Physiological Tolerance Mechanisms of Boreal Forest Tree Species to Climatic and Anthropogenic Stress Factors

by

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Abstract

Boreal plant and tree stress responses under climate change and anthropogenic disturbances have become an important research area due to a strong climate warming signal and large-scale anthropogenic pollution. This thesis investigates tree physiological response mechanisms to a variety of stressors that are particularly relevant to the reforestation and reclamation of boreal forests disturbed by oil sands development.

In my first research chapter I use an experimental approach to observe tree growth and physiological traits under hypoxic or phytotoxic environments using 6 common boreal species that are known to have different sensitivities to hypoxia. The expectation tested is that species that are more sensitive to hypoxia will be compromised disproportionately by additional phytotoxic compounds. However, although chapter results show that root hypoxic conditions further reduced the survival, growth, and physiology of all plants exposed to oil sands tailings water, it was the more salt tolerant species that were disporportionaely affected. Specifically, hypoxia disrupted a salt sequestration mechanism in trembling aspen (*Populus tremuloides*) and may have inhibited an osmoregulative mechanism in black spruce (*Picea mariana*) needles. My results suggest that the preparation of reclamation sites impacted by tailings water should involve efforts to enhance soil aeration to minimize detrimental effects on plants.

Then, to detect physiological responses to specific phytotoxic compounds, in my second research chapter I expose trembling aspen seedlings to a variety of “reconstituted tailings waters” by manipulating the tailings water composition and removing individual potentially phytotoxic compounds. For this experiment trembling aspen (*Populus tremuloides*) was used as it is widely employed in the reclamation of oil sands mining sites because of its early successional life

history, relative tolerances to salinity, and high growth rates. Here, the results corroborated an inference from the first research chapter; that trembling aspen Na tolerance mechanisms at the root level involves the maintenance of membrane selectivity of K over Na and that it is a prerequisite for survival and growth in saline conditions. Furthermore, a pH dependent effect of naphtenic acids on plant roots was inadvertently revealed with results that suggest that lowering the pH of tailings might not be more favorable for plant growth if significant amounts of naphthenic acids are present. Thus, if tailings water is to be adjusted to a more neutral pH, extracting naphthenic acids or minimizing their contact with roots would be important for improving revegetation success.

Low non-negative root temperatures often elicit similar effects on plants as salinity, or drought, yet the subject remains understudied and underexposed. Thus, in my third research chapter I comment on recent findings on the topic and look to examine some of the ways trees may have adapted to avoid or tolerate lower root temperatures effects while maintaining growth and productivity. While lower soil temperatures restrict root hydraulic conductivity, and thus water uptake, often triggering stomatal closure, an increase in transmembrane aquaporin water transporters could partly compensate. Furthermore, though hydraulically risky, it may be that reduced stomatal control and a “willingness” to accept lower xylem water potentials is a strategy used to maintain productivity.

Finally, in my fourth research chapter I further explore the importance of a tradeoff between hydraulic risk and productivity by experimentally investigating needle anatomy, foliar water uptake, and aquaporin expression under variable simulated drought conditions. By using branch samples collected from a white spuce (*Picea* glauca) provenance trial representing seed origins from throughout the range of the species the results can subsequently be related to the

climate of provenance origin. Provenances with source climates characterized by cold and dry climatic conditions from the Yukon and northern Alberta had thinner Casparian strips and hypodermis layers and lost more water during needle dehydration. Foliar water uptake uptake was highest in northern and central Alberta (Boreal Plains), These anisohydric characteristics appear to imply that northwestern white spruce populations are anatomically maladapted to drought. However, this lack of radial foliar hydraulic resistance, along with regulated aquaporin expression, can also allow drought limited western provenances to take advantage of foliar wetting even when precipitation does not meaningfully wet the soil. In these populations foliar water uptake may be a coping strategy that has an overall net benefit to the plant’s water balance.

Preface

This thesis is an original work by Killian Gérardin Fleurial (**KF**). All experiments were carried out and the data analyzed by **KF** under the guidance of Dr. Janusz J Zwiazek (JZ) with intellectual contributions from Dr. Wen Qing Zhang (WZ), Robert Vassov (RV), Dr. Danuta Sztukowski (DS), Dr. Maryamsadat Vaziriyeganeh (MV), Dr. Jaime Sebastian Azcona (JS), and Dr. Andreas Hamann (AH) as detailed below.

A version of Chapter 2 has been published as **Fleurial K. G**., Zhang W. Q., Vassov R., & Zwiazek J. J. “Root hypoxia aggravates the effects of saline tailings water on the growth and physiology of woody plants” *Plant and Soil* (2022), 1-18. This study was conceived by **KF** and JZ. **KF** set up the study with assistance from WZ, then collected the data and performed the analyses. **KF** wrote the manuscript with input and edits from WZ, RV, and JZ.

Chapter 3 is intended for submission as a journal article. It was conceived and designed by **KF** and JZ, and subsequently carried out and analyzed by **KF** with input from JZ and RV. **KF** wrote the manuscript with input from DS, RV, AH, and JZ.

A version of Chapter 4 has been published as **Fleurial K.**, Vaziriyeganeh M., and Zwiazek J.J. "Getting cold feet: tree productivity at the mercy of soil temperature." *Tree Physiology* 42.9 (2022): 1695-1699. The commentary was conceived by JZ. MV contributed to the writing of the paragraph on plant fungal interactions, **KF** wrote the manuscript with input and edits from JZ.

Chapter 5 is currently in preparation for submission to a peer reviewed journal. Building upon anatomical work previously performed by JS, the general idea and experimental design of the study were conceived by **KF**. The field work and experiment were conducted by **KF** and JS with input from JZ and AH. **KF** analyzed the data and wrote the manuscript with input from JS, JZ, and AH.

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1 General Introduction

1.1 Impacts of a changing environment on plants

Anthropogenically influenced changes to our environment have irremediably affected the composition of the Earth's atmosphere, soils, and bodies of water (Ehlers & Krafft, 2006; Lewis & Maslin, 2015). The effect is such that increasing trends in extreme precipitation, flooding, warming temperatures, and drought are all projected to occur (IPCC, 2017). From the perspective of the flora, which has adapted at species and population levels to occupy idiosyncratic terrains and environments over a geological timescale, such comparatively rapid change poses a unique challenge. The proper management and stewardship of remaining forests and the reforestation of disturbed sites such as the Athabasca oil sands mining sites will require a detailed understanding of plant stress physiology down to the molecular level in order to mitigate projected climate change effects by judiciously selecting species for planting and to better advise on such techniques as assisted migration and large-scale genomics projects.

1.2 The boreal forest

The boreal zone encloses 33% of the world's forested area. It is a large reservoir of biogenic carbon and is estimated to sequester 20% of the total forest C sink (Pan *et al.*, 2011). In similar fashion to the tropical rainforest of the Amazon, its health should thus be a global concern (Steffen *et al.*, 2015). Located generally between 50°N to 70°N, though with significant regional variations, boreal forest mean annual temperatures historically vary from -5 to 5°C and freezing temperatures occur for 6 to 8 months. Canada, which contains nearly one third of the boreal zone, or 307 million hectares of forests and woodlands, is considered to host the largest intact undeveloped forest on earth. (Adam & Archibold, 1995; Mather, 2003). As such, the boreal forest of Canada is one of the bio-regions projected to be the most affected by climate change, with potential warming of up to 11°C by 2100 (Gauthier *et al.*, 2015; IPCC, 2017). Declines in common boreal tree species such as trembling aspen have already occurred due to the increase in temperatures (Michaelian *et al.*, 2011; Worrall *et al.*, 2013), and in British Columbia, important conifer species are predicted to lose a large portion of their habitat (Hamann & Wang, 2006). Warming temperatures are also liable to affect forests indirectly, by increasing the incidence of uncontrolled forest fires and facilitating the spread of pathogens and parasites such as bark beetles (Flannigan *et al.*, 2000; Bentz *et al.*, 2010).

Plant species richness in the boreal zone is relatively poor and the boreal forest is largely dominated by a select few conifer species within the *Picea*, *Larix*, *Pinus*, and *Abies* genera, but also by a few types of angiosperms, mostly in the southern parts of the range: *Populus*, *Betula*, and *Alnus* species (Adam & Archibold, 1995; Gauthier *et al.*, 2015). Understanding the underlying physiological tools that species within these genera can use to respond to environmental stresses could therefore have a proportionately large effect on our ability to reclaim disturbed areas and successfully manage forest health in the coming decades across much of the boreal zone.

This thesis focuses on seven common boreal species from within these genera with a particular emphasis on white spruce and trembling aspen which account for much of the dominant vegetation in the Canadian boreal forest:

White spruce [*Picea glauca* (Moench) Voss] is one of the boreal forest’s dominant tree species with a range that spans the entire width of the North American continent encompassing virtually all of the boreal forest. As such its stands grow across a relatively strong gradient of climatic ecozones though it is generally found on well drained soils (Burns & Honkala, 1990a).

Black spruce [*Picea mariana* (Mill.) B.S.P.] is a boreal species widely distributed across Canada though it is also found in parts of the Northeastern United States. It grows mostly on cold wet lowlands and poorly drained sites and can tolerate a wide range of pH. It generally reproduces by seed but can spread vegetatively in swamps or bogs by layering with its lower branches (Viereck & Johnston, 1990).

Distributed over much of Northern North America, tamarack [*Larix laricina* (Du Roi) K. Koch] can form extensive pure stands found in both open and forested bogs, though it does not dominate them. It forms a wide and shallow root system allowing it to grow in moist poorly drained sites as does black spruce, however these sites can be even more wet and nutrient poor (Tilton, 1977). In a similar fashion it is also able to reproduce vegetatively by layering or producing root sprouts; this is an important method of dispersal at the northern end of its range (Johnston, 1990).

Balsam poplar (*Populus balsamifera* L.) is distributed east of the Rocky Mountains in the Canadian boreal zone where it forms extensive floodplain forests. It can also extend past the boreal forest conifer treeline where it grows primarily along streams and in wet sandy areas (Burns & Honkala, 1990b).

Found across most of Canada and much of the United States, lodgepole pine (*Pinus contorta* Dougl.) is one of the most widely distributed species in North America. It generally occurs in pure stands producing a deep root system. Though they grow in a relatively wide range of environments they are not normally considered to be tolerant to waterlogged soils (Lotan & Critchfield, 1990).

Jack pine (*Pinus banksiana* Lamb.), like balsam poplar, is distributed in most of Canada East of the Rocky Mountains as well as in the northern United States. It is a dominant species in the southern boreal and mainly adapted to dry acidic and sandy sites (Rudolph & Laidly, 1990).

Trembling aspen (*Populus tremuloides* Michx.) are found from central Mexico to Canada making it the most widely distributed tree in North America (Perala, 1990). It is also the most abundant deciduous tree in the boreal zone of Canada and makes up large groves interspersed with prairies in the dry transitional biome between the boreal forest and grassland called aspen parkland (Peterson & Peterson, 1992). It propagates itself mainly through root sprouts producing large monoclonal colonies.

In Canada or Alaska all seven of these species can be found growing alongside each other in various combinations of dominant, understory, codominant or successional stages. Because of their growth habits and the ecological niches they occupy, tamarack, black spruce, and balsam poplar are relatively resistant reclamation tree species to flooding and root hypoxia, whereas white spruce, lodgepole pine, jack pine and aspen are generally considered to be relatively sensitive.

1.3 **Plant stress responses**

1.3.1 Impacts of flooding on plants

For plants, the main consequence of soil waterlogging is the decline in O_2 availability. As hypoxic conditions set in, respiration in roots is inhibited and cells undergo an anaerobic metabolic switch over to lactic and or alcoholic fermentation (Agarwal & Grover, 2006). Many plant physiological processes are consequently affected: net photosynthesis, transpiration and stomatal conductance (Pereira & Kozlowski, 1977; Andersen *et al.*, 1984), but also carbohydrate metabolism (Huang & Johnson, 1995). To avoid or mitigate damage from a prolonged switch to fermentation pathways, plants must increase the influx of oxygen to the roots and palliate the accumulation of toxic fermentation products. In various hypoxia tolerant species, morphological changes such as, the production of aerenchyma, hypertrophied lenticels, or adventitious roots occur, yet in others they do not (Islam & Macdonald, 2008; Parent *et al.*, 2008, 2011; Calvo-Polanco *et al.*, 2012; Wang *et al.*, 2013a). Thus, other physiological processes, likely at cellular and molecular levels must take place.

1.3.2 Impacts of phytotoxic soil pollutants on plants

Beyond simply causing hypoxic soil conditions, flooding also largely contributes to the contamination of soils by organic and inorganic compounds (Ciesielczuk *et al.*, 2014). Some of these phytotoxic compounds are transported and deposited by floodwaters and others are produced through the anerobic alteration of organic matter present in the inundated soil (Maliszewska-Kordybach *et al.*, 2008). As might be expected the composition of the soils influences the production of these substances, however it also affects their deposition or release. For instance, previously contaminated soils with tar or soot will have increased sorption of pollutants (Motelay-Massei *et al.*, 2004).Although not a phytotoxic compound, indirectly, soil pH can change significantly because of flooding or environmental pollution. This can consequently affect plant growth by hampering mineral nutrient uptake, even stunting, or stopping it entirely. However, effects greatly depend on the plant species involved (Redfield *et al.*, 2004; Zhang *et al.*, 2013).

Amongst phytotoxic compounds, elevated levels of sodium is widely seen as one of the most common and severe forms of environmental stress. It effectively reduces water availability, causes ionic toxicity and osmotic imbalance, as well as nutrient deficiencies, which can result in plant death (Shannon, 1997). However, many other soil pollutants can have equally deleterious effects. Elevated levels of chloride, boron, fluoride, sulfate, and bicarbonate have all been conclusively linked to reductions in plant growth, and to plant mortality (Eaton, 1942; Rutland & Bukovac, 1971; Arnesen, 1997; Camacho-Cristóbal *et al.*, 2008). Naphthenic acids (Naphs), readily found in bitumen and released during the extraction process (Schramm *et al.*, 1984), have been shown to decrease hydraulic conductivity, and root respiration, resulting in observed reductions in gas exchange and leaf growth in trembling aspen seedlings (Kamaluddin & Zwiazek, 2002b). Bitumen is commonly extracted from oil sand deposits for commercial purposes and bitumen tailings contain relatively high levels of sodium, chloride, boron, fluoride, sulfate, and bicarbonate.

1.3.3 Impacts of low soil temperatures on plants

In addition to a soil's composition, plants must also contend with its temperature. Indeed, reductions in root hydraulic conductivity occur in cool soils even when water is readily available and air temperatures are warm (Running & Reid, 1980; Wan *et al.*, 2001; Kamaluddin & Zwiazek, 2004). Though water is certainly slightly more viscous as it nears zero degrees, this does not account for such large differences and much of the change in hydraulic conductivity in roots appears to be tied to changes in membrane water permeability that is mediated by aquaporin trans-membrane proteins (Lee *et al.*, 2008; Maurel *et al.*, 2015). In parallel, low temperatures also reduce phloem transport (Stanfield & Laur, 2019; Wang & Hoch, 2022), though this may not be the limiting factor for tree growth (Körner & Hoch, 2006). To further compound the reduction in transport, low soil temperatures impede root growth and thus the ability to increase water and nutrient acquisition, even in tree species commonly distributed in cold climates (Landhäusser *et al.*, 2001; Alvarez-Uria & Körner, 2007). Unfortunately, global plant and forest models generally rely on ambient air temperature, even when soil temperatures are lower than recorded air temperatures during the growing season from April to August in the boreal and temperate zones (Lembrechts *et al.*, 2022). As this likely leads to large inaccuracies, the topic of low soil temperature effects on growth merits greater visibility and awareness.

1.3.4 Impacts of drought on plants

In effect, but for very different reasons, drought causes similar reductions in plant hydraulic conductivity as low soil temperatures. Under the lens of the of the cohesion-tension theory under which plant hydraulics are most often viewed, water is pulled through a series of pipes, the xylem, along a water potential gradient stretching from the roots to the leaves (Dixon & Joly, 1895). When this gradient is too severe, the hydraulic chain is snapped, cavitating to form an embolism blocking the water's path (Tyree & Sperry, 1989). When drought reduces the availability of water to the roots, plants can prevent the water potential gradient from reaching critical levels by anatomically managing the transpiration of water through their leaves by opening or closing their stomata at the expense of carbon availability for photosynthesis and thus growth (Sack *et al.*, 2015). Once cavitation has occurred and if drought continues, some trees may also survive by reducing canopy area and thus their transpirational needs in order to return to more favorable hydraulic potentials (Rood *et al.*, 2000; Fleurial, 2017). Given the expected increases in drought incidence and the reductions in precipitation projected for the boreal forest, (Hogg & Hurdle, 1995; Gauthier *et al.*, 2015; IPCC, 2017) a better understanding of boreal tree hydraulic thresholds and responses to drought down to the cellular and molecular levels could be valueable.

1.4 Molecular and tissue specific influences on whole plant water relations.

1.4.1 Role of aquaporins

Aquaporins (AQPs) are membrane integral proteins responsible for the passive transmembrane transport of water and other small neutral molecules across cell or organelle membranes and thus integral to plant-water relations. This is facilitated by their structure: tetrameric holoproteins in biological membranes are formed by four AQP monomers; one monomer consists of 6 transmembrane helices arranged to form a central pore; electrostatic forces caused by the protein's structure allow for specific molecules to traverse the pore (Maurel *et al.*, 2015). Specific membrane permeability can be regulated by AQP expression and translocation to the membranes, but also by AQP gating. This is achieved by the phosphorylation, dephosphorylation, or protonation of residues situated on AQP protein loops extending out from the biological membrane and which lead to a conformational change that effectively closes the AQP central pore (Törnroth-Horsefield *et al.*, 2006). Though their role in water transport has long been recognized (Agre *et al.*, 1993; Maurel *et al.*, 2015), and cytosolic pH is known to regulate water transport during low oxygen stress (Tournaire-Roux *et al.*, 2003), it has only recently been shown that their function in oxygen transport may also be significant (Zwiazek *et al.*, 2017). Of particular interest is the location of aquaporin expression, where, because of a tissue or cells form and function they could play an outsized role.

1.4.2 The endodermis in needles and roots

Localized in roots, in the context of radial hydraulic conductivity, the endodermis is a tissue of singular anatomical and physiological interest. Rather curiously, an endodermis-like bundle sheath is also found in the needles of conifers. Although only one cell layer thick, its importance resides in the fact that the endodermis presents a physical barrier to the radial apoplastic movement of water in and out of the vascularized stele. Often present on the endodermis is a hydrophobic lignified or suberized Casparian strip which apoplastically seals the stele from the cortex (in roots) and the mesophyll in needles (Wu *et al.*, 2003). The water or solute pathway is then entirely dependent on the permeability of the cell membranes. Thus, the gating, expression, and regulation of AQPs in endodermal cell membranes, which might occur in response to flooding, drought, or even foliar wetting, will have whole plant consequences. In contrast with its function in roots, where it prevents the tissue desiccation when soils become too dry, its exact role in conifer needles has yet to be properly defined (Wu *et al.*, 2005). In parallel, there is evidence that in conifers aquaporins may participate in needle water uptake (Sparks *et al.*, 2001; Laur & Hacke, 2014). Given the many occurrences of leaf wetting (Dawson & Goldsmith, 2018) and the dominance and wide range of coniferous species such as white spruce in the boreal forest, foliar water uptake, perhaps linked with tissue specific aquaporin expression, could represent in an important but neglected source of water in tree water budgets (Berry *et al.*, 2019).

1.5 **Oil sands mining**

Central to Canada's boreal forest revegetation efforts and covering over 141 000 square kilometers in the province of Alberta's boreal zone, the Athabasca oil sands are the world's third largest proven oil reserve, accounting for nearly 10% of the oil in the world (Masliyah *et al.*, 2004). As an integral part of Canada's economy, the energy sector accounts for nearly 10% of nominal gross domestic product, they have benefitted from over $300 billion dollars of capital investment to date (Natural Resources Canada, 2014). The oil sands, located beneath a wet layer of muskeg and overburden, are a mixture of sand, clay, minerals, water, and high molar mass viscous petroleum also known as bitumen. Bitumen can be extracted from the ground using two different processes: in-situ extraction, which uses steam and a well to pump deep lying bitumen up to the surface, and open pit mining, similar to traditional mining operations. Here, after harvesting merchantable trees, removing the remaining vegetation, and salvaging upland mineral soil, large shovels excavate and deposit the oil sands into trucks, which then move it to crushers where it is broken down. Unlike more conventional crude oil, since bitumen has very high viscosity it must first be treated with the addition of hot water in order to pump it from the dig site to the extraction plant. At the plant, with the further addition of water, the bitumen is extracted, and the commercially valuable bitumen froth is then separated; the leftover material, tailings, is stored in a pond. (Masliyah *et al.*, 2004; Natural Resources Canada, 2020). Though 80% of water is recycled, every barrel of bitumen still requires approximately three barrels of water sourced from the Athabasca river to be produced (Natural Resources Canada, 2014).

1.5.1 Non-Segregating Tailings technology

The amount of water used by mining operators is only about 0.5% of the Athabasca River flows, however, the more fresh water is used the greater is the amount of wastewater and resulting size of tailings ponds. The larger the ponds develop, the more difficult and slow the reclamation process will become. Thus, one of the primary areas of focus by mining operators in the last 10 years has been to try to reduce the amount of water released back in tailings and to increase the recycle and reuse of wastewater. Canadian Natural Resources Limited (CNRL), the largest producer of heavy crude oil in Canada, has invested more than $3.5 billion in tailings research and resulting technologies. Non-Segregating Tailings (NST) are one such technology.

Tailings are a mixture of water, sand, fine silts, clay, residual bitumen and lighter hydrocarbons, inorganic salts, and water soluble organic compounds (Government of Canada, 2013; Natural Resources Canada, 2020). After transport by pipeline, they are deposited in ponds where many of the solids settle. What remains at the surface are Fluid Tailings (FT). In time, these FT will densify to become Mature Fluid Tailings (MFT), trapping water and thus reducing its availability to be recycled (CNRL, 2020). To avoid generating many FT, CNRL uses NST at their Horizon plant. These are created by removing water from the tailings stream, injecting carbon dioxide (CO_2), and adding thickeners to densify and ultimately reduce the final volume before deposition in the tailings ponds (CNRL, 2020; COSIA, 2020). Water is removed using centrifugal force in cyclones and fines are further consolidated with the addition of polyacrylamide flocculants Flopam A-3331 and A-3342 manufactured by SNF Canada. Finally, CO_2 from Horizon's capture plant is injected, accelerating the settling of fines and lowering the pH of the tailings closer to that of natural river water (Regulator, 2015; CNRL, 2020).

1.5.2 Land reclamation

The government of the province of Alberta requires, that after the oils sands have been extracted, companies remediate and reclaim all of the land they have used for both in-situ and mining operations (Government of Canada, 2013). This means that the disturbed land must be returned to a useable and productive state, similar to which existed prior to any activities. This is accomplished in several steps and over many years: after production ceases and the ground has been re-contoured, the subsoil and topsoil salvaged from before the disturbance are replaced. Seeding and revegetation then takes place in order to begin the establishment of a self-sustaining ecosystem.

However, as mining produces large volumes of tailings which have commonly elevated levels of compounds that are potentially injurious to plants, oil sands companies must use approaches to minimize these effects on the vegetation used for land reclamation. Research has generally focused on the thickness and composition of the soil layers to be placed on top of the tailings during reclamation (Sorenson *et al.*, 2011; Pinno *et al.*, 2012; Macdonald *et al.*, 2015; Zhang *et al.*, 2020), however tailings continually release water which over time may seep into the root zone and which can contain phytotoxic compounds. The elevated levels of sodium and naphthenic acids found in tailings in particular, can adversely affect the growth and establishment of reclamation plants (Renault *et al.*, 1999a; Kamaluddin & Zwiazek, 2002a). However, the existing knowledge of how the chemical composition of oil sands tailings and the water they release affects the physiology of boreal plants, at the organismal and at the cellular level, is limited; more research is needed (Redfield *et al.*, 2003; Zhang *et al.*, 2020). As such, the Athabasca oil sands, much of which are located on peat marshes, and their land reclamation, and provide an excellent case study to assess the effects of phytotoxic soil pollutants and waterlogged conditions on plants and offer up a unique opportunity to investigate science driven best practices for revegetation that fully take biotic factors and abiotic stressors into account.

1.6 **Overall Objectives and Thesis Structure**

Consolidated oil sands tailings are reclaimed by covering them with uncontaminated soil layers that are subsequently replanted with natural vegetation (Renault *et al.*, 1999a; CNRL, 2020; Zhang *et al.*, 2020). The latest data available from Alberta environment and parks shows that 142,807 ha of Athabasca oil sand land have been approved for use, 95,301 ha of which is currently actively disturbed by mining related operations. However, only 6,338.7 ha (20% of which are wetlands) have been permanently reclaimed by mining operators, and 104 ha have been fully certified by regulators and returned to the crown, the final step In the reclamation process (Parks-OSIP, 2017). This constitutes a potentially large environmental liability, as the underlying consolidated tailings continue to release contaminants over time infiltrating the upper layers of reclamation substrates, including the root zone of plants used for reclamation. An initially successful reclamation project could therefore fail in subsequent years, if plants and trees used for reclamation are unable to cope with the phytotoxic compounds commonly found in oilsands tailings. The problem could be exacerbated by flooding as many reclamation sites are located in areas of natural wetlands (Johnson & Miyanishi, 2008), and drought or other climate driven stressors, as under the most severe climate predictions, spruce dominated forests would be replaced by dry aspen parkland or prairie ecosystems in the Athabasca region (Nenzén *et al.*, 2020). Flooding would mobilize phytotoxic compounds contained in oil sands tailings, leading to phytotoxic in addition to hypoxic environments, and both cold-induced drought-like stress and drought stress could lead to the collapse of these budding new ecosystems by the end of the century. The main objectives of this thesis are to: 1) Determine how different boreal forest tree species used in the reclamation of disturbed Athabasca forests respond to environmental stresses induced by the presence of saline oil sand tailings, root hypoxia, low soil temperatures, or drought, 2) Identify potential chemical components in oil sands tailings that may contribute to phytotoxicity, and 3) Examine the role of needle water uptake in overcoming stress conditions that affect soil water uptake.

In **Chapter 2**, I use an experimental approach to observe growth and physiological traits under hypoxic and phytotoxic environments. I contrast six species that are known to have different sensitivities to hypoxia, and I test the expectation that species that are more sensitive to hypoxia will be compromised disproportionately by additional phytotoxic compounds. The hypothesis is that once the metabolism of species that are sensitive to hypoxia is compromised, they accumulate excessive harmful compounds.

In order to observe the physiological response to specific phytotoxic compounds, in **Chapter 3** I expose trembling aspen seedlings to "reconstituted tailings waters" by manipulating the tailings water composition and removing individual potentially phytotoxic compounds. The expectation is that these compounds are not all equally as harmful to the seedlings. I hypothesize that elevated Na levels in tailings water are the principal factor affecting plant responses to tailings water and expect that naphthenic acids and other NST components can aggravate these effects leading to ionic toxicity and nutrient deficiencies.

Low non-negative root temperatures have similar effects on plant hydraulics and transport as salinity, yet the topic remains understudied and underexposed. Thus, in **Chapter 4** I evaluate, compile, and contrast the history and current state of research on the topic and look to examine some of the ways trees may have adapted to avoid or tolerate lower root temperature while maintaining growth and productivity. I expect that the similar whole plant responses to salinity or drought may also lead to similar physiological mechanisms in cool soil adapted trees.

Finally, since salinity in tailings, low root temperatures, and drought trigger similar effects on root water uptake, in **Chapter 5** I examine the role of needle water uptake and its mechanisms as it could contribute to alleviating these effects. Though previous studies have shown that foliar water uptake occurs in white spruce its importance is generally considered to be negligible. Furthermore, unexplained growth differences have been observed between white spruce grown in field conditions vs-controlled growth environments. Thus, I make use of an established common garden experiment and develop an experimental approach to evaluate the importance of foliar water uptake to white spruce water budgets given their wide range of population specific climates. I suspect that specific differences in needle anatomy and aquaporin expression will drive differences in rates of foliar water uptake between populations. I hypothesize that any apparent lack of anatomical adaptation to drought in northern populations may be an adaptation to maintain productivity at colder temperatures whose risk can be partly mitigated by foliar water uptake.

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2 Root hypoxia aggravates the effects of saline tailings water on the growth and physiology of woody plants

2.1 Summary

Oil sands mining in the boreal forest produces large volumes of liquid tailings. Forestry research has generally focused on the thickness and composition of the soil layers to be placed on top of the tailings during reclamation. However, tailings release water, which may seep into the root zone and affect plants. Furthermore, the interactions and combined effects of root hypoxia and root substrate chemistry on plant responses are poorly understood.The effects of the aqueous phase of novel tailings (Non-Segregating Tailings—NST) were studied under well-aerated and hypoxic conditions in three relatively waterlogging tolerant tree species [tamarack (*Larix laricina*), black spruce (*Picea mariana*), and balsam poplar (*Populus balsamifera*)] and three relatively sensitive tree species [lodgepole pine (*Pinus contorta*), jack pine (*Pinus banksiana*), and aspen (*Populus tremuloides*)] by growing them in hydroponic solutions in a controlled environment. Root hypoxic conditions further reduced the survival, growth, and physiology of plants exposed to NST. Our results confirm that NST water produces the same deleterious effects in plants as previously reported in the amalgamated oil sands tailings. Root hypoxia inhibited salt sequestration in trembling aspen and may have inhibited an osmoregulative mechanism in black spruce needles. Our results highlight the potential impact of water seepage from buried tailings on reclamation success. Furthermore, hypoxic conditions can aggravate these effects by inhibiting salt stress mechanisms. I suggest that the preparation of reclamation sites impacted by tailings water should involve efforts to enhance soil aeration to minimize the detrimental effects on plants.

