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SUBLETHAL FREEZING EFFECTS ON NITROGEN UPTAKE AND GROWTH IN POA PRATENSIS

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SUBLETHAL FREEZING EFFECTS ON NITROGEN UPTAKE AND GROWTH IN *POA PRATENSIS*

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Abstract

In northern temperate regions, climate warming is predicted to increase the frequency of soil freeze-thaw cycles (FTC) and reduce plant cold acclimation in late fall and early spring. To test if FTC inhibit plant nitrogen (N) uptake, I exposed *Poa pratensis* tillers to FTC in late fall, mid winter, and early spring, then used a ^{15}N tracer to assess N uptake from a hydroponic solution. To assess the direct effects of FTC on plant growth, I exposed *P. pratensis* tillers to FTC on the same dates, then measured plant biomass the following summer. Freezing of short duration at −10 °C and longer freezing at −5 °C in fall and spring decreased N uptake significantly. Plant growth decreased the most after spring FTC. Reduced plant cold acclimation in fall and spring must thus be coupled with extreme soil freezing to hinder plant N acquisition and growth.

Keywords: climate, freeze-thaw, growing season, ^{15}N uptake, plant productivity, *Poa pratensis*, root damage, winter warming.

Co-Authorship Statement

Dr. Hugh Henry will be a co-author on any manuscript(s) published that include(s) the contents of this thesis.

Dedication

I dedicate this thesis to my family, especially my grandparents, who have instilled in me priceless values in life and whom I have tried to make proud with my work.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

CO_2 – carbon dioxide

FTC – soil freeze-thaw cycle (s)

GHG – greenhouse gas (es)

N – nitrogen

^{15}N - 'heavy' stable isotope of nitrogen, molecular weight of 15

$^{15}NH_4{}^{15}NO_3$ – ammonium nitrate ^{15}N tracer

REL – relative electrolyte leakage

N_2O – nitrous oxide

Chapter 1

General Introduction

1.1 Scientific Rationale

1.1.1 Global climate change

Climate warming predictions

Climate, which is defined as the average 30-year weather pattern of a region, has been warming since the 20th century, and this warming is very likely to have been caused by anthropogenic greenhouse gas (GHG) emissions (IPCC, 2007). Future climate predictions are variable because they are based on assumptions of future GHG emissions, land use changes and consistent behaviour of the climate system. Current climate models predict a global warming of 1.1-6.4 °C over the next century, with the greatest temperature increases occurring on land and at high latitudes (IPCC, 2007). The Earth's average surface temperature has already warmed by 0.6 °C over the past century (IPCC, 2001), causing plant responses such as shifts in phenology (Estrella and Menzel, 2001), lengthening of the growing season (Keeling *et al.*, 1996), changes in species ranges (Walther, 2001), species distribution (Pauli *et al.*, 1996) and species abundance (Smith, 1994), as well as changes in plant growth rates (Graybill and Idso, 1993; Bisgrove and Hadley, 2002). Shifts in species distributions and extinction of some species can in turn lead to changes in community structure and composition (Hughes, 2000; Kreyling, 2010).

Winter climate change

Warming over winter, especially at high latitudes, has been occurring faster than warming during the summer (Saeterdsal *et al.*, 1998) and the annual extent of snow cover over the northern hemisphere has declined by nearly ten percent during the period 1972-2003 (Walsh *et al.*, 2005). Despite this trend, snow cover has generally increased over North America over the last century due to increased precipitation (IPCC, 2007; Groisman *et al.*, 2004). Nonetheless, temperate regions where mean winter temperatures remain close to freezing may receive less snow and experience a higher ratio of rain to snow with climate change (Bélanger *et al.*, 2002; Henry, 2008). Future reductions in snow cover would affect soil temperatures because of reduced insulation provided by snow; for example, a mean annual air temperature of −10.3 °C can be reduced to an average temperature of −1.4 °C at the soil surface by insulative properties of snow (Romanovsky, 2001). Despite less soil insulation, thirty to eighty percent fewer days with air temperatures below freezing that are more scattered over time (Jylha *et al.*, 2008) are predicted to decrease the period of frozen soil in temperate regions. Nevertheless, in northern temperate systems, an increase in the frequency of soil freeze-thaw cycles (FTC) is also expected with reduced snow cover (Groffman *et al.*, 2001; Henry, 2008).

1.1.2 Plant winter survival

Freezing damage in plants

Plants can be damaged by winter stresses such as fluctuating air temperatures and below freezing soil temperatures, excess soil moisture, ice encasement, soil heaving, and

low temperature pathogens (Andrews, 1987). Plant injury and death result primarily from the freezing of water within plant tissues. Ice initially forms in intercellular spaces (Xin and Browse, 2000), partly because the extracellular fluid has a lower solute concentration than does the intracellular fluid (Thomashow, 1999). Internal cell osmotic potential is reduced (Guy, 1990), causing water to move from inside cells across the permeable membrane which becomes more permeable at lower temperature (Vasil'yev, 1961a). Dehydration is the primary cause of freeze-induced damage to cell membranes and, in turn, plant cells (Steponkus, 1984; Steponkus *et al.*, 1993[a]). The physiological consequence of cell dehydration is a loss of compartmentation through a phase change in a fraction of the membrane lipids, from a bilayer to a non-bilayer structure, causing destabilization (Pearce, 2001).

Non-dehydration freeze-damage leads to freeze-induced production of reactive oxygen species (McKersie and Bowley, 1998), cell rupture from formation of intercellular ice adhesions with cell walls and membranes (Olien and Smith, 1977), and protein denaturation at low temperature (Guy *et al.*, 1998). Mechanical damage through ice formation results from tissue rupture rather than cell damage as water expands during freezing (Pearce, 2001).

Fast freezing and warming rates are generally more damaging than are slower rates due to faster pressure changes outside the protoplasm, which the cell membrane cannot sustain (Vasil'yev, 1961b). Warm periods during FTC prevent roots from continuously experiencing dormancy-inducing temperatures (<5 C°), delaying cold acclimation in early winter (Cannell and Smith, 1986). Conversely, exposure to temperatures above 0 °C in winter can cause a loss of cold acclimation, in plants, with the

speed of deacclimation increasing with higher temperatures (Kalberer *et al.*, 2006). Frost vulnerability is then increased by subsequent exposure to subfreezing temperatures (Ouellet and Desjardins, 1981; Suzuki, 1981). Such deacclimation occurs at a much faster rate than does cold acclimation, so winter survival can be affected by exposure to even short periods of warm temperatures during winter (Eagles *et al.*, 1997). Soil heaving damage to fine roots is also induced by FTC by increasing soil movement and ice lens formation which, along with exposing roots to freezing temperatures and dry air, may cause further physical damage (Tierney *et al.*, 2001). In addition, heavy winter rainfall can induce ice-sheet formation at the soil surface, creating anoxic conditions and an accumulation of CO_2, ethanol, lactic acid and ethylene in the soil (Bélanger *et al.*, 2005).

Cold acclimation and freezing tolerance

Cold acclimation (also known as hardening) is a suite of changes in gene expression and physiology that increases plant tolerance to cold temperatures (Kalberer *et al.*, 2006). A reduced photoperiod and declining temperatures initiate the start of winter acclimation in perennial plants (McKenzie *et al.*, 1988; Stout and Hall, 1989); this physiological process is accelerated once air temperatures drop below approximately 5 °C (Paquin and Pelletier, 1980). Cold susceptibility is species-, provenance-, genotype- and tissue-specific, with southern provenances being generally less cold tolerant than northern provenances in the Northern Hemisphere (Kozlowski and Pallardy, 2002). Water stress, short days and low temperature each induce cold acclimation (Fuchigami *et al.*, 1971), the onset of which is also promoted by by an increase in concentration of

abscisic acid, which upregulates genes involved in cold accliation (Thomashow, 1999; Gusta *et al.*, 2005)

The onset of cold acclimation takes place between 10 °C and 20 °C in autumn, with the accumulation of carbohydrates and lipids (Kozlowski and Pallardy, 2002). Acclimation is completed at low and sub-freezing temperatures with the synthesis of anti-freeze and dehydrin proteins and structural changes in membrane lipids (Kozlowski and Pallardy, 1997; Olien and Smith, 1981). Soluble sugars accumulate from starch mobilization during the acclimation period (Sauter *et al.*, 1996) and, together with other solutes, lower the freezing point of the intracellular solution (Poirier *et al.*, 2010). The extent of cellular dehydration is also reduced as the rate of water movement to the outside of cells is lowered by decreased internal osmotic pressure (Xin and Browse, 2000), and solute concentration is further increased as plants decrease their water content (Chen and Gusta 1978; Améglio *et al.*, 2001; Gusta *et al.*, 2004). In addition, sugars also function as cryoprotectants for specific enzymes (Carpenter *et al.*, 1986).

Freezing is also retarded by super cooling, which results from water not freezing inside cells due to the absence of heterogeneous ice nucleating agents (dust, bacterial proteins) and the presence of anti-freeze proteins. Such proteins are transcribed during the cold acclimation stage and increase freezing tolerance by controlling sites of ice formation, the rate at which ice grows, and inhibiting recrystallization (Griffith and Antikienen, 1996). The absolute limit to super cooling of pure water is −40 °C, at which point water freezes inside cells homogeneously (without nucleating agents) (Franks, 2003).

Membrane lipid composition is also modified during acclimation to stabilize membranes and resist freezing (Steponkus *et al.*, 1993[b]; Uemura and Steponkus, 1997[a]). Fatty acids of membranes become more unsaturated, retaining their fluidity at colder temperatures (Xin and Browse 2000), and membranes are further stabilized by hydrophilic polypeptides (Thomashow, 1999).

Winter climate change and plant productivity

The effects of freezing on plant productivity have received little attention in climate change research (Kreyling, 2010), despite the fact that warming and shorter winters are not likely to reduce the risk of frost damage to plants in many temperate regions (Meehl *et al.*, 2000). The frequency of random frost events is predicted to remain stable (IPCC, 2007) and an earlier onset of spring growth can result in greater plant damage by late spring frosts (Hanninen, 1991). Major vegetation die offs, caused by the combination of early spring and late fall frosts can occur over very large spatial scales, as observed in the spring of 2007 across the eastern United States (Gu *et al.*, 2008). The reduction of yellow cedar (*Chamaecyparis nootkatensis*) growth range has also been attributed to reduced snow cover in late winter and early spring, as a result of increased root damage by freezing (Schaberg *et al.*, 2008). In addition to species specific freezing responses, changes in species composition can result from interspecific variation in resiliency to freezing damage (Kreyling, 2010).

Both increased and decreased plant productivity have been attributed to increased winter FTC (Ouellet, 1976; Kreyling *et al.*, 2008). Freeze damage causes substantial yield losses of perennial forage crops in northern temperate regions (Ouellet, 1976). In contrast,

five artificially imposed FTC down to −2 °C *in situ* have led to increased summer biomass in a temperate grassland community, which was attributed to increased plant nitrogen (N) availability throughout the winter and early spring (Kreyling *et al.*, 2008), or possibly as a result of interactions with root parasites (J. Kreyling, pers. comm.). However, the mechanisms remain unclear because none of these studies have documented direct plant freezing responses in the absence of possible indirect effects via changes to physical soil properties or microorganisms.

1.1.3 Impacts of climate warming on plant growth through changes in soil N dynamics

N as a limiting element in ecosystems.

