer through mycorrhizal networks may facilitate shrub expansion in Low-Arctic tundra[1]

Introduction

Human-induced climate change is resulting in non-uniform warming of the earth's surface. Currently, high latitude ecosystems are warming faster than temperate and equatorial regions, and the western North American Arctic is experiencing the highest rate of warming on earth (Anisimov *et al.* 2007). This warming is associated with increased N availability (Chapin 1983, Nadelhoffer *et al*. 1992, Chapin *et al.* 1995, Aerts 2006) and faster C turnover in Arctic soils (Mack *et al.* 2004, Shaver *et al*. 2006, Schuur *et al*. 2009) due to increased microbial activity. Concurrent with these changes is the well documented shift in plant community composition in Arctic tundra, characterized by the expansion of shrubs (Hobbie 1996, Hobbie and Chapin 1998) and the consequent decline of mosses and evergreen ericaceous species (*e.g. Ledum palustre, Vacccinium vitis-ideae, Cassiope tetragona*). This plant community shift has been shown both experimentally (Chapin *et al*.1995, Hobbie *et al*.1999) and observationally at the landscape scale (Sturm *et al*. 2001, Stow *et al*. 2004).

Warming induced plant community change in Arctic tundra has led to positive feedbacks that enhance ecosystem change and lead to greater climate forcing (Sturm *et al*. 2001). For instance, the spread of shrubs onto tundra is associated with increased local snow-trapping in winter, increased soil insulation, higher winter and spring-time soil temperatures, and increased rates of nutrient mineralization. These local conditions beneath shrub thickets impart a competitive advantage that facilitates early season nutrient uptake and consequent increased shrub growth and expansion onto tussock tundra (Sturm *et al*. 2001, Strum *et al*. 2005, Weintraub and Schimel 2005a).

Shrub expansion may cause an increase in C-storage in tundra ecosystems through the increased production of wood, which is slow to decompose, or may cause a decrease in tundra C-storage by releasing the soil microbial community from N-limitation through the input of higher quality leaf litter (Weintraub and Schimel 2005a) and by increasing permafrost thaw and

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microbial decomposition of previously frozen C (Schuur *et al*. 2009). However, changes in decomposition and nutrient mineralization rates cannot be fully understood by considering changes in the plant community alone. Shrub invasion onto tundra necessarily alters the soil microbial community, as all major tundra shrub genera form symbiotic associations with ectomycorrhizal (ECM) fungi. Congruent with above ground plant biomass, both ECM and total fungal biomass appear to increase with long-term warming and fertilization treatments in this ecosystem (Clemmensen *et al*. 2006). Increased ECM fungal abundance should enhance C-storage in surface layers of tundra soils because ECM fungi are a significant sink for plant photosynthate (Simard *et al*. 2002, Hobbie and Hobbie 2006) and fungal hyphae are comprised of complex C compounds that are recycled slowly and degraded only by a subset of the microbial community (de Boer *et al.* 2006). However, the tendancy for ectomycorrhizal fungi to grow prolifically in organic substrates (Bending 2003, Leake *et al*. 2004, Read *et al*. 2004), coupled with their unique enzymatic capabilities, may reduce soil C stores (Langley and Hungate 2003).

The role of mycorrhizal fungi in ecosystem response to climate change warrants more attention than it has received (Treseder and Allen 2000). Ectomycorrhizae are the main pathway for plant access to inorganic and organic soil nutrients (Leake & Read, 1997; Smith & Read, 1997). A model based on the natural abundance of ^{15}N in plant and fungal tissues from Alaskan tundra estimated that 61-86% of the nitrogen in plants is filtered through mycorrhizal fungi (Hobbie and Hobbie 2006). Mycorrhizal networks (MN) occur because most mycorrhizal associations are non-specific, and the mycelium of one or more mycorrhizal fungal species can colonize two or more nearby plants of the same or different species (Molina *et al*. 1992). Mycorrhizal networks have been shown to link plants in ECM, ericoid (ERM) and arbuscular mycorrhizal (AM) plant communities (Fitter *et al*. 1998, Vrålstad *et al*. 2002, He *et al.* 2003, Lerat *et al*. 2002). Arctic tundra plants are likely to be interconnected in a MN because of their low mycorrhizal specificity and dense, intermingling root architecture (Read *et al.* 2004).

Mycorrhizal networks have been shown to provide efficient pathways for belowground carbon and nutrient fluxes among plants in natural systems (Simard *et al.* 1997, Lerat *et al.* 2002, He *et al.* 2003, Teste *et al*. 2009). The magnitude and direction of nutrient transfers among plants has been shown to shift with interplant source-sink gradients for C and N that

occur, for example, through shading or over the growing season (Simard *et al*.1997, Lerat *et al*. 2002), and sink-strength appears to be a stronger determinant of total transfer than source-strength in field settings (Simard *et al*. 1997, He *et al*. 2005a,b, Teste *et al*. 2009). In forest ecosystems, net C-transfer tracks inter-plant photosynthetic differences whereas total C-transfer increases over the growing season as C is increasingly allocated to belowground structures (Philip 2006).

Betula sp. is widespread in temperate, boreal, and Arctic regions of the northern hemisphere. Members of the *Betula* genus are obligate symbionts with ECM fungi in nature (Molina *et al*. 1992) and have been shown to be important sources and sinks for C transferred through MNs in natural ecosystems (Simard *et al.* 1997, Philip and Simard 2008). A deciduous habit leads to periodically high nutrient demands and strong temporal source-sink gradients for C, conditions which promote C-transfer through networks. In Alaskan Low-Arctic tundra, *Betula nana* allocates a high proportion of C below-ground to rhizomes, roots, (Chapin *et al.* 1980) and mycorrhizal structures (Hobbie and Hobbie 2006). It is the strongest competitor for soil N (Bret-Harte *et al*. 2008) and increasingly dominates fertilized tussock tundra in long-term experiments (Chapin *et al*. 1995; Bret-Harte *et al*. 2001; Shaver *et al*. 2001; Mack *et al*. 2004, Bret-Harte *et al*. 2008). *Betula nana* responds to fertilization by generating additional meristems that facilitate rapid new growth under favourable conditions (Bret-Harte *et al*. 2001, 2002). The plasticity with which *Betula nana* responds to changing nutrient conditions suggests that it utilizes effective strategies to reallocate C. However nothing is known of the role of MNs in the C economy of *Betula nana*.

In natural ecosystems, MNs are increasingly shown to influence plant community structure through nutrient transfer or mycorrhizal colonization effects on plant establishment, interspecific competition, and community diversity (Perry *et al*. 1989, Fitter *et al*. 1998, van der Heijden *et al*. 1998, Klironomos 2002, Simard and Durall 2004; Teste *et al*. 2009). To that end, there is growing theoretical and empirical evidence that MNs are drivers of change in plant communities and consequent storage of C in ecosystems (see Read *et al.* 2004, Brooker 2006). To date there has been no quantitative work done on the occurrence or role of MNs in tundra ecosystems.

The objective of this study was to quantify C-transfer through belowground pathways in a Low-Arctic tundra ecosystem. More specifically, we sought to establish whether MNs play a role in C-transfer among plants in this community and if so, to assess the relative ecological significance of C-transfer among different plant species. Our study took place in the relatively well studied, Moist Acidic Tundra adjacent to Toolik Lake, Alaska, where plants that form ECM, ERM, and AM or are non-mycorrhizal, are intermingled in relatively equal abundance (Shaver and Chapin 1991). We studied inter-plant C-transfer by labelling *Betula nana* and *Ledum palustre* with ^{13}C. These species were selected because they are the dominant over-story and mid-canopy species and the ECM and ERM species with the highest biomass at the study site. Our study was designed to test three hypotheses. Firstly, we hypothesized that MNs are involved in the transfer of C among plant pairs in Arctic tundra. We predicted that we would observe greater C-transfer through MNs than through soil pathways. Secondly, we hypothesized that any factor that increased mycorrhizal connectivity among donor and receiver plants, or increased the sink strength of receivers, would result in greater C-transfer between pairs. Thus, we predicted greater C-transfer among conspecific versus inter-specific plant pairs and among plant pairs sharing the same mycorrhizal type (i.e. ECM versus ERM). Likewise, because of their large relative size, we predicted that shrubs would be stronger sinks for C than other tundra plants, and that sink strength of shrubs would increase with their size. Because plant connectivity to a MN should increase with time since establishment, we also predicted that C-transfer between plants would increase from the beginning to the end of the growing season, and from 2007 to 2008. Finally, we hypothesized that the amount of C transferred among plants would be of sufficient magnitude to potentially facilitate shrub expansion in the Low-Arctic Tundra.

Methods

Study site

The study site was located on a gentle (<5^{o}), north-facing slope in Moist Acidic Tundra near Toolik Lake, Alaska, USA (68^{o}38'N, 149^{o}34'W, elevation 780m) approximately 200 m east of the site of the Arctic Long-term Ecological Research (LTER) experiment (see supplementary figures 2.1 & 2.2 for maps of the study site). Mean annual temperature at the study site is -8.5^{o}C, with a mean summertime temperature of 9^{o}C. Mean annual rainfall is 350 mm, and approximately half of this falls as rain in the summer. Moist Acidic Tundra forms on old glacial surfaces (>56,000 years BP) and supports 'heathland'-type tundra plant communities. Regular patterns of vegetation occur in this plant community as a result of the perennial, rhizomatous growth of the tussock-forming sedge, *Eriophorum vaginatum*. The dominant deciduous dwarf shrub, EM *Betula nana*, occupies hollows between the sedge-tussocks in mixture with mid-canopy ericaceous ERM species, such as *Ledum palustre* and *Vaccinium* sp., as well as herbs. Both plurocarpus and acrocarpus mosses are common and form a continuous ground cover that can exceed 40 cm depth in some areas. Soils are characterized by a thick organic horizon, 20-30 cm in depth, with a pH of 5 (Schmidt *et al*. 2002). Mineral soils are essentially unweathered, pale grey in colour, permafrost affected, and show only occasional evidence of cryoturbation. Maximum thaw depth occurs in late July, when the active layer will typically comprise the entire organic horizon and the top 5-10 cm of the mineral soil.

Treatments & $^{13}CO_2$ labeling

Experimental treatment plots were installed in September of 2005 and summer of 2006. Plots were centered on randomly selected *Betula nana* ramets and were a minimum of 3 meters apart. Because of the clonal growth form of many plants, we isolated treatment plots by manually trenching soil to the bottom of the active layer and inserting 150 cm X 20 cm sheets of 24 gauge galvanized steel in rings (approximately 45 cm in diameter). Plant communities within the treatment plot boundaries were left intact. Plot boundaries were maintained over the course of the experiment by ensuring the sheet metal always extended to the bottom of the active layer. Each plot was assigned to one of two donor species (*Betula nana* or *Ledum palustre*) and one of three belowground pathway treatments (mesh, severed, or all-pathways). In all treatment plots, the "donor" *Betula nana* or *Ledum palustre* ramet was selected by choosing an individual

with high leaf area growing to one side of the ringed plot. In the mesh treatment, the donor *Betula nana* or *Ledum palustre* plant was excavated using a serrated kitchen-knife, and the roots and adhering soil carefully placed in a custom made, conical 35 μm nylon mesh bag (Plastok®, Birkenhead, Merseyside UK), preserving the plant's original orientation. The mesh pore size (35 μm diameter) was selected to allow passage of fungal hyphae but prevent penetration of roots or rhizomes, thus allowing formation of mycorrhizal networks between plants inside and outside of the mesh bags (Teste *et al*. 2006). The mesh treatment therefore represented C transferred through MNs and soil pathways alone. In the severed treatment, the entire active layer, including all roots, rhizomes and MNs, was severed midway between the "donor" *Betula nana* or *Ledum palustre* and the "receiver" plant with a serrated kitchen-knife immediately prior to $^{13}CO_2$ labeling. Thus, the severed treatment represented C transferred through soil solution alone. In the all-pathways treatment, no soil manipulation was applied. The all-pathways treatment allowed for carbon transfer from a donor plant to a receiver plant via the soil solution, through MNs, and directly via root to root contact or through rhizomes, in the case of clones of a common rhizome.

Separate sets of plants were labeled with $^{13}CO_2$ at four periods over the course of the experiment: in late-June and mid-August) of 2007 and 2008 (each labeling period hereafter referred to as a 'run'). For each run, ten plants of each donor species (*Betula nana* and *Ledum palustre*) per treatment type (mesh, severing, all-pathways) were randomly selected for labeling (total of 60 plots per run). *Betula nana* and *Ledum palustre* donor plants were sealed in 10 L and 5 L (respectively) custom-made FEP gas sampling bags (Jensen Inert Products) with polypropylene Jaco fittings and silicone stoppers. Gas labeling bags were inflated to capacity using a hand pump. Donor plants were pulse labeled with 1.9-3.2 mmol $^{13}CO_2$ (99.9%; Cambridge Isotope Labs). CO_2 concentrations inside the labeling bags were measured approximately every 30 minutes using a portable infrared gas analyzer (Qubit Systems). Labeling bags were removed when the CO_2 concentration in the bag fell below 300 ppm (4-8 hrs).

After a 7-day chase period, donor and receiver *Betula nana* and *Ledum palustre* plants were harvested from the treatment plots. In 2008, we extended our harvest to include all receiver species in 4 of 10 mesh and severed plots. Plant tissues harvested for all woody or rhizomatous species included leaves, stems, and rhizomes. For herbaceous species, only above-ground biomass was collected. Unfortunately, the very fine roots (<1 mm diameter) and

complex root architecture of *Ledum palustre* limited our ability to effectively collect complete root systems. As a result, the roots of all species were excluded from harvests. Root biomass in *Ledum palustre* and *Betula nana* was small varying seasonally from approximately 9 to 13% of total plant biomass (dry mass) from June (data not shown) to late July, when annual biomass production peaked (S.E. Hobbie, pers. comm.). See Supplementary Figures 2.2, 2.3 and 2.4, for additional information of the experimental design.

Sporocarp collection

Fungal sporocarps were collected at the study site when they occurred throughout the summers of 2006, 2007 and 2008. Sporocarps were photographed in situ, collected, and oven dried at 60^{o}C. Initial identifications of fungal sporocarps were made in the field and from photographs in consultation with Dr. Gary Laursen at University of Alaska Fairbanks. Subsequent confirmation of sporocarp identities were made by sequencing of DNA extracted from sterile internal structures of the sporocarp tissue.

Isotope analysis

Plant tissues were oven-dried at 65°C for a minimum of 48 hours and then weighed. Woody tissues were coarse-ground with a Thomas® Wiley® mini-mill (Thomas© Scientific, NJ, USA). All plant tissues were then ground to a 0.01 mm fine powder using a MM 200 ball mill (Retsch® Newtown, PA, USA) or by hand in a mortar and pestle. All samples were thoroughly homogenized before a ~1 mg subsample was obtained for the measurement of C isotope composition. The CO_2 liberated from the combustion of each sample was analyzed for its $^{13}C/^{12}C$ ratio using either a continuous flow Europa Hydra 20/20 (receivers) or a Europa Integra (donors) isotope ratio mass spectrometer at the UC Davis Stable Isotope Laboratory (Davis, CA, USA). The C isotope ratio ($\delta^{13}C$) was calculated as:

$$\delta^{13}C = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000$$

in ‰ units, where $R = {}^{13}C/{}^{12}C$. The sample ratio is relative to Vienna-PeeDee Belemnite (1.1237 x 10^{-2}), the standard for C. To convert $\delta^{13}C$ into mg of excess ^{12}C-equivalent for plant tissues, we followed the procedure outlined in Teste *et al.* (2009a). Whole plant excess ^{12}C-equivalent

was calculated as the sum of excess ^{12}C-equivalent for all tissues (leaves, stems and rhizomes).

Statistical analysis and meteorological data

Receiver whole plant excess ^{12}C-equivalent data was normalized by applying a natural logarithm (*ln*) transformation after removal of outliers (>2SD from the mean) and receiver plants paired with poorly enriched donors (<5 mg excess ^{12}C-equivalent) (after Teste *et al.* 2009a). General Linear Models (GLM) were used to assess the effects of donor enrichment, donor and receiver species, mycorrhizal type, year, month, and belowground pathway treatment on *ln*-normalized whole receiver excess ^{12}C-equivalent, and to test the relationship between receiver size and enrichment. Barlett's and Levene's tests were used to test for heteroscedascity among groups of categorical factors. Residual plots were used to detect heteroscedascity in continuous predictor variables. Where data violated the assumptions of the GLM, non-parametric tests were used: a Wald-Wolfowitz Runs test, a Mann-Whitney U-test, and a Kolmogorov-Smirnov test were applied to factors with only two categories; a Kruskal-Wallis ANOVA and a Medians test were applied to factors with greater than two categories. To test whether potential plant damage caused by the application of severing and mesh treatments altered patterns of C-transfer in experimental plots, we compared *ln*-normalized receiver enrichments by species from all-pathway plots to those for all treatments using t-tests. To assess the ecological significance of belowground C-transfer pathways in the plant community, we calculated the proportion of fixed C that was transferred from donor to receiver plants, and applied a Kruskal-Wallis ANOVA and a Medians test to determine significant differences among soil treatments, as above. Note that this led to a much higher number of replicates for the mesh treatment than in the calculation of absolute enrichment as plots with <5mg excess ^{12}C-equivalent were not excluded.

The Arctic Long-Term Ecological Research (LTER) network maintains the Toolik Field Station Meteorological Stations at Toolik Lake, Alaska, which are located approximately 600 meters from the study site. Environmental variables are measured every 10 minutes and averaged every three hours. Daily average, maximum, and minimum air and soil temperatures, and the sum of global radiation, photosynthetically active radiation, and precipitation were downloaded from the Arctic Long-Term Ecological Research website (http://ecosystems.mbl.edu/ARC/) for the four pulse-chase runs, as well as for the 7 day periods

that preceded each run. GLMs were used to detect differences among these time periods for all environmental variables. Principal Components Analysis was then used to reduce the number of redundant environmental variables. The effect of remaining environmental factors on *ln*-normalized whole receiver excess ^{12}C-equivalent values were then tested using GLM, as above. All statistical analyzes were performed using STATISTICA version 8.0. (StatSoft, Inc. Tulsa,OK, USA).

Results

Effect of belowground pathways on carbon transfer

Analysis of C-transfer among conspecific *Betula nana* pairs revealed a significant effect of belowground pathway treatment on carbon transfer to receivers (Kruskal-Wallis ANOVA, p=0.0348). *Betula nana* plants received significantly less carbon where they were severed from donors than where all below-ground pathways were left intact (Fig. 1). Where donors were in mesh, receiver plants received intermediate and highly variable amounts of carbon.

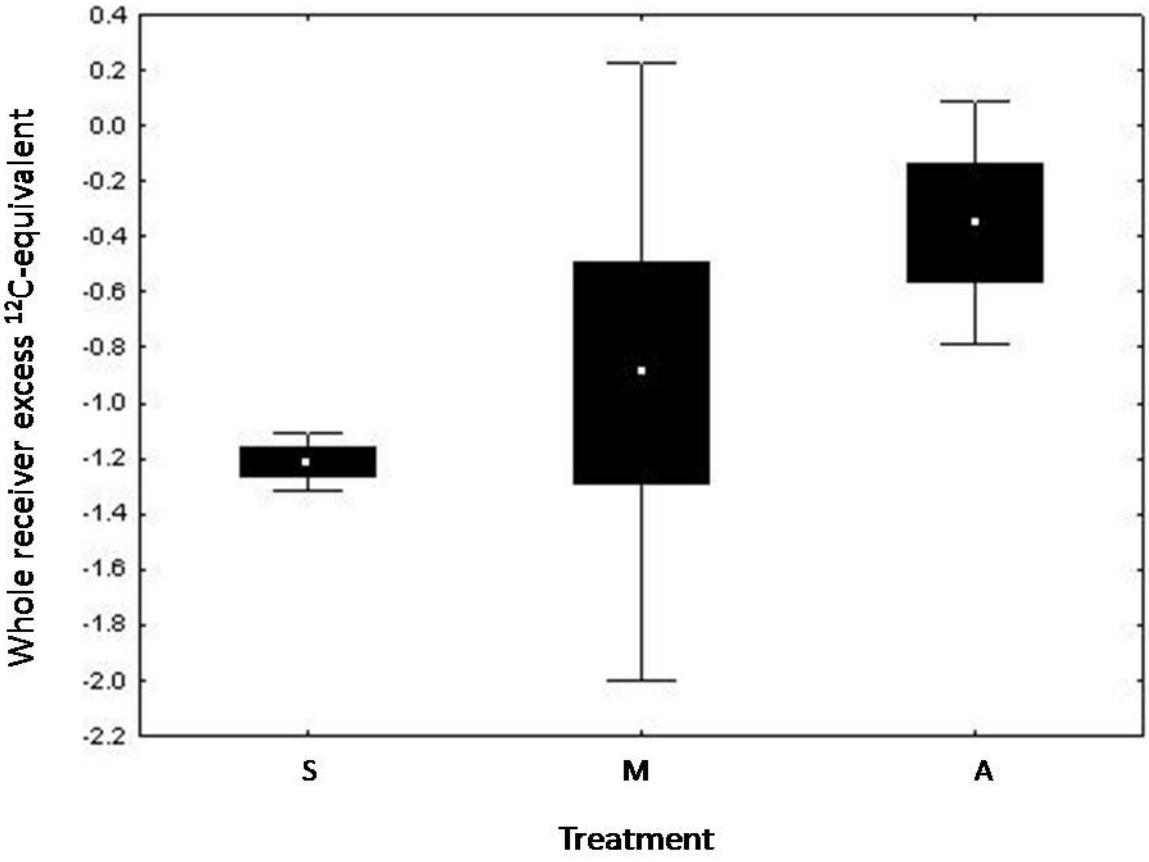

Figure 2.1: Ln-normalized whole receiver enrichment (sum of leaf, stem and rhizome enrichments) by belowground pathway treatment. Treatments symbols: S= severed, A= all pathways intact, M= donor planted in mesh. Inner spread denotes the standard error of the

mean; whiskers denote the 95% confidence interval about the mean; S (n=27); A (n=24); M (n=5).

Given the pattern of C-transfer by treatment for conspecific *Betula nana* pairs, we assessed the relative importance of belowground pathways for C-transfer to *Betula nana*. Mean C transfer through soil solution, represented by the mean tissue enrichment for *Betula nana* receivers in the severing treatment, was $8.8 \times 10^{-2} \pm 0.15$ (S.E.) mg, which was not significantly different from zero. Direct C-transfer through root grafts or joint rhizomes, calculated as the difference in mean enrichment of *Betula nana* receivers between the all-pathway and mesh treatments, was 0.54 ± 0.50 mg; this amount was nearly indistinguishable from zero due to the high variance associated with C-transfer in the mesh treatment. Mean C-transfer through MNs, represented by enrichment of *Betula nana* receivers in the mesh treatment, was 0.40 ± 0.35 mg.

To assess whether the application of severing and mesh treatments may have damaged plants and thus altered patterns of carbon transfer, we compared receiver enrichment by species between all treatments combined and the all-pathway treatment only. We found no significant differences in the patterns of enrichment by species, although the mean excess ^{12}C-equivalent of receivers was generally greater in the all-pathway plots (Table 2.1).

Table 2.1: Comparison of mean whole receiver excess ^{12}C-equivalent by species for all treatment plots and for all-pathway plots only. One standard error of the mean and the sample size are also shown.

Species or Group[1]	**All data** **mg**			**All-pathway plots** **mg**			**T-statistic (p-value)**
	mean	*S.E.*	*N*	*mean*	*S.E.*	*N*	
Betula nana	0.42	5.2×10^{-2}	87	0.59	7.3×10^{-2}	40	-1.27 (ns)
Ledum palustre	-2.7×10^{-2}	5.2×10^{-2}	89	-3.3×10^{-2}	7.9×10^{-2}	34	0.490 (ns)
V. vitis-idaea	6.5×10^{-3}	9.5×10^{-2}	26	1.4×10^{-2}	0.11	19	-0.181 (ns)
Other Ericaceae	-1.6×10^{-2}	7.6×10^{-2}	41	-2.5×10^{-2}	8.9×10^{-2}	27	0.808 (ns)
Salix sp.	-3.0×10^{-2}	0.17	8	-2.3×10^{-2}	0.23	4	-0.341 (ns)
Carex sp.	-6.8×10^{-3}	9.5×10^{-2}	26	-1.3×10^{-2}	0.11	18	0.689 (ns)
Eriophorum. vaginatum	-6.0×10^{-4}	0.15	19	3.7×10^{-3}	0.19	6	-1.04 (ns)
Pedicularis sp.	1.2×10^{-3}	0.11	20	1.1×10^{-3}	0.17	7	0.0338 (ns)
Polygonum sp.	-4.5×10^{-3}	0.11	19	-5.1×10^{-3}	0.12	15	0.0338 (ns)
Other forb	-9.5×10^{-4}	0.12	16	-1.0×10^{-3}	0.13	13	-0.181 (ns)

[1] Plant species that comprise the 'Other Ericaceae' category are: *Andromeda polifolia, Arctostaphylos alpina, Cassiope tetragona, Empetrum nigrum, Loiseleuria procumbens, Pyrola sp.,* and *Vaccinium uliginosum*. Plant species that comprise the 'Other Forb' category are: *Arnica sp., Cardamine bellifolia, Petasites frigidus, Ranunculus sp.,* and *Stellaria longipes*.

Within the mesh and all-pathways treatments, mycorrhizal networks could potentially form between intraspecific plant pairs, or between interspecific pairs provided the plant species host fungal species belonging to the same mycorrhizal type (ECM or ERM). Sporocarp surveys showed that several ECM fungi were common at the site, potentially forming MNs. The most abundant taxa collected were *Cortinarius favrei*, followed by *Leccinum scabrum* and *Cortinarius cf. torvus*. Other common species included *Russula emetic* and *Russula xerampelina*, plus several other species in the *Russula, Lactarius, Hebeloma*, and *Cortinarius* genera. Supplementary table 2.1 provides a complete list of fungal sporocarp taxa collected at the site.

Effect of plant factors on C transfer

Analysis of all species receiver data revealed significantly greater C transfer among conspecific plant pairs than among interspecific plant pairs ($\overline{x}_{conspecific}$ 0.322 ± 0.053 mg ^{12}C-equivalent; $\overline{x}_{interspecific}$ 0.019 ± 0.031 mg ^{12}C-equivalent; $p<0.0001$). Further, when species were categorized by their potential to form mycorrhizal connections, we found that receivers in pairs colonized by the same mycorrhizal type were significantly more enriched than receivers in pairs with different mycorrhizal types ($\overline{x}_{same\ myc.}$ 0.227 ± 0.046 mg ^{12}C-equivalent; $\overline{x}_{different\ myc.}$ 0.024 ± 0.034 mg ^{12}C-equivalent; $p<0.0001$). However, analysis of all receiver data by species shows that significant C transfer was observed only to *Betula nana,* from both *Betula nana* and *Ledum palustre* in this study ($p<0.00001$, Figure 2.2).

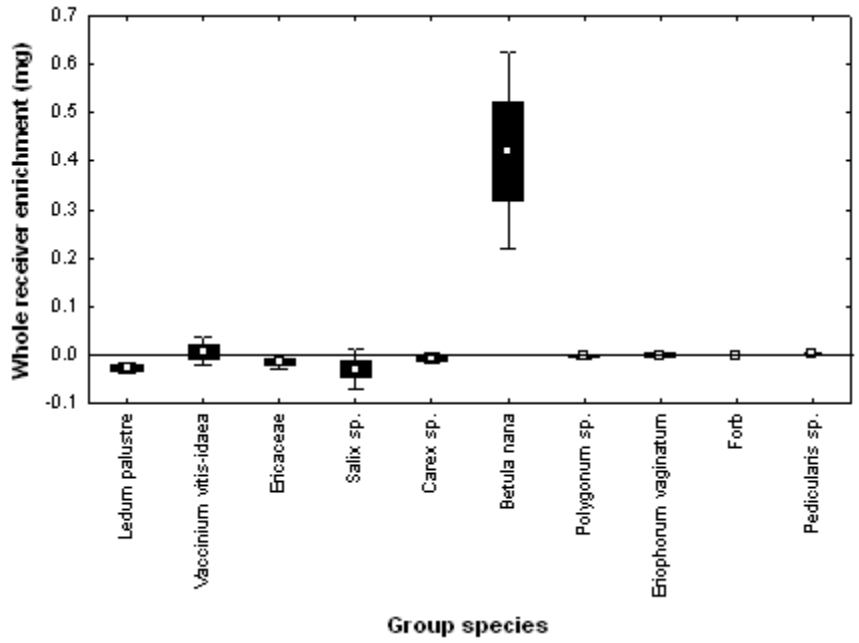

Figure 2.2: Whole receiver enrichment calculated as the sum of leaf, stem and rhizome enrichments in mg of excess ^{12}C-equivalent. Inner spread denotes the standard error of the mean; whiskers denote the 99% confidence interval about the mean.

In *Betula nana* pairs, we observed a significant positive relationship between a receiver's biomass (in Ln-normalized mg dry mass) and its enrichment (Ln-normalized ^{12}C-equivalent in mg; $F= 5.519$; $r^2=0.093$; $p=0.0225$) indicating that larger receivers were stronger sinks for transferred C (Figure 2.3).

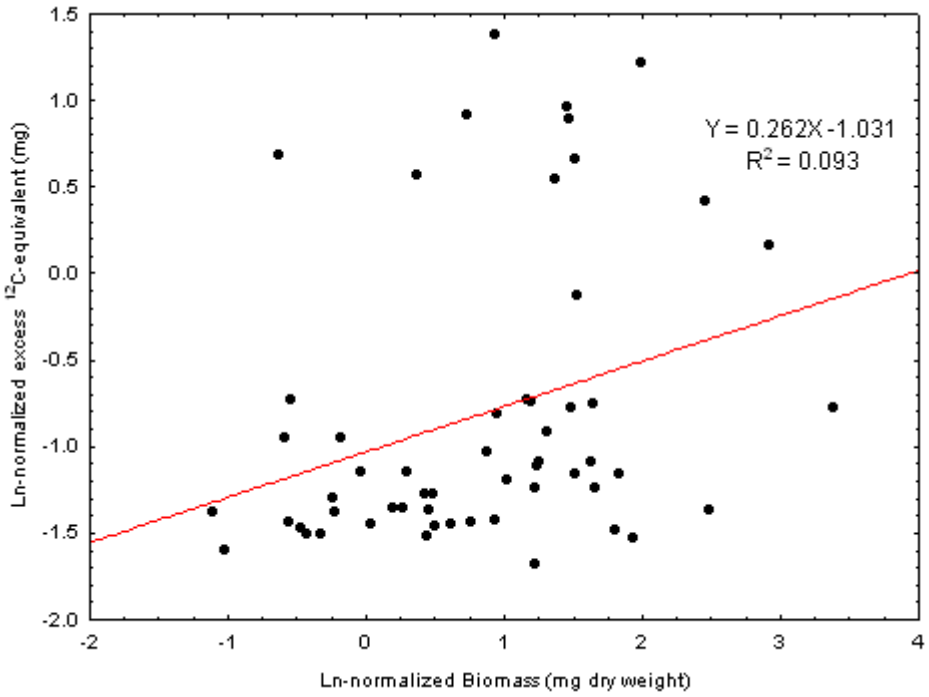

Figure 2.3: Relationship between receiver size (Ln-normalized mg dry biomass) and receiver enrichment (Ln-normalized mg excess ^{12}C-equivalent) for *Betula nana* receivers when all below-ground pathways were intact.

To assess the fate of transferred C, we examined tissue-specific excess ^{12}C-equivalent enrichment as a proportion of total tissue $^{12+13}$C. The proportion of total tissue C that was enriched was two orders of magnitude larger for leaves than for rhizomes or stems, but the difference among tissues was not statistically significant (p=0.0868) due to the high variance of C-transfer to leaves (Table 2.2).

Table 2.2: Mean (± one standard error) proportion of tissue C that was enriched with ^{13}C for *Betula nana* receivers in conspecific pairs.

Tissue	**Proportion of C enriched**
Leaves	$1.17 \times 10^{-3} \pm 7.2 \times 10^{-4}$
Rhizomes	$5.69 \times 10^{-5} \pm 1.8 \times 10^{-5}$
Stems	$1.04 \times 10^{-5} \pm 4.34 \times 10^{-6}$
Whole plant	$6.07 \times 10^{-5} \pm 1.3 \times 10^{-5}$

Effect of environmental factors on carbon transfer

We observed no strong temporal effects on C-transfer in conspecific *Betula nana* pairs. However, a two-factor ANOVA for year and month revealed a trend toward higher C-transfer in June (p= 0.0509). Figure 2.4 shows mean and 95% confidence intervals for whole receiver enrichment (as mg of ^{12}C-equivalent) for *Betula nana* pairs from the four experimental runs.

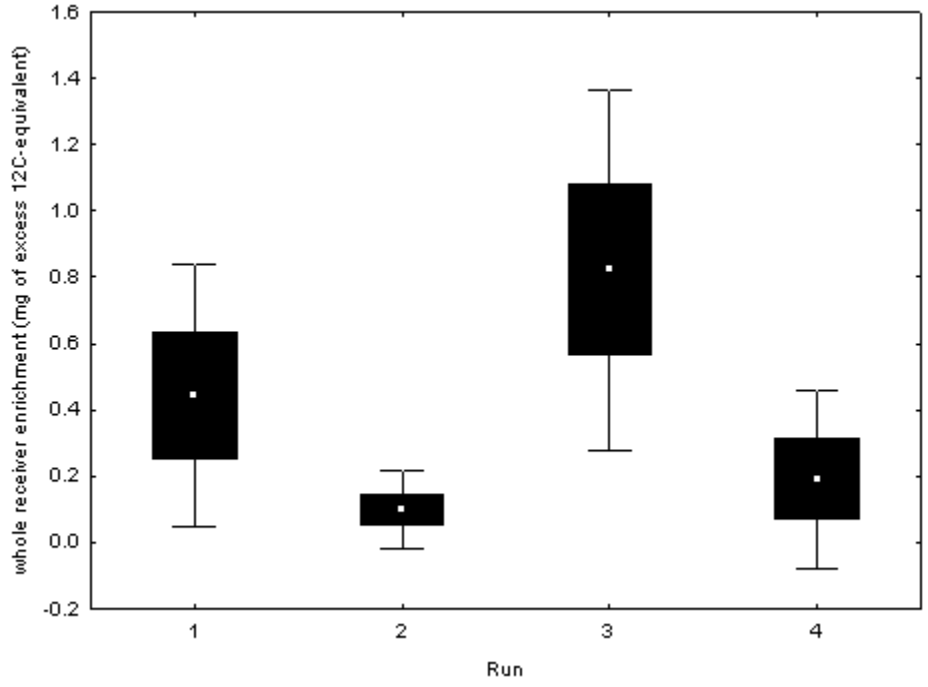

Figure 2.4: Mean and 95% confidence intervals for whole receiver enrichment (mg of excess ^{12}C-equivalent) for *Betula nana* pairs from the four experimental runs (Runs: 1=June 2007; 2= August 2007; 3=June 2008; 4=August 2008). Plants received significantly more C in June than August (p= 0.0509).

