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Soil freezing effects on a grass-dominated old field ecosystem under current and future rates of atmospheric nitrogen deposition

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

SOIL FREEZING EFFECTS ON A GRASS-DOMINATED OLD FIELD ECOSYSTEM UNDER CURRENT AND FUTURE RATES OF ATMOSPHERIC NITROGEN DEPOSITION

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Abstract

Climate change is expected to alter the intensity and dynamics of soil freezing as a result of increased air temperatures and reduced snow cover. Soil freezing can influence ecosystem nitrogen (N) cycling by damaging plants and soil microorganisms, but little is known about how soil freezing effects on ecosystem N cycling may combine or interact with increased atmospheric N deposition, which is also expected to exert a strong influence on terrestrial ecosystems in the coming decades. The objective of my thesis was to examine the combined and possibly the interactive effects of climate induced changes in soil freezing and N addition on plant productivity, soil microorganisms, and soil nutrient cycling in a grass-dominated temperate old field ecosystem. First, using ^{15}N tracer, I investigated N retention by different nitrogen pools (plant, litter, roots, soil and simulated N deposition) in response to soil freezing under current and projected future atmospheric N deposition rates. My results indicated that soil freezing can increase N losses from soil over the winter and from atmospheric N deposition during the growing season, with the latter occurring due to decreased plant productivity. Second, I combined increased freezing (both in controlled environment chambers and in response to snow removal in the field) with N addition to explore whether soil freezing effects are mostly transient (i.e. over winter and spring melt), or whether there are legacy effects of freezing that continue over multiple years. My results indicated that the legacy effect of soil freezing reduced plant productivity over multiple years, but that N addition counteracted these declines in plant productivity. With respect to soil responses, freezing only caused short term (over winter) increases in extractable nitrogen pools, although there were also declines in fungal biomass during the second growing season as a legacy effect of freezing. Overall, my results indicate that intense soil freezing and increased atmospheric N deposition can both alter plant productivity and ecosystem N retention, although there were few significant interactions between these two factors.

Keywords

Climate change, nitrogen deposition, nitrogen leaching, plant productivity, snow removal, soil freezing

Co-Authorship Statement

A version of Chapter 2 was published in the journal *Ecosystems* with Dr. Hugh Henry as a co-author. Dr. Henry provided funding for the project, was involved with the study design, and contributed to the writing and editing of the manuscript.

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List of Abbreviations

ºC = degrees Celsius

^{15}N = ^{15}nitrogen

C = carbon

CO_2 = carbon dioxide

d = days

DOC = dissolved organic carbon

DON = dissolved organic nitrogen

dw = dry weight

g = grams

h = hour

K_2SO_4 = potassium sulfate

m = meter

M = mols

MB = microbial biomass

mg = milligram

mins = minutes

mL = millilitres

mM = millimols

N = nitrogen

NaCl = sodium chloride

NaOH = sodium hydroxide

NH_4^+ = ammonium

NO_3^- = nitrate

N_2O = nitrous oxide

PBS= phosphate buffered saline

ppm = parts per million

PVC= polyvinyl chloride

TDN = total dissolved nitrogen

TOC = total organic carbon

µg = micrograms

µL - microliters

µm = micrometers

y = year

1 Chapter 1: General introduction

1.1 Global climate change

1.1.1 Climate warming predictions

Global surface temperatures have risen by 0.6 ^{o}C over the last hundred years, and they are projected to rise an additional 1.1-6.4 ^{o}C over the next century, with a 2.0-4.0 ^{o}C increase in northern temperate regions (IPCC 2007). Overall, warming is expected to result in earlier snowmelts, later snow accumulation, and extend growing season length relative to recent climate normals (IPCC 2007). Biological responses to warming may include changes in plant species range (Klanderud and Birks 2003, Walther et al. 2005a), distribution (Walther et al. 2005b), phenology (Myneni et al. 1997, Menzel 2000, Chmielewski and Rotzer 2001, Zhou et al. 2001), abundance (Chapin et al. 1995), reproduction (Beggs 2004), and productivity (Chapin et al. 1995, Morgan et al. 2001, Rustad et al. 2001). In addition, climate warming is expected to increase the intensity and occurrence of extreme weather events (Easterling et al. 2000, Meehl et al. 2000), which may have greater impacts on plant productivity, species abundances, and distributions than average warming trends (Jentsch 2006, Jentsch et al. 2007).

1.1.2 Climate change in winter

Climate change research has focused disproportionately on the growing season (Kreyling 2010), despite the prediction that the greatest temperature increases will occur during the winter (IPCC 2007). In addition, the highest increases in winter air temperatures are expected to occur in high latitude and high altitude regions (ACIA 2004, IPCC 2007), which has resulted in a disproportionate amount of winter research in arctic and alpine systems (Kreyling 2010). However, mid-latitude temperate regions, while predicted to experience only moderate increases in winter air temperature, may be particularly sensitive to warming, because their soils often remain close to the freezing/melting point throughout winter (Henry 2008).

Climate warming is expected to decrease the proportion of winter precipitation that falls as snow (IPCC 2007), altering the timing and amount of snow cover during the winter (Kapnick and Delworth 2013). Climate warming has already been linked to declines in snow cover in the northern hemisphere (ACIA 2005), increased winter warm spells across Canada (Shabbar and Bonsal 2003), and an increased annual ratio of rain to snow in the northern United States (Huntington et al. 2004, Feng and Hu 2007), resulting in changes in the timing, duration, and depth of snow cover.

Snow cover can act as a thermal insulator that decouples soil and air temperatures (Sharratt et al. 1992), and when snow cover is absent, soil temperatures tend to be sensitive to changes in air temperatures and solar radiation (Hardy et al. 2001). Despite uncertainty about how increases in air temperatures will affect winter soil temperatures, it has been suggested that decreased snow accumulation and a decreased duration of snow cover will increase the occurrence of soil freezing in temperate regions (Hardy et al. 2001). This suggestion is supported by historical data and snow removal experiments, which indicate that soils will experience increased soil freezing or an increase in the number of freeze-thaw cycles (Henry 2008, Hentschel et al. 2009, Muhr et al. 2009), leading to the apparent paradox of "colder soils in a warmer world" (Groffman et al. 2001a).

However, predicted increases in the occurrence of soil freezing and freeze-thaw cycles are not expected to take place across all regions. While models and historical data for areas in Canada and the northern United States predict soil freeze-thaw cycles will increase in these regions (Henry 2008, Campbell et al. 2010), similar analyses conducted in Germany predict that warmer winters will decrease the frequency of soil freeze-thaw cycles (Kreyling and Henry 2011). This discrepancy between regions is largely explained by differences in mean air temperature over winter in relation to the freezing point. In particular, for the relatively warm sites in Germany, winter warming will not only decrease snow cover, it will result in 'vanishing winters,' where freezing temperatures become increasingly uncommon (Kreyling and Henry 2011). Similarly, Sinha and Cherkauer (2008) predicted from air and soil temperature data that freeze-thaw cycles

will decrease in the southernmost sites of Indiana, but increase in the colder, northern sites.

Soil freezing severity, defined as the minimum soil temperature experienced, and/or by soil frost depth, may also change in a warmer world. Increases in soil freezing severity have been observed in some northern temperate regions (Decker et al. 2003, Isard et al. 2007), however, similar to the trends in freeze-thaw occurrence, areas that currently experience relatively warm winters will likely experience a decrease in the severity of soil freezing (Sinha and Cherkauer 2008, Kreyling and Henry 2011). Likewise, frost depth is expected to vary with warmer winters, with some regions predicted to experience decreases (Venalainen et al. 2001, Sinha and Cherkauer 2008) or no change (Campbell et al. 2010), and others expected to experience increases under winters with less snow precipitation (Isard and Schaetzl 1998). Overall, despite the predicted change in the frequency and severity of soil freezing and freeze-thaw, the number of days of frozen ground is expected to decrease (Venalainen et al. 2001, Campbell et al. 2010).

1.2 Winter plant survival

1.2.1 Winter damage to plants

When temperatures reach 0 °C or below, ice formation is initiated in the intercellular spaces of plants (Pearce 1988), because extracellular fluid has a higher freezing point than the intracellular fluid (Thomashow 1999). Extracellular freezing draws liquid water from cells, as a result of the property that the vapour pressure of ice at any given temperature is less than that of pure water (Guy 2003). Consequently, extracellular ice crystals grow by drawing water from cells until the water potential is equal to that of the cell, dehydrating the cell contents. The water potential of ice declines as temperature falls, and cellular dehydration progressively worsens as temperature decreases (Gusta et al. 1975). Membrane structures are damaged when freezing induced dehydration exceeds cellular dehydration tolerance (Steponkus 1984), with injury becoming more severe the longer the tissue is frozen (Fry et al. 1993, Anderson et al. 2003). Cell death is often caused by dehydration, because too much moisture is lost from the cytoplasm, causing

loss of membrane integrity, protein denaturation, and solute precipitation (Pearce and McDonald 1977).

Plants can also be damaged by soil physical processes such as frost heaving, which causes roots to break as the soil moves (Kinbacher 1956), and creates soil cracks that expose roots directly to cold air temperatures (de Chantal et al. 2007). In addition, freezing and thawing of the snow pack can lead to plants being encased by ice (Tompkins et al. 2004), which limits gas diffusion between the plant and atmosphere (Andrews 1996, Albert and Perron 2000), potentially creating hypoxic and anoxic conditions (Beard 1964, Tompkins et al. 2004). These conditions lead to a buildup of anaerobic metabolites and gases, such as ethanol and lactic acid (Andrews and Pomeroy 1979), resulting in cellular damage and subsequent declines in energy production (Bertrand et al. 2001). However, pasture and perennial cool season grass species can often tolerate ice encasement for 20 to 60 days without significant injury (Beard 1964, Gudleifsson et al. 1986).

Susceptibility to freezing damage varies genotypically (Ebdon et al. 2002, Kreyling et al. 2012), with plant age (Andrews et al. 1960), species type (Bokhorst et al. 2008, Preece et al. 2012), fungal infection (Iriarte et al. 2005) and depends on which plant organ is exposed (Sakai and Larcher 1987). In particular, plant roots are often less cold tolerant than aboveground structures, and consequently are more susceptible to freeze damage (Noshiro and Sakai 1979).

1.2.2 Cold acclimation and deacclimation by plants

Plants have evolved molecular, physiological, and biochemical mechanisms to minimize freeze injury and enhance winter survival; these responses are collectively referred to as cold acclimation or hardening (Thomashow 1999). Cold acclimation is generally achieved through two phases of stimuli. First, plants are exposed to low non-freezing temperatures (5-10 °C) and short periods of daylight (Smithberg and Weiser 1968). Second, plants are exposed to near freezing temperatures (<5 °C) (Olien 1984, Uemura et al. 1995). During these phases, plants change their lipid membrane composition to make their membranes more fluid (Thomashow 1999), induce molecular chaperones (Guy and Li 1998) to protect proteins against denaturation (Anchordoguy et al. 1987), synthesize

new enzymes to increase reactability at low temperatures (Ensminger et al. 2008), and increase carbohydrate reserves to lower the freezing point of water in the cells (Ogren et al. 1997, Ball et al. 2002). In addition, certain species of wheat and cereals can produce cryoprotective (Sarhan et al. 1997) and antifreeze proteins that inhibit freeze damage (Antikainen and Griffith 1997).

Unlike cold acclimation, which can take weeks to months to occur fully, deacclimation can occur over a relatively short period, from days to weeks (Gay and Eagles 1991). Deacclimation is the loss of cold hardiness resulting from increases in air temperatures, and results in phenological changes and reactivation of growth (Kalberer et al. 2006). Deacclimation occurs in two ways: passive, which occurs when fully acclimated plants are exposed to above freezing yet cool air temperatures (~5 °C) for an extended period of time, and active, which is deacclimation that occurs in response to substantial increases in ambient temperature. Active deacclimation occurs rapidly, and causes a wide range of structural and functional changes associated with resumption of growth (Kalberer et al. 2006).

In some cases, deacclimation can be reversed by subsequent exposure to low temperatures, however, it is not always possible to reach the previous cold acclimated state, and reacclimation often becomes less effective the longer the plant is deacclimated (Repo 1991, Kalberer et al. 2006). The mechanism as to why plants cannot reacclimate to previous levels is unclear, but it may be due to a lack of energy producing substrates or irreversible developmental changes following deacclimation (Mahfoozi et al. 2001, Rapacz 2002).

1.2.3 Lethal and sub-lethal effects of freezing on plants

The effects of changes in winter soil temperatures on plants in temperate regions are underrepresented in the literature (Kreyling 2010), despite the strong influence winter climate change may have on growing season plant performance in these regions (Kreyling et al. 2008b, Schaberg et al. 2008, Kreyling et al. 2010). Outside of the areas of forestry and food production, research has focused largely on the lethal temperatures (LT_{50}) needed to kill agricultural and turf grasses (Gudleifsson et al. 1986, Tcacenco et al.

1989, Qian et al. 2001, Hulke et al. 2008, Hanslin and Höglind 2009), due to the associated large economic losses caused by winter injury in these species (Ouellet 1976). However, these lethal temperatures are much lower than soil temperatures typically experienced in the field, particularly in natural and semi-natural systems, where a substantial litter layer is present and insulates the soil (Groffman et al. 2001a, Hardy et al. 2001, Henry 2008). Nevertheless, sub-lethal damage to roots (Malyshev and Henry 2012a) and the apical meristem can occur at less severe freezing temperatures (Eagles et al. 1993), affecting subsequent plant productivity (Weih and Karlsson 2002, Malyshev and Henry 2012a, Malyshev and Henry 2012b, Comerford et al. 2013).

Field studies indicate a wide range of plant and community responses to soil freezing and freeze-thaw. Yellow cedar (*Callitropsis nootkatensis*) die offs have been attributed to increases in soil freezing, and the limited cold tolerance and rapid deacclimation time of this species leaves it susceptible to frost (Schaberg et al. 2005, Schaberg et al. 2008, Schaberg et al. 2011). Weih and Karlsson (2002) found that increased soil freezing decreased the productivity of mountain birch (*Betula pubescens Ehrh.ssp. czerepanovii*), which the authors concluded was due to allocation of resources to replacement roots, reducing nutrient uptake capacity, and growth rate. In contrast to these results, Kreyling et al. (2008) observed that increases in freeze-thaw enhanced the productivity of grassland species, which was suggested to be due to enhanced N availability associated with freeze-thaw exposure. However, in a follow up study, Kreyling et al. (2010) found that this effect only lasted one year, and that heathland shrubs exposed to the same treatment were not affected the first year, but were negatively affected the second year, likely due to sub-lethal root damage decreasing plant N uptake.

Species specific or growth form differences in cold tolerance, nitrogen (N) uptake, and productivity following soil freezing could alter competitive balance and lead to changes in species abundances and composition. Kreyling et al. (2011a) found that eight years of increased soil freezing changed the community composition of understory forest species through the reduction and loss of species that prefer a deep snow cover microclimate. Bokhorst et al (2009) found that mid-winter warming events in the Arctic lead to reductions in plant productivity of certain species, while others were unaffected,

suggesting the potential for long term changes in species abundances. Furthermore, Kreyling et al (2011b) found that plots with at least four species and three functional groups (grass, forb, and legume) were less likely to change following freeze-thaw exposure, likely through increased N in these soils. Collectively, these studies highlight the ecological importance of changes in plant performance in response to altered winter soil temperatures, and the need for studies conducted over multiple growing seasons to identify the short and long term effects of freezing on plant productivity, species abundances, and community composition.

1.3 Winter effects on soil

1.3.1 Soil freezing and freeze-thaw effects on microbial biomass and community composition

In microorganisms, freezing can solidify lipid membranes (Methe et al. 2005), and ice crystal formation can rupture the cell membrane (Rivkina et al. 2000), causing microbial biomass declines at the bulk soil level (Herrmann and Witter 2002, Larsen et al. 2002, Pesaro et al. 2003, Dörsch et al. 2004). In addition, it has been suggested that microbial biomass declines can be caused by changes to soil osmotic pressure, which can dehydrate cells (Jefferies et al. 2010), and prolonged freezing periods can lead to microbial starvation, because the remaining thin water films decrease O_2 diffusion and substrate availability (Clein and Schimel 1995). Soil microbes are particularly susceptible to freeze damage when active and growing (Schimel and Clein 1996).

Despite the potential for freezing damage, many soil microbes remain active during the winter (Grogan and Chapin 1999, Nobrega and Grogan 2007), with bacterial growth occurring down to -2 °C in the thin water films that remain on frozen soil particles (McMahon et al. 2009), and activity has been measured down to as low as -39 °C (Panikova et al. 2006). In the Arctic, microbial biomass increases during the winter (Buckeridge and Grogan 2008; Edwards and Jefferies 2012), which has been suggested to be due to accumulation of intracellular compatible solutes and other physiological adjustments (Jefferies et al., 2010). Furthermore, winter microbial activity and growth are

often greater under deep snow cover than under a relatively thin snowpack, because of the warmer soil temperatures associated with the former (Schimel et al. 2004, Nobrega and Grogan 2007, Buckeridge and Grogan 2008).

Decreases in snow cover can affect microbial activity and damage microbes by increasing the occurrence of soil freezing and freeze-thaw cycles; however, there has been considerable variation among studies with respect to the occurrence of soil microbial biomass resistance to soil freezing and freeze-thaw cycles (Lipson and Monson 1998, Groffman et al. 2001b, Lipson et al. 2002, Grogan et al. 2004, Koponen et al. 2006, Bell et al. 2010, Groffman et al. 2011). Two laboratory experiments conducted with arable and northern forest soils in Norway demonstrated that freeze-thaw cycles can decrease soil microbial biomass (Yanai et al. 2004, Bolter et al. 2005). In contrast, chamber experiments using soil from the Swedish Arctic and Finnish agricultural sites found microbial biomass to be resistant to moderate changes in winter soil temperatures (Grogan et al. 2004, Koponen et al. 2006). Similar lack of changes to microbial biomass in response to altered winter soil temperatures were found with studies using snow removal and overhead heaters in forest and temperate old field systems, respectively (Groffman et al. 2001b, Bell et al. 2010). Although variation among the results of these studies could reflect differences in soil microbial resistance to freezing among regions, the first two studies (e.g. Yanai et al. 2004, Bolter et al. 2005) used much more extreme freeze-thaw cycle amplitudes than the other studies, which suggests that methodological differences among studies may also have contributed to this variation (Henry 2007). In support of this suggestion, Lipson et al. (2000) found that alpine soil microbial biomass was unaffected by realistic freeze-thaw regimes, but decreased when more severe, but unrealistic, freezing regimes were used.

In laboratory studies, freeze-thaw has tended to shift microbial communities towards bacterial dominance (Larsen et al. 2002), because fungal populations are reduced with successive freeze-thaw cycles (Feng et al. 2007, Schmitt et al. 2008, Yergeau and Kowalchuk 2008). Feng et al (2007) hypothesized that bacteria are better competitors for soluble substrate following freeze-thaw, and therefore dominate in the soil afterwards. Schimel et al (2007) suggested that fungal population declines may be due to fungal

hyphal growth being sensitive to physical disruption of the soil structure caused by freezing. On the other hand, fungal populations may dominate soil under prolonged freezing conditions (Schadt et al. 2003, Buckeridge and Grogan 2008). Although there is no clear mechanism to explain the latter, it is suggested that fungi have the ability to tolerate low water potentials and are able to bridge gaps to tap spatially separated resources (Schimel et al. 2007).

Field studies have indicated that despite the potential for short-term microbial community change demonstrated in laboratory studies, soil freezing and freeze-thaw often have very little influence on total microbial, fungal or bacteria biomass in situ during the subsequent growing season (Groffman et al. 2001b, Bell et al. 2010). This lack of effect may result from the fast recovery times of soil microbial populations (Feng et al. 2007), the tendency for winter soil temperatures in the field to be less severe than those used in laboratory studies, or as Aanderud et al. (2012) suggested that the winter to spring transition acts as a 'reset period' for microbial communities, where winter dormant or suppressed bacteria begin to grow under warmer temperatures (Lennon and Jones 2011). However, changes in plant productivity and species richness can influence microbial biomass and microbial community structure and composition (Bardgett et al. 1999a, Hamilton and Frank 2001, Zak et al. 2003, De Deyn et al. 2011, Lamb et al. 2011), which suggests that soil freezing can potentially have indirect effects on microbial biomass and community structure in the field.

1.3.2 Freezing effects on soil nitrogen and trace gas losses

In addition to promoting root and microbial cell lysis, soil freezing can disrupt soil aggregates (Oztas and Fayetorbay 2003, Six et al. 2004) and accelerate litter break down (Harris and Safford 1996), increasing soluble N at a time when root activity is limited by temperature (Henry and Jefferies 2003), which potentially increases ecosystem N losses in the form of leachate or soil trace gas emissions (Groffman et al. 2006, Henry 2007, Joseph and Henry 2008, Matzner and Borken 2008). Factors such as vegetation type, and freezing amplitude and duration appear to influence the amount of N that becomes available both during and following soil freezing and freeze-thaw exposure (Austnes and

Vestgarden 2008, Elliott and Henry 2009, Vestgarden and Austnes 2009). However, enhanced soil N availability and trace gas losses do not always continue with repeated freeze-thaw exposure (Schimel and Clein 1996, Koponen et al. 2004), suggesting the size of the N pool susceptible to freezing-induced losses may be limited (Schimel and Clein 1996, Herrmann and Witter 2002).

In forest ecosystems, increases in soil NO_3^- leaching during the growing season have been observed following experimental snow removal and winters when severe soil frosts have occurred (Boutin and Robitaille 1995, Fitzhugh et al. 2001, Callesen et al. 2007). These increased N losses have not typically corresponded with changes in microbial biomass, net mineralization, and nitrification during the growing season, (Groffman et al. 2001b, Hentschel et al. 2009), but are likely caused by decreased N uptake by frost damaged roots (Matzner and Borken 2008). Similarly, a snow removal experiment conducted in agricultural system demonstrated increases in NO_3^- pool sizes (Maljanen et al. 2007), but at much smaller amounts in the spring time than observed in forest systems (Blankinship and Hart 2012). The mechanisms underlying these NO_3^- concentration differences between systems is unclear, but intraspecific variation in plant responses to freezing may affect recovery times (Kreyling et al. 2011b). In addition, Blankinship and Hart (2012) hypothesized that differences in plant N demand (Bilbrough et al. 2000, Grogan et al. 2004, Larsen et al. 2012) or productivity between woody and non-woody species early in the growing season (Jonas et al. 2008, Wipf and Rixen 2010), may influence N losses following freezing. Interestingly, soil freezing does not always increase N losses during the growing season, despite similar soil freezing temperatures among years. It has been hypothesized that years of high soil N retention coincide with high release of dissolved organic carbon (DOC), which may cause soil microbes to immobilize nutrients (Groffman et al. 2011).

Laboratory experiments have revealed that pulses of CO_2 and N_2O from soil increases following freeze-thaw (Priemé and Christensen 2001, Muller et al. 2002), however, field experiments have shown inconsistent responses of CO_2 fluxes to soil freezing (Dörsch et al. 2004, Groffman et al. 2006, Oquist and Laudon 2008, Groffman et al. 2011), which may result from differences in the intensity of soil freezing exposure, rather than inherent

differences among study sites (Blankinship and Hart 2012). CO_2 respiration rates immediately after snowmelt may be limited by frozen soils and cold temperatures (Groffman et al. 2006). Nevertheless, once soil temperatures rise and the soil thaws, a large pulse of CO_2 release usually occurs (Dörsch et al. 2004, Maljanen et al. 2007), which corresponds with increases in labile carbon (C) substrates available to microbes (Neilson et al. 2001). These pulses are usually short-lived (Maljanen et al. 2007), and overall decreases in CO_2 efflux during the growing season are often observed in plots that experience enhanced soil freezing (Blankinship and Hart 2012). Blankenship and Hart (2012) suggested that soil freezing may create microbial C limitation (Lipson et al. 2000, Fierer et al. 2009, Brooks et al. 2011); however, others have hypothesized that changes to the microbial structure and community (Schmitt et al. 2008), as well as a lack of heterotrophic microbial recovery following soil freezing (Muhr et al. 2009), or potentially due to freeze damage decreasing root growth and function. Although only a limited number of studies have been conducted, these effects on soil CO_2 respiration appear to only last through the growing season immediately following the soil freezing event (Muhr et al. 2009).