N is widely accepted as the most limiting nutrient for plant growth in most terrestrial systems (Tateno and Chapin, 1997). High N demand by plants and low N availability in usable forms are responsible for N limitation (Vitousek and Howarth, 1991). Even though N makes up 78 % of the atmosphere's volume (Chapin *et al.*, 2002; Vitousek *et al.*, 1997), 99.95 % of N exists as inert N_2 gas, which plants are unable to use (Galloway *et al.*, 2004). Specialized bacteria fix N from the air, and other microorganisms mineralize organic N (e.g. proteins, peptides, and amino acids) into inorganic N (NH_3 or NH_4^+ and NO_3^-) (Vitousek and Howarth, 1991; Benbi and Richter, 2002). Some N gets immobilized during mineralization for microbial growth while the rest is secreted as inorganic N, resulting in net mineralization (Chapin *et al.*, 2002). Northern temperate ecosystems are experiencing increasing rates of atmospheric N

deposition (Galloway *et al.*, 2004), and the amount of added N that is retained in the ecosystem will influence primary productivity and plant species composition (Tilman and Downing 1994; Vitousek *et al.* 1997). FTC can increase the amount of N that is lost from soils as leachate following microbial and plant damage (Groffman *et al.*, 2001; Fitzhugh *et al.*, 2001; Tierney *et al.*, 2001), potentially leading to coastal and freshwater eutrophication, as well as a decrease in ecosystem biodiversity through acidification (Galloway *et al.*, 2004; Vitousek *et al.*, 1997).

Physiology of plant N uptake.

Transpiration by plants causes bulk flow of water with nutrients in the soil towards the roots. In addition, when plants take up nutrients and deplete the concentration of nutrients in the rhizosphere, nutrients in the bulk soil move towards the rhizosphere by diffusion (Taiz and Zeiger, 2006). Plants acquire N with low-affinity transport systems, which operate at high nutrient concentrations (>1 mM) and with high-affinity transport systems that predominate in the micromolar nutrient range (Wang *et al.*, 1993).

Nitrate is transported either passively through anion channels (Pouliquin *et al.*, 2000) or through secondary active transport, where it enters the cell along with protons, which are constantly pumped out of cells using H^+ ATPases (Taiz and Zeiger, 2006). The cell's interior is more negatively charged than its exterior, so ammonium is primarily taken up using uniporters that passively transport ammonium along the electrochemical gradient (Ludewig *et al.*, 2002, 2003). Peptides and amino acids are also taken up using H^+ symporters with secondary active transport (Mayer *et al.*, 2006). In addition, plants form associations with plant growth promoting bacteria, which produce plant growth

hormones, inducing root elongation, as well as N fixing bacteria and mycorrhizal networks that enhance N uptake. (Kraiser *et al.*, 2011).

Seasonality of N availability and plant uptake

A large pulse of N is released during snowmelt (Brooks *et al.*, 1998; Lipson *et al.*, 1999), largely due to the release of inorganic and organic N from soil microbes, which have immobilized the N during the winter (Schmidt and Lipson, 2004). The winter microbial population declines due to carbon limitation towards the end of winter and intolerance of warmer temperatures, (Lipson *et al.*, 1999; Lipson *et al.*, 2000; Schmidt and Lipson, 2004).

Spring plant N uptake is influenced by vegetation type. In alpine regions graminoids have been shown to take up 12 % of their season-long nitrogen requirements during spring melt, compared to 7.4 % in perennial forbes, (Bilbrough *et al.*, 2000). Throughout summer, N availability decreases as plants grow actively and take up N. Growing plants input carbon compounds into soil through sloughing of root cells and the exudation of organic molecules into the rhizosphere, driving microbial growth (Rovira, 1969). By late fall, N availability increases again (Henry and Jefferies, 2002) when plants senesce and N uptake rates decline, and microbes mineralize senesced plant biomass, releasing N through net mineralization (Brooks *et al.*, 1998).

Winter microbial communities remain active in temperate regions by virtue of the insulative property of the snowpack (Brooks *et al.*,1998), and sufficient water for microbial growth remains unfrozen in the form of soil water films down to at least −5 °C (Anderson, 1970). Inorganic nutrients also accumulate under snow because net N

mineralization continues and plant uptake is low (Brooks *et al.*, 1998). FTC activity can also contribute to high winter N availability through microbial lysis (Henry and Jefferies, 2002). By late winter, N availability decreases as microbial populations grow, predominantly immobilizing N (Lipson *et al.*, 1999; Schmidt and Lipson, 2004). Spring melt follows with the N flush, completing the cycle.

Climate can influence the seasonality of N availability (Weih, 1998). In temperate and subarctic regions, soil N generally peaks in midwinter (Henry and Jefferies, 2002; Schmidt and Lipson, 2004), although thicker snowpacks at higher elevation and latitudes may cause comparably greater spring N availability due to N released from the melting snow (Haselwandter *et al.*, 1983; Bowman, 1992). In addition, the inorganic soil N content of a moist heath was found to be three times higher than in a dry heath site during the growing season (Weih, 1998).

As discussed by Ueda *et al.*, (2010), few studies have addressed the importance of winter N uptake for deciduous and perennial plants. Uptake of soluble N is slowed when plants are dormant in winter (Laine *et al.*, 1994). Root damage by ice encasement and soil heaving (Ouellet, 1976) can further decrease N uptake in trees found in hardwood forests (Tierney *et al.*, 2001; Weih and Karlsson, 2002). Despite these factors that hinder N uptake, some graminoids can take up N *in situ* over winter (Andersen and Michelsen, 2005) in quantities comparable to summer N uptake in similar grassland species (Nasholm *et al.*, 2000; Bardgett *et al.*, 2003). Although slowed, winter N uptake may be maintained by vascular plants remaining physiologically active and maintaining photosynthesis at subzero air and soil temperatures (Larsen *et al.*, 2007). The amount of winter N uptake seems to be species-specific. For example, peach trees (*Prunus persica*)

take up the majority of N in the spring, with very little uptake over the winter (Munoz *et al.*, 1993), while birch trees (*Betula pubescens*) can take up an amount of N in the winter that enhances the summer growth rate by the same order of magnitude as an increase in growing season soil temperature of 1 to 2 K (Weih, 2000). Winter N uptake may also differ among plant functional groups. In a temperate system, graminoids exhibited a more favorible summer growth response to winter FTC compared to shrubs, possibly due to higher N uptake ability during midwinter N flushes after FTC, (Kreyling *et al.*, 2008). Within graminoids, there is also evidence that winter N uptake can improve summer growth. For example, the growth of *Poa pratensis* was enhanced by N addition as late as December in regions where average temperatures ranged from –1.4 °C to 4.1 °C, with the warmer regions having a more favorable effect of N addition on plant growth (Miltner *et al.*, 2004).

Winter warming effects on microbial communities and N dynamics are confounded by direct plant responses.

Climate warming can increase rates of microbial litter decomposition and N mineralization, in turn increasing plant productivity (Sierra 1997; Rustad *et al.*, 2001). N cycling can also be altered by winter warming through changes in soil freezing dynamics (Henry, 2008). The function and composition of microbial communities is modified by FTC because microbial cells are killed by freezing and thawing of soil, with the surviving microbes benefitting from the released nutrients; in turn, decomposition and plant nutrient supply are increased, potentially positively influencing plant growth through plant specific FTC growth response (Schimel and Clein, 1996). Increases in the

frequency of FTC can lead to ecosystem N losses by promoting the release of soluble N from soil (Henry, 2007). Soluble N in soil increases most in response to rapid FTC with largest temperature fluctuations (Elliott and Henry, 2009). N is then lost through leaching and N_2O emissions as a result of reduced plant N uptake over winter (Sharma *et al.*, 2006; Matzner and Borken 2008). Decomposition, mineralization and nitrification of N compounds from frost-killed fine roots, disruption of soil aggregates (Larsen *et al.*, 2002) and lysis of microbial cells (Yanai *et al.*, 2004) are the proposed mechanisms for increased soluble N supply following FTC (Fitzhugh *et al.*, 2001; Henry, 2007). Therefore, in FTC experiments, plant responses have typically been confounded by the indirect effects of soil responses, and it is unclear to what extent plants might be responding directly to soil freezing.

Plant mortality in response to frost events has been well documented in agricultural systems, and winter climate change is predicted to increase winter crop mortality (Bélanger *et al.*, 2002). However, most studies of plant freezing responses have focused on lethal freezing temperatures (i.e., LD_{50} values), whereas sublethal growth responses to freezing damage have not been addressed in detail. When sublethal effects have been quantified, relative electrolyte leakage has been the most common parameter used as an indicator of freezing damage (Bigras and Dumais, 2005). However, roots cells damaged during freezing treatments can lose electrolytes to the soil prior to the measurement, thereby obscuring the results (Repo and Ryyppo, 2008). Therefore, a functional index of root resistance to frost damage may be more informative. Given that plant N uptake has a strong influence on primary productivity and plant species

composition (Huenneke *et al.*, 1990; Tilman and Downing, 1994; Vitousek *et al.*, 1997), the short term response of root N uptake to freezing may be an ideal response parameter.

Study species.

Poa pratensis is a shallowly rooted, cool-season, perennial grass (Sather, 1996). It starts growth in early spring and comes into bloom in early summer (Stuckey, 1941). It grows best in moist soil (Hoffman *et al.*, 1980), is able to withstand flooding (Schalitz, 1977), and freezing down to −14 °C when acclimated (Gudleifsson *et al.*, 1986). Its growth peaks in spring and fall, but declines in midsummer, with maximum root elongation occuring in April (Stuckey, 1941). The rhizomes constitute a major sink for storage of carbohydrates in *Poa pratensis*, and they grow throughout the year except late winter and early spring (Brown, 1943). *Poa pratensis* is an apomictic species, as its sexually reproducing individuals usually account for less than 20% of populations (Smith *et al.*, 1946). In natural areas *Poa pratensis* competes with native species, reducing species diversity and altering the natural floristic composition (Sather, 1996).

1.2 Objectives and Hypotheses

I selected the grass *Poa pratensis* L. as a model species to evaluate FTC effects on plant growth because it is the dominant species at a field site where a series of plant and soil freezing experiments have been conducted by Dr. Hugh Henry`s research group over the past six years. *P. pratensis* is utilized extensively for turf grass and as a forage grass species (Wieners *et al.*, 2006) and is found in all of the continental states and in Canada from Labrador to the west coast, except in arid regions (Hitchcock, 1950). The main

objective of my thesis was to characterize the direct responses of *P. pratensis* to soil FTC, in isolation from potential indirect effects caused by freezing-induced changes in microbial activity or nutrient availability. I also examined how responses differed among plants exposed to FTC at different stages of cold acclimation, and how responses were modulated by differences in minimum temperature, freezing rate and the length of freezing. With respect to plant responses, I measured the depression of N uptake, an important functional measure, immediately after exposure to freezing. In addition, I examined the longer-term growth response of plants to freezing.

Objective 1. To use short-term N uptake as an indicator of freezing damage in P. pratensis tillers exposed to FTC at different stages of cold acclimation.

I used soil-free roots of intact *P. pratensis* tillers and a ^{15}N tracer in a hydroponic solution to test the hypothesis that short-term N uptake responds directly to variations in root freezing rate, minimum soil temperature, freezing duration and timing of freezing occurrence. I predicted that N uptake would decrease the most in response to FTC in fall and spring, when plants are not fully acclimated to withstand potentially damaging soil temperatures. I also predicted that freezing effects would occur abruptly at a threshold minimum temperature, and that rapid freezing and freezing of long duration would intensify the depression of N uptake in response to freezing.

Objective 2. To determine the direct long-term growth response of P. pratensis to FTC exposure at different stages of cold acclimation.

I used FTC-treated *P. pratensis* tillers transplanted to a common untreated soil to test the hypothesis that summer growth responds directly to the timing of FTC over the previous fall, winter and spring, independent of freezing effects on soil microorganisms and nutrient concentrations. I predicted that plant growth would decrease with increasing freezing intensity (defined by minimum temperature), with the strongest effects exhibited by plants treated in late fall and early spring, when plants are not fully acclimated to withstand potentially damaging soil temperatures. I tested this prediction using both tillers frozen under controlled conditions in the laboratory, and tillers exposed to snow and litter removal treatments in the field.