2.2 **Introduction**

Oil sands mining produces large volumes of tailings, which may potentially affect the growth and survival of plants in reclamation areas if present in the root zone. Therefore, in sites where mining operations have ceased, oil sands companies must employ approaches to minimize the effects of these tailings on the vegetation used for provincially mandated land reclamation to return these sites to a natural, pre-disturbance state with local flora and fauna (Province of Alberta 1993). The latest data available from Alberta Environment and Parks shows that, as of 2020, approximately 160,000 ha of Athabasca oil sand land had been approved for mining use, 105,500 ha of which were actively disturbed by mining related operations. However, only 8,700 ha (15% of which are wetlands) are being reclaimed by mining operators and, of those, only 100 ha (~ 1%) have been fully certified as having completed the process by government regulators and returned to the crown (Parks-OSIP, 2017).

Previous studies have generally focused on the thickness, composition, and layering of the capping material placed on top of the tailings during reclamation (Sorenson *et al.*, 2011; Pinno *et al.*, 2012; Macdonald *et al.*, 2015; Zhang *et al.*, 2020). However, tailings release saline water, which over time may seep into the root zone and affect plants. This potentially constitutes an environmental liability, as the underlying consolidated tailings may continue to infiltrate the upper layers of reclamation substrates, including the root zone of plants used for reclamation. An initially vigorous reclamation project could, therefore, find its species composition significantly altered in subsequent years if some of the species used are unable to cope with the compounds that are commonly found in oilsands tailings.

The elevated levels of Na, boron, and naphthenic acids present in tailings, are especially detrimental to the growth and establishment of reclamation plants (Kamaluddin and Zwiazek, 2002; Renault et al., 1999). However, the existing knowledge of how the chemical composition of oil sands tailings and tailings water affects the physiology of boreal plants is limited and more research is needed (Redfield *et al.*, 2003; Zhang *et al.*, 2020).

In addition to the potentially harmful chemical constituents of tailings water, soil saturation with water may lead to waterlogging, which reduces O_2 availability to plant roots. As hypoxic conditions set in, root respiration is inhibited, and cells undergo an anaerobic metabolic switch over to lactic or alcoholic fermentation (Agarwal & Grover, 2006). Many plant-physiological processes are consequently affected, including photosynthesis, transpiration and stomatal conductance (Pereira & Kozlowski, 1977; Andersen *et al.*, 1984), as well as carbohydrate metabolism (Huang & Johnson, 1995) and root water transport (Kamaluddin and Zwiazek, 2002). To avoid or mitigate damage from a prolonged switch to fermentation pathways, plants must increase the influx of O_2 to the roots and reduce the accumulation of toxic fermentation products. In some hypoxia-tolerant species, morphological changes such as, the development of aerenchyma, hypertrophied lenticels, or adventitious roots are triggered (Islam & Macdonald, 2004; Calvo-Polanco *et al.*, 2012; Wang *et al.*, 2013b). However, the potential interactions and combined effects of root hypoxia and root substrate chemistry on plant responses are poorly understood. This study aims to help fill this knowledge gap.

The present investigation focused on the effects of root hypoxia in combination with Non-Segregating Tailings (NST) developed by Canadian Natural to accelerate tailings consolidation and utilize less water during bitumen extraction. These tailings are created by removing water from the tailings stream, injecting carbon dioxide (CO_2), and adding thickeners to densify and ultimately reduce the final volume before their deposition in the tailings ponds

(CNRL, 2020; COSIA, 2020). Once tailings solids are settled, the top liquid layer is removed and referred to as tailings water.

The study was carried out with six common boreal species which account for much of the dominant vegetation in the Canadian boreal forest where oilsands mines are located. The species included trembling aspen (*Populus tremuloides*), balsam poplar (*Populus balsamifera*), black spruce (*Picea mariana*), jack pine (*Pinus banksiana*), lodgepole pine (*Pinus contorta*), and tamarack (*Larix laricina*). All six of these species are found in the northern boreal forest of North America growing alongside each other in various combinations of dominant, understory, codominant, or successional stages (Burns & Honkala, 1990a). Due to their growth habits and the ecological niches that they occupy, tamarack, black spruce, and balsam poplar are considered to be relatively resistant tree species to flooding and root hypoxia, whereas lodgepole pine, jack pine, and trembling aspen are generally considered to be more sensitive (Johnston, 1990; Lotan & Critchfield, 1990; Perala, 1990; Rudolph & Laidly, 1990; Viereck & Johnston, 1990; Zasada & Phipps, 1990). Furthermore, trembling aspen have previously shown relative tolerance to saline environments in the field (Lilles *et al.*, 2012), and because both black spruce and tamarack are the dominant boreal peatland tree species, they are physiologically adapted to nutrient poor environments (Islam *et al.*, 2003).

The main objectives of this study were to: 1) Assess how well different tree species used in the reclamation of consolidated oil sands tailings can tolerate the presence of oil sand tailings water in the presence and absence of root hypoxia 2) Identify potential chemical components in oil sands tailings water that may contribute to phytotoxicity and interact with hypoxia, and 3) Examine the underlying physiological mechanisms that may help plants tolerate these conditions. Here I evaluate the hypothesis that hypoxic conditions aggravate the effects of NST tailings water more severely in plant species that are less tolerant of root hypoxia and thus, the expectation that the effects of NST water under well-aerated and hypoxic conditions would be less detrimental to the flooding tolerant species (tamarack, black spruce, and balsam poplar) than the flooding sensitive species (lodgepole pine, jack pine, and trembling aspen).

2.3 **Materials and Methods**

2.3.1 Plant material and growth conditions

One-year-old, container-grown trembling aspen (*Populus tremuloides* Michx.) and balsam poplar (*Populus balsamifera* L.) dormant seedlings were obtained from the Smoky Lake Forest Nursery, Smoky Lake, AB, Canada. Tamarack [*Larix laricina* (Du Roi) K. Koch], jack pine (*Pinus banksiana* Lamb.), lodgepole pine (*Pinus contorta* Dougl.), and black spruce [*Picea mariana* (Mill.) B.S.P.] were obtained from the Bonnyville Forest Nursery, Bonnyville, AB, Canada. Fresh undiluted Non-Segregating Tailings (NST) were collected in 20 L airtight buckets directly from a pipe during discharge at the Canadian Natural oil sands mining area near Fort McMurray. After brief settling the liquid phase—NST water—was then collected for use.

The study was conducted in a controlled-environment growth room. Environmental conditions in the growth room were maintained at 22/18°C (day/night), 70 ± 10% relative humidity, and 16-h photoperiod. The photosynthetic photon flux density (PPFD) was 300 μmol m^{-2} s^{-1} at the top of the seedlings provided by full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). Prior to the application of treatments, the seedlings' roots were gently washed free of soil and were then placed in well aerated 50% modified Hoagland's solution (Epstein, 1972) for two weeks to flush buds and acclimate to experimental conditions. Four treatments were then applied to the seedlings for two months: 1) Aerated control (50% Hoagland's solution, 7 – 8 mg O_2 l^{-1}), 2) Hypoxic control (50%

Hoagland's solution, 2 – 3 mg O_2 l^{-1}), 3) Aerated NST (50% NST water in 50% Hoagland's solution at 7 – 8 mg O_2 l^{-1}), 4) Hypoxic NST (50% NST water in 50% Hoagland's solution at 2 – 3 mg O_2 l^{-1}). Each treatment was applied using two separate air pumps and tanks (100 l in volume) containing 50% NST water in 50% Hoagland's solution or 50% Hoagland's solution. Each tank was connected to three containers (30 l in volume) holding 18 randomly placed seedlings (3 plants per species) for a total of 24 containers and 332 plants (Figure 2.S1).

Treatment solutions were aerated using air pumps connected to air stones placed in the tanks and strong solution circulation to maintain 7 – 8 mg O_2 l^{-1} solution. Hypoxia (2 – 3 mg O_2 l^{-1}) was induced by reducing solution circulation, removing air pumps, and ensuring that solution tanks and seedling growth containers below the root collar were near hermetically sealed using plastic film and tape. Solutions were fully replaced one month into the experiment. Dissolved oxygen concentrations were monitored daily using a Fisherbrand Traceable 06-662-66 portable dissolved oxygen meter (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) to ensure the targeted levels were maintained. The pH of the solutions was measured weekly using an Orion STAR A111 pH meter (Thermo Fisher Scientific Inc.) and adjusted when necessary to pH 5.5 in aerated and hypoxic control (no NST water) solutions using KOH.

2.3.2 Growth medium and NST water characterization

Prior to the start of the experiment, undiluted NST water was characterized. The electrical conductivity (EC) was measured with a Traceable 15-077-977 EC meter (Thermo Fisher Scientific Inc.). Elemental analysis (Na, K, Mg, Ca, Cl, P, Fe, S, B) of NST water was conducted at the Natural Resources Analysis Laboratory (NRAL) at the University of Alberta. Elemental concentrations were measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES) with a Thermo iCAP6300 Duo (Thermo Fisher Scientific Inc.) following US EPA methods 6010d and 200.7 (n = 6). To determine naphthenic acids concentrations, NST water samples were first filtered (0.45 µm Teflon membrane), then extracted using dichloromethane and sodium sulphate (Scott *et al.*, 2008). Concentrations were then obtained by Fourier-transform infrared spectroscopy (FTIR; n = 3). The average pH, EC, elemental concentrations, and naphthenic acids concentrations of pure NST and of the treatment solutions used in the experiment are provided in Table 1.

2.3.3 Survival rate, growth, and biomass

The survival rate was calculated by dividing the number of surviving plants at the end of the experiment in each treatment by the total number of plants for each species. The root collar diameter and heights of seedlings from the root collar to the shoot tip were measured at the beginning, after one month, and at the end of the experiment (two months of treatments). The relative root collar diameter growth and shoot height growth over the first month and two months were calculated by dividing the difference between the initial and final measurements by the initial measurements. After fresh weights were determined for 9 individuals per species, harvested roots and stems were dried in an oven at 70 °C for 72 h, whereas leaves were separately freeze-dried for 72 h to avoid heat-induced breakdown of chlorophyll. To determine shoot dry weights, the dry weights of leaves and stems from each plant were added. Water content was determined by dividing the difference between the fresh weight and the dry weight of seedlings by their fresh weight and expressed as a percentage.

2.3.4 Foliar chlorophyll and elemental concentrations

Leaf chlorophyll-a and chlorophyll-b concentrations were determined in seven randomly selected seedlings per species per treatment (n = 7). Living fully expanded uppermost leaves were frozen, freeze-dried, and pulverized with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA).

Chlorophyll was extracted from the pulverized leaf samples (10 mg dry weight) with 8 ml dimethyl sulfoxide (DMSO) at 65°C for 22 h. After filtering, chlorophyll concentrations were measured in DMSO extracts with a spectrophotometer (Genesys 10S-UV-VIS, Thomas Scientific, NJ, USA) at 648 and 665 nm. The total chlorophyll concentration was then calculated using Arnon's equation (Wellburn, 1994).

Elemental (Na, K, Mg, Ca, Zn, Fe, P, Mn, S, Cu, and B) analysis of the pulverized leaf samples was conducted as described above for NST water samples. Total foliar nitrogen of the pulverized leaf samples was determined by flash combustion using a Thermo FLASH 2000 Organic Elemental Analyzer, (Thermo Fisher Scientific Inc.) and following the methods outlined in (Thomas, 1996; EPA, 2002).

2.3.5 Relative electrolyte leakage rate

Leaf electrolyte leakage was measured as a rate using a modified version of Zwiazek and Blake (1991). In brief, two-cm diameter leaf disks were removed with a cork borer from 6 individuals per species per treatment and immediately immersed in collection tubes containing 10 ml milli-Q water. For black spruce, jack pine, lodgepole pine, and tamarack, 6 needle sections, each 1 cm long, were cut from each plant with scissors. An initial electroconductivity reading (EC_0) was immediately taken and samples were set aside to incubate at room temperature. Electrolyte leakage was subsequently measured every hour for the next 5 hours (EC_1-EC_5) and the collection tubes were then immersed in a boiling water bath for 20 min; a final EC_{total} measurement was taken when samples had returned to room temperature.

The relative electrolyte leakage rate was then calculated as follows:

$$EC_{Rate} = \frac{slope(\{EC_1, EC_2, EC_3, EC_4, EC_5\}, \{1,2,3,4,5\})}{(EC_{total} - EC_0)} * 100$$

2.3.6 Net photosynthesis and transpiration rates

Net photosynthesis (A) and transpiration I rates were measured in living, fully expanded leaves between 9:00 and 12:00 h immediately prior to the start (n=6 per species), in the middle (after one month, n = 4 per species per treatment), and at the end of the experiment (after 2 months, n=6 per species per treatment) with an infrared gas analyzer (LI-6400, LI-COR). The reference CO_2 concentration was set to 400 µmol mol^{-1} and the flow rate was 200 µmol s^{-1} in the leaf chamber. The leaf chamber temperature was kept at 20 °C, and PPFD was set to 400 µmol m^{-2} s^{-1}. For black spruce, jack pine, lodgepole pine, and tamarack measurements, a 3-cm distal section of needles was inserted into the leaf chamber and then severed with scissors and scanned. Needle areas were then calculated using Sigmascan Pro 5.0 software (Systat Software, San Jose, USA).

2.3.7 Statistical analysis and graphs

To reveal statistically significant intraspecies differences between treatments ($p \leq 0.05$), data were fitted to linear mixed effect models and analyzed in R using the lme4, lmertest, and emmeans packages (Kuznetsova *et al.*, 2017; Bates *et al.*, 2019; Lenth *et al.*, 2019; Core Development Team, 2020). As relative shoot height and diameter growths were ratios and presented many null values, data were fitted to a zero-inflated beta mixed model using the glmmTMB R package. (Magnusson *et al.*, 2019). Water content was also fitted to a beta mixed model using the same package. Circulating pumps, each of which fed 3 containers holding seedlings, were treated as random effect factors and containers as a random nested factor within pump. Thus, mixed models were constructed as follows: Response variable ~ Aeration * Media + (1 |Pump/Container). When repeated measurements were present, a "Time" fixed effect factor was added. Foliar elements and N were analyzed separately per species per element. However, to investigate aeration type and media type interactions across all elements and N, an “element/N” fixed effect factor was added.

When models were ill fitting, pump was removed as a random effect and the new models were compared using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). Tukey tests were used for post-hoc analysis of estimated means. When data did not meet the assumptions for normality of distribution and homogeneity of variance, they were log or box-cox transformed using the MASS package and assessed further graphically using Q-Q plots (Ripley *et al.*, 2019). Type 3 analyses of variance tables using the Satterthwaite method were constructed for each species and analysis, relative growth and water content excepted. For these, analysis of deviance tables using type 2 Wald chisquare tests were used. For purposes of brevity these tables are not included with this thesis but are available for download at https://doi.org/10.1007/s11104-022-05847-x. Estimated means (except for survival rates where observed values were used) and standard errors are graphed using Sigmaplot 14.5 (Systat Software Inc., San Jose, USA).

2.4 **Results**

2.4.1 Survival rate

Seedling survival was only reduced for trembling aspen, balsam poplar, black spruce, and tamarack in treatments with NST water. When all species were grouped, survival rate was twofold lower in the hypoxic NST water treatment compared with the other treatments (Figure 2.S 2).

2.4.2 Relative root collar diameter growth (RRCDG)

RRCDG differences between treatments with and without NST were greater for the broad-leaf species than the coniferous plants. The effect of medium type on RRCDG was significant for trembling aspen and balsam poplar. This was due to reduced RRCDG in NST water treatments for these species (Figure 1 A). Black spruce also showed differences in RRCDG between treatments. However, this effect was due to hypoxia (Figure 2.1 A). There was a significant time effect on RRCDG for jack pine with a decrease observed in the second month (Figure 2.1 A). There was a significant interaction effect between medium and time factors for trembling aspen, balsam poplar, and lodgepole pine indicating that medium type influenced RRCDG changes over time in these species (Figure 2.1 A). In addition, the medium type to aeration type interaction effect was significant for black spruce seedlings and there was a significant three way medium-time-aeration effect for balsam poplar.

2.4.3 Relative shoot height growth (RSHG)

RSHG differences between seedlings growing with and without NST were markedly greater for the deciduous species (trembling aspen, balsam polar, and tamarack) than the evergreen species (black spruce, jack pine, and lodgepole pine). There was a significant effect of the type of medium on RSHG of trembling aspen, balsam poplar, black spruce, lodgepole pine, and tamarack seedlings and seedlings from these species with their roots in NST solutions had reduced RSHG (Figure 2.1 B). There was a time effect on RSHG for black spruce and tamarack seedlings which is reflected in the significantly smaller RSHG that occurred over the second month of the experiment in the aerated and hypoxic control treatments for black spruce and the hypoxic NST solution treatment for black spruce and tamarack (Figure 2.1 B). Lodgepole pine seedling RSHG had a significant medium-aeration type interaction effect and there was a significant medium-time interaction effect for black spruce and tamarack as RSHG was reduced over the second month in NST treatments (Figure 2.1 B). In parallel, the aeration-time interaction effect was significant for trembling aspen, black spruce, and tamarack as RSHG was also reduced over the second month in hypoxic treatments (Figure 2.1 B).

2.4.4 Root to shoot ratio, dry weight, and water content.

NST water presence increased the root to shoot ratios in the broad-leafed species (Figure 2.2 A). Thus, the effect of medium type on the root to shoot ratio was significant for trembling aspen and balsam poplar. By the end of the experiment, root to shoot ratios in trembling aspen and balsam poplar were significantly higher in treatments where NST water was present in the hydroponic medium, remaining unchanged from the initial ratios (Figure 2.2 A). Black spruce, jack pine, lodgepole pine, and tamarack showed no significant differences or factors for their root to shoot ratios across all treatments (Figure 2.2 A).

Dry weights were significantly lower for trembling aspen and balsam poplar seedlings grown in 50% Hoagland's with NST water. (Figure 2.2 B). Correspondingly, there was a significant effect of medium type on trembling aspen and balsam poplar dry weights. Jack pine dry weights showed a significant aeration effect and an interaction between aeration and medium type and had significantly higher dry weights when grown in the aerated control treatment

(Figure 2.2 B). There was no treatment effect on dry weights in black spruce, lodgepole pine, and tamarack (Figure 2.2 B).

Balsam poplar, black spruce, jack pine, and tamarack seedlings water content was significantly lower in treatments containing NST water (Figure 2.2 C). Medium type thus had a significant effect on the water content of these four species. In addition, trembling aspen, balsam poplar, and black spruce showed a significant effect of aeration and thus had lower water contents when exposed to hypoxic conditions (Figure 2.2 C). Aeration-medium interaction was significant for black spruce and jack pine and seedlings of these species grown in hypoxic NST water did not show significantly lower water content than seedlings in the hypoxic control and aerated-NST media. (Figure 2.2 C).

2.4.5 Foliar relative electrolyte leakage rate (RELR)

Whereas for trembling aspen and balsam poplar seedlings the aerated NST treatment showed the highest rates, tamarack seedlings had significantly higher RELR in the aerated control treatment (Figure 2.3). Thus, the type of hydroponic medium significantly affected the RELR from the leaves of trembling aspen, balsam poplar, and tamarack seedlings. As the RELR of trembling aspen in aerated NST was significantly greater compared with the seedlings in hypoxic NST (Figure 2.3), the effect of aeration was also significant. Finally, there was a significant medium-aeration interaction effect on RELR in lodgepole pine seedlings with significantly higher rates in the hypoxic NST treatment compared with the hypoxic control treatment (Figure 2.3).

2.4.6 Foliar chlorophyll

Although the effect of aeration on chlorophyll concentrations was not significant, there was a significant effect of the type of media on foliar chlorophyll concentrations in all species. In effect, this translated to higher chlorophyll concentrations in the treatments without any NST water for all species except for lodgepole pine (Figure 2.4). In addition, the aeration-medium interaction effect was significant for black spruce and jack pine.

2.4.7 Net photosynthesis (Pn) and transpiration (E)

Pn was lower when seedlings were grown in hydroponic solutions with NST water (Figure 2.5). Correspondingly, in all species, Pn was significantly affected by the type of media. In addition, the Pn of trembling aspen, balsam poplar, jack pine, and tamarack seedlings was significantly influenced by the level of aeration of the growth medium. Thus, in parallel when in the same growth solution, the Pn of seedlings of these 4 species was lower in the hypoxic treatments (Figure 2.5 A, B, D, and F). Time was a significant factor for Pn in trembling aspen, balsam poplar, black spruce, jack pine, and lodgepole pine. There was a significant medium-aeration interaction effect on Pn rates in trembling aspen, balsam poplar, jack pine, and tamarack seedlings. The medium-time interaction also had a significant effect on Pn in balsam poplar and black spruce seedlings. The three-way medium-aeration-time interaction only had a significant effect on trembling aspen Pn.

Transpiration rates (E) in seedlings of all species were significantly affected by the type of media. This was reflected in significantly lower E for seedlings grown in NST treatments (Figure 2.6). In addition, E for trembling aspen, balsam poplar, jack pine, lodgepole pine, and tamarack seedlings was significantly affected by the level of aeration of the hydroponic media. Thus, seedlings grown in hypoxic media showed lower rates of E than their counterparts grown in aerated solutions (Figure 2.6). In addition, time had a significant effect on the E of trembling aspen and black spruce seedlings only. Furthermore, the medium-aeration interaction had a significant effect on trembling aspen, jack pine, and tamarack seedlings E. Likewise, the interaction between hydroponic medium and time was significant for trembling aspen, and black spruce E. Finally, the aeration-time interaction and the three-way medium-aeration-time interaction had a significant effect on jack pine E.

2.4.8 Properties of treatment solutions and foliar elemental analysis

The average pH, EC, elemental concentrations, and naphthenic acids concentrations of pure NST and of the treatment solutions used in the experiment are provided in Table 1. NST water was characterized by a high pH (9.0), and electrical conductivity of 5.5 mS cm^{-1}, largely due to high *a* Na concentration of 1,095.7 mg L^{-1}. It also contained *a* relatively high B concentration (7.5 mg L^{-1}) and 133 mg L^{-1} *of* naphth*e*nic acids (Table 2.1).

All species showed significant foliar accumulation differences between seedlings grown in Hoagland's solution only and those grown with added NST water (Table 2.2). Consequently, the overall media-element interaction, indicative of the effect of medium type on individual elements or N, was highly significant for all species . Broadly, the addition of NST water to growth solutions accounted for large increases in foliar Na and B concentrations in all studied species (Table 2.2). In addition, NST water caused: i) an increase in N and reductions in Zn, Fe, P, S, and Cu concentrations in trembling aspen, ii) a reduction in Ca, Fe, P, Mn, and S in balsam poplar, iii) an increase in N and a reduction in all other elements in black spruce, iv) a decrease in all other elements for jack pine, v) a decrease in K, Mg, Ca, Zn, Fe, Mn, S, and N for lodgepole pine, and vi) a decrease in all other measured elements in tamarack (Table 2.2).

The aeration-elements interaction, i.e., the overall effect of hypoxia on individual elements or N, was significant in balsam poplar, jack pine, and tamarack but not for trembling aspen, black spruce and lodgepole pine. On an element per element basis, hypoxic solutions led to a i) reduction in foliar Mg, Ca, Mn, B, and N in trembling aspen seedlings, ii) a reduction in Na, Mg, Ca, and B, and an increase in Cu in balsam poplar, iii) a decrease in Mg, P, and S in black spruce, iv) an increase in K, S, Cu, in both media and N in the control medium only for jack pine, v) an increase in Cu for lodgepole pine, and vi) a decrease in S and N for tamarack (Table 2.2).

The overall aeration-media-element interaction was significant for trembling aspen, balsam poplar, black spruce, and tamarack. Specifically, NST water influenced the effects of hypoxia, or vice-versa, on i) foliar concentrations of Na, Ca, and B in trembling aspen seedlings, ii) Na, Mg, Ca, Mn, and B in balsam poplar, iii) Zn, Fe, S, and N in black spruce, iv) N only in lodgepole pine, and v) Cu only in tamarack (Table 2.2).

2.5 **Discussion**

Except for electrolyte leakage, the measured stress responses including plant survival, growth, and physiology, were most negatively affected by NST water when root hypoxic conditions were present. Of the two studied factors, growth medium (50% Hoagland's or NST water amended with 50% Hoagland's) and level of aeration (aerated or hypoxic), the type of growth medium had the greatest impact on the parameters measured in the seedlings. The presence of NST water in the growth medium had an overwhelming effect on the proportions and types of mineral elements accumulated in the foliage and, thus, had a significant effect on the chlorophyll concentrations, net photosynthesis, and transpiration of seedlings of all species, which unsurprisingly led to significantly lower root collar diameter, shoot height growth, and dry weights. Balsam poplar, lodgepole pine, and jack pine seedlings, in particular, were on a clear trajectory to carbon exhaustion when exposed to NST water, with transpiration and chlorophyll data indicating both diffusive and light energy limitations to photosynthesis. These results confirm that NST water produces similar deleterious effects in plants as have been previously reported for consolidated NST (Zhang *et al.*, 2020), and underlines the potential risks of water seepage from buried NST layers into the root area to reclamation success. The41nthrted pH, EC, Na, B, and naphthenic acids, that were measured in NST water, have been all previously identified as responsible for reductions in growth and increased mortality in seedlings used for reclamation in prior studies involving oil sands tailings (Croser et al., 2001; Kamaluddin and Zwiazek, 2002; Redfield et al., 2003; Renault et al., 1999; Zhang et al., 2020). These factors were also likely detrimental to plants in the present study. In contrast, the overall effects of hypoxia, though measurable and significant, had an order of magnitude less of an impact on the measured parameters. Nevertheless, hypoxia interacted strongly with the NST water leading to an aggravation of its deleterious effects on plants, notably in aspen and black spruce.

For this study, balsam poplar, black spruce, and tamarack were selected due to their geographical range and their prevalence in many boreal land reclamation projects, and because they are considered to be relatively tolerant to soil waterlogging (Johnston, 1990; Viereck & Johnston, 1990; Zasada & Phipps, 1990). Thus here, as expected, under hypoxic conditions, both balsam poplar and black spruce seedlings were able to acclimate and maintain elevated levels of photosynthesis and transpiration by the end of the experiment. However, unexpectedly, tamarack seedlings were unable to recover, whereas trembling aspen, a relatively hypoxia sensitive species, was able to do so. It has been reported that tamarack relies on stem hypertrophy and adventitious root production to facilitate root aeration and maintain respiration under flooding conditions (Islam *et al.*, 2003; Calvo-Polanco *et al.*, 2012). In our hydroponic setup, seedlings were supported in hydroponic solutions at the root collar by foam inserts in Styrofoam insulation trays, and the experiment only lasted two months; this may have inhibited or provided insufficient time for these morphological responses to successfully develop. Furthermore, hypoxia disrupts root water uptake by inhibiting the function of aquaporins and triggering stomatal closure (Tan *et al.*, 2018). Reactive oxygen species and ethylene are produced in response to oxygen deficiency in the roots and have been linked to flood adaptive mechanisms across a wide range of plants and climates (Voesenek & Bailey-Serres, 2015). Still, ethylene has been recorded to trigger a range of different responses in species subjected to hypoxia. In trembling aspen, under hypoxic conditions ethylene enhances aquaporin expression in the roots, stomatal conductance, and water transport (Kamaluddin and Zwiazek, 2002; Tan et al., 2021). In tamarack, ethylene leads to reductions in net assimilation and stomatal conductance (Islam *et al.*, 2003). Ethylene production may thus explain the responses observed in trembling aspen and tamarack and are likely reflective of different physiological strategies to flooding. Indeed, in our experiment, water use strategies in balsam poplar, tamarack, trembling aspen, and black spruce varied greatly, resulting in different tradeoffs. To maintain water content and thus turgor under hypoxic conditions, balsam poplar and tamarack reduced their transpiration rates, but trembling aspen and black spruce exhibited a more anisohydric behavior leading to a lower water content.

The addition of NST to the media increased foliar Na concentrations in all studied species. However, although no differences due to aeration or factor interactions were observed in any of the conifer species, hypoxic conditions severely aggravated Na uptake in trembling aspen, leading to foliar concentrations more than 10-fold greater than in seedlings exposed to NST under aerated conditions. This suggests effective oxygen-dependent root Na sequestration or exclusion in trembling aspen, similar to what has previously been reported for other poplar species (Chen & Polle, 2009). Indeed, the ability of roots to store salt is a strong indicator in woody perennials of salt resistance (Redfield *et al.*, 2003). However, excluding ions from the xylem sap is an energy dependent process which is inhibited under hypoxic conditions (Buwalda *et al.*, 1988). In parallel, increases in foliar K have been associated with an Na tolerance mechanism in trembling aspen (Yi *et al.*, 2008), as they compete for similar uptake sites and K is crucial for cells to maintain homeostasis in growing tissues (Chen *et al.*, 2014). Our results may corroborate this as foliar concentrations of K were indeed 25% higher in the aerated compared with hypoxic NST water treatments (Table 2). Furthermore, aerated trembling aspen seedlings in NST water were also able to maintain elevated levels of Ca which is essential to maintain selective membrane permeability and thus K/Na selectivity in the roots (Hanson, 1984; Tozlu *et al.*, 2000). This energy-dependent selectivity may confer some amount of protection to root growth under salt stress(Cramer *et al.*, 1987). In our study, at the energy expense of a higher root-to-shoot ratio, elevated electrolyte leakage and reduced growth, this mechanism likely allowed trembling aspen seedlings growing in the aerated NST water to maintain similar levels of photosynthesis, transpiration and water content as the seedlings grown in the control treatments. However, the combination of hypoxic conditions and NST water appear to have prevented the root growth essential for this tradeoff to occur. Nevertheless, this exclusion mechanism was not effective for boron since its foliar concentrations were all significantly greater under aerated conditions, supporting previous findings that show there is generally little control over boron uptake (Apostol *et al.*, 2002). Similarly, in balsam poplar, where no salt exclusion mechanism was evident, greater transpiration driven water uptake led to greater levels of Na and boron in the leaves of seedlings growing in aerated NST water media. Still, it is worth noting that, even when aggravated by hypoxic conditions, the trembling aspen foliar Na levels remained lower than in balsam poplar, suggesting that although trembling aspen may be more sensitive to root hypoxia, it is still more resistant to the salinity in tailings than balsam poplar. This supports earlier reports showing that trembling aspen can be a viable species that meets commercial forestry requirements, even when growing in moderately saline sites (Lilles *et al.*, 2012).