Analysis of meteorological data during the runs revealed that 2008 was significantly colder (p=0.0000 for mean, max, and min temperatures) and darker (p=0.01679 for global irradiation, p=0.04328 for photosynthetically active radiation) than 2007; while June was warmer (p=0.0000 for mean, max, and min temperatures) and brighter (p=0.0000 for global irradiation, p=0.0000 for photosynthetically active radiation) than August. Principal components analysis of the meteorological variables revealed that weather during experimental runs could be

summarized by two significant axes; Factor 1 yielded an eigenvalue of 7.21 and explained 60.1% of the variance in the dataset, Factor 2 yielded an eigenvalue of 3.78 and accounted for 31.5% of the variance in the dataset. Figure 2.5 shows that the four runs and the 7-day time periods prior to each experimental run differed in their relationships to these two factors. The time periods in June of 2007 and 2008 (R1, PR1, R3, PR3) ranked high on axis 1, while the time periods in August 2008 (R4 and PR4) ranked low on axis 1. The time periods in August of 2007 (R2, PR2) ranked lower on Axis 2 than did the other time periods.

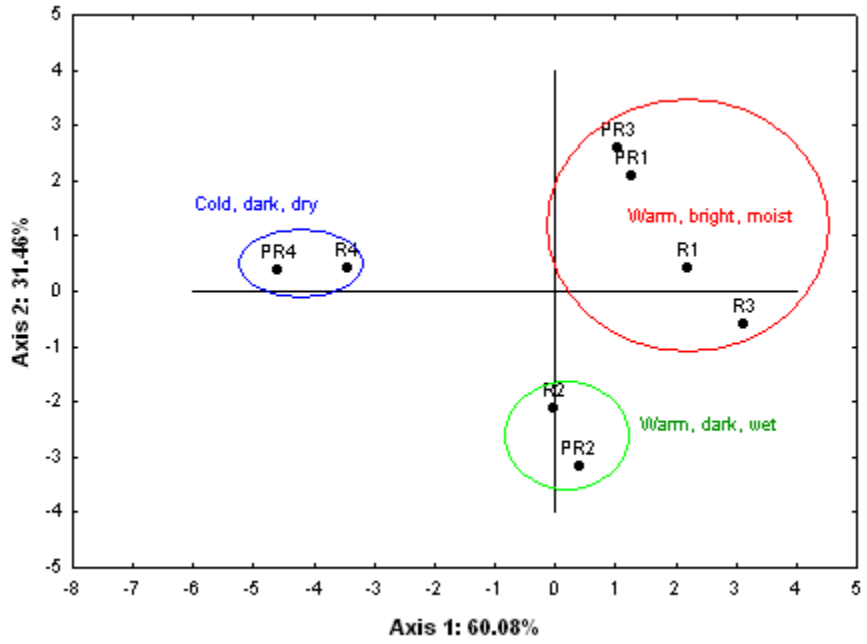

Figure 2.5: Plot of time periods ranked on the first and second axis of a principal components analysis of meteorological data. R1, R2, R3 and R4 represent the time periods of the four experimental runs: June 2007, August 2007, June 2008, and August 2008, respectively. PR1 through PR4 represent the 7-day time periods prior to each run. Ellipses are overlaid on the PCA to emphasize the primary meteorological characteristics of each group of time periods.

A GLM to test the effects of the two synthetic factors (representing the combined environmental variables) on carbon transfer to receivers in *Betula nana* pairs revealed that factor 1 was a significant predictor of receiver enrichment (p= 0.0368).

Ecological significance of carbon transfer

Expressing amount of carbon transferred as a proportion of total carbon fixed by donors provides insight into the significance of belowground carbon transfer to plant community ecology. We found that 10.7 ± 2.4% of the donor's fixed C was transferred to receiver plants where all belowground pathways were intact (Figure 2.6). By comparison, significantly less fixed C (1.4 ± 0.35%) was transferred to receivers through soil solution ($p=0.0007$, from Kruskal Wallis ANOVA). An intermediate amount of donor C (5.5 ± 2.9%) was transferred to receivers through MNs in the mesh treatment.

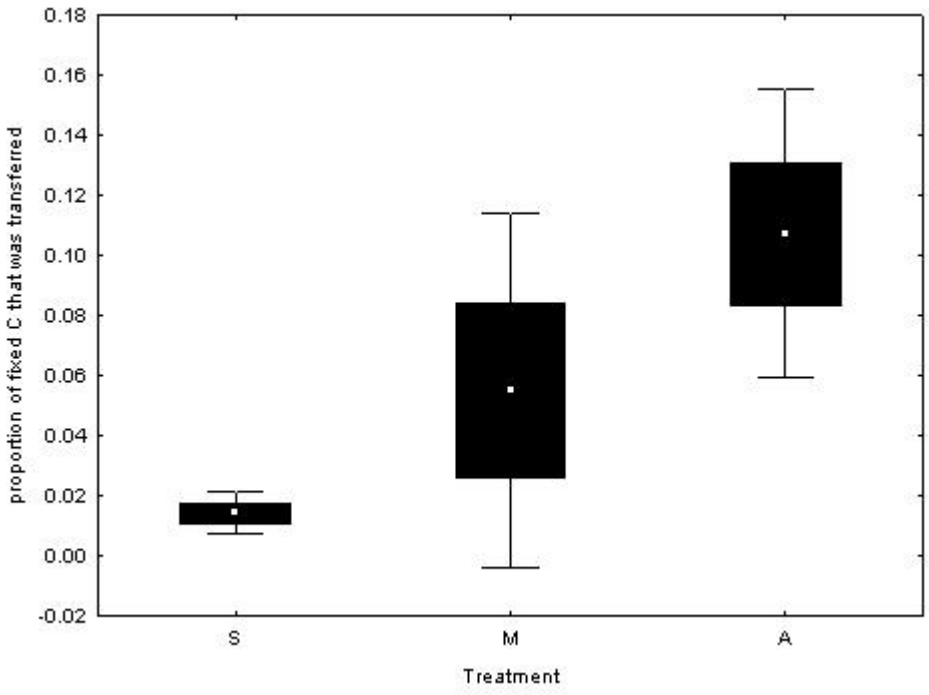

Figure 2.6: Proportion of donor's fixed C transferred to whole receivers (sum of leaf, stem and rhizome enrichments) by treatment type. Treatments symbols: S= severed donor, A= All pathways, M= donor planted in mesh. Inner spread denotes the standard error of the mean; whiskers denote the 95% confidence interval about the mean; S (n=34); A (n=39); M (n=34).

Discussion

Mycorrhizal networks in Low-Arctic tundra

Our results suggest that mycorrhizal networks exist in this Low-Arctic tundra ecosystem and function in the below-ground interplant transfer of C. The amount of C-transferred to receivers in conspecific *Betula nana* pairs varied widely among belowground pathways. *Betula nana* receivers that could only receive C transferred through the soil solution received significantly less carbon than did those in plots where all below-ground pathways were intact. Further, when we considered the proportion of donor's fixed C transferred to receivers we, found that 5.5% was transferred through MNs. This amount was greater than half the total below C-transfer among conspecific *Betula nana* pairs and was significantly greater than the proportion of fixed-C transferred through the soil solution, confirming our prediction that greater C-transfer would occur through MNs than through soil pathways and lending strong support to our hypothesis that MNs are involved in ecologically significant amounts of C transfer among plants in Low-Arctic tundra.

Our experimental design allowed us to partition total C-transfer among the three below-ground pathways. Carbon transfer through MNs was highly variable, however, likely because of the high spatial variability in density and strength of mycelial linkages among neighboring plants (Lian *et al*. 2006, Beiler *et al*., in press). This variability limited our ability to distinguish transfer through MNs from that through all pathways or through soil solution. Nonetheless, mean enrichment of receivers in the mesh treatment was intermediate between severed and all-pathway receivers, reflecting access of mesh receivers to two of three below-ground pathways. The magnitude of C-transfer through the MN (0.40 ± 0.35 mg) was similar to that through rhizomes and root grafts (0.54 ± 0.50 mg), indicating the relatively high importance of MNs in below-ground transfer of C among *Betula nana* in the field. Moreover, our estimate of C-transfer through mesh likely underestimated C-transfer through an intact MN in the field because installation of mesh severed the pre-existing mycelial network, requiring that hyphae re-anastomatose before full C-transfer capacity could be regained (Bebber *et al*. 2007). In our study, we observed a profusion of fungal hyphae associated with *Betula nana* donors within some mesh bags, and occasionally observed fine mycelia protruding from the mesh, suggesting that re-anastomosis had occurred to a certain extent. We did not, however, observe

rhizomorphs (hyphal strands) emanating from mesh bags. Rhizomorphs are fungal structures that appear to rapidly translocate C, nutrients and water to symbiotic plants through MNs (Duddridge *et al*. 1980; Brownlee *et al*. 1983; Egerton-Warburton *et al*. 2007; Warren *et al*. 2008).

Our results indicate very little C was transferred through soil solution in this ecosystem, at least not over the time scale we examined (7-day chase period). This agrees with other field studies showing C-transfer to ECM plants through soil solution to be small relative to transfer through other below-ground pathways (Simard *et al.* 1997, Philip *et al*. in press, Teste *et al*. 2009 a,b). Mechanisms for C-transfer from donor to receiver plants via the soil solution include receiver uptake of C in fungal or root exudates, root-respired CO_2, or sloughed fungal or root cells (Simard *et al.* 2002). At Toolik Lake, levels of *Betula nana* root colonization by ECM are 58-62% (Urcelay *et al.* 2003, Deslippe *et al.* Chapter 2, this volume), and remaining non-mycorrhizal *Betula nana* roots would need to compete with the non-mycorrhizal roots of other dominant plants, such as *Eriorphorum vaginatum* and *Carex sp*., as well as soil bacteria to access C in the organic soil nutrient pool. During the growing season, soil nutrient pool dynamics are tightly controlled by sink processes (Weintraub and Schimel 2005) and non-mycorrhizal plants compete well with ECM fungi and other soil microbes for organic N (Schimel and Chapin 1996, McKane *et al*. 2002).

Plant factors affecting C-transfer

Analysis of C-transfer data for all receiver species provides evidence consistent with our second hypothesis that factors that enhance mycorrhizal connectivity among donor and receiver plants, or enhance receiver sink strength, will result in greater C-transfer. In this study, C-transfer was clearly favoured among conspecific pairs and those that have the potential to share mycorrhizal species. Strikingly, however, we observed significant C transfer only to *Betula nana*. This finding supports the concept of a highly connected below-ground network of plant and fungal structures that provide *Betula nana* with a selective advantage in tundra plant communities by facilitating the rapid redistribution of C at need.

Within the body of a clonal perennial plant, the pressure-flow hypothesis (Turgeon 1991) explains C-translocation along source-sink gradients among ramets. Sink tissues compete for available translocated assimilate and sink strength is a function of sink size and sink activity. Our finding that receiver size was significantly associated with receiver enrichment is in agreement with the pressure-flow hypothesis and may help explain why *Betula* received more transferred carbon than the other species. The weak relationship between receiver ^{12}C-equivalent and receiver biomass resulted from the relatively large number of small receivers that were strong sinks for C. Presumably, rapid relative growth in these individuals caused their high sink activity. Likewise, the observation that leaves tended to receive proportionally more C than stems or rhizomes suggests that leaf expansion was an important mechanism for increasing sink strength.

The potential for mycorrhizal networks to form among conspecific plants of similar age is very high given that there is complete species overlap within the mycorrhizal fungal community (see Chapter 2 for a description of the ECM community on conspecific *Betula nana*). Similarly, C transfer through mycorrhizal networks is likely greater among conspecific than interspecific plant pairs sharing the same mycorrhizal group because of differences in abundance of mycorrhizas shared in common (Simard and Durall 2004, Selosse *et al*. 2006). Despite that many of the common sporocarps collected at our study site were species known to form ectomycorrhizae with both *Betula sp*. and *Salix sp*. (Smith and Read 1997), we found no evidence for interspecific C-transfer from *Betula nana* to *Salix pulchra*. However, *Salix pulchra* suffered almost 90% mortality in 2007 following installation of the sheet metal plant boundaries, a rate that was dramatically higher than among other plant species because it was the largest plant requiring greater cuts into its large diameter rhizomes. Where it remained, *Salix pulchra* was frequently damaged or dying and would have been a very weak sink for C. Thus, we may have underestimated the potential for interspecific C-transfer through ECM networks in this study.

Despite our specific examination of conspecific C-transfer by the ERM mycorrhizal plant species, *Ledum palustre,* we found no significant transfer of C among conspecific *Ledum palustre* pairs. This finding suggests that C-transfer through below-ground pathways is less important for this ericoid mycorrhizal species than it is for *Betula nana*. The apparent lack of below-ground C-transfer among *Ledum palustre* individuals may reflect a selective disadvantage when competing with larger deciduous shrubs that can access a MN to efficiently

reallocate C. We also found no evidence for C-transfer from *Ledum palustre* to other ericaceous plant species, suggesting that MNs comprised of ERM mycorrhizal fungi were not involved in any significant belowground C transfer at the time scales we examined. We know of no studies that have demonstrated C-transfer through ERM mycorrizal networks, although they have been shown to enhance P-transfer from dying to healthy *Calluna vulgaris* plants in a heathland ecosystem (Eason *et al.* 1991). The rapid turnover of ERM hyphal coils within root cells suggests that MNs comprised of ERM fungal species may be ephemeral compared to those comprised of ECM fungal species, which have a long-lasting Hartig-net and mantle (Smith and Read 1997).

We also found no significant interspecific C-transfer between *Betula nana* and *Ledum palustre* plants. C-transfer through a MN may have been possible if a single soil fungus could form ECM with *Betula* and ERM with *Ledum,* as has been shown to occur for *Rhizoscyphus ericae* (formerly *Hymenoscyphus ericae*) with ECM and ERM plants (Vralstad *et al.* 2000, 2002, Villarreal-Ruiz *et al.* 2004), but we found no evidence for this. The absence of C-transfer among these two plant species in the field agrees with other work suggesting they have a predominantly antagonistic relationship. For example, in a removal experiment it was found that ECM fungal colonization in *Betula nana* was reduced when *Ledum palustre* was removed, suggesting that *Ledum* competes for soil nutrients with *Betula* (Urcelay *et al.* 2003). Similarly the removal of *Ledum palustre* has been shown to significantly increase the N accumulation and biomass of deciduous shrubs (Bret-Harte *et al*. 2008), among which *Betula nana* comprised greater than 90% of the initial community (Bret-Harte *et al*. 2004).

When receiver *Betula nana* tissue enrichment is viewed on a per gram C basis, there is a clear trend toward higher enrichments of leaves over rhizomes or stems, indicating that rapidly growing tissues gained greater amounts of carbon. During expansion, leaves would have been strong sinks for C, particularly in the form of amino acids which are necessary for the production of new photosynthetic machinery. In *Betula papyrifera*, protein and nucleic acid concentrations are highest in young leaves and decline in concentration throughout the growing season as organic N is hydrolyzed and translocated elsewhere (Chapin and Kedrowski 1983). The preferential movement of labeled C to leaves has also been reported by Teste *et al.* (2009a) and fits with shifts in net transfer to the strongest sink plants over the growing season (Lerat *et al*. 2002, Philip 2006). Some studies on AM plants report that C-transferred through MNs remain

in receiver's roots structures (Watkins *et al*. 1996, Graves *et al.* 1997, Fitter *et al.* 1998) indicating that roots also periodically act as strong C-sinks, as is possible for example, during autumn translocation of nutrients or during spring growth when root turn-over is high. During August, our study plants were likely allocating a high proportion of their carbon belowground as nutrient uptake continues through the summer to August, and mid and/or late August corresponds with an increase in plant root growth (Shaver & Kummerow 1992). Late August may also be the time of maximum C-allocation to the rhizosphere (Weintraub & Schimel 2005b) as plant acquisition of soil N is required to offset N stores depleted due to growth (Chapin *et al.* 1995). Our exclusion of roots from biomass harvests limited our ability to detect C-transferred to roots and likely resulted in an underestimation of total belowground C-transfer in this study.

Environmental factors affecting C-transfer

Based on previous studies (Simard *et al*.1997, Philip 2006), we expected that C-transfer through below ground pathways would increase over time as plants and mycorrhizal networks in experimental plots recovered from the damage caused by the installation of treatments. However, in our study, we found no strong temporal effects on C-transfer in conspecific *Betula nana* pairs. This result prompted us to investigate whether abiotic conditions could have influenced the patterns of C-transfer we observed. C-transfer was greatest among *Betula nana* pairs in June 2008, which was warmer and brighter than any other labeling period. The PCA of environmental variables revealed that the August 2008 run strongly separated from the other time periods along axis 1 because it was colder and darker. Axis 1 accounted for 60.08% of the variance in the measured environmental parameters and was a significant predictor of C-transfer within *Betula nana* pairs. This result strongly suggests that C-transfer within *Betula nana* pairs was limited by abiotic conditions in August of 2008. Had weather patterns more typical of the other three runs occurred in August of 2008, we would expect to have seen significantly greater C-transfer in 2008 than in 2007 as plants recovered the extent of their MNs.

An interesting extension of our PCA analysis is that the temporal environmental gradient described by axis 1 was dominated by temperature variables, with mean and maximum air temperature at 1 m, moss temperature, and soil temperature at 5 cm depth, all producing factor scores of greater than 0.95. In order of descending importance were light (photosynthetically

active radiation, 0.80; and the sum of global radiation, 0.74) and moisture (cumulative precipitation, 0.66) variables. Because photorespiration is much more sensitive than photosynthesis to temperature change in C_3 plants (Barbour *et al*. 1987, Ryan 1991), warmer temperatures should have increased the C-sink strength of receivers more than the C-source strength of donor plants in our study. The strong loading of temperature parameters on axis 1 relative to variables that would more strongly influence photosynthetic rates (and thus C-source), such as photosynthetically active radiation, in combination with the strong relationship between C-transfer and axis 1, supports the idea that C-transfer within *Betula nana* pairs was controlled most strongly by sink strength in our study. This is further supported by the positive relationship we found between magnitude of C transfer and receiver plant size. The dominant influence of sink versus source strength in governing belowground C transfer agrees with the results of studies in forested ecosystems (Read *et al*. 1985, Simard *et al*. 1997, Fitter *et al*. 1999, Wu *et al*. 2001, Teste *et al*. 2009). Importantly, this finding suggests that C-transfer through belowground pathways will become more important as climate warms.

Implications of MNs for tundra plant communities facing climate change

The potential influence of MNs on plant community structure is complex and variable. Facilitative effects of MNs through mycorrhization or redistribution of nutrients among plants (Simard *et al*. 1997, Lerat *et al*. 2002, Nara 2006) can include increased seedling establishment and survival (Nara and Hogetsu 2004, Teste *et al*. 2009), reduced interplant competition (Perry *et al*. 1989) and increased species diversity within a plant community (van der Heijden *et al*. 1998, Grime *et al*. 1987). Conversely, MNs may lead to the competitive exclusion of non-networked species and a reduction in total community diversity as occurs in mono-dominant stands of tropical forest trees (McGuire 2007). Where MNs act to alter competitive interactions among plants, they will have profound effects on the structure and diversity of plant communities and ultimately on the C-cycling traits of ecosystems (Wilkinson 1999). It has been proposed that climate change will shift dominant plant interactions from facilitative to competitive in Arctic and alpine ecosystems (Brooker 2006).

In this study we report that C-transfer to *Betula nana* receivers through a MN represents approximately 5.5% of isotope uptake through photosynthesis. This value is comparable to the

6% transfer from *Betula papyrifera* to *Pseudotsuaga menziesii* through MNs in mixed temperate forests (Simard *et al.* 1997), but much greater than the 0.064% of fixed C that *Erythronium americanum* transferred to *Acer saccharum* saplings in a mixed hardwood forest, and far greater than the 0.0063% reported for conspecific *Pseudotsuaga menziesii* trees and seedlings in a dry temperate forest (Teste *et al.* 2009). C-transfer through MNs in this tundra ecosystem accounted for slightly more than half of the total belowground C-transfer; 10.7%, which is roughly equivalent to the carbon costs of reproduction (Reekie and Bazazz 1987).

The ability to transfer relatively large quantities of fixed C rapidly through MNs fits with the characteristic plasticity of *Betula nana* noted by others (Bret-Harte *et al.* 2001). For example, the rapid new growth of *Betula nana* after release from nutrient limitation is facilitated by its ability to generate additional meristems (Bret-Harte *et al*. 2001, 2002). During meristem differentiation and shoot elongation, integration within a mycorrhizal network and the ability to draw on large below-ground C-stores (held in both plant and fungal structures) may temporarily sustain growth until leaves are produced. In this way, connection to a mycorrhizal network may provide a critical supplement to the C-economy of expanding *Betula nana* plants, resulting in a competitive advantage relative to non-networked tundra species, and facilitating the expansion of *Betula nana* into tussock-tundra communities.

Conclusions

This study provides evidence, for the first time, that mycorrhizal networks exist in Low-Arctic tundra and act in the below-ground transfer of C among *Betula nana* individuals. Significant C-transfer among other tundra species was not observed in this study. C-transfer through MNs among conspecific *Betula nana* pairs represented 5.5% of plant photosynthesis slightly more than half of the total belowground transfer of fixed C. C-transfer through MNs to *Betula nana* was positively related to receiver size and to ambient air and soil temperatures. Our results suggest that C-transfer through mycorrhizal networks is of sufficient magnitude that it may alter plant interactions in Arctic tundra, possibly leading to an increase in interspecific competition, and the mono-dominance of *Betula nana*. Further, our data suggest that increased air and surface soil temperatures act to enhance C-transfer through below-ground pathways in *Betula nana*, further facilitating shrub expansion, reducing land-surface albedo, and acting as a positive feedback to climate change.

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3 Long-term experimental manipulation of climate alters the mycorrhizal community associated with *Betula nana* in Arctic tundra[1]

Introduction

Arctic regions have warmed rapidly in recent decades (Kaufman *et al.* 2009), highlighting the relevance of Polar Regions as sensitive indicators of global climate trends. Arctic warming is spatially non-uniform, with the western North American Arctic generally warming the fastest, at rates of up to 0.1^{o}C per year in the past 35 years (Anisimov *et al.* 2007). Already this warming is associated with marked changes in terrestrial ecosystems, including increased microbial activity leading to increased plant N availability (Chapin 1983, Nadelhoffer *et al.* 1992, Chapin *et al.* 1995, Aerts 2006) and faster C turnover in Arctic soils (Hobbie and Chapin 1998, Mack *et al.* 2004, Shaver *et al.* 2006, Schuur *et al.* 2009). Differences in the relative productivity of tundra species have led to ecosystem-scale changes in plant community composition characterized by the expansion of shrubs (Hobbie 1996, Hobbie and Chapin 1998, Sturm *et al.* 2001, Strum *et al.* 2005) and the consequent decline of mosses and evergreen ericaceous species. Shrub growth affects positive feedbacks that enhance ecosystem change and lead to greater climate forcing (Sturm *et al.* 2001). Beneath shrub thickets, increased local snow-trapping in winter, increased soil insulation, higher winter and spring-time soil temperatures, and increased rates of nutrient mineralization lead to local conditions that further favour shrub growth and expansion onto tussock tundra (Sturm *et al.* 2001, Strum *et al.* 2005, Weintraub and Schimel 2005).

In Low-Arctic tussock tundra, *Betula nana* is among the species most responsive to climate change. It is the strongest competitor for soil N (Bret-Harte *et al.* 2008), increasingly dominates fertilized tussock tundra in long-term experiments (Chapin *et al.* 1995, Bret-Harte *et al.* 2001, Shaver *et al.* 2001, Mack *et al.* 2004, Bret-Harte *et al.* 2008), and shows positive growth responses to warming treatments (Chapin *et al.* 1995). *Betula nana* is obligately symbiotic with ectomycorrhizal (ECM) fungi in nature (Molina *et al.* 1992), allocates a high

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proportion of C below-ground to rhizomes, roots, (Chapin *et al.* 1980) and mycorrhizal structures (Hobbie and Hobbie 2006), and utilizes mycorrhizal networks to rapidly reallocate C among neighbours according to need (Deslippe and Simard, Chapter 2 this volume). A model based on the natural abundance of ^{13}C and ^{15}N in plant and fungal tissues from Alaskan tundra estimated that 61-86% of the nitrogen in plants is filtered through mycorrhizal fungi (Hobbie and Hobbie 2006). These characteristics suggest an important role for *Betula nana*'s symbiotic ECM fungi in mediating its response to climate change factors. ECM fungi have been shown to be important determinants of plant response to ecosystem change through their dual role as drivers of decomposition processes (Read and Perez-Moreno 2003) and as the main nutrient harvesting structures of plants (Leake & Read, 1997; Smith & Read 1997).

There is growing evidence to suggest that mycorrhizal fungi respond strongly to climate change factors. Enhanced soil nutrient availability is frequently associated with negative impacts on ECM communities. For example, ECM community diversity declined sharply over an increasing N-deposition gradient in boreal Alaska (Lilleskov *et al.* 2002a), and net N mineralization rates have been negatively correlated to ECM fungal richness in the field (Parrent *et al.* 2006). Likewise, the proportion of *Betula nana* root tips colonized my ECM fungi declined after 3 years of fertilization treatment (Urcelay *et al.* 2003). Despite significant increases in ECM mycelial production after 14 years of fertilizer additions and warming treatments in Low-Arctic tundra, these gains were offset by much greater increases in above ground plant biomass, leading the conclusion that ECM fungal biomass represented the *status quo* or a decline relative to plant biomass following warming and fertilization (Clemmensen *et al.* 2006). By contrast, ECM fungal biomass tends to increase following warming without nutrient additions, though measurements are rarely corrected for total plant biomass gains as in the former study. For example, warming led to increased ergosterol content of hair roots of ericoid mycorrhizal plants (ERM) in subarctic Sweden (Olsrud *et al.* 2004), and to an increase in *Salix arctica* root-associated fungal biomass, at a Canadian High Arctic site (Fujimura *et al.* 2008).

Currently, our ability to predict the response of ECM and plant communities to climate change factors is hampered both by the few detailed descriptions of the members of these communities as well as our limited understanding of the ecological role of many fungal species. 5000-6000 fungal species form ECM, and these are phylogenetically diverse, spanning three

phyla (Smith and Read 1997, Agerer 2006). It is unlikely that the response of the ECM community to climate change factors is monotonic. In their characterization of ECM communities across an N deposition gradient, Lilleskov and others (2002) found *Paxillus involutus* and *Lactarius theiogalus* to be "nitrophillic", increasing linearly with net nitrification rate in soils, while some ECM taxa, such as *Laccaria bicolor* and *Hebeloma* spp., were found only at higher N sites. By contrast, several ECM were "nitrophobic" including *Cortinarius* spp., *Piloderma* spp., *Amphinema byssoides* and *Cenococcum geophilum*. Still other ECM species such as *Tomentella sublilacina* reached greatest abundance at sites with intermediate nitrification rates, where nutrient availability was higher but where soil pH had not yet been strongly reduced by N-pollution.

Patterns in the natural abundance of N isotopes (^{15}N:^{14}N ratios, expressed as $\delta^{15}N$) in plants, soils, and fungal sporocarps have provided insight to the ecological role of many ECM taxa. Nitrogen isotopes are useful markers of the mycorrhizal role in plant nitrogen supply because discrimination against ^{15}N during creation of transfer compounds within mycorrhizal fungi leads to low $\delta^{15}N$ in plants and high $\delta^{15}N$ in fungi, relative to N in soil organic matter (Hobbie *et al.* 2005, see Hobbie and Hobbie 2008 for a review). In low nitrogen environments, plants allocate a high proportion of C belowground to ECM and ERM fungi (Högberg *et al.* 2003) and are strongly dependent on the proteolytic functions of their symbionts for N (Hobbie and Hobbie 2008), leading to an 8-10% depletion of ^{15}N plant tissues relative to fungal tissues (Hobbie *et al.* 2005). As N availability increases and plants are released from N-limitation, foliar N concentration increases linearly with $\delta^{15}N$ (Hobbie *et al.* 2000), suggesting that plants access proportionally less N from their fungal symbionts, and leading to a negative relationship between foliar $\delta^{15}N$ and ECM fungal biomass (Hobbie and Colpart 2003). This relationship has proven useful in accessing plant and ECM and ERM response to climate change factors. For example, leaves of the ERM species, *Vaccinium vitis-idaea* and *Vaccinium myrtillus*, became more depleted in ^{15}N with exposure to elevated CO_2 (Olsrud *et al.* 2004). Likewise, $\delta^{15}N$ values of the foliage of two dominant ECM species, *Salix arctica* and *Dryas integrifolia,* were significantly depleted following warming at a High Arctic site (Deslippe 2004). By contrast, long-term fertilizer additions increased mean $\delta^{15}N$ values for *Betula nana* in MAT from -6.32 ± 0.15 ‰ to -2.52 ± 0.21 ‰ (Arctic-LTER data, Gough *et al.* 2000). These findings suggest that, under N-limiting conditions, climate change factors that promote plant photosynthesis, such as elevated temperature and CO_2, will lead to increased C allocation to ECM and ERM fungi; however, the

opposite pattern can be expected from climate change factors that enhance soil nutrient availability.

In addition to field based studies that provide evidence linking ECM taxa with ecological functions, several morphological attributes may suggest roles for species or groups (Agerer 2001). For example, several families in the order Boletales have structurally advanced rhizomorphs and are associated with the efficient transport of water and nutrients in soils (Agerer *et al.* 2006). The wide variability of $\delta^{15}N$ enrichment in ECM fruiting bodies, which is frequently observed in nature, may reflect species specific ecological roles for ECM fungi in regards to N cycling. For example, $\delta^{15}N$ signatures of sporocarps were recently correlated to hyphal growth patterns in ECM species at four sites that differed in latitude and nitrogen availability (Hobbie and Agerer in press). Of the hyphal characteristics measured, hyphal hydrophobicity, the ability to form rhizomorphs, and the extent and pattern of hyphal growth, called "exploration type", were found to most closely correlate with ^{15}N signatures. These hyphal characteristics may occur in common because ECM species with high biomass types may alone have the carbon necessary to produce more complex hyphal patterns, such as rhizomorphs. Further the function of rhizomorphs in long distance transport of water and nutrients would be enhanced by hydrophobic hyphae. ECM that form high-biomass exploration types had high $\delta^{15}N$ values, and dominated low-N sites, while those characterized by lower biomass forms had low $\delta^{15}N$ values and were more prevalent at sites with higher N availability (Hobbie and Agerer, in press).

The objective of this study was to determine the effects of climate change factors on the ECM community associated with the dominant tundra shrub, *Betula nana*. Specifically, we tested the hypothesis that long-term fertilizer addition and warming by greenhouses would alter the composition of the mycorrhizal community of *Betula nana* in Low-Arctic tussock tundra. Given the evidence suggesting generally positive effects of warming on ECM fungi and generally negative effects of increased nutrient availability, we predicted that warming or fertilization would lead to different ECM community composition; while fertilization should result in ECM taxa characteristic of sites with high N availability, warming should lead to ECM taxa adapted to N limitation. We used a molecular approach to achieve the high taxonomic resolution necessary to detect ECM species changes among treatments.

Methods

Study site

The study site was located on a gentle (<5^{o}), north-facing slope in MAT near Toolik Lake, Alaska, USA (68^{o}38'N, 149^{o}34'W, elevation 780m). MAT forms on old glacial surfaces (>11,000 years BP) and supports 'heathland'-type tundra plant communities. Regular patterns of vegetation occur in this plant community as a result of the perennial, rhizomatous growth of the tussock-forming sedge, *Eriophorum vaginatum*. The dominant deciduous dwarf shrub, ECM *Betula nana*, occupies hollows between the sedge-tussocks in mixture with mid-canopy ericaceous ERM species, such as *Ledum palustre* and *Vaccinium* spp., and arbuscular or non-mycorrhizal species such as *Rubus chamaemorous* as well as herbs. Both plurocarpus and acrocarpus mosses are common and form a continuous ground cover that can exceed 40cm depth in some areas. Soils are characterized by a thick organic horizon that is 10-20cm deep and with a pH of 4.4 (Nadelhoffer *et al.* 1991). Mineral horizons are essentially unweathered, pale grey in colour, permafrost affected, and show only occasional evidence of cryoturbation. Maximum thaw depth occurs in late July, when the active layer typically comprises the entire organic horizon and the top 5-10 cm of the mineral soil.

Experimental treatments

The study treatments are maintained as part of the Arctic Long Term Ecological Research (LTER) experiment. Warming and fertilizer treatments with ambient controls are replicated four times in a randomized block design in the Moist Acidic Tundra. Fertilizer treatments consist of 10 g m^{-2} additions of N as NH_4NO_3, and 5 g m^{-2} additions of P as P_2O_5, both applied as pellets immediately after snowmelt annually. Warming is accomplished passively with greenhouses constructed of 0.15-mm polyethylene fixed on permanent wooden frames that are 2.5 X 5 m, and 1.5 m in height. The uneven microtopography of the study site allows for air circulation from the base the greenhouse walls. Greenhouses reduce the photosynthetically active radiation by approximately 20% over the growing season, but have no significant effect on soil water content, and previous shading experiments indicate that plant production and biomass are unresponsive to light reductions of 50-64% at this site (Chapin *et al.*

1995). Soil temperature in treatment plots tends to be cooler than in control plots in summer, because of increased shading by higher plant biomass, but warmer than ambient control plots in winter, due to increased insulation by snow trapped by plants. Mean annual temperature differences among treatments, in 2007 are shown in Table 3.1.

Table 3.1: Mean annual change (treatment – ambient control) in air temperature within the plant canopy and soil temperature at 3 depths. Significance of treatment effects were determined by comparing the control and treatment plot data using paired t-tests; * denotes a significant treatment effect (α=0.05). These data are from the Arctic LTER data archive, file # 2007dlMATO (Shaver and Laundre 2007).

Temperature (°C)	Treatment		
	Fertilizer	Warming	Warming + Fertilizer
Canopy	1.89*	2.09*	3.30*
Soil at 10cm depth	0.65*	1.76*	2.46*
Soil at 20cm depth	0.28*	1.60*	2.13*
Soil at 40cm depth	-0.042	1.26*	1.72*

Betula nana growth responds strongly to the experimental treatments (Shaver *et al.* 2000). Fertilizer addition resulted in significant increases in total above-ground biomass, but smaller and non-significant increases in rhizome biomass, and these changes were further amplified by warming. As a result, fertilization and warming strongly altered *Betula nana's* biomass allocation; from a ratio of above to belowground biomass of 1:4 in control plots, to nearly 1:1 in fertilizer plots, and 2:1 in warming + fertilizer plots (Figure 3.1). Warming alone resulted in a 55% increase in above ground biomass of *Betula nana*. Figure 3.1 shows *Betula nana* above-ground and rhizome biomass after 9 years of fertilization and warming at this study site.

