# Effects of Elevated Root Zone pH and NaCl on the Stress Responses and Recovery of Selected Boreal Forest Tree Species

by

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

Doctor of Philosophy

in

Forest Biology and Management

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## Abstract

Prior to surface mining of bitumen in the Athabasca oil sands region of northern Alberta, all vegetation and soil is removed from the boreal forests where the mines are located. Revegetation following mine closures is challenging, partly due to high soil pH and elevated Na levels present in many reclamation sites. My thesis addresses some of these revegetation concerns. In the first study, trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) were subjected to three levels of pH (5, 7, 9) and three levels of NaCl (0, 30, 60 mM) in a factorial design in hydroponic culture. Aspen was relatively tolerant of 30 mM NaCl treatments at pH 5 and 7, but showed decreases in dry weights, leaf chlorophyll concentrations, photosynthesis, and transpiration. Green alder was sensitive to elevated pH and NaCl because moderate increases of pH and NaCl caused significant physiological decline. Tamarack exhibited declines in dry weights, chlorophyll concentrations, photosynthesis, and transpiration as pH and NaCl levels increased. White spruce showed no changes in dry weights from elevated pH and NaCl; however, elevated NaCl levels caused declines in photosynthesis and transpiration. All species showed decreases in foliar nitrogen, dry weights, foliar chlorophyll concentrations, photosynthesis, and transpiration as a result of increased stress. I hypothesized that seedlings exhibited stunting of growth and downregulation of metabolism as a result of elevated pH and NaCl. In the second study, aspen and white spruce seedlings were maintained at two pH levels (5 and 8) with two levels of NaCl (0 and 30 mM). Another group of seedlings was subjected to the same pH and NaCl levels, but N supply was increased by 4x. Supplementation with 4x N caused a partial recovery of photosynthesis and transpiration in aspen exposed to elevated NaCl but had no effect on seedlings exposed to elevated pH. White spruce seedlings exposed to elevated pH and NaCl exhibited decreases in net

photosynthesis. Supplementation with 4x N had no effects on photosynthesis in seedlings exposed to elevated NaCl but caused further declines in net photosynthesis of seedlings exposed to elevated pH. The study suggests that supplemental N can partially recover photosynthesis and transpiration in aspen exposed to elevated NaCl. In the third study, recovery from NaCl stress was investigated in aspen, tamarack, and white spruce seedlings subjected to 0, 50, and 100 mM NaCl treatments in soil. Most seedlings treated with 50 mM NaCl showed a return to non-stress levels for dry weight, foliar chlorophyll, photosynthesis, and transpiration after 30 days of recovery. Recovery after 60 days from 100 mM NaCl varied between species. Some aspen seedlings completely defoliated during the stress period and re-flushed during the recovery period. After 60 days of recovery, the new leaves exhibited higher levels of chlorophyll, photosynthesis, and transpiration compared to untreated controls. Following 60 days of recovery from 100 mM NaCl treatment, the dry weight, chlorophyll, and photosynthesis values in tamarack were lower compared with control seedlings, whereas white spruce showed no changes. Both aspen and tamarack exhibited increased foliar necrosis and K in response to NaCl stress, suggesting that both processes are important for NaCl stress and recovery. In the fourth study, NaCl stress was applied to aspen, tamarack, and white spruce seedlings, which were grown in soil and were first subjected to non-lethal NaCl stress followed by overwintering. Seedlings were then subjected to NaCl stress in the second year. Plants of all three species exhibited some form of salt injury in the first year. Aspen and tamarack seedlings treated with 50 mM NaCl in year one exhibited lower dry weights compared to non-treated control in year one. Both species exhibited elevated foliar K in response to NaCl treatment in year two. Tamarack seedlings exposed to NaCl in year one exhibited increases in photosynthesis and water use efficiency when exposed to NaCl in year two compared to control seedlings that were not treated

with NaCl in year one. For white spruce, NaCl treatment in year two had no effect on any measured parameters. Taken together, the results suggest that aspen, tamarack, and white spruce can tolerate moderate levels of elevated pH and NaCl and they can also recover from moderate levels of NaCl stress.

**Acknowledgements**

I am grateful for the guidance, support, and encouragement from my supervisor, Dr. Janusz Zwiazek, as well as the members of my supervisory committee, Dr. Simon Landhäusser and Dr. Barb Thomas. I am also grateful for the help and support from members of the Zwiazek lab, including: Ale Equiza, Seong Hee Lee, Wenquing Zhang, Frank Tan, Deyu Mu, Hao Xu and Samantha Olivier. This research was funded by research grants held by Janusz Zwiazek from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Total E&P Canada Ltd.

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## Chapter 1: General Introduction

### 1.1 The boreal forest and oil sands operations in Northern Alberta

The boreal forest biome of Northern Canada is one of the largest intact ecosystems in the world and covers approximately one third of Canada's total land area (Brandt 2009). Ecosystem productivity is primarily limited by long cold winters and short cool summers (Bonan & Shugart 1989). Cold temperatures lead to slow rates of decomposition and nutrient cycling resulting in low soil nutrient availability, particularly N (Vitousek & Howarth 1991; Lupi et al. 2013). In the past century, the boreal forest of Northern Alberta has seen a dramatic increase in human activity because of the extraction of natural resources such as wood, oil and coal. The oil sands deposits of Northern Alberta are one of the largest oil deposits in the world and also have a heavy human footprint because approximately 4800 $km^2$ of boreal forest lands are feasible for surface mining operations (Berkowitz & Speignh, 1975; Masliyah et al., 2004). This process is especially destructive to the boreal forest because it involves the removal of all vegetation and removal of soil for the extraction of bitumen (Berkowitz & Speignh, 1975). Oil producers are required by law to reclaim disturbed lands to equivalent land capability which should ultimately require minimal human intervention (Government of Alberta, 2010). Reclamation is particularly difficult because the extraction of bitumen results in soils with elevated pH and salts compared to soils found naturally in the boreal forest (Howat 2000). The processes which create elevated pH and salts on reclamation sites will be discussed in more detail below.

Surface mining for bitumen first involves the complete removal of boreal forest vegetation followed by the salvaging and stockpiling of soil organic and mineral layers for future reclamation (Mackenzie & Naeth 2010). After soil salvage, overburden material above bitumen deposits is removed and placed in waste dumps and can be used as a subsoil for future reclamation (Grant et al. 2008). Overburden material has natural sources of salts from saline-sodic clay shale from the Clearwater geologic formation (Lazorko 2008). Sands containing bitumen are then mined and transported to an extraction plant. Bitumen is separated from sand using hot water and NaOH which increases the sodicity and pH of the tailings sand (Misliya et al. 2004). Both tailings sands and overburden can be used as subsoil during the reclamation process. Hill slope landforms are created to give sites heterogenous exposure, slope, and hydrology (Leatherdale et al. 2012). The depth of subsoil used on each site is dependent on the

salinity and pH of the overburden material (Rowland et al. 2009). Salvaged organic topsoil such as forest floor mineral mix (FMM) or peat mineral mix (PMM) is placed atop the subsoil to increase water holding capacity as well as nutrient content (Rowland et al. 2009). Sodium from saline-sodic overburden and tailings is known to translocate into cover soil via capillary action (Jorenush & Sepaskhah 2003). Sodium bicarbonate can then form and hydrolyze to further increase soil pH (Marschner 2011). Leaking of tailings ponds may also contribute to elevated soil pH and Na (Tenenbaum 2009). Finally, soil Na levels can be heterogeneous and transient on reclamation sites due to variations in evapotranspiration, precipitation, water table depth, and upward water flux (Kessler et al. 2010; Carrera-Hernandéz 2012). Taken together, these processes create reclamation sites with elevated soil pH and Na which may hinder or preclude revegetation (Howat 2000; Alberta-Environment 2010). For example, surface mining reclamation sites typically exhibit soil pH ranges from 7.0 to 8.5 whereas the pH of undisturbed boreal forest ecosystems are typically below 6.0 (Howat 2000). These adverse conditions have negative physiological impacts on trees commonly used for reclamation of oil sands surface mines in Northern Alberta.