N_2O fluxes measured in the field have been consistent with lab studies, with increases in N_2O fluxes following soil freezing across all ecosystem types examined (Priemé and Christensen 2001, Muller et al. 2003, Dörsch et al. 2004). These pulses are likely driven by high soil water content and increases in inorganic N availability following soil freezing (Muller et al 2004). However, similar to CO_2 emissions, increased N_2O emissions after snowmelt only occur over a short period (Yanai et al. 2004, Groffman et al. 2006, Goldberg et al. 2010), and then quickly dissipate when the large differences in soil water content between ambient and soil freezing plots diminish.

1.4 Nitrogen deposition in temperate regions

1.4.1 Nitrogen deposition inputs into ecosystems

Coinciding with climate warming, many temperate regions will also experience increased atmospheric N deposition over the next century (Vitousek et al. 1997). Atmospheric N

deposition consists mainly of N oxides (NO_X=NO + NO_2), a product of fossil fuel consumption, and ammonia (NH_3), a product of fertilizer use. Once in the atmosphere, the N oxides are transformed to nitric acid (HNO_3), nitrates (NO_3^-) and organic compounds, such as peroxyacetyle nitrate (PAN), while NH_3 is transformed to ammonium (NH_4^+) and transferred to aquatic and terrestrial ecosystems through wet (rain or snowfall) and dry (dust) deposition. Total N deposition is expected to increase from a rate of 1 to 2 g m^{-2} y^{-1} to a rate of 2 to 5 g m^{-2} y^{-1} in northeastern temperate regions of North America by the year 2050 (Galloway et al. 2004), with areas located in the vicinity of intense industrial or agricultural activity experiencing the highest N deposition rates. Because many ecosystems are N-limited, these increased N inputs are expected to alter plant and soil dynamics over the next century (Vitousek and Howarth 1991).

1.4.2 Nitrogen deposition effects on plants

Increased N deposition in the short term usually enhances plant productivity (Vitousek and Howarth 1991, LeBauer and Treseder 2008), increases shoot to root ratio (Tilman and Cowan 1989), and changes the C to N ratio of many plant species (Wedin and Tilman 1996, Aber et al. 1998). When N accumulates in the system, plants are lost through competitive exclusion resulting from the expansion of nitrophilous species (Bobbink and Willems 1987, Bobbink et al. 1988), leading to changes in species composition (Wedin and Tilman 1996, Bobbink et al. 2010), species richness declines (Stevens et al. 2004, Clark and Tilman 2008) and reductions in plant species diversity (Tilman 1987, Tilman 1996). Over the longer term, N deposition leads to soil N saturation, increasing N losses through enhanced leaching and denitrification (Aber et al. 1995). In addition, increased N losses decrease soil base cation availability, which lowers soil pH (Boxman et al 1995, Emmett et al. 1995) and decreases plant productivity (Aber et al. 1989).

1.4.3 Nitrogen deposition effects on soil

Many studies have shown contrasting results regarding long term N addition effects on microbial biomass (Bardgett et al. 1999b, Zeglin et al. 2007, Allison et al. 2008, Allison and Martiny 2008), suggesting that other environmental factors such as limitations to

water, C, and phosphorus (P) could be at play (Johnson et al. 1998, Wardle 2008). A recent meta-analysis concluded that long term N additions reduce microbial biomass (Treseder 2008), most likely as a result of fungal biomass declines (Henriksen and Breland 1999, Frey et al. 2004, Treseder 2008). Treseder (2008) suggested that the declines in fungal biomass seen in long term N addition experiments were caused by the progressive inhibition of growth and liginase activity of white rot fungi (Ander and Eriksson 1977, Keyser et al. 1978, Waldrop and Zak 2006), as well as the loss of mychorrizal fungi, because plants allocate less C to roots under N addition. In contrast, bacterial populations appear to be unaffected by short and long term N additions (Treseder 2008).

The effects of N addition on soil CO_2 emissions have varied; some studies have found decreased soil CO_2 respiration (Bowden et al. 2000, Butnor et al. 2003), while others have observed no difference (Ambus and Robertson 2006).

1.5 Interactive effects of soil freezing and nitrogen deposition

Although climate warming and N deposition are two environmental variables that are expected to play a pivotal role in affecting ecosystem plant and soil dynamics over the next century, their potential interactions remain unclear (Hungate et al. 2003). In particular, changes in winter soil temperatures may influence ecosystem responses to N deposition (Hutchison and Henry 2010) over both the short and long term. For example, increases in winter soil N losses caused by soil freezing, coupled with decreased N uptake by frost damaged roots, may limit plant productivity under current rates of N deposition. While plant productivity often increases in response to increased N deposition, freezing damage to roots may limit the ability of plants to exploit this additional source of N, resulting in a reduced response of plant productivity to N deposition (Figure 1.1). In addition, the combined effect of frost and increased N deposition may exacerbate soil N losses, increasing leaching losses from terrestrial ecosystems to aquatic systems (Figure 1.1).

Testing the above conceptual model, whereby soil freezing and increased N deposition may interact to alter ecosystem N dynamics, was the motivation for a warming and N addition field experiment conducted in a temperate old field by Dr. Hugh Henry's

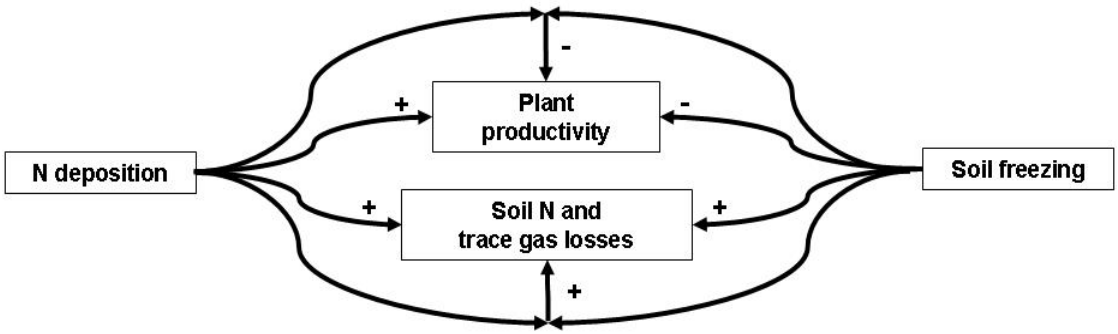

Figure 1.1: The combined and interactive effects of soil freezing and atmospheric N deposition on plant productivity and soil solution N and trace gas losses.

research group at Western University over the past seven years. Although this experiment has revealed an important role of winter weather in influencing both soil N dynamics and primary production over the summer (Hutchison and Henry 2010, Turner and Henry 2010), it has not been useful for testing several other important aspects of this model.

First, while increased soil freezing can enhance N losses, it is unclear which ecosystem N pools are most vulnerable to losses in response to freezing, and when. In particular, the effects of freezing on N derived from N currently in organic form (e.g. from live plants, plant litter, microorganisms and soil organic matter) may differ substantially from the direct losses of N from atmospheric deposition. Second, while the experimental warming indeed increased the frequency of soil freeze-thaw cycles in the experiment, the effect on freezing severity (minimum temperature) was relatively mild and not intense enough to cause root damage (Malyshev and Henry 2012a). Therefore, the interactive effects of freezing damage and N addition, both in the short term and over subsequent growing seasons, has remained untested in this study system. In addition, while laboratory experiments based on soils in other systems have shown considerable effects of freezing on microbial structure and populations in the short term (Schimel and Clein 1996, Sharma et al. 2006, Walker et al. 2006), the examination of longer term effects and experiments under natural conditions are uncommon (Schimel et al. 2007, Borken and Matzner 2009, Aanderud et al. 2012). Finally, short and long term soil N availability, microbial biomass and community structure have not been explored in the context of possible changes in plant productivity and relative species abundances in response to freezing. In particular, changes to soil N availability may continue over multiple growing seasons if soil freezing effects on plant productivity or relative species abundance last more than one growing season.

1.6 Goal and hypotheses

The goal of my thesis was to examine the combined and possibly interactive effects of freezing damage and N deposition on plant and soil N dynamics over multiple years. I hypothesized that (i) while increased N from atmospheric deposition has the potential to increase plant productivity, freezing can counteract this effect by increasing plant damage

and soil N losses, and (ii) significant effects of freezing on plant productivity and microbial biomass will only persist over multiple years if there are changes in plant relative species abundance. I tested my hypothesis by completing the following objectives:

Objective 1: To explore seasonal variation in N retention among different N pools (live plant, plant litter, soil, and simulated N deposition) in response to soil freezing and N addition.

Prediction 1: I predicted that snow removal would increase ^{15}N losses from roots and soil over winter, and that N addition would increase soil ^{15}N losses over the winter and summer. With respect to the short term uptake of ^{15}N pulses, based on the expectation that soil freezing would damage plant roots, I predicted snow removal would decrease ^{15}N interception at snow melt and at peak biomass, and that N addition would exacerbate these losses.

Objective 2: To investigate the effects of a single winter of intense soil freezing (e.g. of sufficient magnitude to damage plants) on the responses of plant productivity and relative species abundance to N addition over two years.

Prediction 2: I predicted that snow removal would both decrease plant productivity and decrease the ability of plant productivity to respond to N addition based on the expectation that it would damage plant roots. However, I also predicted that legacy effects of freezing would only occur in the second growing season if there were significant changes in plant relative species abundance.

Objective 3: To investigate the combined effects of a single year of intense soil freezing and N addition on extractable C and N pools, potential trace gas losses, microbial biomass C and N pools, and fungal and bacterial biomass, over the subsequent two years.

Prediction 3: I predicted that both soil freezing and N addition would increase extractable soil inorganic N pools and potential trace gas losses. In addition, based on the observation that soil microbes have fast recovery times and are generally unresponsive to short term N addition, I predicted that neither treatment would affect microbial biomass C

and N pools and bacterial and fungal biomass unless there were changes in plant relative species abundance.

1.7 Thesis organization

My thesis is written in the integrated article format, and contains three manuscripts. The first chapter is an introductory chapter that provides an overview of the literature related to the topics of soil freezing and N deposition. Chapter 2 (manuscript) addresses Objective 1 by exploring seasonal variation in N retention among different N pools (live plant, plant litter, soil, and N deposition) in response to soil freezing. Chapter 3 (manuscript) addresses Objective 2 by investigating the effects of freezing damage on plant productivity and relative species abundance under N addition equal to current and future rates of N deposition over two growing seasons. Chapter 4 (manuscript) addresses Objective 3 by investigating the interactive effects of intense soil freezing and N addition on extractable soil inorganic and organic C and N pools, potential trace gas emissions, microbial biomass C and N pools and bacterial and fungal biomass over two years. Finally, Chapter 5 is a general discussion that synthesizes the results from all of the chapters and provides ideas for future research.

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2 Chapter 2: Combined effects of soil freezing and N addition on losses and interception of N over winter and summer[1]

2.1 Introduction

Climate warming over the next century is expected to intensify during the winter with increasing latitude (IPCC 2007). In northern temperate regions, warming will increase the ratio of rain to snow over winter (Feng and Hu 2007) and reduce snow cover (Hayhoe et al. 2007, IPCC 2007). Decreased snow cover is expected to alter soil freezing dynamics in many of these regions, and in some cases may increase soil freezing severity (Groffman et al. 2001a, Zhao et al. 2004, Mellander et al. 2007, Henry 2008). These changes in the timing, intensity, and dynamics of soil freezing can exert strong influences over plant and soil processes (Fitzhugh et al. 2001, Kreyling et al. 2008b, Turner and Henry 2009, Hutchison and Henry 2010).

In many northern temperate regions, climate driven changes in nitrogen (N) cycling over the coming decades will occur in the context of increasing atmospheric N deposition (Galloway et al. 2004). N deposition often increases plant productivity, at least over the short term (Vitousek and Howarth 1991, Hutchison and Henry 2010), but it can enhance N leaching losses, particularly when N no longer limits plant growth (Vitousek et al. 1997, Aber et al. 1998, Turner and Henry 2009). While climate warming and increased atmospheric N deposition can both strongly influence plant productivity and ecosystem N cycling, their interactions nevertheless remain unclear (Hungate et al. 2003). Extreme soil freezing and freeze-thaw events can affect soil N directly by lysing soil microbes (Skogland et al. 1988, Larsen et al. 2002), disrupting soil aggregates (Oztas and Fayetorbay 2003, Six et al. 2004) and damaging roots (Tierney et al. 2001, Cleavitt et al. 2008, Gaul et al. 2008). Litter breakdown can also be accelerated by freeze-thaw cycles (Taylor and Parkinson 1988, Harris and Safford 1996), although most of these losses may

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occur in response to the first freeze rather than subsequent cycles (Taylor and Parkinson 1988), and in the field a high proportion of cold season mass loss from fresh litter may occur in late autumn, with little additional mass loss over winter (Bokhorst et al. 2010). Increases in soluble N in soil over winter occur at a time when cold temperatures limit plant N uptake (Henry and Jefferies 2003), which can increase N losses over winter (Matzner and Borken 2008). N losses can be particularly high during snowmelt (Brooks et al. 1998), and N deposition that has accumulated in the snowpack can contribute to these losses. Alternatively, freeze damage to roots might enhance N losses indirectly by decreasing the capacity for plant N uptake over the growing season (Fitzhugh et al. 2001). These reductions in the capacity for ecosystems to retain increased N deposition over summer could outweigh the direct effects of freezing on N losses over winter.

Studies documenting increased ecosystem N losses in response to soil freezing (e.g., Fitzhugh and others 2001) have typically not been able to distinguish between the direct and indirect effects of plant and soil freezing damage, nor has it been clear what N pools are contributing to these losses. Moreover, the use of overhead heaters in field experiments often increases the frequency of soil freeze thaw cycles, but not their intensity (Hutchison and Henry 2010), and mild soil freezing typically causes little damage to plants and microbes (Henry 2007). Therefore, combined studies of winter warming and N addition (e.g., Turner and Henry 2009) have not adequately addressed possible interactions between soil freezing damage and N deposition. Although increased climate variability over winter is expected to decrease the frequency of cold temperature events and increase the occurrence of warm temperature events at this time (Meehl et al. 2000), extreme warming events over winter can expose soils to severe freezing if these warming events are followed by hard frosts in the absence of further snowfall (Bokhorst et al. 2011).

I conducted a field study to investigate ^{15}N losses from plants, litter, and soil in response to the combined effects of enhanced soil freezing (via snow removal) and N addition. I placed a specific emphasis on comparing the extent to which the treatments enhanced the losses of ^{15}N that was already incorporated in the system (e.g., in plants, litter, and soil) *versus* the extent to which they affected the short term interception of ^{15}N pulses designed

to simulate atmospheric N deposition. Based on the observation that enhanced soil freezing can increase root damage and microbial lysis, I predicted that snow removal would increase ^{15}N losses from roots and soil over winter. In addition, based on the observation that added N can cause soil N saturation, I predicted that N addition would increase soil ^{15}N losses over the winter and summer. With respect to the short term uptake of ^{15}N pulses, based on the expectation that root systems would be damaged, I predicted that snow removal would decrease ^{15}N interception at snow melt and at peak biomass, and that N addition would exacerbate these losses.

2.2 Methods

2.2.1 Site description

The study was conducted at an old field site in London, Ontario, Canada (43°01 46 N, 81°12 52 W). The site was previously an agricultural field, but has not been plowed, mowed, or fertilized for at least 27 years. Mean daily air temperature at the site was 7.5 ^{o}C, with a low daily monthly mean of -6.3 ^{o}C (January), a high monthly mean of 20.5 ^{o}C (July), and an average annual precipitation of 818 mm (Canadian Climate Normals 1971-2000, Environment Canada, National Climate Data and Information Archive). Snowfall generally begins in early to mid December with snow melt occurring by mid to late March. Vegetation at the site was dominated by the perennial grasses *Poa pratensis* L. (Kentucky blue grass) and *Bromus inermis* Leyss. (smooth brome). The soil was classified as a well to imperfect drained silt loam glacial till (Hagerty and Kingston 1992), and had an average pH of 7.5 (Bell and Henry 2010).

2.2.2 Experimental design

The experimental design consisted of snow removal and ambient snow treatments, crossed in a factorial design with two levels of N (addition of 0 and 6 g N m^{-2} y^{-1}). Treatments were randomly assigned to 1.69 m × 1.69 m plots within six blocks (n=6 for each treatment combination). The N addition plots received 2 g N m^{-2} of NH_4NO_3 as an aqueous solution on three dates in 2010 (7 June, 19 August, and 18 October) and three dates in 2011 (5 April, 9 June, and 4 October). These addition rates were designed to

approximate projected increases in atmospheric deposition in this region by the year 2050 (Galloway et al., 2004). Each plot contained four circular 30 cm diameter sub-plots used for ^{15}N tracer addition separated by at least 50 cm. Soil temperature sensors (Maxim: Ibutton DS1922L-F5) were placed at 5 cm depth (n=2-3 per treatment combination), and data were logged hourly. Gravimetric water content was measured immediately after snowmelt and two weeks later by taking 2 cm diameter × 12 cm deep soil cores and drying them at 65 ºC for at least three d, to assess treatment effects on soil moisture.

2.2.3 Snow removal

In order to minimize litter disturbance during snow removal, I placed white plastic mesh with 2 × 2 cm spacing (Winter Wrap; Quest Plastic Limited) on top of the vegetation and secured it to the ground just prior to snowfall in early December 2010 (I also added mesh to the ambient snow plots). I removed snow to a depth of 2 cm following each snowfall event from December 2010 to mid-February 2011. Ambient plots accumulated snow naturally all winter, while snow removal plots were allowed to accumulate snow starting in mid-February, in order to minimize differences in soil moisture among snow removal and ambient snow plots in spring.

2.2.4 ^{15}N addition

I applied 250 mL of a 98% enriched $^{15}NH_4{}^{15}NO_3$ solution (0.1 g m^{-2} of N) on 17 May 2010 to the surfaces of two of the 30 cm diameter sub-plots per main plot. I applied this ^{15}N in order to obtain labeled plant biomass, litter, roots, and soil by the end of the plant growing season (initial conditions), allowing N losses from these pools to be tracked over the following winter (over-winter effects) and summer (over-summer effects) (refer to the following section). For the two remaining sub-plots in each plot, I added the same quantity of $^{15}NH_4{}^{15}NO_3$ following snowmelt on 10 April 2011, or at peak plant biomass on 15 June 2011, to simulate N deposition.

2.2.5 Losses of the ^{15}N label from plants, litter, and soil over winter and summer

In the first set of sub-plots, I sampled *Poa pratensis* plant and litter vegetation on 30 November 2010 to establish baseline levels of ^{15}N enrichment. I then resampled the sub-plots immediately after snowmelt on 4 April 2011 to assess over-winter ^{15}N losses, and again on 25 November 2011 (using 4 April 2011 as initial conditions) to assess over-summer ^{15}N losses. For each sampling period, I clipped aboveground vegetation at the soil surface from a 10 cm × 10 cm area, while roots and soil were sampled four to five times within the 10 cm × 10 cm area using a 2 cm diameter corer to a depth of 12 cm.

2.2.6 Interception of simulated N deposition at snowmelt and peak biomass

I sampled plants, litter, roots, and soil from the remaining sub-plots on 17 April 2011 and 22 June 2011, one week after the ^{15}N was added to simulate N deposition. Sampling was performed as described in the previous section.

2.2.7 Plant tissue and soil collection and processing

Within 24 h of sampling, I sorted aboveground vegetation samples into live (green) shoots and litter (brown/senesced leaves). I weighed all soil cores for bulk density measurements and removed the roots. I then rinsed the roots twice in water to remove attached soil and immersed the roots in 0.005 M K_2SO_4 for 5 mins to remove adhering ^{15}N label from exterior surfaces (Kielland 1997). In addition, I dried soil sub-samples (~10 g) at approx. 65 ºC for at least three d to determine gravimetric moisture content. I oven dried aboveground biomass and roots at 65 ^{o}C for 3-5 d.

2.2.8 ^{15}N analyses

I ground oven dried shoots, litter, roots and soil separately for about three mins to a fine powder using a ball mill (SPEX SamplePrep Model 2000 Geno/Grinder, Metuchen, New Jersey, US), weighed out subsamples (~5 mg and ~20 mg for all vegetation components

and soil, respectively) into tinfoil containers (4 × 6 mm, Costech Analytical Technologies, Valencia, CA), and sent the samples for ^{15}N and total N analyses to the University of California at Davis Isotope Laboratory. ^{15}N analyses were performed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

2.2.9 Data analyses

For each sampling period, I subtracted the proportion of ^{15}N in unenriched samples from the proportion of ^{15}N in samples from treated plots for shoots, litter, roots, and soil, then multiplied these differences by total N in the respective pools to estimate the quantity of added ^{15}N (g/m^2 above natural abundance) in each pool. Percent changes over winter and over summer were then calculated by dividing the difference between final excess ^{15}N and initial excess ^{15}N by the initial ^{15}N for the relevant time interval and multiplying by 100. I tested for significant effects of snow removal, N addition, and their interaction on ^{15}N losses from shoots, litter, roots and soil, respectively, using two-way analyses of variance (ANOVAs) (JMP 4.0, SAS Institute). I used separate ANOVAs to test for losses of the ^{15}N label over winter and over the summer. I also used two-way ANOVAs to test for treatment effects on the interception of simulated N deposition in each of the N pools at snowmelt and peak biomass, and to examine treatment effects on the mass of each N pool at each sampling period. I checked all distributions for normality and performed log transformations when necessary.

2.3 Results

2.3.1 Snow removal effects on soil microclimate

There was a strong effect of snow removal on soil temperature over the treatment period relative to that of ambient plots (Figure 2.1) with respect to average temperature (~0.3 °C *vs.* ~1.5 °C), minimum temperature (-3.1 °C *vs.* 0.7 °C), degree days sum >0 °C (35 *vs.* 135) and number of freeze-thaw cycles (5 *vs.* 0) at 5 cm depth. The snow removal treatment had no significant effects on soil moisture measured immediately after snowmelt and two weeks later ($P = 0.85$ and $P = 0.37$, respectively).

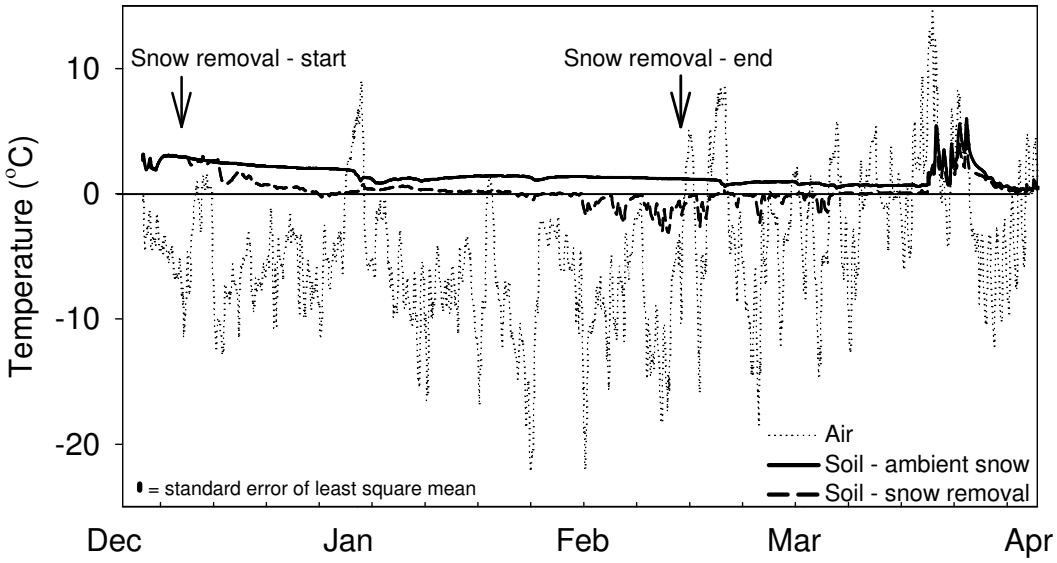

Figure 2.1: Mean air temperature and hourly soil temperature under ambient snow and snow removal plots from early December 2010 to early April 2011 at 5 cm soil depth (nitrogen treatments had no effect on winter soil temperature and thus were pooled). Vertical line indicates magnitude of pooled standard error for soil temperatures in ambient and snow removal plots.