Although the studied conifer seedlings growing in the NST treatments had much greater foliar concentrations of Na and boron than those growing in the control solutions, contrary to the two broadleaf species, they were less prone to media-aeration interaction effects. Only relatively minor differences were apparent in foliar elemental concentrations between conifer seedlings grown in the aerated or hypoxic NST water amended treatments. This could partly be due to their slower growth rates and the overwhelming effects of NST media on all the measured parameters, but also be caused by an inability to prevent the uptake of salts by the roots (Zhang *et al.*, 2020). Nevertheless, black spruce seedlings growing in aerated NST had twofold greater needle N concentrations than those subjected to the other treatments. In tamarack seedlings growing in aerated media. N concentration was 50% greater compared with the hypoxic media, regardless of the presence of NST water. This was in contrast to lodgepole pine needles, which had lower N concentrations when growing in the NST treatments. Flooded tamarack and black spruce have been reported to have less foliar nitrogen than those accessing water from lower in the water table (Lieffers & Macdonald, 1990), which may explain the observed results in tamarack, but not in black spruce. The increased N in black spruce needles could instead be a mechanism associated with increased foliar accumulation of proline and stress protective proteins (El Moukhtari *et al.*, 2020). If this was indeed the case, then hypoxic conditions inhibited the production of these compounds aggravating the hypoxic effects. It would then also seem probable that foliar N differences between the media types did not occur in the broadleaf species and tamarack even if they were salt tolerant as their deciduous nature could allow them to lose their foliage and produce new salt-free leaves making such a mechanism unnecessary (Renault *et al.*, 1999b). More research is needed to investigate the potential mechanisms that allow black spruce to commonly grow in nutrient poor environments.

2.6 **Conclusion**

Prior to the start of this study, I hypothesized that root hypoxic conditions would aggravate the effects of NST tailings less severely in plant species that are more tolerant to root hypoxia. However, I observed that root hypoxia (< 3 mg O_2 l^{-1}) aggravated the effects of the saline NST water more severely in species that are reported to be more tolerant to salt stress. Our analysis of the liquid phase of NST also revealed elevated levels of naphthenic acids along with levels of B and Na, similar to those found in solid NST tailings and which are known to be detrimental to the growth and establishment of plants. Thus, I suggest that the development of hypoxic conditions in root zones where tailings water or soils of similar chemical composition are present, should be avoided or mitigated as hypoxia will interfere with root and leaf ionic toxicity tolerance mechanisms. Although the transpiration driven uptake of phytotoxic ions occurs more greatly under aerobic conditions in species bereft of these tolerance or ionic sequestering mechanisms, the additional deleterious effects caused by hypoxia led to greater mortality, and reductions in photosynthesis, growth, and dry weight. Further research should investigate the precise nature of the increased nitrogen accumulated in the needles of black spruce growing in NST water amended media and the salt sequestration mechanism in trembling aspen.

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2.8 Figures and Tables

Table 2.1: pH, electrical conductivity (EC, mS cm^{-1}), elemental concentrations (mg L^{-1}) and naphthenic acids (Naph, mg L^{-1}) concentrations of 100% Non-Segregating Tailings (NST) water, 100% NST water in 50% Hoagland's solution (1:1, by volume) and 50% Hoagland's solution (n = 8 for pH and EC, n = 6 for 100% NST water elemental concentrations, and n = 3 for 100% NST water naphthenic acids concentrations, standard errors are shown).

	pH	EC	Na	K	Mg	Ca	Zn	Cl	P	Fe	S	B	Naph
100% NST water	9	5.5 ± 0.2	1095.7 ± 2.6	34.0 ± 0.7	7.3 ± 0.3	5.8 ± 0.4	0	NA	0.1 ± 0.1	1.1 ± 0.4	34.9 ± 0.6	7.5 ± 0.1	133.0 ± 5.4
100% NST water in 50% Hoagland's	8.0 – 8.7	4.1 ± 0.1	547.9	75.8	9.7	42.9	0	0.4	15.6	0.8	25.5	3.8	66.5
50% Hoagland's	6 – 5.5	1.2 ± 0.0	0	117.5	12	80	0	0.9	31	0.6	16	0.1	0

Table 2.2: Foliar elemental concentrations (mg kg^{-1} and g 100 g^{-1} for N) after two months of treatments (n = 7 per species per treatment). Estimated menas are shown and different letters indicate significant posthoc differences between treatments (from largest to smallest , $P < 0.05$). Color indicates significant factors after a two-way ANOVA: Yellow for an effect of growth medium, blue for an aeration effect, and green when both medium and aeration effects occurred, with ‘*’ indicating a significant medium-aeration interaction ($P < 0.05$).

		Na	K	Mg	Ca	Zn	Fe	P	Mn	S	Cu	B	N
Trembling Aspen	Aerated	35.8 bc*	17829 a	1850 ab	10061 a*	57.7 a	80.5 a	2934 a	48.7 a	2951 a	7.7 a	38.8 c*	2.3 a
	Hypoxic	9.3 c*	17810 a	1468 b	8498 a*	29.0 ab	72.6 a	2932 a	30.8 a	2407 a	6.0 ab	39.9 c*	2.4 a
	Aerated/NST	264.8 b*	21291 a	2288 a	10923 a*	21.6 b	40.9 b	1770 b	43.8 a	1552 b	3.6 b	292.5 a*	3.0 a
	Hypoxic/NST	3558 a*	15957 a	1313 b	5466 b*	25.9 ab	48.5 ab	1922 b	28.2 b	1734 b	4.8 ab	104.8 b*	2.8 a
Balsam Poplar	Aerated	4.4 b*	28062 a	1902 ab*	10249 a*	28.2 a*	85.2 a	5433 a	34.2 a*	3213 a	1.8 a	26.2 c*	3.2 a
	Hypoxic	47.7 b*	31580 a	2008 ab*	8712 a*	17.3 a*	78.1 a	4037 ab	20.7 b*	3202 a	4.3 a	27.0 c*	2.51 a
	Aerated/NST	19435 a*	27862 a	2528 a*	9263 a*	17.9 a*	29.0 b	2496 bc	15.7 b*	1342 b	1.4 a	314.1 a*	3.07 a
	Hypoxic/NST	7807 a*	24898 a	1595 b*	4580 b*	19.1 a*	34.9 b	2197 c	18.3 b*	1279 b	3.0 a	171.9 b*	3.35 a
Black Spruce	Aerated	38.7 b	9599 a	1094 a	3717 a	35.7 a*	52.9 a*	2194 a	105.3 a	1071 a*	4.4334 a	23.3 b	1.1 b*
	Hypoxic	40.2 b	9213 a	884 a	3210 a	26.5 b*	33.7 a*	1708 b	82.3 a	772 b*	3.1757 a	20.0 b	1.3 b*
	Aerated/NST	2785 a	4966 b	476 b	938 b	12.3 c*	13.5 b*	1089 c	25.3 b	421 c*	0.00 b	57.6 a	2.3 a*
	Hypoxic/NST	2825 a	4336 b	462 b	871 b	14.8 c*	18.3 b*	986 c	21.6 b	470 c*	0.7577 b	56.9 a	1.4 b*
Jack Pine	Aerated	28.9 b	8230 b	1182 a	2362 a	58.0 a	55.2 a	1989 a	124.2 a	1453 a	6.4 a	29.0 b	1.3 a
	Hypoxic	78.8 b	12138 a	1093 a	2404 a	48.6 a	36.1 ab	2271 a	135.5 a	1748 a	8.0 a	25.4 b	1.5 a
	Aerated/NST	5933 a	4408 c	689 b	859 b	30.6 b	18.7 c	1345 b	52.5 b	701 b	4.0 b	49.6 a	1.8 a
	Hypoxic/NST	7977 a	6601 bc	668 b	920 b	26.7 b	20.1 bc	1519 b	44.3 b	900 b	5.7 ab	55.8 a	1.6 a
Lodgepole Pine	Aerated	59.9 b	10838 a	738 a	1800 a	36.7 ab	27.7 a	1749 a	66.9 ab	1098 a	4.6 ab	16.2 b	1.5 a*
	Hypoxic	78.7 b	10145 a	750 a	1926 a	43.8 a	25.3 ab	1665 a	79.3 a	1176 a	5.7 ab	16.0 b	1.4 a*
	Aerated/NST	4993 a	6603 b	551 a	732 b	27.9 b	12.7 b	1410 a	47.7 b	663 b	3.8 b	46.4 a	1.0 b*
	Hypoxic/NST	4612 a	8690 ab	628 a	845 b	27.7 b	17.0 ab	1709 a	56.0 ab	907 ab	6.2 a	41.6 a	1.2 ab*
Tamarack	Aerated	13.0 b	9229 ab	1118 a	2606 a	26.9 a	31.6 a	1996 a	126.6 a	1130 a	3.9 a*	26.4 b	1.7 a
	Hypoxic	26.3 b	10890 a	1054 a	2409 a	20.7 ab	32.4 a	2043 a	100.4 a	840 a	3.9 a*	22.5 b	1.0 b
	Aerated/NST	4227 a	8103 ab	766 ab	1073 b	13.0 b	13.9 b	1538 a	43.8 b	528 b	< 1.5 b*	91.3 a	1.6 a
	Hypoxic/NST	5488 a	5570 b	463 b	691 b	13.7 b	18.6 b	1484 a	37.3 b	506 b	1.7 ab*	74.4 a	1.1 b

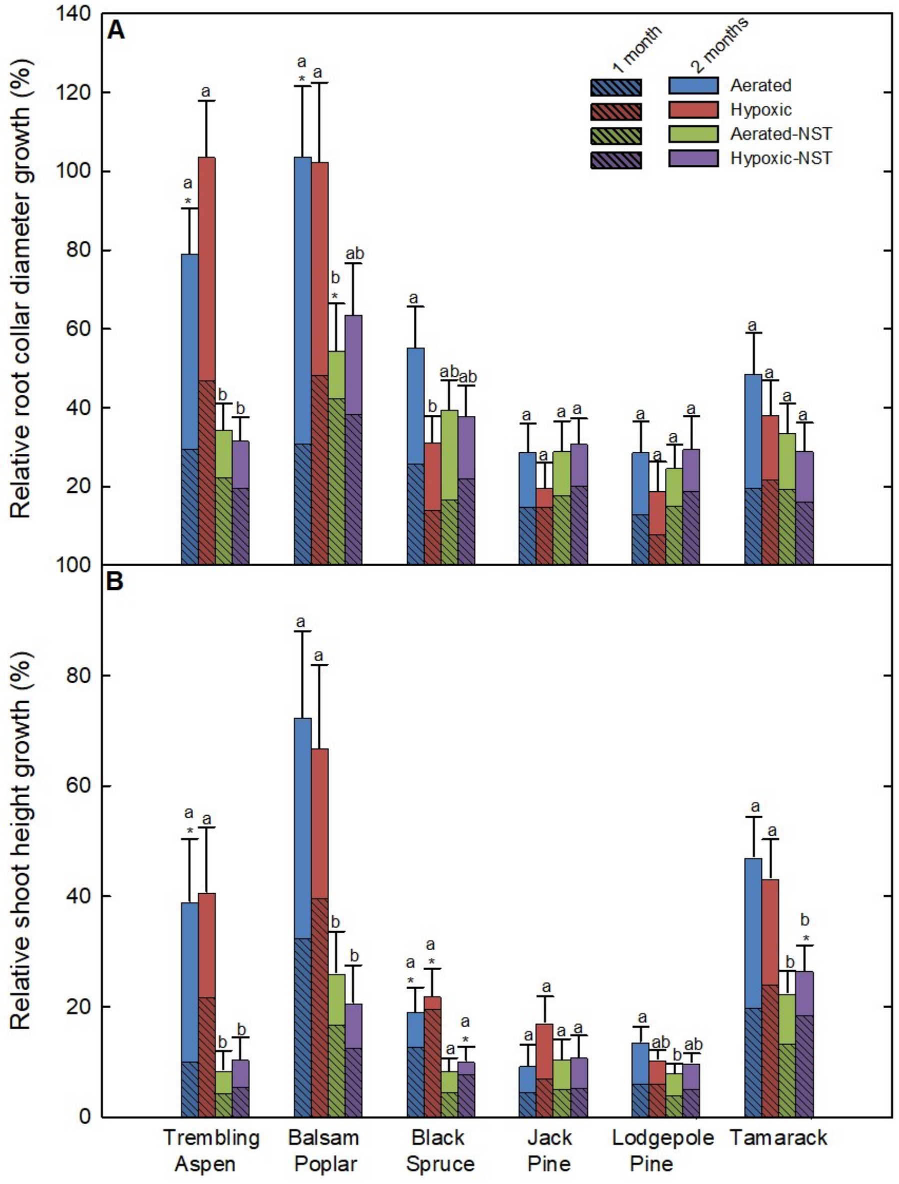

Figure 2.1: Relative root collar diameter growth (A), and relative shoot height growth (B) after the first and second month of the different treatments for plants of the studied species (n = 18 per species per treatment). Values were obtained by dividing the difference between the initial and final measurements by the initial measurements. Estimated means and standard errors are shown. Letters indicate significant differences within species between treatments ($P < 0.05$), and “*” indicates a significant difference between the first and second month of growth ($P < 0.05$)

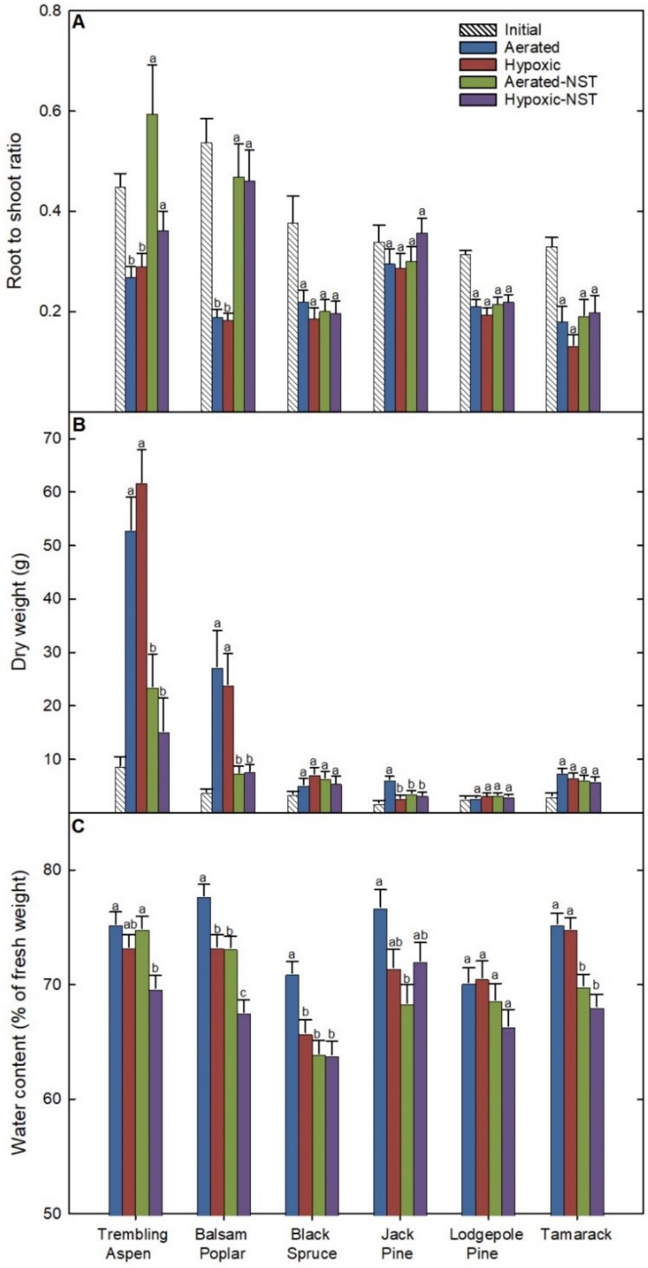

Figure 2.2: Root to shoot ratio (A), Dry weight (B), and Water content (C) after two months of different treatments for the plants of the studied species (n = 9 per species per treatment). A and B include initial (prior to application of treatments, n = 4 per species per treatment) root to shoot ratios and dry weights for reference. Estimated means and standard error bars are shown; different letters indicate significant differences between treatments of the same species ($P < 0.05$).

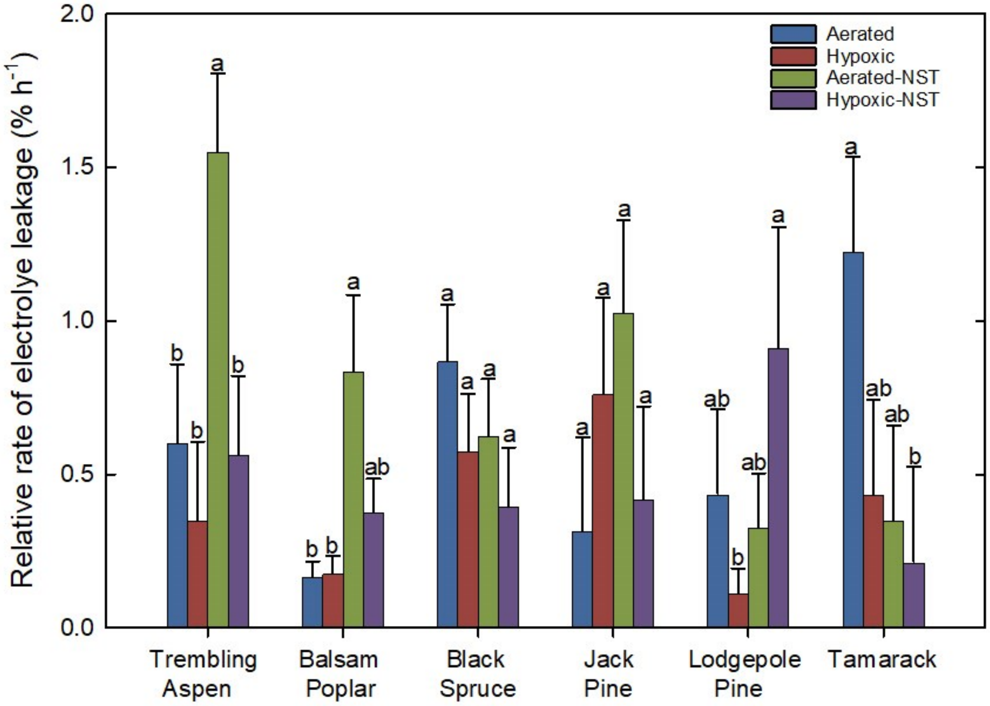

Figure 2.3: Relative rate of electrolyte leakage after two months of different treatments for the plants of the studied species (n = 6 per species per treatment). Estimated means and standard error bars are shown; different letters indicate significant differences between treatments of the same species ($P < 0.05$).

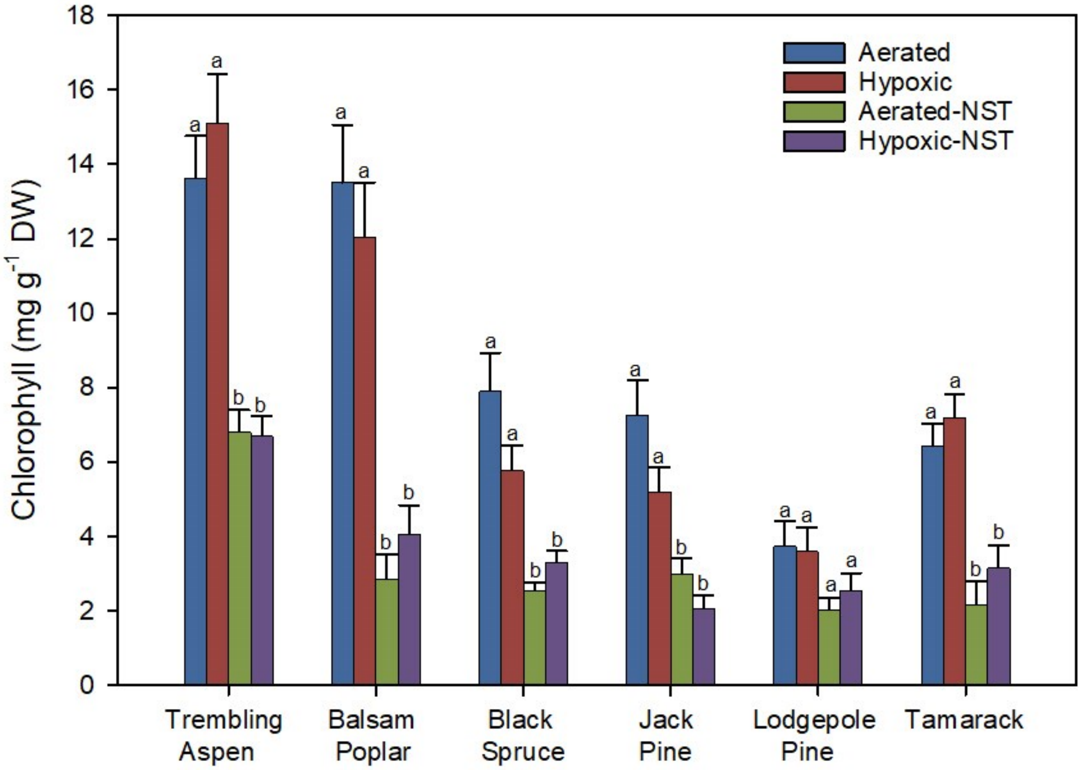

Figure 2.4: Foliar chlorophyll concentration (chlorophyll a + b) after two months of different treatments for the plants of the studied species (n = 7 per species per treatment) Esimated means and standard error bars are shown; different letters indicate significant differences between treatments of the same species ($P < 0.05$).

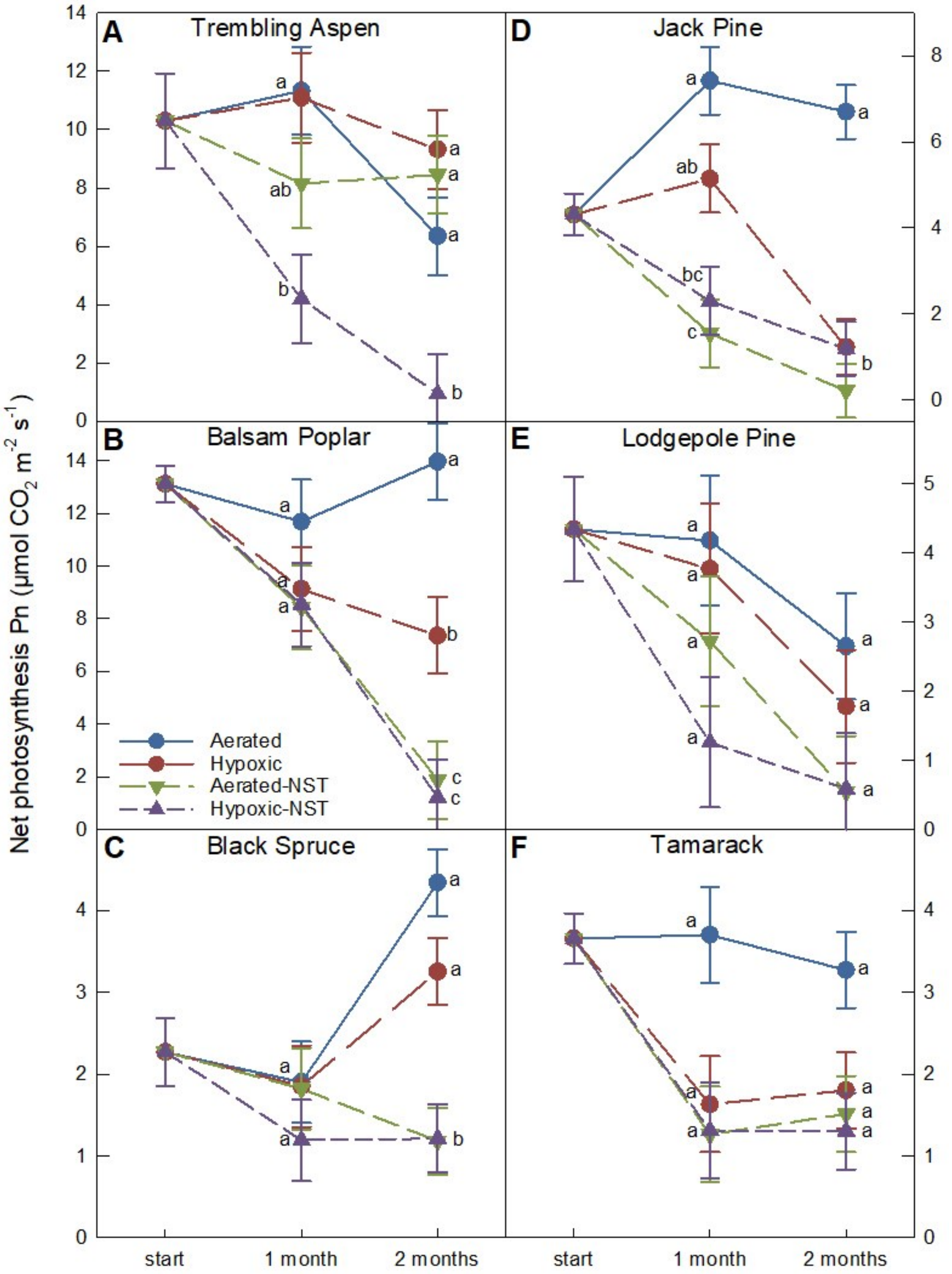

Figure 2.5: Net photosynthesis rate of trembling aspen (A), balsam poplar (B), black spruce (C), jack pine (D), lodgepole pine (E), and tamarack (F) at the start, after 1 month (n = 4 per treatment), and after 2 months (n = 6 per treatment) of the various treatments. Estimated means and standard error bars are shown; different letters indicate significant differences between treatments at the same time period ($P < 0.05$).

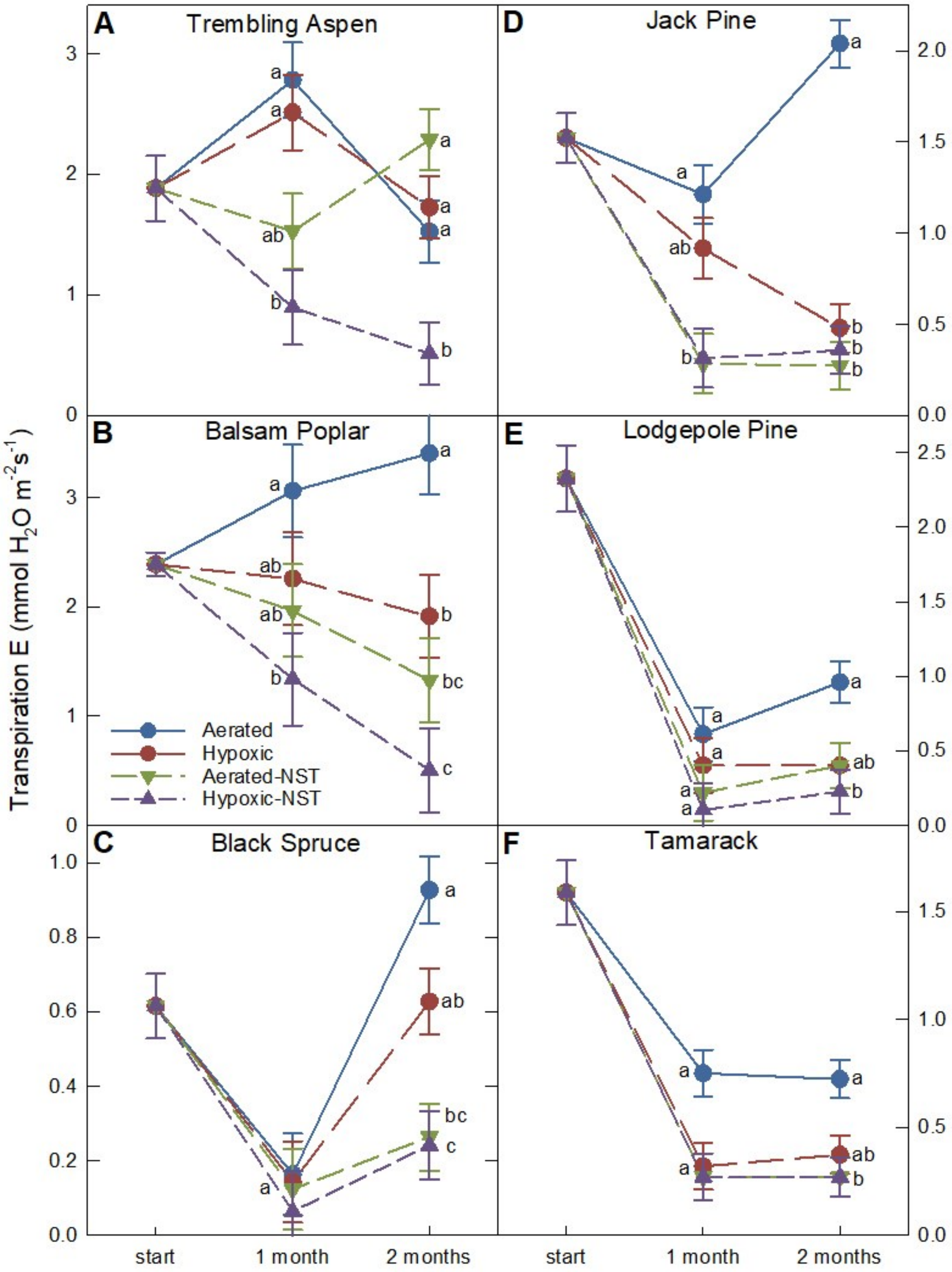

Figure 2.6: Transpiration rate of trembling aspen (A), balsam poplar (B), black spruce (C), jack pine (D), lodgepole pine (E), and tamarack (F) at the start, after 1 month (n = 4 per treatment), and after 2 months (n = 6 per treatment) of the various treatments. Estimated means and standard error bars are shown; different letters indicate significant differences between treatments at the same time period ($P < 0.05$).