1.3 Thesis Organisation

My thesis is written in the integrated article format, and contains two manuscripts. This first introductory chapter included relevant information on the current state of knowledge in the topic of research and an overview of my objectives, hypotheses and predictions. The first manuscript (Chapter 2) addresses my first research objective by describing direct ^{15}N uptake responses to variation in FTC in *P. pratensis*. The second manuscript (Chapter 3) addresses my second research objective by describing how FTC at different stages of cold acclimation directly affect *P. pratensis* biomass production. The general discussion and conclusion (Chapter 4) connect the results of the experiments and summarize implications of my findings in a broader context.

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Chapter 2

Nitrogen uptake responses to soil freeze-thaw cycles at different cold acclimation stages

2.1 Introduction

Changes in plant productivity in response to climate warming over the next century will likely be influenced by changes in the availability of mineral nitrogen (N) (St Clair and Lynch, 2010). Although the effects of increased temperature on N availability during summer have been studied extensively, less is known about how climate warming during winter may affect nitrogen dynamics. Net N mineralization rates may increase with winter climate warming, increasing N availability (Rustad *et al.*, 2001; Aerts *et al.*, 2006). An increased frequency of soil freeze-thaw cycles (FTC) over winter can also increase soil soluble N concentrations (Schimel and Clein 1996; Groffman *et al.*, 2001; Tierney *et al.*, 2001; Matzner and Broken, 2008). Winter N uptake can be significant for some plant species (Andresen and Michelsen, 2005), but it is unclear to what extent N uptake over winter may have implications for summer plant growth.

Plant N uptake decreases at low temperatures due, in part, to reduced apparent hydraulic conductance (Macduff and Jackson, 1991; Laine *et al.*, 1994). N translocation from roots to shoots is further reduced in the winter because of reduced shoot N demand (Engels and Marschner, 1992), which increases root tissue N concentration and causes a negative feedback on further N uptake (Laine *et al.*, 1994). Nonetheless, the potentially reduced but steady N uptake over winter can increase plant N root storage (Svensson and Clarholm, 1994), preventing N leaching from the soil (Catt *et al.*, 1998; Ritter *et al.*,

1998), and benefiting summer plant growth (Weih, 2000). As an application of their N catchment ability, crops such as winter rape (*Brassica napus)* and rye (*Secale cereale*) are grown to immobilize N over winter in temperate regions (Laine *et al.*, 1994).

The overwinter N dynamics of northern temperate systems have received less attention than those of arctic and alpine systems (Campbell *et al.*, 2005). Soils in temperate systems remain close to freezing (at 5 cm depth) for much of winter and thus are highly vulnerable to reductions in snow cover and an increased frequency of FTC (Henry, 2008). There are few empirical data documenting the effects of freezing on plant N uptake, but N uptake is thought to be reduced by freeze damage (Bigras and Dumais, 2005).

Plant freezing damage has been quantified by measuring the leakage of various cellular compounds (Studer *et al.*, 1978; Bigras, 1997), changes in metabolic activity (Lassheikki *et al.*, 1991) and sugar content (Gibbons *et al.*, 1982), as well as other indices of root integrity and morphology. Relative electrolyte leakage (REL) is the most commonly used method to assess root damage (Bigras and Dumais, 2005). Electrolytes leak from the symplast to the apoplast following freeze damage and are used as the primary symptom of cellular damage (Flint *et al.*, 1967). However, assessment of root damage only shows evidence of freezing damage without quantifying the functional responses of roots to freezing. Furthermore, REL values have been shown to be underestimated (Bigras 1997; Stattin and Lindstrom, 1999; Coursolle *et al.*, 2000). This underestimation occurs because electrolytes can be lost from roots during soil freezing and thawing as well as during washing the roots free from soil, before electrolyte leakage is tested (Repo and Ryyppo, 2008). As an alternative to REL, the measure of

short-term N uptake in response to freezing represents a potentially useful functional measure of a plant's freezing response, given the strong influence of N uptake on plant productivity (Huenneke *et al.*, 1990; Tilman and Downing, 1994; Vitousek *et al.*, 1997).

A threshold plant N uptake response may exist at subzero soil temperatures, because plants possess mechanisms that protect them from frost damage. Plants exhibit a suite of changes in gene expression and physiology that increase cold tolerance (Kalberer *et al.*, 2006). Acclimated plants contain a higher carbohydrate and lipid content, antifreeze and dehydrin proteins, as well as structurally modified cell membrane lipids, inhibiting water from freezing within and outside cells, as well as preventing cell membrane damage (Kozlowski and Pallardy, 1997; Kozlowski and Pallardy, 2002; Olien and Smith, 1981; Poirier *et al.*, 2010). Fast freezing and warming rates are generally more damaging than slower rates due to faster pressure changes outside the protoplasm, which the cell membrane cannot sustain (Vasil'yev, 1961). Plants are also likely to sustain greater root damage when not fully cold acclimated in late fall and early spring (Noshiro and Sakai, 1979; Harrison *et al.*, 1997; Bélanger *et al.*, 2002).

In this study I examined how N uptake in the grass *Poa pratensis* is affected directly by variation in freezing temperature, rate and duration. I examined freezing responses both when plants were not fully cold acclimated in late fall and early spring and when plants were acclimated during mid winter. I hypothesised that short-term N uptake would respond directly to variations in root freezing rate, duration, intensity and timing. Specifically, I predicted that a reduction in N uptake would occur abruptly at a threshold minimum temperature, and that rapid freezing and freezing of long duration

would intensify the depression of N uptake in response to freezing. I also predicted that N uptake would decrease the most in response to freezing in fall and spring.

2.2 Methods

2.2.1 Site description

I collected *P. pratensis* tillers from an old field at the Agriculture Canada Southern Crop Protection and Food Research Centre in London, Ontario (43° 04' N, 81° 20' W, elevation 264 m). The site is dominated by the grass species *Poa pratensis* L. The site has not been ploughed, fertilized, or mowed for over 25 years. The soil at the site is classified as well to imperfectly drained silt loam glacial till (Hagerty and Kingston, 1992), with an approximate pH of 7.5 (Bell *et al.*, 2010). Air and soil temperatures during the sampling period are presented in Fig. 2.1 (Environment Canada, National Climate Data and Information Archive).

2.2.2 FTC treatments

I evaluated freezing responses of *P. pratensis* tillers in late fall (3rd of November), mid winter (21st of January), and early spring (24th of March) (Fig. 2.1), representing a range of plant acclimation stages. On each date, I collected six distinct clumps of *P. pratensis* tillers from the field and separated the clumps into individual tillers by hand. A grass tiller is an aerial shoot that develops in the axillary bud of live leaf tissue (Dahl and Nyder, 1977). Grass samples remained in a refrigerator for up to 24 hours at 4-6 °C, after being brought from the field, until it was time for the tillers to be separated and exposed

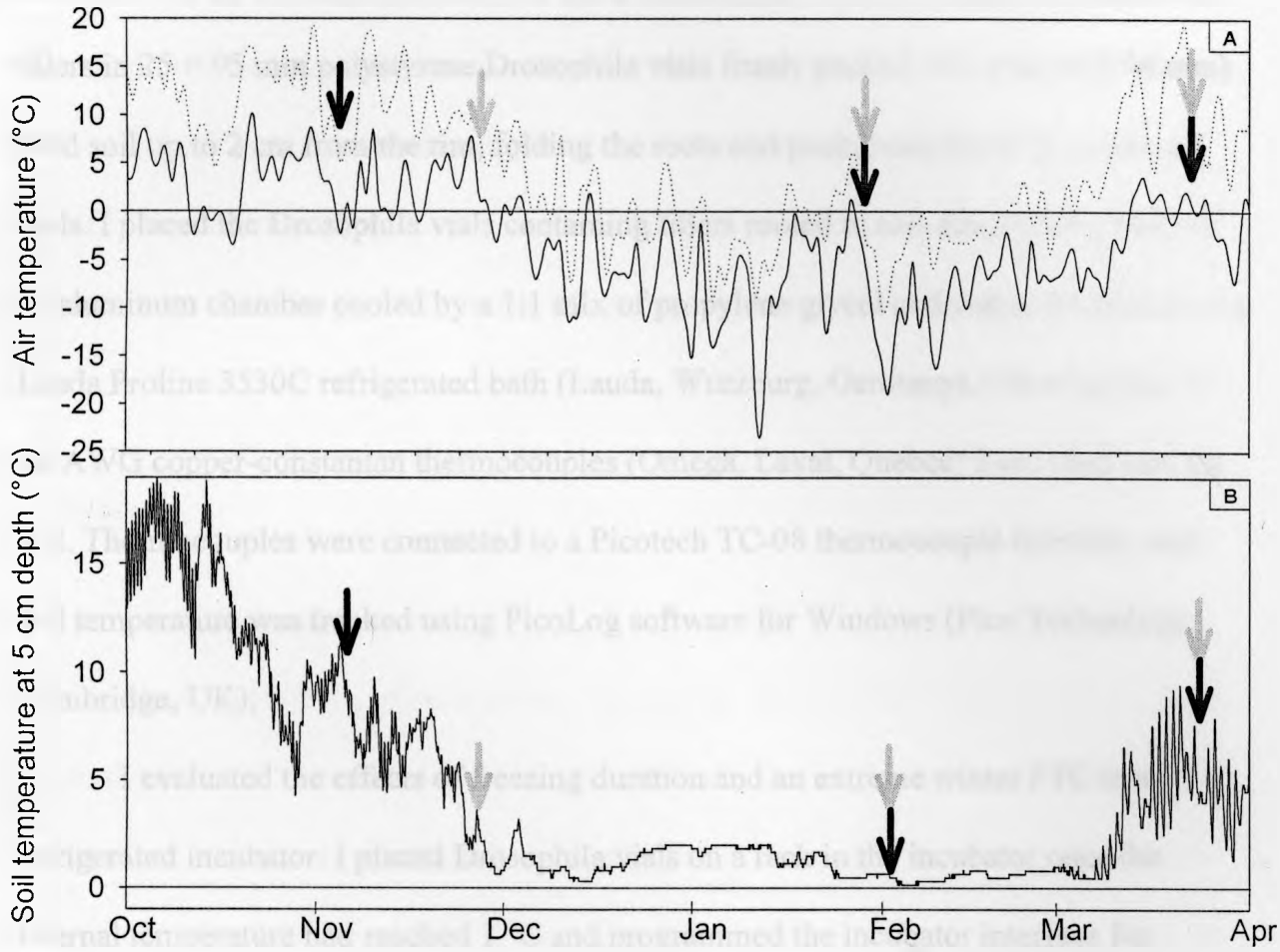

Figure 2.1 A). Daily minimum (solid line) and maximum (dotted line) air temperatures from November 2009 to April 2010 at the London CS weather station (Environment Canada), approximately 5 km from the study site. B). Soil temperature at the Agriculture Canada's Southern Crop Protection and Food Research Centre in London, Ontario. Arrows indicate dates when freezing treatments were carried out to test ^{15}N uptake (black arrows; 3rd of November 2009, 21st of January and 24th of March 2010) and growth response (grey arrows; 24th of November, 1st of February and 26th of March 2010) of *Poa pratensis*.

to freezing treatments. I chose tillers of approximately the same size, measuring 15-25 cm from root tip to leaf edge, and with roots measuring 5-10 cm in length. I placed the tillers in 25 × 95 mm polystyrene Drosophila vials firmly packed with sieved (2.36 mm) field soil up to 2 cm from the rim, folding the roots and positioning them in centers of vials. I placed the Drosophila vials containing tillers rooted in soil directly into wells of an aluminum chamber cooled by a 1:1 mix of propylene glycol and water, circulated by a Lauda Proline 3530C refrigerated bath (Lauda, Wurzburg, Germany). I inserted tips of 36-AWG copper-constantan thermocouples (Omega, Laval, Quebec) 2 cm deep into the soil. Thermocouples were connected to a Picotech TC-08 thermocouple interface, and soil temperature was tracked using PicoLog software for Windows (Pico Technology, Cambridge, UK).