2.3.2 ^{15}N losses from plants, litter, and soil

Approximately 40% of the ^{15}N added in spring 2010 was recovered in fall 2010 (prior to snow removal), with approximately 2%, 3.5%, 4% and 30% recovered in the shoots, litter, roots and soil, respectively. Root ^{15}N enrichment was highest in the N addition plots (P = 0.035), primarily as a result of these plots having ~1.4× more root biomass than ambient N plots (P = 0.040; Table 2.1). Total ^{15}N recovery in shoots, litter, and soil did not change significantly with N addition, despite there being ~1.4× higher shoot and litter mass in these plots relative to ambient N plots (P = 0.010 and P = 0.005, respectively).

Snow removal resulted in almost 4× more total ^{15}N losses than in ambient plots over winter (35% *vs.* 8%, P = 0.004; Figure 2.2a). Snow removal also increased root ^{15}N losses over winter (Figure 2.2a), which was associated with a decline in root biomass (P = 0.007; Table 2.1). Neither snow removal nor N addition had significant effects on ^{15}N losses from shoots or litter over winter.

Over the following summer, total ^{15}N losses were ~5% on average, with no significant treatment effects (Figure 2.2b). However, there was a marginally significant trend of enhanced shoot ^{15}N in snow removal plots (P=0.056), and for root ^{15}N there was a significant interaction between snow removal and N addition (Figure 2.2b), largely explained by a corresponding interactive effect on ^{15}N concentrations (P = 0.019; Table 2.2).

2.3.3 Interception of ^{15}N pulses

At snowmelt, N addition significantly reduced the total interception of added ^{15}N (P = 0.052), primarily as a result of ^{15}N interception by soil, which was 39% on average in ambient N plots, but only 27% in N addition plots (Figure 2.3a). Soil ^{15}N interception was not affected significantly by snow removal. Snow removal reduced root biomass, (P = 0.035), but had no effect on total ^{15}N interception by roots (Figure 2.3a), because ^{15}N concentration was enhanced in these roots (P = 0.039; Table 2.3).

Table 2.1: Biomass (g dw m^{-2}) for plant shoots, litter, and roots (all species pooled) in fall 2010, spring 2011, and fall 2011 for each treatment combination. Means within columns are not significantly different if they share a common lowercase letter (Tukey's test P <0.05). Parentheses indicate standard error (n=6).

	Fall 2010						**Spring 2011**						**Fall 2011**					
	Shoots		Litter		Roots		Shoots		Litter		Roots		Shoots		Litter		Roots	
Ambient	74^{b}	(6)	167^{a}	(19)	285^{a}	(57)	16^{a}	(4)	96^{a}	(12)	212^{b}	(21)	105^{a}	(9)	376^{a}	(31)	379^{ab}	(33)
Ambient + N	108^{a}	(13)	250^{a}	(41)	445^{a}	(50)	23^{a}	(7)	122^{a}	(29)	348^{a}	(64)	106^{a}	(25)	467^{a}	(41)	423^{ab}	(83)
Snow removal	81^{ab}	(2)	176^{a}	(17)	293^{a}	(31)	11^{a}	(2)	104^{a}	(23)	165^{b}	(13)	75^{a}	(11)	167^{b}	(19)	251^{b}	(19)
Snow removal + N	101^{ab}	(10)	235^{a}	(39)	338^{a}	(47)	15^{a}	(4)	98^{a}	(19)	184^{b}	(13)	111^{a}	(13)	493^{a}	(42)	415^{a}	(33)

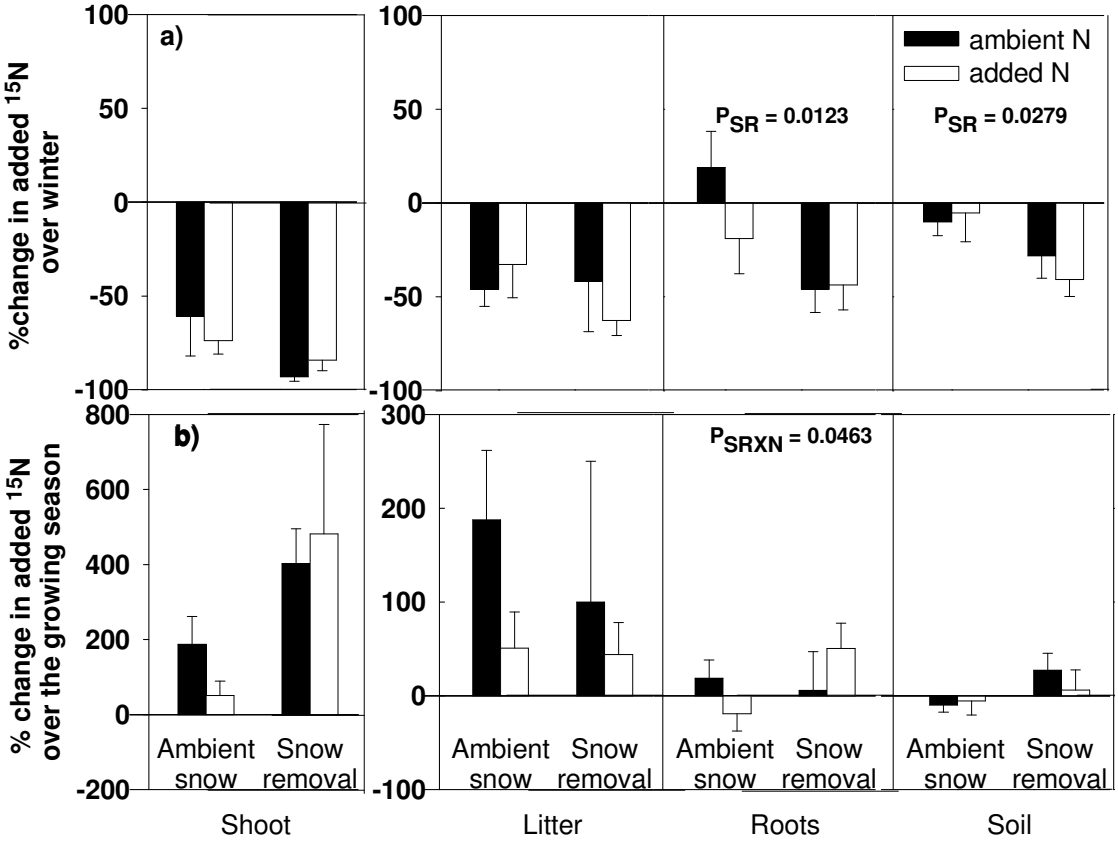

Figure 2.2: a) Over-winter and b) over-summer percent changes in ^{15}N enrichment in *P. pratensis* shoots and litter, roots (all species pooled), and bulk soil. Error bars represent standard error (n = 6 for each treatment combination). Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen).

Table 2.2: ^{15}N concentrations (μg/g dw) in shoots, litter, roots (all species pooled), and soil in fall 2010, spring 2011, and fall 2011 for each treatment combination. Means within columns are not significantly different if they share a common lowercase letter (Tukey's test, P <0.05). Parentheses indicate standard error (n=6).

	Fall 2010								**Spring 2011**								**Fall 2011**							
	Shoots		Litter		Roots		Soil		Shoots		Litter		Roots		Soil		Shoots		Litter		Roots		Soil	
Ambient	31[a]	(4)	25[a]	(3)	11[a]	(2)	0.04[a]	(<0.01)	37[a]	(6)	17[a]	(4)	15[a]	(3)	0.04[a]	(0.01)	10[a]	(1)	5[a]	(1)	9[a]	(1)	0.04[a]	(<0.01)
Ambient + N	21[a]	(3)	20[a]	(3)	12[a]	(2)	0.03[a]	(<0.01)	25[ab]	(3)	17[a]	(2)	12[a]	(2)	0.04[a]	(0.01)	6[a]	(1)	3[a]	(1)	5[a]	(1)	0.04[a]	(0.01)
Snow removal	29[a]	(3)	21[a]	(1)	13[a]	(2)	0.04[a]	(<0.01)	14[b]	(3)	14[a]	(3)	9[a]	(2)	0.04[a]	(0.01)	9[a]	(2)	4[a]	(1)	6[a]	(2)	0.03[a]	(0.01)
Snow removal + N	23[a]	(3)	20[a]	(2)	15[a]	(3)	0.04[a]	(0.01)	18[ab]	(2)	15[a]	(3)	12[a]	(1)	0.03[a]	(<0.01)	7[a]	(1)	3[a]	(1)	8[a]	(1)	0.03[a]	(<0.01)

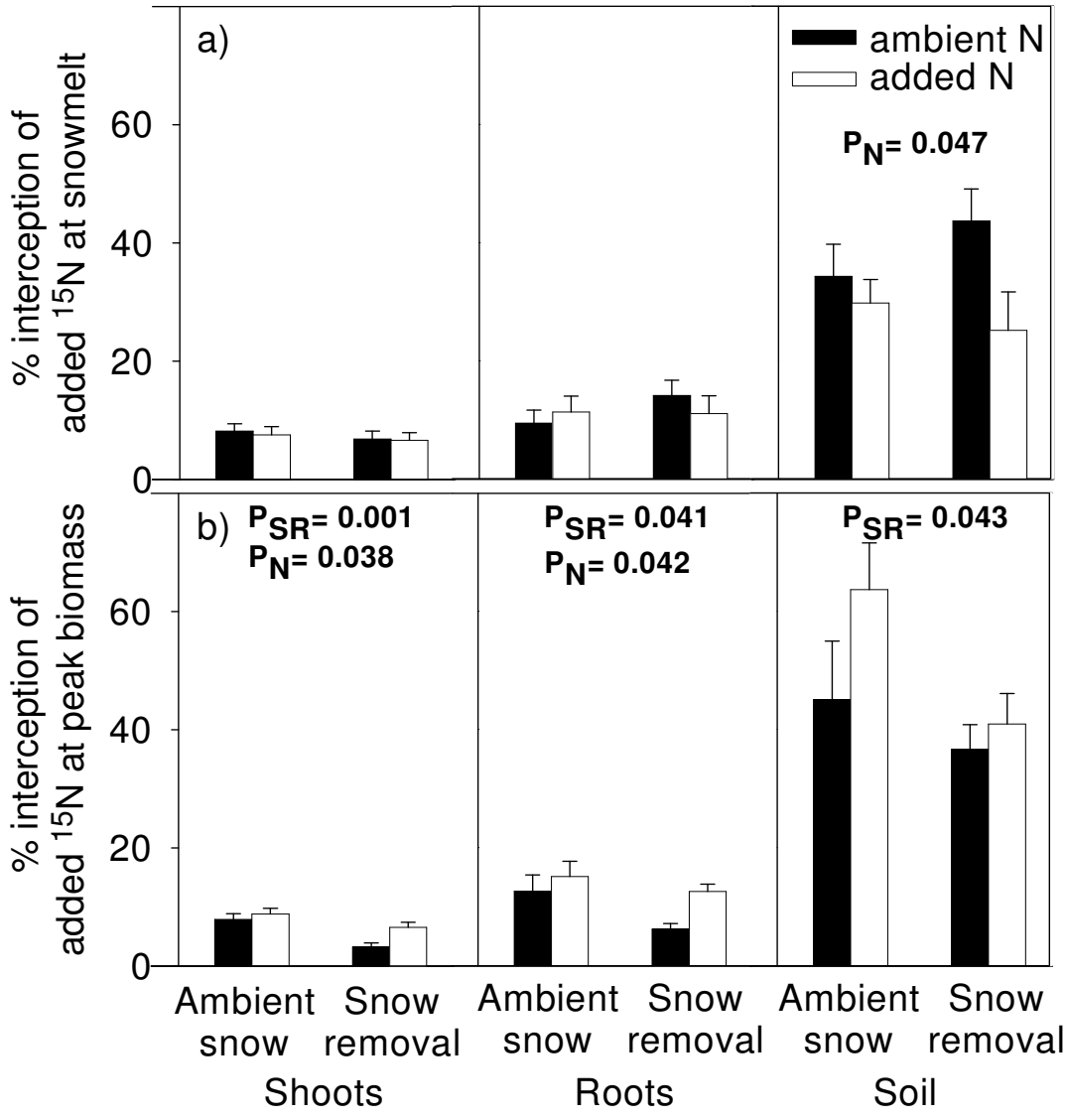

Figure 2.3: Percent ^{15}N intercepted at a) snowmelt and b) peak biomass by shoots, roots (all species pooled), and bulk soil. Error bars represent standard error (n = 6 for each treatment combination). Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen).

Table 2.3: ^{15}N concentrations (µg/g dw) in shoots, roots (all species pooled), and soil at snowmelt 2011 and peak biomass 2011 for each treatment combination. Means within columns are not significantly different if they share a common lowercase letter (Tukey's test, P <0.05). Parentheses indicate standard error (n=6).

	Snowmelt 2011			**Peak biomass 2011**		
	Shoots	Roots	Soil	Shoots	Roots	Soil
Ambient	391[a] (55)	36[a] (6)	0.05[a] (0.01)	47[a] (5)	43[a] (12)	0.07[a] (0.02)
Ambient + N	365[a] (32)	34[a] (4)	0.05[a] (0.01)	24[b] (3)	40[a] (10)	0.10[a] (0.01)
Snow removal	603[a] (88)	70[a] (14)	0.07[a] (0.01)	31[b] (3)	43[a] (13)	0.06[a] (0.01)
Snow removal + N	395[a] (72)	57[a] (15)	0.04[a] (0.01)	31[b] (2)	38[a] (3)	0.07[a] (0.01)

At peak biomass, snow removal reduced total ^{15}N interception by approximately 1.5× compared to control plots (P = 0.007); however, N addition enhanced ^{15}N interception (P = 0.033). Snow removal reduced the interception of ^{15}N found in shoots from 8% on average to 5% (Figure 2.3b), which was associated with lower shoot biomass in the snow removal plots (P = 0.0002), whereas N addition had the opposite effect of significantly enhancing the interception of ^{15}N found in shoots from 5% on average to 8% on average (Figure 2.3b), which was associated with larger shoot biomass in N addition plots (P <0.001; Table 2.4). Similarly, interception of ^{15}N by roots was reduced significantly from 14% on average in ambient snow plots to 9% in the snow removal plots (Figure 2.3b), which was associated with lower root biomass in snow removal plots (P = 0.044), and N addition enhanced root ^{15}N interception on average from 9% in the control plots to 14% in the N addition plots (Figure 2.3b), which was associated with larger biomass in the N addition plots (P = 0.030; Table 2.4). Snow removal also reduced soil ^{15}N interception, from an average of 54% down to 39% (Figure 2.3b).

2.4 Discussion

2.4.1 Snow removal effects on the retention and interception of N

Consistent with my prediction, snow removal increased ^{15}N label losses from roots and soil over winter. Increased leaching and trace gas losses in response to severe soil freezing have often been attributed to increased soluble N caused by the lysis of root and microbial cells (Fitzhugh et al. 2001, Austnes and Vestgarden 2008). While soil temperatures in the snow removal treatment decreased on average to as low as -3.1 ºC at 5 cm depth, which is sufficient to damage grass roots in my system (Malyshev and Henry 2012a), soil microorganisms appear to be relatively insensitive to freezing-induced lysis at these temperatures at my site (Elliott and Henry 2009). However, diminished water film availability, leading to reduced O_2 and substrate diffusion in frozen soils (Clein and Schimel 1995) could lead to microbial starvation (Austnes and Vestgarden 2008). Losses of ^{15}N from aboveground litter were not significantly affected by snow

Table 2.4: Biomass (g dw m^{-2}) of plant shoots and roots (all species pooled) at snowmelt 2011 and peak biomass 2011 for each treatment combination. Means within columns are not significantly different if they share a common lowercase letter (Tukey's test $P < 0.05$). Parentheses indicate standard error (n=6).

	Snowmelt 2011		**Peak biomass 2011**	
	Shoots	Roots	Shoots	Roots
Ambient	21^{a} (3)	247^{a} (32)	168^{b} (12)	315^{a} (27)
Ambient + N	20^{a} (3)	323^{a} (62)	363^{a} (39)	471^{a} (124)
Snow removal	11^{b} (1)	213^{a} (14)	106^{c} (21)	176^{b} (29)
Snow removal + N	17^{ab} (2)	193^{a} (22)	198^{b} (17)	327^{a} (19)

removal, which is consistent with reduced microbial decomposition of the litter layer over winter (Clein and Schimel 1995, Bokhorst et al. 2010).

Contrary to my prediction, snow removal did not decrease the interception of ^{15}N added at snowmelt. My prediction had been based on the assumption that the plant root systems in snow removal plots would be damaged during the winter, thus reducing their capacity for N uptake in the spring, as has been observed in forests (Tierney et al. 2001, Gaul et al. 2008). While root biomass was indeed reduced in these plots, the increased ^{15}N enrichment in the roots of these plots compensated for the reduced root biomass, such that the total interception of ^{15}N by roots did not differ from that of ambient snow plots.

It has been suggested that N uptake by grass roots in early spring, while possibly benefiting future tiller growth, may be sufficiently low that it does not substantially reduce soil N losses at snowmelt (Malyshev and Henry 2012a), at time when soluble N pools are very high (Brooks et al. 1998). Therefore, it appears to be the quantity of soluble N liberated over winter, not the ability of the system to retain N, that primarily determines total N retention in my system. This lack of an effect of snow removal on N interception in spring is consistent with the observation by Blankinship and Hart (2012) that non-forest systems may differ substantially from the forest systems (Boutin and Robitaille 1995, Fitzhugh et al. 2001, Callesen et al. 2007, Matzner and Borken 2008) that have been used extensively to model ecosystem responses to freezing. With respect to possible differences in the dominant mechanisms operating in non-forest systems compared to forest systems, Blankinship and Hart (2012) suggested that differences in plant N demand and productivity at spring between these systems can influence the relative availability of N at this time (Bilbrough et al. 2000, Jonas et al. 2008, Wipf and Rixen 2010). In addition, the shallow rooting depth of grasses may allow them to better synchronize with snow melt and microbial turnover (Mullen et al. 1998, Bilbrough et al. 2000).

Decreased interception of ^{15}N at peak biomass in snow removal plots was associated with decreased root biomass, as I predicted, and was consistent with previous studies of severe frost effects (Weih and Karlsson 2002, Bokhorst et al. 2011, Malyshev and Henry 2012a).

Curiously, ^{15}N interception by plants at this time was not substantially higher than that observed in early spring. A possible explanation for this observation is that C_3 grass productivity declines at this time of the summer, because the soil dries out (Hutchison and Henry 2010), leading to lower plant N interception in the summer than in spring. Alternatively, ^{15}N in fine roots that could not be separated from the bulk soil may have inflated my estimate of ^{15}N interception by soil over summer.

While my ^{15}N tracer results provide estimates of treatment effects on the relative losses of recently added N, it would be of further interest to consider the treatment effects on absolute N losses from the ecosystem. Over winter, losses of 45% of the root ^{15}N on average in snow removal plots relative to losses of 0% on average in ambient snow plots would equal total N losses of approximately 2 g m^{-2} y^{-1}. This estimate was obtained by multiplying the proportion of root ^{15}N lost over winter by the initial root N pool at the beginning of winter. While snow removal also enhanced ^{15}N losses from soil, a similar approach of multiplying the proportion of soil ^{15}N losses by the total soil N pool would be unreasonable (it would provide estimated losses of 55 g m^{-2} y^{-1}), given that the added ^{15}N would have mixed poorly with N in the large recalcitrant soil organic matter pool. With respect to reductions in ^{15}N intercepted over summer, the ~30% decrease in interception by snow removal plots relative to that of ambient plots equates to enhanced losses of approximately 1 g m^{-2} y^{-1} (assuming a future N deposition rate of ~3 g m^{-2} y^{-1} over the snow-free season). Therefore, my estimate of total ecosystem N losses resulting from snow removal (3 g m^{-2} y^{-1} plus the soil N losses, which I could not estimate reliably) is within the same order of magnitude of N loss estimated for a hardwood forest in response to an ice storm event (0.5 to 0.7 g m^{-2} y^{-1} NO_3^-; Houlton et al. 2003) and a soil frost in a hardwood forest (2.1 to 3.4 g m^{-2} y^{-1} NO_3^- and NH_4^+; Fitzhugh et al. 2001).

2.4.2 N addition effects on the loss and interception of N

Although ^{15}N losses did not increase over winter in response to N addition, as I predicted, N addition decreased the interception of ^{15}N at snowmelt, which is consistent with microbial C starvation and reduced microbial N demand at this time (Schimel and Mikan 2005). Nevertheless, contrary to my prediction, both the ^{15}N loss and ^{15}N interception

results indicated that plant N demand remained high over summer in N addition plots (there was increased plant productivity), such that decreases in ^{15}N interception caused by snow removal were balanced out by increases in ^{15}N interception caused by N addition. Therefore, the balance between N demand and N saturation appeared to shift seasonally throughout my experiment. I did not observe any meaningful interactions between snow removal and N addition with respect to ^{15}N losses or interception, but my predictions regarding these interactions were based on the assumption the N addition would consistently decrease N demand. However, N saturation would be expected to become more dominant with chronic N deposition when the stimulation of biomass production by N levels off (Aber et al. 1989, Stevens et al. 2004). There was no effect of N addition on ^{15}N losses from the labeled pools over summer, which is contrary to the expectation that the decomposition of N-rich litter is accelerated (Aber et al. 1990, Aerts and Caluwe 1997, Aerts et al. 2006). However, a recent meta-analysis indicates that across ecosystems, N addition does not increase litter decomposition *in situ* on average, as a result of the direct suppression of microbial activity by the added N (Knorr et al. 2005)

2.5 Conclusions

My results demonstrate that severe soil freezing can enhance over-winter losses of N recently incorporated into ecosystems, while over the summer, N losses resulting from severe soil freezing the previous winter represent an opportunity cost of decreased N interception, rather than enhanced losses from existing ecosystem N pools. Given the strong effects of freezing on soil N retention in my study, further insights could be gained by exploring N losses from specific soil N pools (e.g., soluble, microbial, detrital, etc.) to increase the connection between my results and those that have measured bulk soil N losses in response to freezing. Decreased N interception at snowmelt in N addition plots indicated that the system was sensitive to N saturation induced N losses at that time, although overall, there were few interactive effects of snow removal and N addition.

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3 Chapter 3: Soil freezing and N deposition: transient *vs.* multi-year effects on plant productivity and relative species abundances

3.1 Introduction

Increased atmospheric nitrogen (N) deposition has altered the productivity and plant species composition of numerous terrestrial ecosystems over the last century (Vitousek et al. 1997, Clark and Tilman 2008), and rates of atmospheric N deposition are projected to increase further in the coming decades (Galloway et al. 2004). A critical question that must be answered to predict future plant responses to a changing environment is how increased N availability may interact with climate change over this time (Hungate et al. 2003). While much of the research on this topic has focused on the importance of increases in mean annual temperature and growing season length, the case has also been made for the potential importance of extreme climate events in affecting plant communities and ecosystem processes (Jentsch and Beierkuhnlein 2008). The relative importance of extreme, episodic climate events versus chronic environmental change depends not only on the frequency and intensity of extreme events (Jentsch et al. 2007), but on the resilience and lifespan of the dominant community members. Therefore, while for forest communities the effects of extreme events can be long-lasting, for herbaceous plant communities it is less clear to what extent extreme events may have long-lasting effects (Kreyling et al. 2008a,b,c, Mirzaei et al. 2008, Kreyling et al. 2010, Kreyling et al. 2011).

Change in winter temperatures has been identified as a critical factor that can affect plant performance in temperate regions, but research on this topic has been greatly underrepresented in the literature compared to that of warming effects during the growing season (Kreyling 2010). Paradoxically, climate warming can increase soil freezing by reducing snow cover and increasing the exposure of soil to cold air temperatures (Groffman et al 2001). Warming has already decreased the annual extent of snow cover by 10% in the Northern Hemisphere (ACIA 2005), and increasing air temperatures are

expected to further decrease snow fall in many areas of North America (Kapnick and Delworth 2013). In many temperate regions, climate warming is therefore expected to increase the freeze-thaw exposure of plants that overwinter beneath the snow (Groffman et al. 2001, Hardy et al. 2001, Henry 2008). Sub-lethal freezing damage can have substantial effects on plant productivity during the subsequent growing season, but with a few exceptions (Kreyling et al. 2010, Kreyling et al. 2011), most field studies of freezing effects on herbaceous plants have not extended beyond a single growing season.