2.9 Supplementary Figures

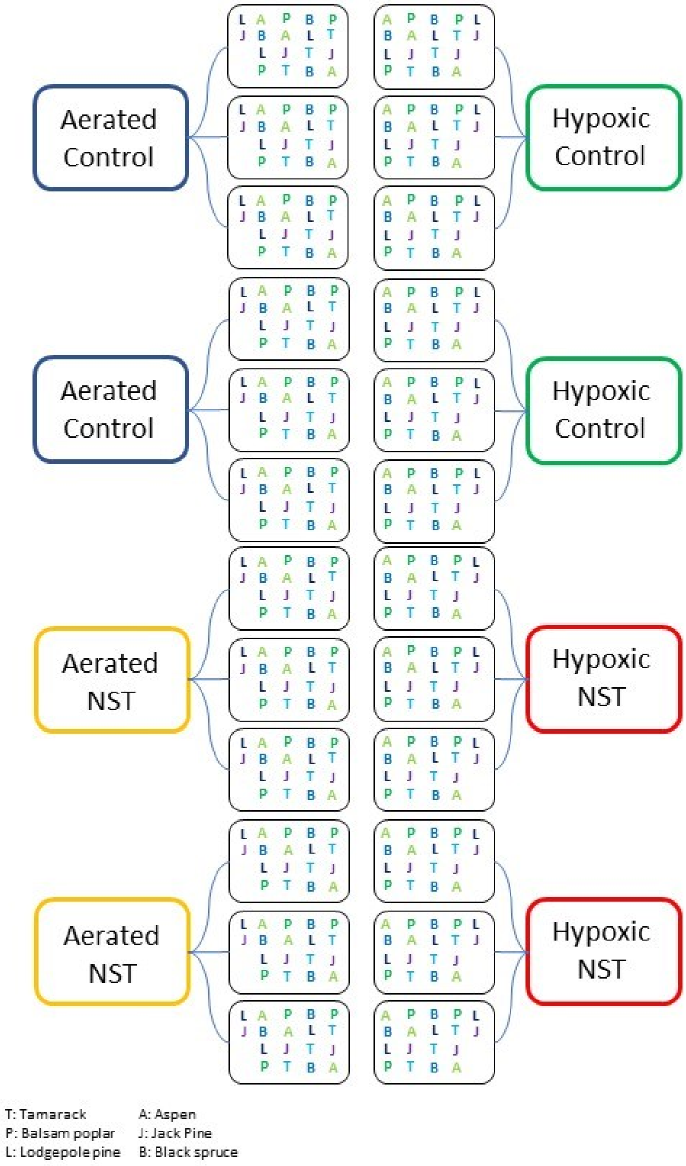

Figure 2.S1: Experimental setup diagram. Aeration (Aerated, Hypoxic) and hydroponic media (Control, NST) were crossed producing four different treatments. Each treatment was applied using two separate pumps and tanks (100 l in volume) each connected to three containers (30 l in volume) holding 18 randomly placed seedlings (3 individuals per species) for a grand total of 24 containers and 332 individuals.

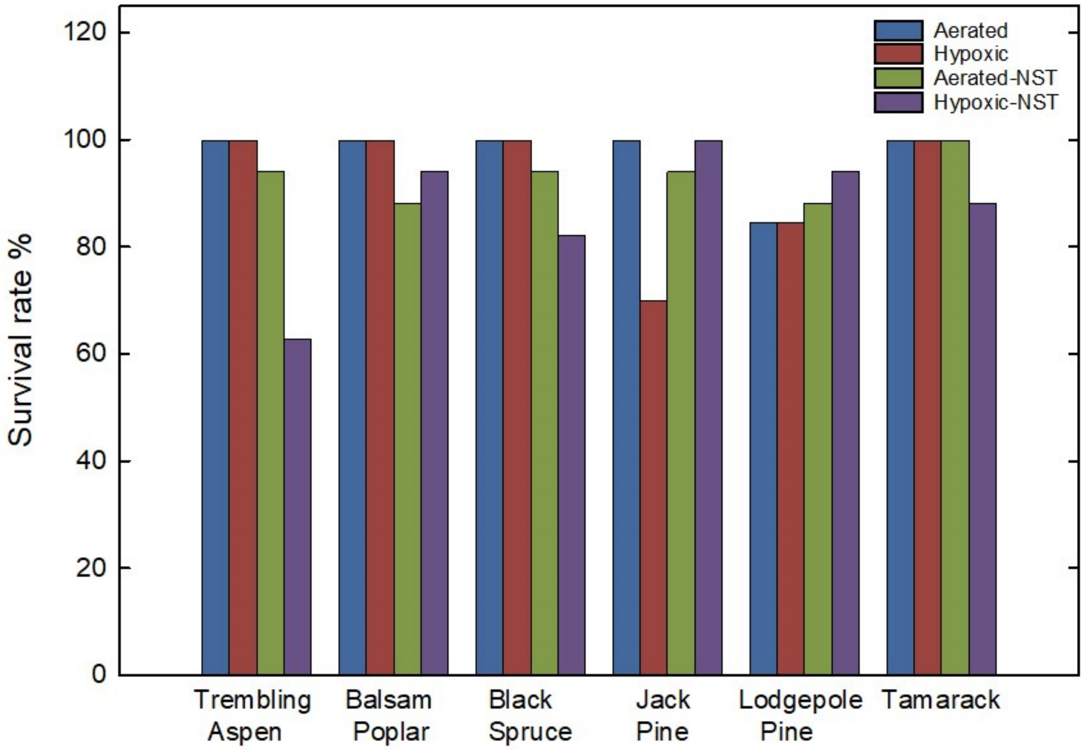

Figure 2.S2: Plant survival rates of the studied species after and after 2 months of the various treatments.

3 Responses of *Populus tremuloides* seedlings to reconstituted saline oil sands tailings water highlight the importance of K/Na membrane selectivity and indicate naphthenic acids are major contributors to tailings phytotoxicity.

3.1 Summary

Trembling aspen (*Populus tremuloides*) is widely used in reclamation of oil sands mining sites because of its early successional life history, tolerances to salinity and high growth rates. Here, I examine the species' tolerancances to pollutants of specific concern in oil sands tailings, namely sodium, and naphtenic acid. To observe physiological responses to specific phytotoxic compounds, I carried out an experiment where I exposed trembling aspen seedlings to real and reconstituted tailings waters and manipulated the reconstituted tailings water composition by removing Na or naphthenic acids. I found that Na tolerance in trembling aspen appears to rely on membrane selectivity of K over Na. Our results corroborate other research that maintaining K uptake in the roots is an essential mechanism for exclusion of Na, and is also a prerequisite for survival and growth. I also found a pH dependent effect of Naphtenic acids on plant roots, which merits further study, as our results suggest that lowering the pH of tailings might not be more favorable for plant growth if significant amounts of naphthenic acids are present. If tailings water is to be adjusted to a more neutral pH, extracting Naphs or minimizing their contact with roots would be important for improving revegetation success.

3.2 Introduction

Bitumen extraction from the Athabasca oil sands in Alberta, Canada, is partly conducted through open pit mining (Natural Resources Canada, 2020). Due to its high viscosity, the crushed excavated ore must first be treated with the addition of hot water in order to pump it as a slurry from the mine pit to the extraction plant, where, with the further addition of water and NaOH when required, the commercially valuable bitumen is collected and the leftover material, tailings, are transferred to a Dedicated Disposal Area (DDA), frequently referred to as tailings ponds (Masliyah *et al.*, 2004; Natural Resources Canada, 2020). When mining operations are completed, the government of Alberta requires companies to remediate and reclaim the disturbed land, including DDAs, to self-sustaining pre-disturbance state (Province of Alberta, 1993). Since tailings are potentially toxic to plants, research has often focused on alleviating these effects through different thicknesses and compositions of soil layers that are placed on top of the tailings during reclamation (Sorenson *et al.*, 2011; Pinno *et al.*, 2012; Macdonald *et al.*, 2015; Zhang *et al.*, 2020, 2023). However, tailings pore water has been shown experimentally to produce similar deleterious effects on plants as the solid tailings (Fleurial *et al.*, 2022b). Thus, given that water can potentially travel upwards between soil layers, the leaching of tailings water into the root zone is a possible concern (Utting, 2021). Therefore, a better understanding of how the specific chemical composition of oil sands tailings release water affects the physiology of boreal plants, at the organismal and cellular levels, is needed.

To reduce the amount of freshwater used during the extraction process and the resulting volume of tailings and size of DDAs, one of the primary areas of focus by mining operators in the last 10 years has been to increase the recycling and reuse of wastewater. One such technological solution is the production of Non-Segregating Tailings (NST) developed by Canadian Natural (CNRL), the largest producer of heavy crude oil in Canada. During NST production, water is removed using centrifugal force in cyclones and fines are further consolidated by gravity in thickeners with the addition of flocculants and the injection of CO_2

from Horizon's capture plant, which also aims to lower the pH of the tailings closer to that of natural river water (Regulator, 2015; CNRL, 2020).

Indeed, though the effects are highly species dependent, the elevated pH (>8) from tailings can result in high plant mortality and reduced growth (Redfield *et al.*, 2004; Zhang *et al.*, 2013). Similarly, elevated levels of sodium (>1000 mg/L) and residual hydrocarbons present in tailings, mostly in the form of naphthenates (Naphs), can adversely affect the establishment and growth of reclamation plants (Schramm *et al.*, 1984; Renault *et al.*, 1999a; Kamaluddin & Zwiazek, 2002a). Both Na and Naphs have also been identified as primary contributors to tailings toxicity (Renault *et al.*, 1998; Kamaluddin & Zwiazek, 2002b; Morandi *et al.*, 2015)..

Trembling aspen (*Populus tremuloides* Michx.) is one of the most commonly used tree species for oil sands revegetation in the Athabasca region. It is the most widely distributed tree in North America, ranging from central Mexico to Canada (Perala, 1990), and the most abundant deciduous tree in the boreal zone of Canada. It also makes up large groves interspersed with prairies in the dry transitional biome between the boreal forest and grassland that is referred to as aspen parkland (Peterson & Peterson, 1992). Significantly, its potential for assisted migration has also garnered interest as climatic predictions show that Northern Boreal and Boreal Plains climates in the Athabasca oil sands region may be replaced by aspen parkland climates (Gray *et al.*, 2011). These characteristics make trembling aspen a good candidate species upon which to evaluate the effects of tailings water chemistry.

To examine physiological responses to specific NST chemical components, I carried out an experiment in which I exposed trembling aspen seedlings to NST water and "reconstituted tailings waters" that matched the previously published NST water composition and that of the composite tailings (CT) water which for many years was the most widely used tailings management technology (Renault et al. 1998, 2001, Franklin et al. 2002, Redfield et al. 2003, 2004). Furthermore, I manipulated the composition of these "reconstituted tailings waters" by removing either Na or Naphs to assess their contributions to NST water phytotoxicity. I hypothesized that elevated Na levels in tailings water would be the principal factor affecting plant responses to tailings water and expected that Naphs and other NST components would aggravate these effects leading to ionic toxicity and nutrient deficiencies.

3.3 **Materials and methods**

3.3.1 Plant material and growth conditions

One-year-old, container-grown trembling aspen (*Populus tremuloides* Michx.) dormant seedlings were obtained from Bonnyville Forest Nursery (Bonnyville, AB, Canada). The study was conducted in a controlled-environment growth room at 22/18 °C (day/night) temperature, 70 ± 10% relative humidity, a 16-h photoperiod, and 300 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the seedlings provided by full-spectrum fluorescent bulbs (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

3.3.2 Experimental design and treatments

Non-segregated tailings water (NST) was obtained from the Canadian Natural oil sands mining site near Fort McMurray and allowed to settle (Fleurial *et al.*, 2022b). Reconstituted NST water, Syncrude composite or consolidated tailings water (CT), and control solutions with and without Na and Naphs were then designed based on the analyses of NST and CT water composition (Renault et al. 1999b; Redfield et al. 2003; 2004; Fleurial et al. 2022) (Table 3.1). Solutions were then constructed by adding the specific salts and naphthenates ((sodium; 3-(3-

ethylcyclopentyl)propanoate), Acros Organics, Geel, Belgium), as shown in Table S3.1, to 50% modified Hoagland's solution (Epstein, 1972).

Prior to the application of the treatments, the seedlings' roots were gently washed free of soil and placed in 50% modified Hoagland's solution (Epstein, 1972) for two weeks to break dormancy and acclimate to experimental conditions. The following treatments were applied: 1) Control (50% Hoagland's mineral solution), 2) Reconstituted complete NST, 3) Reconstituted complete CT, 4) Reconstituted NST without Na, 5) 50% Hoagland's + Na (Na positive control), 6) Reconstituted NST without naphthenates, 7) 50% Hoagland's + NST levels of naphthenates (Naphs positive control) 8) NST release water from CNRL (tailings water control). The pH was adjusted to 8.4 after 48 hours (the pH of NST release water treatment). The hydroponic set up consisted of eight 100 L tanks (one per treatment) each connected to three 30 l containers, with 6 randomly placed plants in each container for a total of 18 plants per treatment (144 plants in total). Solutions were aerated using air pumps connected to air stones placed in the tanks and a strong solution circulation was maintained, to keep dissolved oxygen levels of 7-8 mg/L. The plants were subject to the above treatments for 6 weeks after which final measurements were carried out.

3.3.3 Mortality rates, root collar diameter growth, height growth, and plant fresh and dry weights

The mortality rate was averaged per container and calculated as the mean number of dead plants at the end of the experiment divided by the total number of plants. Two rates were determined: 1) Mortality (complete) where seedlings were considered to be dead when all leaves had abscised or the leaves were completely dry and 2) Mortality (root system), a broader definition, (easily identified in a hydroponic setup where roots are readily accessed and white and pink living roots contrast strongly with dead blackened roots) where root systems were categorized as dead when no visible living root tissue remained regardless of the presence or absence of living leaves.

The root collar diameter and heights of all seedlings from the root collar to the shoot tip were measured at the beginning and at the end of the experiment (after 6 weeks). The relative root collar diameter growth and the relative shoot height growth were calculated by dividing the difference between the initial measurements and final measurements by the initial measurements.

Fresh weights were determined for all living plants at the end of the experiment. Roots and stems were harvested and dried in an oven at 70 °C for 72 h, whereas leaves were freeze-dried for 72 h to avoid the heat induced breakdown of chlorophyll. Shoot dry weights were determined by adding the dry weights of leaves and stems.

3.3.4 Foliar chlorophyll, total nitrogen, and elemental concentrations

Leaf chlorophyll-a and chlorophyll-b concentrations were determined at the end of the experiment in six randomly selected seedlings per treatment (less if fewer than six seedlings survived the treatment). The uppermost living leaves were collected, freeze-dried, and pulverized with a Thomas Wiley Mini-Mill (Thomas Scientific, NJ, USA). Chlorophyll was extracted from the pulverized leaf samples (10 mg dry weight) with 8 ml dimethyl sulfoxide (DMSO) at 65°C for 22 h. After filtering, chlorophyll concentrations were measured in DMSO extracts with a spectrophotometer (Genesys 10S-UV-VIS, Thomas Scientific, NJ, USA) at 648 and 665 nm. The total chlorophyll concentration was then calculated using DMSO specific chlorophyll equations (Wellburn, 1994).

For foliar elemental analysis (Na, Mg, P, K, Ca, Fe, Mn, Cu, Zn, B), pulverized leaf samples of fully expanded uppermost leaves (0.2 g dry weight) were digested using HNO_3. The extracts were then filtered and analyzed at the Natural Resources Analytical Laboratory (NRAL) of the University of Alberta using a Thermo iCAP6300 Duo inductively coupled plasma-optical emission spectrometer (ICP-OES; Thermo Fisher, Cambridge, United Kingdom)(Hanlan *et al.*, 1973). At the same facility, total nitrogen concentration was determined following dry combustion using a Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher, Bremen, Germany)(EPA, 2002).

3.3.5 Relative electrolyte leakage ratio

Two-cm diameter leaf disks were collected from 6 plants per species per treatment using a cork borer and immediately immersed in collection tubes containing 10 ml milli-Q water. An initial EC reading (EC_0) was immediately taken and samples were set aside to incubate at room temperature for one hour, after which EC was measured once again (EC_1). The collection tubes were then immersed in a boiling water bath for 20 min and a final EC_{total} measurement was taken when the samples had returned to room temperature.

The relative electrolyte leakage ratio was then calculated as follows:

$$EC_{Ratio} = \frac{(EC_1 - EC_0)}{(EC_{total} - EC_0)} * 100$$

3.3.6 Net photosynthesis and transpiration rates

Net photosynthesis (A) and transpiration I rates were measured between 9:00 and 12:00 h at the end of the experiment (n=6 per treatment) with an infrared gas analyzer (LI-6400XT, LI-COR). The reference CO2 concentration was set to 400 µmol mol^{-1} and the flow rate was 200 µmol s^{-1} in the leaf chamber. The leaf chamber temperature was kept at 20 °C, and PPFD was set to 400 μmol m^{-2} s^{-1}. After ensuring the F stability indicator was no longer fluctuating, 3 measurements were recorded per leaf.

3.3.7 Data analysis

To compare all treatments, linear mixed-effects models were constructed as follows with the lme4 package (Bates *et al.*, 2015) using the R programming language and environment (Core Development Team, 2020) : Response Variable ~ Treatment + (1|Container). When necessary, data were normalized using box-cox transformations. Mortality count data were transformed using the log(x+1) transformation. Model fit was assessed graphically using boxplots and by selecting for maximum likelihood by comparing model AIC, BIC, and dispersion. Estimated means were obtained using the emmeans package and p-values of pairwise comparisons were then obtained using the Tukey method (Kuznetsova *et al.*, 2017; Lenth *et al.*, 2019). Except for mortality rate, where container means and standard errors were used, the estimated means and standard errors of treatments were used to graph figures in SigmaPlot 14 (Systat Software Inc., San Jose, California). Finally, a principal component analysis of the foliar element concentration results was performed and a multiple factor analysis with hierarchical clustering of treatments using the estimated means obtained from all previously completed analyses was completed using the FactoMineR shiny, FactoInvestigate, and Factoshiny R packages (Lê *et al.*, 2008; Vaissie *et al.*, 2015; Wickham, 2016; Chang *et al.*, 2021).

3.4 **Results**

3.4.1 Foliar elemental and nitrogen concentrations

After two months of growth in the treatment solutions, foliar Na concentrations were significantly higher in the Reconstituted CT treatment plants compared to those growing in the control solution, the control with Naphs added, and the Reconstituted NST solution without Naphs (Table 3).

Potassium was found in significantly higher concentrations in the leaves of trembling aspen growing in the control solution with Na added, the Reconstituted NST without Naphs, and NST water, when compared to Reconstituted NST and the control solution with Naphs added (Table 3.2).

Magnesium foliar concentrations were significantly higher in the Reconstituted NST without Na, than plants growing in the control, Reconstituted CT water, Reconstituted NST without Naphs added, and NST water treatments (Table 3.2).

Zinc concentrations were significantly higher in the leaves of aspen growing in the Reconstituted CT water treatment than for those growing in the control, control with Na added, the control with Naphs, Reconstituted NST without Naphs, and NST water (Table 3.2). Iron foliar elemental concentrations were higher for the control, Reconstituted NST, and Reconstituted NST without Na, than the control with Na added (Table 3.2).

Phosphate foliar concentrations were significantly lower in individuals growing in the NST water treatment than all other treatments except for the Control, Reconstituted Ct, and the Control with Naphs added (Table 3.2).

Manganese foliar concentrations were significantly higher for individuals growing in Reconstituted CT than the control with Na added and NST water treatment, and were significantly lower in the Reconstituted NST without Naphs than all other treatments. (Table 3.2).

Copper foliar concentrations were significantly higher for individuals growing in Reconstituted NST than the control with Na added and NST water (Table 3.2).

Boron concentrations were significantly highest in the NST treatment when compared with all other treatments. Ca, S, and nitrogen foliar concentrations did not significantly differ between treatments (Table 3.2).

The first and second dimensions of the principal component analysis (PCA) of foliar elemental concentrations accounted for 31 % and 19 % of total variation respectively (Fig. 3.S1,). NST, Reconstituted NST and Reconstituted NST without Na were the primary treatments driving the variation in the first dimension of the PCA, whereas Reconstituted CT and reconstituted NST without Naphs drove the second dimension (Fig. 3.S1). Mn, Ca, Zn, and Na were positively correlated with each other and negatively correlated with K foliar concentrations (Fig. 3.S1). Fe, Cu, P and N were strongly positively correlated with each other as were Mg and S, and all were negatively correlated with foliar B concentrations. (Fig. S3.1).

3.4.2 Mortality rate

Mortality rate (complete) was significantly higher in the Reconstituted NST treatment followed by the control with Naphs, Reconstituted NST w/o Na, and Reconstituted CT. The effects of Na and Naphs on mortality were significant ($P < 0.001$ and $P < 0.01$ respectively). (Fig 3.1)

When taking the mortality of the root system into account, the rate was significantly higher in the Reconstituted NST treatment, followed by Reconstituted NST w/o Na, and control with Naphs. There was no mortality in the Control, Control with Na, and Reconstituted NST without Naphs treatments (Fig 3.1).

3.4.3 Relative growth

Relative root collar diameter growth was significantly lower for plants growing in Reconstituted CT, Reconstituted NST without Na and the control treatment with Naphs added when compared to the control treatment grown aspen seedlings (Fig. 3.2 A). Relative root collar diameter growth was also higher in the Reconstituted NST treatment without Naphs than the Reconstituted NST without Na and the control treatment with Naphs added (Fig. 3.2 A).

Relative shoot height growth followed a similar pattern with shorter height growth after 6 weeks for individuals growing in Reconstituted NST, Reconstituted CT, Reconstituted NST without Na, and the control solution with Naphs added than for those growing in both the control solution and the Reconstituted NST solution without Naphs added (Fig. 3.2 B).

3.4.4 Photosynthesis and transpiration

Both net photosynthesis and transpiration rates were lowest in the NST treatment plants (Fig 3.3). Photosynthesis was significantly higher for plants in the control with Naphs added treatment than the control, Reconstituted CT, Reconstituted NST without Na, Reconstituted NST without Naphs added, and NST water (Fig. 3.3 A). Photosynthesis in the Reconstituted NST treatment was also higher than for individuals in the Reconstituted CT, Reconstituted NST without Naphs, and the NST water treatment (Fig. 3.3 A). Transpiration rates were only significantly higher for plants growing in the control solution with Na added compared to those growing in the NST treatment (Fig. 3.3 B).

3.4.5 Dry weight and root-to-shoot ratio

After 6 weeks, trembling aspen seedling dry weights were significantly higher in the control solution treatment compared to all treatments but the Reconstituted NST without Naphs solution

(Fig. 3.4 A). Seedlings growing in Reconstituted NST without Naphs solution had significantly higher dry weights than Reconstituted CT, Reconstituted NST without Na, and the control solution with Naphs added (Fig. 3.4 A).

Trembling aspen seedlings growing in Reconstituted CT had a significantly larger root to shoot ratio than seedlings growing in control, control with Na added, and Reconstituted NST without Naphs added solutions (Fig. 3.4 B). There were no significant differences for aspen seedlings growing in Reconstituted NST, Reconstituted NST without Na, control with Naphs added, and NST water solutions (Fig. 3.4 B).

3.4.6 Relative foliar electrolyte leakage

Foliar electrolyte leakage was significantly higher for plants growing in the Reconstituted CT treatment solution when compared to those growing in the control, Reconstituted NST without Na, Reconstituted NST without Naphs, and control with Naphs (Fig. 5). Plants growing in Reconstituted NST, control with Na added, and NST water treatments were not significantly different from other treatments (Fig. 3.5).

3.4.7 Chlorophyll

Foliar chlorophyll concentrations were significantly greater for trembling aspen seedlings growing in the control and Reconstituted NST solutions than the control with Na added treatment (Fig. 3.6). In addition, seedlings in the Reconstituted NST treatment also had greater chlorophyll concentrations than those growing in Reconstituted CT (Fig. 3.6).

3.4.8 Multiple Factor Analysis

The first and second dimensions of the multiple factor analysis explained 47 % and 19.26 % of the total variation respectively for all the parameters measured (Fig. 3.7). The first dimension was primarily driven by growth, mortality rates, the concentration of Naphs in solution, and foliar potassium concentrations, whereas the second dimension was mainly driven by transpiration rates, the concentration of Na in solution, foliar electrolyte leakage and foliar elemental concentrations of B and Na (Fig. 3.7). Relative growths, dry weight, and tissue water content were strongly negatively correlated with the concentration of Naphs in solution, and positively correlated with greater mortality rates and root to shoot ratios (Fig. 3.7). The concentration of Na in solution and Boron in the leaves was strongly negatively correlated with transpiration, and negatively correlated with net photosynthesis (Fig. 3.7). Foliar electrolyte leakage was positively correlated with foliar concentrations of Na only. Transpiration and photosynthesis were correlated with foliar concentrations of chlorophyll, nitrogen, phosphate, and iron. Mortality rates were correlated with foliar concentrations of Cu, Ca, and Mg, but negatively correlated with foliar potassium concentrations (Fig. 3.7).

3.5 **Discussion**

Although Na present in treatment solutions was a significant stress factor, which was reflected in the physiological responses of plants, contrary to my hypothesis, Naphs led to greater mortality compared with Na. Indeed, reconstituted tailings treatments containing Naphs resulted in significant mortality likely brought on by the initial stages of treatment prior to adjusting the pH of treatment solution to 8.4, (48 h after the start of treatments) when leaf wilting was observed. In effect, this early stress preceded by mortality in the next two to three weeks, explains how seedling relative growth, dry weight, and tissue water content were strongly negatively correlated with the concentration of Naphs in solution throughout the experiment (Fig. 3.7). In contrast with the effects at the higher adjusted pH of 8.4 (the pH of pure tailings water), it appears that Naphs acted as a stronger surfactant at the initial pH of 5.5. Although the lower pH itself was likely less stressful to the seedlings, since Naphs are less soluble at a low pH (Huang *et al.*, 2016) and concentrate at aqueous-non aqueous interfaces (Facanali et al. 2021), this may have enabled them to coat root surfaces.

While the analysis of trembling aspen xylem root exudate has previously shown that they can take up and translocate Naphs, discussion has also centered on their inhibitory effects on root hydraulic conductivity(Kamaluddin & Zwiazek, 2002b). Although the exact mechanisms of Naphs interference with water transport processes is largely unknown, it is plausible that Naphs block aquaporin pores, or indirectly affect cell-to-cell water transport by disrupting cell membrane integrity (Van Overbeek & Blondeau, 1954),. To compound this, at a pH of 5.5, Naphs may also have been present in their more phytotoxic, non-ionized forms (Armstrong *et al.*, 2009). Nevertheless, as the root surface microenvironment is typically acidic (Rudolph-Mohr *et al.*, 2014) these initial effects may provide a better reflection of growth responses under field conditions, particularly when considering that strong solution circulation to maintain elevated root oxygen levels in the hydroponic setup made the development of root microenvironments unlikely. Given the potential role in phytoremediation for plants to translocate specific types of Naphs that are inefficiently degraded by microorganisms (Alberts *et al.*, 2021), it may be worthwhile to further investigate the relationship between different Naphs, their pH specific conformations, and root hydraulic conductivity.

Plants that survived the addition of Naphs to treatment solution had initially visually damaged roots but a subsequent combination of new root growth with reduced shoot growth led to a strong positive correlation between solution Naphs and root to shoot ratios. This initial morphological shift had consequences throughout the remainder of the experiment where the physiological effect of Na was more pronounced than that of the Naphs. Solution concentrations of Na were not only negatively correlated with photosynthesis, transpiration but also with chlorophyll. This may be explained by the limited phosphorus levels and foliar nitrogen present in the leaves of seedlings growing in treatments with elevated Na and thus likely lower Rubisco concentrations (Luo *et al.*, 2021).

It was previously reported that, when growing in saline tailings solutions, trembling aspen appears to be able to utilize K- and Ca-dependent sodium exclusion or sequestration mechanisms (Fleurial et al. 2022), similar to what has been described for other poplar species (Chen & Polle, 2009). Results of the present study confirm these findings and highlight the importance of trembling aspen's ability to impose K-Na uptake site selectivity (Chen *et al.*, 2014) as foliar Na and K levels were inversely correlated with each other (Fig 3.S1). Also, K levels were highest in treatments with elevated Na levels undisrupted by an initial addition of Naphs at pH 5.5 (i.e., Control with Na, Reconstituted NST without Naphs, and NST water) (Table 3). This suggests that Naphs at a low pH may inhibit trembling aspen Na resistance. At the same time, foliar Ca concentrations were inversely correlated with K and positively correlated with foliar Na (Fig. 7) strengthening the notion of the importance of Ca in the maintenance of selective membrane permeability in response to Na stress, as previously reported (Hanson, 1984; Tozlu *et al.*, 2000; Fleurial *et al.*, 2022b).

As foliar electrolyte leakage was only positively correlated with foliar concentrations of Na in part this is likely explained by compared to other ions, sodium has been reported to leak out of tissues nearly completely during incubation in deionized water for electrolyte leakage measurements. (Bajji *et al.*, 2002). Finally, I also observed that the concentration of Na in solution and B in the leaves was negatively correlated with transpiration rates, net photosynthesis, and foliar chlorophyll concentrations (Fig. 8). While these were independent of growth of mortality in this 6 week ex-situ experiment with seedlings, they could have long term consequences in situ. Furthermore, this appears to confirm both the effectiveness of the sodium exclusion mechanism and the apparent lack of control over boron uptake that was also previously reported for trembling aspen exposed to NST water (Fleurial *et al.*, 2022b) and may support the notion that boron in tailings can have a limited effect on its own but aggravates the injurious effects of NaCl leading to strong declines in gas exchange rates (Apostol *et al.*, 2002).