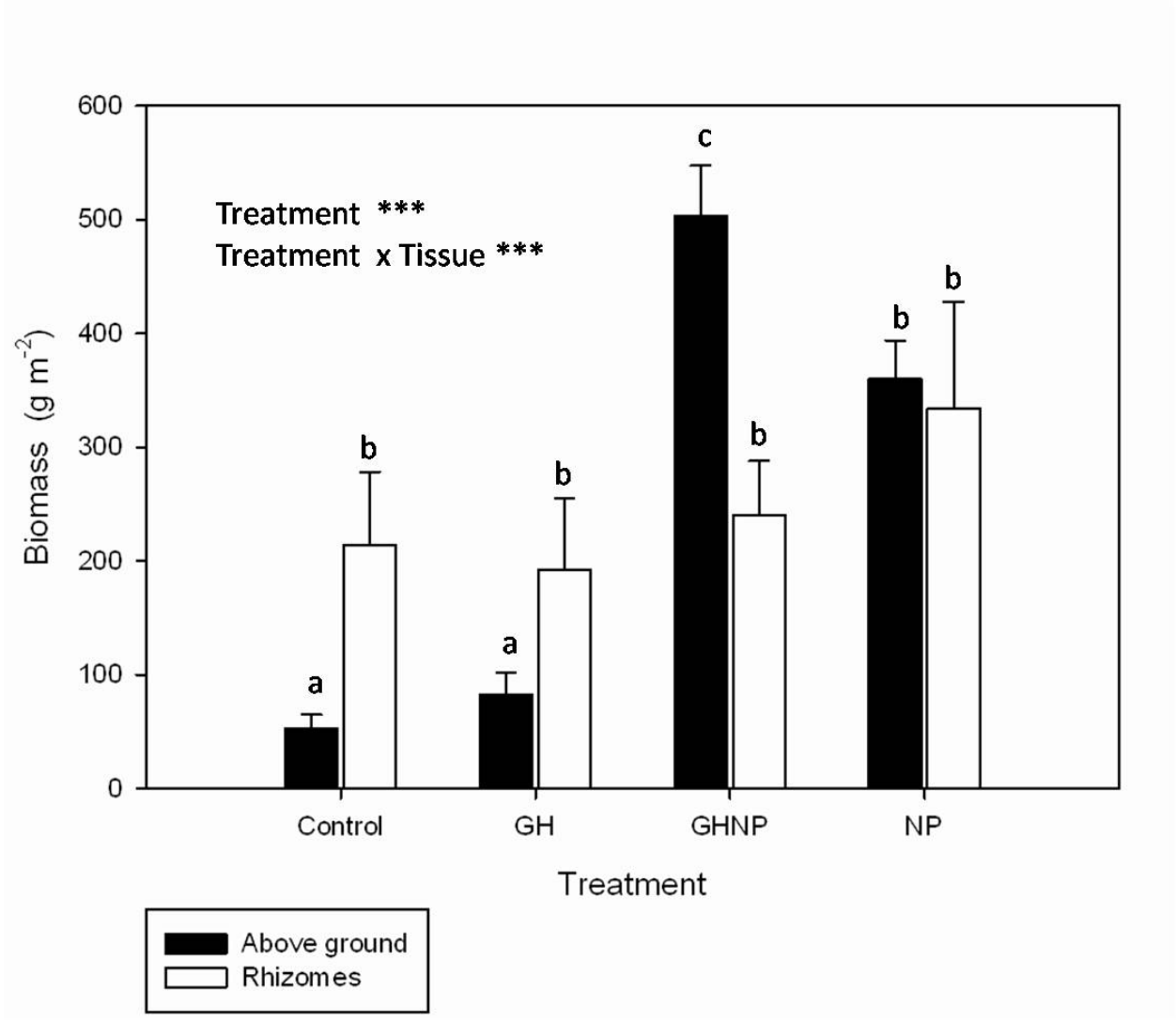

Figure 3.1: *Betula nana* biomass in response to 9 years of fertilizer and warming treatments; GH = warming, NP = fertilizer. Above ground biomass is the sum of stem, leaves and inflorescences. Root biomass was not determined. Significant factor effects as determine by two-way ANOVA are indicated; *** = P<0.001, and letters above bars denote means that are significantly different as determined by a Tukey's post hoc test. All data is from the Arctic LTER data archive, file # 1982_2000gs81tusbm (Shaver *et al.* 2000).

Root sampling and nucleic acid extraction

ECM roots of *Betula nana* were sampled on July 28th, 2007, a time that corresponds to maximum annual above-ground biomass. To obtain root samples, soil cores were sampled from three randomly selected locations in each of the four replicate plots per treatment and control. Soil cores were 5cm in diameter and 20-30 cm in depth from surface to mineral soil, as *Betula nana*'s roots rarely penetrate into the B-horizon. Each soil core was packaged separately in a clean, airtight, plastic bag, and placed immediately on ice. Soil cores were maintained at 4°C until processed for ECM roots.

Each soil core was placed in a 2 mm soil sieve and washed well in tap water. Fine roots of *Betula nana* were removed, floated in a shallow tray, and cut into 2-cm sections. Root sections were selected at random and placed under a dissecting microscope. At least 1000 root tips per soil core (mean = 1120; total = 17927) were assessed for colonization by ECM fungi. Root tips were identified as ectomycorrhizal if a fungal mantle enveloped more than 50% their length and no root hairs were present. For each core, three randomly selected sub-samples comprised of 10 root tips each were removed for DNA extraction (30 root tips per core; 90 root tips per plot). Individual root tips were measured with a micrometer scale ruler and cut with a micro-scalpel so that their length did not exceed 2 mm. Root tips were placed in sterile microfiche tubes and lyophilized at -50°C in a Labconco lyophilizer (Kansas City, Missouri). Lyophilized root tips were stored at -80°C until DNA was extracted.

To extract DNA, lyophilized root tips were placed in Lysing Matrix E® (Qbiogene) tubes with 400µl of Buffer AP1 and 4 µl RNase A stock solution from the Qiagen plant miniprep kit, and shaken at maximum speed for 30 second in a FastPrep® instrument (Qbiogene). Following this step, the Qiagen plant miniprep kit extraction protocol was followed according to the manufacturer's directions (DNeasy Plant handbook, Qiagen). A 5-µl aliquot of each soil extract was run on a 1% agarose gel, stained with ethidium bromide and visualized under UV light to check extraction efficiency. The 3 root tip extractions per core were then pooled for subsequent analysis for a total of 48 samples (3 soil cores * 4 replicate plots * 4 treatments).

PCR and sequencing

Polymerase chain reactions targeted the Internal Transcribed Spacer (ITS) of the fungal rRNA operon. PCRs employed the forward primer ITS1F (Gardes and Bruns, 1993), and the reverse primer, ITS4 (White *et al.*, 1990). PCR reactions consisted of 50 ng genomic DNA, 20µmol dNTPs, 10 nmol primers, 10X PCR Buffer, 50µmol $MgCl_2$, 15 µg BSA, and 2.5 U of Taq DNA polymerase (Fermentas) in a final volume of 25 µl. The thermocycler program consisted of an initial denaturing temperature of 95°C for 5 minutes followed by 30 cycles of: denaturing at 94°C for 1 min, annealing at 55°C for 50 s, and extension at 72°C for 50 s, followed by a final extension period of 7 minutes at 72°C. Subsequently, a 'reconditioning' step, designed to eliminate PCR artifacts formed in late-stage, template-limiting cycles of PCR, was employed (Thompson *et al.* 2002). Here, 5µl of initial PCR product were added as template to 45 µl of fresh PCR reagents and subjected to three additional thermal cycles. PCR products were purified using MiniElute purification columns (Qiagen). Cleaned PCR products were quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and 100ng of DNA from each PCR reaction was pooled according to treatment; for a total of 4 cloning reactions. Pooled templates were cloned using a TOPO TA Cloning® kit with pCR®II-TOPO® vector and DH5α™ competent cells (Invitrogen, Burlington, ON) with blue/white screening. Clones were plated, picked and sequenced at the Genome Sciences Centre (Vancouver, Canada) using the vector specific primers M13for and M13rev.

Phylogenetic analyses

Bidirectional sequence reads were assembled using the ContigExpress function of Vector NTI 10.3 (Invitrogen), manually checked for base-calling errors, and trimmed of the vector sequence. The Bellerphon server (Huber *et al.* 2004) was used to detect chimeric sequences in each of the fungal libraries. Phylogenetic affiliations of fungal ITS sequences were determined using the Fungal ITS Pipeline (Nilsson *et al.* 2009a), which employs multiple alignment programs (Clustal W2 [Larkin *et al.* 2007], MAFFT v6 [Katoh, 2008], and DIALIGN-TX [Subramanian *et al.* 2008] to align ITS sequences, and HMMER http://hmmer.janelia.org/, a hidden Markov model algorithm (Durbin *et al.* 1998), to run similarity searches of the query sequences using a local installation of NCBI-BLAST. The Fungal ITS Pipeline queries both the

entire ITS sequence length and additionally extracts the hyper-variable ITS2 subregion for additional searches to achieve greater phylogenetic sensitivity (Nilsson *et al.* 2009b). The Fungal ITS Pipeline groups like sequences based on 50% identity of the 15 closest NCBI-BLAST hits. Sequence groups and singletons were assigned to taxa based on the following parameters: sequences with greater than 97% identity over more than 90% of the query sequence were considered species-level matches; sequences with 93-97% identity, or sequence groups where there was species level incongruence among member's top blast hits, were assigned to genera; sequences with matches below 93% were considered family-level; and those below 83% were considered order-level.

Fungal sequences were divided into two groups based on their affiliation with mycorrhizal and non-mycorrhizal fungi. Two sequence alignments, one for the putatively mycorrhizal fungi, and one for the entire dataset were constructed using MUSCLE 3.7 (Edgar, 2004) and refined using the "-refine" command. Resulting alignments were visually checked for accuracy and manually edited in ARB (Ludwig *et al.* 2004). Preliminary phylogenetic trees for the putatively mycorrhizal and combined fungal datasets were constructed with Neighbour-joining using Jukes-Cantor correction (ARB). Phylogenetic trees were uploaded to the Unifrac server http://bmf2.colorado.edu/unifrac/ with their corresponding 'environment files'. Unifrac measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree that leads to descendants from either one environment or the other, but not both (Luzopone & Knight 2005). Unifrac significance tests and P-tests, both corrected for multiple comparisons (Boferroni correction), were used to test for significant differences in the composition of paired libraries. The 'environment distance matrix' function was used to calculate pairwise distances between each library. The 'lineage specific analysis' function was used to detect lineage specific differences in the clone libraries by treatment (Lozupone and Knight 2005, Lozupone *et al.* 2006), and to support X^2-tests for phylogenetic groups (see below).

Putatively ECM fungi that could be identified at least to the genus level were categorized according to their hyphal characteristics (presence of rhizomorphs, hyphal hydrophobicity, exploration type) based on previously published reports for the taxon as summarized in Agerer *et al.* (2006) and Hobbie and Agerer (in press). Where data on the $\delta^{15}N$ values of affiliated

genera were available, putatively ECM fungi were further characterized by their relative ^{15}N enrichment (Table 3.2). Because of site specific effects on the isotope signatures of fungi we utilized a relative scale of $\delta^{15}N$ values (Lilleskov *et al.* 2002b, Hobbie 2005), and wherever possible selected fungal isotope values reported for Toolik Lake or other N-limited sites in Arctic and boreal regions. For sporocarp data from Toolik Lake, AK the relative scale of high, medium and low, corresponded to $\delta^{15}N$ values of >7‰, 3-7‰, and <3‰, respectively. Table 3.2 summarizes the data sources utilized for assigning fungal taxa to relative $\delta^{15}N$ enrichment categories.

Table 3.2: Relative $\delta^{15}N$ value, study site, and reference for mycorrhizal fungal taxa found in this study.

Taxon	Relative $\delta^{15}N$ value	Study site	Reference
Cenococcum sp.	High	Boreal, Alaska	Lilleskov *et al.* 2002b[2]
Cortinarius sp.	High	Toolik Lake, AK	Clemmensen *et al.* 2006
Laccaria bicolor	Low	Boreal, Alaska	Lilleskov *et al.* 2002b
Laccaria sp.	Low	Toolik Lake, AK	Hobbie & Hobbie 2006
Lactarius sp.	Medium	Toolik Lake, AK	Hobbie & Hobbie 2006
Russula sp.	Low	Boreal, Alaska	Lilleskov *et al.* 2002b
Thelephora terrestris	Low	Laboratory study	Hobbie and Colpart 2003[1,2]

[1]As reported in Hobbie (2005)

[2] $\delta^{15}N$ values are for hyphae only

Statistical analysis

Paired T-tests were used to assess treatment effects on soil temperatures compared to the control plots. General linear models were used to assess treatment effects on above and belowground biomass (two-way ANOVA), and the proportion of root tips colonized by ECM fungi (one-way ANOVA), in *Betula nana*. Barlett's and Levene's tests were used to test for heteroscedascity among treatments. Tukey's post hoc tests were used to assess significant differences among pairs of treatments. These statistical analyzes were performed using STATISTICA version 8.0. (StatSoft, Inc. Tulsa,OK, USA). Clone library size was standardized to that of the control. The frequency of clones affiliated with phylogenetic groups and hyphal characteristics were compared to the control values using X^2-tests with Yates correction. X^2-tests were performed using a purpose-made Excel® spreadsheet obtained from http://udel.edu/~mcdonald/statchigof.html

Results

We found no significant differences in the proportion of *Betula nana* root tips colonized by ECM fungi among experimental treatments; mean percent colonization was 68% in this study ($p=0.750$). Table 3.3 shows mean percent colonization and the mean number root tips examined for soil cores by treatment. However, analysis of the composition of the clone libraries constructed for root tip ITS gene sequences for the four treatments revealed that a significantly smaller proportion of clones derived from the warming + fertilizer treatment were of putatively mycorrhizal origin ($p=0.0029$, Table 3.4). Similarly, there was a trend toward fewer mycorrhizal-affiliated clones in the fertilizer treatment than the control. The vast majority of members of clone libraries that were not affiliated with mycorrhizal taxa in this study were yeasts, although a few (<1%) basiomycetous mycelium forming saprotrophic fungi were also observed.

Table 3.3: Proportion of root tips colonized by ECM fungi and number of root-tips examined per soil core in this study. Values are reported as mean ± one standard error, a one-way ANOVA revealed no significant differences among treatments.

Treatment	Proportion of root tips colonized	Number of root tips examined per core
Control	0.61 ± 0.06	1130 ± 37
Fertilizer	0.64 ± 0.07	1174 ± 29
Warming	0.67 ± 0.09	1046 ± 89
Warming + Fertilizer	0.71 ± 0.02	1131 ± 31

Table 3.4: Summary of clone libraries constructed for ITS region of rRNA genes of root-tip associated fungi of *Betula nana*. Each treatment was compared to the control where the level of significance was determined by X^2-tests with Yates correction.

Library	Library Size	Proportion of putatively mycorrhizal sequences	*P*-value
Control	248	0.90	-
Fertilizer	248	0.75	0.093
Warming	308	0.95	0.575
Warming + Fertilizer	256	0.61	0.00023

Unifrac analysis of fungal clone libraries by treatment revealed that all pairwise comparisons between libraries were statistically significant at α=0.05, suggesting that each treatment contained a *Betula nana*-associated fungal community with a distinct composition and structure (Table 3.5). This was also true when only the putatively mycorrhizal sequences in each library were tested. Larger Unifrac distances among treatment libraries were observed when the non-mycorrhizal sequences were removed, indicating that the mycorrhizal portion of the fungal community was more sensitive to experimental treatments relative to the entire root-tip fungal community. Comparison of the ranked quartile distances between control and other treatments in both datasets reveals that the control was consistently most similar to the fertilizer treatment whereas the warming + fertilizer treatment occupied an intermediate distance, and the warming treatment was the most different from the control.

Table 3.5: Unifrac distance matrix for pairwise comparisons between of fungal ITS clone libraries for all sequences and for those affiliated with mycorrhizal taxa only. All pairwise comparisons are significant at α=0.05, as determined by the Unifrac significance test and the P-test, both with Bonferroni corrections. Unifrac distances are coloured according to the quartiles of distance each pairwise comparison accounts for with increasing shades of grey corresponding to increasing quartile distance: 0-25%; 25-50%; 50-75%; 75-100%.

Dataset	Treatment	Control	Fertilizer	Warming	Warming + Fertilizer
All	Control	-	0.778	0.817	0.809
	Fertilizer		-	0.740	0.737
	Warming			-	0.790
	Warming + Fertilizer				-
Putatively mycorrhizal	Control	-	0.794	0.862	0.815
	Fertilizer		-	0.854	0.842
	Warming			-	0.852
	Warming + Fertilizer				-

At the family level, the clone compositions of control and fertilizer treatment libraries were similar except that the clone library for the control was unique in containing sequences affiliated with the Clavulinaceae. Clavulinaceae-affiliated clones made up 5% of the control library, but were absent from all other treatment libraries and this change was statistically significant (p=0.0026). By contrast there were large changes in the composition of clone libraries in the warming and warming + fertilizer treatments. Most notable were the large differences in clones affiliated with the Cortinariaceae. Cortinariaceae-affiliated clones increased by a factor of 15 in the warming treatment over the control, and this change was highly significant ($p=3.1 \times 10^{-24}$, Figure 3.2). While clones affiliated with the Cortinariaceae doubled in

the warming + fertilizer treatment compared to the control, this change was not statistically significant ($p=0.21$). These changes were accompanied by significant reductions in the number of Russulaceae-affiliated clones in the warming and warming + fertilizer treatments ($p=0.047$, and 1.5×10^{-8}, respectively), as well as a significant reduction in the number of Helotiaceae-affiliated sequences in the warming treatment ($p=5.9 \times 10^{-4}$). Interestingly, the proportion of Thelephoraceae-affiliated sequences increased in all treatments compared to the control, but this change was only statistically significant in the warming + fertilizer treatment ($p=2.4 \times 10^{-4}$). Clones affiliated with the Tricholomataceae occurred only in the two treatments where fertilizer was added, and their numbers were significantly greater than in the in the warming + fertilizer treatment than the control ($p=0.023$).

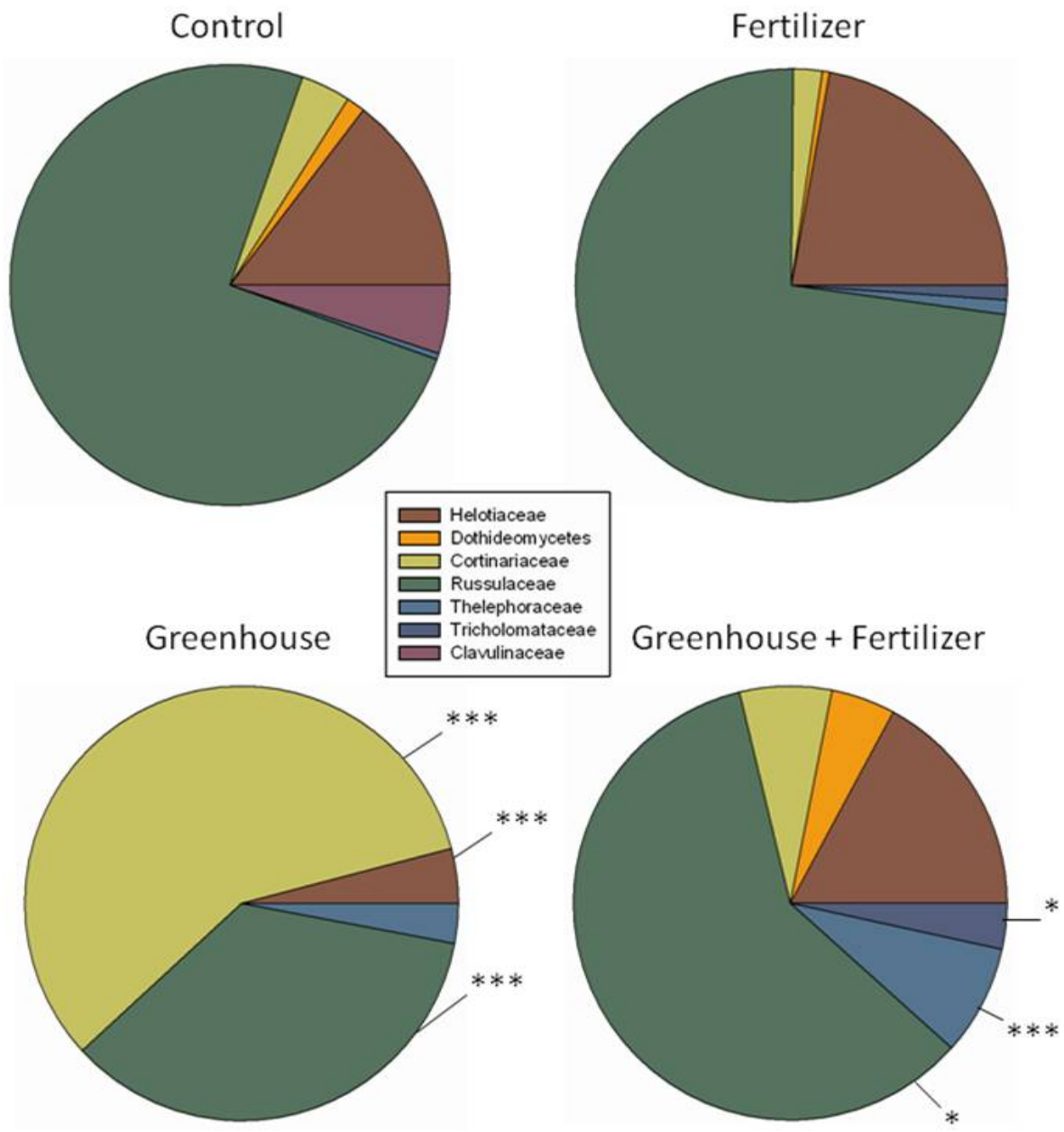

Figure 3.2: Proportion of clones affiliated with ECM fungi families in clone libraries constructed for *Betula nana* root tips from the four treatments. Significant treatment effects are denoted by symbols as follows: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

Seventy percent (597/858) of clones affiliated with mycorrhizal taxa could be identified at least to genus level, compared to 73% (777/1059) of clones overall. When only clones identified to genus or species levels were considered, control and fertilizer treatment libraries remained similar in composition, with no significant differences in the proportion of clones affiliated with different genera or species ($p>0.05$). By contrast, there were large differences in the composition of the clone library of the warming treatment and, to a lesser extent, the warming + fertilizer treatment. The large increase in sequences affiliated with *Cortinarius* remained an important effect of the warming treatment, although the detectable effect was smaller with greater taxonomic resolution, because of the smaller number of sequences that could be positively identified to the genus-level. Interestingly, *Cortinarius favrei* identified from sporocarps (see Chapter 2, supplementary Table 2.1) was the most common species in the control, and this was the only species that fruited in the warming treatment. The change in community composition with warming was also characterized by a significant reduction in the number of clones affiliated with *Rhizocyphus ericae* (formerly *Hymenoscyphus ericae*), and with *Lactarius* spp. Clones affiliated with the genus *Pseudotomentella* occurred only in the warming treatment, and this increase was significant ($p=0.023$, Figure 3.3). Sequences affiliated with *Laccaria bicolor* occurred only in the clone libraries for treatments that received fertilizer additions.

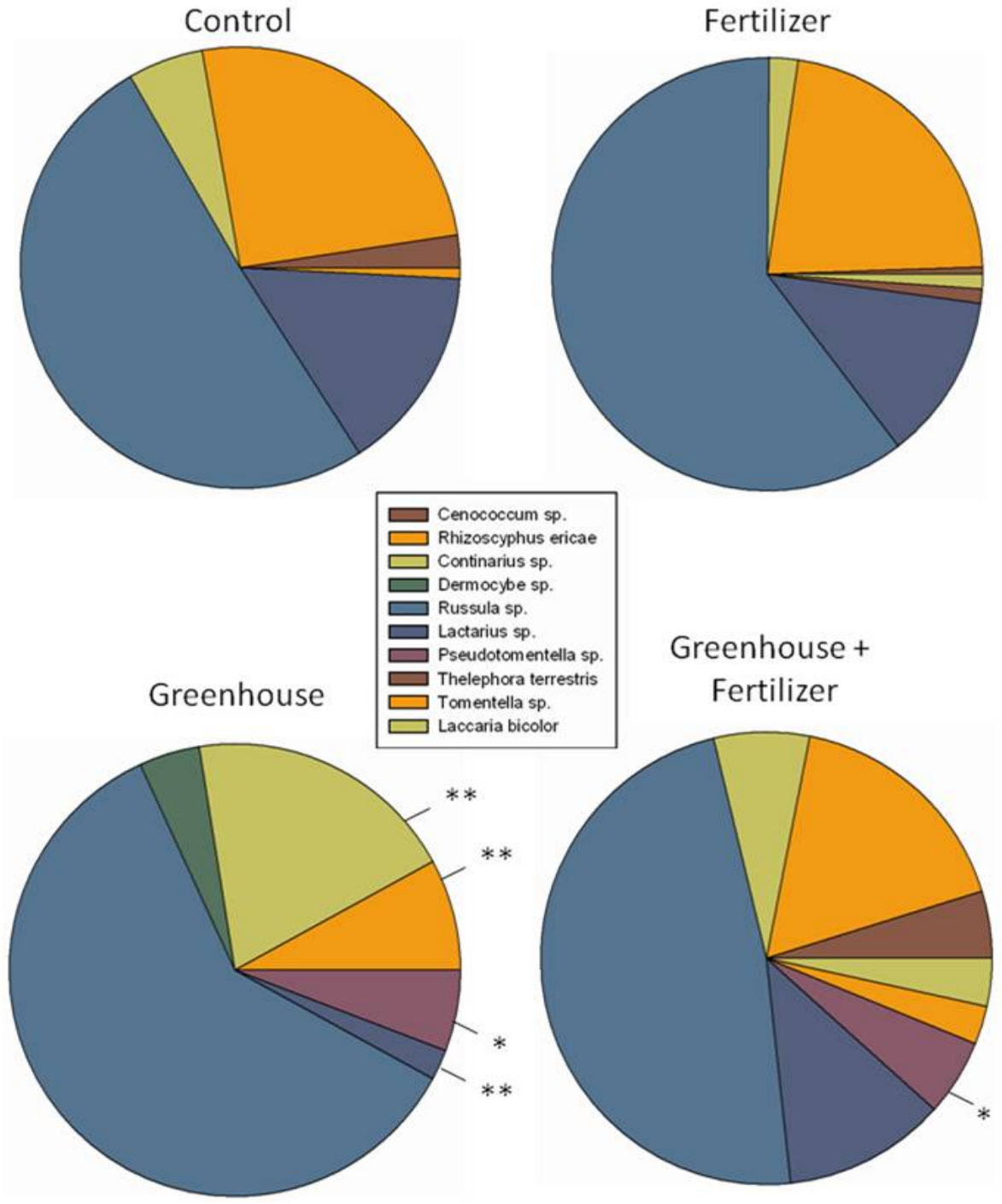

Figure 3.3: Proportion of clones affiliated with ECM fungi genera in clone libraries constructed for *Betula nana* root tips from the four treatments. Significant treatment effects are denoted by symbols as follows; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

When clones were categorized according to the hyphal characteristics of their closest taxonomic identity, a strong pattern emerged. We found that the warming treatment was dominated by clones affiliated with mycorrhizas that have hydrophobic hyphae, form rhizomorphs, have longer distance explorations types, and tend to be enriched in $\delta^{15}N$ relative to other mycorrhizal species (Table 3.6). By contrast, the control, fertilizer and warming + fertilizer treatments had approximately equal proportions of clones affiliated with taxa that form rhizomorphs as those that lack rhizomorphs. Moreover, these treatments had similar numbers of clones with contact, short distance and medium distance-smooth exploration characteristics and, compared with the warming treatment, far fewer clones affiliated with mycorrhizal taxa that exhibit the medium-fringe hyphal growth pattern. Likewise, clone libraries for the control and two fertilizer treatments were dominated by mycorrhizal taxa that have been shown to have intermediate $\delta^{15}N$ values; these treatments also had a high proportion taxa with low $\delta^{15}N$ values.

Table 3.6: Proportion of clones from 4 treatment libraries affiliated with mycorrhizal fungal genera with different hyphal characteristics. Only sequences that could be identified to genus level are included. Significant differences among treatment and control libraries are assessed using X^2-tests with Yates correction, symbols: ** = p< 0.01; *** = p<0.001.

	HYPHAL CHARACTERISTIC							
		Exploration Type				Relative $\delta^{15}N$ value		
Treatment	Presence of Rhizomorphs	Contact /Short-distance	Medium-fringe	Medium-smooth	Hydrophobic hyphae	High	Medium	Low
Control	0.51	0.44	0.06	0.5	0.52	0.11	0.69	0.20
Fertilizer	0.51	0.47	0.03	0.5	0.51	0.03	0.79	0.17
Warming	0.72***	0.06***	0.44***	0.5	0.72***	0.64***	0.35**	0.01***
Warming + Fertilizer	0.54	0.28	0.05	0.66	0.56	0.16	0.68	0.16

Discussion

The proportion of *Betula nana* root tips colonized by ECM fungi did not differ from control levels after 18 years of warming and/or fertilization applications, agreeing with Clemmensen and others (2006), who report no change in ECM colonization as measured by fine root ergosterol content after 14 years of fertilizer amendments in the same treatment plots. However, our findings contrast with a previous study reporting nearly a 50% reduction in the ECM colonization of *Betula nana* after 3 years of fertilization at this site (Urcelay *et al.* 2003). The reasons for this discrepancy are uncertain. The percent colonization by ECM fungi was quite similar in control plots in our study (61%) and the previous study (60%, Urcelay *et al.* 2003), but we observed a tendency for mycorrhization to increase with warming and fertilization treatments over the longer term, as was also found based on ergosterol content of fine roots (Clemmensen *et al.* 2006). The reduction in ECM colonization reported by Urcelay and others (2003) may reflect a short-term response of the ECM community of *Betula nana*, whereby initial reductions in plant C allocation belowground resulted in less mycorrhization, while over time, the ECM community acclimated to the continued fertilizer amendments, and mycorrhization returned to original levels. Species-specific responses to fertilizer additions have also been observed in an arid Pinyon Pine ecosystem; with N addition, *Cenococcum geophillum* contained younger C (as determined by analysis of the natural abundance of ^{14}C) despite the observation that C turnover rates of the whole community did not change significantly (Treseder *et al.* 2004). Likewise, we observed a few species specific responses to fertilizer treatment but relatively small changes in the ECM community as a whole.

Although percent ECM colonization was unchanged by warming or fertilization, we found that fertilizer additions were associated with a significantly smaller proportion of clones of putatively mycorrhizal origin, particularly where fertilization was combined with warming. We selected only ECM root tips and carefully cut large root tips to 2 mm in length in an attempt to equally sample the ECM species in DNA extractions. Thus, the decreased abundance of clones affiliated with mycorrhizal taxa in the fertilizer treatments may reflect an increased abundance of saprotrophic relative to ECM fungal biomass associated with root tips. This result suggests that although mycorrhizal colonization was unaffected by treatment, fertilization enhanced growth of saprotrophic fungi, and that this effect was stronger when fertilizer was combined with warming.

It is interesting to note that methods used to estimate fungal biomass in fine roots that do not distinguish between saprotrophic and mycorrhizal fungi, such as measurement of ergosterol, may be prone to overestimate ECM biomass where the ratio of saprotrophic to mycorrhizal fungal root associates increases with experimental treatments.

Unifrac analysis indicated that clone libraries for each treatment contained a *Betula nana* root-associated fungal community with a distinct composition and structure. The increasing Unifrac distance among libraries when non-mycorrhizal affiliated clones were removed further suggests that changes in the composition of the mycorrhizal community were particularly dramatic. This finding agrees with results for fungal communities in bulk soil (Deslippe *et al.*, Chapter 4 this volume) and suggests that mycorrhizal fungi may be particularly sensitive to climate change factors. The Unifrac distance matrix for the pair-wise comparisons of the putatively mycorrhizal clones clearly shows the high dissimilarity between mycorrhizal communities in the fertilizer versus warming treatments, supporting our prediction that fertilization and warming would to different responses of the ECM community. Similarly, at a tundra heath site in Sweden, long-term fertilizer additions led to a higher relative abundance, and warming treatments to a lower relative abundance, of fungal biomarkers in soil (Rinnan *et al.* 2007).

The most dramatic response observed in our study was the large and significant increase in the proportion of clones affiliated with the Cortinariaceae, and *Cortinarius* in particular, in the warming treatment. Members of the genus *Cortinarius* are strongly proteolytic (Lilleskov *et al.* 2002), a characteristic that is reflected in their generally high $\delta^{15}N$ values (Hobbie 2005). *Cortinarius* spp. are characterized by the presence of rhizomorphs, hydrophic hyphae, and they exhibit a medium distance-fringe exploration type of relatively high biomass. These characteristics suggest that *Cortinarius* is adapted to N-limited conditions, and thrives on relatively high C inputs from its host to fuel the production of biomass and extracellular proteolytic enzymes that act on soil organic matter. For example, Lilleskov *et al.* (2002) found that *Cortinarius* associated with white spruce increased linearly with decreasing net nitrification rates in boreal Alaska. The strong increase in *Cortinarius*-affiliated sequences observed in our study suggests that *Betula nana* responds to warming with an increased growth rate supported by increased dependence on N derived from the proteolytic capabilities of *Cortinarius* to support

its increased growth rate. Although no foliar $\delta^{15}N$ values are available for *Betula nana* in warming plots in the MAT-LTER, $\delta^{15}N$ values for *Salix reticulata*, which is the dominant ECM species in Moist Non-Acidic tundra (MNT), showed significant declines with warming. Mean $\delta^{15}N$ values for *Salix reticulata* in the MNT-LTER treatment plots were -5.18 ± 0.57 in control plots and -6.95 ± 0.39 in warmed plots (values are means ± standard deviations from Arctic LTER, see Gough and Hobbie 2003), supporting the idea that ECM plants in Arctic tundra increasingly depend on their fungal symbionts with warming. In addition to accessing organic N, the increased populations of ECM fungi with longer-distance exploration types may enhance connectivity among *Betula nana* individuals and facilitate nutrient transfer through mycorrhizal networks. Interestingly, warmer soil and air temperatures are associated with increased C transfer through mycorrhizal networks in this ecosystem (Deslippe and Simard Chapter 2, this volume) suggesting that warming affects the C-dynamics of *Betula nana* and its ECM on multiple time scales.

Warming may enhance the growth of high-biomass ECM fungi partly by altering the physiology of their plant hosts. ECM tundra plants, including *Betula nana*, are known to respond to warmer temperatures by producing leaves with significantly higher C to N ratios, when nutrients constrain growth (Tolvanen and Henry 2001, Welker *et al.* 2005). In culture studies of ECM plants, belowground allocation of C ranges from 27-68% of net primary productivity (NPP). Of this C, the proportion allocated to ECM fungi is a linear function ranging from 1-21% of total NPP, and is highest at lowest nutrient concentrations and at lowest plant growth rates (Hobbie 2006). Thus, as warming acts to enhance photosynthesis while N limitation constrains growth, ECM plants may increasingly allocate excess C to ECM fungi, fuelling the growth of high biomass types capable of degrading protein, and supplying their host with much needed N.

Concurrent with the significant increase in *Cortinarius*-affiliated sequences with warming was the decline in taxa that have small genet sizes characterized by contact, short, or medium distance-smooth exploration types, including significant reductions in affiliates of *Rhizocyphus ericae, Lactarius spp.,* and members of the Russulaceae. *Russula* spp. typically have low $\delta^{15}N$ values (Hobbie 2005), and though $\delta^{15}N$ values of *Lactarius* spp. range from low to medium values (Hobbie 2005), they have shown limited ability to degrade protein in culture studies (Abuzindah and Read 1986). Thus, the decline of populations affiliated with these ECM taxa in

the warming treatment provides further support for the idea that *Betula nana* allocates more C to ECM with proteolytic capabilities in response to warming.