Numerous mechanisms have been proposed whereby increased soil freezing can potentially interact with N availability to affect plant growth, including freezing-induced changes in microbial N mineralization (Elliott and Henry 2009), root N uptake (Malyshev and Henry 2012a) and soil N leaching and trace gas losses over the winter (Muller et al. 2003, Goldberg et al. 2010, Vankoughnett and Henry 2013). The responses of grasses to the combined effects of winter warming and N addition have been explored previously in the field using overhead heaters that supply a constant input of warming (Turner and Henry 2009, Hutchison and Henry 2010). While warming in the latter studies increased the frequency of freeze-thaw cycles at the soil surface, the intensity of these cycles was mild (a minimum temperature of -2 ºC was reached at 2 cm soil depth), and not sufficiently cold to damage the dominant plant species (Malyshev and Henry 2012a). Therefore, the combined effects of freezing damage and increased N availability on plant productivity and relative species abundance in the field remain unexplored.

I conducted an experiment in a grass-dominated old field to investigate the combined effects of enhanced freezing (via a single winter of snow removal) and N deposition on plant productivity and relative species abundance over two growing seasons. In addition, I exposed plant-soil mesocosms treated with three levels of N addition to a range of controlled freezing treatments in a growth chamber, and then monitored the plant responses over two years in the field. Based on the expectation that increased soil freezing severity would damage roots, I predicted that snow removal would both decrease plant productivity and decrease the ability of plant productivity to respond to N addition. In addition, I predicted that significant treatment effects of freezing on plant productivity

would only occur in the second growing season if there were changes in plant relative species abundance.

3.2 Methods

3.2.1 Site description

The experiments were carried out in a former agricultural field at the Agriculture and Agri-Food Canada research station in London, Ontario, Canada (43°01 46 N, 81°12 52 W). The site was dominated by the perennial grasses *Poa pratensis* L. and *Bromus inermis* Leyss., and the forbs *Cirsium arvense* L. and *Lotus corniculatus* L. were also common, but patchy. The forbs *Asclepias syriaca* L., *Aster ericoides* L. and *Solidago altissima* L. were also present, but at low densities. The soil was classified as a well to imperfect drained silt loam glacial till (Hagerty and Kingston 1992), with a mean pH of 7.6 (Bell et al. 2010), and the site had not been ploughed, fertilized, or mowed for over 28 years. According to local climate records (Canadian Climate Normals 1981-2002 or 2006, Environment Canada, National Climate Data and Information Archive) the mean annual air temperature was 7.9 °C, with a low monthly mean of -5.6 °C (January) and a high monthly mean of 20.8 °C (July). The mean annual precipitation was 1011.5 mm. Snowfall at the site typically begins in early to mid-December, with snow cover occurring from late December to mid-March. Mid-winter snowmelts are common, but are usually followed by new snow accumulation.

3.2.2 Snow removal experiment

The snow removal experiment consisted of twenty four plots (1 m × 1 m) divided into six spatially distinct blocks. Snow was removed from half of the plots in each block, and half of the snow removal and ambient snow plots in each block were fertilized with 6 g N m^{-2} y^{-1}, for a total of four treatment combinations with six spatially replicate plots each. The N additions were divided equally among three dates in 2010 (8 June, 21 August, and 19 October), 2011 (13 April, 10 June, and 5 October), and 2012 (15 March, 6 June, and 1 October), and the addition rate approximated projected increases in atmospheric N deposition in the region of the study by the year 2050 (Galloway et al. 2004). Soil

temperatures at 5 cm soil depth were recorded using Ibutton DS1922L-F5 loggers (Maxim: San Jose, CA; n=3 for each treatment).

Prior to snowfall, white plastic mesh (2 cm × 2 cm; Winter Wrap, Quest Plastic Limited) was placed on top of the vegetation of both ambient snow and treatments plots to prevent litter and soil disturbance during snow removal in the latter. Snow was removed to a depth of approximately 2 cm following each snowfall event from 9 December 2010 to 16 February 2011, and periodically when snow accumulated in the plots due to wind. After 16 February 2011, snow was allowed to accumulate in the plots to avoid differences in soil moisture between the ambient snow and snow removal plots at snow melt. Snow was not removed over the subsequent winter. Thus, any effects observed during the second growing season were interpreted as legacy effects of the snow removal during the first winter. Gravimetric soil moisture content was measured immediately after snow melt and four weeks after, by taking 2 cm × 12 cm deep soil cores and drying them for three d at 65 ºC and then weighing them.

3.2.3 Mesocosm experiment

Intact plant-soil mesocosms (10 cm diameter ×15 cm deep) were collected by inserting sections of PVC pipe into the ground on 25 May 2010. The mesocosms were collected from six spatially distinct blocks in the field and incubated in the holes from which they had been collected. The mesocosms were randomly assigned to one of three freezing treatments (0, -5, and -10 °C) and one of three N addition treatments (0, 2 and 6 g N m^{-2} y^{-1}), with three mesocosms per treatment combination in each block, for a total of 108 mesocosms. N additions were administered on 1 June, 29 July, and 11 October in 2010, 13 April, 10 June, and 5 October in 2011, and 15 March, 6 June, and 1 October in 2012. On 12 March 2010, all mesocosms were removed from the field and placed in incubation chambers at 2 °C for 3 h, then brought down at a rate of 0.5 °C h^{-1} to 0, -5 or -10 °C for six d. Subsequently, the mesocosms were returned to their holes in the field. A freezing rate of 0.5 °C h^{-1} was chosen because it was similar to what has previously been observed at the field site (Elliott and Henry 2009).

3.2.4 Productivity estimates

Throughout the first and second growing seasons, I estimated cumulative aboveground plant biomass production the first week of May, mid-June, early July, and October from plots in the snow removal experiment non-destructively using leaf length to mass allometry, following the methods adapted from Hutchison and Henry (2010). A 10 cm × 10 cm sampling ring was placed randomly in each plot, all *P. pratensis* leaves within the ring were counted, and 9-15 leaves were selected at uniform locations for height measurements. The number and height of inflorescences were recorded separately. 30 to 50 leaves of varying height were collected from the field outside of the plots on the same day, the heights were recorded, and the leaves were individually dried and weighed. The logarithm of height was then plotted against the logarithm of mass, and a regression line was fit to describe the allometric relationship. For *P. pratensis*, r^2 ranged from 0.72 to 0.89 over the two sampling years. A similar approach was taken for *B. inermis,* except the allometric equations were calculated on a per tiller basis, using the height of the tallest leaf. For *B. inermis*, r^2 ranged from 0.79 to 0.94 over the two sampling years. The *B. inermis* tillers senesced in mid-summer following seed set, then a new round of growth occurred in the late summer. Therefore, for the purpose of estimating cumulative productivity across the growing season, the productivity values for the first round of growth were added to the late summer fall productivity estimates to obtain a cumulative measure of productivity. *P. pratensis* did not experience a phase of abrupt leaf senescence in mid-summer, so it was not possible to account for any leaf turnover in the cumulative productivity estimates for this species. The biomass of the dominant forbs (*C. arven*se, *A. ericoides,* and *L. corniculatus*) were estimated using the same method as *B. inermis,* but because these species were less common and patchily distributed, measurements were made for all individuals in each 1 m × 1 m plots for each sampling date (r^2 ranged from 0.72 to 0.91 for *C. arven*se, 0.71 to 0.89 for *L. corniculatus*, and was 0.81 for *A. ericoides*). For the mesocosms, the same method was used, but all tillers in a given mesocosm were recorded.

Root biomass was estimated at snowmelt, the first week of May, mid-June, and October by sampling the soil in each plot four or five times using a 2 cm diameter corer to a depth

of 12 cm, then sieving the roots to separate them from the bulk soil. A similar approach was used for the mesocosms, except the soil was sampled one to two times at snowmelt and in June, and three times in October. In addition, I dried soil sub-samples (~10 g) at approx. 65 ºC for at least three d to determine gravimetric moisture content.

3.2.5 Data analyses

I tested for significant effects of snow removal, N addition, and their interaction on total and species-specific cumulative aboveground production and root biomass using repeated measures two-way analyses of variance (ANOVAs) (JMP 4.0, SAS Institute). Similar statistical analyses were conducted for the plant-soil mesocosms, but tested for significant differences for temperature, N addition, and their interaction on aboveground production and root biomass. Tukey's post-hoc tests were used to assess treatment combination effects on root biomass. All data were checked for normality and log- or Boxcox-transformed as necessary. For the *C. arvense* data and the other species in the second growing season, which could not be normalized, I used the Scheirer–Ray–Hare non-parametric two way test in R-3.0.1 (R Development Core Team 2013). There was not sufficient representation of the other species in the plots to test their responses in the first growing season.

3.3 Results

3.3.1 Snow removal experiment

The mean air temperature (1 December to 31 March) was ~1 °C cooler and total snow precipitation was almost double over the first winter (2010-2011) than compared to climate normals (Table 3.1). Snow removal decreased soil temperatures on average, with soil temperatures in these plots dropping below 0 °C from early to mid February, and continuing to stay cooler than those of ambient snow plots until mid-March (Figure 3.1). The snow removal plots reached a minimum average temperature of -3.1 ºC at 5 cm depth and experienced 5 freeze-thaw cycles, whereas the ambient snow plots experienced a minimum average temperature of 0.3 ºC, and thus 0 freeze-thaw cycles over the winter. Gravimetric soil moisture measured immediately after and four weeks following snow

Table 3.1: Mean air temperature, total precipitation, and snow precipitation over the first (2010-2011) and second (2011-12) winters (1 December to 31 March) and mean air temperature and total precipitation during the first (2011) and second (2012) growing seasons (1 April to 31 November) relative to the climate normals for London, Ontario.

	Climate normals	**First year**	**Second year**
Mean winter air temperatures (ºC):	-3.2	-4.4	1.2
Total winter precipitation (mm):	298.7	371.8	306.6
Total snow precipitation (mm):	164.9	304.0	128.9
Mean growing season air temperatures (ºC):	13.4	14.6	14.3
Total growing season precipitation (mm):	712.9	829.0	455.5

Data from Environment Canada, National Climate Data and Information Archive. Mean winter and growing season air temperature and total winter and growing season precipitation climate normals range from years 1981-2002 or 2006.

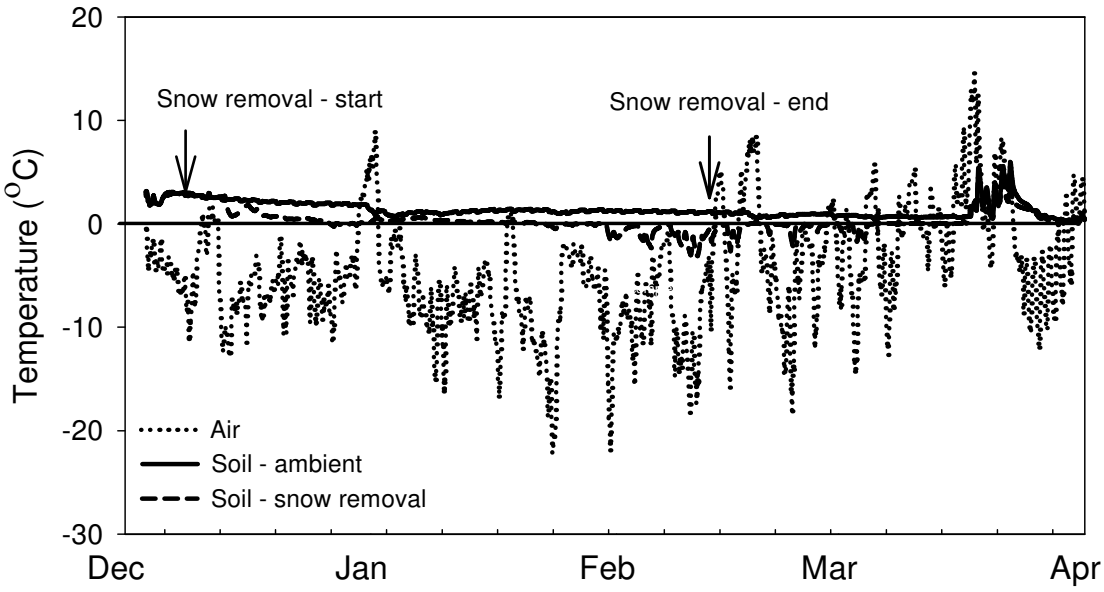

Figure 3.1: Mean air temperature and soil temperature under ambient snow and snow removal plots from early December 2010 to early April 2011 at 5 cm soil depth (nitrogen treatments had no effect on winter soil temperature and thus were pooled).

melt during the first growing season was not significantly affected by snow removal (P = 0.66 and P =0.20, respectively; snow removal was not conducted over the second winter). The mean air temperature during the second winter (2011-2012; 1 December to 31 March) was relatively mild (1.2 °C), with similar total winter precipitations as climate normals, but less precipitation falling as snow (Table 3.1). Winter soil temperatures in the plots never went below 0 ºC and did not differ among treatment plots (Appendix A). Low snow accumulation over the second winter coupled with low precipitation over the following plant growing season resulted in gravimetric soil moisture being ~25% lower in mid-June compared to the same sampling period in the previous growing season (Appendix B).

By the end of the first growing season, snow removal alone reduced cumulative aboveground production by ~45%, N addition alone increased production by ~24%, with the two effects interacting doubling production in the snow removal plots (Table 3.2; Figure 3.2). The grass responses at the species level largely reflected those of total production, with increased production for N addition, decreased production for snow removal and a significant interaction between the two factors for *P. pratensis* (Table 3.2; Figure 3.2). *B. inermis* exhibited similar responses, except there was no interaction between N addition and snow removal (Table 3.2; Figure 3.2). The forb *C.arvense* contributed to less than 1% of the total biomass in the plots, and was not significantly affected by the treatments during the first growing season (Table 3.2; Figure 3.3). Snow removal reduced root biomass, but N addition had no significant effect (Tables 3.2, 3.3).

By the end of the second growing season, cumulative production was reduced by 21% in response to the legacy effect of snow removal, N addition increased production by ~18%, and these effects were additive (Table 3.4; Figure 3.2). At the species level, *P. pratensis* exhibited significant decreases in cumulative production in the former snow removal plots, and significant increases with N addition that trended towards an interaction (P=0.054), but there were no significant treatment effects for *B. inermis* (Table 3.4; Figure 3.2). The biomass of *C. arvense* increased during the second growing season in response to the legacy effect of snow removal, although this species still only contributed

Table 3.2: Summary of ANOVA P-values for the effects of treatment and date on cumulative net aboveground production (total, *Poa pratensis*, *Bromus inermis* and *C. arvense*; the latter was only sampled in October, and a non-parametric two way test (Scheirer-Ray-Hare) was used for analysis) and root biomass (all species pooled) for the first growing season (2011) in the snow removal experiment. Degrees of freedom are displayed in parentheses after the treatment. Significant effects (P<0.05) are in bold.

Effect	**Aboveground production**				**Roots (all species)**
	Total	***P. pratensis***	***B. inermis***	***C. arvense***	
Block $_{(5,55)}$	0.063	0.48	**0.018**		0.07
Date $_{(3,55)}$	**<0.001**	**<0.001**	**<0.001**		0.23
Snow $_{(1,20)}$	**<0.001**	**<0.001**	**0.002**	0.32	**0.021**
Date×Snow $_{(3,55)}$	0.29	0.78	0.43		0.86
N $_{(1,20)}$	**<0.001**	**<0.001**	**0.003**	0.83	0.20
Date×N $_{(3,55)}$	0.17	**0.036**	0.27		0.22
Snow×N $_{(1,20)}$	**0.005**	**0.002**	0.24	0.44	0.68
Date×Snow×N $_{(3,55)}$	0.99	0.99	0.99		0.36

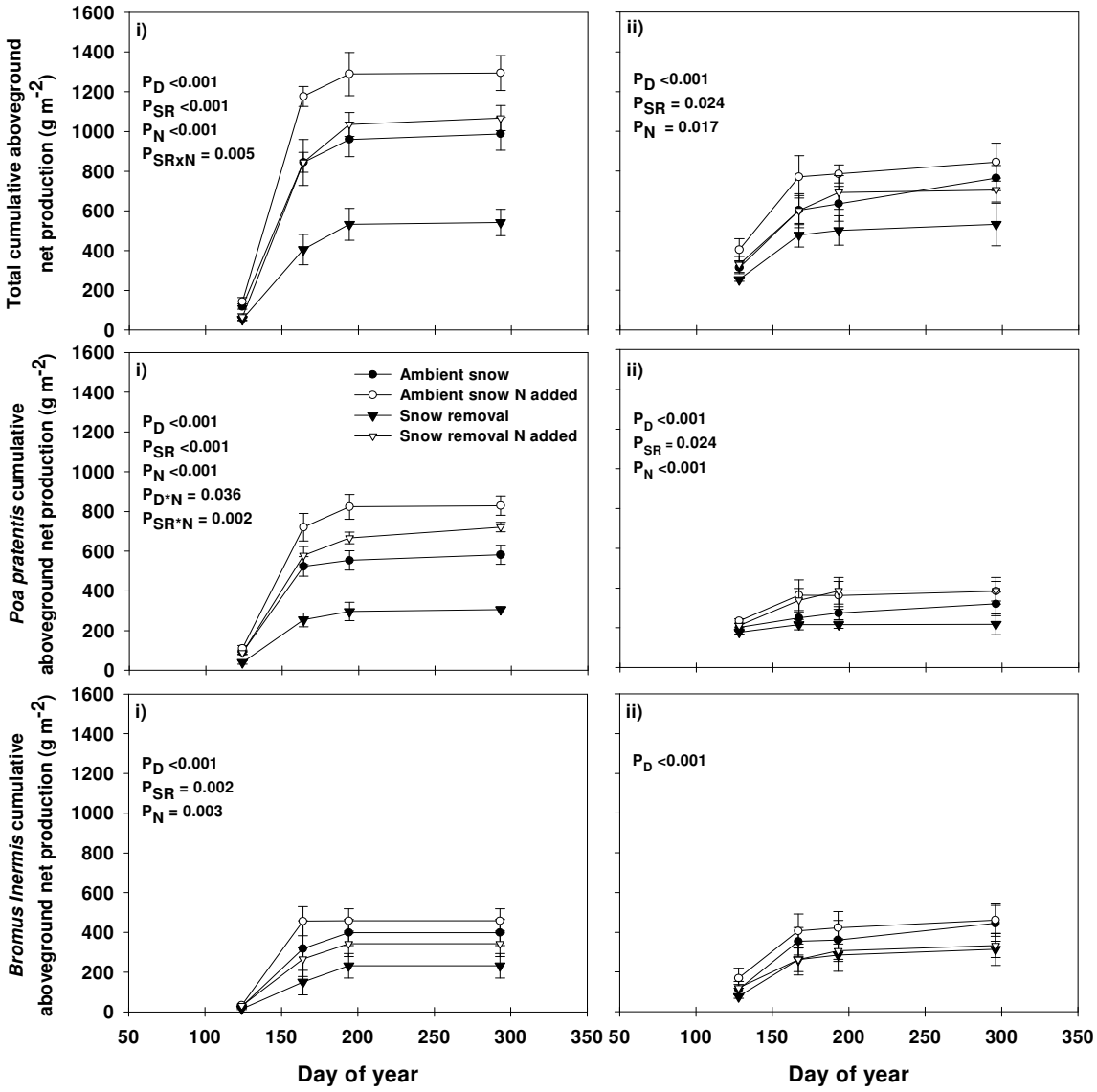

Figure 3.2: Total cumulative net aboveground production (g m^{-2}), and cumulative production of *P. pratensis* and *B. inermis* for sampling dates during the first (2011; i) and second (2012; ii) growing seasons in the snow removal experiment. Data are presented for ambient snow plots (filled circles), snow removal plots (filled triangle), and N addition plots (open symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen; D - Date).

Table 3.3: Root (all species pooled) biomass (g dw m^{-2}) for each treatment combination during the first (2011) and second (2012) growing seasons in the snow removal experiment. Corresponding statistical analysis can be found on Table 3.2 and 3.4 for the first and second growing seasons, respectively. Means within columns for each growing season are not significantly different if they share a common lowercase letter (Tukey's test, $P < 0.05$). Parentheses indicate standard error (n=6).

	Snowmelt		**May**		**June**		**October**	
First growing season								
Ambient	497[a]	(58)	443[b]	(29)	612[a]	(95)	462[a]	(40)
Ambient +N	454[a]	(41)	655[a]	(69)	538[a]	(132)	686[a]	(62)
Snow removal	390[a]	(54)	442[b]	(20)	430[a]	(61)	548[a]	(104)
Snow removal + N	463[a]	(71)	518[ab]	(71)	398[a]	(45)	524[a]	(75)
Second growing season								
Ambient	236[a]	(77)	312[a]	(53)	283[a]	(33)	480[a]	(53)
Ambient +N	293 [a]	(67)	330[a]	(54)	312[a]	(27)	547[a]	(122)
Snow removal	221[a]	(47)	185[a]	(24)	259[a]	(23)	508[a]	(105)
Snow removal + N	224[a]	(33)	216[a]	(42)	352[a]	(27)	486[a]	(89)

Table 3.4: Summary of ANOVA P-values for the effects of treatment and date on cumulative net aboveground production (total, *Poa pratensis*, *Bromus inermis, C. arvense* and other species (pooled); the latter two were only sampled in October, and a non-parametric two way test (Scheirer-Ray-Hare) was used) and root biomass (all species pooled) for the second growing season (2012) in the snow removal experiment. Degrees of freedom are displayed in parentheses after the treatment. Significant effects (P<0.05) are in bold.

Effect	**Aboveground production**					**Roots (all species)**
	Total	***P. pratensis***	***B. inermis***	***C. arvense***	**Other species**	
Block $_{(5,55)}$	0.59	**0.001**	0.22			0.065
Date $_{(3,55)}$	**<0.001**	**<0.001**	**<0.001**			**<0.0001**
Snow $_{(1,20)}$	**0.024**	**0.024**	0.11	**0.047**	0.52	**0.059**
Date×Snow $_{(3,55)}$	0.68	0.77	0.76			0.18
N $_{(1,20)}$	**0.017**	**<0.001**	0.50	0.13	0.39	0.09
Date×N $_{(3,55)}$	0.95	0.44	0.97			0.86
Snow×N $_{(1,20)}$	0.71	**0.054**	0.91	0.48	0.72	0.99
Date×Snow×N $_{(3,55)}$	0.65	0.42	0.93			0.89

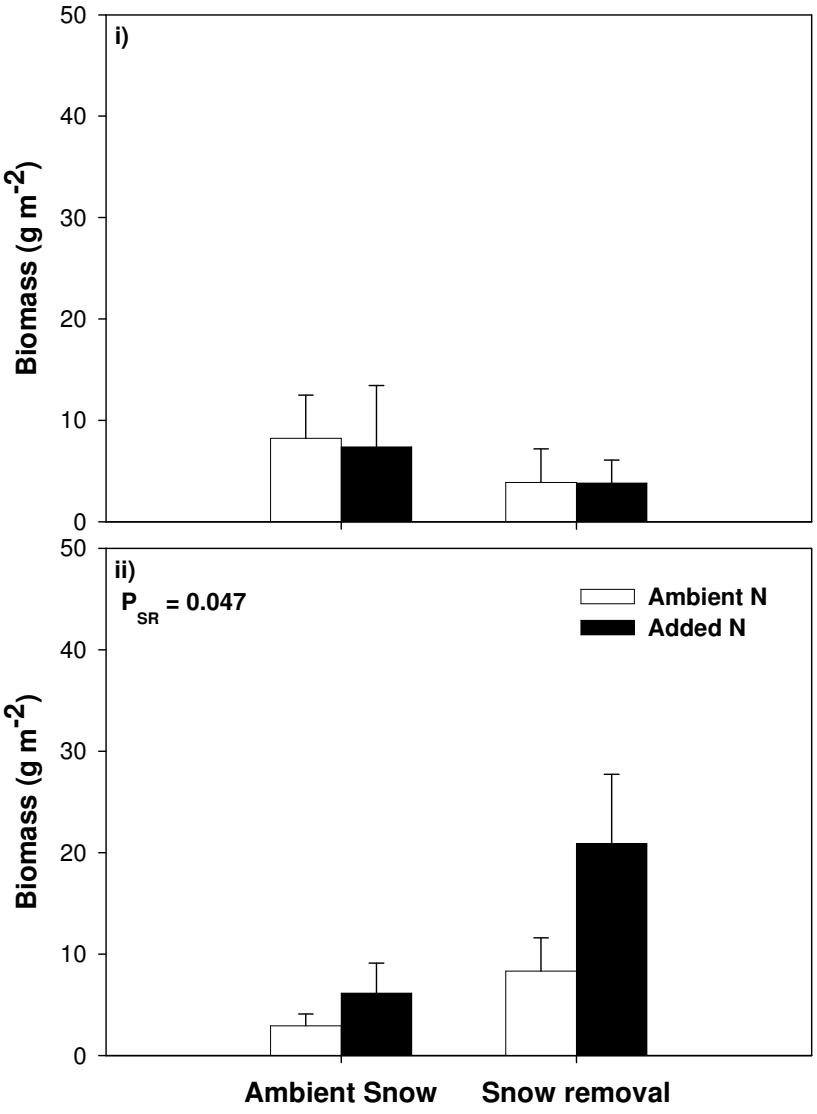

Figure 3.3: Biomass (g m^{-2}) of *C. arvense* for the first (2011; i) and second (2012; ii) growing seasons in the snow removal experiment. Data are presented for ambient N (open bars) and added N (filled bars). Raw means are displayed (n=6) and error bars denote standard error. Significant effects (Scheirer-Ray-Hare two way non parametric tests) are displayed in the panel (SR- Snow removal)

2 and 0.5 % of the total production in the snow removal and ambient snow plots, respectively (Table 3.4; Figure 3.3). N addition had no effect on *C. arvense* biomass (Table 3.4; Figure 3.3), and both the legacy effect of snow removal and N addition had no significant effects on the biomass of other forbs. There was a trend towards decreased root biomass in the snow removal plots during the second growing season (P=0.059; Table 3.3, 3.4), but no significant effect of N addition.