3.6 **Conclusion**

This study's results corroborate an inference from Ch 2; that trembling aspen Na tolerance mechanisms at the root level appear to involve the maintenance of membrane selectivity of K over Na and are also a prerequisite for survival and growth. Naphs in all synthetic treatment solutions caused high mortality and was associated with low leaf K and high leaf Na. This indicates that the K selectivity in roots may be compromised by Naphs. Low leaf K was associated with low growth and high mortality. The only exception was the in-situ collected NST water, which always had a high pH (due to an experimental design artifact), and likely lower Naphs viscosity. Here, high leaf K levels were maintained and there was no plant mortality, low root mortality and decent growth. This accidental finding could indicate that lowering tailings pH to more "beneficial" values could have unintended consequences in the presence of Naphs.

This is of particular importance given the strong evidence that various plant species such as trembling aspen, sandbar willow, or slender wheatgrass can take up and incorporate different Naphs into their tissues thus making them important for the phytoremediation of oil sands affected soils (Kamaluddin & Zwiazek, 2002b; Alberts *et al.*, 2021). However, given that past reclamation trials using only bioremediation techniques have often reported limited success in reducing Naphs to levels below 19 mg/L (Quagraine *et al.*, 2007), further investigation of pre-reclamation treatments that would partly degrade or remove Naphs would be beneficial. Ozone treatment to degrade Naphs is one such promising avenue as it produces little waste and is effective at alkaline conditions.(Brown & Ulrich, 2015). Nevertheless, since Naphs are less soluble and more easily extracted at a lower pH (Huang *et al.*, 2016), further research into the filtration of Naphs using porous materials (Mohamed *et al.*, 2008) after a neutralizing of the pH could also improve revegetation success of the oil sands sites affected by the mine tailings.

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3.8 Figures and Tables

Table 3.1: Target elemental and naphthenic acid (Naph) concentration additions for each treatment solution (mg/L). Gray indicates base 50% Hoagland's solution concentrations and no experimental modifications.

	Na	K	Mg	Ca	F	Cl	P	Fe	S	B	Naph
Control	None	117.5	**12**	80	None	0.885	31	0.56	16	0.135	None
Reconstituted NST*	+1095.7	+34.0	+7.3	+5.8	+2.6		+0.1	+1.1	+34.9	+7.5	+133.0
Reconstituted CT**	+1127	+22.5	+28.3	+55.8	+1.8	+702.5	+1.5		+1346.7	+3.6	+53.0
Reconstituted NST – Na	None	+34.0	+7.3	+5.8	+2.6		+0.1	+1.1	+34.9	+7.5	+133.0
Control + Na	+1095.7	117.5	**12**	80	None	0.885	31	0.56	16	0.135	None
Reconstituted NST – Naph	+1095.7	+34.0	+7.3	+5.8	+2.6		+0.1	+1.1	+34.9	+7.5	None
Control + Naph	None	117.5	**12**	80	None	0.885	31	0.56	16	0.135	+133.0
NST***	+50% pure NST water										

*Reconstituted NST water was designed to match the composition of NST water (Fleurial *et al.*, 2022b).
**Reconstituted CT water was designed using averages established from selected publications (Renault et al., 1999; Redfield et al., 2003; Redfield et al., 2004).
*** This has the same concentrations of elements as *, but other elements and compounds as well from in-situ collection.

Table 3.2: Foliar element concentrations (mg kg^{-1} and g 100 g^{-1} for N) in trembling aspen after 6 weeks of treatments ($n = 6$). Means and standard errors are shown. Different letters indicate significant post hoc Tukey test differences between treatments ($P < 0.05$) with cells shaded using a Red-Yellow-Blue diverging color scale to highlight differences from highest (red) to lowest (blue) values. Colors also distinguish letter groups.

	Na	K	Mg	Ca	Zn	Fe	P	Mn	S	Cu	B	N
Control	17.1 ± 3.2 bc	23473 ± 1919 abc	1443 ± 203 b	9866 ± 1135 a	32.3 ± 3.91 d	42.6 ± 3.48 ab	2234 ± 249 ab	47.0 ± 5.31 ab	2623 ± 238 a	5.08 ± 0.899 ab	32.8 ± 2.68 cd	2.73 ± 0.172 a
Reconstituted NST	249.5 ± 118 ab	12359 ± 3323 bc	2106 ± 315 ab	9987 ± 1755 a	92.3 ± 27.82 ab	50.2 ± 4.63 a	3115 ± 490 a	25.3 ± 4.95 ab	2819 ± 361 a	9.95 ± 1.299 a	44.6 ± 6.06 bc	3.50 ± 0.474 a
Reconstituted CT	958.1 ± 427 a	17047 ± 1919 abc	1909 ± 203 b	11272 ± 1135 a	148.6 ± 30.53 a	27.3 ± 3.35 bc	2297 ± 256 ab	49.1 ± 5.55 a	2512 ± 238 a	4.83 ± 0.899 ab	53.1 ± 4.34 b	2.45 ± 0.140 a
Reconstituted NST – Na	25.2 ± 2.3 abc	24289 ± 1799 ab	2927 ± 196 a	11429 ± 1095 a	68.2 ± 10.03 abc	43.8 ± 3.3 ab	3081 ± 337 a	40.1 ± 4.25 ab	2346 ± 231 a	6.34 ± 0.878 ab	43.2 ± 3.36 bc	2.68 ± 0.174 a
Control + Na	51.7 ± 14.2 abc	26216 ± 1919 a	2082 ± 203 ab	10603 ± 1135 a	36.5 ± 4.60 bcd	24.1 ± 3.35 c	3047 ± 340 a	27.0 ± 3.05 b	2149 ± 238 a	4.14 ± 0.899 b	40.8 ± 3.34 bc	2.83 ± 0.185 a
Reconstituted NST – Naph	45.9 ± 33.6 bc	26047 ± 1919 a	1687 ± 203 b	6899 ± 1135 a	18.5 ± 1.84 e	29.3 ± 3.35 bc	2762 ± 308 a	10.8 ± 1.22 c	1854 ± 238 a	4.52 ± 1.060 ab	55.7 ± 4.56 b	2.88 ± 0.201 a
Control + Naph	14.5 ± 2.6 c	14194 ± 1799 c	1936 ± 196 ab	9927 ± 1095 a	35.4 ± 4.15 cd	29.7 ± 3.3 bc	2115 ± 242 ab	32.4 ± 3.43 ab	2202 ± 231 a	4.80 ± 0.878 ab	26.0 ± 2.02 d	2.68± 0.166 a
NST	87.1 ± 65.6 abc	25847 ± 1919 a	1598 ± 203 b	8353 ± 1135 a	33.1 ± 4.03 d	28.1 ± 3.35 bc	1563 ± 174 b	27.9 ± 3.16 b	1913 ± 238 a	3.94 ± 0.899 b	142.1 ± 12.73 a	2.47 ± 0.148 a

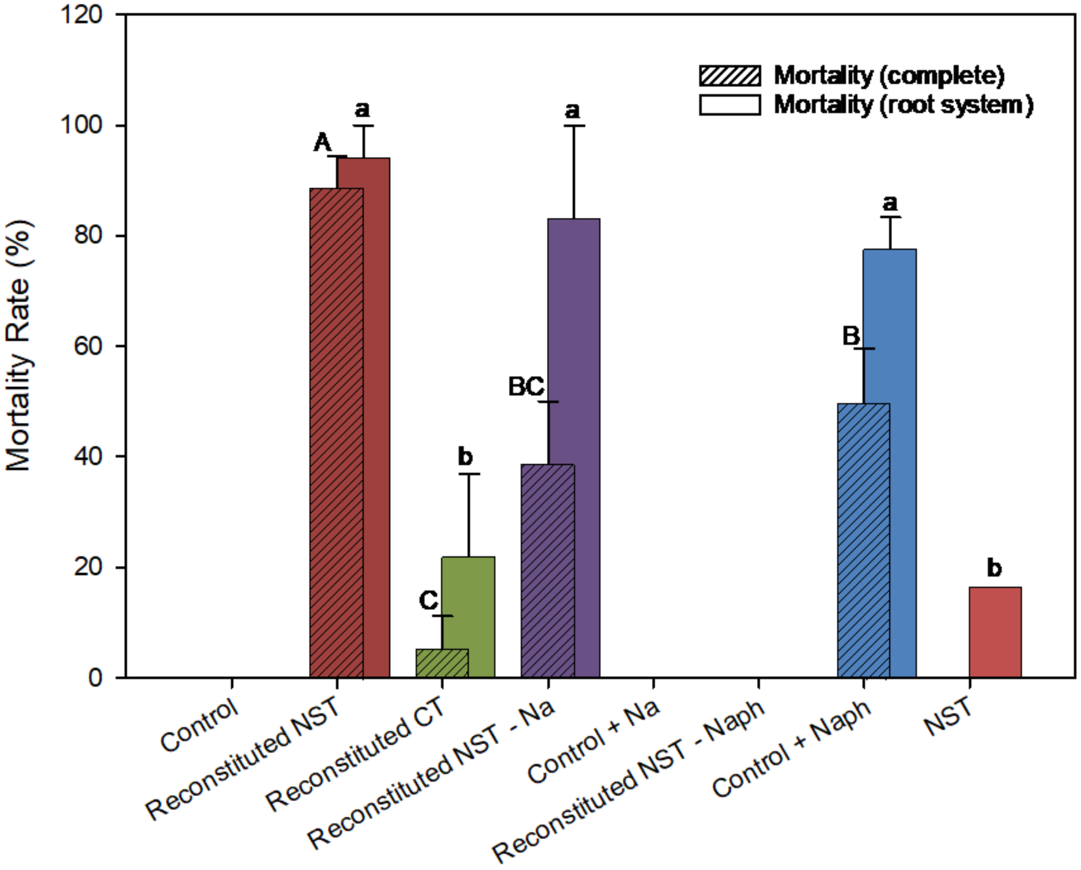

Figure 3.1: Average mortality rate per container of aspen seedlings (%) at the end of the experiment determined with no visible living tissue remaining (Mortality complete) and including plants with some above ground living tissue remaining but no visible living roots (Mortality root system). Standard error bars are shown (6 seedlings per container, n = 3 containers per treatment. Different letters indicate significant differences between treatments for mortality of the entire plant or the root system exclusively obtained using Tukey' tests (Capitalized and lowercase letters respectively; $P < 0.05$).

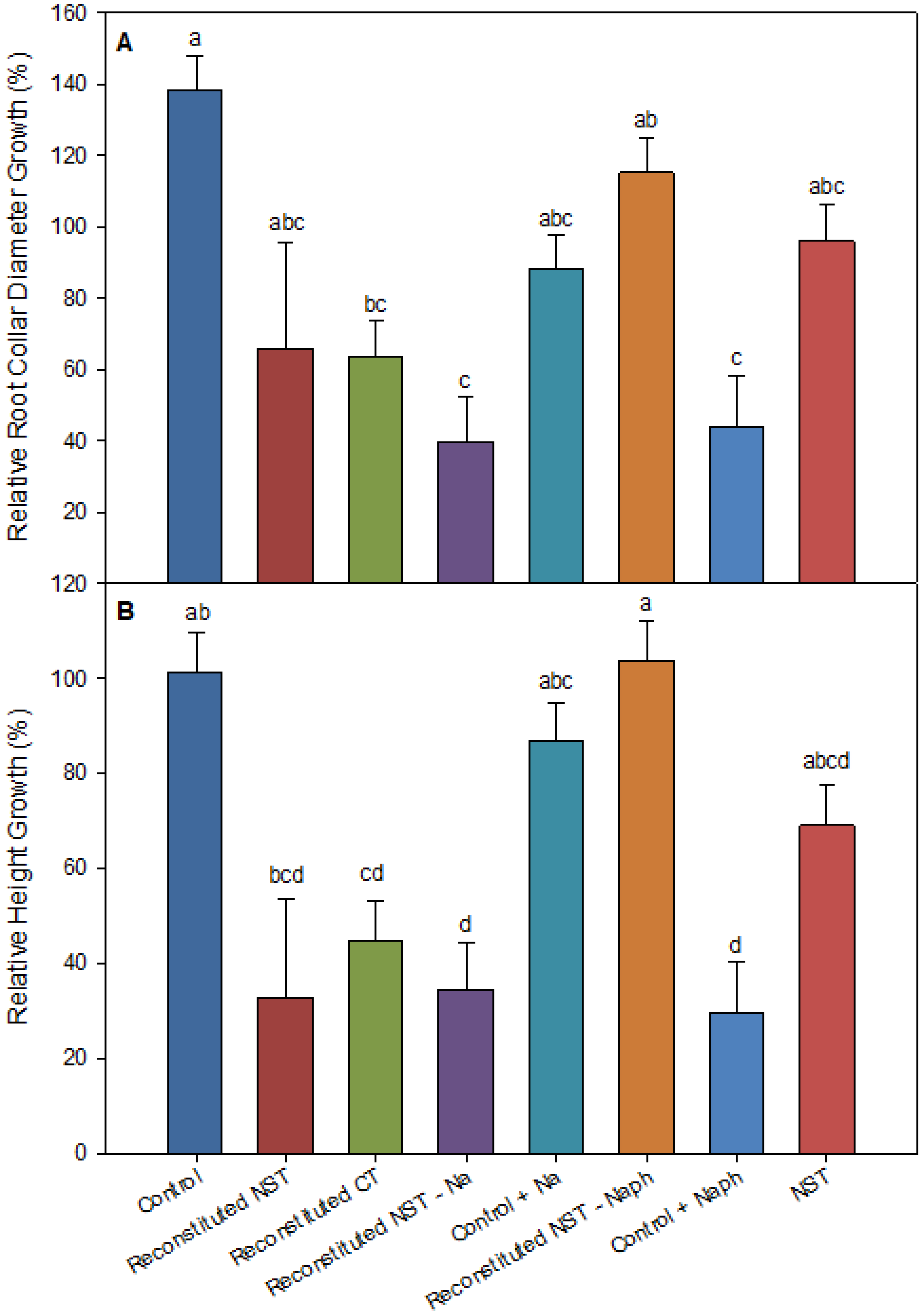

Figure 3.2: Relative root collar diameter growth (A), and relative height growth (B) of trembling aspen seedlings after 6 weeks of treatments (n=18). Estimated means and standard error bars are shown; different letters indicate significant differences between treatments obtained using Tukey tests ($P < 0.05$).

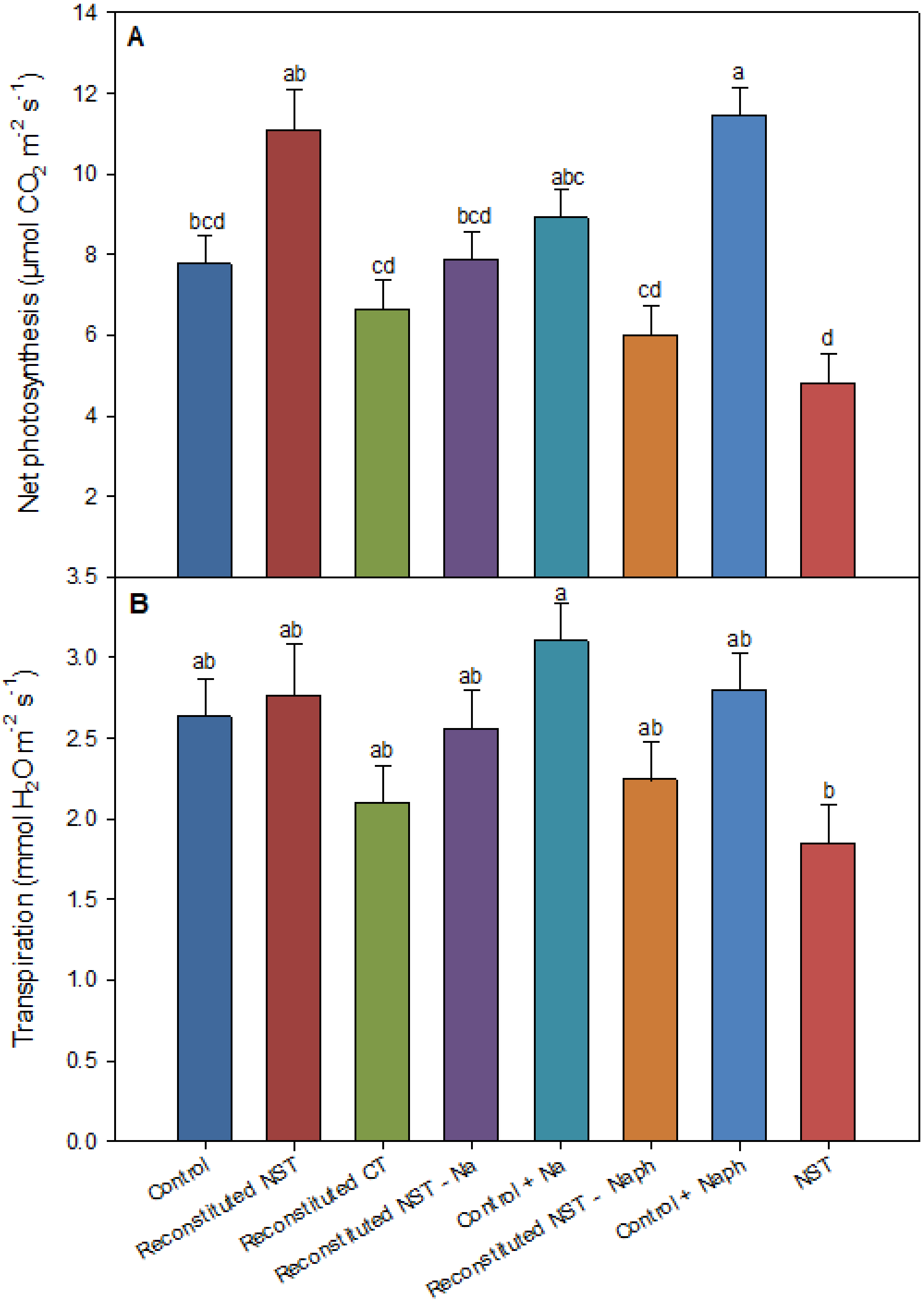

Figure 3.3: Net photosynthesis (A) and transpiration (B) rates of trembling aspen seedlings after 6 weeks of treatments (n=6). Estimated means and standard error bars are shown; different letters indicate significant differences between treatments obtained using Tukey tests ($P < 0.05$).

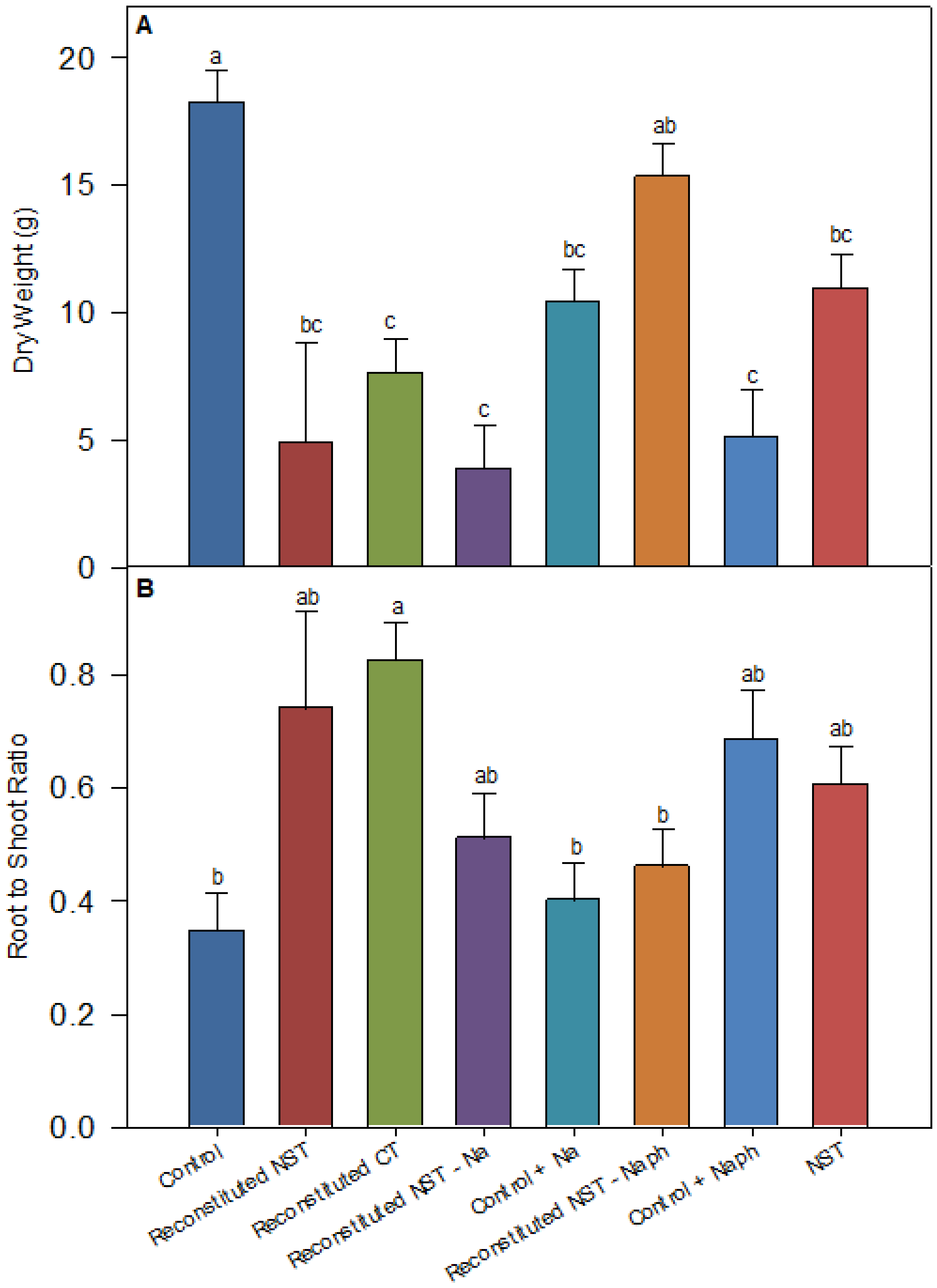

Figure 3.4: Dry weight (A) and root to shoot ratio (B) of trembling aspen seedlings after 6 weeks of treatment (n=18). Estimated means and standard error bars are shown; different letters indicate significant differences between treatments obtained using the Tukey tests ($P < 0.05$).

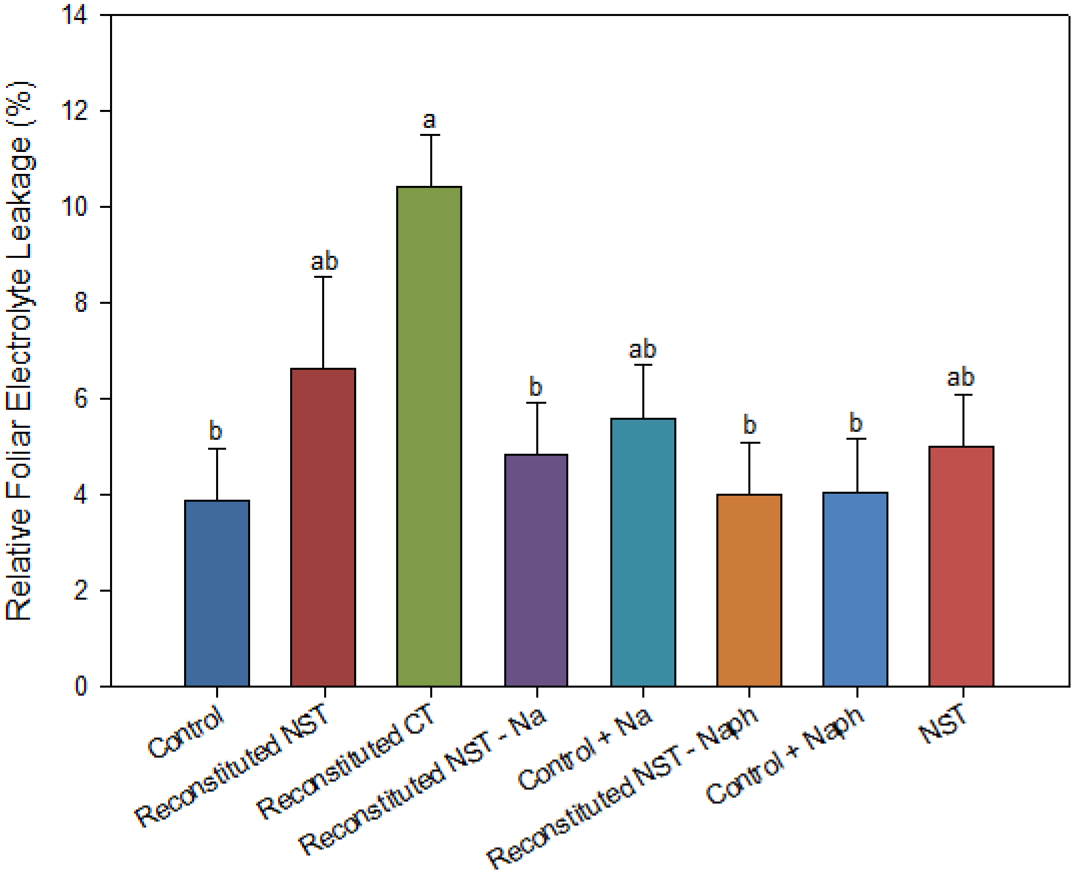

Figure 3.5: Relative foliar electrolyte leakage (%) of 2 cm discs incubated over a 1-hour incubation at the end of the experiment (n=6). Estimated means and standard error bars are shown; different letters indicate significant differences between treatments obtained using the Tukey tests ($P < 0.05$).

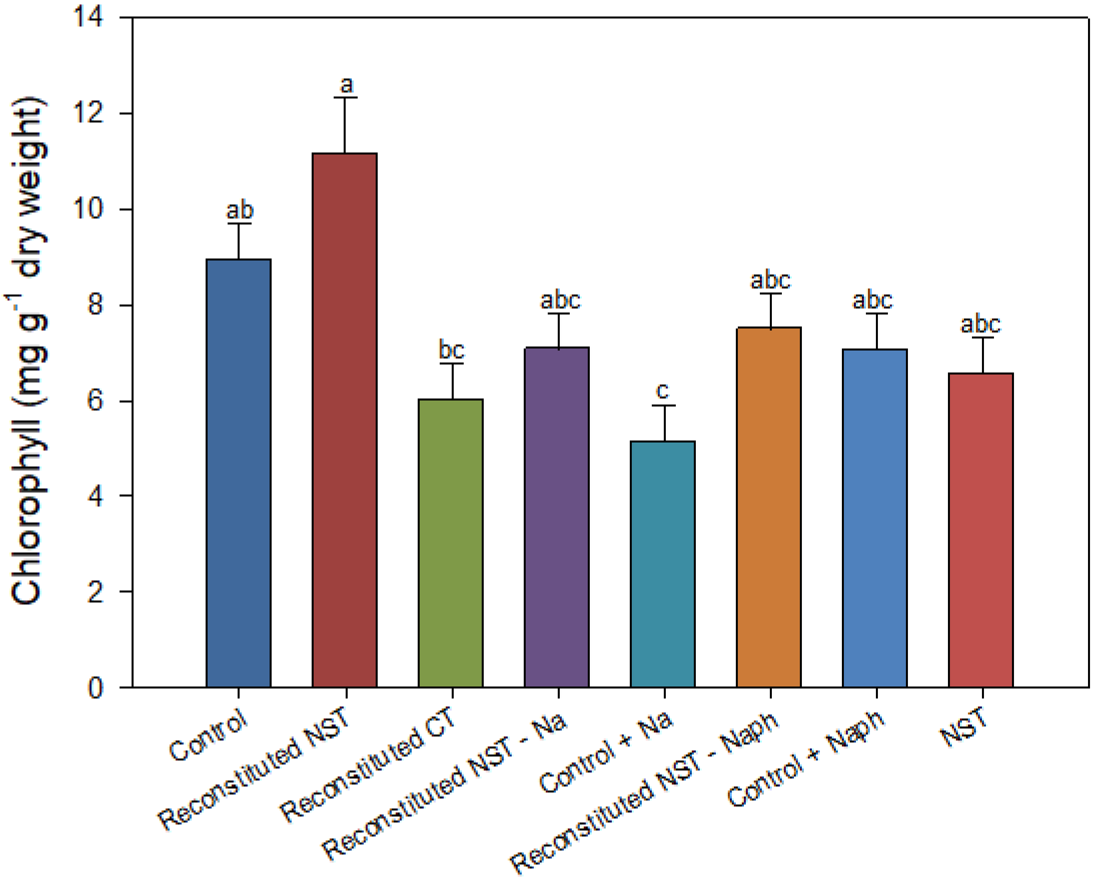

Figure 3.6: trembling aspen seedling foliar chlorophyll (n=6) extracted after 6 weeks of treatment in mg g^{-1} of dry weight. Estimated means and standard error bars are shown; different letters indicate significant differences between treatments obtained using Tukey tests ($P < 0.05$).