I evaluated the effects of freezing duration and an extreme winter FTC in a refrigerated incubator. I placed Drosophila vials on a rack in the incubator once the internal temperature had reached 1 °C and programmed the incubator interface for temperature decreases of either 0.5 °C/h or 0.2 °C/h. I placed temperature loggers (StowAway Tidbit Temp Logger, onset computer corporation, Cape Cod, Massachusetts, US) in 80 ml specimen collection containers (Starplex Scientific, Etobicoke, Ontario, Canada), filled with soil to track the temperature experienced by the plant roots.

I evaluated the effects of minimum temperature and freezing rate by exposing each sample to one of 9 treatments in either the refrigerated bath (specifications above) or in a refrigerated incubator (REVCO model # BOD50A16, Thermo Electron Corporation, Gormley, Ontario, Canada), which included all combinations of freezing rates (2 °C/h, 1 °C/h, 0.5 °C/h, 0.2 °C/h) and minimum freezing temperatures (−2 °C, −5 °C, −10 °C) in

the refrigerated circulator (n=6 for each treatment combination), with the exception of −5 °C at 0.2 °C/h, and −10 °C at 0.2 °C/h and 0.5 °C/h, which were not included because they could not be completed in a single diel cycle. The samples were always cooled from 1 °C and the freezing rates were chosen to reflect realistic rates of soil freezing that can occur at the field site (Elliott and Henry, 2009). I froze another set of tillers for 1 and 3 days down to −5 °C at 0.5 °C/h in the fall and spring to examine the effect of freezing duration. In addition, I froze a set of tillers to −15 °C in the winter at 0.2 °C/h to simulate an extreme freeze-thaw event. For all treatments, tillers underwent a single FTC.

2.2.3 ^{15}N uptake measurements

Upon completion of each cycle, I thawed the samples for 1 hour at 4-6 °C, then washed soil from the roots by hand in 0.5 mM $CaCl_2$. I used this salt concentration in all solutions to maintain root membrane integrity. I then immersed the tillers in an aerated solution of 100 μM ^{15}N as $^{15}NH_4{}^{15}NO_3$. After 20 minutes, I removed the tillers from the ^{15}N solution and immediately immersed them in 5 mM KCL for 5 minutes to remove label left in the Donnan free space, before a final rinse with $CaCl_2$. This method was modified from Epstein *et al.*, (1963). Pilot trials indicated that the relationship between total uptake and time was linear over the course of the uptake experiments (Fig. 2.2)

I excised the roots with scissors after the final rinse and dried them at 65 °C to constant weight in a forced air oven. I then ground the dried root material to a fine powder with a laboratory mill (SPEX SamplePrep Model 2000 Geno/Grinder, Metuchen, New Jersey, US) and weighed it into tin capsules. Isotopic analyses were performed by

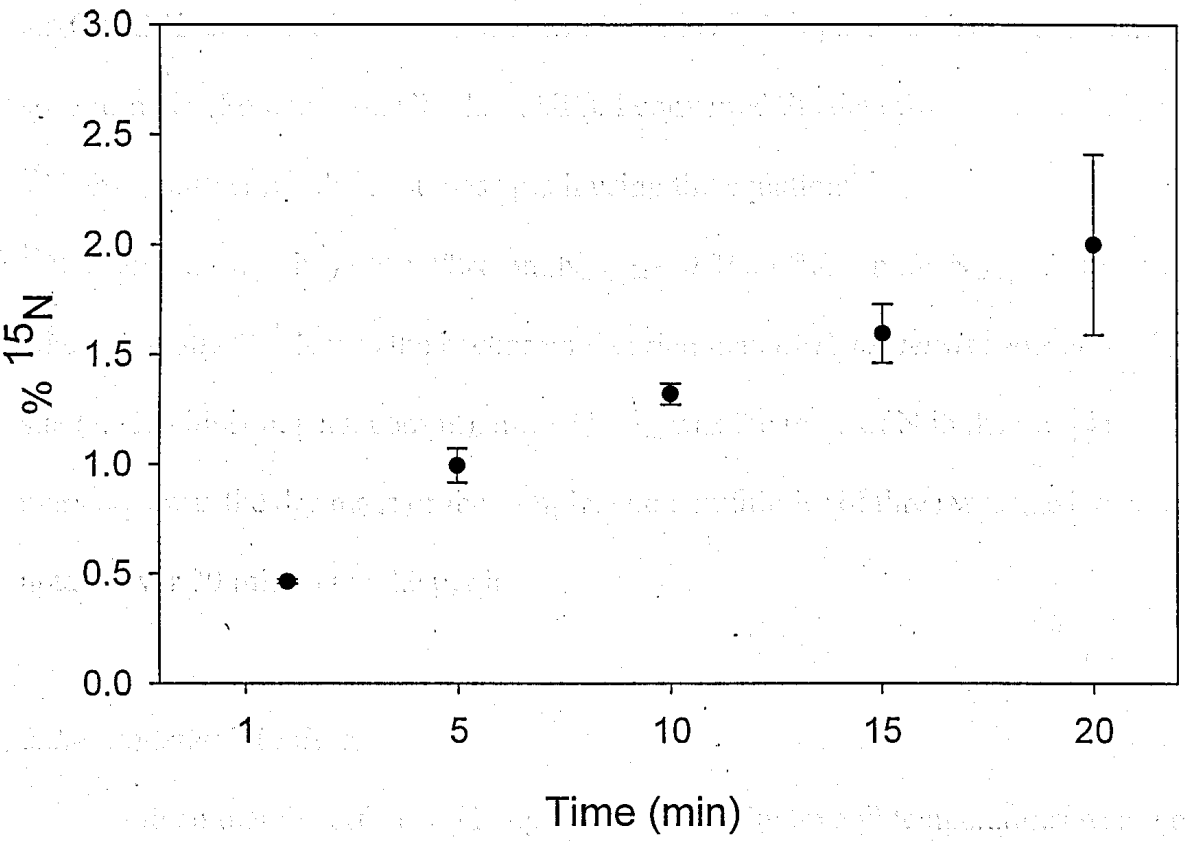

Figure 2.2 ^{15}N uptake by roots 1 to 20 minutes after exposure to a solution of 100 µM ^{15}N as $^{15}NH_4{}^{15}NO_3$ (n=2). The y axis shows percent of N in root samples that was enriched (^{15}N) by mass, and the x axis shows the length of time that roots had been exposed to the solution containing the labeled N. Error bars represent standard error.

the University of California Davis Stable Isotope Laboratory, who used a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). I converted the data from % atom ^{15}N to mg ^{15}N absorbed per mg dry root mass per h using the equation:

$$^{15}N \text{ absorbed } (mg^{-1}\ h^{-1}) = 3 \times (\%\ \text{atom } N_{sample} - 0.36809\%) \times \text{mass } N_{sample} / \ mass_{sample}$$

where 0.36809 % ^{15}N was the background enrichment of *P. pratensis* roots at our field site (J. Hutchinson, pers. comm.), mass N_{sample} was the mass of N in the sample, $mass_{sample}$ was the dry mass of the sample, and a multiplier of three was used to convert uptake over 20 min to uptake per h.

2.2.4 Statistical Analyses

Given that the effects on N uptake by all minimum soil temperatures were not examined over all freezing rates, I could not examine the three factors (minimum soil temperature, freezing rate, and season) using a single factorial ANOVA. Therefore, I used two-way ANOVAs to examine the effect of rate and its interaction with season on N uptake for each minimum temperature. I then pooled N uptake data from all freezing rates as rate had an insignificant effect on N uptake and used a two-way ANOVA to examine the interactive effects of minimum soil temperature and season on N uptake with the pooled data from all freezing rates. I used one-way ANOVAs to examine the effect of minimum temperature on N uptake for each season. After running the ANOVAs, I used Tukey's post-hoc tests to distinguish between differences in plant N uptake among minimum temperature treatments within a specific season and freezing rate. Some

treatments had less than 6 replicates (5 minimum) due to insufficient dry root biomass that was generated for ^{15}N analysis.

I used a two-way ANOVA and then student's t-tests to examine N uptake differences after 1 day and 3 day freezing treatments at 0.5 °C/h in the fall and spring. I log-transformed the N uptake data prior to analyses to satisfy the assumptions of normality and homogeneity of variances. An alpha value of 0.05 was used for all statistical tests. I conducted all statistical analyses using JMP version 4.0 (SAS Institute Inc., Cary, NC, USA).

2.3 Results

There were interactive effects of season and minimum soil temperature on N uptake, (Table 2.1, Fig. 2.3 A). Minimum soil temperature had a significant effect on N uptake in fall and spring, but not in winter (Table 2.1). Under mild to moderate freezing (minimum temperatures of −2 °C and −5 °C), N uptake was generally higher in spring than in fall or winter (Fig. 2.3 A, B, C). N uptake decreased significantly in response to severe freezing (minimum temperature of −10 °C), but only in fall and spring (Fig. 2.3 A). However, in winter, a very severe freezing temperature of −15 °C decreased N uptake (Fig. 2.3 C). The effects of rate and all interactions between rate and season on N uptake were not significant over the range of minimum temperatures examined (Table 2.2).

Increased freezing duration (3 days instead of 1 day), which was examined in both fall and spring, also decreased N uptake ($p=0.019$ and $p<0.001$ respectively; Fig. 2.4),

Table 2.1. Results of two-way and one-way ANOVAs, summarizing effects of season, and minimum soil temperature on ^{15}N uptake in *Poa pratensis*. Bold p values represent significant differences. Some treatments had less than 6 replicates (5 minimum) due to insufficient dry root biomass that was generated for ^{15}N analysis.

Season	Source	DF	F	P
All seasons	Season	2	5.336	**0.006**
	Minimum temp	1	55.303	**<0.001**
	Season x Minimum temp	2	4.619	**0.011**
	Error	145		
Fall	Minimum temp	2	9.514	**<0.001**
	Error	47		
Winter	Minimum temp	2	2.642	0.082
	Error	46		
Spring	Minimum temp	2	21.325	**<0.001**
	Error	49		