### 1.2 Effects of elevated pH and NaCl on boreal forest tree species

Alkaline soils have generally been linked to problems with reduced plant growth and nutrient availability. Elevated soil pH negatively affects plants by increased levels of [$OH^-$], which affects the solubility of some nutrients (Zieslin & Snir 1989; Kopittke & Menzies 2004; Comerford 2005; Brady and Weil 2008). Soil N can be also negatively affected by high soil pH due to decreased microbial mineralization (Marschner 2011). Plants grown in alkaline soils are known to develop chlorotic young leaves due to low Fe solubility at high soil pH (Kosegarten et al., 2001; Boukhalfa & Crumbliss 2002; Tang et al., 2006). Alkaline soils also negatively affect the availability of P, Mn, and Zn (Melton et al., 1973; Parker and Walker 1986; Brady and Weil 1996; Valentine et al. 2006; Marschner 2011). In several species of boreal forest plants, elevated soil pH has been shown to decrease root water flux, net photosynthesis, transpiration, and growth (Renault et al. 1999; Kamaluddin & Zwiazek, 2004; Siemens & Zwiazek, 2011; Zhang et al. 2013).

Elevated soil NaCl imposes two types of stresses on plants; an immediate osmotic stress followed by an ionic stress, which occurs when NaCl enters the shoot tissues (Munns & Tester,

2008). Osmotic stress upsets water balance in plants, which results in a relatively rapid inhibition of shoot growth, reduced emergence of new leaves as well as decreased leaf expansion, lateral bud development, net photosynthesis, and transpiration. The consequences of ionic stress take longer time to manifest and are characterized by chlorosis and senescence of older leaf tissues (Munns & Tester, 2008). The topic of NaCl stress in plants is covered in more detail in Appendix five.

Studies on the combined effects of elevated soil pH and Na are limited. A study on barley showed that elevated soil Na caused lower rates of net photosynthesis, transpiration, and growth. However, the combined effects of elevated soil pH and Na caused further declines in these parameters with increased foliar Na and root electrolyte leakage (Yang et al. 2009). A study testing the combined effects of elevated soil pH and NaCl on American elm seedlings showed that elevated NaCl caused declines in transpiration and chlorophyll concentration, but elevated soil pH had relatively no effect on these parameters (Calvo-Polanco et al. 2009). Clearly, more studies are needed to tease out the combined effects of elevated pH on NaCl stress on plants.

### 1.3 Biology of studied tree species

Several predominant boreal forest tree species were examined in this project including trembling aspen (*Populus tremuloides* Michx.), green alder (*Alnus viridis* (Chaix.) D.C.), tamarack [*Larix laricina* (Du Roi) K. Koch], and white spruce [*Picea glauca* (Moench) Voss]. Their biology and tolerance are briefly described below.

Trembling aspen (*Populus tremuloides*)

Trembling aspen is a small to medium sized, fast-growing, shade intolerant, pioneer species that is widely distributed throughout North America. It is short lived and can propagate through seeds or suckers. Due to rapid growth and nutrient uptake, it has high ecosystem value for recycling nutrients (Boyle 1973; Alban 1982; Shepperd 1986; Perala 1990). It grows best on well drained loamy soils with high organic matter and nutrient content as well as soils with silt plus clay content of 80% or more. It grows poorly on sandy soils due to limited moisture and nutrients and heavy clay soils due to limited water availability and oxygen availability (Perala 1977; DeByle 1985). It often dominates in an early successional ecological phase and is slowly replaced by conifer species over time. Mixedwood stands of trembling aspen and white spruce

are common in Northern Alberta where aspen acts as a nurse species for white spruce (Perala 1990; Man & Lieffers, 1999).

Green alder (*Alnus viridis*)

Green alder is a fast growing, light demanding, large deciduous shrub or small tree with a height ranging from three to 12 m. It is widely distributed throughout cooler climates in North America and can be found in harsh locations such as course texture soils or sandy hills and near wetlands or streams. It is semi shade tolerant and can establish well after a fire (Soper and Heimburger 1994; Straker 2010). It is especially useful on nutrient poor soils as it can fix nitrogen in root nodules. This process can add significant amounts of nitrogen into the soil through leaf litter or decomposition of roots (Crocker and Major 1955; Tarrant and Trappe 1971). These qualities make it a good candidate for further study for reclamation.

Tamarack (*Larix laricina*)

Tamarack is a medium sized deciduous conifer distributed throughout North America. It is a hardy species and can grow in a variety of ecozones, soil types, and soil moisture regimes. It is fast growing in full sunlight but is very shade intolerant and is not found on sites with high competition for light from other species. Therefore, tamarack is commonly found on poor sites such peatlands, extremely dry and calcareous soils, or burnt organic soils after a fire (Rowe 1973; Eyre 1980; Johnston 1990). Tamarack can be found with many other tree species such as: balsam fir, balsam poplar, black spruce, jack pine, trembling aspen, red-osier dogwood, and white spruce. When tamarack is present on poor sites with full sunlight, they can outgrow many other species (Fowells 1965; Johnston 1990). In the boreal forest, it is typically succeeded by black spruce (Fowells 1965).

White spruce (*Picea glauca*)

White spruce is a slow growing medium to large sized tree with moderate shade tolerance that is distributed throughout Canada and Alaska. It is found in a variety of soil types and nutrient regimes throughout the boreal forest where it endures temperature extremes from -50 to 34 °C (Maini 1966). White spruce can grow on a wide range of soil pH, including alkaline soils. It is known to lower soil pH by introducing acidic needles to the forest floor (Sutton 1969; Stiell 1976; Brand et al. 1986; Nienstaedt and Zasada 1990). White spruce begins producing cones and

viable seeds as early as four years old and can also reproduce clonally by layering (Sutton 1969; Densmore 1980). White spruce is commonly shallow rooted with a rooting depth range of 90 to 120 cm. Tap roots can reach a depth of three m (Nienstaedt and Zasada 1990). White spruce can be found with co occurring species such as balsam fir, black spruce, jack pine, lodgepole pine, snowberry, red-osier dogwood, and trembling aspen (Sutton 1969).

### 1.4 Objectives and Hypotheses

The following objectives and hypotheses are explored in the following chapters:

Chapter 2:

The overall objective of this study was to generate fundamental knowledge concerning the effects of elevated root zone pH and NaCl on the physiology of the selected boreal forest tree species. In particular, this study focused on separating both pH and NaCl stresses by subjecting seedlings to various pH and NaCl levels that are commonly found at oil sands reclamation sites in Northern Alberta. It was hypothesized that elevated pH and NaCl would cause physiological declines for all species.

Chapter 3:

From Chapter two, elevated root zone pH and NaCl caused decreases in foliar N, leaf chlorophyll concentrations, net photosynthesis, transpiration rates, and growth for all species tested. Therefore, supplemental N was given to trees subjected to elevated root zone pH and NaCl. It was hypothesized that supplemental N would recover the physiological functions of trembling aspen and white spruce from elevated root zone pH and NaCl.

Chapter 4:

The objective of this study was to examine the recovery of physiological functions in trembling aspen, tamarack, and white spruce following exposure to NaCl stress. It was hypothesized that physiological functions would increase for all species during the recovery period; however, the degree and timing of recovery was unknown.

Chapter 5:

The objective of this study was to apply non-lethal levels of NaCl to trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) seedlings in one growing season. Seedlings were overwintered and again subjected to NaCl stress in a second growing season. I then studied weather sub-lethal levels of NaCl during the first year of growth caused acclimation or cumulative salt injury when exposed to overwintering followed by NaCl treatment in the second year. It was hypothesized that NaCl stress and overwintering in year one would hinder the ability of seedlings to recover from NaCl stress in year two. The question of the importance of chlorosis and necrosis of foliar tissue for plant survival was especially interesting since both trembling aspen and tamarack are deciduous plants and lose their foliage before winter whereas white spruce can maintain needles for many years. The process of chlorosis was studied in more detail in trembling aspen leaves.

Appendix 5

Finally, in Appendix five that is placed following the main body of the thesis, I put together my thoughts based on the literature review that was focused on linking whole plant physiology to cell physiology in relation to NaCl stress in glycophytes. In this review, I focus on plant responses that may constitute salinity survival mechanisms that I would like to explore in my future research work. Furthermore, I describe a potential cell signalling cascade that is shared by many cell types to elicit the physiological responses.

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## Chapter 2: Elevated pH and NaCl in the root zone decrease foliar nitrogen, chlorophyll, and physiological performance in trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*).

### 2.1 Introduction:

The Athabasca oil sands region of north-eastern Alberta contains one of the largest deposits of bitumen in the world (Berkowitz and Speignh, 1975; Masliyah et al., 2004). Surface mining for bitumen is preceded by a removal of all vegetation and soil from the native boreal forest land (Berkowitz and Speignh, 1975). Oil producers are required by law to reclaim disturbed lands to equivalent land capability which should ultimately require minimal human intervention to sustain (Government of Alberta, 2010). However, reclamation soils are challenging to revegetate because some soils are left with elevated pH and Na levels after the bitumen extraction process (Dai and Chung, 1996; Masliyah et al., 2004). Consequently, soils within reclamation areas are reported to have pH values which exceed eight and salinity values exceeding four dS $m^{-1}$ (Howat 2000; Kessler et al., 2010). These unfavorable soil conditions pose a major problem for revegetation because boreal forest plants are sensitive to both elevated soil pH and NaCl (Howat, 2000).