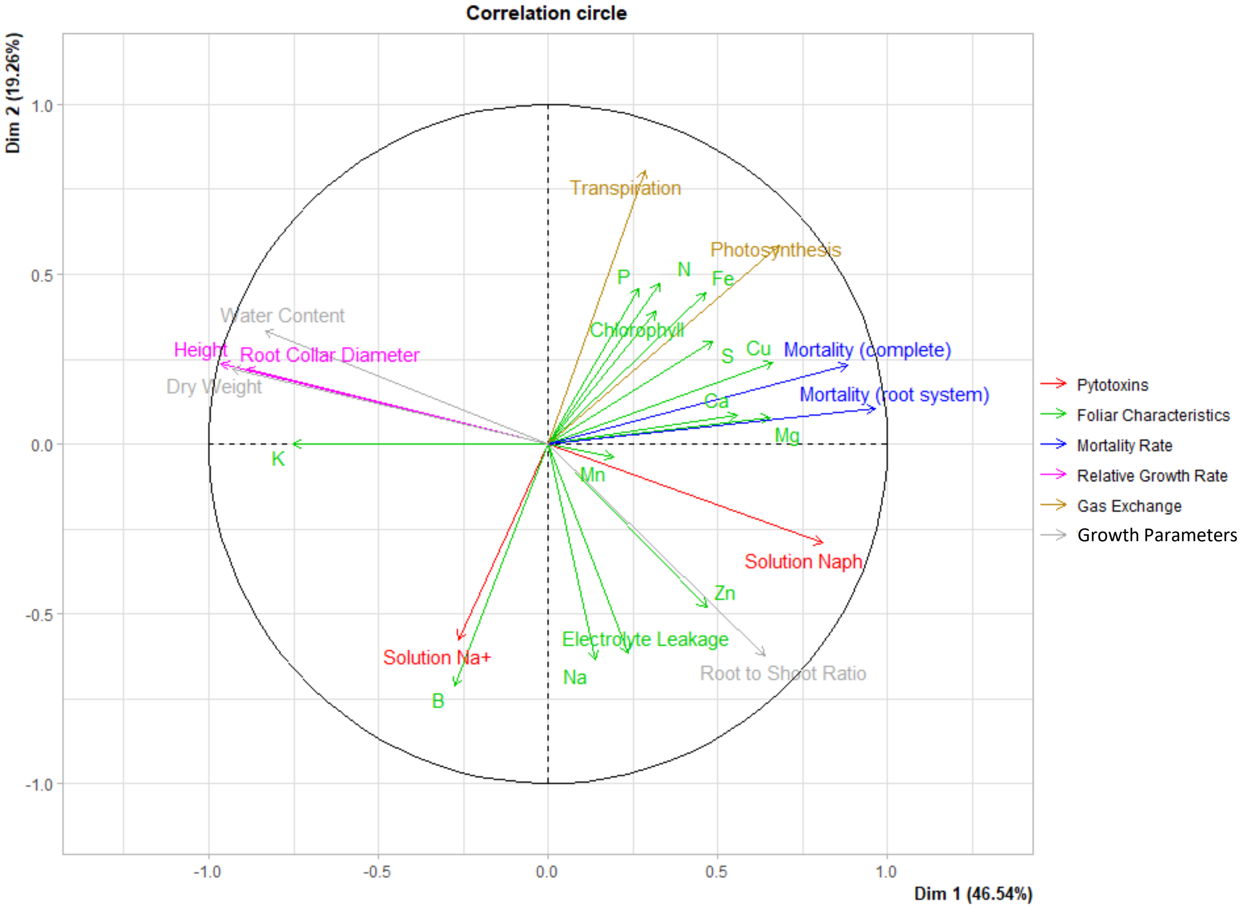

Figure 3.7:Correlation circle of multiple factor analysis variables measured in trembling aspen seedlings after 6 weeks of growth in 8 different treatment solutions. Factor groups include phytotoxins (the concentration of Na or naphthenic acids in treatment solution), foliar characteristics (elemental concentrations of Na, Mg, P, K, Ca, Fe, Mn, Cu, Zn, B, and total N, electrolyte leakage, and chlorophyll concentrations), mortality rate (complete or root system only), relative growth rates (shoot height, root collar growth), gas exchange (transpiration, net photosynthesis), and growth parameters (dry weight, root to shoot ratio, and water content).

3.9 **Supplementary Information**

3.9.1 Tables

Table 3.S1: Actual concentrations of salts added in g/100 L (ml/100 L of 5M KOH for pH adjustment) to 50% Hoagland’s solution to prepare different treatment solutions.

	NaCl	5M KOH	Mg SO_4	$CaSO_4$	NaF	NaH_2PO_4	FeEDTA	Na_2SO_4	H_2BO_3	$C_{10}H_{17}NaO_2$ (Naph)
Control	None	None	None	None	None	None	None	None	None	None
Reconstituted NST	177.106	12.109	3.608	1.959	0.575	0.020	0.656	None	0.754	15.107
Reconstituted CT	137.996	8.021	13.999	18.955	0.391	0.246	None	162.831	0.360	6.020
Reconstituted NST – Na	None	12.109	3.608	1.959	0.575	0.020	0.656	None	0.754	15.107
Control w/ Na	180.608	None	None	None	None	None	None	None	None	None
Reconstituted NST – Naph	180.084	12.109	3.608	1.959	0.575	0.020	0.657	None	0.754	None
Control + Naph	None	None	None	None	None	None	None	None	None	15.107
NST	None	None	None	None	None	None	None	None	None	None

3.9.2 Figures

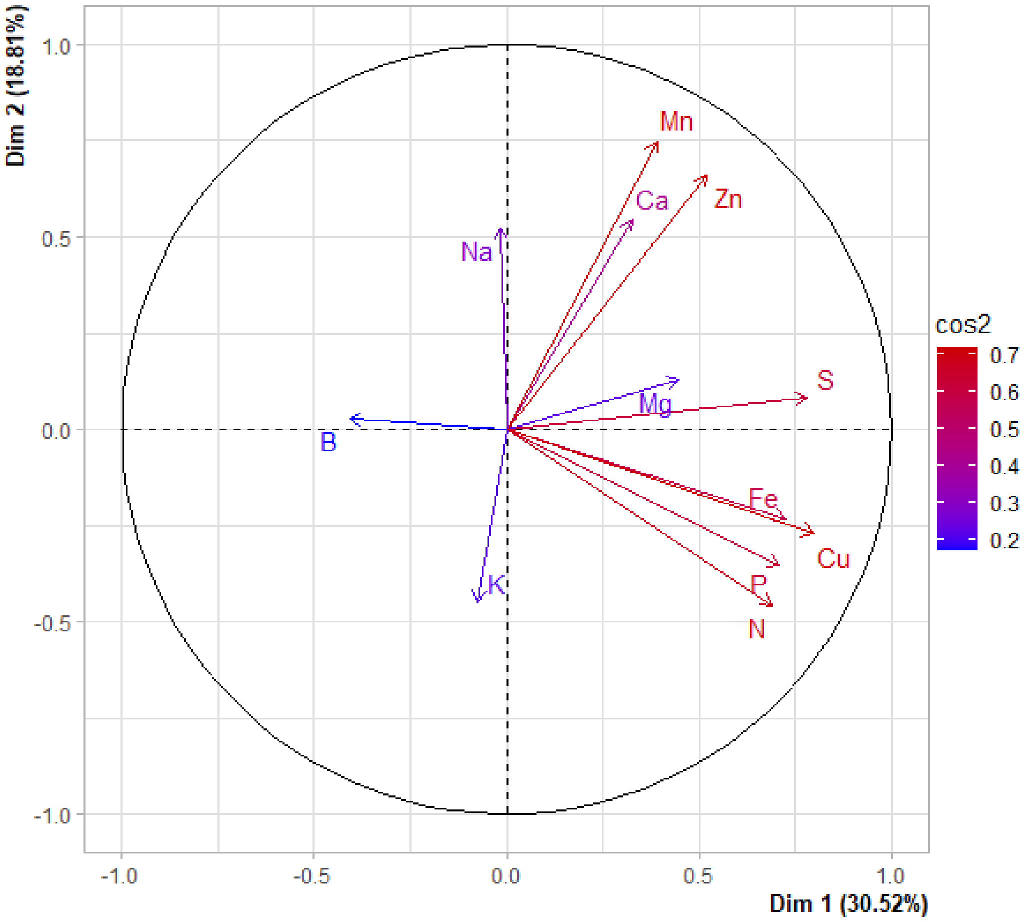

Figure 3.S1: Correlation circle of principal component analysis of trembling aspen foliar elements (Na, Mg, P, K, Ca, Fe, Mn, Cu, Zn, B, and N). The foliar elemental concentrations were determined after 6 weeks of growth in the 8 different treatment solutions (n=6).

4 Getting cold feet: tree productivity at the mercy of soil temperature.

4.1 Summary

Tree root hydraulic conductivity is reduced at low non-negative soil temperatures even when water is readily available and air temperatures are warm. Unfortunately, global plant and forest models generally rely on ambient air temperature, even when soil temperatures are lower than recorded air temperatures during the growing season from April to August in the boreal and temperate zones. As this likely leads to large inaccuracies, in this commentary the topic of low soil temperature effects on growth is discussed for greater visibility and awareness. Though water is more viscous at lower temperatures much of the change in hydraulic conductivity in roots appears to be tied to changes in membrane water permeability. In addition, while low root temperatures also reduce phloem transport, carbon transport does not appear to be as limiting a factor for tree growth as hydraulic conductivity. Nevertheless, to alleviate the effects of low soil temperature some trees may be able to take advantage of extensive mycorrhizal networks to increase water uptake surface area and root hydraulic conductivity, upregulate aquaporin transmembrane proteins to increase membrane permeability for water uptake or to release water from the phloem to resume transport flow, and make greater use of aboveground branch storage reserves. Under certain conditions, anisohydric behavior, though risky, might also allow trees to maintain growth at low soil temperatures.

4.2 Commentary

In his pioneering 1868 *Lehrbuch der Botanik* (*Textbook on Botany*) Julius Sachs, oft-considered to be the father of plant physiology (Kutschera & Niklas, 2018), noted that “the absorption of water through the roots is also confined to certain limits of temperature ... Tobacco plant and Gourd [sic] no longer absorb sufficient water to replace a small loss by evaporation in a moist soil of from 3 to 5 °C" (Sachs, 1868). At the time, Sachs also noted that these low temperatures are limiting to other processes in plants such as the growth of green tissue or the exchange of oxygen and carbon dioxide. With considerable hindsight, we now know that water uptake is inextricably linked to turgor pressure and thus essential for cell expansion (Lockhart, 1965). As such, turgor is a major limiting factor in tree growth and scaling up its effects on forest biomass production are key to carbon sink and climate modeling (Friedlingstein *et al.*, 2020; Cabon & Anderegg, 2022). Yet, global models still overwhelmingly rely on ambient air temperatures for their modeling even when soils show negative temperature offsets from recorded air temperatures from April to August in boreal and temperate zones, and nearly year round in the tropical forested regions of the globe (Lembrechts *et al.*, 2022). These differences are highly dependent on both anthropogenic land use and climate driven changes in ground cover(Lembrechts & Nijs, 2020). Clearly, more attention needs to be given to the effects of low soil temperatures on plant roots and how they may impact these tree productivity models and, thus, projected climate change simulations.

Reductions in root hydraulic conductivity occur in cold soils even when water is readily available and air temperatures are warm (Running & Reid, 1980; Wan *et al.*, 2001; Kamaluddin & Zwiazek, 2004). Although this effect has long been observed and reported, it can appear to run counter to the cohesion-tension paradigm under which plant hydraulics are most often viewed as a purely physical process with water pulled through a series of pipes along a water potential gradient stretching from the roots to the leaves (Dixon & Joly, 1895). Although this simplified physical concept considers increased water flow resistance due to higher water viscosity at lower temperatures, it ignores important biological factors. Indeed, as Wang and Hoch clearly show in their paper "Negative effects of low root temperatures on water and carbon relations in temperate tree seedlings assessed by dual isotopic labelling", water uptake is strongly reduced at 7°C, well above freezing, even in seedlings from cold tolerant species (*Alnus*, *Ulmus*, *Picea*, and *Pinus* species), with water plentily available, and warm air temperatures (24/18°C day night). Where does the increased resistance come from? If water viscosity is the sole factor, the calculations of parameters such as the diameters of tracheary elements, vessel perforation plates, bordered pits, etc., would let us rank the species according to their low soil temperature tolerance levels. In this case, one would logically expect that conifer trees, due to their inherently less conductive hydraulic architecture, would be found at the bottom of the list, but many years of observations and research have proven that this is not necessarily the case.

Though water is certainly slightly more viscous at positive temperatures nearing zero, its effect does not account for such large differences and much of the change in hydraulic conductivity in roots appears to be tied to changes in membrane water permeability that is mediated by aquaporins—small intrinsic membrane proteins that can act as water channels (Ionenko *et al.*, 2010; Maurel *et al.*, 2015). To illustrate this, in figleaf gourd (*Cucurbita ficifolia*) plants, when a cell pressure probe was used to gauge membrane permeability, low root temperature strongly reduced the hydraulic conductivity of root cortical cells and cotyledon midrib cells (Lee *et al.*, 2008). Underlining these results, when high irradiance levels were further used to induce elevated transpiration, the permeability of the cell membranes did not change and was imperfectly compensated by a higher increase of water transport over the apoplastic pathway (Lee et al. 2008). In a subsequent study, it was then shown that overexpressing aquaporins in *Arabidopsis* increased root water uptake in cold conditions (Lee *et al.*, 2012). The two key factors that can affect gating properties of water channels, and that can be affected by soil temperature, are phosphorylation of aquaporins and cytoplasmic pH. Many aquaporins must be phosphorylated to function as water channels (Maurel *et al.*, 1995) and the channels close in response to cytoplasm acidification (Tournaire-Roux *et al.*, 2003). Low root temperature reduces root respiration rates which, in consequence, could deplete the energy required for protein phosphorylation and the maintenance of a proton gradient (Wan & Zwiazek, 1999; Maurel *et al.*, 2015). Maintaining root hydraulic conductivity despite low soil temperatures could, therefore, be key for plants to sustain growth and productivity if these conditions are to persist over extended periods of time, though it may not prove sufficient if plants are unable to avoid the oxidative damage that accompanies chilling (Aroca *et al.*, 2005).

To further compound the reduction in water uptake, low soil temperatures impede root growth, even in tree species commonly distributed in cold climates (Landhäusser *et al.*, 2001; Alvarez-Uria & Körner, 2007), consequently reducing the root surface area that could be available for the uptake of water and nutrients. As such, the presence of an increased absorbing surface area brought about by certain mycorrhizal associations in roots could have a direct effect on water and nutrient uptake under low soil temperature conditions (Lehto & Zwiazek, 2011). In addition, mycorrhizae are widespread throughout boreal zones of the world and many are freezing resistant, persisting in the soil throughout the winter months. Some ericoid mycorrhizae may be able to maintain nutrient acquiring capacity at low soil temperatures through the secretion of cold active enzymes (Tibbett & Cairney, 2007). Indeed, root hydraulic conductance (K_r, the extrinsic capacity of the whole root system to transport water) was enhanced approximately three-fold at all root temperatures between 4°C and 20°C when the roots of American elm (*Ulmus americana*) seedlings were colonized by the mycorrhizal fungus *Hebeloma crustuliniforme* (Muhsin & Zwiazek, 2002). Furthermore, fungal colonization resulted in approximately the same values of K_r at 4°C as those measured in non-mycorrhizal seedlings at

20°C (Muhsin and Zwiazek 2002). Large increases in K_r compared with non-mycorrhizal control were also reported for other northern tree species including white spruce (*Picea glauca*) and trembling aspen (*Populus tremuloides*) (Landhäusser *et al.*, 2002). However, the most significant impact of mycorrhizal fungi on root water transport may not in fact be through their increase of root area of roots but rather through an enhancement of root hydraulic conductivity (L_{pr}, the intrinsic capacity of the roots to transport water that is independent of their size) (Muhsin & Zwiazek, 2002; Siemens & Zwiazek, 2008; Xu *et al.*, 2015). Closer examination of the factors responsible for the effect of mycorrhizal fungi on root hydraulic conductivity revealed that both plant roots (Marjanović *et al.*, 2005) and the associated mycorrhizal fungal aquaporins (Xu *et al.*, 2015) may be responsible for this effect. The fungus increases gene expression of root aquaporins (Marjanović et al. 2005), through a yet undetermined process, and this, in turn, enhances cell hydraulic conductivity (L_{pc}) in plant roots (Lee et al. 2010, Xu et al. 2015).

In parallel, low temperatures also affect phloem transport. As early as in 1919, Child and Bellamy reported a blockage of translocation in the phloem induced by chilling a small part of a stem, petiole, or runner to 3-6°C (Child & Bellamy, 1919). Wang and Hoch reported a 60% reduction in the phloem transport of carbon to the roots at just 7°C (Wang & Hoch, 2022). This loss of phloem conductivity due to chilling may also be partly due to the decreased fluidity of the cytoplasm and the plasma membrane (Alonso *et al.*, 1997). In cow thistle stems, it was hypothesized that aquaporins or other transport molecules in the sieve element plasma membranes are disrupted by the low temperature (Gould *et al.*, 2004). Nevertheless, after localized chilling a rapid aquaporin upregulation was shown in balsam poplar sieve cell membranes (Stanfield & Laur, 2019). This dynamic response may serve to counteract the reduced passive permeability of the plasma membrane, as the water released from the sieve tubes consequently adjusted pressure allowing flow to resume (Stanfield and Laur 2019). At a larger scale, there is evidence of a greater use of whole tree non-structural carbohydrate (NSC) pools in boreal rather than in temperate paper birch populations (*Betula papyrifera*) (Fermaniuk *et al.*, 2021). These differences are primarily driven by the branch pools for spring growth (Fermaniuk et al. 2021) and could partly help avoid the impact of a potentially slowed root phloem transport in cold springtime soils. Furthermore, the role played by the conversion of starch to soluble carbohydrates for cold protection has long been documented (Sakai & Yoshida, 1968). However, it was shown that carbon assimilation and, thus, its transport may not be the limiting factor for productivity in cold soils as Norway spruce trees growing in soil cooled by pockets of permafrost maintained high levels of carbon despite their severely stunted growth (Körner & Hoch, 2006; Hoch, 2008).

Indeed, although low temperature effects and limitations on productivity are often discussed in terms of cell growth with respect to limitations in nutrients and reduced photosynthesis, evidence is now mounting that root water uptake may be the most important limiting factor. As cold soils immediately lead to an apparent restriction of the water supply in plants (Fig. 1 Primary Response), growth is rapidly reduced leading to reduced productivity and stunted growth (Fig. 1 Secondary Response; Wan et al. 1999, Landhäusser et al. 2001). As such, low soil temperature stress is often considered to elicit a drought-like stress, a dual adaptation to both types of stress could plausibly be expected to occur. Coastal Douglas fir in the Pacific Northwest from populations subjected to lower winter temperature gradients have been recorded to be more drought resistant than those from provenances with milder winters (Bansal *et al.*, 2016). However, contrary to expectations, Arctic tree populations were reported to be physiologically maladapted to drought (Isaac-Renton *et al.*, 2018). In their provenance trial, in addition to thinner cell walls, Isaac-Renton et.al (2018) observed consistently poor stomatal control in lodgepole pine from northern provenances. Considering that a common response to low soil temperatures is an immediate closure of the stomata due to decreased water delivery (Fig 1 Secondary Response; Landhäusser et al. 1996, Wan et al. 2004), anisohydric plants should be better adapted to prolonged low soil temperatures in terms of maintaining their productivity. Further research is undoubtedly needed, but when viewed from this prism, the results observed in Douglas fir and lodgepole pine would no longer seem so contradictory. An adaptation to withstand seasonal lower temperatures is different from an adaptation to grow at low soil temperatures where maintaining productivity could be crucial. The first could confer drought resistance and the other drought vulnerability. More research is also needed to better understand the effects of plant and fungal aquaporins and their associations on root transport dynamics in trees exposed to low soil temperatures. It seems likely that all these factors may contribute to make certain species or populations more productive at low soil temperatures (Fig 1 Resistance and/or Acclimation).

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4.4 Figures

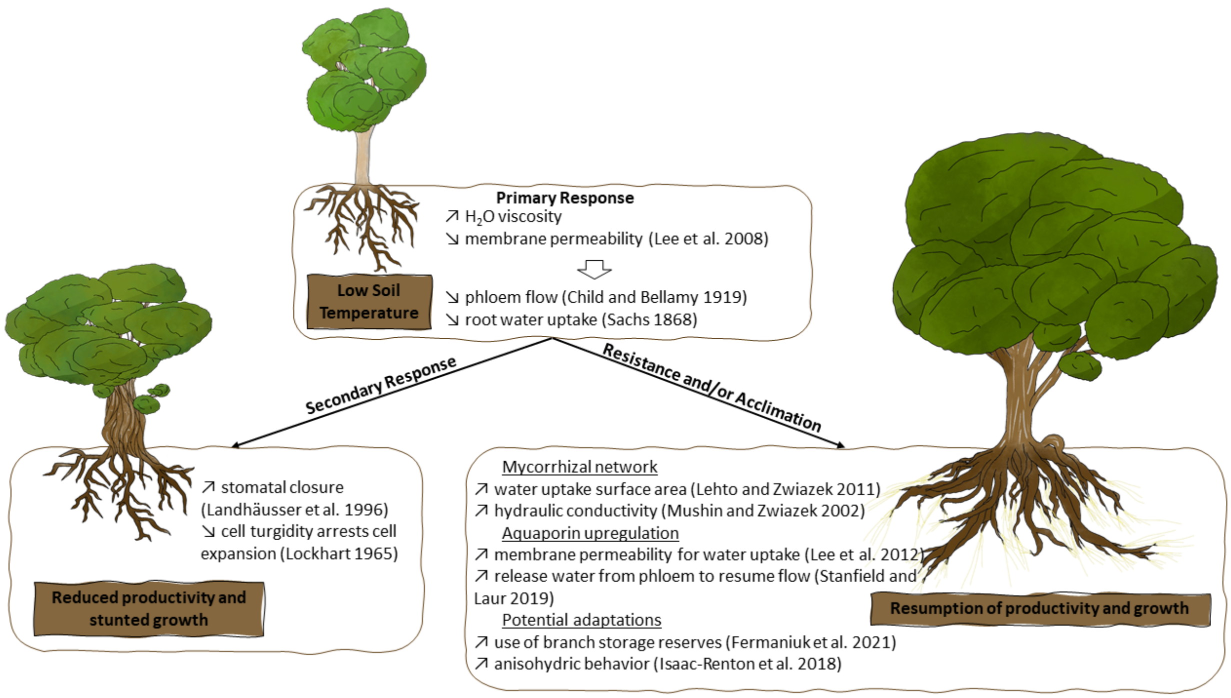

Figure 4.1: Commentary summary diagram. As soil temperatures decrease, the viscosity of water increases. In plant roots a primary response to lower temperatures is a reduction in membrane permeability (Lee et al. 2008). The combination of these effects leads to a reduction in the flow of photosynthates in the phloem (Child and Bellamy 1919) and water uptake (Sachs 1868). This apparent reduction in available water to the roots leads to two notable secondary responses, stomatal closure (Landhäusser et al 1996) and a drop in turgidity affecting cell expansion (Lockhart 1965). In time, these secondary responses reduce productivity and stunt growth. Nevertheless, resistance or acclimation to non-negative low soil temperatures likely occurs in certain species or populations. Certain mycorrhizal associations in the roots can significantly increase water uptake by providing a greater surface area (Lehto and Zwiazek 2011) and increased root hydraulic conductivity (Mushin and Zwiazek 2002). The upregulation of certain aquaporins can increase membrane permeability not only for water uptake (Lee et al. 2012), but also for water release from the phloem to resume flow (Stanfield and Laur 2019). I also suggest that the greater dependence on branch carbohydrate reserves and anisohydric behavior observed in certain northern tree populations (Isaac-Renton et al. 2018, Fermaniuk et al. 2021) may help trees resume and maintain productivity and growth at lower soil temperatures.

5 White spruce population differences in needle anatomy, foliar water uptake, and aquaporin expression indicate trade-offs between hydraulic safety and productivity

5.1 Summary

Drought impacts on western boreal forests and white spruce in particular are a significant concern and have been investigated through dendroecology and physiological research as well as provenance testing for drought resistant genotypes for future reforestation. Here I take advantage of such a provenance trial to investigate the underlying mechanisms of drought tolerance strategies. To investigate needle anatomy, foliar water uptake, and aquaporin expression under variable simulated drought conditions, a dehydration and foliar water uptake experiment was conceived using branch samples representing seed origins from throughout the range of the species to subsequently relate results to the climate of provenance origin.

Provenances from the Yukon and northern Alberta (Boreal cordillera and northern boreal plains) had thinner Casparian strips and thinner hypodermis layers and lost more water during needle dehydration. The climate of origin also had strong negative correlations with the width of the Casparian strip and the thickness of the hypodermis. Foliar water uptake uptake was highest in northern and central Alberta (Boreal Plains) provenances, with source climates characterized by cold and dry climatic conditions. Expression in four of six aquaporins was significantly positively correlated with relative water content during experimental drought and subsequent foliar water uptake.

Our results suggest that populations from the northwest apparently lack anatomical traits that would typically be associated with drought adaptation (thick hypodermis and casparian

strips), which adds to other research on water use efficiency and stomal control. This anisohydric behavior appears to imply that northwestern white spruce populations are anatomically maladapted to drought. However, this lack of radial foliar hydraulic resistance can also allow drought limited western provenances to take advantage of foliar wetting even when precipitation does not meaningfully wet the soil. In these populations foliar water uptake may be a coping strategy that has an overall net benefit to the plant's water balance.

5.2 **Introduction**

Western boreal forests are water limited ecosystems which climate change aggravates (Hogg & Hurdle, 1995; Gray *et al.*, 2011). Large amounts of research have shown the vulnerability of western boreal species to drought (Hamann & Wang, 2006; Michaelian *et al.*, 2011; Sang *et al.*, 2019). Some of this research identified an apparent lack of drought adaptations that may be somewhat unexpected (Isaac-Renton *et al.*, 2018; Sinclair, 2019). However, drought coping strategies varying widely from drought avoidance to drought tolerance and can be based on a broad array of trait combinations from rooting depth and anatomical resistance to hydraulic failure to CAM plants that only open their stomata at night and can take advantage of foliar water uptake (McDowell *et al.*, 2008; Reyes-García *et al.*, 2012). Nevertheless although some western boreal forest species have been show to be able to use their foliage for water uptake its relative importance to water budgets and relationship with climate and needle anatomy has not been investigated(Laur & Hacke, 2014).

Indeed, although evidence of foliar water uptake has been recorded in many tree species (Dawson & Goldsmith, 2018; Berry *et al.*, 2019), its functional importance has only been acknowledged for trees exposed to saturated water vapor conditions such as fog in coastal communities of *Sequoia sempervirens* (Koch *et al.*, 2004; Limm *et al.*, 2009). Generally, foliar water uptake is considered to be of insignificant eco-physiological importance to tree water budgets and remains largely ignored and excluded from forest management practices and ecological modeling (Matos *et al.*, 2022). This may be a large oversight, as the potential to benefit from a directional change in the xylem hydraulic tension gradient in aboveground organs and absorb atmospheric water appears to be a trait shared by a broad variety of plant species of diverse growth forms spanning nearly all climate zones: In dry deserts succulent cacti can collect dew and take up water using spines, highly modified leaves (Ju *et al.*, 2012; Malik *et al.*, 2016). In the canopies of wet tropical rainforests and montane cloud forests it is vital to a diversity of epiphytes—tank bromeliads (North *et al.*, 2013), orchids, and *Tillandsia* (Ohrui *et al.*, 2007). More recent evidence points to its occurrence in mangroves growing in saline waterlogged soils (Fuenzalida *et al.*, 2019). In more temperate climates there is evidence that timberline conifers utilize snow melt on branches in the spring to refill embolisms (Mayr *et al.*, 2014), and that foliar water uptake enables the tallest living trees to reach their towering heights (Koch *et al.*, 2004).

In epiphytic bromeliads the principal foliar water uptake entry pathway is through specialized water absorbing trichomes (Benzing *et al.*, 1976). However, for most plants, evidence supports the model that only minor diffusion occurs through the more resistive cuticle and epidermis, and that stomata are the main point of entry (Berry *et al.*, 2019). Once within the leaf, water must travel along the same intra- or extra-cellular pathways to the xylem that it might follow in reverse under a transpiration driven hydraulic gradient. The resistivity and plasticity of this hydraulic pathway is therefore logically similarly determined by foliar tissue anatomy and cell membrane permeability.

In vascular plants, in addition to the cuticle, the epidermis, and the hypodermis (if present), the bundle sheath is a significant control point which strongly influences leaf hydraulic conductance and thus whole plant hydraulic conductance (Sack *et al.*, 2015). In conifer needles, bundle sheath cells are arranged around a central vascular cylinder as a root-like endodermis and, at maturity, may contain suberized hydrophobic Casparian strips in their radial cell walls that restrict radial apoplastic water flow (Wu *et al.*, 2003). Indeed, during freezing and thawing cycles in pine needles, tissues outside and inside the endodermis react independently, shrinking and swelling with little radial transfer of water between the vascular cylinder and the mesophyll (Roden *et al.*, 2009). A strong body of evidence now shows that, in roots, Casparian strip permeability and suberin deposition are tightly regulated and directly influence hydraulic conductivity, nutrient transport, and solute leakage (Barberon, 2017; Wang *et al.*, 2019; Calvo-Polanco *et al.*, 2021). Given the anatomical similarities of the root and conifer needle endodermis, this may also hold true for conifer needles.

Likewise, by encouraging water to follow the cell-to-cell pathway to cross the endodermis, the Casparian strip increases the relative importance of cell membrane hydraulic permeability. Indeed, PIP (plasma membrane intrinsic proteins) aquaporins, which largely control plant hydraulic conductivity (Javot & Maurel, 2002; Lee *et al.*, 2010, 2012), appear to be dynamically regulated during foliar water uptake in foliage (Ohrui *et al.*, 2007; Laur & Hacke, 2014; North *et al.*, 2019). Thus, specific differences in the cuticle, epidermal cells, the hypodermis, the endodermis, the Casparian strip, and aquaporins may all play a role in enabling or restricting foliar water uptake in conifer needles.

White spruce (*Picea glauca*) is one of the boreal forest’s dominant tree species with a range that spans the entire width of the North American continent and stands growing across a relatively strong gradient of climatic ecozones (Burns & Honkala, 1990a). As such, it provides an ideal candidate to investigate the relationship between climatic conditions, drought tolerance, and foliar water uptake in boreal conifers. In addition, it has previously been reported that droughted white spruce seedlings can absorb atmospheric water through their needles when exposed to high relative humidity while concurrently up-regulating the expression of aquaporins in the needle endodermis and the transfusion parenchyma of the vascular cylinder (Laur & Hacke, 2014). This suggests that white spruce may have a measure of plasticity over the degree of resistance of the radial movement of water to and from the vascular cylinder and the mesophyll in their needles.

Furthermore, in 2017, in a controlled growth environment, white spruce seedlings were grown using seed stock that was used to establish the provenance trial in Calling Lake, AB, Canada (Sinclair, 2019). The results of the trial showed that when subjected to drought, seedlings grown from seeds sourced from more northern colder and drier climates (the Boreal Cordillera and Boreal Plains) had poor stomatal control compared to those from more southern and wetter climates (Sinclair, 2019). Furthermore, strong height differences were observed between control growth environment seedlings and adult provenance trial trees from the Boreal Plains provenances where the field trial was also located (Sinclair 2019), suggesting that other environmental factors, not occurring in the controlled growth environment, were affecting growth under field conditions. Likewise, these results also appeared to partly corroborate that population specific changes of white spruce growing patterns in the Brooks Range of Alaska are not only explained by drought induce stomatal closure (Brownlee *et al.*, 2016).