In general, responses of the ECM community of *Betula nana* due to fertilizer were small relative to those due to warming. The dominance of clones affiliated with the Russulaceae was common to the control and fertilization treatment libraries, suggesting that ECM with limited proteolytic capacity are common symbionts of *Betula nana* at this site, and remain so with fertilizer additions. Forming ECM with these species may provide *Betula nana* with better access to labile soil N, as small size and profuse growth of hyphae in soils often lead to as much as 60 times the absorptive area of hyphae relative to fine roots (Simard *et al.* 2002) given this difference, hyphal N uptake should dominate root uptake in tundra and forest soils (Hobbie and Hobbie 2008). The persistence of a fungal community specialized in the uptake of inorganic forms of N fits with *Betula nana*'s characterization as the strongest competitor for soil N (Bret-Harte *et al.* 2008) and likely predisposes *Betula nana* to increasingly dominate tussock tundra in long-term fertilization experiments (Chapin *et al.* 1995, Bret-Harte *et al.* 2001, Shaver *et al.* 2001, Mack *et al.* 2004, Bret-Harte *et al.* 2008).

Both fertilizer treatments increased the presence of *Laccaria bicolor* sequences. *Laccaria* spp. have been characterized as "nitrophilic" (Lilleskov *et al.* 2002a), "non-protein" (Abuzinadah & Read 1986) fungi. Congruently, sporocarp production by *Laccaria proxima* increased significantly in response to N fertilization of a *Pinus sylvestris* stand in the Netherlands (Termorshuizen 1993). *Laccaria bicolor* utilizes three assimilatory pathways for ammonium and possesses highly active aspartate and alanine aminotransferases (Iftikhar *et al.* 1990). Pure cultures of *Laccaria bicolor* can sustain exponential growth on ammonium (Lilleskov *et al.* 2002b) as well as aspartate and alanine (Iftikhar *et al.* 1990), but grow poorly on BSA as their sole N source (Lilleskov *et al.* 2002b). When *Laccaria bicolor* forms ECM, one of its ammonia transporters is greatly up-regulated (Martin *et al.* 2008), suggesting that mycorrhization enhances the potential for N uptake in this species. *Laccaria* spp. show no host specificity (Molina *et al.* 1992) and are known to fruit prolifically from relatively few ECM root tips, suggesting a high relative allocation of resources to reproduction (Lilleskov *et al.* 2002a and references therein). These characteristics suggest that *Laccaria* spp. may be opportunistic, competing effectively with other soil organisms for inorganic N and responding quickly to

increased N by sporulating and colonizing new hosts. The increase in *Laccaria bicolor*-affiliated clones with fertilization treatments fits with this species' role as an opportunistic nitrophile.

We observed a relatively small change in the composition of clone libraries when fertilizer alone was added, which is consistent with other studies showing relatively little effect of N fertilization on belowground ECM communities (reviewed in Wallenda and Kottke 1998, Jonsson *et al.* 2000, Peter *et al.* 2001, Treseder *et al.* 2007). A recent meta-analysis of 82 published field studies revealed that fungal communities were not significantly altered by N additions, although total microbial biomass was reduced by 15% (Treseder 2008). Although ECM morphotype richness often declines with increasing N availability (Read and Perez-Moreno 2003, Allison *et al.* 2007), all studies that have found ECM communities to respond strongly to increasing N availability were conducted on conifer-associated ECM communities (Lilleskov and Bruns 2001). Along an N-deposition gradient in Europe, there were stronger declines in diversity of conifer-associated than deciduous-associated ECM taxa (Arnolds 1991). Likewise, while a negative relationship between ECM morphotype richness and soil inorganic N was found in *Picea abies* stands in Europe, a weaker and positive relationship between these variables was found in *Fagus sylvatica* stands (Taylor *et al.* 2000). These findings may suggest that the ECM communities of deciduous plants are more stable with increasing N than are the ECM communities of conifers (Lilleskov and Bruns 2001), which may reflect greater relative below-ground C allocation in deciduous ECM plants compared to conifers (Steele *et al.* 1997, Coleman *et al.* 2000) or greater baseline levels of N availability in many deciduous than coniferous forests (Jerabkova *et al.* 2006).

The fundamentally different trajectories of ECM communities in the warming and fertilizer treatments confirm our prediction that these treatments apply different selective pressures on the ECM community of *Betula nana*. This finding agrees with the contrasting responses of microbial communities to warming and fertilization treatments observed in a Swedish sub-Arctic heath (Rinnan *et al.* 2007), and suggests that direct fertilization of tundra ecosystems does not simulate the increase in mineralization rates, due to warming (Hobbie 1996). In this study, 18 years of fertilization caused relatively small changes in the ECM community of *Betula nana*, suggesting that this community is already well adapted to take advantage of higher nutrient conditions if they occur. By contrast, warming led to a dramatic

shift in the composition of the ECM community of *Betula nana* toward species with high biomass and an ability to degrade protein, changes in the ECM community of *Betula nana* resulting from climate warming will lead to increased decomposition of soil organic matter.

Conclusions

Here we show that 18 years of warming by greenhouses led to significant changes ECM community composition of *Betula nana* in Low Arctic tussock tundra. Warming was associated with a significant increase in fungal taxa with proteolytic capacity, particularly *Cortinarius* spp., and a reduction in fungal taxa with high affinity for inorganic N, especially *Russula* spp. By contrast, the change in ECM community composition resulting from fertilization was relatively small, although we observed a significant increase in the nitrophillic species *Laccaria bicolor* with fertilizer treatments. These findings suggest that warming elicits changes in the composition of ECM communities that enhance decomposition of soil organic matter and may increase the connectivity of *Betula nana* individuals through mycorrhizal networks of larger size. These changes will act to enhance N acquisition and nutrient redistribution to *Betula nana,* further facilitating its expansion onto Arctic tundra.

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4 Long-term warming reduces diversity and alters the structure of soil microbial communities in Arctic tundra[1]

Introduction

Despite steady orbitally driven reductions in summer insolation to Arctic regions over the past 2000 years, recent meta-analysis of temperature proxy data for 23 Arctic sites reveals that cooling in Arctic regions has reversed during the present century, with four of the five warmest decades occurring between 1950 and 2000 (Kaufman *et al.* 2009). Currently the western North American Arctic is experiencing the fastest rate of warming on earth. Temperature has increased in the region up to 0.1^{o}C per year over the last 35 years (Anisimov *et al.* 2007), highlighting the relevance of Polar regions as sensitive indicators of global climate trends. Of particular concern are the globally significant C and nutrient stores, which have accumulated in tundra soils in response to cold and short growing seasons and the presence of permafrost, which restricts drainage. Current warming in Arctic regions is associated with increased microbial activity leading to higher N availability for plants (Chapin 1983, Nadelhoffer *et al*. 1991, 1992, Chapin *et al*. 1995, Hobbie and Chapin 1998, Schmidt *et al.* 2002, Aerts 2006) and faster rates of C turnover in soils (Mack *et al*. 2004, Shaver *et al*. 2006, Schuur *et al*. 2009). These findings have led to a growing concern that warming threatens the stability of Arctic C stores, which upon release, are likely to result in significant additional positive climate forcing (Cox *et al*. 2000, Friedlingstein *et al.* 2006, Schuur *et al.* 2009).

Soil microbes are central to the C and nutrient balance of tundra ecosystems because of their dual role as decomposers of soil organic matter and as determinants of plant community diversity (van der Heijden *et al.* 2008), which in turn controls the quality and quantity of C inputs to soils (De Deyn *et al.* 2008, Bardgett *et al.* 2008). The stability of Arctic soil C stores may not only be sensitive to changes in the activity of soil microorganisms but also to changes in the composition of microbial communities. For example, temperature-induced changes in the structure of soil bacterial communities in an American hardwood forest soil led to a non-linear relationship between soil temperature and microbial respiration and to the hypothesis that

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increased temperature leads to shifts in microbial community composition, wherein dominant populations at higher temperatures have access to substrates that are not utilized by members of the community at lower temperatures (MacDonald *et al.* 1995, Zogg *et al.* 1997). However, our current understanding of the relationships between temperature and microbial community composition and function in Arctic soils is very limited (Nemergut *et al.* 2005).

By contrast, we have a good understanding of the strong responses of tundra plant communities to warmer temperatures. Earlier spring thaw and warmer summer temperatures result in alterations to plant phenology that includes earlier flowering, earlier fruit set, and increased plant productivity at high latitudes (Hartley *et al.* 1999, Aft *et al.* 1999, Walker *et al.* 2006). In more southerly Arctic regions, changes in the relative productivity of tundra species have led to ecosystem-scale changes in plant community composition characterized by the expansion of shrubs (Hobbie 1996, Hobbie and Chapin 1998) and the consequent decline of mosses and evergreen ericaceous species. These changes in the timing of plant processes or plant community composition can be expected to feed back to the microbial community through changes in the timing and quality of plant derived C substrates (Bardgett *et al.* 1999).

Despite the intimate relationship between plants and soil microbial communities, factors that influence the diversity of these organisms appear to be fundamentally different. A study of the diversity of angiosperms and acidobacterial communities over an elevational gradient reported that acidobacterial communities were phylogenetically clustered and had high spatial structure, and that their richness and phylogenetic diversity decreased monotonically with altitude; by contrast, angiosperm communities showed no phylogenetic structure corresponding to altitude and had a unimodal distribution pattern, achieving greatest richness and diversity at middle elevations (Bryant *et al.* 2008). Similarly, a continental scale assessment found that soil bacterial diversity was unrelated to site factors that commonly control the diversity of macroorganisms, including site temperature and latitude. Differences in the diversity and richness of bacterial communities instead corresponded to ecosystem type and could be explained in large part by soil pH, with highest diversity in pH neutral soils, and lowest in acidic soils (Fierer and Jackson 2006).

Our ability to predict the response of soil microbial communities to climate change is partly hampered by the many interacting factors that influence the structure of soil microbial communities. For instance, compositional shifts in Arctic microbial communities may be a direct result of increased temperature, or may be mediated by changes in plant communities or other soil organisms, and the timescales of these interactions may differ, complicating observed trends. For example, a significant increase in the density of fungal feeding nematodes was observed after 6 years of warming a Swedish tundra heath (Ruess *et al.* 1999), but a significant decline in the abundance of fungal biomarkers was observed after 15 years of warming in this experiment (Rinnan *et al.* 2007). Likewise, 7 years of warming at a High-Arctic site resulted in no change in the composition of the root-associated fungal community of *Salix arctica,* but there was an increase in root-associated fungal biomass (Fujimura *et al.* 2008). Similarly, 13 years of warming an Alaskan tussock tundra site led to a significant increase in all fungal biomarkers, and a significant increase in the mycelial production of mycorrhizal fungi. However, the increase in mycorrhizal biomass was associated with a strong increase in the biomass of the host shrub *Betula nana* and was interpreted to represent an overall reduction of mycorrhizal fungal biomass relative to plant root biomass (Clemmensen *et al.* 2006).

Until recently, our ability to predict the response of Arctic tundra microbial communities to climate warming was hampered by relatively little baseline data on the organisms that thrive in these important habitats (see Nemergut *et al.* 2005 for a review). In recent years, however, a concerted effort to describe the microbial communities of Arctic soils has greatly increased our understanding. For example, studies that have employed PCR amplification, cloning, and sequencing of small subunit (16/18S) rRNA genes of bacteria and/or fungi have now been reported from Siberian tundra (Zhou *et al.* 1997), the Canadian High-Arctic (Neufeld *et al.* 2005), Alaskan tundra (Wallenstein *et al.* 2007), and Finnish Lapland (Männistö *et al.* 2007), as well as several studies of permafrost microbial communities (see Wagner 2007 for a review). Although these reports vary considerably in terms of the relative abundances of phyla, common phyla appear to include the Proteobacteria, Actinobacteria, Acidobacteria, Bacteroidetes, Verrucromicrobia, Gemmatimonadetes, Firmicutes and the Chloroflexi, all of which are typical soil bacteria (Jassen 2006). From these studies, we have learned that Arctic soil microbial communities are diverse (Neufeld *et al.* 2004, Kobabe *et al.* 2004) and spatially variable at the microsite (Kobabe *et al.* 2004) and landscape scales (Männistö *et al.* 2007, Wallenstein *et al.* 2007). Further, Männistö *et al.* (2007) report that Arctic soil bacterial communities were

structured most strongly by pH differences among sites, a finding that appears to hold at continental scales for soil bacteria (Fierer and Jackson 2006).

Seasonal cycles in temperature and substrate availability have also been shown to have strong impacts on the structure of soil microbial communities in alpine tundra (Lipson *et al.* 2000, 2002). Winter communities are dominated by saprotrophic fungi and members of the Bacteroidetes, many of which are cold-tolerant degraders of complex C-compounds. In contrast, spring snow-melt and summer communities are dominated by bacterial assemblies that undergo a succession from Acidobacteria, which may include anaerobic organotrophs adapted to growth on cells lysates in wet snow-melt soils, to Verrucomicrobia- and β-proteobacteria-dominated summer soil communities, many of which may be oligotrophs adapted to conditions of limited labile-C availability (Lipson *et al*. 2002, Schadt *et al.* 2003, Lipson and Schmidt 2004, Nemergut *et al.* 2005).

The strong seasonal cycles in microbial community structure observed in alpine tundra, as well as the accelerated phenology of Arctic plants treated with warming, raise the question of whether Arctic warming will lead to sustained changes in microbial community assemblages, or merely accelerate normal seasonal succession among microbial groups in Arctic tundra soils. The two studies that included a temporal component to their sampling of Arctic tundra soil reported fairly stable microbial community assemblages (Männistö *et al.* 2007, Wallenstein *et al.* 2007), but to date there has been no effort to characterize microbial community response to warming in Arctic tundra soils. In this study, we tested the hypothesis that long-term warming will alter the composition of soil bacterial and fungal communities in Low-Arctic tundra. We predicted that microbial community change resulting from warming treatments would be greater than that due to normal seasonal succession in this ecosystem. Further, we predicted that the response of microbial communities to warming would be mediated, in part, by changes in plant community composition that result from warming treatments. We show, for the first time, that warming leads to a reduction of microbial diversity in Arctic tundra that is similar in scale to the loss of plant community diversity at this site.

Methods

Study site

The study site was located on a gentle (<5^{o}), north-facing slope in Moist Acidic Tundra near Toolik Lake, Alaska, USA (68^{o}38'N, 149^{o}34'W, elevation 780m). Moist Acidic Tundra forms on old glacial surfaces (>11,000 years BP) and supports 'heathland'-type tundra plant communities. Regular patterns of vegetation occur in this plant community as a result of the perennial, rhizomatous growth of the tussock-forming sedge, *Eriophorum vaginatum*. The dominant deciduous dwarf shrub, ectomycorrhizal *Betula nana*, occupies hollows between the sedge-tussocks in mixture with mid-canopy ericaceous ericoid mycorrhizal species, such as *Ledum palustre* and *Vaccinium* sp., and arbuscular or non-mycorrhizal species such as *Rubus chamaemorous* as well as herbs. Both plurocarpus and acrocarpus mosses are common and form a continuous ground cover that can exceed 40 cm depth in some areas. Soils are characterized by a thick organic horizon, 10-20 cm in depth, with a pH of 4.4 (Nadelhoffer *et al*. 1991). Mineral hoizons are essentially unweathered, pale grey in colour, permafrost affected, and show only occasional evidence of cryoturbation. Maximum thaw depth occurs in late July, when the active layer typically comprises the entire organic horizon and the top 5-10 cm of the mineral soil. Characteristics of the organic and mineral horizons at the study site are shown in Table 4.1.

Table 4.1: Soil characteristics of moist-acidic tundra at Toolik Lake, Alaska

Soil Properties	Organic	Mineral	Source
Depth (cm)	7-15	15-40	This study
Texture			
Gravel (%)		16	Walker and Walker 1995, in Marion *et al*. 1997
Sand (%)		46	
Silt (%)		26	
Clay (%)		28	
Bulk density (g/c^3)	0.12	0.60	*Ibid.*
pH	4.4	4.5-4.7	Nadelhoffer *et al*. 1991, Walker and Walker 1995, in Marion *et al*. 1997
Organic matter (%)	72	6.5	Walker and Walker 1995, in Marion *et al*. 1997
Soil C ($g\ m^{-2}$)	5655	ND	Schmidt *et al.* 2002
Soil N ($g\ m^{-2}$)	182	ND	*Ibid.*
Soil P ($g\ m^{-2}$)	19.5	ND	*Ibid.*
CEC ($cmol\ kg^{-1}$)	29.0	6.9-7.9	Nadelhoffer *et al*. 1991, Walker and Walker 1995, in Marion *et al*. 1997
Exchangable Ca ($cmol\ kg^{-1}$)	11.1	1.7	Walker and Walker 1995, in Marion *et al*. 1997
Exchangeable Mg ($cmol\ kg^{-1}$)	4.8	0.68	*Ibid.*
Exchangeable K($cmol\ kg^{-1}$)	1.7	0.14	*Ibid.*

Warming treatments

The study treatments are maintained as part of the Arctic Long Term Ecological Research (LTER) experiment. Warming treatments and ambient controls are replicated four times in a randomized block design. Warming is accomplished passively with greenhouses constructed of 0.15mm polyethylene fixed on permanent wooden frames that are 2.5 X 5m, and 1.5 m in height. The uneven microtopography of the study site allows for air circulation from the base the greenhouse walls. Greenhouses reduce the photosynthetically active radiation by approximately 20% over the growing season, but have no significant effect on soil water content, and previous shading experiments indicate that plant production and biomass are unresponsive to light reductions of 50-64% at this site (Chapin *et al*. 1995). Soil temperatures in greenhouses tend to be cooler than ambient controls in summer because of increased shading by higher plant biomass, but warmer than ambient controls in winter due to increased insulation by snow trapped by plants. Mean soil temperature increase due to warming over the two year period (2006 and 2007) was 1.33^{o}C at 10 cm depth; 1.21^{o}C at 20 cm depth; and 0.96^{o}C at 40 cm depth. Mean daily ambient air temperature and soil temperatures at 20 cm depth in warming and control plots, during the study period, are shown in Figure 4.1 (Shaver and Laundre 2009).

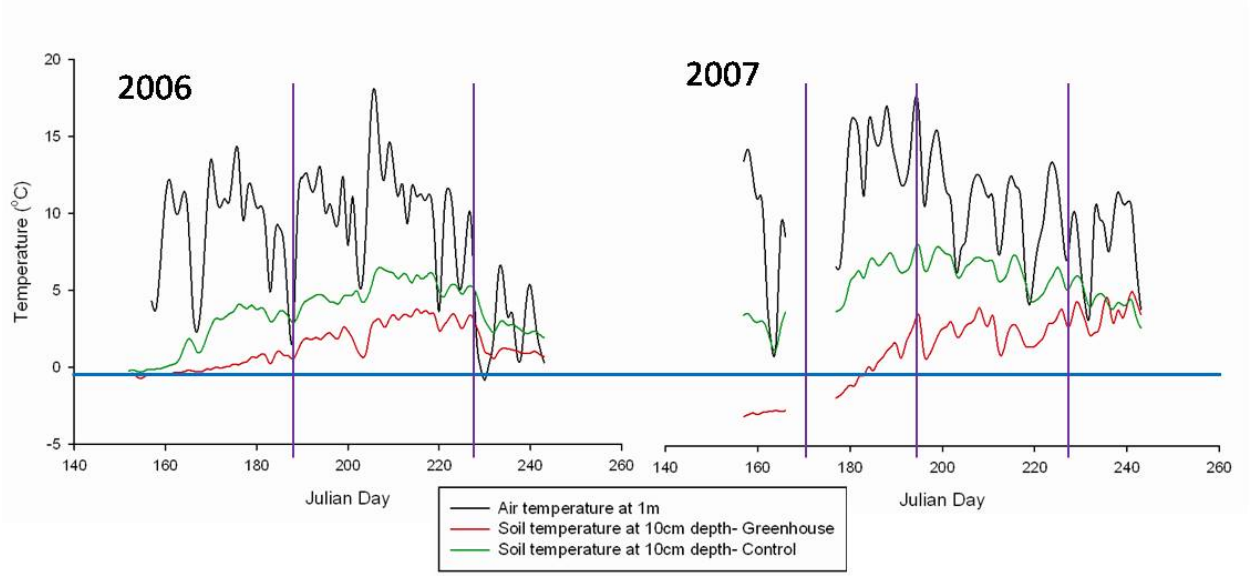


Figure 4.1: Mean daily air temperature at 1 m, and soil temperatures at 20 cm depth for warming and control plots for June, July and August of 2006 and 2007. Soil sampling dates are denoted by purple vertical bars. Data is from the Arctic LTER (see Shaver and Laundre 2009)

Plant communities have responded to warming treatments at this site and other Moist Acidic tundra sites through strong shifts in community composition (Chapin and Shaver 1986, Chapin *et al.* 1995). While total plant biomass was unchanged after 9 years of warming treatment at this site, vascular plant biomass increased, while non-vascular plant biomass declined significantly. Among vascular plants deciduous and evergreen plants increase, while forbs decline significantly; by contrast all mosses and lichen decline significantly with warming treatment. Table 4.2 summarizes the response of total plant biomass to 9 years of warming at this site.

Table 4.2: Total plant biomass (excluding roots) by species in control and warming treatments after 9 years. Significant treatment effects are denoted by the following symbols; * = p<0.05; ** = p<0.001. All data is from the Arctic LTER data archive, file # 1982_2000gs81tusbm (Shaver *et al.* 2000).

Species	Control	Warming
Eriophorum vaginatum	50.78 ± 2.7	101.03 ± 7.2
Carex bigelowii	54.09 ± 3.3	21.6 ± 1.8
Grasses	0.26 ± 0.05	0.08 ± 0.02
Betula nana	267.26 ± 14.3	273.88 ± 12.9
Rubus chamaemorus	14.33 ± 0.9	33.42 ± 2.6
Salix spp.	3.66 ± 0.5	0 ± 0
Other deciduous	4.53 ± 0.7	0.076 ± 0.02
Ledum palustre	184.77 ± 6.9	271.01 ± 7.7
Vaccinium vitis-idaea	129.4 ± 3.6	159.69 ± 5.1
Other Evergreen	30.86 ± 2.0	26.37 ± 2.0
Forbs spp.	3.08 ± 0.3	0.28 ± 0.02
Mosses spp.	235.96 ± 20.4	59.24 ± 15.2**
Lichens spp.	44.00 ± 12.5	9.33 ± 5.1*

Soil sampling and nucleic acid extraction

Soil samples consisted of three randomly placed soil cores in each of the four replicate warming and control plots at each sampling date. Soil cores were 2 cm in diameter and extended to the entire depth of thaw. Soils were sampled on July 9th and August 17th in 2006 and on June 20th, July 13th, and August 14th, in 2007. Each soil core was divided into organic

and mineral horizons, packaged separately in clean, airtight, plastic bags, and placed immediately on ice. All soil cores were frozen to -80^{o}C within 2 hours of sampling and remained frozen during transport.

In the laboratory, each frozen soil core was placed on a sterile bench top and scraped with a sharp sterile knife to remove its outer surface. The core was then sub-sampled to select the central portion of each horizon, where soil of uniform colour could be obtained. One gram of frozen soil was weighed into a small beaker containing 200ul of CTAB buffer and mixed by hand until the soil had thawed and a thick slurry was formed. For organic horizons, a 0.5 g portion of this slurry was subjected to nucleic acid extraction using the protocol described by Griffiths *et al*. (2000). Mineral horizons had high clay content (28% clay), requiring modification of the Griffiths protocol to reliably yield high quality nucleic acids, whereby 0.1% pyrophosphate and 200 µg poly A were added to the CTAB buffer-soil slurry and incubated at room temperature for 5 minutes prior to extraction.. A 5 µl aliquot of each soil extract was run on a 1% agarose gel, stained with ethidium bromide and visualized under UV light to check extraction efficiency. The three nucleic acid extracts per sampling plot were then pooled for downstream analysis. Thus, subsequent procedures were applied to eighty DNA extracts (5 dates * 2 treatments * 4 plot replicates * 2 soil horizons).

PCR and automated ribosomal intergenic spacer analysis

Polymerase chain reactions for ARISA analysis of bacteria used the flurophore labeled forward primer 6FAM-1541F and the reverse primer 115R (both primers Normand *et al*. 1996), yielding products that ranged in size from 300-1200 bp. PCR reactions consisted of 50 ng genomic DNA, 10µmol dNTPs, 5 nmol of each primer, 10X PCR Buffer, 50µmol $MgCl_2$, 15 µg BSA, and 2U of Taq DNA polymerase (Fermentas) in a final volume of 25 µl. The thermocycler program consisted of an initial denaturing temperature of 95°C for 5 min followed by 35 cycles of: denaturing at 94°C for 45 s, annealing at 57°C for 45 s, and extension at 72°C for 2 min. A final extension period of 10 min at 72°C completed the program. PCR reactions were kept on ice until the thermocycler block temperature reached 95^{o}C and then quickly loaded in a simplified 'hot-start' procedure. Positive PCR amplifications were confirmed by running 5 µl of

the PCR products on a 1% agarose gel. In order to reduce the occurrence of multiple RIS peaks from single phylotypes due to partial 3' adenylation of DNA polymerase, an 'A-tailing' step was employed. 'A-tailing' reactions consisted of 2 µl 10X PCR Buffer, 20 µmol $MgCl_2$, 2 µmol dATP, and 1U of Taq DNA polymerase (Fermentas) to a final volume of 10 µl, which was added to 20 µl of the PCR product, and incubated for 30 min at 72^{o}C. A PTC-200 Peltier Thermal Cycler (MJ Research Inc., Reno, NV, USA) was used for all thermal reactions. All PCR products were cleaned purified using a Qiagen Mini Elute PCR clean up kit following the manufacturer's directions.

Similarly, PCR amplifications for fungal ARISA employed the flurophore labeled forward primer FAM-ITS1F (Gardes and Bruns, 1993) and the reverse primer ITS4 (White *et al*., 1990). PCR reactions consisted of 50 ng genomic DNA, 20µmol dNTPs, 10 nmol primers, 10X PCR Buffer, 50µmol $MgCl_2$, 15 µg BSA, and 2.5 U of Taq DNA polymerase (Fermentas) in a final volume of 25 µl. The thermocycler program consisted of an initial denaturing temperature of 95°C for 5 minutes followed by 35 cycles of: denaturing at 94°C for 1 min, annealing at 55°C for 50 s, and extension at 72°C for 50 s, and a final extension period of 7 minutes at 72°C. Fungal PCR products were verified on an agarose gel, subjected to 'A-tailing', and purified using a Qiagen kit as for bacteria.

ARISA of bacteria and fungal communities was accomplished using an Applied Biosystems 3730S, 48 capillary sequencer. An injection voltage of 1.6 kV was applied for 15 seconds. 1 µl of each sample and 0.15 µl of a GeneScan™ 1200 LIZ® size standard (Applied Biosystems) were loaded onto each capillary. Resulting ARISA profiles were analyzed using GeneMarker® v1.70 software (Software Genetics, State College, PA). ARISA fragment lengths were determined using the internal size standard and the local Southern algorithm. To avoid binning errors and subsequent data loss that are the inevitable result of applying binning procedures to highly diverse ARISA community profiles, we employed the approach described by Hartmann *et al*. (2009) to compare the topographies of each profile's trace data. Briefly, trace data were sized and then exported from GeneMarker in bins of 0.5 relative migration units. To remove background noise, a threshold of 300 relative fluorescence units (rfu) was applied. To normalize profiles to the same total signal, which compensates for differences in amplification efficiency as well as sample load, each data point was divided by the sum signal of its own

profile and multiplied by the mean sum of signals from all profiles. Threshold and normalization steps were iterated until the data set was stable (Dunbar *et al*., 2001). Normalized fungal and bacterial ARISA profile data were used for subsequent statistical analyses.

Nonmetric Multidimensional Scaling (NMS) was chosen to visualize treatment plots in phylotype-space. All ordinations were run using PC-ORD version 5.0 in the 'auto-pilot' mode, which used random starting configurations and assessed dimensionality by minimizing stress. Sørensen distance was selected as the distance measure for each initial matrix (McCune and Mefford 1999). Initial ordinations produced unstable solutions when bacteria from organic and mineral horizons were included together, indicating that the disparity among these communities prevented NMS from finding stable local minima. Consequently, bacteria from mineral and organic horizons were ordinated separately.

Cloning and sequencing

We targeted the 3' end of the 16S ribosomal RNA gene adjacent to the ITS. Initial PCRs for bacteria applied the forward primer 1073F (Garcia-Martinez *et al*. 1999) and the reverse primer 115R (Normand *et al*. 1996) in reactions conditions similar to those used for ARISA-PCRs, except that the annealing temperature was increased to 60^{o}C and the number of cycles reduced to 30. Subsequently, a 'reconditioning' step, designed to eliminate PCR artifacts formed in late-stage, template-limiting cycles of PCR, was employed (Thompson *et al*. 2002). Here, 5 µl of initial PCR product was added as template to 50 µl of fresh PCR reagents and subjected to three additional thermal cycles. Fungal PCR for cloning and sequencing were as described above with the addition of the reconditioning step as applied to bacteria. We retained fungal primers that target the ITS region of the rRNA operon rather than using primers that would target the small subunit (18S) because of reports that many primer sets that target the 18S are rarely fungal specific, and amplify non-target eukaryotes as a result of the high similarity among eukaryotic 18S gene regions (Smit *et al.* 1999, Anderson *et al*. 2003). All PCR products were purified using MiniElute purification columns (Qiagen). These PCR products were quantified using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). Amplicons were pooled, combining 100 ng of DNA from each PCR same domain, treatment, and soil horizon. Consequently, 8 cloning reactions were performed: bacteria/fungi *

warming/control * organic/mineral horizon. Pooled templates were cloned using a TOPO TA Cloning® kit with pCR®II-TOPO® vector and DH5α-T1™ competent cells (Invitrogen, Burlington, ON) with blue/white screening. Clones were plated, picked and bidirectionally sequenced at the Genome Sciences Centre (Vancouver, BC) using the vector specific primers M13for and M13rev.

Phylogenetic analyses

Bidirectional sequence reads were assembled using the ContigExpress function of Vector NTI 10.3 (Invitrogen), manually checked for base-calling errors, and trimmed of the vector sequence. The Bellerphon server (Huber *et al.* 2004) was used to detect chimeric sequences in each of the bacterial and fungal libraries. Bacterial sequences were aligned using the on-line Alignment tool available from the Ribosomal Database Project (Cole *et al*. 2009) at http://rdp.cme.msu.edu. Fungal sequences were aligned using MUSCLE 3.7 (Edgar, 2004) and refined using the "-refine" command. Both alignments were visually checked for accuracy and manually edited in ARB (Ludwig *et al*. 2004). Preliminary phylogenetic trees of the combined fungal and bacterial datasets were constructed with Neighbour-joining (ARB). Fungal and bacterial trees were uploaded to the Unifrac server http://bmf2.colorado.edu/unifrac/ with their corresponding environment files. Unifrac measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree that leads to descendants from either one environment or the other, but not both (Luzopone & Knight 2005). Unifrac significance tests and P-tests, both corrected for multiple comparisons (p-value is multiplied by the number of pairwise comparisions), were used to test for significant differences in the composition of paired libraries. The 'environment distance matrix' function was used to calculate pairwise distances between each library. The 'lineage specific analysis' function was used to detect lineage specific differences in the clone libraries by environment (Lozupone and Knight 2005, Lozupone *et al.* 2006), and to support Chi^2-tests for phylogenetic groups (see below). Phylogenetic affiliations of bacterial 16S rRNA sequences were determined using the naïve Bayesian classifier (Wang *et al.* 2007) available through the Ribosomal database project. Phylogenetic affiliations of fungal ITS sequences were determined using the Fungal ITS Pipeline (Nilsson *et al.* 2009a), which employs multiple alignment programs (Clustal W2 [Larkin *et al.* 2007], MAFFT v6 [Katoh, 2008], and DIALIGN-TX [Subramanian *et al.* 2008] to align ITS

sequences, and HMMER http://hmmer.janelia.org/ a hidden Markov model algorithm (Durbin *et al.* 1998), to run similarity searches of the query sequences using a local installation of NCBI-BLAST. The Fungal ITS Pipeline queries both the entire ITS sequence length and additionally extracts the hyper-variable ITS2 subregion for additional searches to achieve higher phylogenetic sensitivity (Nilsson *et al.* 2009b). The Fungal ITS Pipeline grouped like sequences based on 50% identity of the 15 closest NCBI-BLAST hits; only groups that contained a minimum of two sequences (ie. no singletons) were retained for analysis. Chi^2-tests with Yates corrections were used to determine whether phylogenetic groups differed among control and warming treatments.

Diversity indices

To estimate the diversity of bacterial 16S and fungal ITS rRNA gene sequences in clone libraries we used two indices of alpha diversity. We applied the Shannon's index (H), as

$$H = -\sum \left\{ \left(\frac{n_i}{N}\right) \ln \left(\frac{n_i}{N}\right) \right\}$$

Where *ni* is the abundance of taxon i and *N* is the total number of all phylotypes. The Shannon's index of diversity measures the evenness of the distribution of taxa in each library and can vary from 0 to high numbers, reaching maximum values at maximum entropy of the species distribution. We applied Simpson's index (1-D), which is also a measure of taxon diversity. Simpson's index is calculated as 1-D (dominance) where:

$$D = \sum \left\{ \left(\frac{n_i}{N}\right)^2 \right\}$$

Since D varies from 0 (where all genotypes are equally present) to 1 (where a single genotype completely dominates the library), Simpson's index inversely ranges from 0 to 1, where higher

values indicate higher genotype evenness and richness. Whereas Shannon's index emphasizes evenness, Simpson's index emphasizes the richness portion of diversity, placing greater on the abundance of the most common species. We applied diversity indices only to clones that could be identified to phylum for bacteria and to class for fungi. Singleton ITS sequences were not included in diversity calculations for fungi, but are reported separately as an indication of genotype diversity within fungal clone libraries.

Results

NMS ordinations of ARISA profiles for ITS regions of rRNA genes for bacteria and fungi revealed that the structure of both communities was strongly changed by warming treatments, and that little seasonal succession in these microbial communities occurred (Figures 4.2, 4.3, 4.4). The NMS plot for organic soil samples in bacterial ARISA profile space (Figure 4.2a) produced a 2D solution after 270 NMS iterations, with a final stress of 11.45, and a final instability of zero; the total r^2 for this ordination was 0.814. Figure 4.2a reveals that 58.7% of the variation in ARISA profiles was explained by axis 2. All organic soil samples that ranked high on axis 2 originated from the warming treatment plots and did not cluster according to the time of sampling.

The NMS ordination for mineral soil samples in bacterial ARISA profile space (Figure 4.2b) produced a 3D solution with a final stress of 10.49 and a final instability of zero. Axes 1 and 2 (Figure 4.2b) accounted for 33.7% and 41.0% of the total variance in the dataset (cumulative $r^2 = 0.747$), while the third axis (not shown) accounted for 15.2% (total $r^2 = 0.899$). A comparison of the NMS plots between organic and mineral soils highlights the declining effect of warming on microbial community structure with soil depth. The warming effect was still greater, however, than the effect of sampling date.