3.3.2 Mesocosm experiment

Similar to the snow removal experiment, cumulative aboveground grass production in the mesocosms was reduced during the first growing season with increasing freezing severity, and N addition increased production, but unlike the snow removal experiment, the latter effects were additive (Table 3.5; Figure 3.4). Root biomass also decreased with increased freezing severity (Table 3.5, 3.6). Similar responses were observed for aboveground production and root biomass during the second growing season, but only the legacy effect of the most severe freezing treatment (-10 ºC) elicited significant responses (Table 3.5; Figure 3.4). Likewise, the significant N addition effect was driven primarily by increased production in the 6 g N y^{-1} treatment both years (Table 3.5; Figure 3.4). There were no significant interactions between N addition and the legacy effect of freezing in the second growing season (Table 3.5; Figure 3.4),

3.4 Discussion

Contrary to my prediction that the response of plant productivity to N addition would be reduced in the snow removal plots, there was instead a larger increase in productivity in response to N addition in the snow removal plots than in the ambient snow plots. My prediction had been based on the assumption that freezing damage to roots in the snow removal plots would decrease the capacity for root N uptake, as has been observed in forest systems (Fitzhugh et al. 2001). In my study, snow removal reduced soil temperatures to -3.1 ºC at 5 cm depth, which is sufficient to damage the *Poa pratensis* roots (Malyshev and Henry 2012a). Consistent with this observation, snow removal decreased plant production over the following growing season. The explanation for why

Table 3.5: Summary of ANOVA P-values for the effects of treatment and date on the cumulative net aboveground production of grasses (*Poa pratensis* and *Bromus inermis* pooled) and roots (all species pooled; the latter sampled in October) for the first (2011) and second (2012) growing seasons in the mesocosm experiment. Degrees of freedom are displayed in parentheses after the treatment. Significant effects (P<0.05) are in bold.

	First growing season		**Second growing season**	
Effect	**Grass aboveground production**	**Roots**	**Grass aboveground production**	**Roots**
Block $_{(5,40)}$	0.62	0.63	0.13	0.58
Date $_{(3,135)}$	**<0.001**		**<0.001**	
Temp $_{(2,40)}$	**<0.001**	**<0.001**	**0.044**	**0.011**
Date×Temp $_{(6,135)}$	**0.026**		0.33	
Nitro $_{(2,40)}$	**0.001**	0.48	**0.029**	0.17
Date×Nitro $_{(6,135)}$	0.80		0.99	
Temp×Nitro $_{(4,40)}$	0.62	0.55	0.50	0.49
Date×Temp×Nitro $_{(12,135)}$	0.91		0.45	

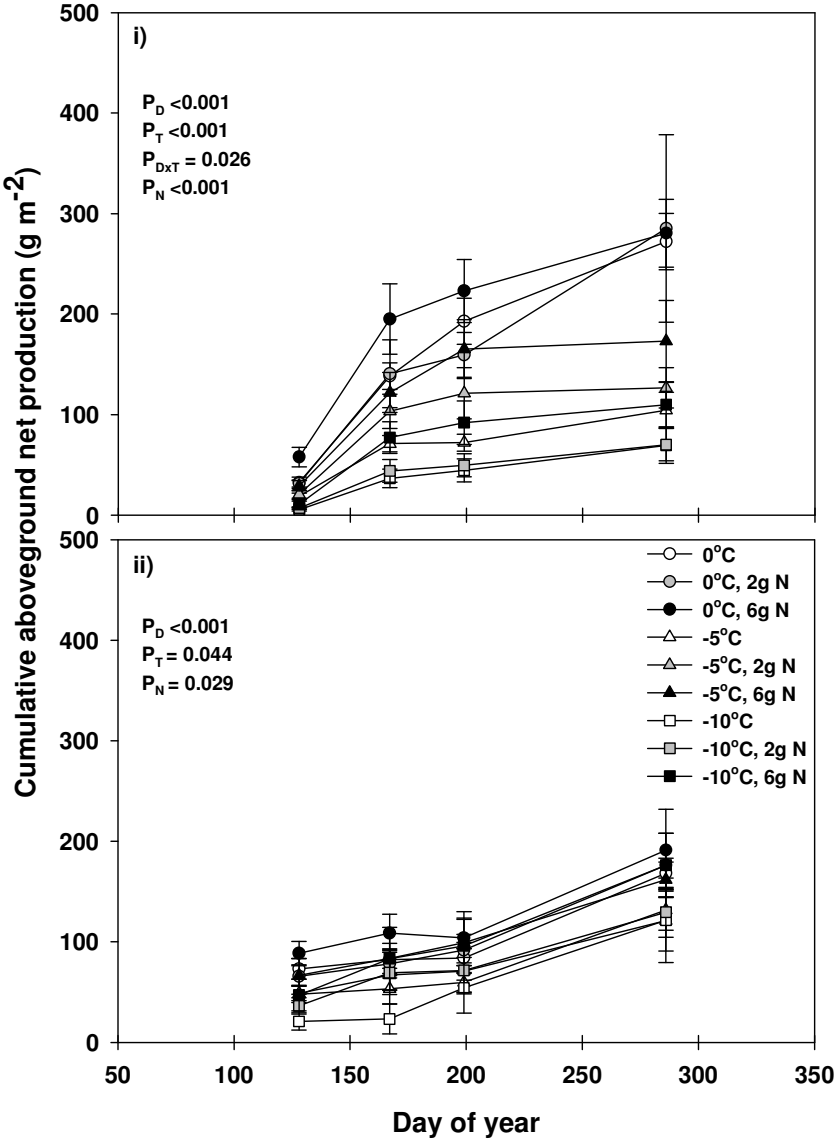

Figure 3.4: Total grass cumulative production (g m^{-2}) during the first (2011; i) and second (2012; ii) growing seasons in the mesocosm experiment. Data are presented for 0 °C (open circles), -5 °C (open triangles), and -10 °C (open squares), and 2 g m^{-2} y^{-1} of NH_4NO_3 (grey symbols), and 6 g m^{-2} y^{-1} of NH_4NO_3 (filled symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (T - Temperature; N - Nitrogen; D - Date).

Table 3.6: Mesocosm root biomass (g dw m^{-2}; all species pooled) for each treatment combination for the first (2011) and second (2012) growing seasons. Corresponding statistical analyses are displayed in Table 3.5. Means within columns for each growing season are not significantly different if they share a common lowercase letter (Tukey's test, $P < 0.05$). Parentheses indicate standard error (n=6).

	First growing season		**Second growing season**	
0°C	241^{ab}	(40)	137^{a}	(28)
0°C, 2g N	257^{ab}	(37)	126^{ab}	(59)
0°C, 6g N	336^{a}	(62)	102^{ab}	(19)
-5°C	130^{ab}	(25)	97^{ab}	(22)
-5°C, 2g N	159^{ab}	(62)	68^{ab}	(28)
-5°C, 6g N	212^{ab}	(70)	67^{ab}	(28)
-10°C	102^{b}	(35)	30^{b}	(10)
-10°C, 2g N	95^{b}	(25)	34^{ab}	(10)
-10°C, 6g N	98^{b}	(33)	55^{ab}	(15)

plants in the ambient snow plots were less responsive to N is likely based on growth limiting factors other than N. Specifically, with N addition, the plants in ambient snow plots exhibited relatively high aboveground biomass, and thus they may have experienced increased light limitation (or possibly phosphorus limitation) relative to the plants in the snow removal plots. While the mechanism why elevated N promoted freezing damage recovery is unknown, increased aboveground production in N addition plots likely allowed for faster regrowth of damaged tissue.

Although the interaction between N addition and snow removal only lasted a single season, the legacy effects of snow removal on plant productivity carried over to the second growing season, which is consistent with the few other studies that have examined multiyear responses to frost events (Kreyling et al. 2008b, Kreyling et al. 2011). Furthermore, I observed increases in *C. arvense* productivity during the second growing season. Episodic events are an important catalyst that can change competitive interactions among plants and alter successional pathways (Jentsch and Beierkuhnlein 2003, Jentsch et al. 2007). Accordingly, beyond several years, longer-term effects of freezing damage in herbaceous systems may be most evident when changes in species composition or relative species abundance occur. *Cirsium arvense* is an opportunistic species that often increases biomass in response to reduced competition (Thrasher et al. 1963, Wilson and Kachman 1999, Edwards et al. 2000). Spread from seed can occur with this species, but germination is often low and erratic (Tiley 2010); thus it is most likely that the increase in biomass observed in the snow removal plots was caused by vegetative growth. Although increases in *C. arvense* in the snow removal plots were relatively minor as a proportion of the total plant biomass in these plots, this species can have prolonged negative effects by annually reducing grass yields due to changes in resource competition (O'Sullivan et al. 1982, O' Sullivan et al. 1985, Grekul and Bork 2004).

The mesocosm experiment confirmed that the recovery of the grasses over multiple years is contingent on freezing severity, based on the observation that plant productivity only remained depressed during the second growing season in response to the most severe freezing treatment (-10 ºC). There are often large differences in freezing severity

experienced by grasses in unmanaged versus managed systems; at our field site, the combination of snow and a thick litter layer kept soil temperatures near 0 ºC, and even when the snow was removed, soil temperatures only reached a minimum of -3.1 ºC on average during the winter, despite air temperatures periodically reaching -20 ºC (Figure 3.1). Only when litter and snow removal have been conducted simultaneously have soil temperatures approached -10 ºC at our field site (Malyshev and Henry 2012a). The latter approximates conditions experienced in highly managed systems, where grasses are mowed or grazed, leaving behind a relatively thin litter layer and exposing overwintering shoot bases and roots to the air.

In the first year of the mesocosm experiment, there was not a threshold response of freezing damage over the range of temperature examined, rather freezing damage intensified from 0 to -5 to -10 °C. However, the effect of one week at -10 °C was the only temperature treatment to persist during the second growing season. Freezing damage can increase in severity at increasingly severe sub-zero temperatures because the concomitant decrease in water potential exacerbates cellular dehydration (Gusta et al. 1975). However, in addition to minimum temperature, other factors such as duration of freezing can influence damage (Malyshev and Henry 2012a), which likely explains why the plants exposed to a minimum of -3.1 °C in the snow removal experiment exhibited reduced productivity during the second growing season, while those exposed to -5 °C over the short term in the mesocosms only showed a significant response after one year. The timing of freezing can also be important; winter warming spells have increased in some regions (Shabbar and Bonsal 2003), which can prematurely deacclimate plants and leave them susceptible to freeze damage if snowmelt is followed by the return of severe frost (Bokhorst et al. 2008, Bokhorst et al. 2009, Bokhorst et al. 2011).

Much like the temperature response, N addition level was influential in the mesocosm experiment, with productivity increasing significantly in response to 6 g N m^{-2} y^{-1}, but not in response to 2 g N m^{-2} y^{-1}. This distinction is important, given that 2 g N m^{-2} y^{-1} represents the lower bound on estimates of N deposition increases in our region, whereas 6 g N m^{-2} y^{-1} is slightly above the upper bound (Galloway et al. 2004). However, the chronic effects of moderate increases in the rates of N deposition could potentially affect

productivity through changes in species composition over the longer term (Wedin and Tilman 1996, Isbell et al. 2013).

3.5 Conclusions

My results revealed an interaction between plant freezing damage and N addition after one year, with N addition promoting recovery from freezing damage in the snow removal experiment. However, in the subsequent year, the additive effects of increased freezing and N addition were dominant in defining the overall plant responses. Moderate rates of N addition did not elicit a significant plant response, although unlike extreme frost, N deposition is a chronic effect, and N responses may thus accumulate over time. Episodic soil freezing events can change relative species abundances over the short term, but it remains to be tested to what extent that changes may alter successional trajectories over time in grass dominated systems. Overall, my results emphasize that while both increased N deposition and freezing damage have the potential for multiyear effects in grass-dominated systems, the potential interactions between these global change factors are highly contingent on the intensities of the treatments.

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4 Chapter 4: Soil freezing and N deposition: transient *vs.* multi-year effects on extractable C and N, potential trace gas losses, and microbial biomass

4.1 Introduction

Many herbaceous plants overwinter at or below the soil surface. The temperatures they experience are influenced by soil thermal properties, and soil freezing dynamics are in turn a function of air temperature fluctuations and the timing, duration, and amount of snow cover (Henry 2008). In the northern United States, climate warming has already increased the annual ratio of rain to snow in recent decades (Huntington et al. 2004, Feng and Hu 2007), and continued decreases in snow cover are expected throughout much of Canada and the United States over the next century (Kapnick and Delworth 2013). Reduced snow cover exposes soils to cold air temperatures over winter (Hardy et al. 2001), and historical data and snow removal experiments support the prediction that an altered snow regime over the next century will increase the intensity of soil freezing and/or the frequency of freeze-thaw cycle exposure in some regions (Groffman et al. 2001a, Henry 2008).

While the potential economic impacts of soil freezing as a result of damage to perennial crops have been studied for decades by agronomists (Ouellet 1976), more recent work by ecologists has demonstrated that soil freezing can have important implications not only for declines in wild plant populations (Schaberg et al. 2008, Schaberg et al. 2011), but for increased soil nutrient loss *via* leaching (Mitchell et al. 1996, Fitzhugh et al. 2003) and transformation to trace gases (Groffman et al. 2006, Maljanen et al. 2010). Therefore the effects of soil freezing on plant productivity may not only occur directly, but also may occur indirectly as a result of changes in the cycling and availability of soil nitrogen (N) (Kreyling et al. 2010, Comerford et al. 2013). However, knowledge regarding the ecological effects of soil freezing remains sparse for temperate regions relative to arctic and alpine regions (Kreyling 2010), despite a renewed interest in this topic in the context of climate change.

Several studies have demonstrated that the effects of soil freezing on plant productivity and community changes can persist over multiple years (Kreyling et al. 2010, Kreyling et al. 2011). Changes to plant productivity and relative species abundances can alter microbial biomass and community structure, leading to changes in soil C and N dynamics (Bardgett et al. 1999, Hamilton and Frank 2001, De Deyn et al. 2011, Lamb et al. 2011). However, freezing effects on soil C and N cycling have only been studied for a single growing season following the freezing stress (Groffman et al. 2001b, Groffman et al. 2006, Maljanen et al. 2007), thus it remains unclear whether these effects are only transient (over-winter and spring melt) or whether the legacy effects of freezing can persist over multiple years.

Winter climate-driven changes in soil C and N dynamics could also interact with increased atmospheric N deposition, which is predicted to increase substantially in temperate regions over the next century (Galloway et al. 2004). Increases in N availability, at least in the short term, often enhance plant productivity (Vitousek and Howarth 1991, LeBauer and Treseder 2008), but increased N leaching and trace gas losses can occur when N no longer limits plant growth (Vitousek et al. 1997, Aber et al. 1998). Soil freezing can lyse microbial cells (Skogland et al. 1988) and damage plant roots (Tierney et al. 2001, Gaul et al. 2008, Malyshev and Henry 2012), which not only increases soluble N, but can decrease the capacity for plant N uptake (Malyshev and Henry 2012), increasing soil NO_3^- losses over the growing season (Boutin and Robitaille 1995, Fitzhugh et al. 2001, Callesen et al. 2007). The combined effects of winter warming and N addition have been explored previously for soil N availability and microbial biomass (Bell et al. 2010, Turner and Henry 2010). Nevertheless, while winter warming in the previous studies increased the frequency of soil freeze-thaw cycles, freezing intensity did not increase to the point where it could cause measurable damage to the plants or soil microorganisms (Elliott and Henry 2009, Malyshev and Henry 2012). Therefore, the combined effects of freezing damage and increased N availability in the field remain unexplored.

I conducted an experiment in a grass-dominated old field to investigate the combined effects of enhanced soil freezing (*via* a single winter of snow removal) and N addition on

soil extractable C and N pools, and bacterial and fungal biomass, over two growing seasons. In addition, I exposed plant-soil mesocosms treated with three levels of N addition to a range of freezing treatments in a controlled environment chamber, then returned the mesocosms to the field and monitored soil responses over two years. I predicted that both soil freezing and N addition would increase extractable soil inorganic N pools and potential trace gas losses. In addition, based on the observation that soil microbes have fast recovery times and are generally unresponsive to short term N addition, I predicted that neither treatment would affect microbial biomass C and N pools and bacterial and fungal biomass unless there were changes in plant relative species abundance.

4.2 Methods

4.2.1 Site description

The snow removal experiment was carried out in a former agricultural field in London, Ontario, Canada (43°01 46 N, 81° 12 52 W). Vegetation at the site was dominated by *Poa pratensis* L. and *Bromus inermis Leyss*, while the forbs *Cirsium arvense* L. and *Lotus corniculatus* L. (a legume) were also common, but patchy. Soil at the site had not been ploughed, fertilized, or mowed for over 28 years and was classified as imperfect drained silt loam glacial till (Hagerty and Kingston 1992), with a mean pH of 7.6 (Bell et al. 2010). Local climate records (Canadian Climate Normals 1981-2002 or 2006, Environment Canada, National Climate Data and Information Archive) the mean annual air temperature was 7.9 °C, with a low monthly mean of -5.6 °C (January) and a high monthly mean of 20.8 °C (July). The mean annual precipitation was 1011.5 mm. Snowfall begins in early to mid December, with continuous snow cover from late December to mid-March. Mid-winter snowmelts are common; however they are usually accompanied by subsequent snow accumulation.

4.2.2 Experimental design - snow removal experiment

The experiment consisted of twenty four square plots (1 m × 1 m) divided into control and snow removal treatments, combined with N addition treatments (0 and 6 g N m^2 y^1),

with one plot of each treatment combination in each of six spatially-separated blocks (n=6 for each treatment combination). For the N addition treatment, 2 g N m^{-2} of NH_4NO_3 as an aqueous solution was added to each plot on three dates in 2010 (8 June, 21 August, and 19 October), 2011 (13 April, 10 June, and 5 October), and 2012 (15 March, 6 June, and 1 October). The N additions were designed to simulate projected increases in atmospheric N deposition by the year 2050 (Galloway et al. 2004).

Shortly prior to snowfall, white mesh with 2 cm × 2 cm holes (Winter Wrap; Quest Plastic Limited) was placed on top of the vegetation in the plots to prevent soil and litter disturbance during snow removal. Snow was removed to a depth of 2 cm whenever snow accumulated in the plots from 9 December 2010 to 16 February 2011. After the 16 February 2011, snow was allowed to accumulate for the remainder of the winter, and no snow was removed from the plots in the subsequent year. Thus, any effects of snow removal over the second growing season would be interpreted as legacy effects of snow removal over the first winter. Snow in the control plots was left undisturbed in both years. Soil temperatures were recorded by placing temperature loggers (Maxim: Ibutton DS1922L-F5) at 5 cm depth within the plots (n=3 for each treatment combination).

4.2.3 Experimental design - controlled freezing experiment

On 25 May 2010, plant-soil mesocosms (sections of PVC pipe: 10 cm diameter × 15 cm long) were inserted into the ground in six spatially-separated blocks and assigned randomly to different combinations of freezing treatments (0, -5, -10 °C), N addition treatments (0, 2 and 6 g N m^{-2} y^{-1}), and one of two harvest dates, for a total of 108 mesocosms. For the N addition treatment, 50 mL of either 2 g N m^{-2} or 0.66 g N m^{-2} of NH_4NO_3 was added to each mesocosm on each of three dates in 2010 (1 June, 29 July and 11 October), 2011 (13 April, 10 June and 5 October), and 2012 (15 March, 6 June and 1 October). On 12 March 2010, all mesocosms were removed from the field and divided evenly among three temperature chambers set at 2 °C for 3 h, then the temperature was decreased at a rate of 0.5 °C per h to 0, -5, or -10 °C. After six d, chamber temperatures were increased at a rate of 0.5 °C per h to 2 °C. However, to allow for uniform thawing among all temperature treatments, increases in the -10 °C and -5 °C

chambers started 20 and 10 hours, respectively, prior to those of the 0 °C chamber. Mesocosms remained at 2 °C for 3 h, then were transported back to the field and returned to their original locations.

4.2.4 Soil sampling

For each sampling period, up to five soil cores and up to three soil cores were collected using a 2 cm diameter corer to a depth of 12 cm from the snow removal and mesocosm plots, respectively. For the snow removal experiment, within 24 hours, soil cores from each plot were bulked, cleared of visible root fragments, then divided up into sub-samples to measure bacterial and fungal biomass, potential trace gas emissions, soil moisture, and extractable soil and microbial C and N. A similar approach was conducted for the mesocosm experiment except soil was separated for soil moisture and extractable soil and microbial C and N only.

4.2.5 Soil moisture and microbial biomass C and N analyses

To determine soil moisture content, soil sub-samples (~10 g) were weighed and then dried at ~65 ºC for 3-4 d and then reweighed. Soil microbial biomass C and N (MBC and MBN) contents were determined by the rapid direct chloroform-fumigation extraction method (Witt et al. 2000). Soil sub samples (~10 g) were placed in a 250 mL glass jar with 0.6 mL of ethanol free chloroform, soil was spread thin using a glass rod, and then incubated for 24 hours in the dark. After 24 hours, the chloroform was allowed to evaporate for 30 mins, then 50 mL of 0.5 M K_2SO_4 was added, samples were shaken for 1 hour, and then samples were filtered through pre-rinsed Whatman # 1 paper. Separate non-fumigated (~10 g) samples were extracted with 50 mL of 0.5 M K_2SO_4 immediately after sorting. Two blanks without sample were included to detect possible contamination during extraction and filtration. All extracts were frozen until further analysis.

4.2.6 Extractable C and N analyses

Extractable total dissolved N (TDN) and total organic C (TOC) in the K_2SO_4 extracts were determined by oxidative combustion and infrared and chemiluminesence analyses,

respectively (TOC-TN auto analyzer, Shimadzu Japan). NH_4^+–N and NO_3^--N were determined colorimetrically (NH_4^+-N: EPA method 353.2; NO_3^--N: EPA method 350.1) using a SmartChem 140 discrete auto-analyzer (Westco Scientific Instruments, Brookfield, Conn.). Extractable organic N (DON) was calculated by subtracting inorganic forms of N from the TDN in the non-fumigated samples. Microbial biomass C and N contents were calculated as the differences between fumigated and non-fumigated extractable C and N samples. All extract concentrations were corrected for initial soil moisture contents.