Unfavorable soil conditions such as alkaline soils and elevated soil NaCl can negatively affect plant growth and survival. Plants grown in alkaline soils have generally been linked to problems P and Fe uptake as well as the development of chlorotic leaves within younger tissue due to decreased Fe uptake (Kosegarten et al., 2001; Tang et al., 2006). In boreal forest species, elevated pH causes decreased root water flux, photosynthesis, transpiration, and growth (Renault et al. 1999; Kamaluddin and Zwiazek, 2004; Siemens and Zwiazek, 2011; Zhang et al. 2013). Plants grown in elevated soil NaCl imposes two types of stress on plants; an immediate osmotic stress followed by an ionic stress which occurs when NaCl enters the shoot tissue (Munns and Tester, 2008). Osmotic stress upsets water balance in plants, which results in a relatively rapid inhibition of shoot growth, reduced emergence of new leaves as well as decreased leaf expansion, lateral bud development, photosynthesis, and transpiration. The consequences of ionic stress take longer time to manifest and are characterized by chlorosis and senescence of older leaf tissues (Munns and Tester, 2008). Although boreal trees are considered relatively

sensitive to elevated soil pH and NaCl, different tree species vary in their tolerance levels of these soil conditions. Studies on the combined effects of elevated soil pH and Na are limited. A study on barley showed that elevated soil Na caused lower rates of photosynthesis, transpiration, and growth. However, the combined effects of elevated soil pH and Na caused further declines in these parameters with increased foliar Na and root electrolyte leakage (Yang et al. 2009). A study testing the combined effects of elevated soil pH and NaCl on American elm seedlings showed that elevated NaCl caused declines in transpiration and chlorophyll concentration, but elevated soil pH had relatively no effect on these parameters (Calvo-Polanco et al. 2009). More studies are needed to tease out the combined effects of elevated pH on NaCl stress on plants.

Trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) were chosen for this study because they are commonly found in the boreal forest region of northern Alberta. Moderate tolerance to elevated pH has been reported for trembling aspen, tamarack, and white spruce which have shown physiological decline between root zone pH 7.5 and 9 (Maynard et al. 1997; Renault et al. 1999; Zhang et al. 2013. Additionally, moderate tolerance to elevated salinity has been reported for trembling aspen, tamarack, and white spruce. Trembling aspen and white spruce grown in liquid culture showed 100% survival after four weeks of exposure to 60 mM NaCl (Renault et al. 1999). Tamarack was shown to withstand 60 mM NaCl for 40 days (Renault, 2005). Little is known about the effects of elevated pH and NaCl on green alder. However, it has been shown that green alder slightly lowers soil pH and increases soil N concentration, which subsequently fosters understory plant growth (Rhoades et al. 2001).

The overall objective of this study was to generate fundamental knowledge concerning the effects of elevated root zone pH and NaCl on the physiology of trembling aspen, green alder, tamarack, and white spruce. This information is needed to facilitate successful revegetation of boreal forest lands disturbed by bitumen mining operations in northern Alberta. In particular, this study focused on separating both pH and NaCl stresses by subjecting seedlings to pH levels of 5, 7, and 9 as well as NaCl levels of 0, 30, and 60 mM in a factorial design commonly found at reclamation sites (Howat 2000; Kessler et al., 2010). It was hypothesized that both elevated pH and NaCl would cause physiological declines for all species.

## 2.2 Materials and Methods:

### 2.2.1 Plant material and growth conditions

One-year-old dormant trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) seedlings were obtained from Smoky Lake Forest Nursery (Smoky Lake, AB, Canada). Plant material was produced from seeds collected from open-pollinated wild tree stands in various locations within Alberta seed zone CM 2.2 by Tree Time Services Inc. (Edmonton, AB, Canada). Trembling aspen and white spruce were transported to the University of Alberta on December 19$^{th}$, 2013 and placed into liquid culture on December 21$^{st}$, 2013.Tamarack and green alder were transported to the University of Alberta on March 21$^{st}$, 2014 and placed into liquid culture on April 4$^{th}$, 2014. Seedlings were stored in a refrigerated room at 4°C in the dark until the experiment began. Seedlings of uniform height and root collar diameter were selected (Tables 3.S2, 3.S3), their roots were washed free of potting medium and placed in aerated solution culture maintained at pH 5 and 0 mM NaCl for two weeks before treatments began to break dormancy. Experiments were conducted in a controlled environmental growth room maintained at 22/18 °C (day/night) temperature, 65 ± 5 % relative humidity, and 16-h photoperiod with 300 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) using full spectrum fluorescent lights (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). The solution culture set-up was previously described by Zhang et al. (2013). An individual unit consisted of two 30 L opaque plastic tubs with Styrofoam covers containing 20 holes so seedlings could be placed in the nutrient solution. Each tub was attached to an aerated 120 L opaque plastic reservoir with a circulating pump (Model 9.5 950GPH, Danner MFG Inc., NY, USA). Solution medium consisted of 120 L of 25 % Hoagland's solution and was changed once every two weeks throughout the experiment (Epstein 1972). Solution pH was continuously maintained at the desired level with a pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) and Orion 9106 BNWP gel-filled combination pH electrode that were immersed in the nutrient solution (Thermo Scientific, Rochester, NY). The pH was automatically adjusted using small volumes of 5% KOH with an electrode valve (Model 8260G071 120/60 ASCO Valve Inc., Florham Park, NJ, USA) connected to a plastic ball valve (Model R-01377-84, Cole-Parmer Canada Inc., Montreal, QC). The pH fluctuations were

approximately ±0.2 from their set values. A photograph and overhead schematic of the experimental setup can be found in Appendix six (Figure a6.1).

### 2.2.2 Treatments

Experiments were run separately with two tree species at a time. Trembling aspen and white spruce were tested in one experiment whereas green alder and tamarack were tested in a second experiment. Each treatment started with 20 replicates per species. Individual seedlings were placed into the Styrofoam cover holes so the roots were submerged in Hoagland solution. Foam plugs were placed in the holes to hold the stems in place. Species were alternated when placed in the Styrofoam cover holes (Figure a6.1 B). Replicates that died during the experiment because of stress were immediately removed. At the onset of the experiment, both pH and salinity were gradually elevated over seven days to reduce shock to the trees. Seedlings were maintained at three pH levels (5, 7, and 9) with three NaCl levels (0, 30, and 60 mM) in a factorial design for a total of nine treatments. Seedlings were then maintained at their respective treatments for 50 days prior to harvest. It should be noted that balsam poplar, jack pine, black spruce, and paper birch were tested in this study but performed poorly in liquid culture. It was decided to not include these species in further analysis because of high mortality in control treatments.

### 2.2.3 Measurements

#### 2.2.3.1 Gas exchange

After 50 days of treatment, seedlings were randomly taken from each treatment for gas exchange measurements. Typically, six seedlings were taken from each treatment; however, higher stress treatments produced increased mortality in trembling aspen and green alder. In some cases, only three seedlings remained and were used for measurements. Net photosynthesis (Pn) and transpiration (E) rates were measured using an infrared gas analyzer equipped with a standard 6 $cm^2$ leaf chamber (Li-Cor 6400XT, Li-Cor Inc., Lincoln, NE, USA). Foliar tissue samples were removed from plants and placed in the leaf chamber for measurement. Measurements were conducted in the experimental growth room. Samples were allowed to equilibrate to a steady state for approximately two minutes and measurements were taken no

later than 5 minutes after the foliar tissue was removed from plants. The light intensity for all measurements was 300 μmol $m^{-2}$ $s^{-1}$ PPFD provided by a red-blue light source (6400-02, Li- Cor Inc., Lincoln, NE, USA). The [$CO_2$] was maintained at 400 μmol for all measurements. Light intensity and [$CO_2$] values were chosen to be the same as plant growth conditions in the experimental growth room. For white spruce and tamarack, needle area was calculated using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA, USA).