Speculating that the striking differences between the controlled growth environment and field trial phenotypes for an entire subset of genotypes were caused by diverging foliar water use strategies, I designed an experiment using trees at the same Calling Lake common garden trial to investigate how traits affecting radial needle water movement—anatomy and aquaporin expression patterns—might differ between white spruce adults from different provenances of origin. Specifically, I hypothesized that provenances from colder climates have less hydraulically resistive foliar anatomies in line with their previously reported anisohydric behaviors, and that provenances having shown superior growth performance in the field than in a controlled environment have greater rates of foliar water uptake. Specific aims were to 1) relate population specific foliar anatomical traits with climatic parameters of their seed source, 2) assess differences in rates of foliar water uptake between provenances 3) gauge the effects of foliar anatomy and aquaporin expression on foliar dehydration and water uptake, and 4) evaluate the range-wide implications of these contrasting foliar water use strategies.

5.3 **Materials and Methods**

5.3.1 Study site and plant material

The Calling Lake Genetics Experimental Site is located approximately 60 km north of Athabasca, Alberta, Canada, in the boreal forest (55°17′N, 113°10′W). The site contains a white spruce (*Picea glauca* [Moench] Voss) provenance trial where 4-year-old containerized seedlings germinated from seeds from 43 natural stands collected from different Canadian ecozones (Fig. 1 a) were planted in 1982 and arranged in a randomized complete block design (Rweyongeza *et al.*, 2007). There are 5 blocks each containing rows with 5 trees from each provenance all evenly grid-spaced 2.5 meters apart.

5.3.2 Needle anatomy

5.3.2.1 Anatomical measurements

Of the 43 provenances at the common garden site, 11 representative provenances from across the white spruce geographic range and Canadian ecozones were chosen to investigate potential differences in their foliar physiological anatomies. New growth needles were collected from four random trees—one tree per experimental block—per provenance from healthy, exposed upper canopy branches using an extensible pole pruner and a ladder. Fresh needle cross sections were obtained using a Leica RM2125 rotary microtome (Wetzlar, Germany) and stained with safranin and fast green. Micrographs were obtained at 20x magnification using a Leica MC 170 HD camera mounted to a Leica DM3000 microscope (Wetzlar, Germany). Measurements (at least 30 per section for the linear measurements) of a broad ranging number of anatomical parameters were then obtained using Image J (Rasband, 2011). The following parameters were measured: total area of the section, mesophyll area, xylem area, phloem area, endodermis radial width, Casparian strip radial width, the ratio of Casparian width to endodermis width, hypodermis radial width, and the radial width of the epidermis with the cuticle.

5.3.2.2 Provenance climates of origin and Canadian ecozones

Climate data normals for the provenances were obtained using ClimateNA v.5.21 (Wang *et al.*, 2016) using the standard reference period of 1961-1990, after sufficient weather stations were deployed and before anthropogenic warming skewed the data. Climatic variables considered were mean annual temperature (MAT), mean annual precipitation (MAP), mean summer precipitation (May to September; MSP), the annual heat moisture index (MAT/MAP*1000; AHM), and summer heat moisture index (mean warmest month temperature/MSP*1000; SHM). Provenance climates and locations were categorized following the Canadian ecozones classification (Ecological Stratification Working Group, 1996).

5.3.3 Foliar water uptake experiment

5.3.3.1 Provenance selection

To investigate functional differences in foliar water uptake relative to provenance climate of origin, 6 representative provenances were used in total, 3 pairs, each from a different ecozone.

After running a Pearson’s R correlation on all available climatic variables and anatomical variables measured, the Boreal Cordillera, the Boreal Plains, and Temperate Mixedwood ecozones were selected to best capture a gradient of differences in latitude and marked differences in climate of origin and needle anatomy.

5.3.3.2 Experimental setup

To evaluate the potential differences in the rates of foliar water uptake between the selected provenances and ecozones, the experiment was run twice on the three ecozones selected: first as a mild dehydration followed by foliar uptake (a 5% average reduction in relative water content—RWC—from fresh) using provenances 1385, 12, and 1333, and then as a severe dehydration (10 % average reduction in RWC from fresh) followed by foliar water uptake using provenances 1386, 10, and 1383.

To obtain plant material, one healthy tree was randomly selected per 5-tree row plots per block for a total of 5 trees per provenance. For each selected tree, one healthy south facing and sun exposed branch from near the top of the canopy was then severed at its base around midday with a telescopic pruning pole. Branches were labeled and immediately placed in large plastic bags with wet paper towels at the base of the cut stems to avoid water loss during transport to the laboratory.

There, three twigs per branch (15 per provenance), approximately 8 to 12 cm long and containing only new growth needles, were severed using a hand shear and immediately weighed on a Mettler Toledo analytical scale (Columbus, Ohio, United States) to determine their fresh weight. The twigs were then left overnight (14-16 hours) to dehydrate on the benchtop to their target RWC reduction. The following morning dehydrated weights were determined on the scale and the twigs were then misted with deionized water using a hand pumped garden sprayer and placed in labeled plastic bags. To ensure water uptake did not occur through the cut at the base of the twig, mineral oil and parafilm were used to fully seal the cut. At 30, 60, and 240 minutes after first being misted with water, the twigs were surface dried by patting them down with clean paper towels and brushing them with thin wide jets of cool dry air to ensure no water drops remained before being weighed, re-misted, and replaced in the plastic bags. To obtain a turgid weight the bags were then filled with deionized water and the twigs were allowed to float for 48 hours before being surface dried and weighed once more. The twigs were then dried at 70°C in an oven for 72 hours to obtain their dry weight; at this point the dry weight of the needles was also obtained. At all timepoints twigs were measured randomly but always in the same sequence of blocks to minimize time variations between measurements.

5.3.3.3 Relative water content

The relative water content (RWC) for the various timepoints was determined as follows:

$$RWC\ at\ Timepoint_{\,n} = \frac{Twig\ weight\ at\ Timepoint_{\,n} - Total\ twig\ dry\ weight}{Turgid\ twig\ weight - Total\ twig\ dry\ weight}$$

5.3.3.4 Water flux through the epidermis

To gauge the amount of water lost or gained per needle area over time, for each dry twig sample the needles were carefully removed and then scanned and projected area was measured using Sigmascan Pro 5.0 software (Systat Software, San Jose, USA). The water flux through the epidermis was then calculated as follows:

$$Water\ Flux = \frac{Twig\ weight\ at\ Timepoint_{\,n+1} - Twig\ weight\ at\ Timepoint_{\,n}}{Twig\ dry\ needle\ area * (Timepoint_{\,n+1} - Timepoint_{\,n})}$$

5.3.3.5 RNA extraction, cDNA synthesis, and RT-qPCR analysis of aquaporins

From one twig per branch (5 per provenance—severed and dehydrated at the same time as the twigs for the foliar water uptake experiment), 4-5 needles were collected and immersed in liquid nitrogen before placing them in a -80°C freezer for storage prior to RNA extraction. The same was repeated immediately prior to misting, and 30, 60, and 240 minutes after misting with deionized water.

After retrieval from storage, the needles were ground in liquid nitrogen and RNA was then extracted from the needle tissue using the following adjustments to the modifications brought by (Pavy et al., (2008) to the original protocol developed for pine needles (Chang *et al.*, 1993). In brief, 750 µl of CTAB extraction buffer (prepared as in Pavy et al., 2008, but without the addition of spermidine) was preheated to 65°C and added to ~400 µg of frozen ground needle tissue. After 10 minutes of incubation in a water bath at 65ºC with periodic vortexing, 500µl of 24:1 chloroform: isoamyl alcohol was added to the samples and the aqueous top layer containing RNA was allowed to separate by centrifugation at 21,000g at 4°C for 5 minutes. This upper layer was then transferred to a clean tube and the addition of chloroform:isoamyl alcohol followed by centrifugation was repeated once more. In a fresh tube, the RNA in the aqueous layer was precipitated at -20°C for 1 hour after adding an equal volume of 7.5 M LiCl-50 mM EDTA solution. The RNA was then pelleted by centrifugation 21,000 g at 4°C for 20 minutes, rinsed with 500 µl of 80% ethanol, collected anew by centrifuging for 5 min at 20°C and 21,000g, and finally resuspended in 20 µl of 60°C RNase free water by incubating for 2 minutes at 60°C.

RNA quality and concentrations were determined using a Thermo Scientific NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and by running a 1% agarose gel electrophoresis. A QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) was then used for cDNA synthesis using 500 µg of RNA per sample. Aquaporin transcript levels were measured using a qRT-PCR Applied Biosystems 7500 Fast system (Waltham, MA, USA) as described in (Khan *et al.*, 2019) using *P. glauca* aquaporin primers for *PgPIP 1;1*, *1;2*, *1;3*, *2;1*, *2;2*, and *2;8* (Laur & Hacke, 2014) and expressed relative to *P. glauca* endogenous genes, Alpha and ELF (Mageroy *et al.*, 2017; Boyd & Nkongolo, 2021).Differences in expression between provenances and timepoints were determined using ΔC_T, the relative PCR cycle threshold difference between target aquaporins and endogenous genes for each sample. The primer sequences are detailed in Table 5.S1.

5.3.4 Statistical analysis and graphing software

Data were fitted to linear mixed effect models and analyzed in R using the lme4, lmertest, and emmeans packages (Bates et al. 2019, Kuznetsova et al. 2019, Lenth et al. 2019, R Core Team 2019). As three twigs were selected from one branch from one tree per block per provenance and the experiment was repeated on three new provenances from the same three ecozones originally selected but with a stronger level of dehydration, the following models were constructed for analysis:

To compare the provenances within each dehydration level:

$$\text{Variable} \sim \text{Provenance} * \text{Timepoint} + (1|\text{Block}).$$

To compare ecozones and dehydration levels:

$$\text{Variable} \sim \text{Dehydration Severity} * \text{Ecozone} * \text{Timepoint} + (1|\text{Block}).$$

Type III analysis of variance tables were constructed using the Satterthwaite's method to compare provenances, dehydration severity, and ecozones. The Kenward-Roger degrees of freedom method was used for the emmeans, and Tukey tests were used for post-hoc analysis. Estimated means and standard errors were graphed using SigmaPlot 14.5 (Systat Software, San Jose USA). Compact letter displays of estimated means pairwise comparisons for the anatomical table (Table 2) were obtained using the multcomp package (Hothorn *et al.*, 2008). The correlation plot of anatomical variables and climate variables was obtained using the corrplot package (Wei & Simko, 2021). Generalized additive models were developed using the mgcv R package (Wood, 2011) and graphed along with regressions and the map of provenances using the ggplot2 package (Wickham & Sievert, 2016).

5.4 **Results**

5.4.1 Needle anatomy

Micrographs showing representative cross sections of spruces needles from the study are shown in Figs 1 b and c. The total needle cross-sectional area was greater in provenance 1333 compared with 10 and 1315. Provenance 1365 also had greater cross-sectional area than 1315. Mesophyll area was only significantly greater in needles of provenance 1365 than 1315. The endodermis was only significantly wider in provenance 1333 when it was compared to provenance 12. However, the hypodermis was radially thicker in provenances 1315 and 1383 compared with 1385, 1381, and 10. The radial width of the epidermis including the cuticle was significantly thicker in provenances 1333, 1355, 1365 1376 1385 compared with provenance 10. There were no significant differences in xylem area, phloem area, Casparian strip width, and Casparian strip to endodermis width ratio (Table 5.2).

5.4.2 Correlations of provenance climate of origin and needle anatomy

Radial Casparian strip thickness in white spruce trees was significantly positively correlated with mean annual temperature and mean annual precipitation in the provenances of origin (Fig. 5.1 d). The ratio between radial Casparian strip thickness and radial endodermis thickness was significantly positively correlated with mean annual temperature, mean annual precipitation, and mean summer precipitation, and significantly negatively correlated with the summer heat: moisture index ratio (Fig. 5.1 d). Needle radial hypodermis width was also significantly positively correlated with mean annual temperature, mean annual precipitation, and mean summer precipitation, and significantly negatively correlated with the summer heat: moisture index ratio of the provenances of origin (Fig. 1 d).

5.4.3 Relative water content status and water flux, before and during the foliar water uptake experiment

The fresh RWC of the spruce twigs was not statistically different between provenances, levels of dehydration, or between ecozones at the start of the experiment (Fig. 5.2 a and c). The water outflux during the overnight dehydration period was then significantly greater in the severely dehydrated provenances (Fig. 5.2 b and c), which led to their significantly lower relative water contents for the remainder of the experiment (Fig. 5.2 a and c). In addition, when considering both levels of dehydration, from this moment onwards the twigs from the Boreal Cordillera ecozone provenances had a significantly lower RWC than their counterparts from the Boreal Plains and the Temperate Mixedwood (Fig. 5.2 a and c). After the misting of the dehydrated twigs with deionized water, the influx of water over the first 30 minutes was significantly greater for Boreal Plains ecozone spruce needles than for those from the Boreal Cordillera or the Temperate Mixedwood (Fig. 5.2 b and c). The water flux into the needles then gradually decreased from 30 to 60 minutes and from 60 to 240 minutes reaching final rates similar to those observed during the fresh to dehydrated outflux (Fig 5.2 b).

5.4.4 Aquaporin expression in needles

5.4.4.1 Relationship with Relative Water Content

PgPIP 1;1, 1;2, 2;1, and *2;2* ΔC_T was significantly positively associated with needle relative water content (Fig. 5.3 a, b, d, and e). *PgPIPs 1;1* and *2;2* had strong coefficients of determination—0.53 and 0.81 respectively—with few differences between provenances (Fig. 5.3 a and e). *PgPIPs 1;3* and *2;8* ΔC_T was more loosely associated with relative water content abundance with larger discrepancies present in expression behavior between provenances (Fig. 5.3 c and f)

5.4.4.2 Relative transcript abundance over wetting time

PgPIP 1;1 was significantly upregulated after 60 min in mildly dehydrated Boreal Cordillera needles whereas it was downregulated in the mildly dehydrated Boreal Plains provenances after 240 minutes (Fig. 5.S1 a). *PgPIP 1;2* was always less expressed in the Boreal Plains provenances during water uptake but was only downregulated by 240 min in the mildly dehydrated Boreal Cordillera and Temperate Mixedwood provenances (Fig. 5.S1 b). *PgPIP 1;3* was strongly downregulated between 30 and 60 minutes after rewetting in the mildly dehydrated Boreal Cordillera provenance and strongly upregulated by 240 min of wetting in the mildly dehydrated Boreal Plains provenance (Fig. 5.S1 c). *PgPIP 2;1* was significantly upregulated from 30 to 60 min in provenance 1385 and upregulated by 240 min of wetting in provenances 1383, 12, and 10 (Fig. 5.S1 d). *PgPIP 2;2* was downregulated at 240 min in all mildly dehydrated needles (Fig. 5.S1 e). *PgPIP 2;8* was upregulated from 30 to 60 minutes in the mildly dehydrated provenances 12 and 1333, and downregulated over the same period in the severely dehydrated twigs from provenances 1383 and 1386 (Fig. 5.S1 f). *PgPIP 2;8* was then downregulated by 240 min of wetting in provenances 10, 1385, and 1333 (Fig. 5.S1 f).

5.4.4.3 Specific relationships between aquaporins and with anatomy

PgPIP 1;1 ΔC_T was significantly negatively associated with *PgPIP 1;3* expression (Fig. 5.4 a). In effect, at *PgPIP 1;1* ΔC_T threshold of 4 and above, the transcript abundance of PIP *1;3* drastically increased (Fig. 5.4 a). Radial hypodermis thickness was significantly negatively correlated with *PgPIP 1;2* ΔC_T, with lower levels of *PgPIP 1;2* expression during water uptake occurring in provenances with needles containing a thinner hypodermis (Fig 5.4 b).

5.5 **Discussion**

5.5.1 Spruce from colder and drier climates have less resistive radial foliar anatomies.

Our results support the more recent concept that anisohydric water use strategies do not underlie a more resistant xylem architecture (Kannenberg *et al.*, 2019). The results also confirm our first hypothesis, as in line with their previously reported anisohydric behaviors (Sinclair, 2019), provenances from the colder and drier Boreal Cordillera and Boreal Plains provenances had thinner hypodermal and endodermal layers compared to the more isohydric Temperate Mixedwood provenances. Our results echo what has been observed in lodgepole pine planted in a common centrally located (relative to the range) site where northern lodgepole pine populations were reported to have poor stomatal control, thinner cell walls, and reduced total height and diameter growth compared to central and southern populations (Isaac-Renton *et al.*, 2018). It may be that the more resistive hydraulic radial foliar traits observed in trees from wetter seed-source climates of origin developed in response to their growth in the drier climates of the provenance trials, as dry conditions can lead in conifer trees to the development of drought resistant foliar anatomy; more hypodermis cell layers, less mesophyll, and smaller needles (Grill *et al.*, 2004). Nevertheless, our results strongly indicated that irrespective of needle size, the thickness of the Casparian strip and the hypodermis was positively correlated with higher temperatures and increased precipitation, and negatively correlated with summer drought indices of provenance climates. Given that needles with these less resistive foliar anatomical traits experienced not only greater water loss but also greater influx, I can report that, similarly to montane cloud forest communities (Gotsch *et al.*, 2015), there appears to be a clear trade-off in white spruce needles between the potential for foliar water uptake and drought resistance.

5.5.2 Foliar water uptake is greater in white spruce from the Boreal Plains ecozone.

I observed a significantly higher foliar water influx over the first 30 minutes of wetting in the twigs and needles of both Boreal Plains provenances compared to those from white spruce seed stock from the Boreal Cordillera and Temperate Mixedwood ecozones (Fig 2 c outlined box). Following the 5% reduction in RWC prior to wetting, Boreal Plains provenance twigs not only fully recovered their initial fresh RWC but even surpassed it after 30 min of surface wetting. This confirms our second hypothesis and may potentially explain results of the study which showed that Boreal Plains provenances exhibited much greater growth in the field than in a controlled growth environment where they were manually watered at their base (Sinclair, 2019). Indeed, field conditions expose trees to many instances of occult precipitation such as light rain, dew, or fog which wet the canopy without meaningfully increasing soil water content. (Monteith, 1976). In the boreal forest foliar wetting (> 0.1 mm of precipitation) has been estimated to occur on average 151 days per year (Dawson & Goldsmith, 2018). Given that all the white spruce twigs recovered ~5% of their RWC after 240 minutes of wetting, our results underline the importance of these events as a water source in white spruce hydraulic budgets and particularly so for Boreal Plains tree populations.

5.5.3 Foliar water uptake appears to involve tightly regulated aquaporin expression.

Water influx was greater at a mild dehydration level than severe dehydration, suggesting that foliar water uptake is an actively controlled mechanism in white spruce, not just determined by the severity of the hydraulic gradient. In Boreal Plains twigs, greater rates of foliar water uptake also coincided with an immediate decrease in the expression levels of *PgPIP 1;2* in needle tissues after foliar misting, whereas this downregulation only occurred after 4 hours in the Temperate Mixedwood and Boreal Cordillera provenances and only for twigs subjected to a mild dehydration (Fig 5 b dashed lines). In the absence of clear anatomical differences (hypodermis thickness, endodermis and Casparian strip width) observed between the Boreal Plains and Boreal Cordillera provenances (Table 2 Fig 5b) this suggests that *PgPIP 1;2* regulation differences may be connected to the significantly greater foliar water uptake rates observed in the needles of white spruce from the Boreal Plains provenances over the first 30 minutes of the experiment. In addition, given the rapid downregulation, it likely occurred before the mesophyll water potential became less negative than that of the vascular cylinder implying that it likely occurs in the mesophyll of Boreal Plains needles. Likewise, I observed that the downregulation of *PgPIP 1;2* only occurred after 4 hours and only at the mild level of dehydration in the provenances from other climatic ecozones. Thus, it may be that in contrast to Boreal Plains trees, other provenances require a tissue RWC threshold to be reached prior to decreasing cell membrane hydraulic permeability through *PIP 1;2* downregulation. In these provenances expression could thus be regulated by the mesophyll water potential being equal to or greater than that of the vascular cylinder. Furthermore, our results also show that *PgPIP 1;1* and *PgPIP 2;2* expression is directly correlated with RWC as expression levels linearly decrease with greater needle water content irrespective of the provenance or prior level of dehydration (Fig 3a and e).

5.5.4 Diverging water use strategies—is anisohydric behavior climate driven?

Like lodgepole pine (Isaac-Renton *et al.*, 2018), white spruce from northern and colder climate populations appear to exhibit more anisohydric stomatal behavior (Sinclair, 2019) and less drought resistant leaf hydraulic anatomy. However, hydraulic risk is likely mitigated by their reduced specific leaf areas and thus lower water demand as white spruce tree height is strongly linked with the length of the growing season (Sebastian-Azcona *et al.*, 2018). A shift between these climatically diverging water use strategies has been artificially induced in *Picea abies* before. Norway spruce trees that were subjected to the Kroof summer rainfall exclusion experiments initially closed their stomata and had pre-dawn water potentials nearing -2 MPa over the first two years of drought stress. However, they were able to significantly recover their water potentials by reducing their leaf area index over the next three summer droughts (Pretzsch *et al.*, 2020; Grams *et al.*, 2021). It may be that less stomatal sensitivity to more negative water potentials is important to maintain productivity over shorter growing seasons and at colder soil temperatures (Fleurial *et al.*, 2022a) and the low heat sum requirements of northern white spruce populations have indeed been shown to allow them to effectively increase the length of their growing season at the potential risk of spring frost damage (Casmey *et al.*, 2022). Nevertheless, while anisohydric behavior may be advantageous at mild stress conditions it does leave plants significantly more vulnerable to severe drought stress (Sade *et al.*, 2012). Our experiment results indicate that, at mild dehydration stress, foliar water uptake may help mitigate this risk in white spruce grown in field conditions as thinner hypodermis and Casparian strip allows for greater water uptake to not only offset but surpass the water lost. However, at severe dehydration levels, our results suggest that western spruce populations may be significantly vulnerable to severe drought.

5.5.5 Other considerations

It has been suggested that the conifer endodermis plays a role in protecting the mesophyll from freezing as when conifer needles are subject to freezing temperatures ice crystal formation is first compartmentalized to the vascular cylinder (Kaku, 1971; Stegner *et al.*, 2023). Nevertheless, our results showed no evidence that the thickness or permeability of the Casparian strip plays any role in this, as the most freezing tolerant populations in the provenance trial (Sebastian-Azcona *et al.*, 2019) were found to broadly have a thinner Casparian strip and hypodermis. I suggest that although the endodermis appears to present a barrier to freezing spreading to the mesophyll cells, the lack of conductive tissue and osmotic adjustments in the mesophyll may be more important to needle frost tolerance than the permeability of the endodermis.

Finally, although the difference in foliar water uptake between Boreal Plains and Cordillera needles may partly be explained by differences in *PgPIP* 1;2 aquaporin expression, other factors may also have an influence. White spruce stomata are obstructed by a plug of crystalline wax tubules, similar to those found in other spruce species (Jeffree *et al.*, 1971). Though this plug remains the same during the growing season it appears to deteriorate over the summer leading to older foliage having less blocked stomata (Despland *et al.*, 2016). Recent results showcased that both smaller wax caps and wax wear appear to allow for greater foliar water uptake in older conifer needles compared to current growth needles in different pine species (Roth-Nebelsick *et al.*, 2023). It could be that differences in stomatal cap shape or composition between white spruce provenances influenced foliar water uptake rates. Given the strong indications that foliar water uptake is widespread in conifers and the uncertain functional role of stomatal crypts and plugs (Roth-Nebelsick et al., 2009), future research could investigate if crypt shape or wax cap microtubules create a gradient of surface free energy and Laplace pressure to guide water droplets towards stomata similar to what has been shown to occur on the ribbed spine surface of cactus needles (Ju *et al.*, 2012).

5.6 **Conclusions—Range wide implications**

Our results, build on a now substantial body of work on the Calling Lake white spruce range wide provenance trial (Rweyongeza *et al.*, 2007) to reveal a pattern of *Picea glauca* intraspecific differences with significant implications for assisted migration, forest vulnerability, and the resilience of the boreal forest. Despite strong climatic differences, white spruce populations throughout the continent-spanning species range show similar resilience to drought (Sang *et al.*, 2019), nevertheless their adaptive strategies widely differ. Populations from the colder and drier northern and western range appear to have less hydraulically resistive radial foliar anatomies, less stomatal control (Sinclair, 2019), are less vulnerable to freezing damage during dormancy (Sebastian-Azcona *et al.*, 2018, 2019), but wake more rapidly from dormancy (Casmey *et al.*, 2022) and reach bud burst earlier (Sinclair, 2019), at the expense of lower rates of water use partly through reduced leaf area and biomass to mitigate hydraulic risk. At the other end of the climatic spectrum white spruce from the wetter eastern and southern range have greater stomatal control, more efficient xylem yet more hydraulically restrictive radial foliar anatomy, and greater leaf areas and biomass (Sebastian-Azcona *et al.*, 2018, 2019; Sinclair, 2019). I reveal that the northern Boreal Plains ecozone populations, which are subject to cold and dry conditions at a more southern latitude, show most of the same anisohydric and anatomical characteristics of the northwestern populations. However, they are also able to maintain leaf area and biomass equal to that of the eastern populations in the field likely through foliar water uptake facilitated by their less hydraulically resistive radial foliar anatomy and mediated by a tight control of *PIP* aquaporins (*1;1, 1;2, 1;3, 2;1, 2;2*) expression patterns. In this respect, the ability to benefit from foliar water uptake appears to skew the water use trade off curve towards anisohydric traits and behaviors which may be advantageous under moderate drought stress but lead to significantly more vulnerability to future severe or prolonged drought conditions (Sade *et al.*, 2012). Foliar wetting, which has been conservatively estimated to occur on average 151 days per year in the boreal forest (Dawson & Goldsmith, 2018), appears to be important to maintain coniferous forest growth and biomass, particularly under cold and moderately dry conditions, and merits greater consideration in boreal forest management and modeling.

5.7 **References**

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5.8 **Figures and Tables**

Table 5.1:Common garden provenance seed lot origin location, ecozone, and climate characteristics from 1960-1990—Mean annual temperature (MAT), mean annual precipitation (MAP), mean summer precipitation (MSP), annual heat moisture index (AHM), and summer heat moisture index (SHM). Provenance rows in color indicate they were selected for the foliar water uptake experiments, lighter shades of color indicate provenance samples mildly dehydrated prior to treatments, darker shades were severely dehydrated.

Provenance ID	Ecozone	Province	Latitude	Longitude	Elevation	MAT	MAP	MSP	AHM	SHM
1381	Northern Cordillera	YUKON TERRITORY	60.6833	-135.133	762	-1.5	309	180	27.5	72.6
1385	Northern Cordillera	YUKON TERRITORY	61.35	-139	792	-3.9	285	203	21.3	61.3
1386	Northern Cordillera	YUKON TERRITORY	64.0167	-139	609	-4.6	375	215	14.5	69.5
10	Boreal Plains	ALBERTA	59.8833	-111.717	183	-2.9	351	209	20.2	77.3
12	Boreal Plains	ALBERTA	54.6333	-110.217	610	0.7	456	314	23.5	51.9
1355	Boreal Plains	MANITOBA	54.1667	-99.1667	243	-0.8	473	307	19.4	57.8
1365	Boreal Plains	SASKATCHEWAN	51.6333	-101.667	621	0.3	460	303	22.4	56.7
1315	Atlantic Maritime	NEWFOUNDLAND	49.0167	-57.6167	45	4.4	1113	434	13	38.5
1376	Montane Cordillera	BRITISH COLUMBIA	52.0833	-122.933	1280	1.7	509	234	23	53.6
1333	Temperate Mixedwood	ONTARIO	44.4333	-77.8333	180	6.2	839	361	19.3	55.1
1383	Temperate Mixedwood	ONTARIO	45.5	-77.0167	121	5.6	817	378	19	54

Table 5.2: White spruce needle anatomical characteristics for provenances measured. Provenance rows in color indicate they were selected for the foliar water uptake experiments. Colors are paired by ecozone, with lighter shades indicating were mildly dehydrated prior to treatments, darker shades were severely dehydrated.