4.2.7 Trace gas analysis

Soil sub-samples (30-35 g) were placed in 1 L sealed mason jars with lids fitted with female lures attached to two-way male connection stopcocks (Cole Parmer), and incubated at room temperature (~25 °C). After the 36 hours, the chamber headspace gas was mixed four times using a 30 mL syringe to ensure adequate mixing of the air, followed by the removal of 30 mL of headspace gas. A 36 hour incubation time was chosen because previous measurements determined containers become saturated at 72 hours. For each gas sample, 5 mL of gas was pushed through the sampling needle and then the remaining 25 mL was injected into a sealed 12.1 mL exetainer containing a small amount of magnesium perchlorate as desiccant. Samples were then analyzed for CO_2 and N_2O by gas chromatography using a SRI 8610C (SRI 8610C, Las Vegas, NV). The samples were loaded on a Combi-Pal autosampler fitted with a 2.5 mL syringe with automatic injection of sample into the gas chromatograph. For N_2O samples an electron capture detector (ECD) was used. CO_2 samples were also analyzed on the SRI 8610C (SRI 8610C, Las Vegas, NV) equipped with Flame Ionization Detector (FID) with helium as carrier gas and extra dry air and hydrogen for ignition. CO_2 and N_2O concentrations were determined using one point calibrations of 1500 ppm and 5 ppm, respectively.

4.2.8 Total bacterial and fungal biomass

Slide preparation and analysis was adapted from Buckeridge and Grogan (2008) which was modified from Bloem et al. (1995). In short, soil sub samples (~10 g) and blanks were blended for 1 min with deionized water (90 mL), allowed to settle (~1 min), and then 9 mL of the soil solution was fixed with filter-sterile formalin (3.7%). Fixed samples were stored at 4 °C for up to 5 d.

Slides were prepared by 10× diluting fixed soil solutions with deionized water to a final concentration of 0.2 g soil l^{-1}. To reduce masking of bacteria cells by soil particles, after mixing, each sample solution was allowed to settle for 30 seconds, then 20 µL was pipetted into duplicate 6 mm wells (one slide each for fungi and bacteria), and dried in the dark. The polysaccharide stain fluorescent brightener 28 (FB 28) ($C_{40}H_{44}N_{12}O_{10}S_2$; 2.18 mM with 2 drops 1 M NaOH in water), was used to stain total fungi, and the nucleic acid stain DTAF (5-([4,6-dichlorotriazin-2-yl]amino) fluorescein hydro-chloride, 0.038 M in a phosphate buffer solution (PBS; 0.05 M Na_2HPO_4 and 0.15 M NaCl, pH 9), was used to stain total bacteria. After drying, 20 µL of stain was pipetted into each dried soil well and left for 2 h (FB 28) or 30 mins (DTAF). After the allocated time, fungal slides were rinsed in deionized water for 20 mins, whereas bacteria slides were rinsed for 20 mins in PBS then another 20 mins in deionized water. After rinsing, slides were dried, and covered with immersion oil (Type A, Cargill) and a cover slip. All slides were viewed on an epifluorescent microscope (Zeiss-AxioObserver), and images were captured using a Q Imaging (Retiga 1300) camera and Q Capture software.

Fungal slides were viewed at 400× magnification filter set for UV illumination (350 nm peak/50 nm wide excitation filter, 460 nm peak/50 nm wide emission filter) while the bacterial slides were viewed at 1000× magnification with a filter set for blue light (470 nm peak/40 nm wide excitation filter, 525 nm peak/50 nm wide emission filter). Bacterial cell counts and volumes were based on 10-12 random fields of view per slide resulting in at least 300 enumerated. Fungal biomass was based on 25-30 random fields of view per slide. ImageJ software was used to quantify the length (L) and width (W) in pixels of each selected fungi or bacteria for each image. Pixels were then converted to µm using a

conversion factor obtained by using a stage micrometer. There was no difference in the measured width for fungi among treatments and dates, so all widths were assumed to be 1.56 µm (average across dates and treatments). Bacterial and fungal volume per cell (V) were calculated as $V = \pi/ 4 \times W^2 \times (L \times W/3)$, and converted to mass of C assuming a specific carbon content of 1.3×10^{-13} g C μm^3 and 3.1×10^{-13} g C μm^3 for fungi and bacteria, respectively (Bloem et al. 1995).

4.2.9 Data analyses

I tested for significant effects of snow removal, N addition, and their interaction on extractable inorganic and organic C and N pools and microbial biomass C and N pools, potential soil trace gas headspace concentrations, and total fungal and bacteria biomass using repeated measures two-way analyses of variance (ANOVAs) (JMP 4.0, SAS Institute). Similar statistical analysis were conducted for the plant-soil mesocosms, but tested for significant differences for temperature, N addition, and their interaction on extractable inorganic and organic C and N pools and microbial biomass C and N pools. All data were checked for normality and log- or Boxcox- transformed as necessary.

4.3 Results

4.3.1 Snow removal effects on field soil microclimate

Over-winter soil edaphic conditions for the snow removal experiment were previously reported in Chapter 3. In brief, snow removal reduced soil temperatures to an average minimum of -3.1 ºC and caused 5 freeze-thaws cycles, while soils under ambient snow stayed above 0 ºC and thus experienced 0 freeze-thaw cycles throughout the first winter (Chapter 3, Figure 3.1). Soil moisture measured at snowmelt and four weeks later showed no significant treatment effects (Chapter 3: P = 0.66 and P =0.20, respectively). During the second winter, snow removal was not conducted in order to investigate the legacy effects of a single freezing event. Soil temperatures stayed above 0 ºC throughout the second winter with no significant differences among treatments (Appendix A). Furthermore, during the second growing season there was an extended drought period

that reduced soil gravimetric moisture content in the mid-summer by 25% compared to that of the same time in the previous growing season (Chapter 3, Table 3.1; Appendix B).

4.3.2 Snow removal experiment – extractable inorganic and organic C and N and microbial biomass C and N

During the first year, snow removal increased soil NH_4^+, NO_3^-, DON, and DOC pools, with the greatest increase occurring in late February, immediately after snow removal ceased (Table 4.1; Figure 4.1, 4.2). N addition increased NH_4^+ pools, with this effect being particularly pronounced in late February (Table 4.1; Figure 4.1, 4.2). During the second year, NO_3^- pools were elevated in October 2012, but unaffected by the legacy effect of snow removal during any other sampling dates. There was no significant legacy effect of snow removal on any other soil parameter measured during the second year, other than DOC being particularly pronounced under N addition (Table 4.1; Figure 4.1, 4.2). N addition increased NH_4^+ and NO_3^- (Table 4.1; Figure 4.1, 4.2).

Similar to extractable inorganic and organic C and N, during the first year snow removal increased microbial biomass C and N, with particularly high pool sizes occurring in late February (Table 4.1; Figure 4.3). N addition increased microbial biomass N in the ambient snow plots in May of the first year. Snow removal and N addition had no significant effect on microbial biomass C and N during the second year (Table 4.1, Figure 4.3).

4.3.3 Snow removal experiment - potential soil CO_2 and N_2O production

During the first year, potential N_2O production increased in response to snow removal, particularly in late February, whereas potential CO_2 production only increased in response to snow removal in late February (Table 4.1; Figure 4.4). The legacy of snow removal had no significant effect on CO_2 and N_2O production during the second year (Table 4.1; Figure 4.4). N addition had no effect on these variables both years (Table 4.1; Figure 4.4).

Table 4.1: Summary of ANOVA P-values for effects of treatment and date on production of potential N_2O and CO_2, extractable inorganic and organic C and N, and microbial biomass C and N for the first (2011) and second (2012) year in the snow removal experiment. Degrees of freedom are displayed in parentheses after the treatment. Significant effects (P<0.05) are in bold.

Effect								
First year	**N_2O**	**CO_2**	**NH_4^+**	**NO_3^-**	**DON**	**DOC**	**MBN**	**MBC**
Block $_{(5,75)}$	0.38	0.30	0.88	0.14	0.088	**0.013**	0.29	0.12
Date $_{(4,75)}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**
Snow $_{(1,20)}$	**0.042**	0.66	**0.041**	**0.005**	**0.022**	**0.001**	**0.018**	**<0.001**
Date×Snow $_{(4,75)}$	**0.001**	**0.032**	**<0.001**	**0.010**	0.07	**<0.001**	**0.001**	**<0.001**
Nitro $_{(1,20)}$	0.38	0.69	**0.001**	0.21	0.08	0.24	0.35	0.71
Date×Nitro $_{(4,75)}$	0.96	0.11	**0.013**	0.80	0.35	0.60	0.53	0.55
Snow×Nitro $_{(1,20)}$	0.41	0.84	0.22	0.87	0.95	0.42	0.96	0.83
Date×Snow×Nitro $_{(4,75)}$	0.58	0.55	0.084	0.98	0.70	0.97	**0.017**	0.086
Second year								
Block $_{(5,75)}$	0.069	0.31	0.48	0.60	0.29	0.15	0.28	0.54
Date $_{(4,75)}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Snow $_{(1,20)}$	0.56	0.32	0.43	0.44	0.37	0.86	0.47	0.33
Date×Snow $_{(4,75)}$	0.48	0.12	0.73	**0.001**	0.21	0.33	0.85	0.97
Nitro $_{(1,20)}$	0.61	0.43	**0.024**	**0.003**	0.42	**0.037**	0.07	0.42
Date×Nitro $_{(4,75)}$	0.10	0.14	0.18	0.19	0.73	0.51	0.27	0.44
Snow×Nitro $_{(1,20)}$	0.79	0.38	0.14	0.52	0.11	**0.021**	0.46	0.86
Date×Snow×Nitro $_{(4,75)}$	0.37	0.58	0.67	0.83	0.98	0.88	0.40	0.84

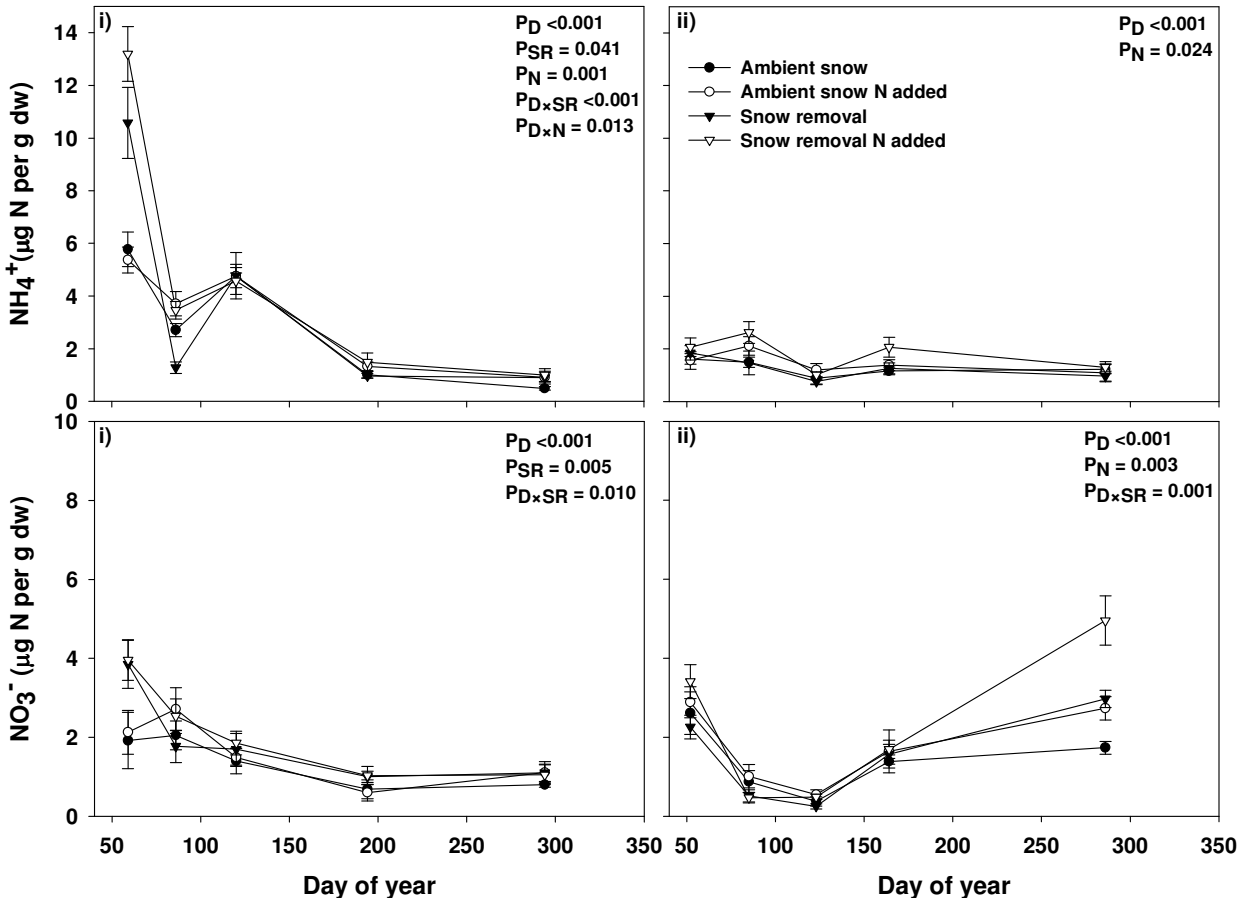

Figure 4.1: Extractable ammonium and nitrate pools (µg N per g dw) during the first (2011; i) and second (2012; ii) year in the snow removal experiment. Data are presented for ambient snow plots (filled circles), snow removal plots (filled triangle), and N addition (open symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen; D - Date).

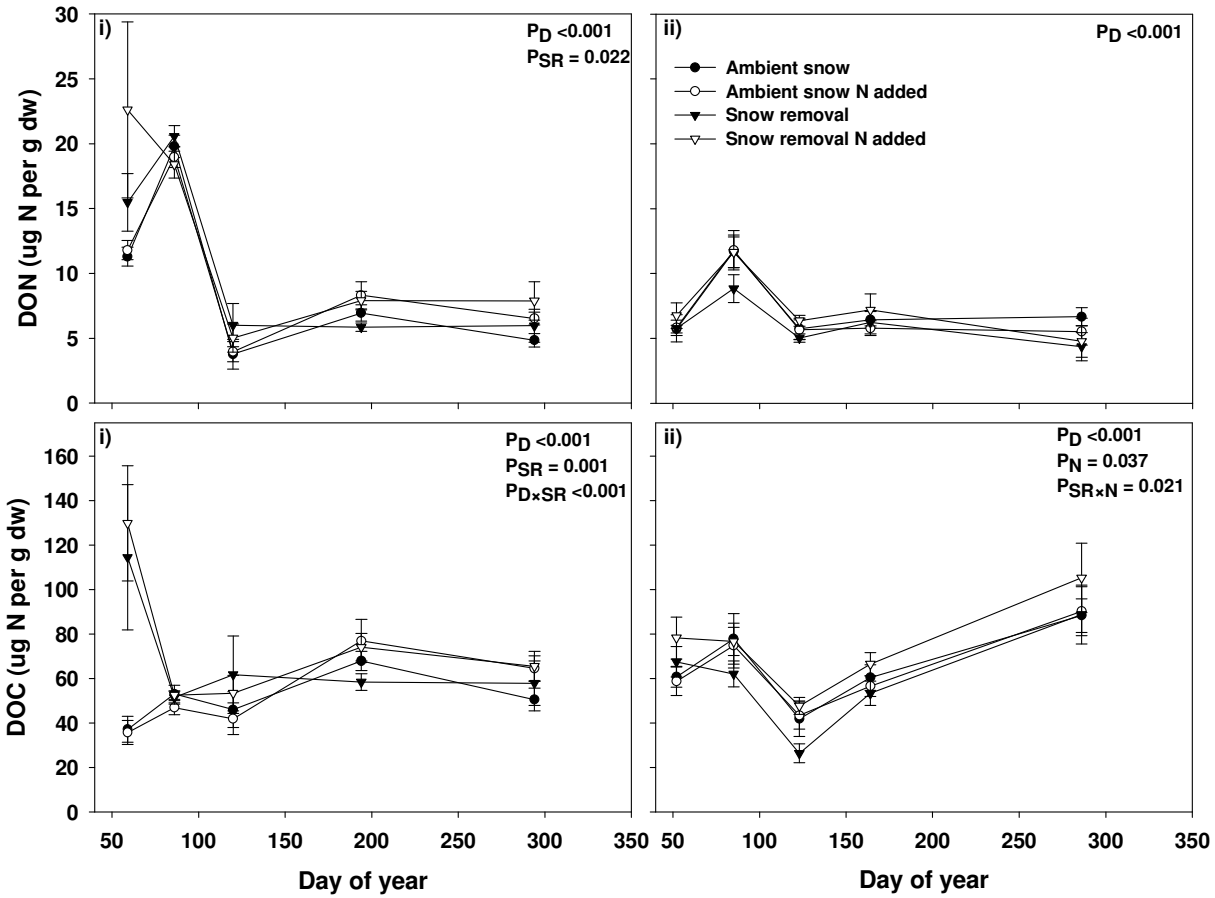

Figure 4.2: Extractable dissolved organic nitrogen and carbon pools (µg per g dw) during the first (2011; i) and second (2012; ii) year in the snow removal experiment. Data are presented for ambient snow plots (filled circles), snow removal plots (filled triangle), and N addition (open symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen; D - Date).

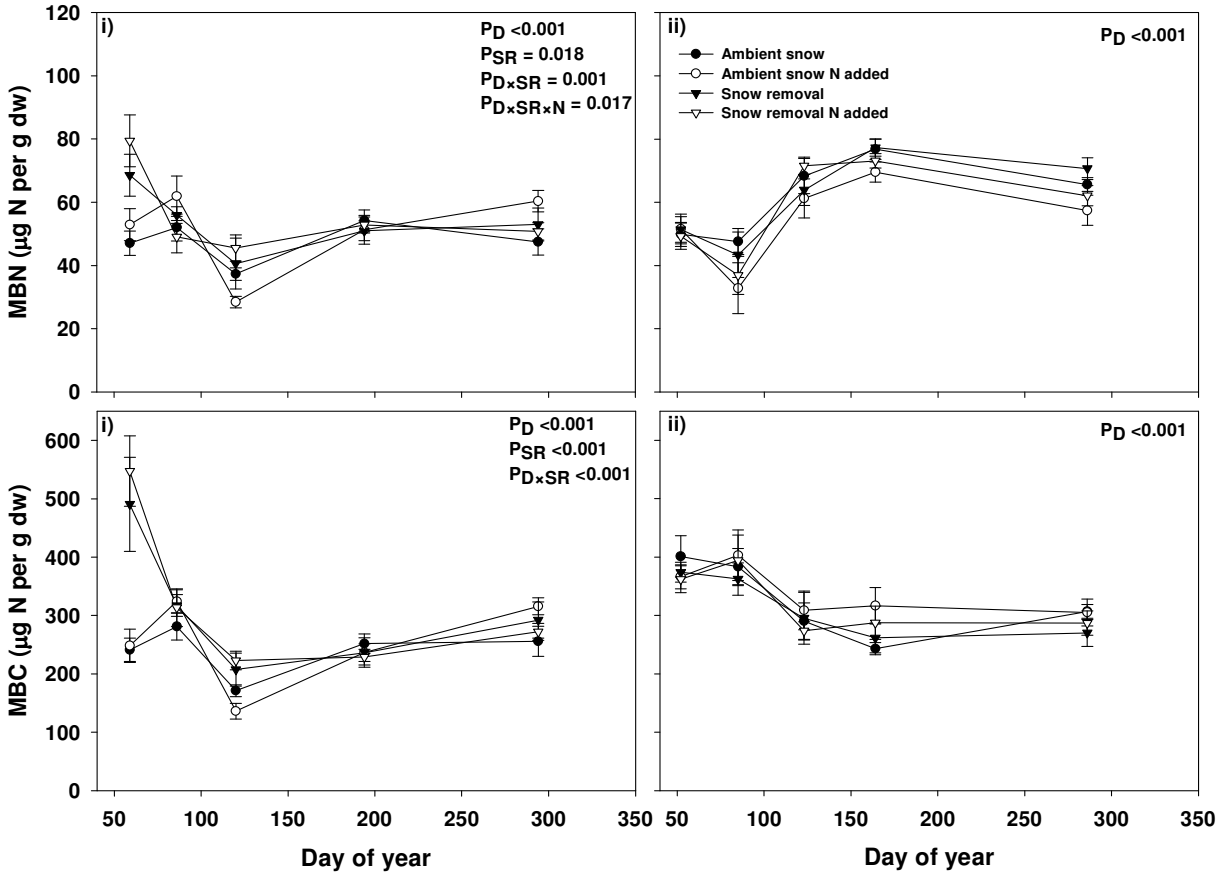

Figure 4.3: Microbial biomass nitrogen and carbon pools (µg per g dw) during the first (2011; i) and second (2012; ii) year in the snow removal experiment. Data are presented for ambient snow plots (filled circles), snow removal plots (filled triangle), and N addition (open symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen; D - Date).

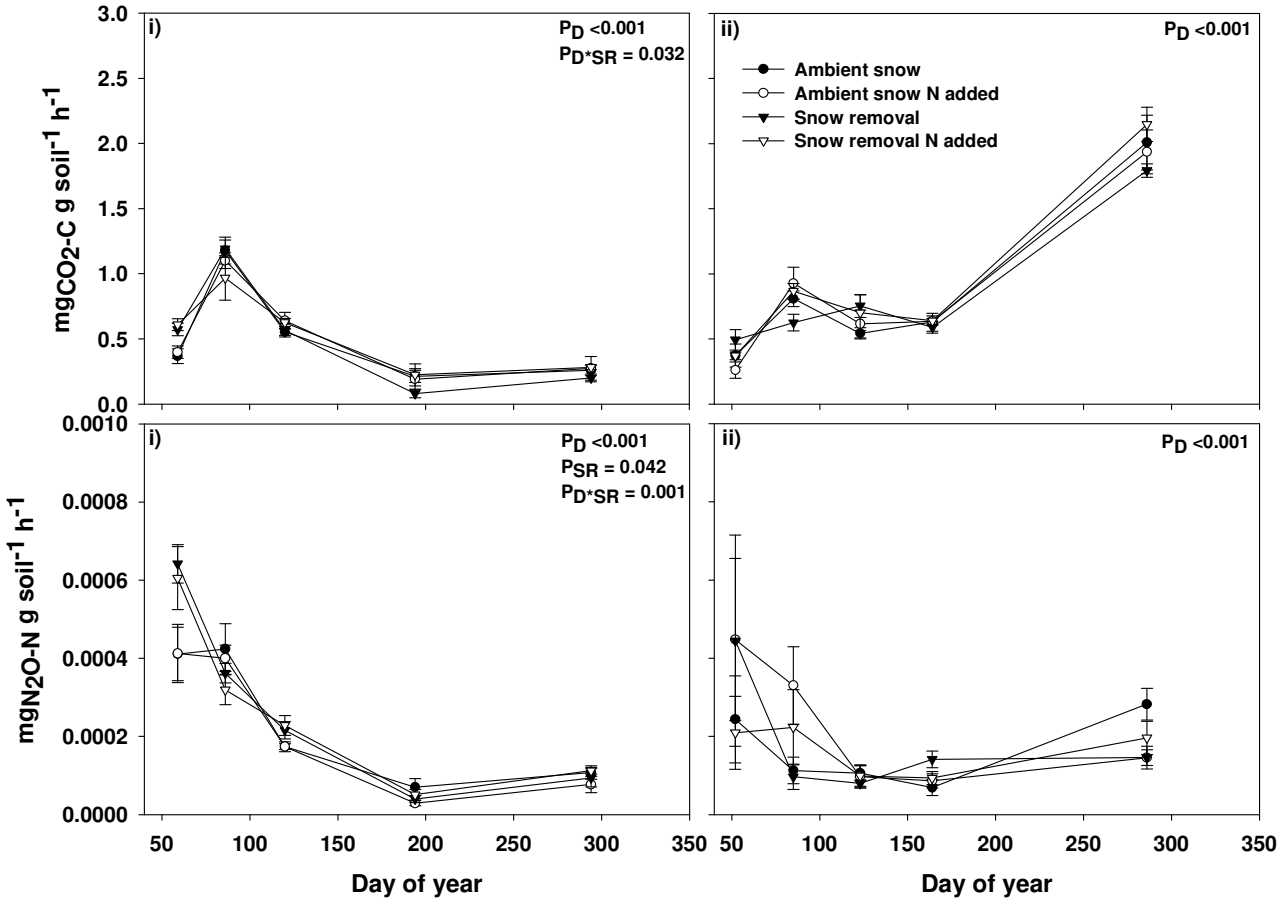

Figure 4.4: Potential soil CO_2 and N_2O production (mg $soil^{-1}$ h^{-1}) during the first (2011; i) and second (2012; ii) year in the snow removal experiment. Data are presented for ambient snow plots (filled circles), snow removal plots (filled triangle), and N addition (open symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen; D - Date).