#### 2.2.3.2 Dry weights and foliar chlorophyll concentration

After gas exchange measurements, leaves or needle samples collected from live seedlings and lyophilized, ground to a powder, and used to measure chlorophyll concentrations. Chlorophyll was extracted from ground tissue (ten mg DW) with eight mL DMSO at 65ºC for 24 h. Extracted chlorophyll was then measured with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden) at 648 nm for chlorophyll-a and 665 nm for chlorophyll-b. Total chlorophyll was calculated using Arnon's equation (Sestak et al. 1971).

Seedlings were then sacrificed and separated into foliar tissue, stems, and roots and oven dried at 70ºC for 72 h before weighing. Lyophilized foliar used for chlorophyll analysis was also weighed and added to the total dry weight measurement. Foliar tissue was ground to a fine powder using a Wiley mill (screen no. 40) and used for elemental analysis.

#### 2.2.3.3 Foliar elemental analysis

Foliar concentrations of Fe, Na, Mg, P, K, and Ca were determined in dried and ground tissue (200 mg) digested with ten mL 70% $HNO_3$ and diluted with DI water up to 50 mL. Samples were then analyzed by ICP-MS in Radiogenic Isotope facility at the University of Alberta (Zarcinas et al. 1987). For the determination of foliar N concentration, approximately two mg of dried ground samples were analyzed for percent N using a CE 440 CHN Elemental Analyzer (Exeter Analytical, MA, USA). Total foliar Cl was analyzed after extracting dried and ground tissue (200 mg) with ten mL of boiling deionized water. Samples were placed in a water bath at 90ºC for one hour. Five mL of liquid extract was combined with 5 mL of deionized water and 200 μL of ion strength adjuster (Thermo-Fisher Scientific, CA, USA). Total Cl was determined using a Cl electrode (Accumet Cl half cell electrode, Thermo-Fisher Scientific, CA,

USA) and reference electrode (Accumet double junction reference electrode, Thermo-Fisher Scientific, CA, USA) attached to a pH meter (Accumet 925 pH/ion meter, Thermo-Fisher Scientific, CA, USA).

### 2.2.4 Statistical analysis

All data were analyzed using univariate and multivariate statistical techniques with the R software (https://www.R-project.org). A significant p value of $P \leq 0.05$ was chose for all analyses. Univariate analysis was performed on all dependent variables using a type III two-way ANOVA linear fixed-effects model with NaCl and pH treatments as fixed independent variables. The model equation is: $Y_{ijk} = \mu + S_i + P_j + (S * P)_{ij} + \mathcal{E}_{ijk}$ where $Y_{ijk}$ is the $_k$th observation of the $_i$th and $_j$th treatments, μ is the sample mean, $S_i$ is the $_i$th NaCl treatment, and $P_j$ is the $_j$th pH treatment. The variable in parenthesis is the interaction between NaCl and pH (ANOVA tables can be found in Appendix 1). It should be noted that green alder exhibited 100% mortality in all 60 mM NaCl treatments as well as pH 9 30 mM NaCl treatment. These treatments were not included in the analysis. A Tukey's HSD test was used when significant differences were detected. Data that did not meet the normality of distribution and homogeneity of variance assumptions were log10 transformed before analysis. Correlations between total chlorophyll and Pn as well as total chlorophyll and foliar N were tested using a two-tailed Pearson's correlation. Correlation analysis between foliar elemental concentrations (N, Fe, Na, Cl) and physiological parameters (dry weight, Pn, E, total chlorophyll, RWR) were tested using a two-tailed Pearson's correlation and can be found in the supplemental information (Table 2.S1). Multivariate analysis was performed using a distance-based redundancy analysis (RDA) with foliar elemental concentrations as the predictor variables (blue vectors) and physiological parameters as the response variables (red vectors). Data from each treatment were averaged to create a single response point for each variable per treatment. Treatment effect P values for single physiological vectors were determined by two-way ANOVA whereas multiple physiological vectors (red ovals) were determined using permutational distance-based two-way MANOVAs. Only significant ($P \leq 0.05$) results were reported on the RDA biplot.

## 2.3 Results:

### 2.3.1 Trembling aspen

Trembling aspen seedlings exhibited a significant disordinal interaction between NaCl and pH for total dry weight which was primarally attributed to a drastic decrease in total dry weight at pH 9 0 mM NaCl compared to pH 5 0 mM NaCl. Seedlings showed moderate decreases in total dry weight at pH 7 when treated with 30 mM NaCl, but drastic decreases in total dry weight at pH 5 for both NaCl concentrations, pH 7 at 60 mM NaCl, and all pH 9 treatments compared to pH 5 0 mM NaCl (Figure 2.1 A). A significant ordinal interaction between NaCl and pH was detected in total chlorophyll. Moderate decrease in total chlorophyll were detected at pH 5 in the 30 mM NaCl treatment, but drastic decreases for all other pH and NaCl combinations compared to pH 5 0 mM NaCl (Figure 2.1 B). A significant disordinal interaction between NaCl and pH was found for Pn as differential decreases were observed across NaCl and pH treatments. Net photosynthesis exhibited moderate decreases for most stress treatments and drastic decreases at pH 5 and 9 when treated with 60 mM NaCl compared to pH 5 0 mM NaCl (Figure 2.1 C). Transpiration rates were unaffected by most pH and NaCl treatments but drastic decreases were observed at pH 5 in 30 mM NaCl and all 60 mM NaCl treatments compared to pH 5 0 mM NaCl (Figure 2.1 D). A significant ordinal interaction between NaCl and pH was detected for foliar N concentrations primarally due to significant decreases for all pH 9 treatments. Foliar N exhibited moderate decreases at pH 5 with 30 & 60 mM NaCl and in all pH 7 treatments and drastic decreases for all pH 9 treatments compared to pH 5 0 mM NaCl. A significant disordinal interaction between NaCl and pH was detected for foliar Fe concentrations which showed differential decreases for all NaCl and pH treatment combinations compared to pH 5 0 mM NaCl. A significant ordinal interaction between NaCl and pH was found for foliar Na concentrations. These values increased for all NaCl treatments and were highest in the 60 mM NaCl treatments. It was noted that Na concentrations were lower at the pH 7 treatment with 60 mM NaCl compared to other pH treatments with 60 mM NaCl. A significant ordinal interaction between NaCl and pH was observed for foliar Cl concentrations because these values exhibited an additive effect at 60 mM NaCl treatments as pH increased. However, treatments of 30 mM NaCl did not show an additive effect (Table 2.1).

### 2.3.2 Green alder

Green alder seedlings exhibited 100% mortality for all 60 mM NaCl treatments as well as pH 9 30 mM NaCl. Total dry weight exhibited drastic decreases in all NaCl and high pH treatments compared to pH 5 0 mM NaCl (Figure 2.2 A). Total chlorophyll and Pn exhibited moderate decreases at pH 5 with 30 mM NaCl and pH 7 with 0 mM NaCl, and more severe decreases for pH 7 30 mM and pH 9 treatments compared to pH 5 0 mM NaCl (Figure 2.2 B,C). Transpiration rates exhibited drastic decreases for all NaCl and high pH treatments compared to pH 5 0 mM NaCl (Figure 2.2 D). Foliar N concentrations exhibited moderate decreases at pH 7 for both NaCl treatments but a drastic decrease at pH 9 0 mM NaCl compared to pH 5 0 mM NaCl. Foliar Fe concentrations showed a slight decrease at pH 7 regardless of NaCl treatment and a relatively moderate decrease at pH 9 with 0 mM NaCl compared to pH 5 0 mM NaCl. Significant ordinal interactions between NaCl and pH was detected for foliar Na and Cl. Foliar Na and Cl concentrations exhibited moderate increases at pH 5 treatment with 30 mM NaCl and drastic increases at pH 7 when seedlings were treated with 30 mM NaCl (Table 2.1).

### 2.3.3 Tamarack

Tamarack seedlings exhibited a significant disordinal interaction between NaCl and pH for total dry weights. Seedlings showed moderate decreases at pH 5 treatment with 30 mM NaCl and all pH 7 treatments, and significant decreases at pH 5 treatment with 60 mM NaCl and all pH 9 treatments compared to pH 5 0 mM NaCl. It was noted that seedlings at pH 7 exhibited slight increases in total dry weight as NaCl treatment concentration increased (Figure 2.3 A). A significant disordinal interaction between NaCl and pH was detected for total chlorophyll concentrations. Seedlings exhibited slight decreases at pH 5 treatment with 30 mM NaCl and pH 7 treatment with 0 mM NaCl, and drastic decreases in all other treatments compared to pH 5 0 mM NaCl (Figure 2.3 B). Net photosynthesis and E exhibited moderate decreases at pH 5 for both NaCl treatments and pH 7 treatment with 0 mM NaCl, and significant decreases for all other pH and NaCl treatment combinations compared to pH 5 0 mM NaCl (Figure 2.3 C,D). A significant disordinal interaction between NaCl and pH was observed for foliar N concentrations. These values exhibited slight decreases at pH 5 and 7 at 60 mM NaCl but moderate decreases at pH 7 30 mM and all pH 9 treatments combinations compared to pH 5 0 mM NaCl. Foliar Fe concentration had moderate decreases at pH 5 60 mM NaCl, pH 7 60 mM, and all pH 9

treatments compared to pH 5 0 mM NaCl. Significant ordinal interactions between NaCl and pH were found in foliar Na and Cl concentrations. Both Na and Cl had significant incremental increases as NaCl concentration increased. However, foliar NaCl concentrations were highest at pH 7 60 mM NaCl treatments (Table 2.3).