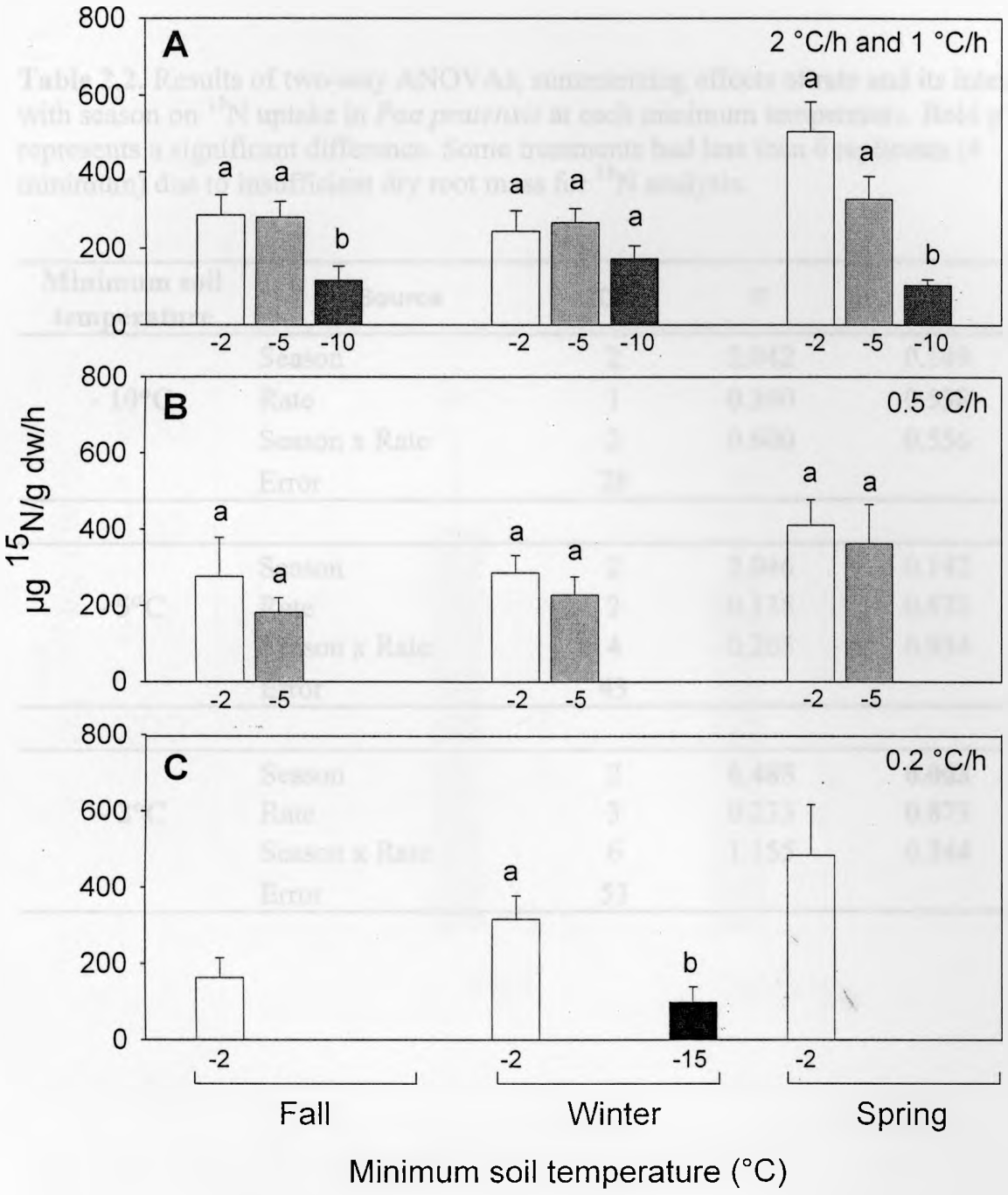

Figure 2.3 ^{15}N uptake by *Poa pratensis* collected in different seasons and frozen at a range of minimum temperatures and freezing rates. The y-axis indicates amount of ^{15}N that was recovered from the roots that had been frozen and then immersed in a 100 µM ^{15}N as $^{15}NH_4{}^{15}NO_3$ and the x-axis indicates minimum soil freezing temperatures. A). Pooled ^{15}N uptake results from treatments that underwent 1 °C/h and 2 °C/h freezing rates. B and C). ^{15}N uptake results from treatments that underwent 0.5 °C/h and 0.2 °C/h freezing rates respectively. Different lower case letters above bars represent significant differences among treatments within each season and freezing rate. C) Fall, winter and spring freezing were induced on 3rd of November 2009, 21st of January 2010 and 24th of March 2010, respectively. Error bars represent standard error.

Table 2.2. Results of two-way ANOVAs, summarizing effects of rate and its interaction with season on ^{15}N uptake in *Poa pratensis* at each minimum temperature. Bold p value represents a significant difference. Some treatments had less than 6 replicates (4 minimum) due to insufficient dry root mass for ^{15}N analysis.

Minimum soil temperature	Source	DF	F	P
- 10°C	Season	2	2.042	0.149
	Rate	1	0.390	0.538
	Season x Rate	2	0.600	0.556
	Error	28		
- 5°C	Season	2	2.046	0.142
	Rate	2	0.138	0.871
	Season x Rate	4	0.205	0.934
	Error	43		
- 2°C	Season	2	**6.485**	**0.003**
	Rate	3	0.233	0.873
	Season x Rate	6	1.155	0.344
	Error	53		

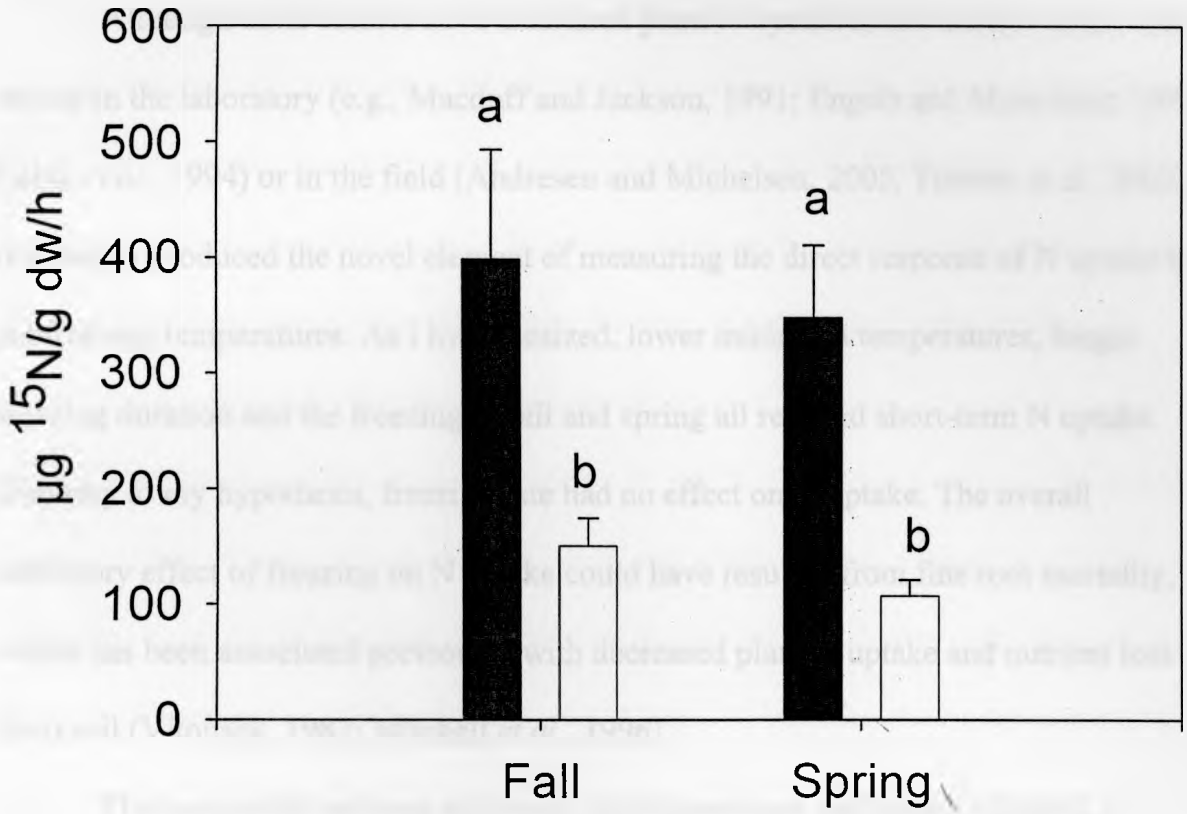

Figure 2.4 ^{15}N uptake by *Poa pratensis* collected in fall (24th of November 2009) and spring (24th of March 2010) after freezing exposure to −5 °C for 1 day (filled bars) and 3 days (open bars). Different lower case letters above bars represent significant differences between treatments. Error bars represent standard error.

while season, and its interaction with freezing duration was nonsignificant (p=0.301 and p=0.453 respectively).

2.4 Discussion

Although other studies have examined plant N uptake at low temperatures during winter in the laboratory (e.g., Macduff and Jackson, 1991; Engels and Marschner, 1992; Laine *et al.*, 1994) or in the field (Andresen and Michelsen, 2005, Tierney *et al.*, 2001), my study introduced the novel element of measuring the direct response of N uptake to subfreezing temperatures. As I hypothesized, lower minimum temperatures, longer freezing duration and the freezing in fall and spring all reduced short-term N uptake. Contrary to my hypothesis, freezing rate had no effect on N uptake. The overall inhibitory effect of freezing on N uptake could have resulted from fine root mortality, which has been associated previously with decreased plant N uptake and nutrient loss from soil (Vitousek, 1982; Mitchell *et al.*, 1996).

The interaction between minimum soil temperature and season (Table 2.1) resulted from plants being more susceptible to freezing effects in fall and spring than in winter (Fig 2.3), likely due to plants not being fully acclimated during the fall and spring (Noshiro and Sakai, 1979). I carried out fall freezing in early November, and the acclimation period for forage crops (including *P. pratensis*) in the Guelph forage producing region for 2010-2039 is predicted to end around the beginning of December (Bélanger *et al.*, 2002). Likewise, I administered spring freezing in late March, and spring deacclimation in our region usually occurs from the beginning of March to early April (Bélanger *et al.*, 2006). Consistent temperatures above zero cause plants to

deacclimate (Kalberer, 2006) much faster that they acclimate (Guy, 1990), and thus increase the susceptibility to injury by subsequent exposure to subfreezing temperatures (Ouellet and Desjardins, 1981; Suzuki, 1981). High daytime temperatures may be particularly influential in causing irreversible cold deacclimation in plants (Rapacz, 2002). My spring freezing treatments were preceded by a week of daytime temperatures averaging around 15 °C degrees, which was likely responsible for fast deacclimation that led to high N uptake at mild freezing (−2 °C and −5 °C; Fig 2.3). High spring N uptake after mild freezing temperatures can be an indication of the start of plant growth, which is driven by changes in the balance between the rates of storage and consumption of assimilates accumulated over fall and winter (Klimov, 1997).

The significant reduction in N uptake at −10 °C following a warm early spring period (−10 °C; Fig 2.3 A) agrees with previously observed root damage at −10 °C, which has been shown to occur when 90% of the osmotically-active water moves out of the cells (Thomashow, 1999). Total extractable soil N has been shown to increase in soils exposed multiple times to −10 °C at my study site, likely as a result of microbial lysis (Elliott and Henry, 2009). This N may be lost as leachate if freeze-damaged plants are unable to take up this newly available N during thaw periods.

My prediction that a threshold freezing temperature is required for a significant reduction in N uptake was partially supported, as significant N reduction occurred only below −5 °C (Fig 2.3 A), although more temperature treatments would be required to narrow this threshold down to a critical damaging temperature. My finding that mild freezing temperatures (−2 °C and −5 °C) were not strong enough to reduce N uptake

ability (Fig. 2.3) supports the literature as even deacclimated *P. pratensis* does not exhibit significant freeze damage at −5 °C (Noshiro and Sakai, 1979).

Freezing rate did not have an effect on N uptake, likely due to the omission of extremely fast freezing rates. Fast freezing rates have been shown to increase damage to plant cells as a result of the increased speed at which water is withdrawn from cells, leading to dehydration (Finkle *et al.*, 1974; Livingston and Tallury, 2009). For example, beet (*Beta vulgaris*) root cells frozen down to −4 °C at 3.3 °C/hr exhibited close to five times the amount of damage compared to cells frozen at 0.16 °C/hr (Finkle *et al.*, 1974). I did not administer freezing rates faster than 2 °C/h, so it is possible that a threshold damaging freezing rate was not reached. It is also possible that the handling of roots during the washing resulted in fine root damage that caused only extreme differences in plant N uptake at lowest minimum temperatures to be distinguishable. Regardless, freezing rates faster than 1 °C/h are rarely encountered at my study site (Elliott and Henry, 2009), which suggests that variation in freezing rate does not influence N uptake in *P. pratensis* within the range of freezing rates experienced in the field. At my field site, leaf litter reduces freezing rates and minimum temperatures, whereas in agricultural and turfgrass systems that are frequently mowed, plants may be exposed to more extreme freezing.