4.3.4 Snow removal experiment - bacterial and fungal biomass

During the first year, fungal biomass was not affected by the snow removal treatment, but decreased in these plots in the second year (Table 4.2; Figure 4.5). Bacterial biomass was unaffected by the snow removal treatment both years, and N addition had no effect on bacterial or fungal biomass both years (Table 4.2; Figure 4.5).

4.3.5 Mesocosm experiment - extractable inorganic and organic C and N and microbial biomass C and N

During the first year, NH_4^+, and NO_3^- pools increased linearly with increased freezing intensity, with the highest increase in pool sizes immediately after the freezing treatment. DON and DOC pools were only affected by temperature immediately after the freezing treatment (Table 4.3; Figure 4.6, 4.7). N addition had no significant effect on extractable NH_4^+ or DOC, but did increase extractable NO_3^- and DON trended (P=0.58) towards elevated levels in response to the -10 °C freezing treatment. During the second year, the legacy effect of intense freezing (-10 °C) still elevated NH_4^+ and DOC pools significantly; however, this effect mostly occurred in late October, and there was a significant interaction with N addition, which increased DOC pools disproportionately in combination with intense freezing at that time. N addition increased NH_4^+ and NO_3^- pools, but for NH_4^+, this effect occurred predominately right after snowmelt. N addition had no effect on any other soil parameter (Table 4.3, Figure 4.6, 4.7).

During the first year, microbial biomass C pools decreased with increasing freezing intensity, but only on the last two sampling dates and in response to -10 °C mesocosms (Table 4.3, Figure 4.8). N addition increased microbial biomass C pool sizes immediately after the freezing treatment, and this effect was most pronounced at the highest rate of N addition. Microbial biomass N pools were not significantly affected by N addition or freezing intensity (Table 4.3, Figure 4.8). During the second year, freezing intensity decreased microbial biomass C and N; however this only occurred in response to -10 °C

Table 4.2: Summary of ANOVA P-values for effects on estimates of bacterial and fungal biomass for the first (2011) and second (2012) year in the snow removal experiment. Degrees of freedom are displayed in parentheses after the treatment. Significant effects (P<0.05) are in bold.

Effect	First year		Second year	
	Bacteria	**Fungi**	**Bacteria**	**Fungi**
Block $_{(5,75)}$	0.41	0.28	**0.001**	**0.043**
Date $_{(4,75)}$	**0.031**	**<0.001**	**0.011**	**<0.001**
Snow $_{(1,20)}$	0.81	0.68	0.54	**0.036**
Date×Snow $_{(4,75)}$	0.74	0.67	0.74	0.17
Nitro $_{(1,20)}$	0.58	0.32	0.62	0.96
Date×Nitro $_{(4,75)}$	0.31	0.87	0.44	0.90
Snow×Nitro $_{(1,20)}$	0.96	0.76	0.57	0.56
Date×Snow×Nitro $_{(4,75)}$	0.99	0.38	0.84	0.36

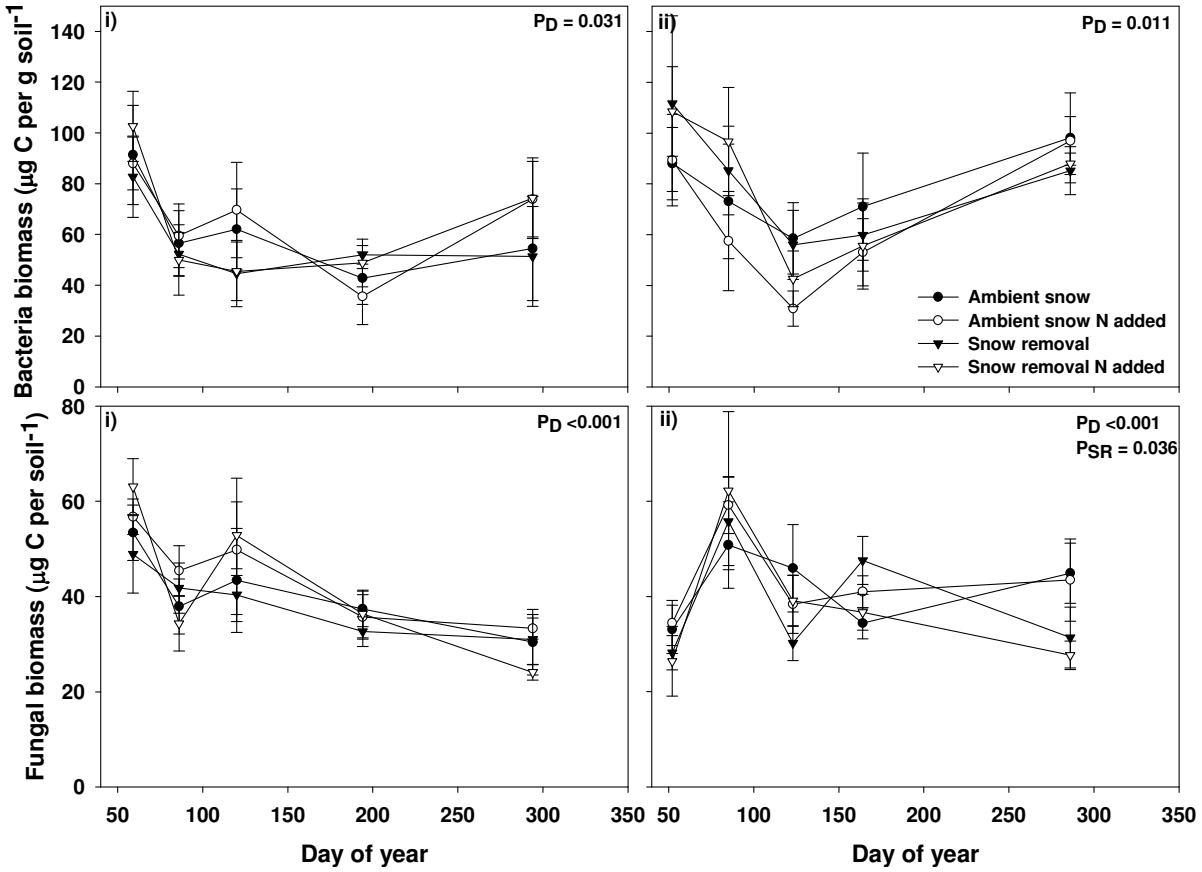

Figure 4.5: Bacterial and fungal biomass estimates (µg per g dw) during the first (2011; i) and second (2012; ii) year in the snow removal experiment. Data are presented for ambient snow plots (filled circles), snow removal plots (filled triangle), and N addition (open symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (SR - Snow removal; N - Nitrogen; D – Date).

Table 4.3: Summary of ANOVA P-values for effects treatment on extractable inorganic and organic C and N and microbial biomass C and N for the first (2011) and second (2012) year in the mesocosm experiment. Degrees of freedom are displayed in parentheses after the treatment. Significant effects (P<0.05) are in bold.

Effect						
First year	**NH_4^+**	**NO_3^-**	**DON**	**DOC**	**MBN**	**MBC**
Block $_{(5,85)}$	**0.026**	0.91	0.21	**0.033**	**0.004**	**0.002**
Date $_{(2,85)}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Temp $_{(2,45)}$	**0.022**	**0.005**	0.29	0.11	0.46	0.11
Date×Temp $_{(4,85)}$	**<0.001**	**0.008**	**0.008**	**0.007**	0.41	**0.017**
N $_{(2,45)}$	0.32	**0.035**	**0.058**	0.55	0.28	**0.059**
Date×N $_{(4,85)}$	0.94	0.21	0.81	0.93	0.13	0.089
Temp×N $_{(2,45)}$	0.80	0.44	0.062	0.17	0.43	0.64
Date×Temp×N $_{(8,85)}$	0.99	0.11	0.85	0.86	0.67	0.40
Second year						
Block $_{(5,85)}$	**0.031**	0.87	**0.033**	0.16	**0.053**	**0.013**
Date $_{(2,85)}$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Temp $_{(2,45)}$	**0.001**	0.097	0.068	0.075	**0.045**	**0.030**
Date×Temp $_{(4,85)}$	**0.003**	0.36	0.067	**0.036**	**0.033**	**0.017**
N $_{(2,45)}$	**<0.001**	**0.001**	0.48	0.60	0.16	0.45
Date×N $_{(4,85)}$	**0.018**	0.24	0.94	0.88	0.44	0.99
Temp×N $_{(2,45)}$	0.13	0.41	0.092	**0.005**	0.93	0.66
Date×Temp×N $_{(3,85)}$	0.81	0.39	**0.020**	**0.014**	0.13	**0.018**

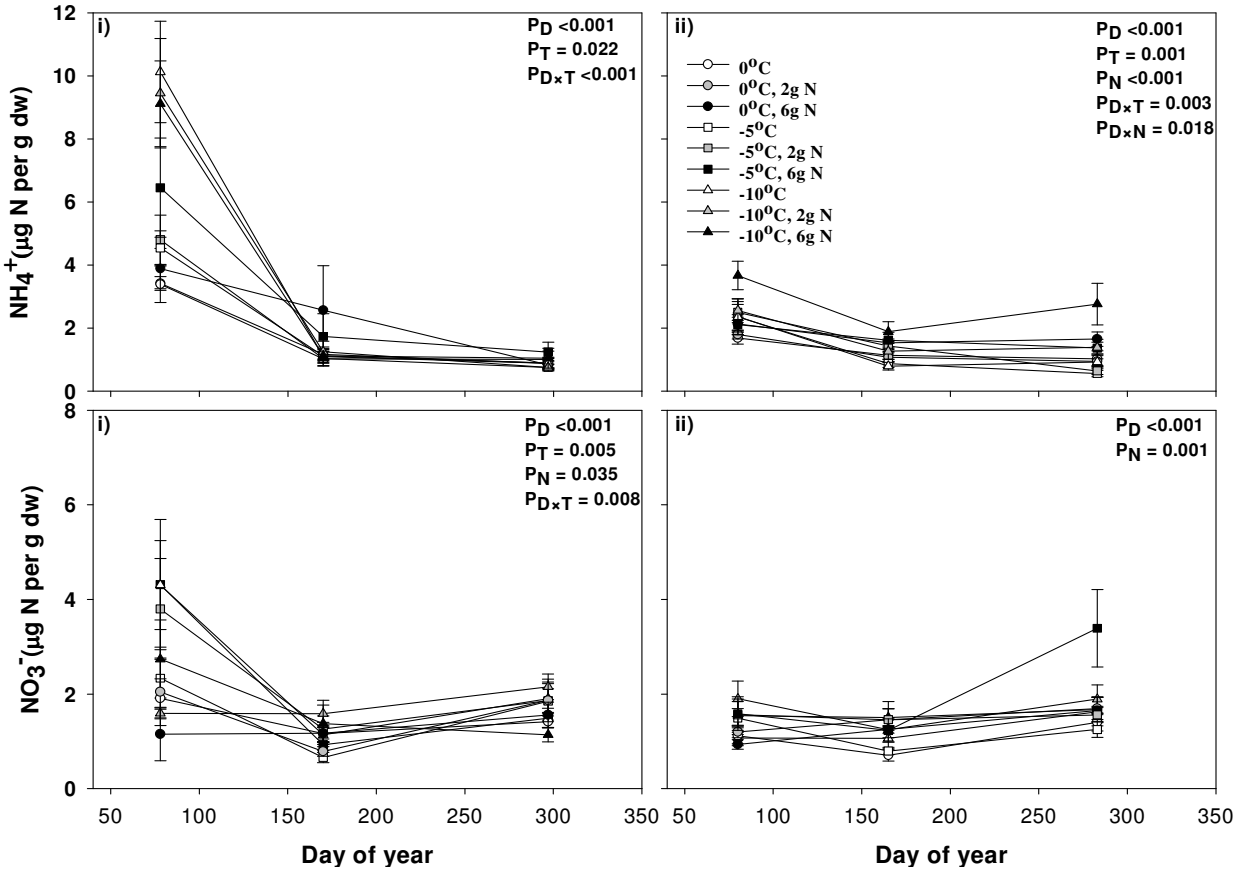

Figure 4.6: Extractable ammonium and nitrate pools (µg N per g dw) during the first (2011; i) and second (2012; ii) year in the mesocosm experiment. Data are presented for 0 °C (open circle), -5 °C (open triangle) and -10 °C (open square) temperatures, and 2 g y^{-1} of NH_4NO_3 (grey symbols) and 6 g y^{-1} of NH_4NO_3 (filled symbols) N additions. Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (T - Temperature; N - Nitrogen; D - Date).

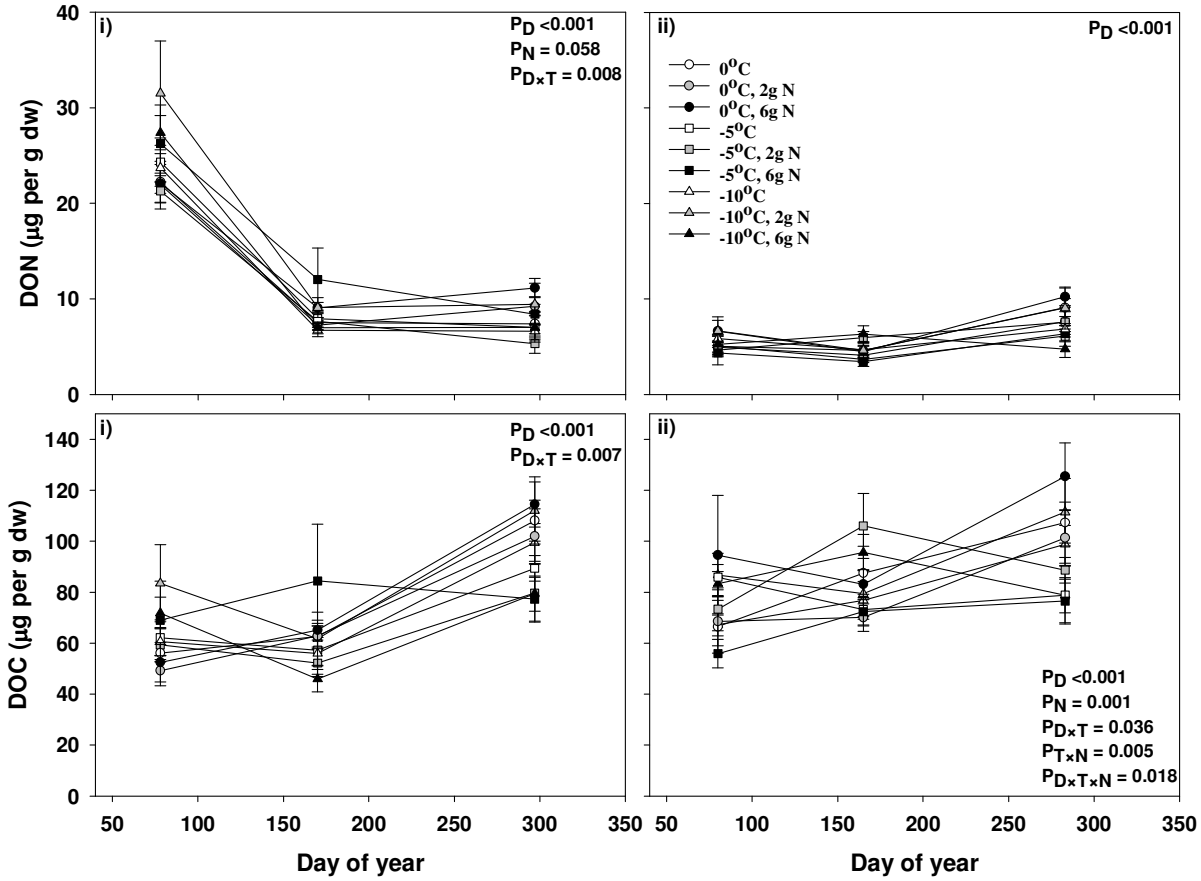

Figure 4.7: Extractable dissolved organic nitrogen and carbon pools (µg per g dw) during the first (2011; i) and second (2012; ii) year in the mesocosm experiment. Data are presented for 0 °C (open circle), -5 °C (open triangle), and -10 °C (open square), and 2 g y^{-1} of NH_4NO_3 (grey symbols), and 6 g y^{-1} of NH_4NO_3 (filled symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (T - Temperature; N - Nitrogen; D - Date).

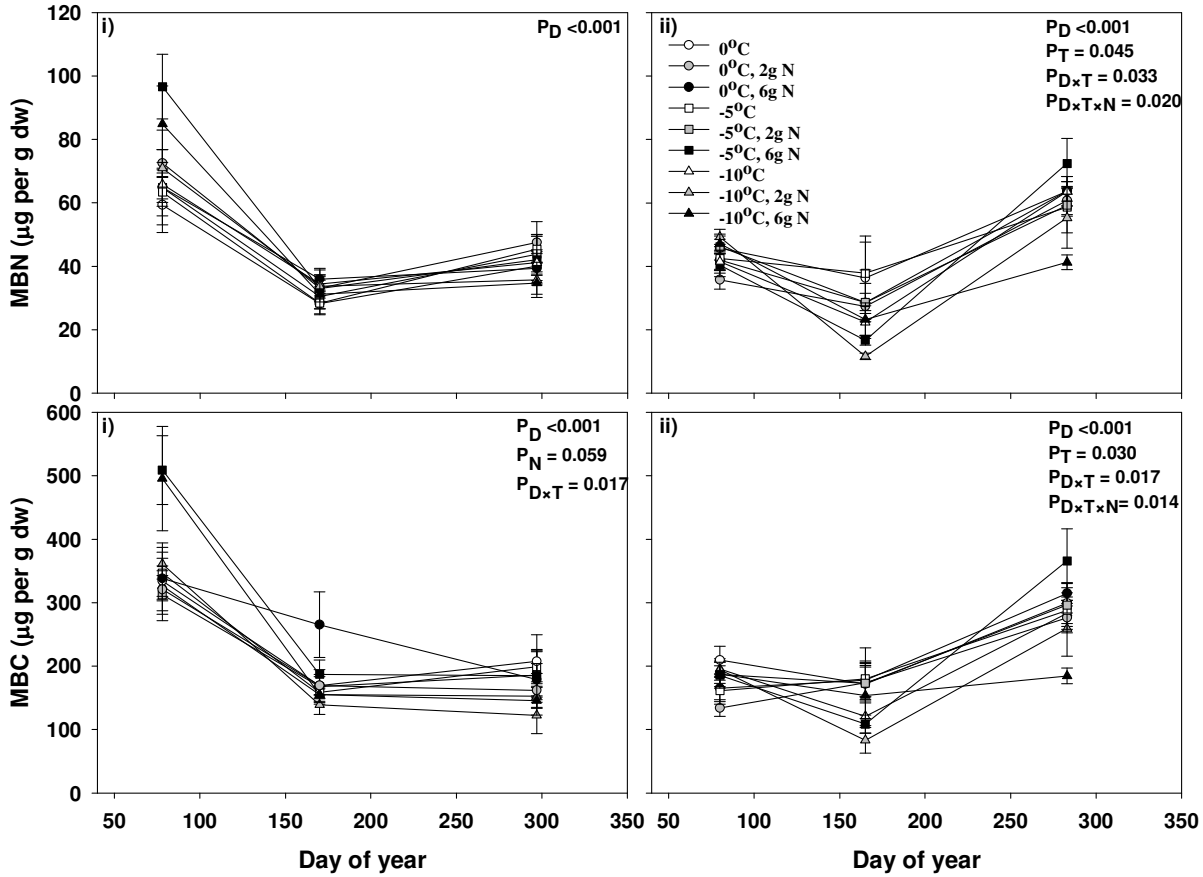

Figure 4.8: Microbial biomass nitrogen and carbon pools (µg per g dw) during the first (2011; i) and second (2012; ii) year in the mesocosm experiment. Data are presented for 0 °C (open circle), -5 °C (open triangle), and -10 °C (open square), and 2 g y^{-1} of NH_4NO_3 (grey symbols), and 6 g y^{-1} of NH_4NO_3 (filled symbols). Raw means are displayed (n=6) and error bars denote standard error. Significant ANOVA effects are displayed in each panel (T - Temperature; N - Nitrogen; D - Date).

for the latter two sampling dates for the mesocosms that received the highest N addition rate (6 g m^{-2} $y^{-}1$; Table 4.3, Figure 4.8).

4.4 Discussion

4.4.1 The effects of soil freezing on extractable inorganic and organic C and N, microbial biomass C and N pools, and bacterial and fungal biomass

Overall, snow removal increased soil extractable inorganic and organic C and N pools, potential CO_2 and N_2O production, and microbial biomass C and N pools in the winter, but many of these effects dissipated by the following growing season (Table 4.1; Figure 4.1, 4.2, 4.3). The most surprising result was a lack of growing season effect on NH_4^+ and NO_3^- pools over summer, because I predicted that freezing damage to roots would decrease the capacity for plant N uptake at this time (Malyshev and Henry 2012), leading to the build-up of these pools in the soil solution (Maljanen et al. 2007). It is possible that leaching or denitrification losses increased concomitantly in the snow removal plots, resulting in no net change in the extractable N pools; however, in previous studies, soil freezing did not increase soil N losses during the growing season at my site (Vankoughnett and Henry 2013), and in the present study, it did not increase potential soil N_2O losses during the growing season (Table 4.1; Figure 4.4). Thus, as a possible alternative, early season microbial immobilization (Table 4.1; Figure 4.3) and high plant N demand may have reduced extractable inorganic N pools across all treatments at this time.

Despite the lack of an increase in extractable inorganic N pools through the first and most of the second growing season, there was a large increase in NO_3^- pools in the snow removal plots at the end of the second growing season (Table 4.1; Figure 4.1). This increase corresponded to a rewetting event that followed an extended drought period (Appendix B). Microbial activity, including N mineralization, often increases following rewetting periods (Birch 1958, Bloem et al. 1992, Borken and Matzner 2009), and the

reduced plant biomass as a legacy of snow removal (Chapter 3: Table 3.4; Figure 3.2) may have delayed the uptake of the NO_3^- pulse in these plots.

Similar to the soil extractable C and N pools, soil freezing enhanced microbial biomass C and N pools during the first winter (Table 4.1; Figure 4.3). These results contrast other studies that have reported decreases in or no effect of soil freezing and freeze-thaw on microbial biomass C and N (Groffman et al. 2001b, Larsen et al. 2002, Grogan et al. 2004, Yanai et al. 2004, Bolter et al. 2005). The increase in microbial biomass C and N that I observed in response to freezing may have resulted from the microbes capitalizing on the pulse nutrients released following the disruption and breakdown of soil aggregates and litter (Harris and Safford 1996, Oztas and Fayetorbay 2003, Six et al. 2004), as well as root and microbial lysis (Gaul et al. 2008; Skogland et al. 1988). By snowmelt, this increase in microbial biomass C and N was no longer present, suggesting that over winter soil N losses in my system in response to freezing may be in part caused by a large turnover in the microbial community at snowmelt (Edwards and Jefferies 2012, Vankoughnett and Henry 2013).

Snow removal had no consistent effect on bacterial or fungal biomass during the first year. Previous studies have documented that increased soil freezing intensity and freeze-thaw cycles can reduce fungal biomass (Feng et al. 2007, Schmitt et al. 2008, Yergeau and Kowalchuk 2008), potentially by physically disrupting hyphal networks (Schimel et al. 2007); however these decreases have generally only occurred with extreme temperatures exposure, below that which was experienced in the current study (Chapter 3: Figure 3.1). Despite bacterial and fungal biomass being unaffected by snow removal during the first year, fungal biomass was reduced during the second year as a legacy of snow removal in the previous winter, corresponding to my prediction. However, fungal declines at this time may have been caused indirectly by reduced litter carbon inputs associated with the reduced plant productivity over the previous growing season or possibly due to freezing reducing fungal colonization (Klironomos et al. 2001), rather than caused by the relatively small changes in plant species abundances.