### 2.3.4 White spruce

White spruce exhibited no changes in total dry weights for all treatments (Figure 2.4 A). A significant disordinal interaction between NaCl and pH was found in total chlorophyll concentrations. Seedlings showed moderate decreases at pH 5 and 7 at 60 mM NaCl and pH 9 with 0 mM NaCl, but drastic decreases at pH 9 in the presence of NaCl compared to pH 5 0 mM NaCl (Figure 2.4 B). Net photosynthesis showed no changes as a result of increased pH but significant decreases when NaCl was added (Figure 2.4 C). Transpiration rates slightly decreased with increasing pH, but drastically decreased in the presence of NaCl (Figure 2.4 D). A significant disordinal interaction between NaCl and pH was observed in foliar N concentrations. Values exhibited moderate decreases at pH 9 with 0 mM NaCl and drastic decreased at pH 9 with 30 and 60 mM NaCl. A significant disordinal interaction between NaCl and pH was found for foliar Fe concentrations. In general, the greatest decreases in foliar Fe were observed at pH 7 & 9 when NaCl was added. Values exhibited slight decreases at pH 5 when NaCl was added as well as pH 7 with 0 mM NaCl and drastic decreases at pH 7 when NaCl was added and all pH 9 treatments. Foliar Na and Cl increased with increasing NaCl treatment concentrations (Table 2.4).

### 2.3.5 Multivariate analysis

Although all species showed strong positive correlations of foliar N and Pn to total chlorophyll (Table 2.5), in most cases analysis using univariate statistics (Pearson's correlation and ANOVA) proved to be confounding. For example, all species showed positive correlations between total dry weight, total chlorophyll, Pn, and E to foliar N and Fe but negative correlations with foliar Na and Cl concentrations (Table 2.S1). Second, a total of 18 significant interactions were detected when analyzing the data using two-way ANOVAs with few solid trends emerging. In order to determine the strongest linear correlations between physiological parameters and foliar elemental concentrations, a multivariate redundancy analysis (RDA) was performed for

further analysis. In most cases, increased pH and NaCl caused decreases in foliar N, total dry weight, chlorophyll, Pn, and E but increase in root weight ratio (RWR). Second, permutational distance-based two-way MANOVAs were used on groups of vectors related to physiological parameters to determine interaction terms. Significant interactions between pH and NaCl were detected for physiological parameters in green alder and tamarack (Figures 3.5 & 3.6).

## 2.4 Discussion:

The current study examined the responses of trembling aspen, green alder, tamarack, and white spruce seedlings to separate and combined stressors of elevated root zone pH and NaCl. It was hypothesized that both elevated pH and NaCl would cause physiological declines for all species. Elevated root zone pH is generally associated with decreased root water flux, photosynthesis, transpiration, growth and mineral deficiencies in foliar tissue (Renault et al. 1999; Kosegarten et al., 2001; Kamaluddin and Zwiazek, 2004; Tang et al., 2006; Siemens and Zwiazek, 2011; Zhang et al. 2013). Elevated root zone NaCl is generally known to causes decreases in photosynthesis, transpiration, and growth as well as accelerated foliar senescence (Munns and Tester, 2008). The current study focused on separating both pH and NaCl stresses by exposing seedlings to various pH and NaCl levels.

### 2.4.1 Effects of pH and NaCl stress on physiological parameters

Trembling aspen and green alder exhibited the greatest decreases in total dry weight as a result of elevated root zone pH and NaCl compared to seedlings exposed to pH 5 0 mM NaCl. Trembling aspen total dry weight exhibited a significant disordinal interaction and was sensitive to NaCl at pH 5 and 7 and to all pH 9 treatments. Green alder seedlings exhibited drastic decreases in total dry weight from low levels of elevated root zone pH and NaCl as well as 100% mortality at the highest levels of root zone pH and NaCl. Tamarack showed a significant disordinal interaction between NaCl and pH where it exhibited modest declines in total dry weight as a result of elevated root zone pH and NaCl where the greatest declines occurred at pH 5 60 mM NaCl and all pH 9 treatments. Elevated root zone pH and NaCl caused no changes in total dry weight in white spruce. Declines in total dry weight from NaCl stress is well reported for other plant species (Parida and Das; Munns and Tester, 2008; Julkowska and Testerinc, 2015; Parihar et al. 2015). The decreases in total dry weight from elevated pH are congruent with

previous reports where declines were observed at pH 7.5 and 7 in trembling aspen and tamarack, respectively, but little changes in white spruce (Zhang et al. 2013). The effects of elevated NaCl on total dry weight of the tested species is limited; however, it has been reported that NaCl concentrations of 30 and 60 mM caused incremental declines in tamarack total dry weight (Renault, 2005).

Total chlorophyll concentrations decreased as a result of interactions between elevated root zone NaCl and pH for trembling aspen, tamarack, and white spruce. Total chlorphyll concentrations decreased in green alder as a result of elevated root zone NaCl and pH but no interactions were detected. For trembling aspen, an ordinal interaction was detected as all treatments with elevated root zone pH and NaCl caused declines in chlorophyll concentrations. Both green alder and tamarack exhibited incremental decreases in chlorophyll concentration as root zone pH and NaCl increased. For white spruce, chlorophyll concentrations exhibited declines at pH 5 and 7 60 mM NaCl and all pH 9 treatments. The greatest declines for all species were at pH 9 in the presence of NaCl. Declines in total chlorophyll from elevated pH is well known to be linked to a decrease in root zone Fe availability (Miller et al. 1984). Declines in total chlorophyll from NaCl stress is also a commonly reported phenomenon; however, no clear mechanism for this decline has been elucidated (Parida and Das, 2005; Parihar et al. 2015). It should be noted that several studies have reported that NaCl stress induces an upregulation of chlorophyllase which degrades chlorophyll (Stivsev et al. 1973; Reddy, 1986; Santos, 2004). Interestingly, all tested species exhibited strong negative correlations between total chlorophyll concentration with Pn and foliar N. Considering that total chlorophyll is known to decline because of elevated root zone pH and NaCl, it may be a reliable indicator of total plant health. Total chlorophyll has been proposed to be used as estimation of N status in hardwoods as well as a biomarker for anthropogenic stress in aquatic plants (Chang and Robison, 2003; Ferrat et al., 2003). Interestingly, total leaf chlorophyll concentration can be measured remotely using hyperspectral imaging (Schlemmer et al. 2005; Darvishzadeh et al. 2008), suggesting it can be a valuable tool to detect both pH and NaCl stress in planted seedlings.

Elevated root zone pH and NaCl caused declines in gas exchange parameters for all species tested. For trembling aspen, a disordinal interaction was observed as elevated root zone pH and NaCl caused declines in Pn for all treatments. The declines were the most severe at pH 5

and 9 with 60 mM NaCl. Transpiration in trembling aspen was only sensitive to NaCl stress but not at pH 7 with 30 mM NaCl. Green alder and tamarack exhibited incremental decreases in Pn and E as root zone pH and NaCl increased. For white spruce, NaCl stress caused significant declines in Pn and E, but elevated pH alone only caused slight declines in E. Declines in Pn and E are commonly reported as a result of NaCl stress for the majority of glycophytic plants. Declines in Pn is believed to be caused by decreased water potential, limited $CO_2$ supply, and Na toxicity around the chloroplast (Parida and Das, 2005; Munns and Tester, 2008; Parihar et al. 2015). The NaCl-induced decrease in E is linked to stomatal closure and is known to rapidly reduce water uptake as well as stunt plant growth (Munns and Tester, 2008). Interestingly, emerging evidence shows that the decreases in both processes may be linked to a self-propagating calcium wave sent from the roots through the vascular tissue and into the leaves. This process raises xylem pH and causes ABA to be released from cell walls to trigger stomatal closure (Pei et al. 2000; Wilkinson and Davies, 2002; Choi et al. 2014; Jiang et al. 2016). Other evidence suggests that electrical signals alone trigger decreases in Pn (Sukhov, 2016). Considering that elevated xylem pH is a major factor for NaCl-induced declines in Pn and E, it appears plausible that seedlings experiencing elevated root zone pH will trigger a similar response in the leaves. Interestingly, in a study comparing the pH sensitive paper birch to the more tolerant red-osier dogwood, the tolerant species had lower xylem sap pH and higher gas exchange rates and root hydraulic conductivity when exposed to elevated pH (Zhang and Zwiazek, 2016). This suggests that tolerance to elevated pH may be linked to maintaining a lower xylem sap pH, perhaps to inhibit the signaling effects of elevated xylem pH.