Increased freezing duration significantly reduced plant N uptake (Fig. 2.4), possibly explained by cellular death from extracellular ice formation and cell dehydration, which can increase with longer freezing periods (Jacobsen *et al.*, 2005). In a previous study, the percentage of freeze-damaged cultivars of quinoa (*Chenopodium quinoa*) at −8 °C doubled with an increase in freezing time of only two hours (Jacobsen

et al., 2005). *P. pratensis* is very cold tolerant, able to survive cold exposure at least down to −14 °C (Gudleifsson *et al.*, 1986) and it is not surprising that only a severe cold exposure down to −15 °C (Fig 2.3 C) resulted in significantly reduced N uptake in fully acclimated tillers during winter. Nevertheless, this species was vulnerable at prolonged mild freezing (−5 °C) in fall and spring.

The vulnerability of perennial grasses to experience reduced N uptake in the fall could lead to reduced root N storage over fall and winter (Laine *et al.*, 1994), at a time when many plants otherwise remain physiologically active (Steenberg-Larsen *et al.*, 2007), and are able to maintain photosynthesis to respiration ratios greater than one, even at temperatures close to freezing (Klimov, 2003). Maximum N loss from soils within northern hardwood forests occurs in early spring (Muller and Bormann, 1976). Reduced N uptake by perennial grasses in the spring may increase soil N losses during spring melt and delay the start of plant growth, given that graminoids can take up as much as 12 % of their season-long N requirements during spring melt (Bilbrough *et al.*, 2000).

2.5 Conclusions

N uptake in *P. pratensis* in response to freezing was sensitive to the degree of cold acclimation, but even deacclimated *P. pratensis* was able to maintain rapid N uptake after short freezes down to −5 °C, although freezing of longer duration (3 days) or greater intensity (− 10 °C) decreased N uptake. Freezing rates did not play a critical role in influencing N uptake, at least across the range of rates I tested, which spans the range of the freezing rates that occur naturally in the field. Even though shorter fall acclimation and spring deacclimation periods are predicted with climate change (Bélanger *et al.*,

2002), as well as longer winter plant deacclimation periods, the continued occurrence of episodic frost events will likely reduce plant N uptake only if frost events are unusually severe.

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Chapter 3

Direct growth responses of *Poa pratensis* to soil freeze-thaw cycles at different cold acclimation stages

3.1 Introduction

Climate warming is expected to increase global net primary terrestrial production (Nemani *et al.*, 2003), primarily as a result of increased growing season length (Bisgrove and Hadley, 2002; Menzel *et al.*, 2006). Nevertheless, the highest temperature increases resulting from climate warming are predicted to occur over winter (IPCC, 2007). Even though some year-round warming experiments have led to increased plant biomass (Rustad *et al.*, 2001), most have not simulated frost damage, which may not decrease or even increase with warmer winters (Hanninen, 1991; Meehl *et al.*, 2000; Gu *et al.*, 2008). In particular, temperate regions, where mean winter temperatures currently remain close to freezing, may experience considerably less snow cover (Bélanger *et al.*, 2002), leading to an increase in the frequency of soil freeze-thaw cycles (FTC) (Groffman *et al.*, 2001; Henry, 2008), as well as lower minimum soil temperatures (Romanovsky, 2001).

Freezing effects on plant productivity in temperate systems have been underrepresented in the climate change literature (Kreyling, 2010), although losses of agricultural and forage crop productivity (Ouellet, 1976; Allan *et al.*, 1992), as well as widespread vegetation die offs (Gu *et al.*, 2008), have been attributed to frost events. Unacclimated plants are especially vulnerable to frost damage in late fall or early spring (Gu *et al.*, 2008). As frost days are predicted to be more scattered over time (Jylha *et al.* 2008), with potentially increased temperature variability (Schär *et al.* 2004), the chance

of late fall and early spring frost is expected to stay the same (IPCC, 2007; Eccel *et al.*, 2009), increasing plant vulnerability to frost due to the shortened acclimation and deacclimation periods (Bélanger *et al.*, 2002). Frost damage may increase as a result, although Schwartz *et al.*, (2006) concluded that both increases and decreases in frost risk are possible due to large regional climate variations. Extreme freezing and FTC events over winter can also damage plants (Strimbeck *et al.* 1995, Bourque *et al.* 2005, Lazarus *et al.* 2006), and warm periods cause plants to deacclimate and become more susceptible to freeze damage (Larcher and Bauer, 1981; Strimbeck *et al.*, 1995; Eagles *et al.*, 1997).

Despite the presence of many studies on plant freezing responses in the agricultural literature, most of these studies have focused on plant survival at critical temperatures (i.e., LD_{50} studies). In contrast, sublethal plant growth performance following winter freezing has rarely been studied (Kreyling *et al.*, 2008). Sublethal frost damage can be very important from an ecosystem perspective, because it leads to reduced terrestrial primary production (Gu *et al.,* 2008), resulting in reduced forest carbon uptake, and altered surface energy balance (Gu *et al.*, 2006). Frost can cause sublethal damage to fine roots either through physiological damage or through physical damage as a result of soil heaving (Goulet, 1995), which can result in decreased ability of roots to take up nitrogen (N) (Chapter 2). The mechanisms contributing to changes in plant growth after FTC are unclear because plant responses have rarely been examined in the absence of possible soil effects. Increased decomposition, mineralization and nitrification of N compounds from frost-killed fine roots, disruption of soil aggregates (Larsen *et al.*, 2002), and lysis of microbial cells (Yanai *et al.*, 2004) can all potentially increase soluble N availability following FTC (Fitzhugh *et al.*, 2001; Henry, 2007). Freezing also

influences interactions between plant roots, soil particles and soil organisms (Goulet, 1995; Klironomos *et al.*, 2001; Larsen *et al.*, 2002), the latter including microbial and mycorrhizal communities, which influence plant nutrient uptake (Bardgett *et al.*, 2003; Kraiser *et al*,. 2011) and growth (Clarholm, 1985). Plant responses to FTC are thus influenced by their ability to take advantage of the newly available N (Kreyling *et al.*, 2008), in addition to the amount of sustained root damage (Tierney *et al.*, 2001; Weih and Karlsson, 2002). Increases in productivity in response to FTC are possible, as a result of increased N availability for plants or freeze damage to root parasites (Kreyling *et al.*, 2008; J. Kreyling, pers. comm.).

In this study, I evaluated the sublethal growth response of the grass *P. pratensis,* exposed to FTC at different stages of cold acclimation, both at controlled temperatures in an incubator, and in response to snow and litter removal in the field. Following the freezing treatments, indirect freezing effects mediated through changes in soil organisms and soil chemistry were controlled by growing the plants in untreated field soil over the growing season. I hypothesized that summer growth would respond directly to the timing of FTC over the previous fall, winter and spring. I predicted that plant growth would decrease with increasing freezing intensity (defined by minimum soil temperature), with the strongest effects exhibited by plants frozen in late fall and early spring.

3.2 Methods

3.2.1 Site description

Please refer to section 2.2.1.

3.2.2 Experiment 1: FTC exposure in the field.

I created variation in soil FTC number, FTC amplitude and duration of freezing through snow manipulation and leaf litter removal from grass plots in the field. The most extreme freezing was initiated by the removal of both litter and snow simultaneously, snow removal provided an intermediate freezing treatment, and snow addition was added to impose the mildest freezing. The experiment was designed with 6 blocks, and each block was divided into 4 circular plots, 30 cm in diameter, one for each treatment and a control (n=6 for each treatment). I removed snow by hand to a depth of ~ 1 cm above the ground within 24 hours of the conclusion of each snowfall throughout the winter every time it snowed more than 2 cm over a 24 hour period,. In the snow and leaf litter removal plot, I removed leaf litter on the 8th of December 2009, 2 days prior to the first snowfall. Snow depth was measured with a ruler in the center of control and snow addition plots after every snowfall. I maintained snow depth in the added snow treatments to be twice the depth of control plots. I gently scooped the loose snow from the ground and slowly let it fall on the snow addition plots as evenly as possible to minimize compaction of snow, which decreases the snow's insulative property (Grundstein *et al.*, 2005). I replaced the litter on the snow and litter removal plots in the 14th of March to ensure even warming of plants in all treatments in the spring. I measured the soil temperature from 8th of December 2009 to 28th of March 2010 by temperature loggers (iButton®, DS1922L, Maxim Integrated Products, Sunnyvale, California, US), inserted into the ground at 5 cm depth.

At spring thaw, I dug up 5-7 tillers from each plot and removed the soil, as described in Experiment 1, and I transplanted the tillers into untreated soil in the field. I

harvested above and belowground biomass on the 14th of July and dried the washed roots and leaves to a constant weight in a forced air oven at 65 °C.

3.2.3 Experiment 2: FTC induced under controlled conditions

I evaluated the growth response of *P. pratensis* tillers to FTC exposure in late fall (24th of November 2009), mid winter (1st of February 2010), and early spring (26th of March 2010), representing a range of acclimation stages (Fig. 2.1). Six distinct clumps of *P. pratensis* tillers were collected from the field and separated into bunches of 5-7 tillers by hand. Tillers with an average of three leaves each were chosen. Tillers ranged from 15-25 cm from root tip to leaf tip and roots were 5-10 cm long. After being transported from the field, grass samples remained in a refrigerator for up to 24 hours at 4-6 °C until it was time for the tillers to be separated and exposed to freezing treatments. I placed the tillers in 80 ml specimen collection containers (Starplex Scientific, Etobicoke, Ontario, Canada), packing the roots with sieved soil from the field site. I then wrapped the containers with perforated Saran-wrap film with holes 1-2 mm in diameter to minimize moisture loss. I froze the tillers from 1 °C to –5 °C at 0.5 °C /h in a refrigerated incubator (REVCO model # BOD50A16, Thermo Electron Corporation, Gormley, Ontario, Canada), and they were maintained at the minimum temperature (–5 °C) for three days. Controls were held at 4-6 °C in a refrigerator (n=6 for each treatment). In mid winter, I added an additional treatment where a set of tillers was frozen to –15 °C at 0.2 °C/h. All treatments consisted of one FTC.

After the soil thawed, I washed it from the roots by hand in 0.5 mM $CaCl_2$, with the latter added to maintain root membrane integrity, then transplanted the tillers into

non-treated, sieved (2.36 mm), field soil. On 28th of March 2010, I transplanted the tillers into larger plastic pots (8 cm diameter x 9 cm tall cylinders) at the field site, ensuring that the soil level in the pots matched the surrounding soil, then clipped all tillers to a uniform length of 1 cm, to measure the growth of new tillers. I harvested above and belowground biomass on the 14th of July and dried the washed roots and leaves to a constant weight in a forced air oven at 65 °C.

3.2.4 Statistical analyses

For plants frozen in the field, I used one-way ANOVAs to examine treatment differences in aboveground, belowground and total plant biomass. Similarly, for the plants frozen under controlled conditions, I used one-way ANOVAs to examine treatment differences in aboveground, belowground and total plant biomass for each sampling period. I log-transformed data prior to analysis to satisfy the assumptions of normality and homogeneity of variances, and conducted all statistical analyses using JMP version 4.0 (SAS Institute Inc., Cary, NC, USA). An alpha value of 0.05 was used for all statistical tests. Some treatments had less than 6 replicates (4 minimum) due to grazing by herbivores.