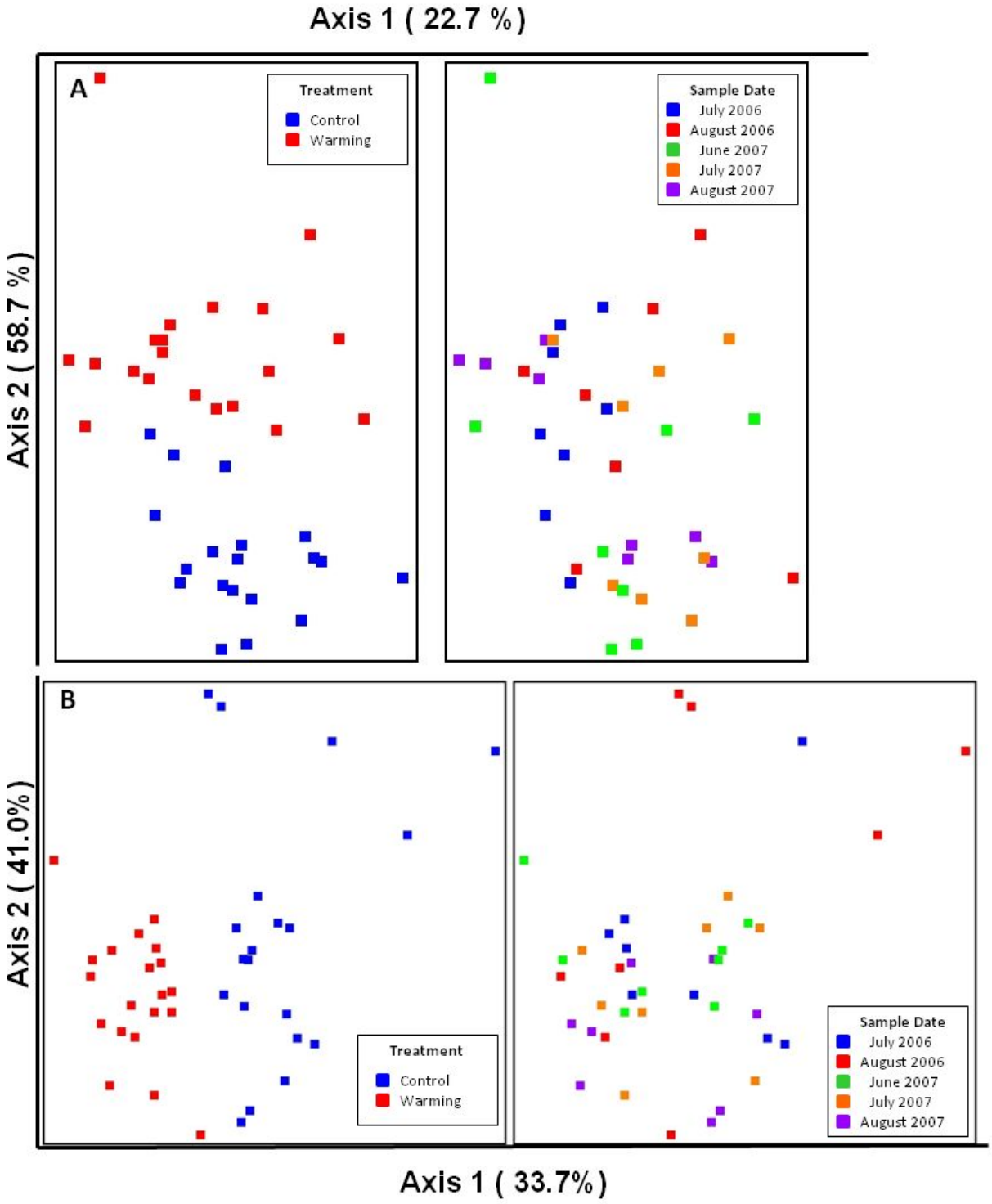


Figure 4.2: Non-metric multidimensional scaling ordinations of soil samples in bacterial RIS space; A) Organic soil samples; B) Mineral soil samples. Each ordination appears twice; plots on the left hand side are overlaid with symbols representing their origin from warming

treatments or control plots; plots on the right hand side are overlaid with symbols representing their sampling date.

We found that the spatial scale of change in fungal communities was greater than in bacterial communities. Consequently, the fungal community response to warming was better observed at the plot than at the sample scale. The NMS ordination for organic soil samples in fungal ARISA profile space (Figure 4.3a) produced a 3D solution after 110 iterations, with a final stress of 14.789 and a final instability of zero. Axes 1 and 2 accounted for 19.0% and 33.4% of the total variance in the dataset (cumulative $r^2 = 0.424$), while the third axis (not shown) accounted for 24.4% (total $r^2 = 0.668$). Similarly, the NMS ordination of mineral soil samples (Figure 4.3b) required 500 iterations to achieve a stable 3D solution with a relatively high final stress of 16.67 and a final instability of 0.00210. Axes 1 and 3 accounted for 22.8% and 31.3% of the total variance in the dataset (cumulative $r^2 = 0.541$), while axis 2 (not shown) accounted for 16.2% (total $r^2 = 0.703$). The relatively high instabilities of the fungal ARISA ordinations compared to those for bacterial ARISA profiles prompted us to examine whether the spatial scale of the fungal response to warming was greater than that observed for bacteria.

When samples from each plot were pooled, the NMS ordinations of fungal ARISA space revealed that warming accounted for a greater proportion of the variance in fungal community structure than when samples were considered separately, and that this increase was associated with NMS solutions with lower stress and higher stability. The NMS ordination for treatment plots in ARISA space for organic soil (Figure 4.4a) produced a 3D solution in only 79 iterations, with a low final stress of 2.83 and a final instability of zero. Axes 1 and 2 accounted for 23.8% and 36.2% of the total variance in the dataset (cumulative $r^2 = 0.600$), while the third axis (not shown) accounted for 32.2% (total $r^2 = 0.922$). Similarly, the NMS ordination for treatment plots in ARISA space for mineral soil (Figure 4.4b) produced a 3D solution in only 83 iterations, with a low final stress of 3.68 and a final instability of zero. Axes 2 and 3 account for 34.9% and 44.2% of the total variance in the dataset (cumulative $r^2 = 0.791$), while the third axis (not shown) accounted for 10.8% (total $r^2 = 0.899$). A comparison of the percent variance explained by the axes associated with warming in Figures 4.3 and 4.4 reveals that the spatial scale of fungal response to warming is better viewed at the plot than at the sample scale. For example, in organic soils the percent variance explained by warming increased from 19% to 36.2% from

the sample to plot scale, while in mineral soils the percent variance explained by warming increased from 22.8% to 44.2%.

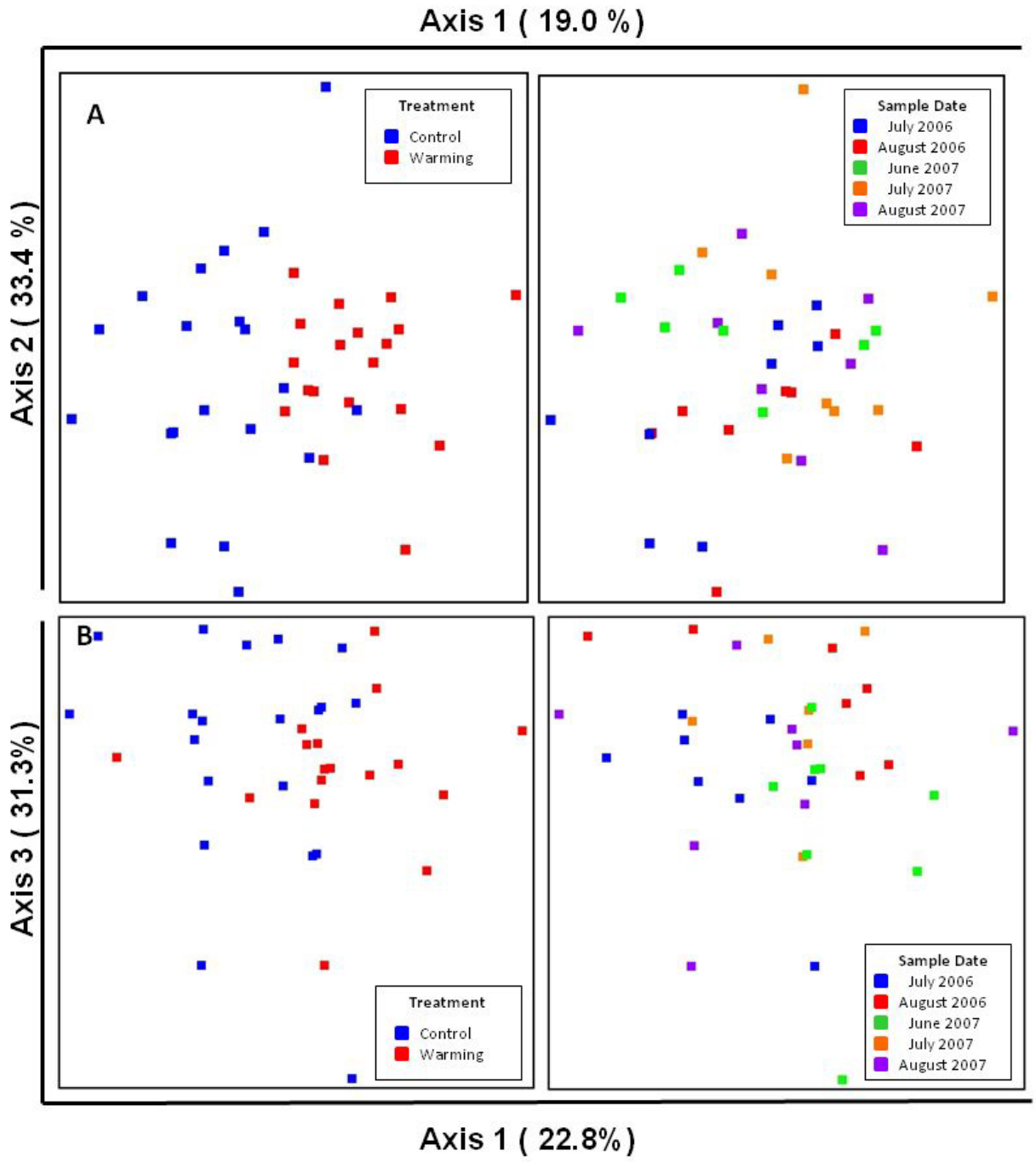


Figure 4.3: Non-metric multidimensional scaling ordinations of soil samples in fungal ITS space; A) Organic soil samples; B) Mineral soil samples. Each ordination appears twice; plots on the left hand side are overlaid with symbols representing their origin from warming treatments or control plots; plots on the right hand side are overlaid with symbols representing their sampling date.

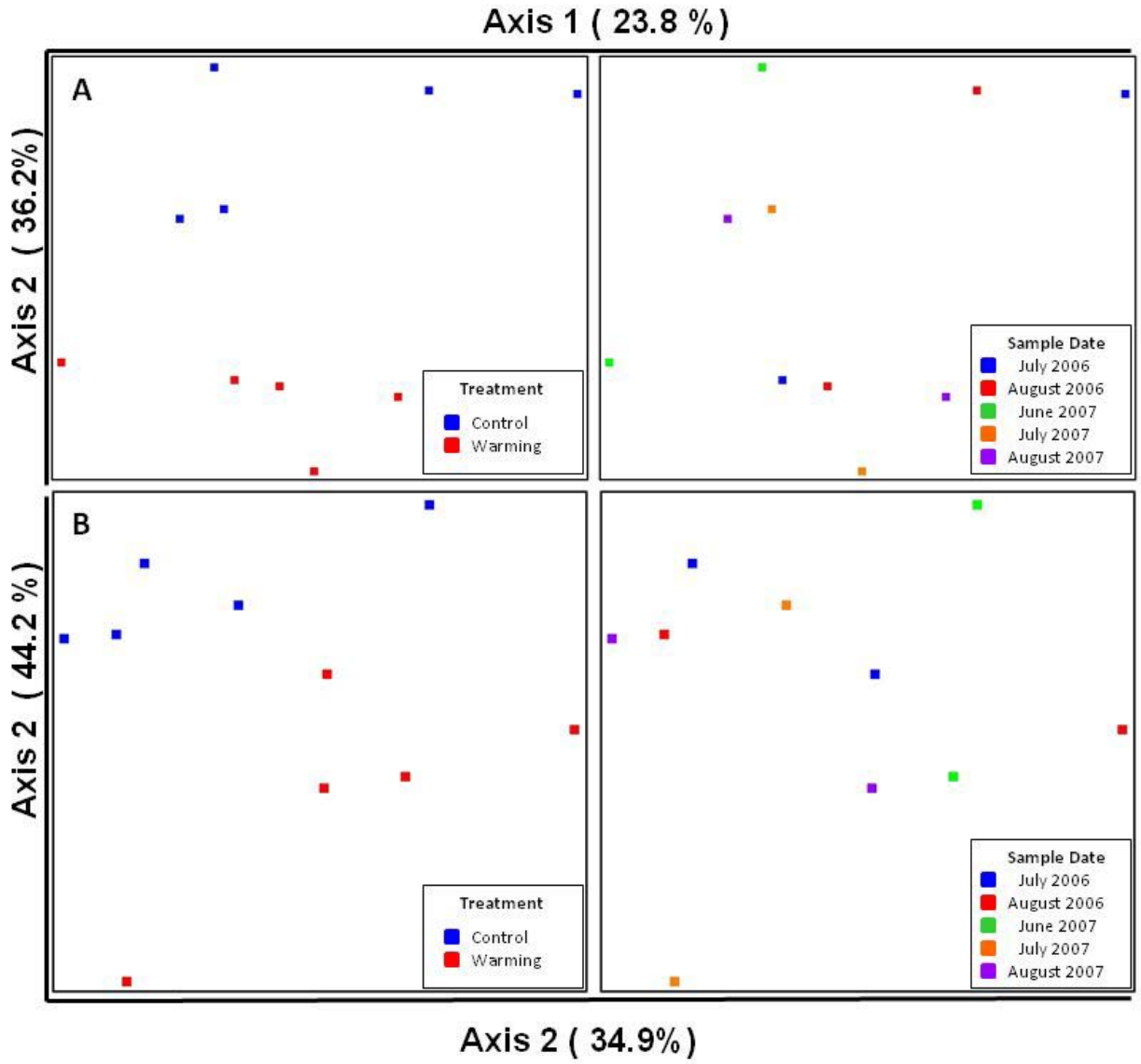


Figure 4.4: Non-metric multidimensional scaling ordinations of treatment plots in fungal ITS region of rRNA gene space; A) Organic soil; B) Mineral soil. Each ordination appears twice; plots on the left hand side are overlaid with symbols representing their origin from warming treatments or control plots; plots on the right hand side are overlaid with symbols representing their sampling date.

A comparison of the NMS ordinations for bacteria and fungal ARISA profiles reveals that bacterial communities responded more strongly to warming treatment than did fungal communities in organic soils. Also, although the effect of the warming treatment declined

sharply with depth for bacteria, no such pattern was found for fungal communities. Interestingly, among mineral soil samples, the percent variance in community structure explained by warming was similar for bacteria (41%, Figure 4.2b) and fungi (44.2%, Figure 4.4b), while, among organic soil samples, the percent variance in community structure explained by warming was two thirds greater for bacteria (58.7%, Figure 4.2a) than for fungi in organic soil plots (36.2%, Figure 4.4a).

Table 4.3: Final number of sequences for the libraries constructed in this study.

	Library size	
Library	Bacteria	Fungi
Control -Organic	366	309
Control-Mineral	357	177
Warming-Organic	355	199
Warming-Mineral	370	273
Total	1448	958

Unifrac analysis of bacteria clone libraries by environment revealed that all pairwise comparisons between libraries were statistically significant at $\alpha=0.05$, indicating that each environment contained a bacterial community with a distinct composition and structure. The distance matrix for pairwise comparisons between libraries, shown in Table 4.4, reveals that the greatest difference in bacterial community structure was found among organic soils from warming and control plots, while the two mineral soils were the most similar.

Table 4.4: Unifrac distance matrix for pairwise comparisons between of bacterial 16S clone libraries, all pairwise comparisons are significant at α=0.05, as determined by the Unifrac significance test and the P-test, both with Bonferroni corrections. Unifrac distances are coloured according to the quartiles of distance each pairwise comparison accounts for with increasing shades of grey corresponding to increasing quartile distance: 0-25%; 25-50%; 50-75%; 75-100%.

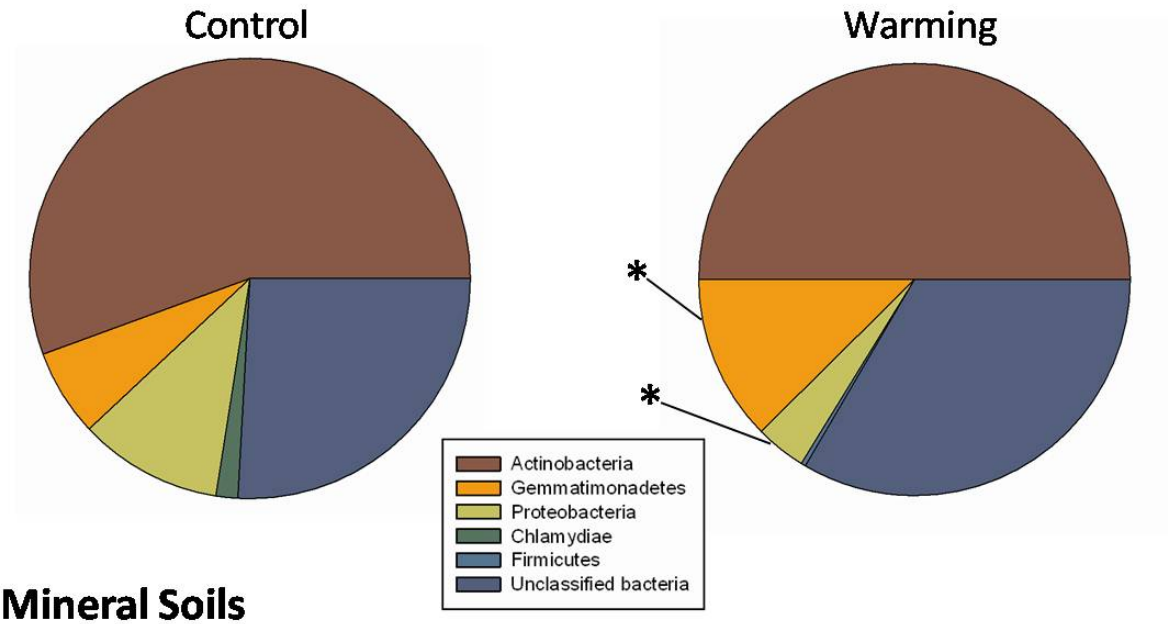
	Control Organic	Control Mineral	Warming Organic	Warming Mineral
Control Organic	0	0.832	0.871	0.865
Control Mineral		0	0.847	0.804
Warming Organic			0	0.805
Warming Mineral				0

Clone libraries of bacterial 16S rRNA genes from organic and mineral soils in control and warmed soils were dominated by sequences affiliated with the Actinobacteria (Figure 4.5), with this group representing approximately half of each of the four clone libraries. Also abundant were members of the Gemmatimonadetes and the Proteobacteria. Twenty to thirty percent of sequences obtained in each library could not be identified beyond the kingdom level. Bacterial groups often responded oppositely to warming in organic versus mineral soils at the phylum and the class level. Thus, we present the analysis of our bacteria clone library data separately for organic and mineral soils.

At the phylum level in the organic layer, warming was associated with a significant increase in sequences affiliated with the Gemmatimonadetes (Chi^2= 4.016; p=0.045), and a significant reduction (Chi^2= 10.023; p=0.0015) in sequences affiliated with the Proteobacteria (Chi^2= 10.023; p=0.0015) (Figure 4.5a). In contrast, in mineral soil libraries (Figure 4.5b), warming led to a significant increase in Actinobacteria (Chi^2= 3.867; p=0.049) and a significant reduction in the Gemmatimonadetes (Chi^2= 4.61; p=0.032). Phylum richness was greater in

mineral soil than in organic soil, with 11 bacterial phyla represented in libraries of mineral soils and only 5 phyla represented in organic soil libraries (Figure 4.5).

A) Organic Soils



B) Mineral Soils

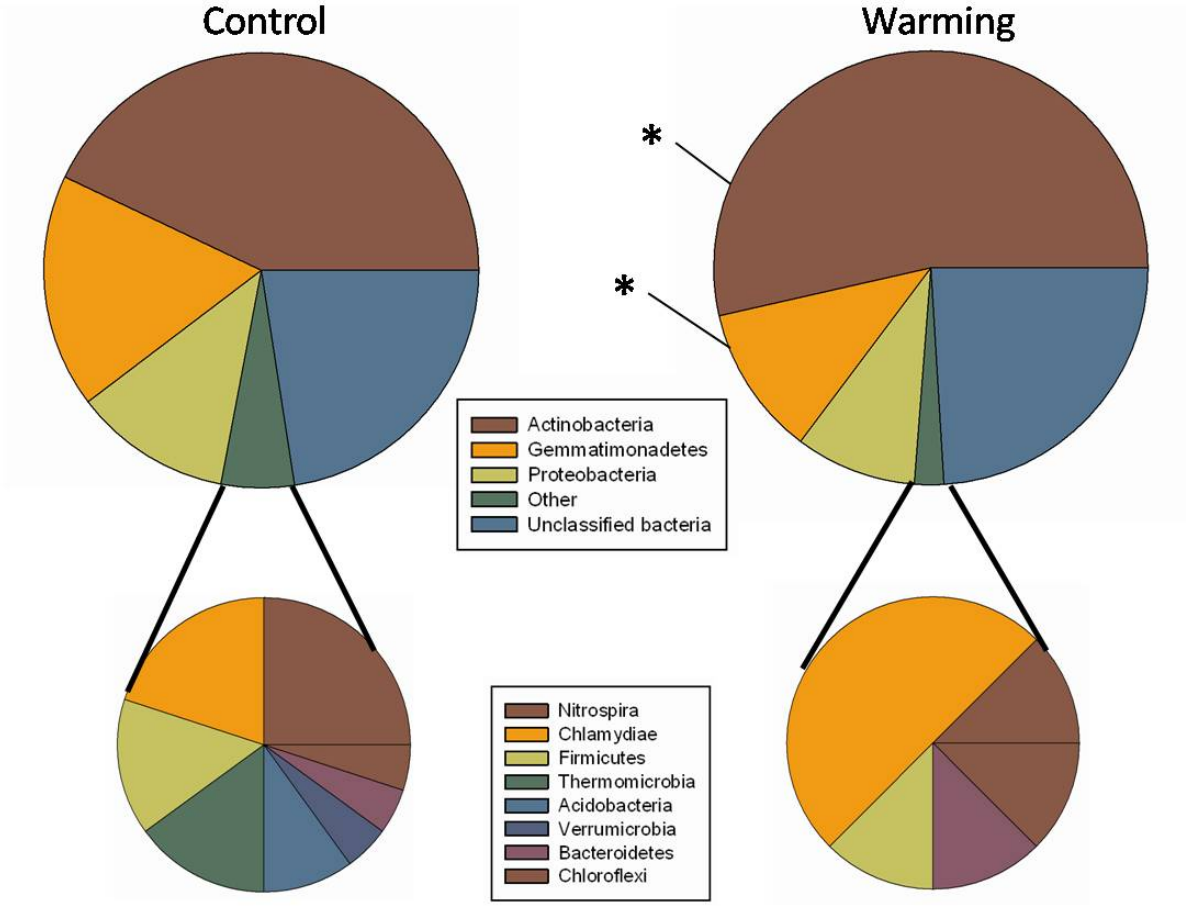


Figure 4.5: Proportion of clones in A) Organic soils, and B) mineral soils; in warming and control treatment libraries affiliated with the bacterial phyla. Results are based on naïve Baysian classification of 16S rRNA gene sequences according to the RDP Green Genes database. Asterisks indicate phyla that significantly differ between control and warming treatment.

At the class level (Figure 4.6) warming led to a significant reduction in the frequency of clones affiliated with the Beta-proteobacteria in the organic layer (Chi^2= 6.722; p=0.0095) (Figure 4.6). Likewise, in mineral soil libraries, warming led to a significant reduction in the Beta-proteobacteria (Chi^2= 6.722; p=0.0095), and also in the Actinobacteridae (Chi^2= 4.306; p=0.038), while the Rubrobacteriadae increased in frequency (Chi^2= 5.684; p=0.0017). Unifrac lineage specific analysis by environment of the Neighbour-Joining tree confirmed these differences were significant. These changes corresponded to a 22% decline in bacterial richness (number of taxa) at the class level, and were associated with an overall reduction in the diversity (Shannon and Simpson's index) of clone libraries based on the bacterial sequences that could be identified to phylum or class (Table 4.5).

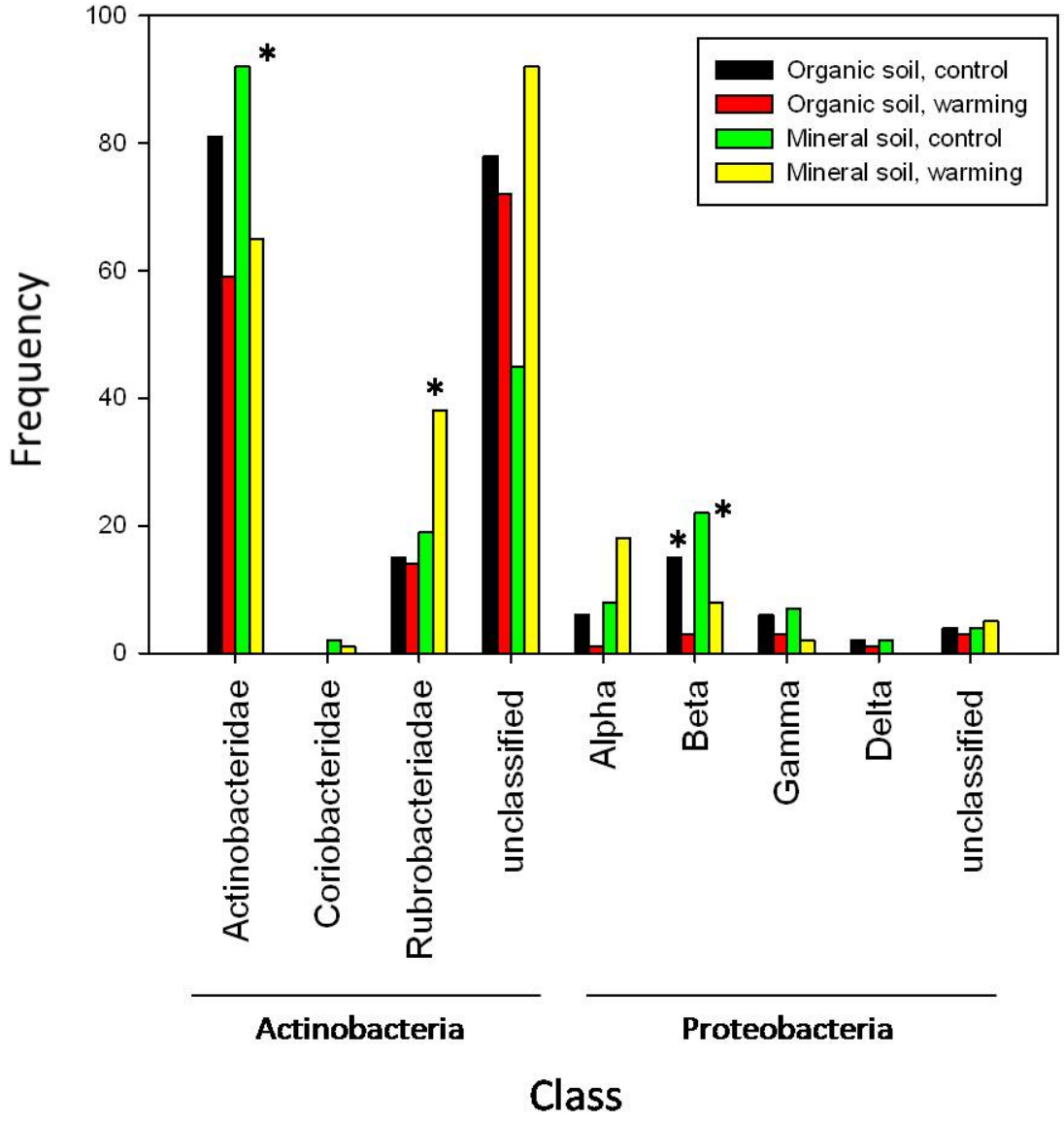


Figure 4.6: Frequency of clones affiliated with bacterial classes in the phyla Actinobacteria and Proteobacteria for clone libraries from warming and control treatment plots. Results are based on naïve Baysian classification of 16S rRNA gene sequences according to the RDP database. Significant treatment effects ($p<0.05$) are denoted by asterisks, as determined by Chi^2 tests with Yates corrections.

Table 4.5: Diversity indices for bacterial clone libraries from organic and mineral soil in warmed and control plots. Only sequences that could be identified to bacterial phylum, or to class in the Actinobacteria and Proteobacteria, are included.

Library	N	Diversity Index	
		Shannon's	Simpson's
Organic - Control	228	1.68	0.750
Organic - Warming	190	1.51	0.730
Mineral - Control	285	2.01	0.810
Mineral - Warming	278	1.81	0.793

As for bacteria, Unifrac analysis of fungal clone libraries by environment revealed that all pairwise comparisons between libraries were statistically significant at $\alpha=0.05$, suggesting that each soil environment contained a fungal community with a distinct composition and structure. The greatest differences in fungal community structure occurred between the mineral layer of control plots and either of the organic layers, while the mineral layers in the warming and control treatments were the most similar (Table 4.6).

Table 4.6: Unifrac distance matrix for pairwise comparisons between of fungal ITS clone libraries. All pairwise comparisons are significant at α=0.05, as determined by the Unifrac significance test and the P-test, both corrected for the number of pairwise comparisons. Unifrac distances are coloured according to the quartiles of distance each pairwise comparison accounts for with increasing shades of grey corresponding to increasing quartile distance: 0-25%; 25-50%; 50-75%; 75-100%.

	Control Organic	Control Mineral	Warming Organic	Warming Mineral
Control Organic	0	0.892	0.853	0.852
Control Mineral		0	0.869	0.745
Warming Organic			0	0.816
Warming Mineral				0

Unlike bacteria clone libraries, patterns in the frequency of clones affiliated with different fungal phylogenetic groups did not change significantly between organic and mineral layers with treatment. As a result, we pooled fungal sequence data from clone libraries of organic and mineral soil and present only differences due to the warming treatment. At the phylum level, clone libraries of fungal ITS region genes did not differ in abundance between the control and warming treatment (Figure 4.7). Libraries were dominated by sequences affiliated with the Ascomycota, with this group comprising almost two thirds of the clones in the warming treatment. The Basiomycota represented one quarter to one third of the composition of the clone libraries, and the Glomeromycota and the Basal Fungal Lineages of uncertain placement representing approximately 1 and 5% of the sequences, respectively. Sequences that grouped together, but were only affiliated with uncultured fungi from environmental samples and thus could not be classified to the level of phylum, represented 3.9% of the sequences in the library. Sequences affiliated with the Zygomycota did occur in the libraries, but these sequences only occurred as singletons and are not shown in Figure 4.7.

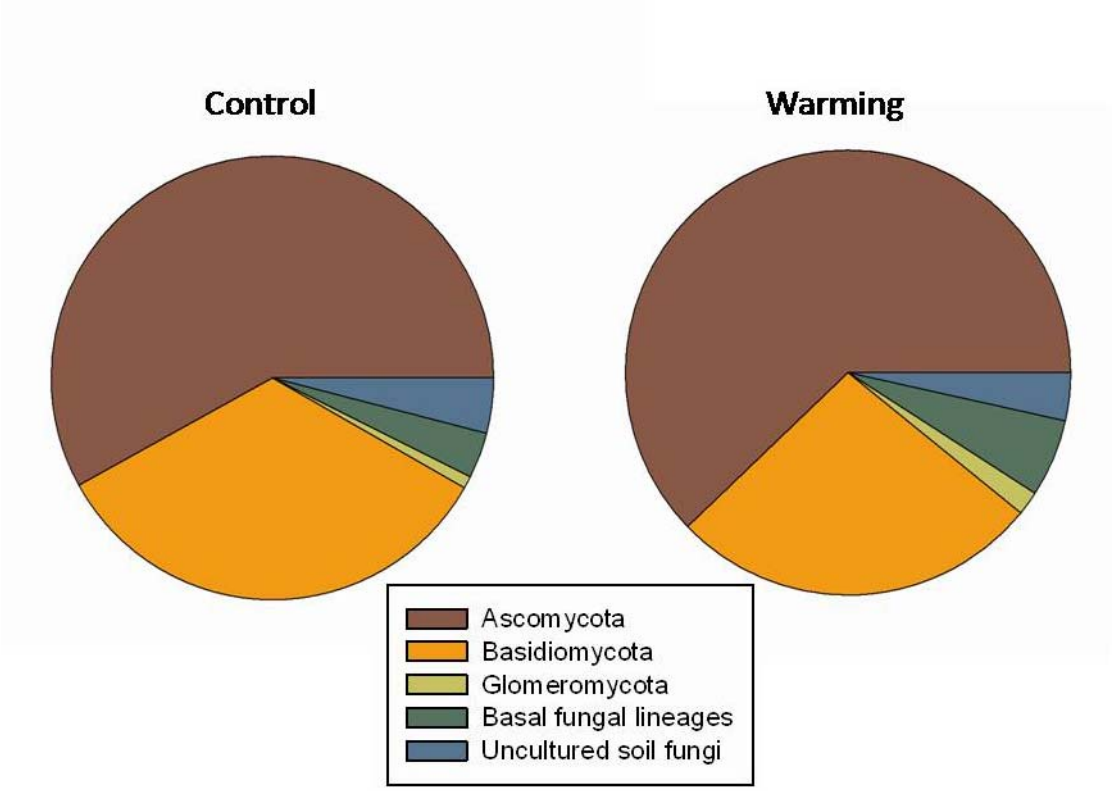


Figure 4.7: Proportion of clones in warming and control treatment libraries affiliated with the fungal phyla. Results are based on BLAST searches of internal transcribed spacer (ITS) sequences.

When fungal orders within the Ascomycota and the Basidiomycota were compared for clone libraries between control and warming treatments, we found that warming was associated with a significant reduction in the frequency of clones affiliated with the Agaricales (Chi^2= 70.92; p«0.00001) and the Atheliales (Chi^2= 5.143; p=0.023) (Figure 4.8). A group of Ascomycota that had very high identity with other uncultured Alaskan soil fungi also decreased significantly with warming (Chi^2= 25.04; p«0.00001). Conversely, warming was associated with a significant increase in clones affiliated with the Cantharellales (Chi^2= 22.40; p«0.00001) and the largest fungal order, the Helotiales (Chi^2= 5.734; p=0.017). Unifrac lineage specific analysis by environment of the Neighbour-Joining tree confirmed that the differences in fungal order membership were significant. Warming was associated with an overall reduction in the diversity of fungal clone libraries based on fungal sequences from groups containing more than 2 members, and as supported by the decline in the number of singleton sequences recovered

from the warming treatment. This change corresponds to a 28% reduction in fungal richness at the order level (Table 4.7).