Soil freezing and freeze-thaw studies have often used treatments much more severe than those likely to be experienced in the field, possibly resulting in a disproportionate reporting of freezing effects in the literature (Henry 2007, Henry 2008). As a complement to the snow removal experiment, I used the mesocosm experiment to examine the effects of freezing intensity in a controlled manner. Although the freezing treatments had no affect on microbial biomass C and N pools, my results indicate that even under relatively moderate freezing, changes in winter soil temperatures can have a strong effect on short term inorganic and organic N pools. Nevertheless, there was a threshold response to the extractable inorganic N increase following freezing, with the coldest freezing treatment (-10 ºC) not having a significantly larger effect than the moderate freezing treatment (-5 ºC).

The lack of an effect of freezing severity on the microbial biomass may have been due to the slow rate of freezing (0.5 ºC h^{-1}) that the mesocosms were exposed to. Decreases in microbial biomass C and N following soil freezing have often only been observed in studies with fast freezing rates (Lipson et al. 2000, Henry 2007). There was a decrease in microbial biomass C and N later in the first year in the mesocosms that had been exposed to -5 ºC and -10 ºC, and this effect persisted throughout the entire second year in the -10 ºC treatment, but these delayed responses likely reflected the reduced plant productivity in these mesocosms.

4.4.2 N addition effects

N addition did not interact with snow removal to further elevate the extractable inorganic N pool (Table 4.1; Figure 4.1); however, this response was consistent with the observation that plant productivity increased in response to N addition in the snow removal plots (Chapter 3: Figure 3.2). Only during the second year, when drought limited plant productivity, did N addition increase soil extractable NO_3^- (Table 4.1; Figure 4.1; Chapter 3: Figure 3.2). Bacterial and fungal biomass were not significantly affected by N addition throughout the experiment. This result was contrary to previous studies that have observed decreases in fungal biomass in response to N addition, although such responses may only occur following chronic N addition over many years

(Treseder 2008). Therefore, although I observed very few interactions between snow removal and N addition, I cannot rule out that such interactions would not occur with chronic N addition (Aber et al. 1989).

4.5 Conclusions

Our results reveled that the direct impacts of severe soil freezing can cause short-term increases in extractable soil N pools and microbial biomass N and C, and these increases could potentially affect soil N losses over winter, while over the longer term, changes to soil C and N cycling are indirectly affected by the legacy effects of soil freezing through changes in plant productivity or relative species abundance.. Although I observed few significant effects of N addition on soil responses, effects on soil in response to multiple years of chronic N deposition cannot be ruled out. Soil freezing impacts on soil N cycling are relatively transient, despite changes to aboveground plant productivity, however, potential changes to soil carbon inputs (litter or root exudates) could lead to longer term impacts on N cycling through changes in microbial community structure.

4.6 Acknowledgements

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5 Chapter 5: General discussion

5.1 Summary

The overarching goal of my thesis was to explore the combined and possibly interactive effects of soil freezing and N deposition on plant productivity, soil microorganisms, and soil N cycling, both transiently (over winter and spring melt) and over multiple growing seasons. In order to investigate these potential combined or interactive effects, I used a combination of snow removal experiments and controlled freezing of plant-soil mesocosms. These experiments provided insight into the consequences and mechanisms underlying how increases in soil freezing and N deposition may combine or interact to affect plant and soil N dynamics. The results presented in Chapter 2 indicate that soil freezing increases the contribution of root and soil N pools to ecosystem N losses over the winter. In contrast, over-growing season losses largely occur from decreased retention of N deposition being added to the system, rather than losses from existing ecosystem N pools. The research findings in Chapter 3 highlight the potential transient and multiyear legacy effects of sub-lethal soil freezing on plant productivity and relative species abundances and how increased atmospheric N deposition may counter decreases in plant productivity. The research findings in Chapter 4 indicate that soil freezing impacts on soil N cycling are relatively transient (gone by late spring), but there is potential for longer term impacts on N cycling through changes in microbial community structure. The potential consequences of soil freezing and atmospheric N deposition for plant and soil N dynamics and the importance of freezing severity for ecosystem responses to variability in winter conditions are discussed below.

5.2 Soil freezing and N deposition effects on ecosystem N losses

A key result in Chapter 2 was that soil freezing increased over-winter N losses from root and soil N pools. The increased contributions by these two specific N pools was not surprising, based on evidence that the over winter soil temperatures experienced in the snow removal plots were severe enough to cause root damage (Malyshev and Henry

2012), and the assumption that freezing periods can lead to resource depletion and microbial starvation (Clein and Schimel 1995). However, it is possible that the latter assumption regarding microbial responses may be incorrect in the context of my study. The field experiment described in Chapter 4 was conducted over the same winter as that described in Chapter 2, and in the former, snow removal increased late winter microbial biomass C and N. Similar increases in late winter microbial biomass (under deepened snow cover) observed in the Arctic enhanced extractable soil N relative to control plots during snowmelt, which corresponded with declines in microbial biomass C and N (Buckeridge and Grogan 2010). A similar snowmelt pulse of extractable soil N may have occurred at my site, although such a pulse could only have been detected through daily soil sampling at this time. Such a pulse would indicate that the over winter soil N losses were caused by the turnover of the winter microbial community at this time, rather than soil freezing damage to microbes.

Another key finding in Chapter 2 was that soil freezing did not increase growing season N losses, which contradicts the findings of similar experiments conducted in temperate forests (Boutin and Robitaille 1995, Fitzhugh et al. 2001, Callesen et al. 2007). However, the lack of such an effect in my study supports the suggestion that vegetation type may control growing season ecosystem N losses in response to soil freezing (Blankinship and Hart 2012). While the mechanisms explaining variation in the responses observed among studies are unclear, Blankinship and Hart (2012) suggested that differences among systems in plant N demand and productivity in the spring may be major factors. Specifically, the rapid growth of grasses in the spring suggests that plant N demand is high in my system at this time, and Blankinship and Hart (2012) proposed that small statured, non-woody plants, which feature rapidly-growing and shallow rooting systems, may be more effective than trees of exploiting early spring N pulses (Mullen et al. 1998, Bilbrough et al. 2000, Starr and Oberbauer 2003). Furthermore, Blankinship and Hart (2012) stated that high soil water availability coupled with the presence of recalcitrant organic matter may enhance microbial activity and N mobilization in forest ecosystems (Oquist et al. 2009, Harrysson Drotz et al.).

The final surprising result from Chapter 2 was that the effects of N addition on retention of the simulated N deposition pulse varied seasonally. N addition decreased the retention of simulated N deposition at snowmelt, but increased retention at peak biomass. This seasonal shift in retention appeared to be driven by N addition effects on plant biomass. Therefore, while future increases in soil N may cause large N losses of N deposition at snowmelt, these increases in soil N could lead to increased plant productivity later in the growing season, which may increase ecosystem N retention.

5.3 The ecological importance soil freezing and N deposition

Although there is widespread evidence for soil freezing effects on plant productivity, species losses, nitrate leaching, and trace gas losses (Fitzhugh et al. 2001, Muller et al. 2003, Groffman et al. 2006, Schaberg et al. 2008, Kreyling 2010, Schaberg et al. 2011, Comerford et al. 2013), most soil freezing experiments have not monitored effects beyond a single growing season, leaving the longer term implications for plants and soil unclear. A key finding in Chapter 3 was that legacy effects of soil freezing on plant productivity and relative species abundance were still apparent after two growing seasons. These findings are consistent with studies where a single soil freezing event has had multiyear impacts on plant productivity and species abundances (Kreyling et al. 2010, Kreyling et al. 2011). Over the longer term, the changes in relative species abundances may prove the most influential component of these legacy effects. In particular, freezing damage to plants can decrease litter layer thickness or open gaps in the plant canopy, which could facilitate succession by increasing tree seedling germination and establishment (Vandenberghe et al. 2006), or allow for the propagation of invasive species (Diez et al. 2012). Either scenario could change the successional trajectories of the plant community (White and Jentsch 2001, Jentsch et al. 2007).

Despite soil freezing decreasing plant productivity over two growing seasons (Chapter 3), as observed in Chapter 4, this effect had little consequence on extractable soil C and N pools and microbial C and N pools over the first and much of the second growing season. However, at the last sampling point there was an increase in extractable NO_3^- during the second growing season in the snow removal plots. As described previously, this increase

in NO_3^- pools coincided with a soil rewetting period after a drought. Rewetting periods following extended droughts can increase microbial activity, and in particular N mineralization (Birch 1958, Bloem et al. 1992, Borken and Matzner 2009). Although the fate of this increased N is unknown, due to the high mobility nature of NO_3^-, it is possible it will be leached from these plots. Nevertheless, this result could have important implications in the context of projected changes in precipitation patterns over the next century. Climate change is predicted to cause heavier rainfall, but with a lower frequency of rain fall days and a longer duration between rainfall periods (Easterling et al. 2000, IPCC 2007), which will lead to extended drought periods. When these precipitation changes follow a soil freezing event in the future, increased N leaching losses could occur during the growing season.

Another important finding in Chapter 3 was that freezing severity (i.e., minimum temperature) played a critical role in determining plant and soil responses. In the mesocosm experiment, I used three different temperature treatments (0, -5, and -10 ºC), and as soil freezing severity increased, plant productivity during the growing season decreased, with only the most severe treatment (-10 °C) carrying over to the second growing season. As for soil, there was a threshold response, where moderate freezing (-5 ºC) increased extractable inorganic N, but this effect was not enhanced further with more severe (-10 ºC) freezing. However, during the second growing season, the most severe freezing treatment (-10 ºC) decreased microbial biomass C and N throughout the growing season. These microbial responses during the second growing season are likely a reflection of soil freezing legacy effects on plant productivity (with resulting effects on microbial community composition or structure), because microbial biomass was also declined during the first growing season in the mesocosms that had decreased plant biomass.

Although I used different freezing temperatures to assess the impacts of soil freezing severity on plant productivity and soil N dynamics, plant and microbial freezing damage can also be enhanced by freezing rate. Plant and soil microbial biomass studies have both found that more rapid freezing generally leads to greater damage to plants and microbes (Finkle et al. 1974, Lipson et al. 2000), because physiological acclimation is too slow to

acclimate to rapid freezing. In unmanaged systems, such as my field site, the thick litter layer provides insulation from the air, and soil freezing rates faster than 1 ºC h^{-1} do not occur (Elliott and Henry 2009). This observation may be of particular importance, because studies where plants and soil microbes have been exposed to realistic freezing rates (i.e. less than 1 ºC h^{-1}) have reported few significant freezing effects (Lipson et al. 2000, Malyshev and Henry 2012). However, in managed systems, where the thick litter layer is removed by mowing or grazing, more rapid freezing could occur.

Increased freezing duration can also increase soil freezing damage severity. For example, Malyshev and Henry (2012) found that root N uptake became more impaired following longer freezing periods, and Austnes and Vestgarden (2008) found soluble N increased under prolonged freezing. These effects are likely driven by plant cellular dehydration damage becoming more severe the longer the tissue is frozen, and frozen water films limit diffusion, leading to resource depletion and microbial starvation (Clein and Schimel 1995). In addition, timing of freezing events is another important factor that influences freezing damage; in particular, there has been a recent interest in the ecological consequences of mid-winter warming events and spring frosts (Bokhorst et al. 2009, Augspurger 2011, Kreyling et al. 2012a, Kreyling et al. 2012b), because warm air causes cold deacclimation in plants and enhances microbial activity, leaving these organisms susceptible to the return of frost (Schimel and Clein 1996, Bokhorst et al. 2008, Gu et al. 2008, Bokhorst et al. 2011, Malyshev and Henry 2012). Therefore, although my results indicated that multiyear impacts of soil freezing only occur in response to very severe freezing intensity, the legacy effects of lower intensity freezing may occur if such events coincide with periods of low plant and microbial cold acclimation.

With respect to potential interactions between soil freezing and N deposition, my results support the prediction that these can occur in the context of plant productivity. As described in Chapter 3, although soil freezing decreased plant productivity, N addition increased plant growth, and thus counteracted the soil freezing effect over both growing seasons. Moreover, there was an interaction between soil freezing and N addition during the first year, whereby the N addition effect was higher in the snow removal plots than in the ambient snow plots. In the Chapter 3 discussion, I attributed such an effect to

limitation of productivity in the ambient snow plots to another factor such as light or phosphorus. Although the results in Chapter 4 indicated that there were no interactions for soil responses, collectively, the plant and soil responses could have important implications for ecosystem C dynamics. In Chapter 4, I observed equal potential soil CO_2 respiration among treatments during the first growing season, but if these CO_2 losses are coupled with plant productivity decreases due to soil freezing (Chapter 3), net ecosystem CO_2 exchange may have differed among treatments. It follows that the interactive effects of soil freezing and N addition on plant productivity could have altered net ecosystem CO_2 exchange.

Another finding in Chapter 3 was that the amount of N added was important in determining plant productivity responses. In the mesocosm study, there were three levels of N addition (0, 2, and 6 g N m^{-2} y^{-1}), with 2 g N m^{-2} y^{-1} corresponding with the lower bound on deposition estimates for the study area by 2050 and 6 g N m^{-2} y^{-1} corresponding with slightly above the upper bound (Galloway et al. 2004). Plant productivity only responded to the 6 g N m^{-2} y^{-1}, despite 2 g N m^{-2} y^{-1} being double the amount of N deposition the system currently receives. This suggests that in order for N deposition to have significant effects on plant productivity or species composition, N deposition rates would have to reach the extreme values expected for this region. However, longer term effects at chronic N addition 2 g N m^{-2} y^{-1} cannot be ruled out.

5.4 The model for interactions between soil freezing and N deposition revisited

In Chapter 1, I presented a composite model of soil freezing mechanisms whereby freezing can lyse the cells of microbes (Skogland et al. 1988), disrupt soil aggregates, breakdown litter (Harris and Safford 1996), and damage roots (Tierney et al. 2001, Gaul et al. 2008), with the latter reducing the capacity for plant N uptake (Malyshev and Henry 2012), which increases N leaching losses (Fitzhugh et al. 2001) and decreases plant productivity over the growing season (Malyshev and Henry 2012, Comerford et al. 2013). When combined with increased atmospheric N deposition, soil N losses may be exacerbated, increasing the transfer of terrestrial N to aquatic ecosystems (Figure 5.1a). While this model provided the conceptual framework for my research objectives and

predictions, the findings presented in my thesis highlight potential alterations needed to this model (Figure 5.1b). Specifically, in contrast to my prediction, despite soil freezing leading to decreased plant productivity, plants were responsive to N addition. As a result, soil freezing only enhanced ecosystem N losses during the winter, not during the growing season, although N deposition losses occurred as a result of decreased plant productivity (Figure 5.1b).

5.5 Future research

Despite an increase in temperate ecosystem winter research over the last 20 years, several questions still remain that need further investigation.

1. Winter photosynthesis

Many northern plants continue to have green leaves over winter (Chabot and Hicks 1982). Due to this adaptation, these plants can continue to photosynthesize during the winter down to 0 ºC or below (Day et al. 1989, Starr and Oberbauer 2003, Skinner 2007, Tuba et al. 2008, Hoglind et al. 2011), helping replenish carbohydrate reserves lost through respiration (Schaberg et al. 2000). During the winter, expected decreases in snow cover will therefore have a considerable impact on plant photosynthesis (Taulavuori et al. 2011). Some plants species may benefit disproportionately from increased sunlight exposure over winter, because they can gain carbohydrates to enhance growth over winter or in early spring, which may allow them to have a competitive advantage over non-winter photosynthetic species. Nevertheless, there are tradeoffs to photosynthesis at this time, because as temperatures approach 0 ºC photosynthetic reactions slow down, water may become limiting, and under high light conditions, the captured light energy cannot be used productively, increasing photooxidative damage and photoinhibition (Oquist and Huner 2003). Under these circumstances, plants may deplete their carbohydrate reserves, decreasing frost hardness. Therefore, future studies should investigate how predicted changes in snow cover may influence winter photosynthesis, and how this will affect growing season plant productivity.

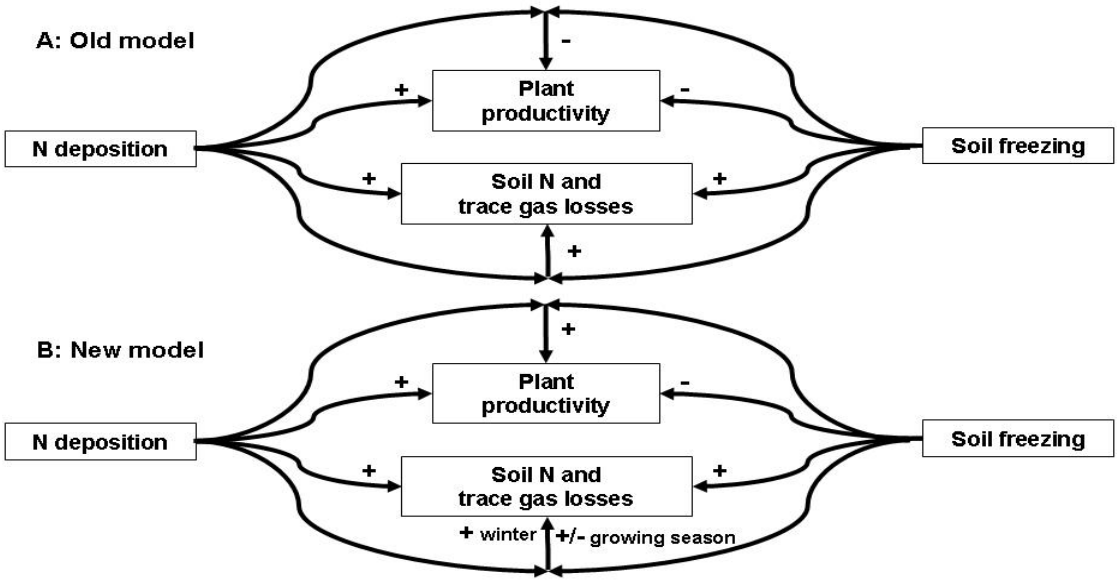

Figure 5.1: (A) The predicted combined and interactive effects of soil freezing and atmospheric N deposition on plant productivity and soil solution N and trace gas losses. (B) Actual observed combined and interactive effects of soil freezing and atmospheric N deposition on plant productivity and soil solution N and trace gas losses.

2. Seedling and grass competition in response to soil freezing

Over the last six decades, more than 35% of grasslands that had been converted to pasture production in eastern Canada have been abandoned (Parson 1999). Succession from grass-dominated old fields to forest communities represents a key transition in ecosystem structure, however, climate change may alter this transition by affecting the ability of seedlings to survive and grow, and in particular, it could alter resource competition with grasses (Davis et al. 1998, Hovenden et al. 2008, Classen et al. 2010). Projected changes to snow cover and frost regimes could influence seedling and grass competition by altering soil N and affecting grass and tree seedling productivity during the growing season (Kreyling et al. 2008, Schaberg et al. 2008, Turner and Henry 2009, Hutchison and Henry 2010, Kreyling et al. 2010, Turner and Henry 2010, Drescher and Thomas 2013). Furthermore, the creation of gaps or decreases in the litter layer caused by soil freezing could decrease grass productivity and promote tree seed germination success, thus facilitating succession. Future studies should investigate how decreased snow cover may influence seedling resource and tree seed germination success in competition with grasses, effectively extending the multiyear context of my study (effects up to two years post freezing) up to that of multiple decades.

3. Microbial community composition

Laboratory studies have confirmed that microbial community composition can change as a result of freeze-thaw exposure (Walker et al. 2006), and these changes are often correlated with changes in microbial function (Pesaro et al. 2003, Sharma et al. 2006). However, there are very few field studies that have investigated the transient *versus* multiyear effects that soil freezing could have on microbial community composition. In particular, changes in plant productivity and species abundances play a key role in influencing changes in microbial community composition.

5.6 Concluding remarks

My thesis has provided considerable insight into the transient *versus* multiyear effects of soil freezing on a grass-dominated old field ecosystem, and how projected increases in atmospheric N deposition may alter plant and soil responses. I found that soil freezing can increase losses of N currently in the system, but this only occurred during the winter, while summer N losses resulted from the inability of plants to retain N being added to the system (i.e. simulated N deposition). Furthermore, I found that while soil freezing and atmospheric N deposition can have a considerable impact on plant productivity over multiple years, in general, outside of very short term responses, changes in soil properties such as soil inorganic and organic N were minimal, and did not correspond with the aboveground changes observed over the longer term. However, it is possible that more drastic changes in plant relative species abundances in the future could lead to changes in soil responses beyond those observed over the time course of my experiments. Therefore, extreme soil freezing events can have short term impacts on soil N cycling with long term implications for plant productivity and species abundances.

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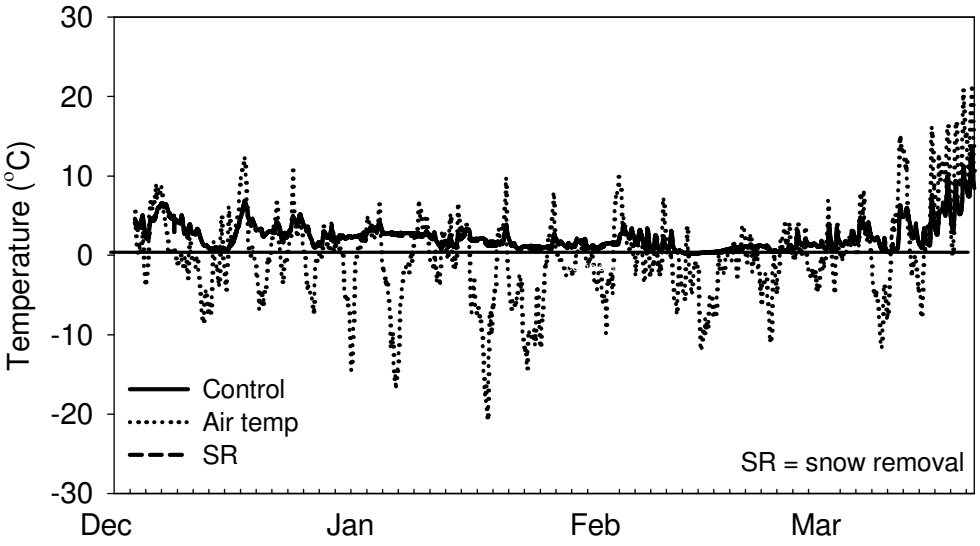
Appendices



Appendix A: Mean air temperature and soil temperature under ambient snow and snow removal plots from early December 2011 to mid-March 2012 at 5 cm soil depth (nitrogen treatments had no effect on winter soil temperature and thus were pooled).

Appendix B: Gravimetric soil moisture (g water/g soil dw^{-1}) for each treatment combination during the first (2011) and second (2012) year in the snow removal experiment. Means within columns for each year are not significantly different if they share a common lowercase letter (Tukey's test, $P < 0.05$). Parentheses indicates standard error (n=6).

	February		Snowmelt		May		June		October	
First year										
Ambient	0.31[b]	(0.03)	0.33[a]	(0.02)	0.40[a]	(0.03)	0.26[a]	(0.02)	0.33[a]	(0.03)
Ambient +N	0.34[b]	(0.03)	0.38[a]	(0.02)	0.36[a]	(0.03)	0.30[a]	(0.03)	0.31[a]	(0.03)
Snow removal	0.67[a]	(0.08)	0.34[a]	(0.03)	0.46[a]	(0.07)	0.27[a]	(0.01)	0.34[a]	(0.01)
Snow removal + N	0.73[a]	(0.04)	0.35[a]	(0.02)	0.41[a]	(0.02)	0.23[a]	(0.01)	0.31[a]	(0.01)
Second year										
Ambient	0.37[a]	(0.02)	0.35[a]	(0.02)	0.29[a]	(0.02)	0.20[a]	(0.01)	0.27[a]	(0.01)
Ambient +N	0.37[a]	(0.02)	0.34[a]	(0.02)	0.34[a]	(0.02)	0.18[a]	(0.01)	0.28[a]	(0.01)
Snow removal	0.41[a]	(0.04)	0.35[a]	(0.01)	0.32[a]	(0.01)	0.20[a]	(0.01)	0.28[a]	(0.01)
Snow removal + N	0.38[a]	(0.02)	0.34[a]	(0.01)	0.31[a]	(0.02)	0.19[a]	(0.01)	0.31[a]	(0.04)

Appendix C: Permission to reproduce published material

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