3.3 Results

3.3.1 Experiment 1: growth response to FTC exposure in the field

Soil temperature responses of the field plots to snow and leaf litter manipulation treatments are presented in Table 3.1 and Fig. 3.1. Relative to control plots, the snow

Table 3.1 Mean soil temperature (± standard error) characteristics of plots in response to snow and litter manipulation treatments from 8th of December 2009 to 29th of March 2010 (n=3). A FTC was defined as an event during which the soil temperature dropped below 0 °C and then rose above 0 °C. The added snow treatment had twice the snow depth of the control treatment. The snow and snow and litter removal treatments had snow removed down to ~ 1 cm above the ground within 24 hours of snow falls that accumulated more than 2 cm of snow in a given 24 hour period.

	Added snow	Control	Snow removal	Snow and litter removal
Average temperature (°C)	1.71±0.10	1.66 ± 0.29	0.98 ± 0.02	0.70 ± 0.18
Minimum temperature (°C)	0.42 ± 0.16	0.14 ± 0.02	–1.09 ±0.16	–3.91±0.77
Number of days soil stayed frozen	0	0	12.4 ± 2.6	18.2 ± 8.0
Total number of FTC	0	0	8 ± 2	7 ± 5
Number of FTC attaining a minimum temperature of less than 1°C	0	0	1	2

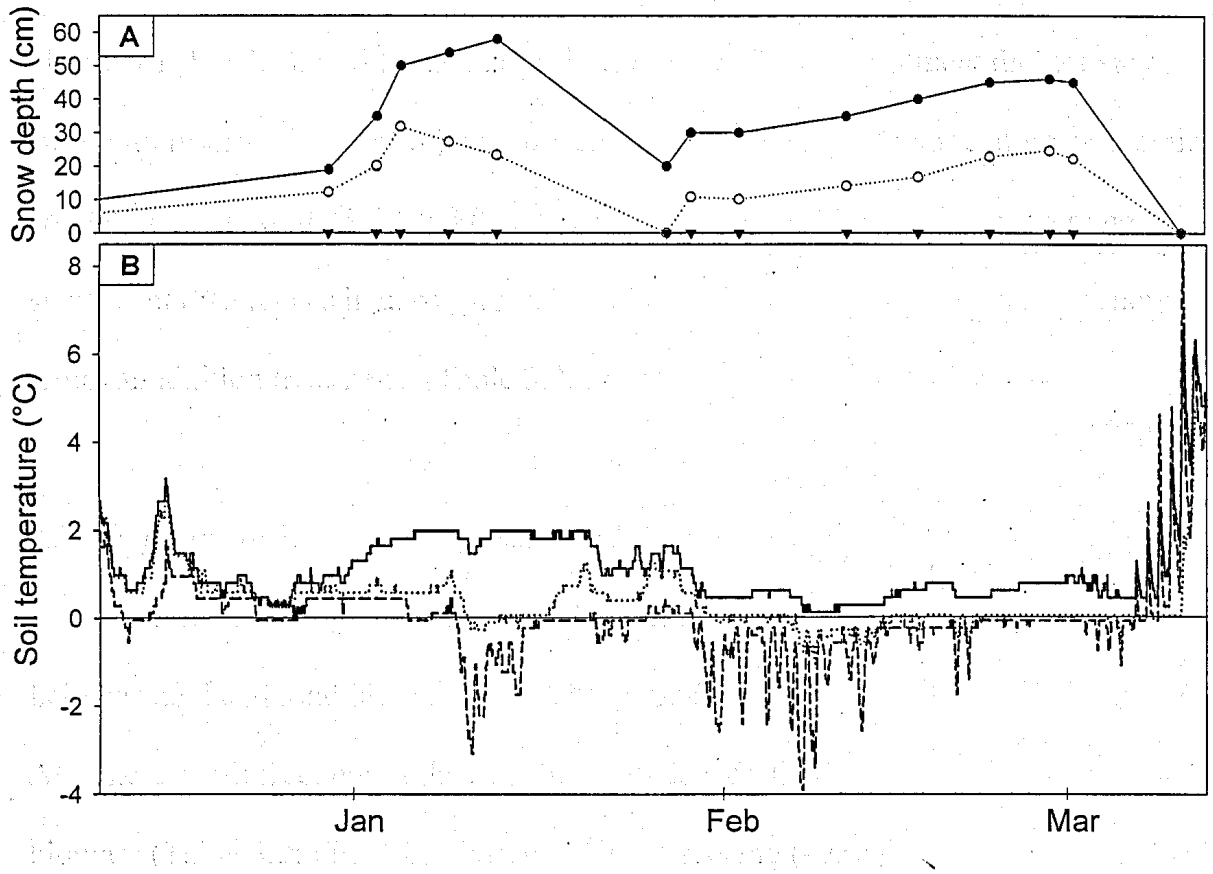

Figure 3.1 Snow depth in field plots and their corresponding soil temperatures (5 cm depth) during the 2009-2010 winter. A). Filled circles represent plots that had snow added, open circles represent control plots, and filled triangles represent snow removal plots. B). Solid, dotted and dashed lines represent the temperature of the soil in the control plots, snow removal plots, and snow and litter removal plots, respectively. The snow addition soil temperature profile is omitted as it did not differ from the control treatment.

removal treatment and the snow and litter removal treatment lowered the minimum soil temperature by 1.2 °C and 4.1 °C, increased the number of days of frozen soil by 12.4 and 18.2 days, and increased the number of FTC (< –1 °C) by 1 and 2, respectively (Table 3.1, Fig 3.1). Soil temperatures in the snow addition treatment did not vary substantially from the control, with differences in average soil temperature and minimum soil temperature of 0.05 °C and 0.28 °C, respectively (Table 3.1). There were no significant differences in aboveground and belowground biomass among any snow removal/addition treatments (Table 3.2, Fig. 3.2).

3.3.2 Experiment 2: growth response to FTC exposure under controlled conditions

Spring freezing under controlled conditions significantly reduced aboveground, belowground and total biomass ($p=0.029$, $p=0.033$ and $p=0.024$, respectively, Table 3.2 A), whereas fall freezing of the same magnitude (–5 °C for 3 days) had no effect on biomass (Table 3.2; Fig. 3.2). Extreme winter freezing (–15 °C at 0.2 °C/h) decreased leaf biomass significantly ($p=0.014$), but not root biomass, and the decrease in total biomass was marginally non-significant, ($p=0.059$; Fig. 3.2; Table 3.2).

Table 3.2 A). Results of one-way ANOVAs summarizing biomass responses of *Poa pratensis* to freezing treatments in different seasons (–5 °C at 0.5 °C/h for 3 days in fall and spring and –15 °C at 0.2 °C/h for 6 days (total) in winter), compared to their respective controls. B). Results of one-way ANOVAs summarizing biomass responses of *P. pratensis* to the snow manipulation treatments. Bold numbers represent significant treatment effects. Some treatments had less than 6 replicates (4 minimum) due to grazing by herbivores.

A) Seasonal freezing vs. controls	**Source**	**d**	**F**	**p**
Fall	Leaf biomass	1	0.042	0.843
	Root biomass	1	0.299	0.599
	Total biomass	1	0.089	0.774
	Error	8		
Winter	Leaf biomass	1	**9.794**	**0.014**
	Root biomass	1	1.112	0.322
	Total biomass	1	4.834	0.059
	Error	8		
Spring	Leaf biomass	1	**6.691**	**0.029**
	Root biomass	1	**6.332**	**0.033**
	Total biomass	1	**7.332**	**0.024**
	Error	9		
B) Field snow manipulation treatments	**Source**	**d**	**F**	**p**
	Leaf biomass	3	0.221	0.881
	Root biomass	3	0.457	0.716
	Total biomass	3	0.249	0.861
	Error	20		

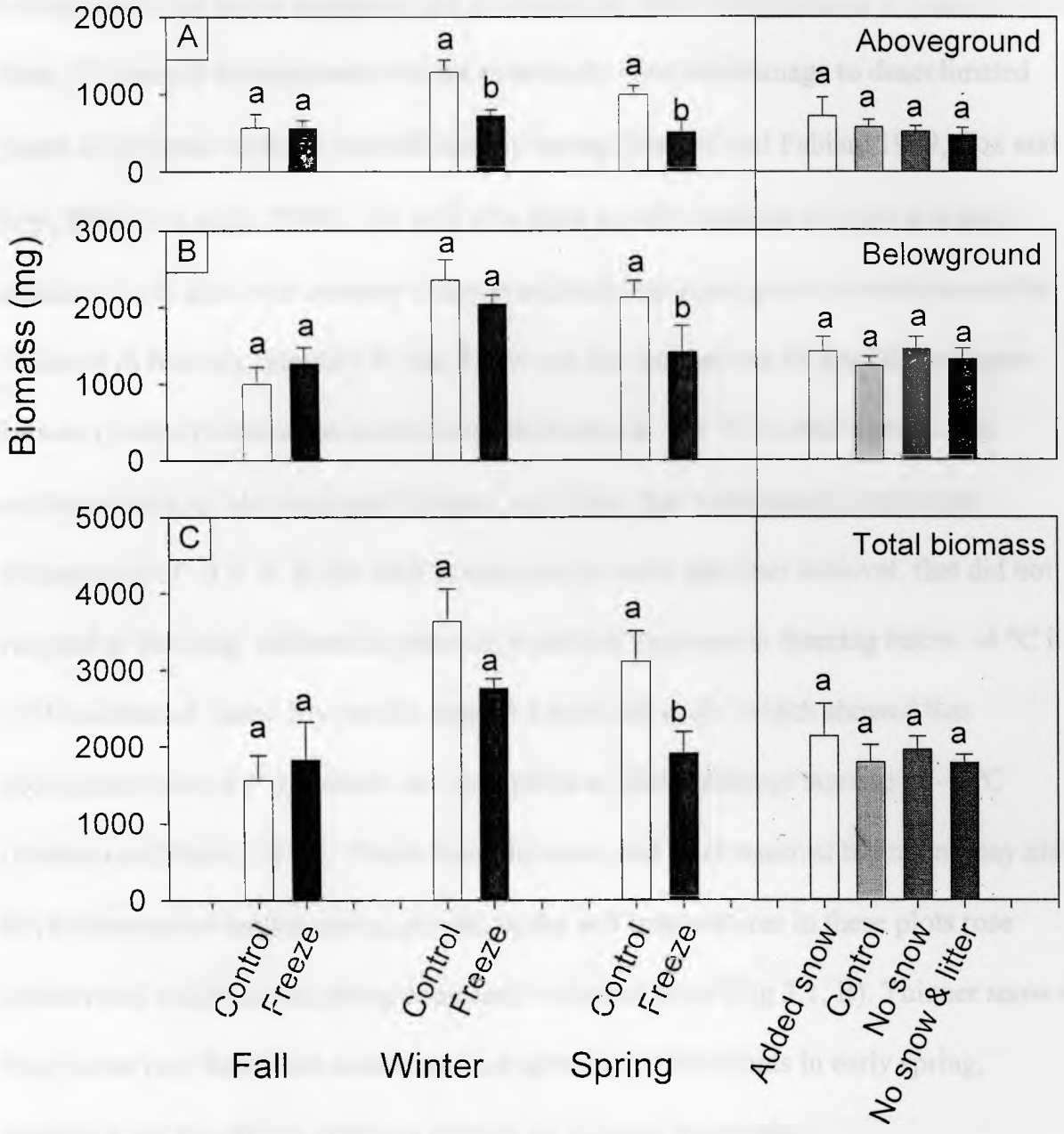

Figure 3.2 Dry biomass of *Poa pratensis* on 14th July 2010 in response to freezing treatments administered in different seasons (–5 °C at 0.5 °C/h for 3 days in fall and spring and –15 °C at 0.2 °C /h for 6 days (total) in winter) under controlled conditions (left of divider), and following winter snow/litter manipulations in the field. Different lower case letters represent significant differences among treatments.