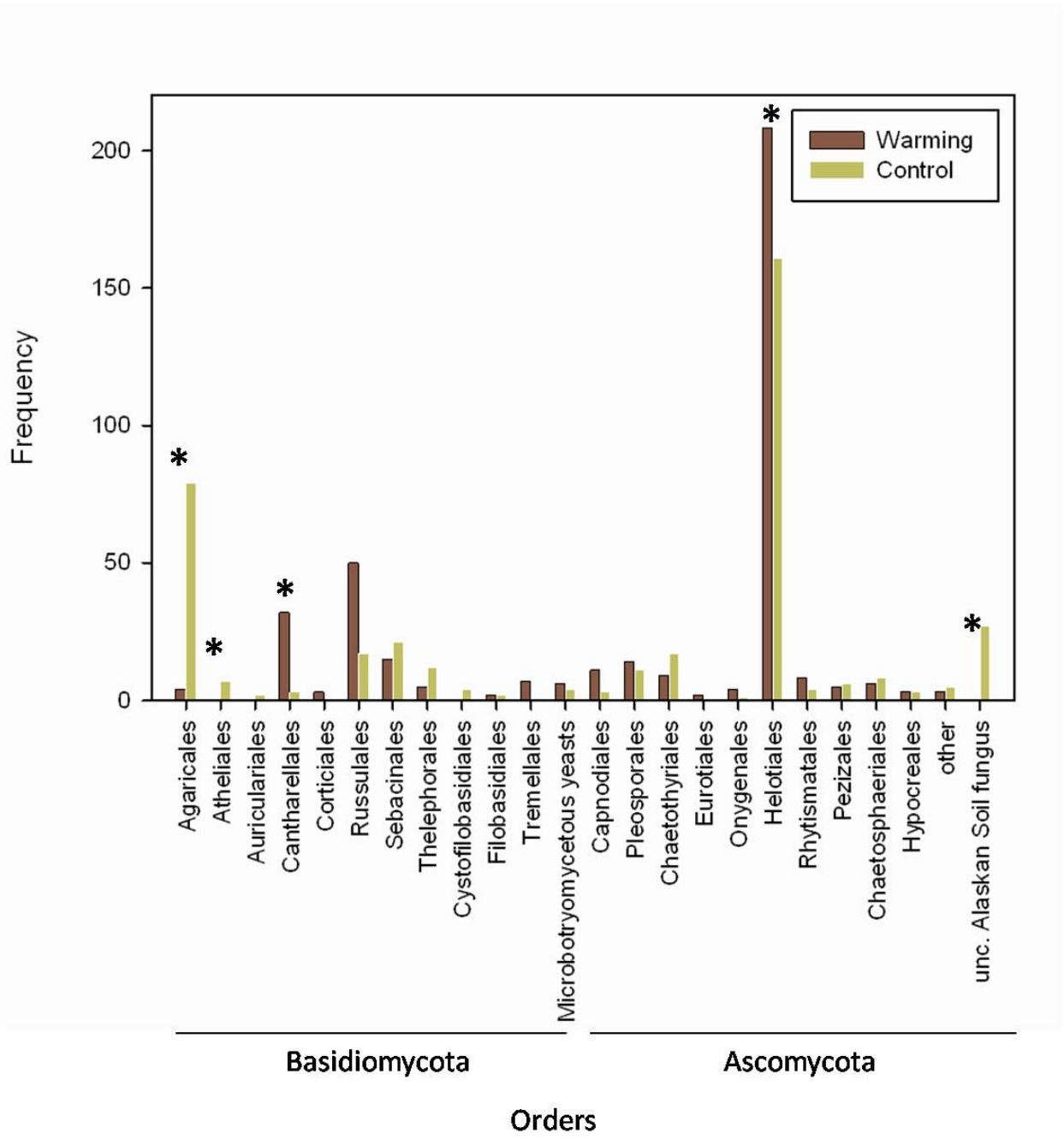


Figure 4.8: Frequency of clones affiliated with fungal orders in the phyla Basidiomycota and Ascomycota for clone libraries from warming and control treatment plots. Results are based on BLAST searches of internal transcribed spacer (ITS) sequences. Significant treatment effects ($p<0.05$) are denoted by an asterisk, as determined by Chi^2 tests with Yates corrections.

Table 4.7: Number of singleton sequences, total numbers of sequences that formed phylogenetic groups (>2 members), and diversity indices for fungal pooled clone libraries (organic + mineral horizons) from warmed and control plots. Only sequences that could be identified to fungal order are included in calculations of diversity indices.

Library	Singletons	N	Diversity Index	
			Shannon's	Simpson's
Control	47	387	1.99	0.772
Warming	38	382	1.74	0.675

Discussion

Our ARISA analysis of bacterial and fungal ITS sequences provides clear evidence that warming elicits a change in microbial community structure that far exceeds that due to normal seasonal succession within these moist Acidic tundra soils. This conclusion is further supported by our independent assessment of microbial community composition by the construction of clone libraries for warming and control soils, which revealed significant changes in bacterial and fungal communities with warming. These findings agree with work from other Arctic tundra climate change experiments. For example, Rinnan *et al.* (2007) found that warming by warming treatment caused a significant decline in the ratio of fungi: bacteria PLFAs in soils from a Swedish subarctic heath, and Deslippe *et al*. (2005) found that warming by Open-Top chamber caused a significant change in the composition of the nitrogen-fixing bacterial community, as determined by analysis of *nif*H gene sequences, in a Canadian High-Arctic tundra oasis.

Our results suggest that Arctic microbial communities are seasonally stable, agreeing with other recent work in Arctic tundras, but contrasting with alpine tundra microbial communities, which undergo strong seasonal patterns of succession (Nemergut *et al.* 2005). For example, Männistö *et al.* (2007) found that bacterial communities in Finnish fjelds, as characterized by analysis of 16S rRNA genes and PLFAs, were seasonally stable over three years of sampling. Likewise, at our own study site, Wallenstein *et al.* (2007) sampled ambient tussock tundra soils twice (August 19th, 2004 and June 6th, 2005) and found little change in the composition of clone libraries of bacterial and fungal sequences between these dates.

We found that warming effects on the structure of soil microbial communities declined with soil depth. This finding fits with the soil temperature data collected at the study site, which indicates a declining difference in the mean warming-induced increase in soil temperature with depth. While warming treatments raised mean soil temperature by 1.33^{o}C at 10 cm depth, this increase was reduced to only 0.96^{o}C at 40 cm depth. The declining effect of warming on soil microbial communities over depth has also been noted by others (Rinnan *et al.* 2007).

Our ARISA data revealed that the warming induced shift in fungal communitity structure occurred at a greater spatial scale than that observed for bacteria. This finding implies that the fungi that responded most strongly to warming had relatively large genet sizes. The ARISA data also suggest that unicellular fungi, such as yeasts, changed less with warming than did fungi that form extensive mycelial networks, as is the case for many mycorrhizal genera (Selosse *et al*. 2006, Fricker *et al*. 2008). Indeed, our analysis of fungal clone libraries supported this observation, with no significant changes observed in any of the orders dominated by yeasts: the Cytofilobasidiales, Filobasidiles, the Microbotryomycetous yeasts, the Capnodiales, Eurotiales, Onygenales, Pleosporales, or the Chaetothryriales; but with significant changes in the abundance of clones affiliated with several known mycorrhizal orders: the Agaricales, Cantharellales, and the Helotiales. Further, there were noticeably more Russulales and members of the Glomeromycota in the clone libraries constructed for warmed soils, although these changes were not statistically significant at $\alpha=0.05$. The two plant species that dominated the warmed plots were *Betula nana* and *Rubus chamaemorus,* which are known to form mycorrhizae with members of the Russulales and Glomeromycota, respectively (Molina *et al*. 1992).

The observation that the percent variance in community structure explained by warming was similar among bacteria and fungi in the mineral horizon, but was much greater for bacteria than fungi in the organic horizon, suggests that fungal community structure in the organic soil horizon was influenced more strongly by factors that may be indirectly related to soil temperature. In Moist Acidic Tundra near Toolik Lake, only roots of the non-mycorrhizal sedge, *Eriophorum vaginatum,* regularly penetrate into mineral soils. Roots of mycorrhizal plants are largely confined to the organic horizon. Given the larger spatial scale of structure in fungal communities, it seems reasonable that fungal response to warming treatment in the organic horizon were mediated by the presence of plant roots. Plant roots, may influence the structure of soil fungal communities directly; by acting as hosts for mutualistic or parasitic fungal species, or by supplying labile-C substrates through rhizodeposition or turnover processes; or indirectly, through secondary rhizosphere effects such as an increased abundance of fungal-feeding nematodes, or through their effects on soil structure, including soil aggregation and aeration.

Analysis of the clone libraries revealed a soil bacterial community composition quite different than that reported previously for the same Moist Acidic tundra site (Wallenstein *et al.* 2007); the most striking difference is the relative abundances of Actinobacteria and Acidobacteria. In our study, sequences affiliated with the Actinobacteria made up 40-55% of the four clone libraries, the previous study found less than 5% Actinobacterial sequences from tussock tundra soils. Conversely, we found Acidobacteria-affiliated sequences to comprise less than 1% of our clone libraries while the previous study reported 30% of sequences belonging to this group. The reasons for this discrepancy are uncertain, but several explanations, or a combination of these, are possible.

Firstly, the different primer sets employed by the two studies could explain the differences in the relative abundance of these two groups. We used the forward primer 1073F (Garcia-Martinez *et al.* 1999) and the reverse primer 115R (Normand *et al.* 1996) while Wallenstein *et al.* (2007) used 8F and 1492R (both, Lane 1991). It is likely that differences in primer biases contributed to differences in the two views of soil bacterial community structure. In a study of the effects of timber harvesting on forest soil microbial communities, Hartmann *et al*. (2009) employed the same bacterial primer set as we did, and likewise, report a predominance of the Actinobacteria, Gemmatimonadetes and the Proteobacteria. Further Hartmann *et al.* (2009) noted that several bacterial phyla that are typically common in soils, such as the *Acidobacteria*, *Chloroflexi*, *Firmicutes*, *Nitrospira*, and *Verrucomicrobia,* each made up less than 1% of the sequences in their libraries, a result that closely matches our own. Alternatively, a second possible reason for the observed differences may be that different DNA extraction techniques were utilized. Actinobacteria are Gram-positive cells that may be difficult to lyse during DNA extraction, whereas Acidobacteria are Gram-negative cells (Kishimoto *et al.* 1991, Barns *et al.* 1999, Eichorst *et al.* 2007); if the Mobio kit used by Wallenstein *et al.* (2007) lysed bacterial cell walls less effectively than the modified Griffiths protocol we employed, we would expect that this could result in an underestimation of Actinobacteria in their study. Finally, it is possible that differences in the clone libraries reflect a directional shift in microbial community composition with warming towards a more ‘shrub influenced’ microbial community. In addition to tussock tundra soil, Wallenstein *et al.* (2007) report on clone libraries constructed for shrub tundra soil. In shrub tundra libraries, there was a marked reduction in abundance of Acidobacteria affiliated sequences (4-12%) and a marked increase in the number of Actinobacterial affiliated sequences (up to 10%) compared with tussock tundra libraries. In our study, warming strongly

increased the dominance of *Betula nana* (Chapin *et al*. 1995, Bret-Harte *et al.* 2001, van Wijk *et al.* 2004), which is a major component of the shrub tundra ecosystem studied by Wallenstein *et al.* (2007). It is possible that the presence of *Betula nana* favours Actinobacteria, while selecting against Acidobacteria. However, given the discrepancy among clone libraries of our control soils and those reported by Wallenstein *et al.* (2007) for tussock tundra soils, the latter explanation is the least compelling.

The UniFrac distances we observed between paired clone libraries of warmed versus control treatments in both organic and mineral horizons clearly indicated distinct bacterial community structures. Interestingly, the strongest difference in bacterial community structure was among the two organic soils, suggesting that warming treatment resulted in a shift in bacterial community structure exceeding the difference due to organic vs. mineral soil envirnments. Our analysis of the clone libraries further revealed a shift in community structure toward dominant groups, and the absence of clones from less abundant groups. This change led to decreased community diversity and a 22% reduction in bacterial richness at the class level.

A comparison of the reduction in library diversity with warming, as measured by Shannon's and Simpson's diversity indices leads to important observations about how the bacteria community changes with warming in the two soil horizons. With warming treatment, Shannon's diversity in the library constructed for both organic and mineral soil horizons declined by 10%, however, Simpson's diversity was reduced by 3% and 2% in the organic and mineral soil horizons, respectively. Thus, it would appear a decrease in genotype evenness, as affected through an increase in dominant classes of bacteria, was more important than the 22% decline in species richness, in driving the overall decline in genotype diversity with warming treatment.

Warming was also associated with significant changes in the structure of bacterial communities at the phylum and sub-phylum levels, as indicated by Unifrac analyses and Chi^2-tests. For instance, there was a significant decline in the abundance of the Beta-Proteobacteria with warming in both organic and mineral horizons. Beta-proteobacteria have been shown to be positively correlated to C-mineralization rates across broad geographic ranges, and to increase

in abundance in sucrose-amended soils (Fierer *et al*. 2007). We thus expected the opposite result, given that (i) shrub-dominated tundra generally supports higher rates of C-mineralization than tussock tundra (Weintraub and Schimel 2003), (ii) our warmed plots supported higher shrub biomass (Chapin *et al*. 1995), (iii) and C-mineralization rates have been shown to increase with soil warming (Luo *et al.* 2001, Melillo *et al.* 2002, Schmidt *et al.* 2002, Shaver *et al*. 2006). Interestingly, however, in alpine tundra, Beta-proteobacteria reach their highest abundance in summer (Lipson and Schmidt 2004) when plants are active and litter decomposition slows (Nemergut *et al.* 2005 and references therein). Many Beta-proteobacteria are known to be important components of plant rhizospheres as root exudate fermenters and nitrogen fixers, and this pattern appears to hold true for Arctic tundra plants (Deslippe and Egger 2006). Experimental warming treatments have been associated with elevated C:N ratios in arctic dwarf-shrubs when nutrients constrain growth (Tolvanen and Henry 2001), and this pattern holds true for *Betula nana* at this site (Welker *et al.* 2005). Thus, the significant reduction of Beta-proteobacteria in warmed soils observed in this study may reflect a reduction in rhizosphere adapted-organisms relative to those specialized in the degradation of deciduous leaf litter of relatively low substrate quality.

In contrast to our bacterial clone libraries, our fungal clone libraries were fairly similar to those reported by Wallenstein *et al.* (2007). Both studies report dominance of sequences affiliated with the Ascomycota and the Basidiomycota. Within the Ascomycota the dominance of the Helotiales (*NB*. class Leomycetes in Wallenstein *et al*. 2007), is clear. Although the Basidiomycota were less abundant in our clone libraries than in the previous study, we a report higher diversity of basidiomycetous orders. Nonetheless, the two studies share commonalities among the orders. For example, sequences affiliated with the Agaricales and Cantharellales were reasonably abundant in both studies.

In addition to the ARISA and clone library data suggesting a plant-mediated response of the fungal community to warming, the significant decrease in the number of clones affiliated with the Atheliales also supports this idea. Although not mycorrhizal, many members of the Atheliales are important lichenized species, and others are major lichen parasites (Kirk *et al.* 2001). The decline of lichens in response to warming at this study site is well known (Chapin *et al.* 1995, Van Wilk et 2003). Lichen decline is attributed to the large increase in litter input from

the significant biomass increase of the two dominant deciduous species at the site, *Betula nana* and *Rubus chamaemorus*. Thus, the significant decline in abundance of Atheliales-affiliated sequences in our clone libraries of warmed soils may reflect a second plant-mediated response of the fungal community to warming.

As with our bacterial clone libraries, analysis of our fungal clone libraries strongly suggests a reduction in fungal diversity with warming. This corresponded to a 28% decline in fungal richness at the order and sub-order level, and a 20% decline in the number of singleton sequences. Interestingly, this change led to equal reductions in fungal diversity with warming, as measured by Shannon's and Simpson's diversity indices (both 13%). Given that Shannon's index emphasizes evenness while Simpson's index emphasizes richness, it appears that the decline in diversity in fungal communities due to warming is driven equally by an increase in dominant fungal orders and by the reduction or disappearance of fungi affiliated with rare orders. Thus, our study suggests that, in concordance with plant species richness, which declined by one third in response to 9 years of warming treatment at this site (Chapin *et al.* 1995), microbial community diversity will decline as climate continues to warm. Further, patterns in our data suggest that this change will, at least in part, be mediated by changes in the plant community.

Conclusions

Here we show that 18 years of warming by greenhouses led to significant changes in the structure of soil bacterial and fungal communities in Low-Arctic tussock tundra that far exceed those due to normal seasonal variation. Greenhouse-treated microbial communities were characterized by a strong increase in bacteria and fungi affiliated with dominant groups, and a decline or disappearance of rare groups. This change led to a 22% and 28% reduction in community richness for bacteria and fungi, respectively, and a corresponding reduction in community diversity for both groups. These changes are in accordance with similar reductions in plant community richness with warming at this site and suggest that future climate change will reduce community diversity in Arctic tundra on decadal timescales. To our knowledge, our study is the first to report that warming leads to a reduction in the diversity of soil microbial communities.

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5 Stable isotope probing of shrubs provides insights to C-cycling in Low-Arctic tundra[1]

Introduction

Globally, northern soils account for nearly twice as much C as is currently present in the atmosphere (Schuur *et al.* 2009 and references therein), and respiration of this C may lead to CO_2 emissions several times greater than those generated though the anthropogenic burning of fossil fuels (Schimel 1995). C exchange between the soil and atmosphere is dynamic, with an estimated exchange of 60 Pg C yr^{-1}among soil and atmospheric pools (Filley and Boutton 2006). Of recently respired soil C, approximately half originates from living plant roots, their mycorrhizal fungi, and other root-associated microorganisms, and is fueled directly by recent photosynthesis (Hogberg and Read 2006), emphasizing the dynamism of the plant-soil continuum. Plant C flux to the rhizosphere is a critical process that couples plant and microbial productivity in terrestrial ecosystems. Yet, despite its importance, it remains among the least understood aspects of the global carbon cycle (Staddon *et al*. 2003, Trumbore 2006, Paterson *et al*. 2008, 2009).

Recent methodological innovations, including the application of stable and radioactive C isotopes in field settings, have provided terrestrial ecologists unprecedented insight to the role of the microbial community in cycling plant C. These studies have revealed that the fate of newly photosynthesized C is of critical importance to its residence time in soils. For example, analysis of the ^{14}C residence time of an arbuscular mycorrhizal (AM) mycelium suggested a mean life span of only 4-5 days (Staddon *et al*. 2003), agreeing with findings in a grassland indicating an initial acquisition of plant C by AM fungi and a subsequent dissipation into the rhizosphere bacteria by Stable Isotope Probing (SIP) of microbial phospholipid fatty acids (PLFA-SIP) (Olsson and Johnson 2005). By contrast, it is becoming increasingly clear that fine roots of ECM plants represent a much more stable C sink than previously assumed (Högberg and Read 2006, Högberg *et al*. 2008) and while C in microbial cytoplasm had a half-life of <12 days, C in ectomycorrhizal (ECM) root tips turned over much more slowly, in a $^{13}CO_2$ pulse-chase experiment of an intact sapling pine stand in Sweden (Högberg *et al.* 2008).

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Mycorrhizal type appears to greatly influence the longevity of plant-derived C in soils. Differences in morphology and chemistry suggest that ECM and ericoid mycorrhizal (ERM) hyphae may be more recalcitrant than AM hyphae (Langley and Hungate 2003). ECM and ERM fungal hyphae are comprised of complex C compounds, that are recycled slowly and are degraded only by a subset of the microbial community (de Boer *et al.* 2006), and melanized cell wall materials may be precursors to stable soil organic matter and recalcitrant soil N (Clemmensen *et al.* 2006 and references therein). The presence of mycorrhizal fungal biomass may further slow C-cycling in terrestrial ecosystems if hyphae are produced at the expense of rapidly decomposing root exudates (Langley and Hungate 2003). Indeed, the presence of ECM fungi in sandy soil has been associated with lower bacterial numbers and reduced rates of bacterial activity in microcosm experiments (Olsson *et al.* 1996). The issue may be further complicated by morphological differences among ECM fungal species, as ECM with well developed mantles release fewer soluble root exudates than those with underdeveloped mantles (Priha *et al.* 1999), leading to a need for high taxonomic resolution of the fate of plant-C.

In Moist Acidic Tundra (MAT) plant growth is tightly constrained by the acquisition of soil N (Chapin and Shaver 1985a, Shaver and Chapin 1980, 1986, Shaver and Kummerow 1992), and the plant community exists by partitioning the soil N pool (McKane *et al.* 2002). Consequently, plants that form ECM, ERM, and AM or are non-mycorrhizal, are intermingled in relatively equal abundance (Shaver and Chapin 1991). Here dominant tundra plants are typically slow-growing perennials that spread by cloning and allocate a high proportion of photosythate C below-ground to rhizomes, roots, (Chapin *et al.* 1980) and mycorrhizal structures (Hobbie and Hobbie 2006). This high allocation of C belowground suggests that tundra plants may be good candidates for studies that attempt to link the fate of plant C to specific microbes in soils through the use of SIP methods, as these studies can be limited by insufficient incorporation of labeled substrates into labeled biomarkers (Griffiths *et al.* 2004, Neufeld *et al.* 2007a).

In this study, we used stable-isotope probing of phospholipid fatty acids (PLFA-SIP) and DNA (DNA-SIP) to examine the fate of root exudates from tundra plants in the field. We hypothesized that C acquisition patterns of rhizosphere organisms are a function of plant mycorrhizal type, and we selected plant species based on their mycorrhizal strategies: *Betula nana* (ectomycorrhizal), *Salix pulchra* (ecto- and arbuscular mycorrhizal), and *Ledum palustre* (ericoid mycorrhizal). Given that C turnover in ECM and ERM hyphae is typically slower than for AM hyphae, we predicted that AM biomarkers would be labeled sooner, with more ^{13}C appearing in ECM and ERM fungi over time. Further, we predicted that the pattern of C-allocation observed in AM species, characterized by the movement of C from the plant to mycorrhizal fungi, and then to soil bacteria (Olssen and Johnson 2005), would not hold true for ECM and ERM plants in Arctic tundra.

Methods

Study site

The study site was located on a gentle (<5^{o}) north-facing slope in Moist Acidic Tundra near Toolik Lake, Alaska, USA (68^{o}38'N, 149^{o}34'W, elevation 780m). Moist Acidic Tundra forms on old glacial surfaces (>11,000 years BP) and supports 'heathland'-type tundra plant communities. Regular patterns of vegetation occur in this plant community as a result of the perennial, rhizomatous growth of the tussock-forming sedge *Eriophorum vaginatum*. The dominant deciduous dwarf shrub, *Betula nana*, occupies hollows between the sedge-tussocks in mixture with mid-canopy Ericaceous species such as *Ledum palustre* and *Vaccinium* sp., as well as herbs. Both plurocarpus and acrocarpus mosses are common and form a continuous ground cover that can exceed 40 cm in thickness in some areas. Soils are characterized by a thick organic horizon, 20-35 cm in depth, with a pH of 4.5 (Schimidt et al. 2002). Mineral soils are essentially unweathered, pale grey in colour, permafrost affected, and show only occasional evidence of cryoturbation.

$^{13}CO_2$ labeling and soil sampling

Ten replicate *Betula nana*, *Salix pulchra* and *Ledum palustre* plants were randomly selected for $^{13}CO_2$ labeling within a total area of approximately 0.6 hectare. Because of the clonal growth form of these plants, individual ramets selected were a minimum of 3 m in distance from any other plant. On July 10 and August 11, 2007, plants were pulse labeled with 1.3-2.2 mmol $^{13}CO_2$ (99.9%; Cambridge Isotope Labs) in FEP gas sampling bags (Jensen Inert Products) fitted with silicone stoppers. CO_2 concentrations inside the labeling bags were measured approximately every 30 minutes with a portable infrared gas analyzer (Qubit Systems). Labeling bags were removed when the CO_2 concentration in the bag fell below 250 ppm (2-4 hrs). Three leaves near the apical meristem of each plant were immediately sampled for determination of plant ^{13}C-uptake. On July 11th and August 12th, 2007 after a 24-hour chase period, five replicate plants of each species were harvested. On July 17th and August 18th, 2007 after a 168-hour (7-day) chase period, five replicate plants of each species were harvested. At

each sampling, two replicate unlabelled plants per species were also harvested to act as ambient controls.

Roots of all plants extended into the dead moss layer and organic soil horizons only, with the occasional exception of deeper *Salix pulchra* roots. For *Salix pulchra* and *Betula nana*, rhizosphere soil samples were obtained by tracing individual roots from the base of the labeled stem and collecting root clusters with adhering soil particles. The fine roots of *Ledum palustre* occurred predominantly in the dead moss layer. Rhizosphere samples for this species consisted of the entire fine root system as well as adjacent partially decomposed mosses. All soil samples were placed immediately on ice, frozen to -80^{o}C within 2 hours of sampling, and remained frozen during transport to the laboratory.

PLFA-SIP

Soil samples designated for PLFA extraction were lyophilized (Labconco) for 3 days until dry and stored in airtight bags. Phospholipid fatty acid extraction was accomplished using the method described by Grayston *et al*. (2004). Briefly, lipids were extracted from 0.5 g portions of the rhizosphere soil samples using the procedure described by Frostegård *et al*. (1991). The separated fatty acid methyl esters were identified and quantified by chromatographic retention time and mass spectral comparison on a Hewlett Packard 5890 II gas chromatograph equipped with a 5972A mass selective detector (MSD II), using standard qualitative bacterial acid methyl ester mix (Supelco UK, Dorset, UK) that spanned the range of C11 to C20 compounds.

Fatty acid nomenclature followed Frostegård (1993a, b). The fatty acids, 15:0, 17:0, cy17:0, cy19:0,16:0,16:1ω7c, 16:1ω9c, i17:1ω8c, 18:1ω7c, 18:1ω7c7, 18:1ω9c, a15:0, i15:0, i16:0, a17:0 and i17:0, 10Me16:0, 10Me17:0, and 10Me18:0 were chosen to represent bacterial PLFAs (Federle 1986, Frostegård 1993a, b, Tunlid *et al.* 1989, Hill *et al.* 2000). The polyenoic, unsaturated fatty acid 18:2ω6 was used as an indicator of total fungal biomass (Federle 1986, Zelles 1999) while the monoenoic unsaturated fatty acid 16:1ω5c was used as an indicator of arbuscular mycorrhizal fungi (Olsson 1999). The cyclopropane fatty acids cy17:0, cy19:0 (Burke et al. 2003), the saturated fatty acid 16:0 (Crossman *et al.* 2005), and the monoenoic

unsaturated fatty acids 16:1ω7c, 16:1ω9c, i17:1ω8c, 18:1ω7c, 18:1ω7c7, 18:1ω9c were chosen to represent Gram-negative bacteria (Ratledge and Wilkinson 1988, Wilkinson 1988, Staddon 2004). The branched, saturated fatty acids a15:0, i15:0, i16:0, a17:0 and i17:0 (O'leary and Wilkinson 1988, Staddon 2004), as well as the methyl-branched saturated fatty acids 10Me16:0, 10Me17:0 and 10Me18:0 (Burke *et al.* 2003, Waldrop *et al.* 200, Zelles 1999), were chosen to represent Gram-positive bacteria. The saturated fatty acids 18:0 and 19:0 were chosen as general indicators of soil biomass. For each sample, ^{13}C-incorporation of individual fatty acid methyl-esters was expressed as ng ^{13}C-PLFA g^{-1} dry soil.

Statistical analysis

General Linear Models (GLM) were used to assess the effects of plant species (*Betula nana*, *Salix pulchra*, or *Ledum palustre*), month of ^{13}C-CO_2 labeling (July or August), and incubation time (24 hours or 7 days) on ^{13}C-PLFA enrichment of individual PLFAs, the ratio of fungal to Gram-negative bacterial or fungal to total bacterial ^{13}C-PLFA. GLM was also used to test the effect of microbial group on total ^{13}C-PLFA enrichment. Barlett's and Levene's tests were used to test for heteroscedascity among groups of categorical factors. Where data violated the assumptions of the GLM, non-parametric tests were used: a Wald-Wolfowitz Runs test, a Mann-Whitney U-test, and a Kolmogorov-Smirnov test were applied to factors with only two categories; a Kruskal-Wallis ANOVA and a Medians test were applied to factors with greater than two categories. Tukey's post hoc test was used to detect significant difference among groups when GLM was possible, when a Kruskal-Wallis ANOVA was necessary, differences among groups were assessed using a multiple comparisons Z-value and a multiple comparisons two-tailed p-value test. All statistical analyses were performed using STATISTICA version 8.0 software (STATISTICA).

DNA-SIP

We focused our DNA-SIP efforts on the August labeling period because the study plants were senescencing and we expected that they would be allocating relatively more carbon below

ground at this time. Two replicate plants of each species per chase period were selected for DNA-SIP (12 total).

Nucleic Acid Extraction. One-gram aliquots of the fresh-frozen rhizosphere samples were thawed at room temperature while mixing in 200 µl of hexadecyltrimethylammonium bromide (CTAB) buffer. Total nucleic acids were extracted from 0.5 g portions of the resulting paste using the method described by Griffiths *et al.* (2000). Two replicate extractions per soil sample were produced, run on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light to check extraction efficiency, before pooling to create a single sample.

Ultracentrifugation. The DNA-SIP protocol employed closely followed that described by Neufeld *et al.* (2007b) for fractionation of ethidium bromide-free CsCl gradients except that synthetic linear polyacrylamide was used rather than glycogen as a DNA co-precipitant to avoid contamination by *Acinetobacter lwoffii* (Bartram *et al.*, in press). Briefly, 1-2 µg of DNA from each sample was added to a CsCl solution (average density ~1.720 g ml^{-1}) and subjected to a 40-hour ultracentrifugation at 44,100 rpm (177,000 g_{av}), at 20^{o}C in a Vti 65.2 rotor, Beckman-Coulter (Fullerton, CA). The gradient medium was completely fractionated into 440 µl fractions using distilled water and bromophenol blue. DNA was quantitatively recovered from each fraction by precipitation with 20 µg linear polyacrylamide and polyethylene glycol (30% PEG 6000 and 1.6 M NaCl), resuspended in 30 µl sterile TE buffer.

One control tube containing an equal mixture of ^{13}C-DNA and ^{12}C-DNA were included in every ultracentrifugation run. This control was used to verify both the proper formation of the gradient, as well as to indicate the fraction containing the gradient buffer with a buoyant density at which the ^{13}C-DNA or ^{12}C-DNA could be maximally recovered (Neufeld *et al.* 2007b). We produced ^{13}C-DNA by growing *E. coli* strain DH5α with $^{13}C_6$ glucose (Cambridge Isotope Labs) as a sole C source. We produced DNA with approximately natural abundance ratios of ^{13}C:^{12}C by growing *E. coli* strain DH5α in Luria Broth. DNA was fractionated and quantitatively recovered from control tubes, as described above. Five-µl aliquots of each DNA fraction from samples and from control tubes were visualized on a 1% agarose gel. This allowed

quantification of sample DNA and identification of “heavy” and “light” DNA fractions for characterization by denaturing gradient gel electrophoresis (DGGE).

PCR and DGGE. Polymerase chain reactions (PCR) for DGGE analysis of bacteria targeted the V3 region of ribosomal small subunit genes with the bacterial specific primer 357F and the universal primer 518R (Muyzer et al. 1996). A GC clamp was added 5'-end of 357F to for use in DGGE fingerprints. PCR reactions consisted of 10 µM of each primer, 5 mM dNTPs, 1 mM $MgCl_2$, 2.5 U Qiagen Taq Polymerase, and 3 µg BSA, in 10X PCR Buffer (Qiagen) to a final volume of 50 µl. Thermocycler conditions consisted of an initial denaturing step of 94^{o}C for 10 minutes followed by 35 cycles of: denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension period of 7 minutes at 72°C. All PCR reactions were kept on ice until the thermocycler (MJ Research Inc., Reno, USA) had reached 95^{o}C in a simplified “hot start” procedure.

Similarly, PCR amplification for DGGE analysis of fungi targeted the first internal transcribed spacer (ITS) of the ribosomal rRNA gene. However, a half-nested PCR protocol was used. The primary amplification employed the forward primer ITS1F (Gardes and Bruns 1993) and the reverse primer ITS4 (White et al. 1990). PCR cocktails consisted of 50 ng genomic DNA, 20 µmol dNTPs, 10 nmol primers, 10X PCR Buffer (Qiagen), 50 µmol $MgCl_2$, 15 µg BSA, and 2.5 U of Qiagen Taq Polymerase in a final volume of 25 µl. The thermocycler program consisted of an initial denaturing temperature of 94°C for 5 minutes followed by 35 cycles of: denaturing at 94°C for 1 min, annealing at 55°C for 50 s, and extension at 72°C for 50 s, and a final extension period of 7 minutes at 72°C. The half-nested amplification employed the same forward primer, but with the addition of a GC-clamp at the 5'-end (GC-ITS1F), and the reverse primer ITS2 (White et al. 1990). PCR cocktails for the nested amplifications were similar to those used in the primary amplifications with the exception that 3.75 U of Qiagen Taq Polymerase, and 0.5 µl of template from the primary amplifications were added to a final reaction volume of 50 µl. Thermocycler conditions consisted of an initial denaturing temperature of 94°C for 5 minutes followed by 26 cycles of: denaturing at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s, and a final extension period of 7 minutes at 72°C. Five-

µl aliquots of all PCR products were run on 1% agarose gels and stained with ethidium bromide. DNA yields were quantified under UV light using Alpha Imager software.

DGGE gels were made of 8% polyacrylamide with a 30% to 70% denaturing gradient (100% denaturant is 7.0 M urea and 40% deionized formamide). 100-110 ng of DNA were added to each lane and run at 85 V for 14 hours on a C.B.S. Scientific DGGE System according to the manufacturer's instructions. DGGE gels were then stained with SYBR Green I (Invitrogen) for 1 hour and 15 minutes, rinsed in deionized water, and visualized using a Typhoon Imager using the 100 µm lazer power set to 500 V. DGGE bands were excised from gels under UV light and reamplified using the PCR conditions described above. PCR products were cleaned using a Qiagen MiniElute PCR purification kit, and eluted in deionized water.

Sequencing and Phylogenetic Analysis. Bacterial and most fungal PCR products were sequenced directly by cycle sequencing using their forward primers (without GC-clamp) and the BigDye Terminator v3.1 Cycle Sequencing Kit and run on an AB3130xl Genetic Analyzer (Applied Biosystems). The PCR products from DGGE bands that failed to produce clean sequence reads were cloned using the TOPO-II PCR cloning Kit (Invitrogen) with blue-white screening. Prior to ligation to the pCR®2.1 vector PCR products were 'A-tailed'. 'A-tailing' reactions consisted of 10X PCR Buffer, 20 µmol $MgCl_2$, 2 µmol dATP, and 1U of Taq DNA polymerase (Fermentas) to a final volume of 10 µl, which was added to 20 µl of the PCR product, and subjected to a 30-minute elongation step at 72^{o}C. Eight white colonies per cloning reaction were selected and placed directly into PCR cocktails for reamplification of the fungal sequence and flanking vector regions using the primers M13F and M13R. PCR cocktails were the same as those described for bacterial DGGE, above. PCR products were then cleaned using a Qiagen MiniElute PCR purification kit, and eluted in deionized water. Sequencing of these PCR products was accomplished using the M13F primer and the BigDye Terminator v3.1 Cycle Sequencing Kit run on an Applied Biosystems 3730S capillary sequencer.