### 2.4.2 Effects of pH and NaCl stress on foliar elemental concentration

All species examined in this study exhibited decreases in foliar N and Fe as a result of elevated root zone pH and NaCl. With the exception of both green alder measurements and tamarack Fe, significant interactions were detected between foliar N and Fe as a result of elevated NaCl and pH. For trembling aspen, the declines in both elements were most prominent at pH 7 with 60 mM NaCl and all pH 9 treatments. Green alder exhibited incremental decreases in both foliar N and Fe as root zone pH and NaCl increased. Tamarack exhibited slight declines in foliar N from elevated NaCl at pH 5 and 7, but the greatest declines in foliar N were seen at pH 9. Elevated root zone pH and NaCl caused decreases in foliar Fe for tamarack; however, the

decreases were less severe compared to other species. For white spruce, decreases in foliar N was most evident at pH 9, especially with the addition of NaCl. Decreases in foliar Fe was caused by elevated pH and NaCl, especially at pH 9. For all tested species, the decline in foliar N and Fe was seen at pH 9 for all NaCl treatment levels, suggesting that trees planted in soils at or above pH 9 may experience poor health. Zhang et al. (2013) reported that soluble Fe was severely reduced at pH 9 in mineral solution culture. Furthermore, the authors noted that tamarack may have the ability to translocate Fe into younger tissue under pH stress. In the current study, increased stress caused only modest decreases in foliar Fe for tamarack. This suggests that tamarack may possess a physiological mechanism to overcome stress-induced Fe deficiencies. However, it should be noted that Hoagland solution contained Fe in the chelated form and is not the same as the Fe found in reclaimed soils.

In general, Na and Cl increased incrementally as NaCl treatment level increased for all species. Interestingly, foliar Na and Cl concentrations for trembling aspen, green alder, and tamarack exhibited significant ordinal interactions between elevated NaCl and pH. Interestingly, trembling aspen had significantly lower foliar Na compared to foliar Cl for all treatments. This suggests that trembling aspen possesses physiological adaptations to keep foliar Na concentrations relatively low. Ion exclusion at the roots via suberin deposition and senescence of older leaves are both plausible mechanisms (Franke and Schreiber, 2007; Munns and Tester, 2008). Both tamarack and white spruce had higher foliar Na concentrations compared to Cl for all NaCl treatments, suggesting that ion exclusion of Na did not occur in these species. Since Na is most dangerous to photosynthetic processes in the mesophyll cells, it is possible that toxic Na ions are being stored in the cell walls or vacuoles as a protective mechanism (Munns and Tester, 2008; Parihar et al. 2015).

### 2.4.3 Is the similar response to stress by all species tested evidence of a deliberate stunting of growth?

All species exhibited a similar underlying pattern in response to elevated NaCl and pH. With little exception, all species showed decreases in foliar N, total dry weight, chlorophyll, Pn, and E but increased RWR in response to elevated stress. Interestingly, multivariate analysis revealed that interactions between pH and NaCl existed for physiological parameters in green alder and tamarack but not in trembling aspen and white spruce. However, these results should

be interpreted with caution due to the large number of treatments and low sample size. The physiological response of plants to elevated NaCl is well studied and includes rapid decreases in root water flux, transpiration, net photosynthesis, and growth. Prolonged periods of NaCl stress will lead to decreased chlorophyll concentration but increased root growth (Volkmar et al. 1998; Munns, 2002; Parida and Das, 2005; Munns and Tester, 2008; Parihar et al. 2015). In a review by Julkowska and Testerink (2015), the authors stated that NaCl stress induces an immediate halt of plant growth followed by a slower, more conservative growth form with enhanced lateral root formation. The altered growth varies by organs and fundamentally alters plant morphology. It has been proposed that this phenomenon is induced by long distance electrical signals produced by plants in response to stress (Gilroy et al. 2016). Essentially, upon the perception of abiotic stress such as drought or increased soil Na, a self-propagating wave of $Ca^{2+}$ and ROS is produced from the root tip which leads to a rapid and systemic electrical signal within the vascular tissue. This signal can have numerous effects on plant physiology such as: decreases in net photosynthesis, transpiration, and $CO_2$ assimilation among other responses (Gilrow et al. 2016). Considering that a similar stunting of growth and decrease of many physiological parameters were observed because of elevated NaCl and pH for all species tested, it is possible that these trends represent a stress resistance mechanism by the seedlings rather than an inadvertent consequence of elevated NaCl and pH. However, significant testing is needed to verify this hypothesis. All species showed decreases in foliar N as a result of elevated NaCl or pH. Studies focusing on the effects of NaCl stress on N metabolism are relatively limited. However, decreases in nitrate uptake as well as decreases in leaf and root nitrate reductase activity have been reported (Silveira et al. 2001; Meloni et al. 2004; Debouba et al. 2006). It may be possible that the lower foliar N concentrations due to NaCl and pH in this study are a result of similar mechanisms. The effects of elevated pH on the stress physiology of plants is primarily focused on mineral nutrition. However, the declines in physiological parameters is similar to NaCl stress and may be governed by a physiological downregulation of metabolism. A more detailed description of this hypothesis can be found in Appendix five. More studies focused on these topics should be conducted to better elucidate how plants maintain homeostasis under adverse environmental conditions.

### 2.4.4 How can this study be used to improve land reclamation in northern Alberta?

The objective of this study was to examine seedling responses to the separate and combined effects of elevated pH and NaCl. Liquid culture was used in order to maintain pH in a controlled and precise manor. It should be noted that many other factors are present under field conditions which may complicate revegetation. Some factors not tested in this study include drought, freezing, soil compaction, and rhizosphere microorganisms. Therefore, the results reported in this study only reflect a portion of the stresses encountered by field-grown seedlings.

In terms of overall tolerance, green alder was very sensitive to elevated root zone pH and NaCl whereas trembling aspen showed moderate tolerance at some treatments with elevated root zone pH and NaCl but high mortality and decreased growth at 60 mM NaCl and all pH 9 treatments. Both tamarack and white spruce had limited mortality but did show decreases in physiological parameters at higher levels of root zone pH and NaCl, which may affect physiological performance of planted seedlings. Evidence that elevated pH aggravated the effects of NaCl stress is seen in green alder, tamarack, and white spruce. For example, these species exhibited further declines in leaf chlorophyll concentrations from NaCl stress as pH treatment level increased. Green alder and tamarack both exhibited incremental declines in Pn, E, and foliar Na as root zone pH and NaCl increased. For green alder, no seedlings survived at pH 9 in the presence of NaCl.

Successful reclamation of boreal forest land is dependent on many factors. If unfavorable site conditions are found, tamarack and white spruce have the best chance of survival. Elevated root zone pH and NaCl will lead to the stunting of growth, limited physiological function, high mortality in trembling aspen, and an uncertain prognosis for long-term success. The inoculation of seedlings with mycorrhizal fungi should be investigated as these associations could potentially enhance nutrient uptake and stress tolerance for seedlings in stressful environments (Evelin et al. 2009). Secondly, total leaf chlorophyll concentration could be used as a biomarker of sublethal stress. Finally, trembling aspen and white spruce could potentially be planted together because trembling aspen may as a nurse species to protect white spruce (Man and Lieffers, 1999).