3.4 Discussion

A significant reduction of biomass response to freezing at –5 °C under controlled conditions in the spring supported my prediction of direct frost damage to plants at this time. This result is consistent with the previously observed damage to deacclimated plants in response to warm periods in early spring (Menzel and Fabian, 1999, Cox and Arp, 2001, Gu *et al.*, 2008). The lack of a plant growth response to snow and litter addition in the field was contrary to my prediction that plant growth would respond to variation in freezing intensity *in situ*. However, the comparison of growth responses between plants frozen under controlled conditions (at –15 °C in mid-winter), that exhibited reduced aboveground biomass, and those that experienced a minimum temperature of –3.9 °C in the field in response to snow and litter removal, that did not respond to freezing, indicated a potential threshold response to freezing below –4 °C in cold acclimated tillers. My results support a previous study, which showed that acclimated roots of *P. pratensis* are susceptible to freeze damage starting at –7 °C (Noshiro and Sakai, 1979). Plants from the snow and litter removal treatment may also have experienced earlier spring growth, as the soil temperatures in these plots rose quicker and earlier in the spring compared to control plots (Fig 3.1, B). Thinner snow and litter cover may have thus accelerated the growth of these plants in early spring, canceling out the effects of freeze damage on biomass production.

Although plants can also deacclimate in midwinter in response to long thaw periods and suffer subsequent frost damage (Shabbar and Bonsal, 2003), I did not attempt to simulate the latter in my experiment. Such periods could result in a significant

reduction in plant N uptake at higher minimum soil temperatures than those that reduced plant summer growth in my experiments.

Contrary to my prediction, fall freezing did not result in reduced summer growth, possibly because the plants were already cold acclimated by the time freezing was administered. The freezing date (November 24th) was selected with the aim of stressing the plants during a late acclimation stage, when fall soil frosts are most likely to occur. Temperatures of 2 °C to 5 °C are considered to be optimal for cold acclimation (Jacobsen *et al.*, 2004), and such temperatures occurred for around two weeks prior to the fall freeze. The plants may have thus acclimated sufficiently to withstand freezing at –5 °C.

Despite the rapid re-growth of roots often observed following soil freezing damage (Tiery *et al.*, 2001; Kreyling, 2010), belowground biomass did not fully recover by the end of the growing season in the plants frozen in spring, as it did in the winter-frozen plants. The damage plants experienced in response to spring freezing may have been particularly severe because the roots were actively growing and had fully deacclimated. Fine roots of grasses including *P. pratensis* are more susceptible to freeze damage than are aboveground tissues, (Noshiro and Sakai, 1979). Accordingly, the significantly reduced aboveground biomass in the winter-frozen plants could be explained by the allocation of plant resources to root repair instead of shoot growth. Another possible mechanism for reduced summer growth could be lower nutrient stores in plant tissues due to the reduced ability of damaged roots to take up and store N, directly through root freezing damage and through the disruption of root - mycorrhizal associations (Klironomos *et al.*, 2001; Tierney *et al.*, 2001). Resorption of nutrients, particularly of N and phosphorus from plant tissues, plays a significant role in shoot

growth in the spring (Karlsson, 1994; Cheng and Fuchigami, 2002). Fine roots contain high amounts of stored N (Pregitzer *et al.*, 1997), and their loss to freeze damage can lead to lower reserves of N for plants to draw upon at the start of growing season. Lack of N resorption in spring can be particularly detrimental, because root N uptake may already be reduced as a result of freezing damage (Chapter 2).

In the snow removal experiment, the control treatment accumulated a maximum of approximately 30 cm of snow, yet it did not differ in soil temperature from the snow addition treatment, where snow depth was doubled. This result confirms the observation that a snow depth of 30-40 cm can effectively decouple soil and air temperatures (Edwards *et al.,* 2007). The observation that snow and litter removal resulted in a minimum soil temperature almost 3 degrees lower than the snow removal treatment alone similarly demonstrates the importance of litter for protection from frost.

The absence of significant effects on plant growth in response to snow and litter removal implies that reductions in snow cover with climate warming (Henry, 2008) may have little effect on frost damage in *P. pratensis* in northern temperate regions in the absence of extreme freezing events and prolonged mid-winter thaw periods.

3.5 Conclusions

While frost damage to partially deacclimated plants in spring resulted in significant reduction of biomass, increased midwinter freezing through snow and litter removal did not affect plant biomass, likely due to high cold tolerance of acclimated *P. pratensis* (Noshiro and Sakai, 1979) and a lack of prolonged midwinter thaw periods that could have deacclimated the plants. *P. pratensis* is thus likely to be most vulnerable to

frost damage during spring deacclimation periods in the future, provided the frequency of random freeze events stays the same (Shaver *et al.*, 2000).

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Chapter 4

General Discussion & Conclusions

4.1 Research findings

Both immediate (nitrogen (N) uptake) and long term (summer growth) plant responses to freezing were detrimentally affected by extreme soil freeze-thaw cycles (FTC), with the most detrimental effects being evident when plants that were not fully acclimated were exposed to freezing. Prolonged freezing in the winter and spring also significantly reduced both plant response parameters, with winter and spring freezes down to –15 °C and –5 °C, respectively, resulting in decreased N uptake and summer growth. Two likely explanations emerge for similar responses of N uptake and growth to FTC. The short term reduction in plant N uptake after freezing could have persisted until spring, causing *Poa pratensis* to take up less N over winter and spring compared to undamaged plants. The plants would then have less N stored in its tissues at the start of spring growth. As N is the most limiting nutrient plant growth (Tateno and Chapin, 1997), and a significant portion of spring growth results from utilizing stored N in the plants (Karlsson, 1994; Cheng and Fuchigami, 2002), lower N stores would lead to lower productivity, at least at the initial growth stage. Another possible explanation is that the primary cause of reduced summer growth was the energy cost of re-growing freeze-damaged roots, in which case the reduced short term N uptake was merely a symptom of freezing damage, similar to electrolyte leakage, but not a cause (Bigras and Dumais, 2005). The latter would imply that either winter N uptake is insignificant

compared to spring and summer N uptake, or that plants can regain N uptake capability within a short time period. Differentiating between these two alternative explanations would require knowing whether reduced N uptake following freeze stress is sustained over winter, and if N uptake during winter increases plant biomass over the following growing season.

N uptake was significantly reduced in the fall after freezing down to –5 °C, as I predicted, while no significant growth reduction was observed in the summer, contrary to my initial prediction. This inconsistency may be attributed to the fact that the N uptake experiment took place earlier in the fall (3rd of November), when plants were less acclimated, than the plants that had been frozen later in the fall (24th of November) to measure the growth response.

Increased soil N content resulting from microbial lysis (Henry and Jefferies, 2002) and increased root damage (Tierney *et al.*, 2001; Weih and Karlsson, 2002) can both occur in response to FTC. My results indicate that root damage after FTC hinders N uptake and it can thus be hypothesised that the net effect of FTC on plant growth may be dependent on the balance between survival of the microbial community and plant roots. If microbes are damaged less compared to plant roots, their populations may expand as they feed on the frost-killed roots. If the reverse happens, plants may benefit by taking advantage of the increased N availability resulting from microbial cell lysis. This balance may shift depending on the specific microbial community present and the cold tolerance of roots of specific plant species. The resulting summer growth, therefore, likely depends on the interactions among the soil, microbial, and plant responses to freezing.

My results also indicate that FTC can interact with increasing atmospheric N deposition (Galloway *et al.*, 2004) by limiting the amount of N that can be immobilized by plants during the winter and spring. With future predicted increases in midwinter thaw periods (NRCan, 2008), plants may deacclimate and become more vulnerable to FTC, reducing ecosystem N retention. These midwinter thaws may function similarly to spring thaw events, with the release of N pulses, primarily due to microbial turnover (Lipson *et al.*, 2000; Schmidt and Lipson, 2004). Furthermore, because plant productivity over summer can also be reduced following FTC (Chapter 3), summer immobilization of N would be further reduced.

In a more global context, more frequent FTC (Henry, 2008) of short duration and mild freezing temperatures, resulting from warmer winters, are unlikely to decrease plant productivity. Plant productivity could decrease however, if midwinter thaw periods increase in duration, and the frequency of random frost events remains unchanged (IPCC, 2007). Therefore, even though plant death may not increase with warmer winters, increased deacclamation of plants in the future could still increase sublethal plant damage, reducing forest carbon uptake, and altering surface energy balance through reduced plant productivity (Gu *et al.*, 2006).

Plant responses to FTC may also be modified by large scale environmental changes such as the increasing atmospheric carbon dioxide (CO_2) concentration (IPCC, 2007). Plant exposure to increased CO_2 has been shown to affect plant cold acclimation, resulting in both increased and decreased cold tolerance (Hanslin *et al.*, 2010). It can also increase plant N uptake and growth (He *et al.*, 2010; Tingey *et al.*, 1997), which makes

future FTC experiments incorporating a CO_2 concentration factor useful in getting a more complete understanding of winter climate change impacts on plants.

4.2 Potential limitations

Plants were frozen in soil in the laboratory to simulate the natural root disturbance that occurs by soil heaving during FTC. Freezing in the field occurs starting from the soil surface and propagates downwards, while in test tubes, the freezing occurred evenly from all sides around the roots. The deepest roots may have thus experienced more damage than they would have experienced in the field. However, larger scale soil heaving and ice formation in the field can cause vertical (frost heaving) and lateral (frost thrusting) soil disruptions, increasing seedling mortality (Regehr and Bazzaz 1979). Such detrimental effects were likely lessened by the loose packing of soil around the roots in the laboratory. Also, the initial separation of roots from soil prior to freezing may have damaged fine roots and diminished their contribtion to N uptake, but this effect would have been consistent among treatments.

Although indirect effects on plants mediated through soil freezing were controlled by growing plants in untreated soil, a separate treatment where plants were grown in treated soil for comparison was not included in the experimental design due to time constraints. Inclusion of this additional treatment would have shown the extent to which indirect soil effects modify plant freezing responses relative to direct effects. In addition, only single FTC were reproduced in the laboratory, whereas multiple FTC can occur in the field. Multiple FTC have been shown to intensify freezing effects on soil N content (Joseph and Henry, 2008), microbial biomass (Larsen *et al.*, 2002), and plant growth

response (Kreyling *et al.*, 2008). Lastly, during the field experiment, neither prolonged midwinter thaw periods nor late spring frost events were examined, and the increased occurrence of these events in the future could result in substantial plant deacclimation and increased freezing damage.

4.3 Directions for future research

Freezing responses should be evaluated with more species to allow for more general conclusions regarding plant winter climate change responses and to enable predictions of how plant community composition may change in response to freezing, as different species may experience differential freezing impacts (Gu *et al.*, 2008). In addition, even within one species such as *P. pratensis*, individuals of local and regional populations develop traits that cause local adaptation to their specific environment (ecotypic variation). Evaluating differences among ecotypes would show the importance of local adaptation in modifying plant responses within a species. There is evidence that ecotypic variation can play a large role in plant responses to climate change, for example in response to drought (Beierkuhnlein *et al.*, 2011). In addition, long term studies are scarce in winter climate change research, comprising only 4 % of all cases (Kreyling 2010). Longer term studies on the responses to FTC would be beneficial, as plant responses have been shown to vary between 1 and 2 years after FTC treatments (Kreyling *et al.*, 2008).

Although the frequency of frost events is predicted to remain the same (IPCC, 2007), midwinter thaw periods are predicted to increase in frequency and duration (NRCan, 2008), and future winter climate change studies should therefore simulate FTC

of variable durations that occur immediately after or during thaw events. Lastly, interactions with freezing and increased N availability following FTC should be addressed. During extended midwinter thaws, plants may deacclimate (Kalberer, 2006) and increase their N uptake ability. Few studies have addressed the impact of N uptake on the amount of sustained FTC damage and subsequent plant growth, although the amount and timing of N availability in relation to the cold acclimation process is thought to play a critical role (Bélanger *et al.*, 2006).

4.4 References

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