Provenance ID	Total area (μm^2)	Mesophyll area (μm^2)	Xylem area (μm^2)	Phloem area (μm^2)	Endodermis width (μm)	Casparian width (μm)	Casparian : Endodermis width ratio	Hypodermis width (μm)	Epidermis + Cuticle width (μm)
1381	641000 ± 80600 abc	549000 ± 76800 ab	2190 ± 351 a	3580 ± 567 a	17.6 ± 0.5 ab	6.1 ± 0.4 a	0.35 ± 0.03 a	9.7 ± 1.2 b	15.6 ± 0.7 ab
1385	703000 ± 69900 abc	613000 ± 65200 ab	2270 ± 375 a	3450 ± 663 a	16.2 ± 0.4 ab	5.6 ± 0.4 a	0.35 ± 0.03 a	10.7 ± 0.7 b	17.7 ± 1.5 a
1386	655000 ± 53200 abc	576000 ± 50000 ab	2490 ± 366 a	3660 ± 474 a	16.3 ± 1.0 ab	6.0 ± 0.4 a	0.37 ± 0.03 a	12.0 ± 1.2 ab	15.5 ± 0.4 ab
10	444000 ± 40300 bc	465000 ± 82500 ab	1520 ± 84 a	2170 ± 82 a	15.9 ± 0.9 ab	5.7 ± 0.2 a	0.36 ± 0.02 a	9.9 ± 0.3 b	11.1 ± 0.2 b
12	490000 ± 39700 abc	410000 ± 37100 ab	1560 ± 319 a	2770 ± 439 a	15.1 ± 1.0 b	6.2 ± 0.3 a	0.41 ± 0.02 a	12.2 ± 0.6 ab	15.4 ± 1.0 ab
1355	714000 ± 97200 abc	612000 ± 88000 ab	1710 ± 259 a	3300 ± 802 a	16.0 ± 1.0 ab	6.2 ± 0.8 a	0.38 ± 0.03 a	12.9 ± 0.8 ab	16.5 ± 0.4 a
1365	746000 ± 77700 ab	646000 ± 68900 a	2340 ± 523.4 a	3680 ± 718 a	18.5 ± 0.3 ab	6.7 ± 0.4 a	0.36 ± 0.02 a	12.7 ± 0.7 ab	15.7 ± 1.1 a
1315	413000 ± 49600 c	335000 ± 41700 b	1530 ± 138 a	2050 ± 203 a	16.6 ± 0.5 ab	6.9 ± 0.3 a	0.42 ± 0.03 a	15.3 ± 1.4 a	15.5 ± 0.6 ab
1376	681000 ± 25900 abc	578000 ± 26300 ab	2510 ± 459 a	3860. ± 603 a	17.9 ± 1.0 ab	6.7 ± 0.8 a	0.37 ± 0.03 a	12.6 ± 0.4 ab	18.3 ± 1.0 a
1333	753000 ± 45900 a	630000 ± 43200 ab	2160 ± 247 a	3160 ± 381 a	18.8 ± 0.5 a	7.8 ± 0.4 a	0.42 ± 0.02 a	13.3 ± 1.1 ab	19.9 ± 0.2 a
1383	621000 ± 62600 abc	521000 ± 57500 ab	2240 ± 441 a	3890 ± 514 a	17.1 ± 0.6 ab	6.5 ± 0.3 a	0.38 ± 0.02 a	15.9 ± 1.1 a	15.3 ± 0.9 ab

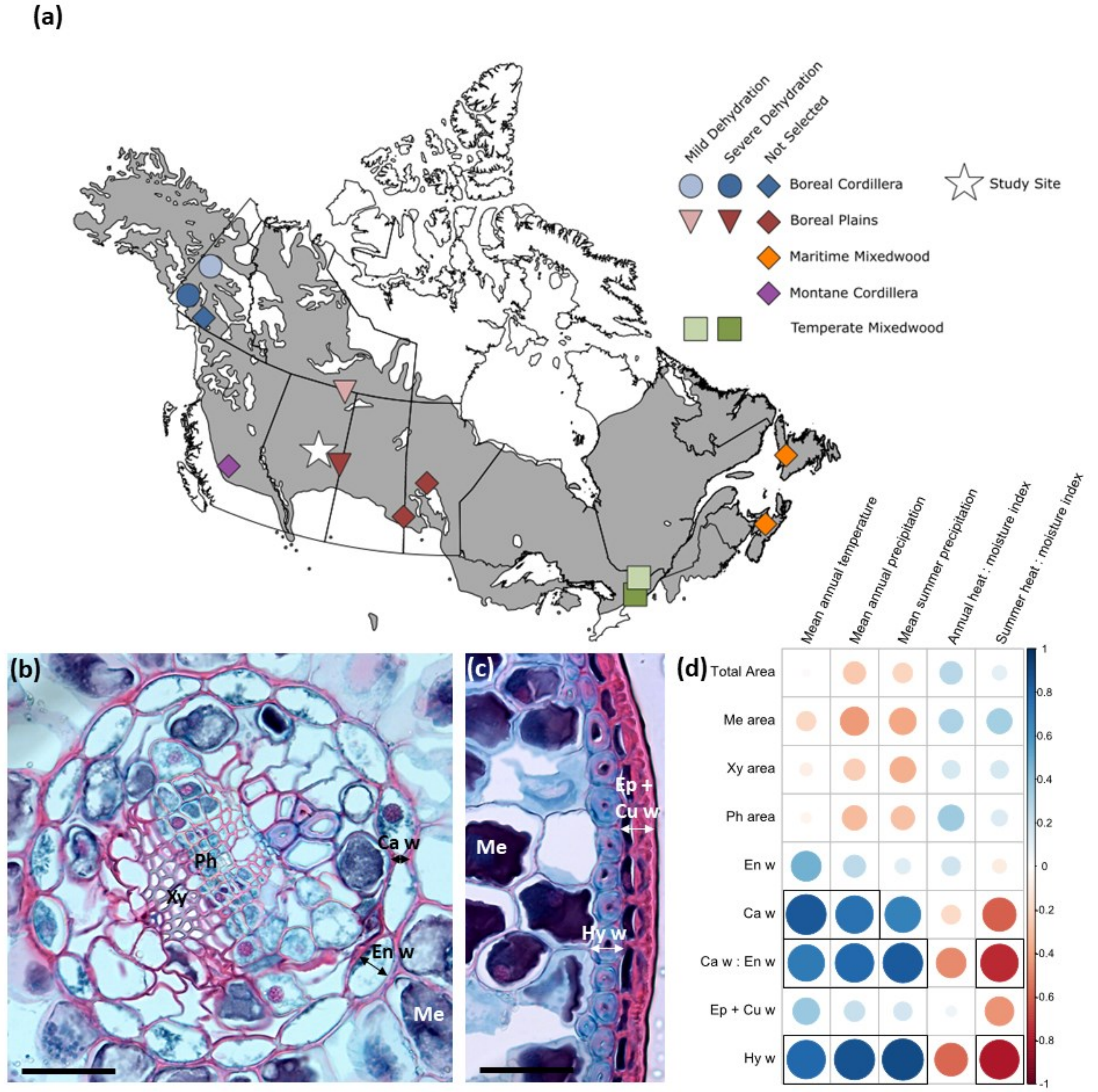
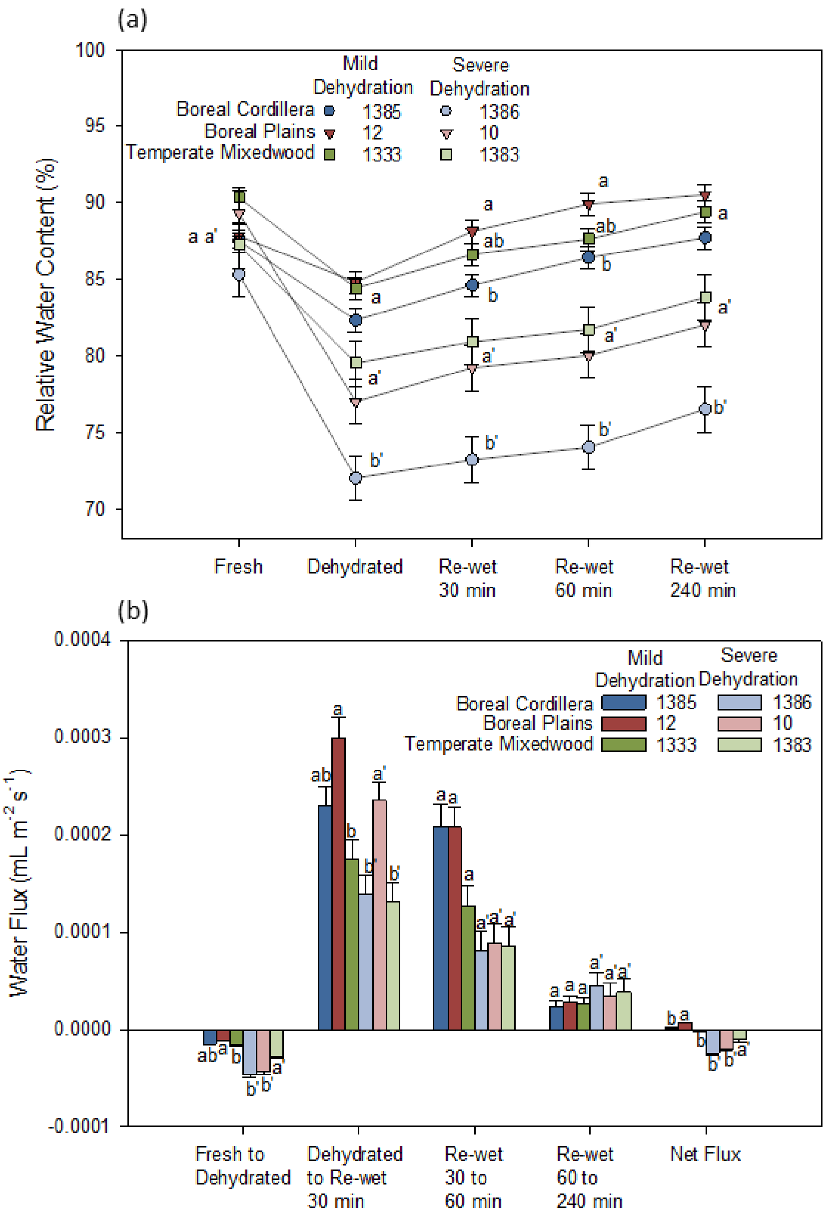


Figure 5.1: (a) Location of the common garden experiment (star) and the location of origin and ecozone of the seedlots for which needle anatomical variables where measured (n = 4). Triangles indicate provenances that were selected for the needle water uptake experiment and the white spruce species distribution is delineated in grey. (b and c) Micrographs of a cross section of a white spruce needle central vascular cylinder and outer cell layers respectively. Sections were stained with safranin and fast green, scale bars are 50 µm (d) Pearson's r correlations of needle anatomical variables and climate of provenance, dark boxes indicate significant correlations after adjustment using Benjamini & Hochberg's correction for multiple comparisons.



(c)

Experiment progression

	Dehydration Severity	Ecozone
Fresh	N/A	
15-hour dehydration		
→**Outflux**	Mild < Severe	
Dehydrated	Mild > Severe	BC < BP&TM
Foliar misting with DI water		
←**Influx**	Mild > Severe	BP > BC&TM
Re-Wet 30 min	Mild > Severe	BC < BP&TM
←**Influx**	Mild > Severe	
Re-Wet 60 min	Mild > Severe	BC < BP&TM
←**Influx**	Mild < Severe	
Re-Wet 240 min	Mild > Severe	BC < BP&TM
↔ **Net flux**	Mild > Severe	

Figure 5.2: (a) Relative water content (%) of spruce needles at various timepoints; (b) water flux (mL m^{-2} s^{-1}) between timepoints. Different letters indicate significant differences ($p < 0.05$) between provenances within the same level of dehydration; and (c) summary of the significant differences ($p < 0.05$) observed between the two dehydration levels and the ecozones for both relative water content and water flux for Boreal Cordillera (BC), Boreal Plains (BP), and Temperate Mixedwood (TM) A black cell outline delineates a putative ecophysiological difference highlighted in the discussion.

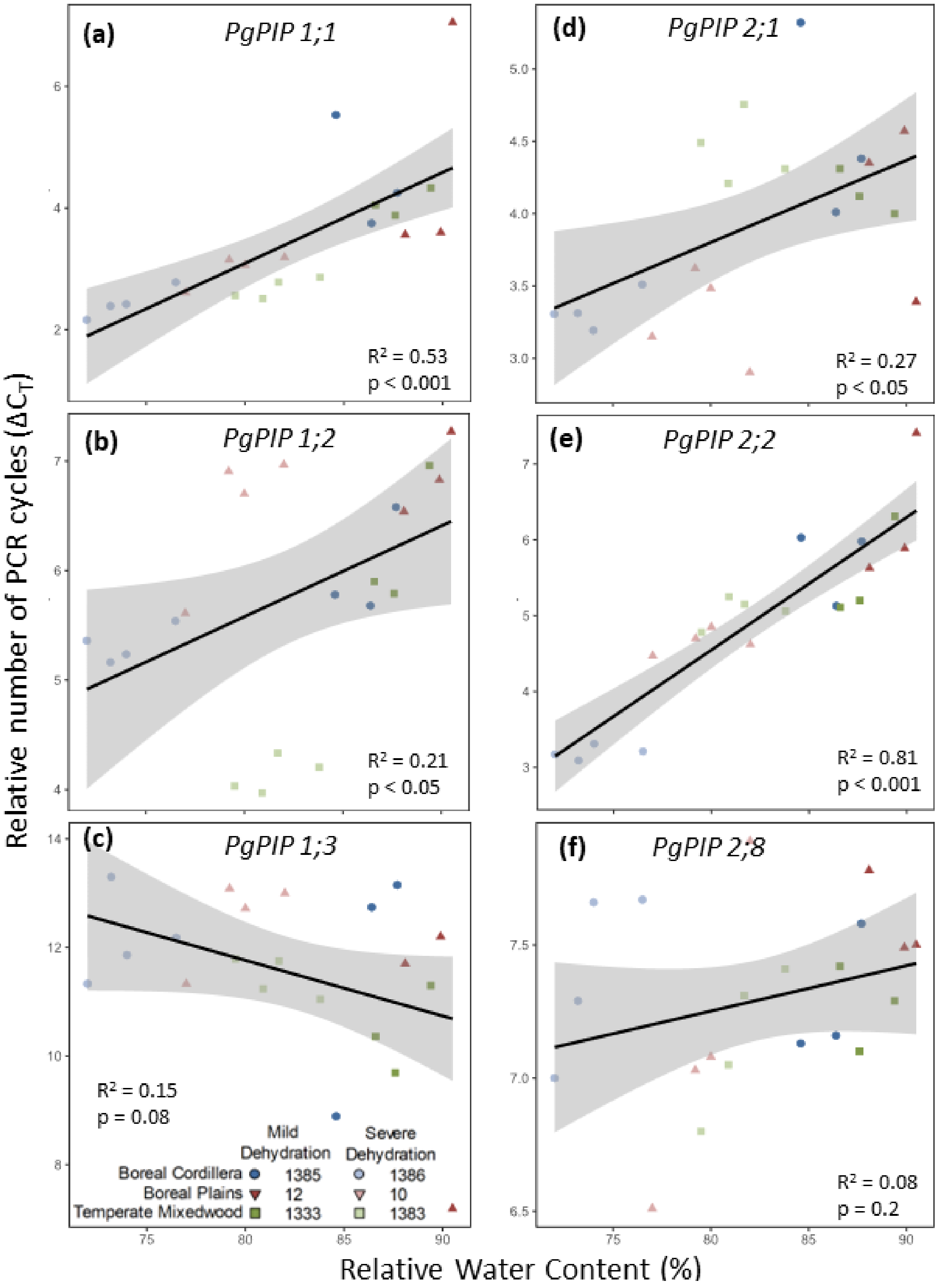


Figure 5.3: Linear regressions of the relationship between the relative transcript abundance of aquaporins expressed in white spruce needle tissue during foliar water uptake and needle relative water content. Needle tissue was obtained from common garden grown Boreal Cordillera, Boreal Plains, and Temperate Mixedwood provenance twigs subjected to two different levels of dehydration prior to foliar misting. Aquaporins are *PgPIPs 1;1, 1;2, 1;3, 2;1, 2;2*, and *2;8* (a-f respectively), regression p-values and coefficients of determination are shown.

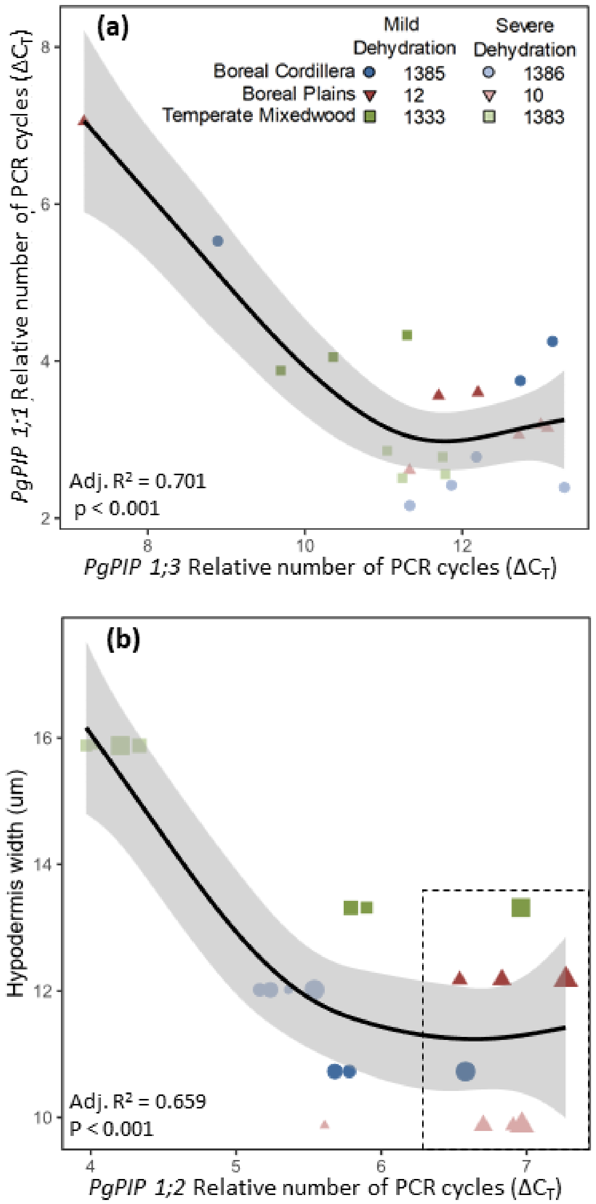


Figure 5.4: Generalized Additive models of the relationship between the relative transcript abundance of aquaporins *PgPIP 1;1* and *PgPIP 1;3* (a) and between the radial thickness of the hypodermis and the relative transcript abundance of aquaporin *PgPIP 1;2* (b) in white spruce needles. Tissue originates from common garden grown Boreal Cordillera, Boreal Plains, and Temperate Mixedwood provenance twigs subjected to two different levels of dehydration prior to foliar misting. Adjusted coefficients of determination are shown, and in (b) symbols scale with wetting time (0,30, 60, and 240) and the dashed box highlights downregulated expression of *PgPIP 1;2*.

5.9 **Supplementary Information**

5.9.1 Tables

Table 5.S1: Primer sequences of targeted PIP aquaporins and housekeeping genes used for RTqPCR analysis.

Target Gene	Direction	Primer (5' to 3')	Publication of origin
PgPIP 1;1	Forward	TGCAACAATTCCCATCACCGGAAC	(Laur & Hacke, 2014)
	Reverse	TGATGGCAGCTCCCAAACTTCGAG	
PgPIP 1;2	Forward	TCCTAGAAACAGCCCAGCGTATCG	
	Reverse	ACACATGCGCTAACAGACCTCAGC	
PgPIP 1;3	Forward	TCATCAGCTCATCATCCGAGCCATAC	
	Reverse	AACAGCCCAAACGAGAAGAGACTGA	
PgPIP 2;1	Forward	TAGGCAGCAGCTAATGCAGCTCC	
	Reverse	GCCACAAACAATCCTGGGATGCC	
PgPIP 2;2	Forward	AGGGTAGCTTCTCTCCGAACCTTGA	
	Reverse	AAACATCCATCGCCCTCTCTGACG	
PgPIP 2;8	Forward	TGCTGCGATTGCATCAGCCTAC	
	Reverse	AACACTGCGGAAAGAACCCAAGG	
ELF-1α	Forward	CCCTTCCTCACTCCAACTGCATA	(Mageroy *et al.*, 2017)
	Reverse	TCGGCGGTGGCAGAGTTTACATTA	
Alpha-tubulin	Forward	GGGCGATGAGGATGAGGGCG	(Boyd & Nkongolo, 2021)
	Reverse	GCAAGCCCATGTCCCAAAACCA	

5.9.2 Figures

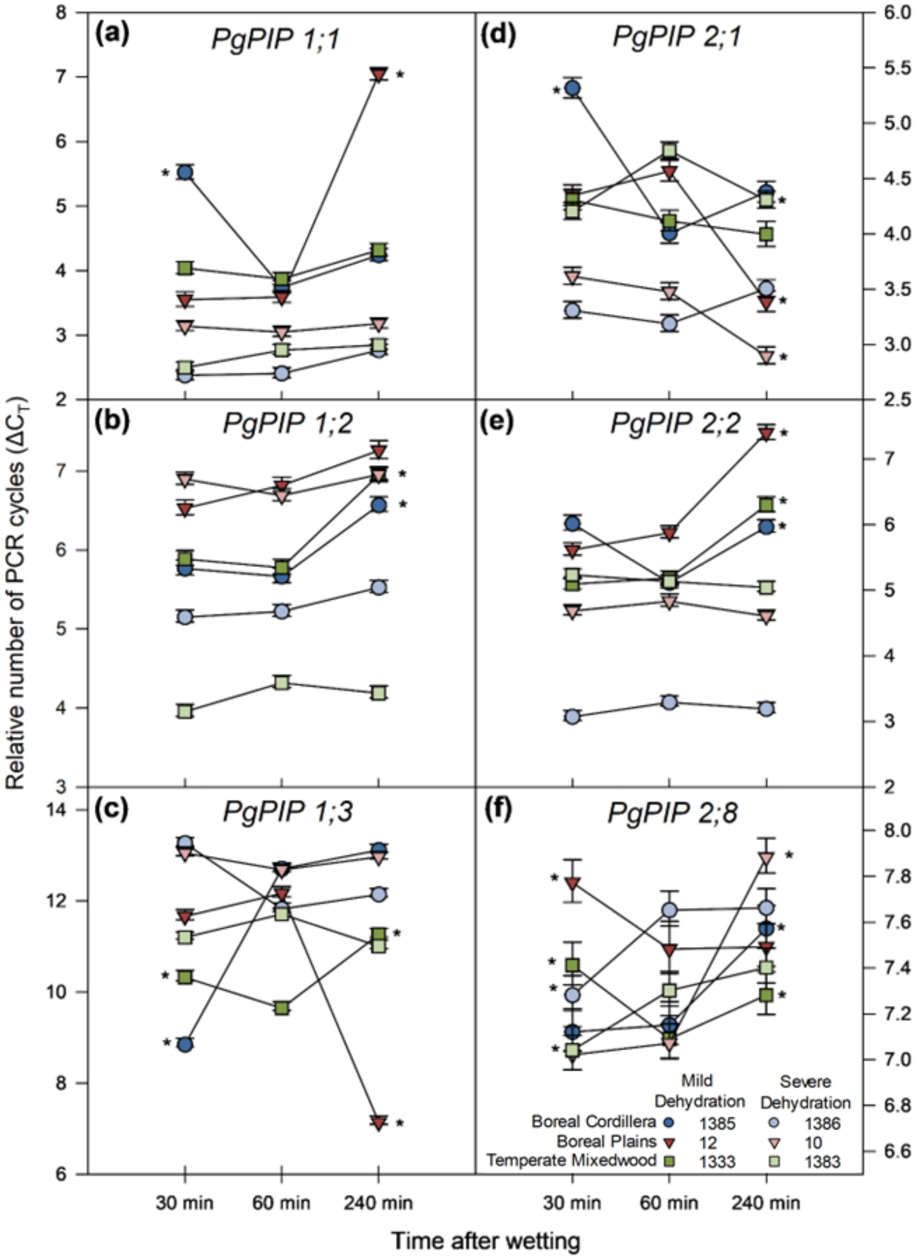


Figure 5.S1: Relative transcript abundance of aquaporins expressed in needle tissues during foliar water uptake. Tissue originates from common garden grown Boreal Cordillera, Boreal Plains, and Temperate Mixedwood provenance twigs subjected to two different levels of dehydration prior to foliar misting. Aquaporins are *PgPIPs 1;1, 1;2, 1;3, 2;1, 2;2,* and *2;8* (a-f respectively), “*” indicates significant down- or up-regulations ($p < 0.05$) of aquaporins relative to the 60 min after wetting timepoint within individual provenances.

6 General Discussion and conclusions

6.1 The effects of oil sands pore water on tree stress physiology.

Prior research into plant and tree stress responses to oil sands tailings has often focused on the depth and composition of substrate layers to be placed on top of the tailings for reforestation (Sorenson *et al.*, 2011; Pinno *et al.*, 2012; Macdonald *et al.*, 2015; Zhang *et al.*, 2020, 2023). However, as the upwards leaching of tailings water into the root zone remains a possible concern particularly in waterlogged soils (Utting, 2021), both *Chapters 2* and *3* of this thesis examined responses of various boreal forest tree species to oil sands release water. The analysis of the liquid phase of NST revealed levels of naphthenic acids, boron, and sodium, similar to those found in the solid NST tailings, and which are known to be detrimental to the growth and establishment of plants (Zhang *et al.*, 2020).

To simulate waterlogged conditions, the hypoxia treatment (< 3 mg O_2 l^{-1}) tested in *Chapter 2*, showed that, although greater transpiration-driven uptake of sodium and boron occurs under aerobic conditions, the additional deleterious effects caused by hypoxia lead to greater mortality and reductions in photosynthesis, growth, and plant dry weights. In addition, root hypoxia appears to disproportionately aggravate the effects of NST in the more salt stress tolerant tree species such as trembling aspen (*Populus tremuloides*). Given that trembling aspen is the most wide-ranging tree species in North America and future warming climatic predictions show that the aspen parkland climates could become dominant in the Athabasca oil sands region (Gray *et al.*, 2011), this Na sequestration mechanism was also further investigated in *Chapter 3*. The results show that Na sequestration appears to involve a Ca-dependent membrane selectivity of K over Na at the root level, and foliar iron levels suggest a potential role of vacuolar Fe transporters in the maintenance of cell homeostasis. Finally, when trembling aspen plants are subjected to hypoxic soil conditions, this moderate Na tolerance appears to be diminished by the levels of boron and naphthenic acids that are present in tailings.

6.2 Factoring climatic variables into tree seed source selection for oil sands land reclamation.

Given the ongoing and expected future increase in average air temperatures across the Boreal Forest, successful long-term reclamation of disturbed Athabasca oil sands areas may also involve the climatic resilience of plants. Here, research has often focused on the strong potential of southern seed provenances for planting further north with the only caveat that they may be potentially less cold hardy and thus more vulnerable to frost events. For instance, while southern white spruce (*Picea glauca*) populations have generally been shown to have superior growth in common garden experiments, an important intraspecific and potentially limiting tradeoff between fall cold hardiness and tree growth was reported (Sebastian-Azcona *et al.*, 2019). As more southern seedstock tend to exhibit shorter winter dormancy and thus can take advantage of a longer growing season, they become more vulnerable to late fall or early spring frost events. However, the scientific debate has largely ignored the impact of soil temperatures on growth. It should be expected that tree growth may be restricted by soil temperature, particularly in the early spring, and that trees from southern provenances with shorter dormancy may thus not be able to take full advantage of the growing season when planted further north. In this thesis, the topic of soil temperature as a limiting factor for tree growth was discussed at length in *Chapter 4*.

Indeed, regardless of availability, low non-freezing temperatures reduce aquaporin trans-membrane water transporter activity and membrane permeability which limits root water uptake, reducing productivity and, if maintained over a prolonged period, lead to stunted growth (Wan *et al.*, 1999; Landhäusser *et al.*, 2001; Körner & Hoch, 2006). Low soil temperature stress is often considered to be a drought-like stress, as secondary responses include an immediate closure of the stomata due to decreased water delivery (Landhäusser *et al.*, 1996; Wan *et al.*, 2004). However, *Chapter 4* suggests that, contrary to a drought, as water in the soil remains present, anisohydric plants could be better adapted to prolonged low soil temperatures in terms of maintaining their productivity. If so, then low soil temperature adaptation could explain the lack of stomatal control that has been observed in northern boreal *Pinus contorta* (lodgepole pine) populations (Isaac-Renton *et al.*, 2018). Under the hypothetical scenario of an assisted migration using a more drought resistant isohydric southern population, this tradeoff could be a limiting factor to their productivity.

Chapter 5 digs further into the interaction between seed source provenance and climatic variables through an investigation of the foliar anatomy and foliar water uptake abilities of white spruce, an important species for oil sand reclamation, using a range-wide common garden experiment established near Athabasca. The results show that, in contrast with the south and eastern end of the range, populations from the colder and drier northern and western regions appear to have less hydraulically resistive radial foliar anatomies, and less stomatal control (Sinclair, 2019). In these populations hydraulic risk appears to be mitigated through lower rates of water use partly through reduced leaf area and biomass. However, northern Boreal Plains ecozone populations appear to circumvent this tradeoff as they are also able to maintain leaf area and biomass equal to that of the eastern populations in the field despite showing most of the same anisohydric and foliar anatomical characteristics of the far northwestern populations. My results suggest that this could partly be due to greater use of foliar water uptake facilitated by their less hydraulically resistive radial foliar anatomy and mediated by a tight control of *PIP* aquaporins expression patterns.

6.3 **Implications for the Athabasca oil sands and boreal reforestation.**

Taken all together, the results of this thesis provide important information about potential reclamation risks and future avenues of boreal reforestation research. First, avoiding or mitigating the development of hypoxic conditions in root zones where tailings water or soils of similar chemical composition are present is vital, as hypoxia will interfere with root and leaf ionic toxicity tolerance mechanisms. Then, lowering the pH of tailings may not be more beneficial for plant growth if elevated concentrations of naphthenic acids (> 60 mg/L) are present. Furthermore, the phytotoxicity of naphthenic acids at these levels is severe, even in trembling aspen which has been shown to take up and incorporate it into the tissues (Kamaluddin & Zwiazek, 2002b). Thus, establishing pre-reclamation treatments, such as ozone treatments (Brown & Ulrich, 2015), that would partly degrade or remove naphthenic acids, would likely significantly improve revegetation success of the oil sands sites affected by mine tailings. Finally, it is hypothesized that productivity maintenance at low soil temperatures, particularly at the start of the growing season may be an evolutionary driver skewing the water use trade off curve towards anisohydric traits and behaviors in northern tree populations at the expense of leaf area and biomass. In this respect, the ability to benefit from foliar water uptake in certain populations appears to be a potential way to “cheat” this tradeoff and maintain elevated biomass. Indeed, foliar water uptake likely merits greater consideration in boreal forest management and foliar wetting days should also be considered for forest modeling purposes. Nevertheless, while this behavior may remain advantageous under moderate drought stress, given that it requires a less hydraulically resistant foliar anatomy it could lead to significantly more vulnerability under future severe or prolonged drought conditions. Under the lens of a northwardly assisted migration, the less hydraulically risky strategies exhibited by more southern and eastern white spruce populations appear to remain a safer bet for long-term revegetation success.

6.4 References

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