**2.5 References:**


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## 2.6 Tables

Table 2.1. Foliar concentrations of selected elements in trembling aspen. Values represent the mean ± SEM (n=6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

| Treatment | N (% DW) | Fe (mg/kg DW) | Na (mg/kg DW) | Cl (mg/kg DW) |
|---|---|---|---|---|
| pH 5 | | | | |
| 0 mM NaCl | 3.22 ± 0.08 a | 193.05 ± 16.10 a | 94.41 ± 11.49 c | 421.73 ± 49.91 c |
| 30 mM | 2.85 ± 0.11 b | 92.04 ± 13.64 c | 646.26 ± 164.99 b | 3668.48 ± 304.89 b |
| 60 mM | 2.45 ± 0.31 b | 139.51 ± 5.33 b | 3238.09 ± 904.37 a | 4308.28 ± 462.32 b |
| pH 7 | | | | |
| 0 mM | 2.83 ± 0.08 b | 115.14 ± 23.29 b | 76.46 ± 29.47 c | 661.54 ± 35.09 c |
| 30 mM | 2.72 ± 0.10 b | 106.29 ± 12.02 b | 599.93 ± 98.31 b | 3774.98 ± 288.74 b |
| 60 mM | 2.54 ± 0.14 b | 38.48 ± 21.06 d | 718.30 ± 82.68 b | 6194.45 ± 294.23 a |
| pH 9 | | | | |
| 0 mM | 1.96 ± 0.08 c | 5.20 ± 2.42 e | 41.30 ± 13.34 c | 426.97 ± 16.48 c |
| 30 mM | 1.98 ± 0.09 c | 40.74 ± 16.93 d | 398.07 ± 166.16 b | 3747.27 ± 304.74 b |
| 60 mM | 2.04 ± 0.03 c | 9.56 ± 6.89 e | 4735.40 ± 1666.35 a | 7712.41 ± 1363.68 a |

Table 2.2. Foliar concentration of selected elements in green alder. Values represent the mean ± SEM (n=6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

| Treatment | N (% DW) | Fe (mg/kg DW) | Na (mg/kg DW) | Cl (mg/kg DW) |
|---|---|---|---|---|
| pH 5 | | | | |
| 0 mM NaCl | 3.09 ± 0.11 a | 134.89 ± 4.02 a | 80.03 ± 12.30 c | 765.25 ± 89.53 a |
| 30 mM | 2.8 ± 0.05 a | 140.3 ± 7.39 a | 4942.73 ± 884.65 b | 8464.43 ± 797.21 c |
| 60 mM | N.A. | N.A. | N.A. | N.A. |
| pH 7 | | | | |
| 0 mM | 2.24 ± 0.15 b | 125.26 ± 3.82 ab | 118.84 ± 9.19 c | 1740.89 ± 191.28 b |
| 30 mM | 2.35 ± 0.10 b | 120.70 ± 9.09 ab | 11495.80 ± 2157.79 a | 11862.0 ± 1885.19 d |
| 60 mM | N.A. | N.A. | N.A. | N.A. |
| pH 9 | | | | |
| 0 mM | 1.52 ± 0.05 c | 110.98 ± 2.82 b | 134.98 ± 21.04 c | 428.72 ± 25.79 a |
| 30 mM | N.A. | N.A. | N.A. | N.A. |
| 60 mM | N.A. | N.A. | N.A. | N.A. |

Table 2.3. Foliar concentration of selected elements in tamarack. Values represent the mean ± SEM (n=6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

| Treatment | N (% DW) | Fe (mg/kg DW) | Na (mg/kg DW) | Cl (mg/kg DW) |
|---|---|---|---|---|
| pH 5 | | | | |
| 0 mM NaCl | 1.74 ± 0.10 a | 103.85 ± 3.17 a | 90.70 ± 7.35 d | 401.13 ± 34.66 d |
| 30 mM | 1.73 ± 0.11 a | 95.95 ± 5.99 a | 1536.46 ± 295.45 c | 1610.83 ± 96.30 c |
| 60 mM | 1.58 ± 0.12 ab | 88.26 ± 4.71 b | 6593.17 ± 1325.66 b | 3096.86 ± 325.43 b |
| pH 7 | | | | |
| 0 mM | 1.88 ± 0.09 a | 95.14 ± 5.08 a | 134.97 ± 7.29 d | 498.10 ± 23.23 d |
| 30 mM | 1.34 ± 0.06 b | 83.61 ± 4.54 b | 2803.43 ± 316.57 c | 1527.35 ± 161.52 c |
| 60 mM | 1.61 ± 0.09 ab | 87.19 ± 6.03 b | 10697.10 ± 1132.13 a | 4282.78 ± 501.36 a |
| pH 9 | | | | |
| 0 mM | 1.21 ± 0.10 b | 83.83 ± 5.31 b | 145.35 ± 10.84 d | 348.41 ± 52.73 d |
| 30 mM | 1.21 ± 0.06 b | 83.06 ± 3.91 b | 3544.13 ± 398.58 c | 1703.66 ± 241.59 c |
| 60 mM | 1.27 ± 0.06 b | 91.95 ± 6.38 ab | 5864.73 ± 911.98 b | 2440.08 ± 300.59 bc |

Table 2.4. Foliar concentration of selected elements in white spruce. Values represent the mean ± SEM (n=6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

| White Spruce | N (% DW) | Fe (mg/kg DW) | Na (mg/kg DW) | Cl (mg/kg DW) |
|---|---|---|---|---|
| pH 5 | | | | |
| 0 mM NaCl | 2.01 ± 0.12 a | 236.01 ± 11.42 a | 117.95 ± 11.48 d | 250.78 ± 18.34 c |
| 30 mM | 1.55 ± 0.13 b | 109.62 ± 19.21 b | 1568.98 ± 208.92 c | 933.65 ± 72.98 b |
| 60 mM | 1.95 ± 0.12 a | 82.63 ± 17.06 bc | 6642.7 ± 727.04 a | 2842.34 ± 334.17 a |
| pH 7 | | | | |
| 0 mM | 2.03 ± 0.07 a | 135.61 ± 14.66 b | 124.71 ± 22.12 d | 334.37 ± 27.17 c |
| 30 mM | 2.12 ± 0.08 a | 52.01 ± 8.81 c | 2877.91 ± 487.54 b | 1255.41 ± 95.19 b |
| 60 mM | 1.84 ± 0.07 ab | 76.75 ± 8.62 bc | 7336.81 ± 1016.83 a | 2786.23 ± 126.46 a |
| pH 9 | | | | |
| 0 mM | 1.54 ± 0.04 b | 89.79 ± 24.56 bc | 71.67 ± 21.68 d | 222.42 ± 28.28 c |
| 30 mM | 1.19 ± 0.07 c | 52.41 ± 19.27 c | 2904.76 ± 437.57 b | 1090.44 ± 125.07 b |
| 60 mM | 1.31 ± 0.07 c | 22.60 ± 11.09 c | 6407.04 ± 891.17 a | 2333.68 ± 407.35 a |

Table 2.5. $R^2$ values for two-tailed Pearson's correlations comparing total chlorophyll to Pn and foliar N in trembling aspen, green alder, tamarack, and white spruce.

$P < 0.05$ = * $P < 0.01$ = ** $P < 0.001$ = ***

| Species | Total Chlorophyll VS. Pn | Foliar N |
|---|---|---|
| Aspen | 0.207*** | 0.261*** |
| Green Alder | 0.669*** | 0.557*** |
| Tamarack | 0.573*** | 0.444*** |
| White Spruce | 0.082* | 0.326*** |