Sequence reads were manually checked for base-calling errors, and trimmed of the vector sequence using Vector NTI 10.3 (Invitrogen). Phylogenetic affiliations of fungal ITS

sequences were determined using the BLASTn nucleotide-nucleotide search function in GenBank (http://www.ncbi.nlm.nih.gov).

Results

Three-way ANOVAs on the ^{13}C-enrichment data of specific PLFAs revealed no significant effects of plant species (*Betula nana*, *Salix pulchra*, or *Ledum palustre*) or the month of ^{13}C-CO_2 labeling (July or August) on the uptake of carbon by different members of the soil microbial community. However, 16:1ω7c was significantly more enriched in ^{13}C after 7 days than after 24 hours ($p= 0.04795$), indicating that Gram-negative bacteria became significantly more enriched over time. Similarly, both a Mann-Whitney U-test, and a Kolmogorov-Smirnov test revealed that 18:2ω6,9 was significantly more enriched at 7 days than at 24 hours ($p=0.00027$, and $p<0.001$, respectively), indicating that fungal biomass also became more enriched over time. Interestingly, when the ratio of fungal to Gram-negative bacterial biomarkers was considered, a one-way ANOVA for incubation time revealed a trend toward increasing enrichment of the fungal biomarker relative to the Gram-negative bacterial biomarkers with time ($p=0.05431$). Further, the ratio of fungal to total bacterial biomarkers was significantly greater after 7 days than after 24 hours ($p=0.04566$). However there was no corresponding change in the ratio of Gram-negative bacterial to Gram-positive bacterial ^{13}C-enrichment over time ($p=0.2495$).

When all PLFAs were considered simultaneously, a Kruskal-Wallis ANOVA of total ^{13}C-PLFA enrichment per sample revealed a highly significant ($p=0.00021$) effect of the microbial group acquiring the ^{13}C at 24 hours and 7 days, for all samples (Figure 5.1). Post-hoc testing by multiple comparison p-values and multiple comparison z-values, revealed a strong pattern of carbon acquisition by Gram-negative bacteria, with the fungal biomarker 18:2ω6 showing approximately one-quarter and one-half the enrichment of the Gram-negative bacterial biomarkers, at 24 hours and 7 days, respectively. Figure 5.1 shows ^{13}C-PLFA values by microbial indicator for rhizosphere samples after 24 hours and after 7 days. Although ^{13}C-enrichment of the fungal PLFA was an order of magnitude greater than the enrichment of biomarkers general to all biomass after a 7-day chase period, and two orders of magnitude greater than those specific to Gram-positive bacteria, these means were not statistically different due to the high variability of enrichment in the fungal biomarker, and the relatively low power of the non-parametric tests. The ^{13}C-PLFA incorporation of the total bacterial community (TB) was not significantly different from the value for Gram-negative bacteria, indicating that

Gram-negative bacteria were responsible for the vast majority of the ^{13}C-acquisition by rhizosphere bacteria in our study.

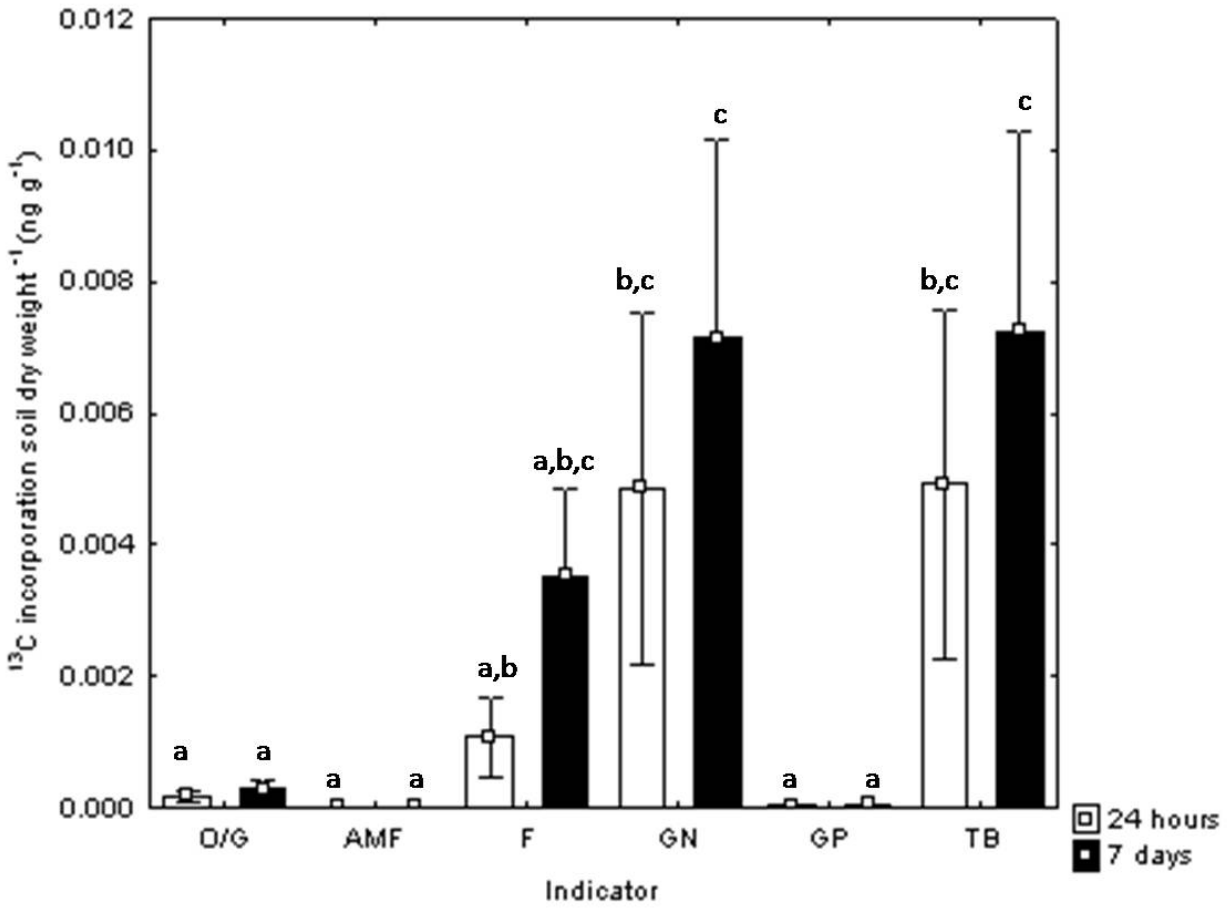

Figure 5.1: Total ^{13}C-incorporation into fatty acids representative of microbial groups in the rhizosphere of three Arctic tundra shrubs, expressed per gram of soil dry weight: O/G= other/ general biomarkers; AMF= arbuscular mycorrhizal fungi; F= fungi; GN= Gram-negative bacteria; GP= Gram positive bacteria; TB= total bacterial biomass. Values are means ± one standard error (N=53) means that share the same letter were not significantly different as determined by a multiple comparison p-values test and by multiple comparison z-values test.

DNA-SIP

DNA-SIP of bacteria from August samples clearly indicate ^{13}C-enrichment in two bacterial phylotypes (Figure 5.2). Phylotype 1, highlighted in red, was common to all plant rhizospheres and showed the strongest enrichment. Phylotype 2, highlighted in green, was less commonly present in DGGE profiles, and showed highest enrichment in *Betula nana* and *Ledum palustre* profiles, appearing enriched only in a single *Salix pulchra* DGGE profile. Phylotype 1 and phylotype 2 were gel-extracted and re-amplified and will be sequenced in Josh Neufeld's lab at the University of Waterloo, Waterloo, Canada imminently.

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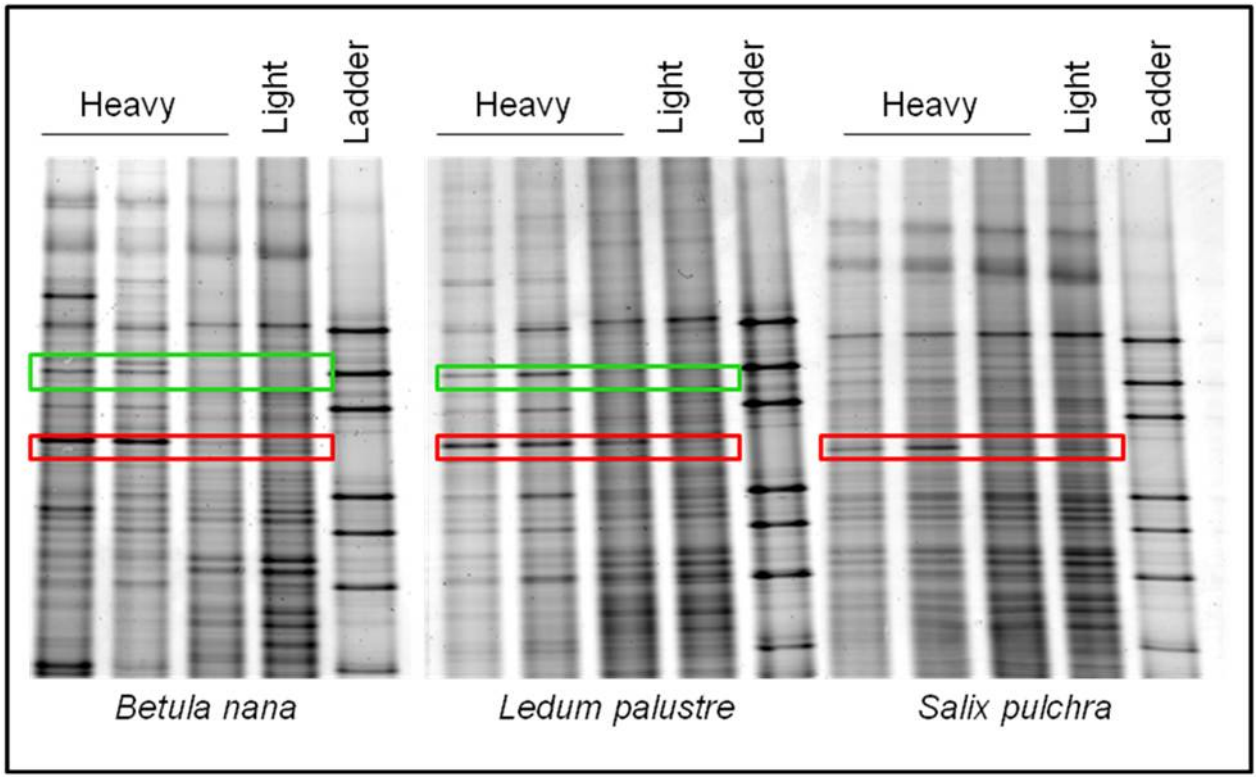

Figure 5.2: Denaturing gradient gel electrophoresis (DGGE) for bacteria from "heavy" (5,6 and 7) and "light" (10) DNA-SIP fractions, obtained by ultracentrifugation in CsCl gradients. Green and red rectangles indicate phylotypes that show ^{13}C enrichment and that are being further characterized by sequencing.

In contrast to the strong enrichment observed in all DNA-SIP DGGE gels for bacteria, fungal DGGE revealed enrichment of only one sample each of *Betula nana* and *Ledum palustre*, and two samples of *Salix pulchra*, with lower enrichment of individual fungal phylotypes. For *Betula nana* and *Ledum palustre* the samples that showed fungal enrichment were from the 7 day sampling, while the enriched *Salix pulchra* samples were from the rhizospheres collected after 24 hours. Fungal DGGE patterns were also more species specific than bacterial DGGE patterns, indicating more host specification among fungal than among bacterial communities. Figure 5.3 shows the enriched DGGE gels for each plant species (only 1 gel is shown for *Salix pulchra*). Table 5.1 summarizes the phylogenetic affiliations of all enriched fungal phylotypes.

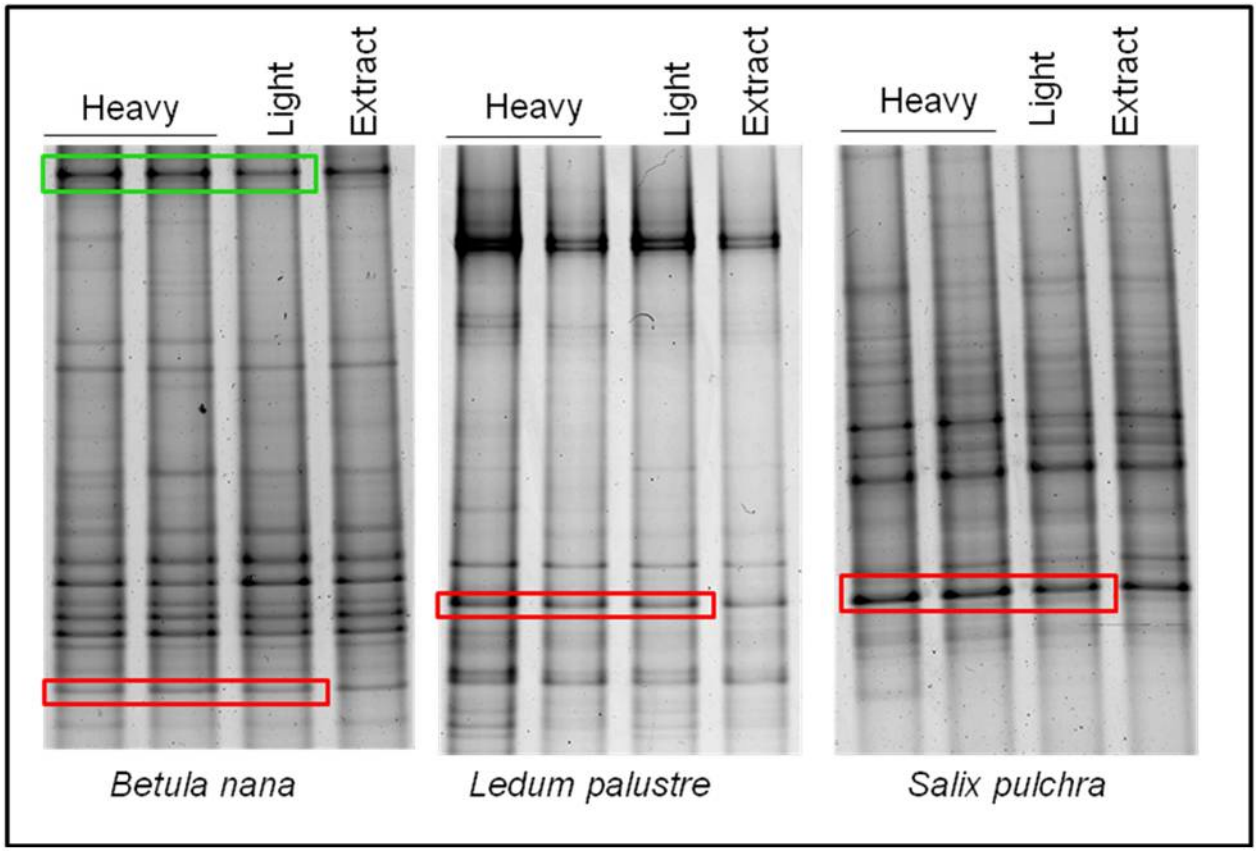

Figure 5.3: Denaturing gradient gel electrophoresis from fungi from “heavy” (6 and 7) and “light” (10) DNA fractions, obtained by ultracentrifugation in CsCl gradients, and the corresponding DNA extract (non-fractionated, [E]) showing the total fungal community fingerprint for the sample. Green and red rectangles indicate phylotypes that show ^{13}C enrichment and selected for sequencing.

Table 5.1: Phylogenetic affiliation of ^{13}C-enriched fungal phylotypes as determined by DNA-SIP.

Plant Species	Sample	Closest Genbank relative[1] (Accession #)	Identity (%)	Score	E-value	Putative phylogenetic affiliation
Betula nana	184G3	Unc. soil fungus, Boreal forest, Alaska (EF434069)	278/278 (100%)	551	10^{-156}	Agaricomycetes
Betula nana	184H1	Unc. ECM mycelium, Spruce forest, Sweden (EF521247)	191/201 (95%)	330	10^{-82}	Unknown
Salix pulchra	143B1	*Russula aeruginea* (EU819421)	298/304 (98%)	549	10^{-155}	*Russula aeruginea*
Salix pulchra	307J5	*Russula vinosa* (AJ534938)	306/308 (99%)	587	10^{-167}	*Russula vinosa*
Ledum palustre	343N3	Unc. ECM mycelium, Spruce forest, Sweden (EF521217)	272/286 (95%)	448	10^{-125}	Helotiales

[1]Unc. - uncultured

Discussion

Contrary to our expectation that C acquisition patterns of rhizosphere microbes would be a function of plant mycorrhizal type, we found little evidence for different ^{13}C-PLFA enrichment patterns in the three plant species studied. Further, we found no evidence for C allocation to AM fungi in *Salix pulchra* suggesting that in this ecosystem *Salix pulchra* may be more heavily dependent on its ECM symbionts. Overall, these data suggest similar C-allocation patterns among ECM and ERM plant species in the field, which agrees with the similar rates of ecosystem C turnover for these two plant functional types (Cornelissen *et al*. 2001).

We found that ^{13}C-enrichment was greatest in Gram-negative bacterial and fungal PLFAs as was also found in an AM grassland (Treonis *et al.* 2004) and in AM microcosms (Paterson *et al.* 2008). These patterns fit with the rapid growth rates of Gram-negative bacteria in rhizosphere soils (Paul and Clark 1996) and with the high C allocation to mycorrhizal fungi by ECM and ERC plant species (Simard *et al.* 2002, Hobbie 2006, Hobbie and Hobbie 2006). However, while we found higher ^{13}C-enrichment in Gram-negative bacteria than in fungi, the opposite was true in the grassland study (Treonis *et al.* 2004). Also, while we found increasing total ^{13}C-enrichment of fungal and Gram-negative bacterial biomarkers from 24 hours to 7 days lower total enrichment was observed in these groups at 8 days than at 4 days in the grassland (Treonis *et al.* 2004). Confirming our prediction that the patterns of C-allocation observed in other AM systems, whereby ^{13}C is initially acquired by fungi and subsequently dissipated to the rhizosphere bacteria (Olssen and Johnson 2005), would not hold true for ECM and ERM plants in Arctic tundra. These differences likely reflect ecosystem and mycorrhizal-type differences in C turnover among Arctic tundra and temperate grassland ecosystems. The slower enrichment of microbial biomarkers likely reflects lower growth rates of microbial biomass in cold tundra soils compared to those of temperate grasslands. Likewise, the slower turnover of ERM and ECM hyphae relative to AM fungal hyphae in soils (Langley and Hungate 2003), likely explains the differences in relative enrichment of Gram-negative bacterial and fungal biomarkers among the two studies, as opposed to possible lower C-allocation to ECM and ERM fungi relative to Gram-negative bacteria by plants.

DNA-SIP of bacteria supported our PLFA data, indicating that the high enrichment of two bacterial phylotyes, which we predict will be Gram-negative species, was common to the rhizosphere of all plant species. The commonality of the single most abundant phylotype was particularly striking. The highly similar bacterial DGGE profiles among replicates and among plant species suggests a very high diversity of bacterial phylotypes, as *in silico* models simulating the complexity of DGGE profiles suggest that any community with diversity exceeding 1000 units will lead to a maximum profile complexity that saturates at 35 bands (Loisel *et al.* 2006). Despite the high diversity of these communities, only one or two phylotypes acquired plant C. This suggests that in terms of primary consumption of rhizodeposits, bacteria communities may be highly specialized and that these phylotypes may represent key species in the cycling of plant C in Arctic tundra, although the identity of these organisms still needs to be confirmed.

As with DNA-SIP of bacteria, fungal DNA-SIP supported our PLFA data. Agreeing with the high error associated with enrichment of the fungal biomarker 18:2ω6, only 4 of the 12 plants sampled for DNA-SIP indicated enriched fungal phylotypes, and differences among DGGE band intensity in heavy and light density gradient fractions were smaller than those for bacteria. In addition to slower turnover of fungal biomass relative to bacteria, the lower and more variable DNA content per unit biomass of fungi relative to bacteria (Harris 1994, Leckie 2004), may have contributed to our limited ability to detect enrichment in fungal samples. Given that we observed both a greater enrichment of 18:2ω6 and a greater enrichment of fungal to bacterial biomarkers over time, we may have observed greater enrichment of fungal phylotypes if we had conducted a harvest at a later date. However, of the 4 plant rhizospheres that showed ^{13}C-enrichment in fungal DNA, those of *Ledum palustre* and *Betula nana* were collected 7 days after labeling while those of *Salix pulchra* were collected after only 24 hours, suggesting that differences in plant specific leaf area and photosynthetic rates, which are higher in *Salix pulchra* (Matthes-Sears *et al.* 1988), may have also influenced ^{13}C-DNA enrichment in fungal phylotypes.

Identification of enriched fungal phylotypes using DNA-SIP revealed that fungi that received rhizodeposits were mainly mycorrhizal species or mycorrhizal-associated. ^{13}C-enriched fungal phylotype in the rhizosphere of *Salix pulchra* were both *Russula spp. Russula spp.* are known to be an important component of the ECM community at Toolik Lake. For example, in a

clone library of ECM root tips on *Betula nana* at this site, *Russula spp*. made up approximately half of the total community. *Russula spp*. appears to be important in securing inorganic N for *Betula nana* at this site, and may predispose shrubs to outcompete neighbouring plants when climate conditions favour the increased availability of inorganic N (Deslippe *et al.* Chapter 3, this volume). Given that *Salix pulchra* has more than twice the average leaf area, higher leaf N, and higher net photosynthesis per leaf area than *Betula nana* (Matthes-Sears *et al.* 1988), and given that leaf area explains a high proportion of the observed variation in gross primary productivity in tundra landscapes, with the GPP of *Betula nana*-dominated tundra only exceeded by that of *Salix pulchra*-dominated tundra (Street *et al.* 2007), it seems reasonable that the relatively rapid ^{13}C-enrichment of *Russula spp*. in the rhizosphere of *Salix pulchra* reflects higher belowground C-allocation by this plant, which as for *Betula nana*, may be a competitive strategy to secure inorganic soil N, facilitating the expansion of this shrub onto tundra under conditions of enhanced N availability.

The two ^{13}C-enriched phylotypes associated with *Betula nana* rhizospheres were both most closely affiliated with uncultured fungi. The first was most similar to an uncultured soil fungus clone from boreal Alaska (Taylor *et al.* 2007). Among its closest Genbank matches were uncultivated members of the Agaricomycotina and the Agaricomycetes, and its most similar named Genbank entry was *Clavulina sp*. suggesting possible affiliation with the Cantharellales although this designation is not possible given the overall low similarity among the two sequences. Members of the Cantherelles are known ECM symbionts with *Betula* spp. (Molina *et al*. 1992) suggesting a mycorrhizal role for this phylotype.

The second ^{13}C-enriched phylotype associated with *Betula nana* showed sequence similarity with a single fungal clone obtained from a mesh in-growth bag buried in a phosphorus-poor spruce forest in Sweden (Hedh *et al.* 2008). Although the mesh in-growth bag method is thought to select for ECM fungi (Wallander *et al.* 2001) and clone libraries were constructed from DNA extracts of hyphae contained in each bag, this particular clone was found to have no match in genetic databases, and was considered to be a non-ectomycorrhizal sequence by the authors.

Two possible explanations may account for the occurrence of these sequences. It is possible that both sequences represent a lineage distantly related to know fungal groups. Alternatively, given that both studies used the same primer sets, both sequences may represent PCR artifacts of remarkable similarity.

The single ^{13}C-enriched fungal phylotype associated with *Ledum palustre* was most closely affiliated with another clone from a mesh in-growth bag buried in a phosphorus-poor spruce forest in Sweden (Hedh *et al.* 2008). Other top Genbank relatives were uncultivated members of the Helotiales and the Peziomycotina, or sequences from environmental samples of ERM plant root tips, suggesting a mycorrhizal role for this fungus as well. Members of the Helotiales are common mycorrhizal symbionts of other ERM plants at this site, including *Vacciunium vitis-idaea, Cassiope tetragona and Empetrum nigrum* (unpublished data Aldrich-Wolfe and Jumpponen 2009).

In contrast to the bacterial results, patterns of C-allocation to ERM and ECM fungal communities appear to be much more species specific; enrichment of fungal phylotypes differed among plant species and among replicates, and fungal DGGE patterns showed species-specific community assemblages. These data agree with the results of another study, indicating that the response of fungal communities to warming was partly mediated by changes in plant community, while no similar pattern was observed for bacteria (Deslippe *et al.* Chapter 4, this volume). Taken together, these data suggest that fungal and bacterial communities have fundamental different relationships to plants. While the factors that influence the diversity and community structure of plants and soil fungi are intimately linked, especially in the case of mycorrhizae, the factors that influence the structure and diversity of bacterial communities appear to be fundamentally different.

The fate of newly photosynthesized C is of critical importance to its residence time in soils (Högberg and Read 2006, Högberg *et al*. 2008). As *Betula nana* and *Salix pulchra* expand onto Arctic tundra, bringing with them their associated mycorrhizae, and displacing AM plants, the increased abundance ECM fungal hyphae should enhance C-storage in surface layers of tundra soils. Coupled with the increased production of wood and the higher primary production

of shrub tundra relative to tundra dominated by other plant growth forms (Street *et al.* 2007), the increase of ECM fungal biomass should help to dampen the rapid rates of C-loss that result from the thawing of frozen soils, which is enhanced, in part, by the expansion of the shrubs themselves (Sturm *et al*. 2001, Strum *et al*. 2005, Weintraub and Schimel 2005a). However, it appears that the period of time while shrub growth offsets the losses of older carbon from soils is sub-decadal, and the net effect of warming of tundra ecosystems within our lifetimes will be massive losses of carbon from tundra soils (Schuur *et al*. 2009).

It is important to acknowledge two current limitations of these data. Firstly, it would be useful to have the initial ^{13}C-enrichment values of the plant foliage so that we are assured that all plant replicates incorporated sufficient label to explain rhizosphere enrichment patterns. These data would also help us to assess plant species differences in C uptake, which may have resulted in species specific rhizosphere allocation patterns; however, since patterns of microbial ^{13}C-uptake were similar among plant species, this is of low concern. Nonetheless, the initial ^{13}C-content of plant foliage data are forthcoming. Of greater concern is our current lack of ambient DNA-SIP controls. The purpose of the ambient DNA-SIP controls is to assure us that darker bands in the heavy fractions of the DGGE gels, which indicate ^{13}C-enriched phylotypes, do not also appear sporadically in unlabelled samples. Without ambient DNA-SIP controls all DNA-SIP results are currently preliminary. However, the inclusion of the DGGE profile of the total extract (without density gradient ultracentrifugation) in the fungal profiles, assures us that at least enriched phylotypes do not appear to be over represented in the total extract. Ambient DNA-SIP controls for this experiment are currently being analyzed in Josh Neufeld's lab at the University of Waterloo, Canada.

Conclusions

Here we use both PLFA-SIP and DNA-SIP methods to examine C acquisition patterns in the rhizosphere of three dominant Arctic tundra shrubs plants that differ in their mycorrhizal status. PLFA-SIP and DNA-SIP indicated similar patterns of C-allocation to bacteria for all three plant species, characterized by the rapid enrichment of two Gram-negative phylotypes. However, while PLFA-SIP revealed increasing enrichment of rhizosphere fungi over time in all three plant species, DNA-SIP revealed plant species-specific differences in the fate of fungal ^{13}C-enrichment. *Salix pulchra* was associated with the relatively rapid enrichment of two *Russula* species, while enrichment of phylotypes most closely related to uncultivated fungi occurred after one week in *Betula nana* and *Ledum palustre* rhizospheres. Four of the five enriched fungal sequences appear to represent mycorrhizal species, while one clone associated with *Betula nana* may represent a novel fungal lineage. These conclusions are preliminary because of our current lack of ambient DNA-SIP controls. To our knowledge, this is the first study to apply SIP techniques to Arctic plants in the field. It provides unprecedented insight to the key microbial players in the carbon cycle of this Low Arctic tundra ecosystem.

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6 Conclusions

Summary

Effects of anthropogenic climate change have now been reported in all of the earth's biomes (Rosenzwieg *et al.* 2008) but Arctic regions, which sustain the highest rates of warming (Trenberth *et al.* 2007), have been particularly perturbed (Post *et al.* 2009). The stability of terrestrial Arctic ecosystems is of immediate concern because tundra soils store a disproportionally large share of the earth's C, and increased microbial decomposition of this C will feedback to climate on decadal timescales (Schuur *et al.* 2009). Soil microbes are central to the C balance of terrestrial ecosystems because of their dual role as decomposers of soil organic matter and as determinants of plant community diversity (van der Heijden *et al.* 2008), which in turn controls the quality and quantity of C inputs to soils (De Deyn *et al.* 2008, Bardgett *et al.* 2008). In this thesis, I address critical gaps in our understanding of the role of soil microbial communities in the response of an Arctic ecosystem to climate change.

My main objectives were:

1) To assess the role of mycorrhizal networks (MNs) in plant-plant interactions in a Low-Arctic tundra ecosystem.
2) To determine the effects of warming and increased nutrient availability on the ectomycorrhizal (ECM) and root-associated fungal community of a tundra shrub that is responsive to these climate change factors.
3) To determine the effects of warming on soil fungi and bacteria over time.
4) To assess the role of the mycorrhizal symbiosis in determining C-allocation to rhizosphere organisms.

My most important findings were:

Mycorrhizal networks exist in the Low-Arctic tundra, and facilitate below-ground transfer of C among *Betula nana* individuals, but not between or within the other tundra species examined (Chapter 2). C-transfer among conspecific *Betula nana* pairs through MNs represented 5.5 ± 2.2% of plant photosynthesis, slightly more than half of the total belowground transfer of fixed C (10.7 ± 2.1%). The remaining C was transferred predominantly through clonal

rhizomes, with very little through soil pathways. Intraspecific C-transfer among *Betula nana* through MNs was positively related to receiver size and to ambient air and soil temperatures.

In natural ecosystems, MNs are increasingly shown to influence plant community structure through nutrient transfer or mycorrhizal colonization effects on plant establishment, interspecific competition, and community diversity (Perry *et al*. 1989, Fitter *et al*. 1998, van der Heijden *et al*. 1998, Klironomos 2002, Simard and Durall 2004; McGuire 2007, Teste *et al*. 2009). The potential influence of MNs on plant community structure is complex and variable. Facilitative effects of MNs through mycorrhization or redistribution of nutrients among plants (Simard *et al*. 1997, Lerat *et al*. 2002, Nara 2006) can include increased seedling establishment and survival (Nara and Hogetsu 2004, Teste *et al*. 2009), reduced interplant competition (Perry *et al*. 1989) and increased species diversity within a plant community (Grime *et al*. 1987, van der Heijden *et al*. 1998). Conversely, MNs may lead to the competitive exclusion of non-networked species and a reduction in total community diversity as occurs in mono-dominant stands of tropical forest trees (McGuire 2007). Where MNs act to alter competitive interactions among plants, they will have profound effects on the structure and diversity of plant communities and ultimately on the C-cycling traits of ecosystems (Wilkinson 1999). In Chapter 2, I provide the first evidence that C-transfer through MNs is of sufficient magnitude that it may alter plant interactions in Arctic tundra, possibly leading to an increase in competitive ability and the mono-dominance of *Betula nana*. Thus, I provide a hitherto unknown mechanism that may help explain the observed expansion of *Betula nana* in tundra ecosystems (Sturm *et al*. 2001, Stow *et al*. 2004). It has been proposed that climate change will shift dominant plant interactions from facilitative to competitive in Arctic ecosystems (Brooker 2006). Likewise, my results suggest C-transfer through MNs will be enhanced under warmer climate conditions, leading to the increased competitive ability of *Betula nana.* Thus, my data are part of a growing body of evidence that MNs are drivers of change in plant communities and consequent storage of C in ecosystems (Read *et al.* 2004, Brooker 2006).

Warming, but not nutrient addition, leads to strong changes in the composition of the ECM community of *Betula nana* (Chapter 3). Warming resulted in a significant increase in the presence of fungal taxa with proteolytic capacity, particularly *Cortinarius* spp., and a reduction in fungal taxa with high affinity for labile N, especially *Russula* spp. By contrast, the change in ECM community composition with fertilization was relatively small, although I observed a significant increase in the nitrophilic species, *Laccaria bicolor*.

My findings suggest that climate warming will profoundly alter the ECM community and nutrient cycling dynamics in Low Arctic tundra. Warming led to a dramatic shift toward fungal species with high biomass and an ability to degrade protein, suggesting that warming-induced changes in the ECM community of *Betula nana* will lead to increased decomposition of soil organic matter as climate warms. *Betula nana* is expanding onto tundra (Sturm *et al*. 2001, Stow *et al*. 2004) and its expansion is known to cause changes in snow accumulation patterns that lead to higher winter and spring-time soil temperatures, and increased rates of nutrient mineralization under thickets resulting in a positive feedback that further enhances its own growth (Sturm *et al.* 2001, Strum *et al.* 2005, Weintraub and Schimel 2005). My ECM community data provide an additional and hitherto unknown mechanism that may help to explain observations of increased microbial activity leading to increased plant N availability (Chapin 1983, Nadelhoffer *et al.* 1992, Chapin *et al.* 1995, Aerts 2006) and faster C turnover in Arctic soils (Hobbie and Chapin 1998, Mack *et al.* 2004, Shaver *et al.* 2006, Schuur *et al.* 2009) with warming.

These findings also support and extend results from Chapter 2. The observation that warming leads to the increased abundance of fungi with high biomass types and the ability to form rhizomorphs, which may be utilized in the rapid reallocation of C at need, complements the observation that warming leads to increased C-transfer though ECM-MN in *Betula nana* (Chapter 2), because ECM with these characteristics are more likely to be important in C-transfer through MNs (Simard *et al*. 2002). Thus, these data provide another mechanism by which the ECM community may affect plant-plant interactions that further feedback to climate through changes in the structure of plant communities and consequent storage of C in ecosystems (Read *et al.* 2004, Brooker 2006).

The fundamentally different trajectories of ECM communities treated with warming versus fertilizer additions agrees with the contrasting responses of microbial communities to warming and fertilization treatments observed in a Swedish sub-Arctic heath (Rinnan *et al.* 2007), and together these studies suggest that direct fertilization of tundra ecosystems do not simulate the increase in mineralization rates that follow warming (Hobbie 1996). Although the response of above ground plant biomass to warming and nutrient additions are similar and

additive (Chapin *et al.* 1995), my findings for the ECM community indicate that the mechanisms that ellicit these changes are not. Thus, a full understanding of the response of Arctic ecosystems to climate change will not be gained without inclusion of the below-ground community response. To my knowledge, my data provide the first evidence that ECM community change in response to climate change factors can alter nutrient cycling in Arctic tundra.

Long-term warming leads to a reduction in the community diversity of soil fungi and bacteria in Low-Arctic tundra that is stable at annual timescales (Chapter 4). Warming for 19 years was associated with an increase in bacteria and fungi affiliated with dominant groups, and a decline or disappearance of rare groups at the class and order level, leading to a 28% and 22% reduction in community richness of fungi and bacteria, respectively.

Despite the importance of Arctic soils in the global C cycle, we know very little of the impacts of long-term warming on the soil microbial communities that drive C and nutrient cycling in these ecosystems. Although other studies have reported strong effects of warming on the structure of soil microbial communities (Deslippe *et al.* 2005, Rinnan *et al.* 2007), to my knowledge, Chapter 4 is the first study to document a reduction in the diversity of bacteria and fungi in response to warming. Warming is associated with reductions in the diversity of plants at the species level in Low Arctic tundra (Chapin *et al.* 1995) and changes in plant community composition can be expected to feed back to the microbial community through changes in the timing and quality of plant derived C substrates (Bardgett *et al.* 1999). Indeed, patterns in my data, including the relatively larger spatial extent of change in mycorrhizal- and lichen-associated phylotypes, suggest the reductions in diversity observed are mediated in part by changes in the plant community. Likewise, in a separate study conducted after 13 years of warming at this site, there was a significant increase in mycorrhizal biomass that was outpaced by a strong increase in the biomass of the host shrub, *Betula nana,* and was interpreted to represent an overall reduction of mycorrhizal fungal biomass relative to plant biomass (Clemmensen *et al.* 2006).

These data also form part of a small but growing body of evidence to suggest that Arctic microbial communities are temporally stable at the annual scale. For example, Männistö *et al.* (2007) found that bacterial communities in Finnish fjelds, as characterized by analysis of 16S rRNA genes and PLFAs, were temporally stable over three years of sampling. Likewise, at Toolik Lake, Wallenstein *et al.* (2007) sampled ambient MAT tundra soils twice (August 19th, 2004 and June 6th, 2005) and found little change in the composition of clone libraries of bacterial and fungal sequences between these dates. These finding are in stark contrast to Alpine tundra microbial communities which undergo strong seasonal patterns of succession (Lipson *et al*. 2002, Schadt *et al.* 2003, Lipson and Schmidt 2004, Nemergut *et al.* 2005).

The reductions in diversity due to warming reported in Chapter 4 were not matched by a reduction in diversity of *Betula nana* ECM and root-associated fungi due to warming in Chapter 3. However, given the very different sampling strategies employed in the two studies (i.e., *Betula nana* root tips versus soil cores), direct comparisons are not possible. Overall, fungal richness in Chapter 3, where I considered a single plant host (*Betula nana*) at a single point in time, was lower than in Chapter 4. Given the much greater temporal extent of soil core sampling in Chapter 4, and that soil cores may have included roots of many species, it seems reasonable that my increased sampling in Chapter 4 provided the power necessary to detect reductions in microbial diversity that are due to warming.

Gram-negative bacteria and a species-specific community of ECM and ERM fungi are the primary consumers of rhizodeposit C among three dominant tundra shrubs of different mycorrhizal types (Chapter 5). Irrespective of plant species, two Gram-negative bacteria rapidly acquired plant C. Over time, fungi consumed a greater proportion of C in all plants tested, but the fate of C within the fungal community was plant species specific. *Salix pulchra* was associated with the rapid ^{13}C-enrichment of two *Russula* species, while ^{13}C-enrichment of phylotypes most closely related to uncultivated fungi occurred more slowly in *Betula nana* and *Ledum palustre* rhizospheres. Four of the five ^{13}C-enriched fungal sequences appear to represent mycorrhizal species, while one clone associated with *Betula nana* may represent a novel fungal lineage.

Although preliminary, my results suggest that ERM and ECM plants have similar patterns of C-acquisition by rhizosphere organisms at the domain level, which are different from those of AM plants. This difference may be driven by the relatively high rate of hyphal turnover in AM fungi (Staddon *et al*. 2003, Treonis *et al.* 2004, Olsson and Johnson 2005) in contrast with ECM and ERM hyphae, which turn over much more slowly (Langley and Hungate 2003, de Boer *et al.* 2006, Högberg *et al.* 2008) leading to slower ^{13}C-enrichment of fungi in ERM and ECM systems compared to AM systems. By contrast, Gram-negative bacteria appear to be important primary consumers of plant C in the rhizospheres of all three mycorrhizal types (Treonis *et al.* 2004, Olsson and Johnson 2005, this study). Strikingly, and despite the very high diversity of bacterial communities, my data suggest that only two Gram-negative bacterial phylotypes dominate C-acquisition in the rhizosphere of all three plant species studied. These phylotypes may represent key species in the cycling of plant C in Arctic tundra, although the identity of these organisms still needs to be confirmed. To my knowledge, my study represents the first comparison of the patterns of C-acquisition in the rhizospheres of ERM and ECM plants.

In contrast to the bacterial results, patterns of C-allocation to ERM and ECM fungal communities appear to be much more species-specific; enrichment of fungal phylotypes differed among plant species and among replicates, and fungal DGGE patterns showed species-specific community assemblages. These data agree with my RISA and clone library results in Chapter 4, which indicated that the response of fungal communities to warming was partly mediated by changes in plant community, while no similar pattern was observed for bacteria. Taken together, my data suggest that fungal and bacterial communities have fundamentally different relationships to plants. While the factors that influence the diversity and community structure of plants and soil fungi are intimately linked, especially in the case of mycorrhizae, the factors that influence the structure and diversity of bacterial communities appear to be fundamentally different.

The fate of newly photosynthesized C is of critical importance to its residence time in soils (Hogberg and Read 2006, Hoberg *et al*. 2008). As *Betula nana* and *Salix pulchra* expand onto Arctic tundra, displacing AM plants and bringing with them their associated mycorrhizae, the increased abundance ECM fungal hyphae should enhance C-storage in surface layers of tundra soils. This may help to dampen the rapid rates of C-loss that result from the thawing of

frozen soils, which is enhanced, in part, by the expansion of the shrubs themselves (Sturm *et al*. 2001, Strum *et al*. 2005, Weintraub and Schimel 2005a).

Taken together, the results of these four experiments strongly suggest that **soil microbes play a critical role in plant community dynamics and carbon cycling in Arctic tundra, and that this role will become increasingly important as climate warms.**

Strengths and limitations

In addition to the strengths of individual chapters that were already discussed above, a major strength of my thesis arises from the connections among the studies. Integration of results from all chapters helps to overcome some of the inherent limitations of each experiment. For example, the very high mortality of *Salix pulchra* in experimental treatment plots in Chapter 2 limited my ability to examine interspecific C-transfer through MNs because *Salix pulchra* is the second most common ECM species at the study site, after *Betula nana*. Thus, the lack of replication of *Betula nana*-*Salix pulchra* pairs likely limited my ability to detect C transfer to *Salix pulchra* in Chapter 2. However in Chapter 4, I found that *Russula spp.* represented nearly 50% of *Betula nana*'s ECM community, while in Chapter 5, I observed rapid ^{13}C-enrichment of *Russula spp*. in the rhizosphere of *Salix pulchra.* Taken together, these data suggest a high potential for the formation of ECM-MNs among *Betula nana* and *Salix pulchra* at this Arctic tundra site. Based on these observations, I would expect that interspecific C-transfer through MNs is likely to occur, but the high mortality of *Salix pulchra* in Chapter 2 limited my ability to detect it.

A second limitation of Chapter 2 was that I was unable to harvest plant roots. This likely resulted in my underestimation of C-transfer through belowground pathways in this study, as transferred C may have remained in plant roots if growth or high turnover rates made them strong sinks for C. However, plant roots in tussock tundra are highly intermingled, and the time involved in detangling them among species and from rhizomes had to be balanced against the need for the large number of replicates required to detect inherently variable C-transfer through MNs. Quantitatively extracting roots from highly organic natural soils will always present a challenge to tundra ecologists. However, researchers conducting future studies of belowground C-transfer in this plant community may be well advised to consider focusing their efforts on the relatively large roots of the ECM plant species, and to assemble large field crews.

A major limitation of Chapters 3, 4 and 5 was the difficulty and potential error in assigning environmental sequences to fungal taxa. Although growing, the availability of tools for this task is currently limited relative to similar tools available for bacterial 16S rRNA genes. Moreover, there is no widespread agreement on which criteria should be used to assign an

environmental sequence to a fungal lineage. Adding to the problem is the relatively small number of known fungal sequences in public genetic databases, and the relatively large number of sequences with no associated taxonomic metadata, and often even extremely limited environmental metadata. This situation has arisen partly because recent innovations in sequencing technology has made the generation of large environmental sequence datasets increasingly possible, but biologists and bioinformaticians have only begun to rise to the challenge of how to interpret all this data. In my view, one of the most exciting aspects of the large amount of sequence data that I generated in these studies is the high probability that these data will become more meaningful as genetic databases grow, and as microbial ecologists increasingly link fungal species to their ecological functions.

Future directions

Whenever they are quantitatively examined, it appears that MNs are critical to the structure and function of terrestrial ecosystems, and are often drivers of change within plant communities. Yet we still know very little about MNs in nature. Until recently, the opacity of soil made it impossible to study MNs directly, and their occurrence and functions were implied by the use of stable and radio isotope tracers in the field. These studies necessarily focused on the transfer of labeled C to plant tissue, and though the ultimate fate of this C is important, the way it got there may also be. Stable Isotope Probing of MNs is now possible and, in my view, is among the most promising ways to gain greater insight to the role of MNs in terrestrial ecosystems. I plan to use rhizosphere samples that I collected from ^{13}C-enriched donor and receiver *Betula nana* plants in mesh treatments (Chapter 2), in a SIP-PLFA and SIP-DNA experiment, similar to that in Chapter 5. Simultaneous characterization of the donor and receiver plant's ^{13}C-enriched rhizosphere community will provide insight to which organisms actually mediate the transfer of C among plants in this ecosystem. Given the preliminary results of Chapter 5, I expect that C-transfer through MN between *Betula nana* pairs may involve shared ECM fungi or at least two bacterial phylotypes.

In Chapter 3, I examined the response of the ECM community of *Betula nana* to warming and fertilizer additions and learned that these treatments had significantly different effects on the composition of the ECM community. My data suggests that the ECM community at this site is well adapted to enhanced nutrient availability but that warmer soil temperatures will lead to radical changes in the composition of ECM communities. Because greenhouses only result in warmer wintertime soil temperatures, it should follow that a thicker snowpack will lead to a similar response of the ECM fungal community. Ina Timling, a PhD student with Lee Taylor at the University of Alaska Fairbanks, USA is currently examining the effects of snow additions on the mycorrhizal community structure of plants at Toolik Lake. Direct comparisons among our results would be useful to confirm my results and to enhance our overall understanding of how climate change will alter ECM communities.

Among the most interesting results of my thesis is the indication that a future, warmer, climate will select for ECM fungi that have high biomass and proteolytic capacity. To my

knowledge, this result is novel, and warrants further investigation. Certainly, similar additional studies are needed to confirm it. In Chapter 3, I relate a large sequence dataset to previously published literature $\delta^{15}N$ values and hyphal characteristics for fungal genera and species. A limitation of my approach is that I relied on literature values for these parameters. Future work should involve directly linking ECM species that respond to warming treatments to their ^{15}N values in the field. This could be accomplished by carefully dividing single ECM root tips into two subsamples, one destined for DNA extraction and the other destined for mass spectrometry of the ^{15}N it contains. However, in order to achieve the depth of sampling that mass sequencing facilitated in my study, this approach would become quite costly.

We know that the presence of shrubs is associated with greater snow accumulation, higher soil temperatures, and increased rates of N mineralization (Sturm *et al.* 2005). My Chapter 3 results suggest that a future, warmer, climate will select for ECM fungi that have high biomass and a high potential to mineralize C as they produce exoenzymes that act to release N held in organic matter. This leads to the question of whether or not these ECM fungi are directly responsible for the observed increase in N mineralization rates under thicker snow packs in *Betula nana* thickets or, alternatively, whether the increased rates of mineralization are related to the activities of soil bacteria or saprotrophic fungi. Subnivian sampling of soils and measurement of gene expression by soil bacteria and fungi would reveal if ECM fungi are directly responsible for this change, and may provide even stronger evidence that ECM fungi are critical drivers of Arctic ecosystem change as climate warms.

The knowledge that a warmer climate will lead to an increase in ECM taxa with proteolytic abilities should also be taken into account in models of Arctic C budgets, as warming effects on ECM community structure may represent a positive-feedback to C-mineralization in Arctic soils. These below-ground changes will also feedback to above-ground communities in several ways. Firstly, changes in ECM abundance will likely be reflected in above-ground sporocarp production. This may have important implications for Arctic ground squirrels and other small mammals, as well as for caribou, because mushrooms are a seasonally important food for these animals (Julee Shamhart *pers. comm.* 2008, Barnett 1994). Secondly, my data suggest that changes in the ECM community will influence plant community dynamics by further enhancing the spread of shrubs, which is also likely to have consequences for forage availability

to herbivores. Thus my data may be useful to northern communities as they assess the effects of rapid climate change on their lands and make plans to mitigate its impacts on traditional food supplies and cultural resources.

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Appendix 1: Supplementary materials to chapter 2

Supplementary Table 1: Fungi identified in association with plant roots or from sporocarps at the study site

Root associates of *Betula nana*	**Root associates of ericoid mycorrhizal plants[3]**	**Sporocarps**
Mycorrhizal species		**Mycorrhizal species**
Cenococcum sp. 1	*Alternaria alternata*	*Amanita arctica*
Cenococcum sp. 2	*Amyloathelia sp.*	*Amanita sp.*
Clavulinaceae unk. 1	*Archaeospora leptoticha*	*Cortinarius cf. torvus sp. 1*
Cortinariaceae unk. 1	*Articulospora sp.*	*Cortinarius cf. torvus sp. 2*
Cortinariaceae unk. 2	*Aspergillus oryzae*	*Cortinarius cf. torvus sp. 3*
Cortinarius boulderensis	*Caloplaca sp.*	*Cortinarius croceus*
Cortinarius fennoscandicus	*Capronia sp.*	*Cortinarius favrei*
Cortinarius obtusus	*Chalara sp.*	*Cortinarius heuronenis*
Cortinarius sp. 1	*Chloridium sp.*	*Cortinarius paragaudis*[2]
Cortinarius subsertipes	*Coccomyces sp.*	*Cortinarius sg. Telemonia*
Dermocybe crocea	*Cordyceps militaris*	*Cortinarius sp. 1*
Dermocybe sp. 1	*Cortinarius sp.*	*Cortinarius sp. 2*
Gymnomyces sp.	*Cryptosporiopsis ericae*	*Cortinarius sp. 3*
Helotiales unc. 1	*Didymosphaeria futilis*	*Cortinarius sp. 4*
Helotiales unc. 2	*Eupenicillium pinetorum*	*Cortinarius sp. 5*
Helotiales unc. 3	*Gloeosporium sp.*	*Entoloma sp. 1*
Helotiales unc. 4	*Gyoerffyella sp.*	*Entoloma sp. 2*
Helotiales unc. 5	*Helicoma sp.*	*Hebeloma pussilum*
Helotiales unc. 6	*Humicola sp.*	*Hebeloma sp. 1*
Helotiales unc. 7	*Hymenoscyphus sp.*	*Hebeloma sp. 2*

Laccaria bicolor	*Lachnum sp.*	*Hebeloma sp. 3*
Lactarius glyciosmus	*Lactarius torminosus*	*Hebeloma sp. 4*
Lactarius sp. 1	*Lasiodiplodia sp.*	*Hebeloma sp. 5*
Lactarius sp. 2	*Leohumicola sp.*	*Hebeloma sp. 6*
Lactarius sp. 3	*Leohumicola verrucosa*	*Helvella lacunosa*
Pseudotomentella tristis	*Leptodontidium orchidicola*	*Hygrophorus borealis*
Rhizoscyphus ericae	*Meliniomyces sp.*	*Inocybe leucoblema*[1]
Russula decolorans	*Meliniomyces variabilis*	*Inocybe sp.1*
Russula nana	*Microglossum viride*	*Laccaria lanceolatus*
Russula sp. 1	*Mollisia sp.*	*Laccaria montana*[1]
Russula sp. 2	*Mycena epipterygia*	*Laccaria pumila*[1]
Russula vinosa	*Nectria mauritiicola*	*Laccaria ruffus*
Russulaceae unk. 1	*Oidiodendron maius*	*Laccaria tortilis/proxima*
Russulaceae unk. 2	*Periconia macrospinosa*	*Lactarius aspideoides*
Thelephora terrestris	*Phialocephala europaea*	*Lactarius dryadophyllous*
Tomentella sp. 1	*Phialocephala fortinii*	*Lactarius scrobiculatus*
Tomentella sublilacina	*Phialocephala turiciensis*	*Lactarius sp. 2*
Tomentellopsis submollis	*Phialophora sp.*	*Lactarius sp. 3*
Tulasnella sp.	*Phoma sp.*	*Lactarius sp. 4*
Other root associates	*Phyllosticta sp.*	*Lactarius sp. 5*
Chaetothyriales	*Physcia sp.*	*Lactarius subcircelatus*
Cladophialophora chaetospira	*Pseudofusicoccum sp.*	*Leccinum rotundifoliae* [2]
Cordyceps sp.	*Pseudozyma aphidis*	*Leccinum scabrum*
Cryptococcus terricola	*Rhizoscyphus ericae*	*Leccinum sp.*
Galerina fallax	*Russula decolorans*	*Russula aeurginea*
Hypocreales	*Sebacina vermifera*	*Russula emetica*
Leptodontidium sp.	*Trichocladium opacum*	*Russula lutea*
Leucosporidium scottii	*Trichoderma asperellum*	*Russula sp. 1*

Meliniomyces sp.	*Venturia sp.*	*Russula sp. 2*
Meliniomyces variabilis	*Xenostigmina sp.*	*Russula sp. 3*
Microbotryomycetes		*Russula sp. 4*
mitosporic Ascomycota		*Russula sp. 5*
Mortierella sp.		*Russula zerampellina*
Pezizomycetes		**Saprotrophic species**
Phialocephala fortinii		*Agrocybe praecox*[1]
Phialophora sp.		*Aleuria aurantia*
Rhodotorula sp.		*Calvatia sp.*
		Clavulina sp.
		Clitocybe polygonarium
		Clitocybe sp. 1
		Clitocybe sp. 2
		Clitocybe sp. 3
		Clitocybe sp. 4
		Clitocybe sp. 5
		Collybia cf. maculata
		Collybia dryophila[1]
		Cystoderma amianthinum
		Galerina sp. 1
		Galerina sp. 2
		Galerina sp. 3
		Gymnopus aquosus[1]
		Hygrocybe nitidus
		Hygrocybe vitellinus
		Hypholoma sp.1
		Hypholoma udum[2]
		Lapista multiformis

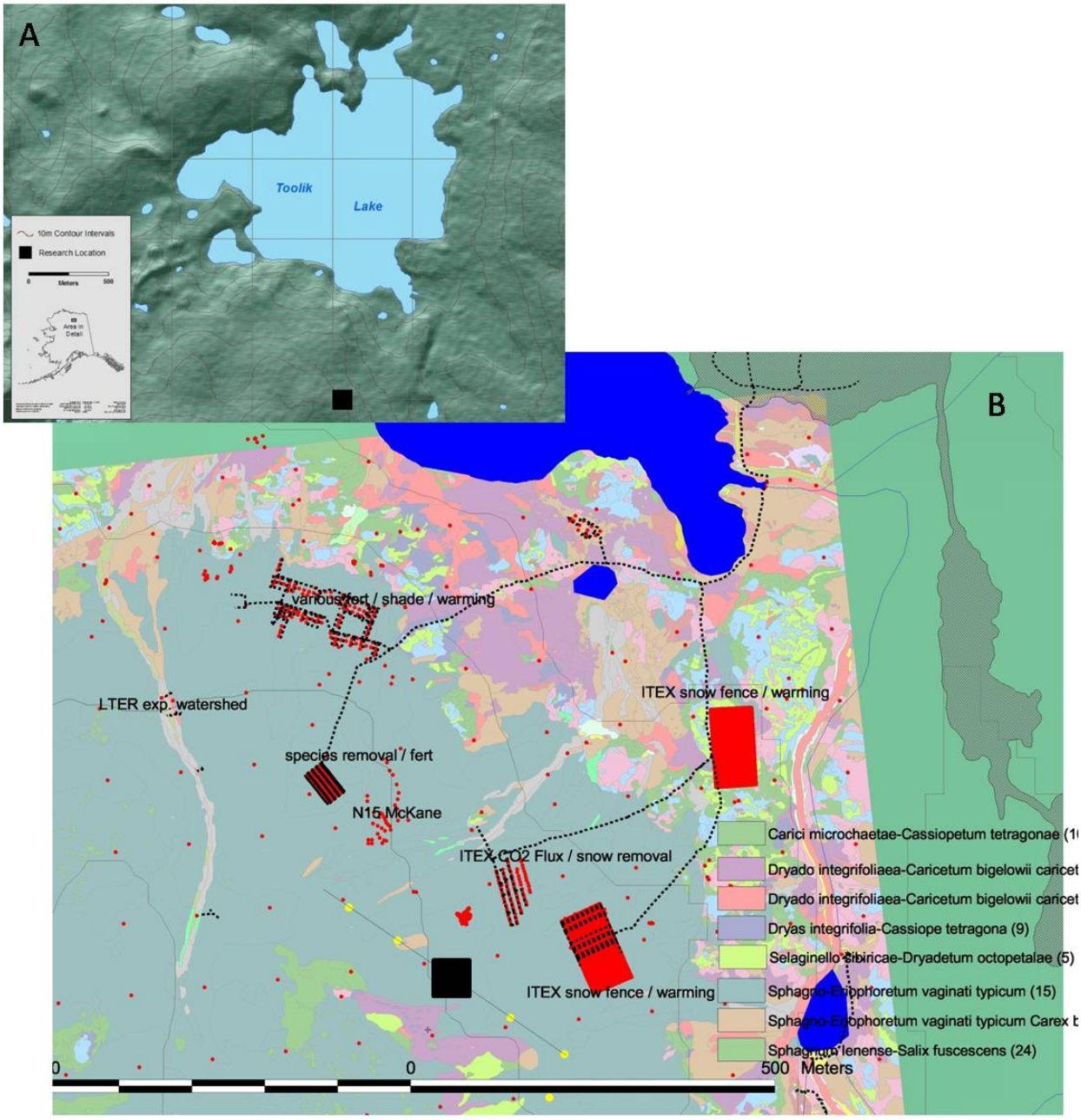
	Marasmius androsaceus [2]
	Microcollybia cirrata
	Mycena pura
	Mycena simia [2]
	Omphalina postii
	Psilocybe sp.
	Rhodophyllus sp.
	Rickella sp.
	Rumaria cf. fumosa
	Stropharia sp.
	Urnula sp.
	Lichenized species
	Lichenomphalia alpina
	Lichenomphalia hudsoniana
	Lichenomphalia umbellifera

[1]Sporocarp identification courtesy of Julee Shamhart, Erik Hobbie, John Hobbie, and Lee Taylor, *pers comm*. 2009.

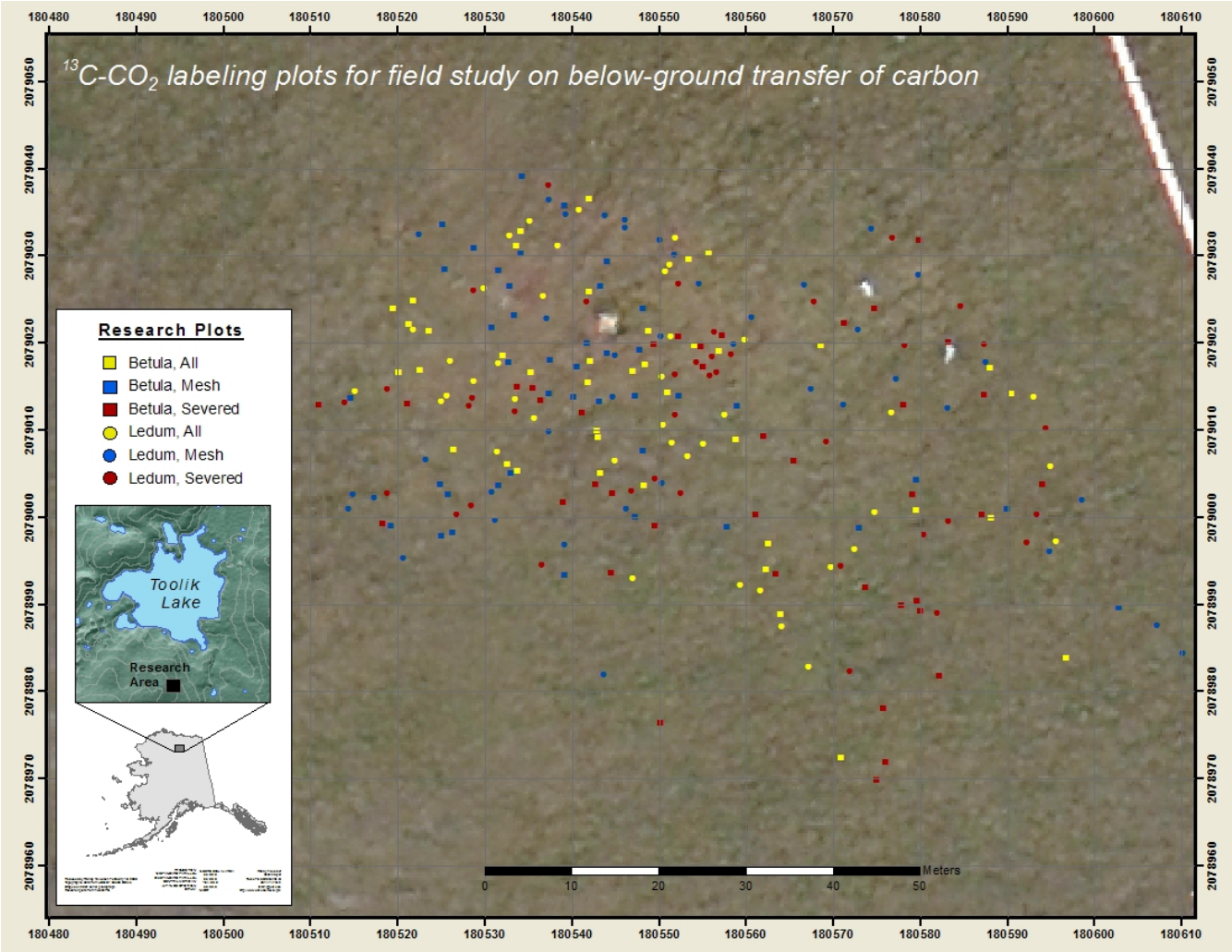
[2] From: Clemmensen KE, Michelsen A, Jonasson S, Shaver GR (2006) Increased ectomycorrhizal fungal abundance after long-term fertilization and warming of two Arctic tundra ecosystems. New Phytologist 171:391–404

[3] All root associates of ericoid mycorrhizal plants are courtesy of Laura Aldrich-Wolfe and Ari Jumpponen *pers comm*. 2009.

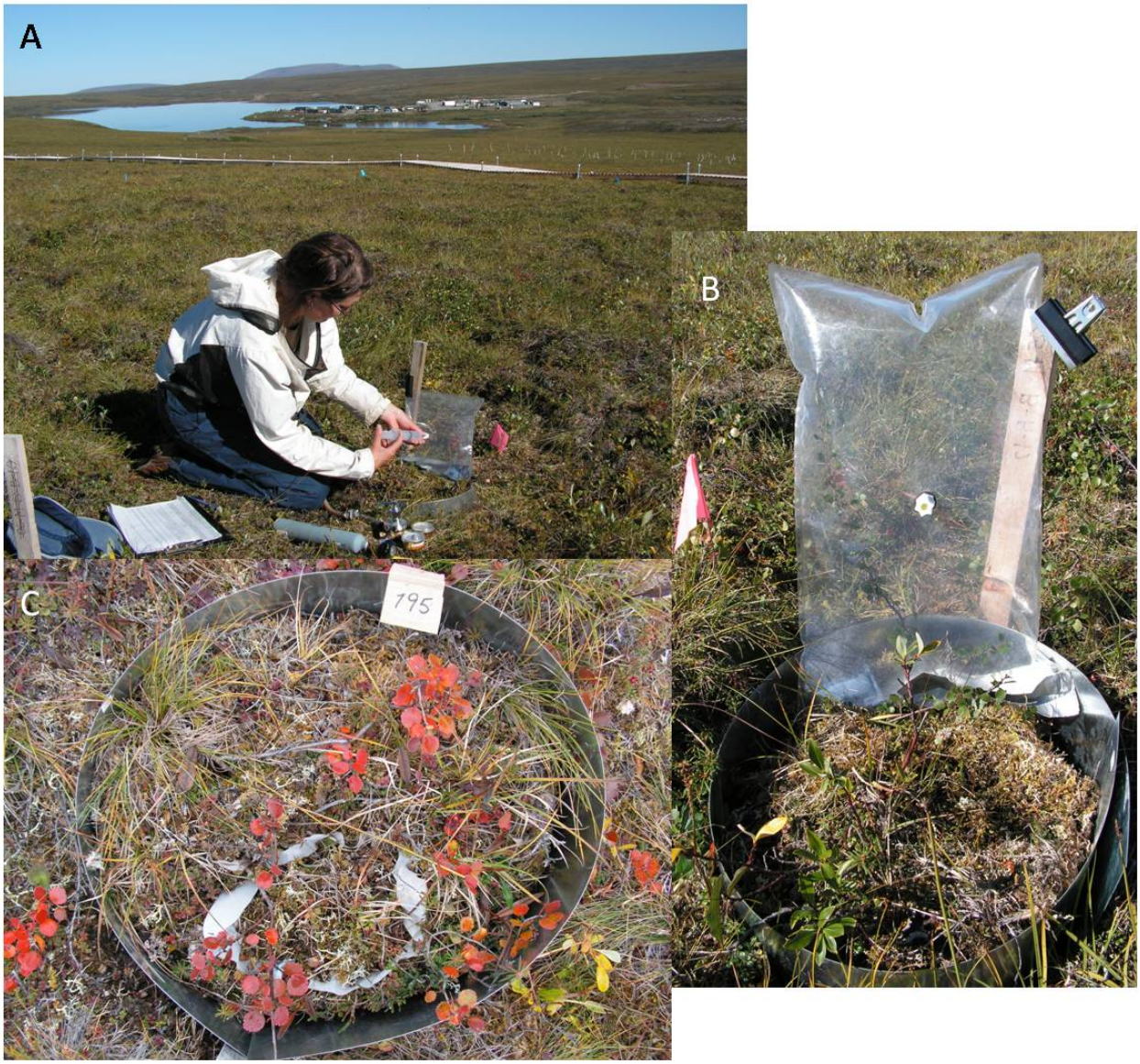
Supplementary Figure 1: Maps of study site, a black square depicts the location of the study site in both images. A) Study site location relative to Toolik Lake. B) Vegetation zones of study site and adjacent areas, and location of study site relative to the Arctic Long-term Ecological Reseach and International Tundra Experiment experimental plots.



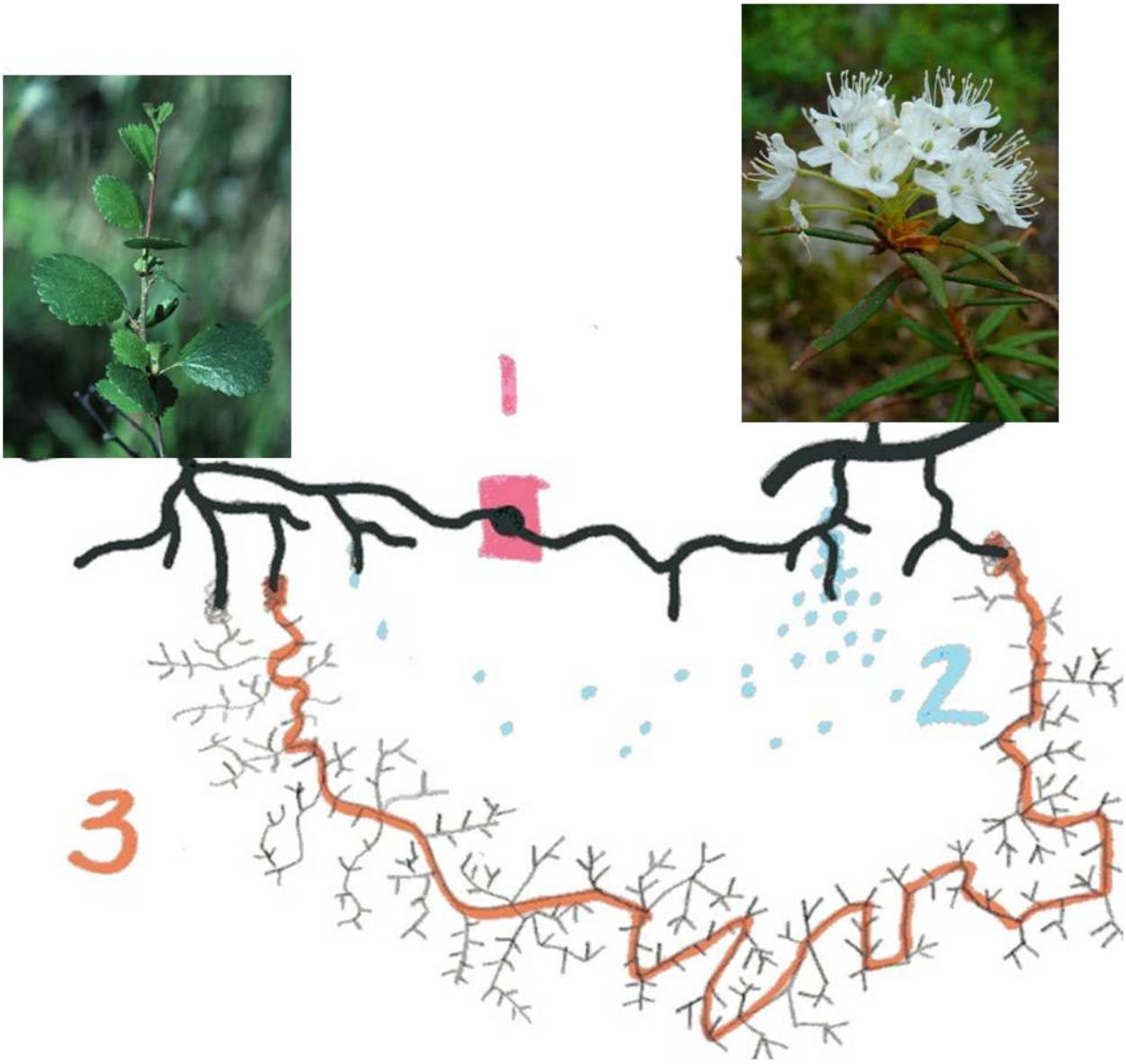
Supplementary Figure 2: Map of study site.



Supplementary Figure 3: Experimental ^{13}C-CO_2 labeling of a *Betula nana* and *Ledum palustre* near Toolik Lake, Alaska, USA. A) Injecting ^{13}C-CO_2 to a *Ledum palustre* donor in August of 2007. B) A *Betula nana* donor during a ^{13}C-CO_2 pulse, August 2007. C) An experimental plot during the August 2008 chase period, showing advancing senescence of *Betula nana* in response to an unusually cold, wet, and dark August.



Supplementary Figure 4: Simplified schematic diagram of below-ground pathways of carbon transfer among plants. 1) direct transfer through root grafts or rhizomes (conspecific transfer only); 2) transfer of carbon containing compounds via the soil solution; 3) transfer of carbon through a mycorrhizal network.



Supplementary Figure 5: Schematic representation of the treatments applied in this study to control for below ground pathways of C. 1) Mesh; prevents direct C transfer through rhizomes or root grafts (A, conspecific transfer only); 2) Severing; cuts roots & rhizomes (A) and ECM hyphae (B), allowing C-transfer only through the soil solution (C); 3) Stainless steel sheet metal plot boundary prevents the transfer of C through all pathways from outside of the plot (X; X= A+B+C).