## 2.7 Figures

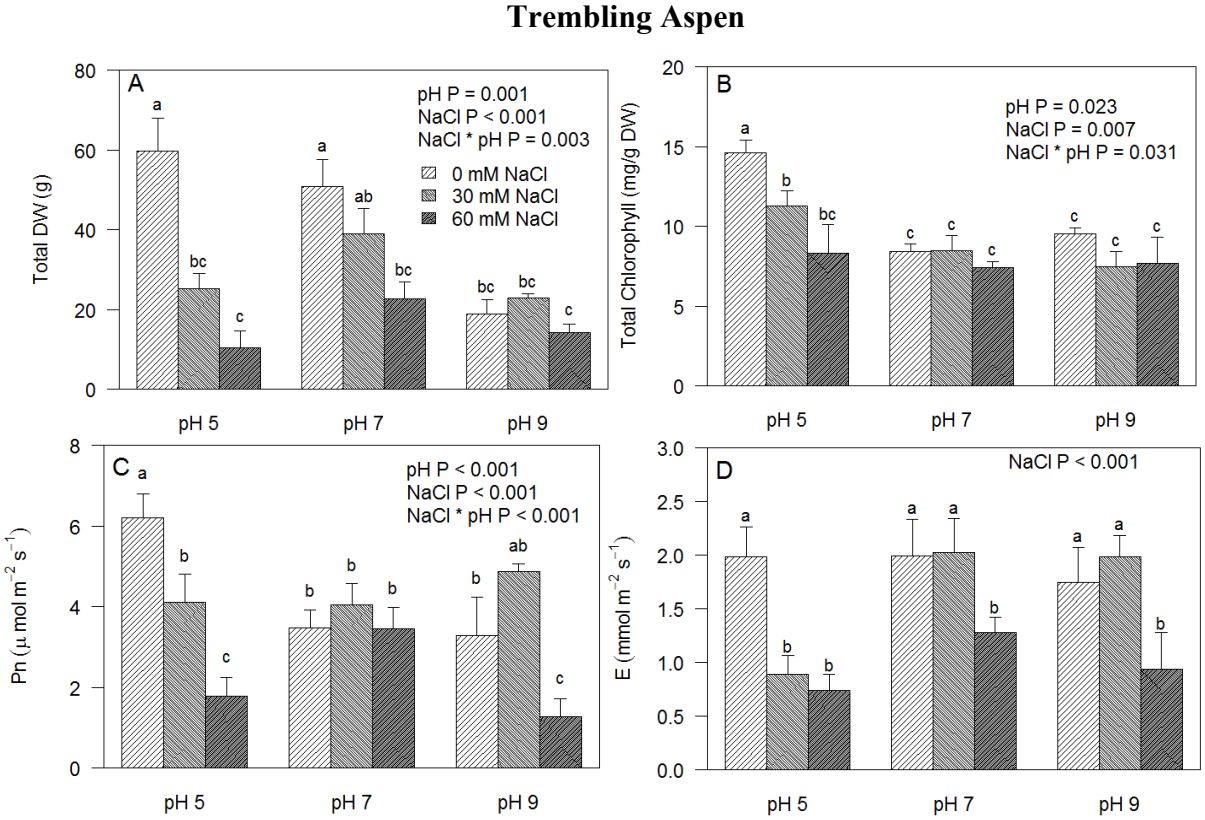


Figure 2.1. Effects of 50-day treatments with elevated pH and NaCl on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rate (E) (D) for trembling aspen. Values represent the mean + SEM (n=3-6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

**Green Alder**

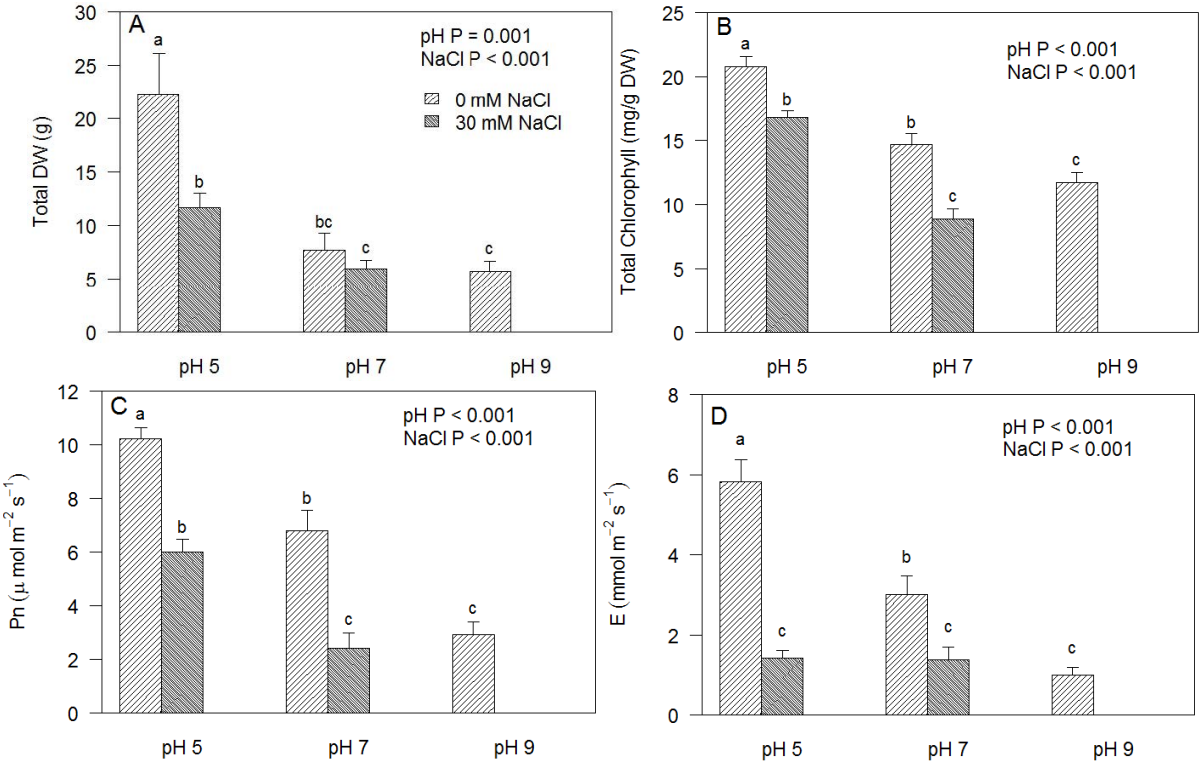


Figure 2.2. Effects of a 50-day treatment with elevated pH and NaCl on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rate (E) (D) for green alder. Values represent the mean + SEM (n=3-6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

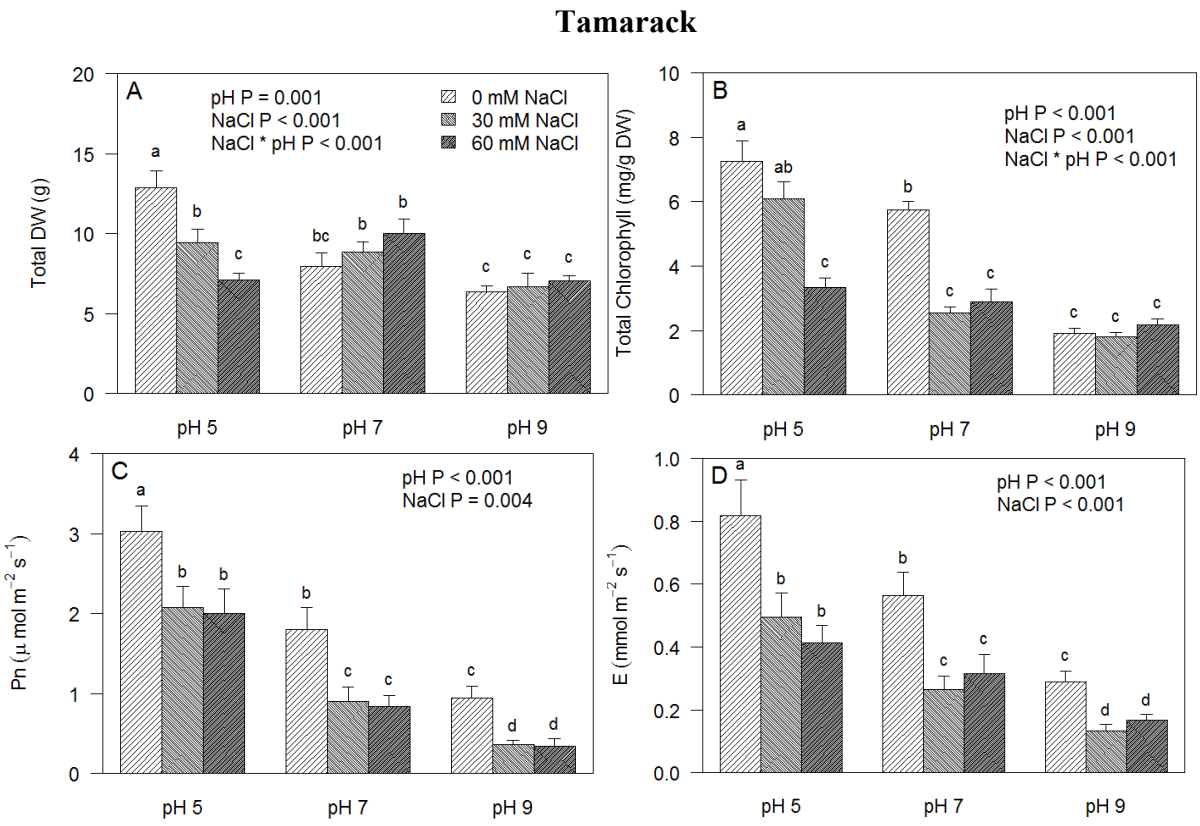


Figure 2.3. Effects of a 50-day treatment with elevated pH and NaCl on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rate (E) (D) for tamarack. Values represent the mean + SEM (n=6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

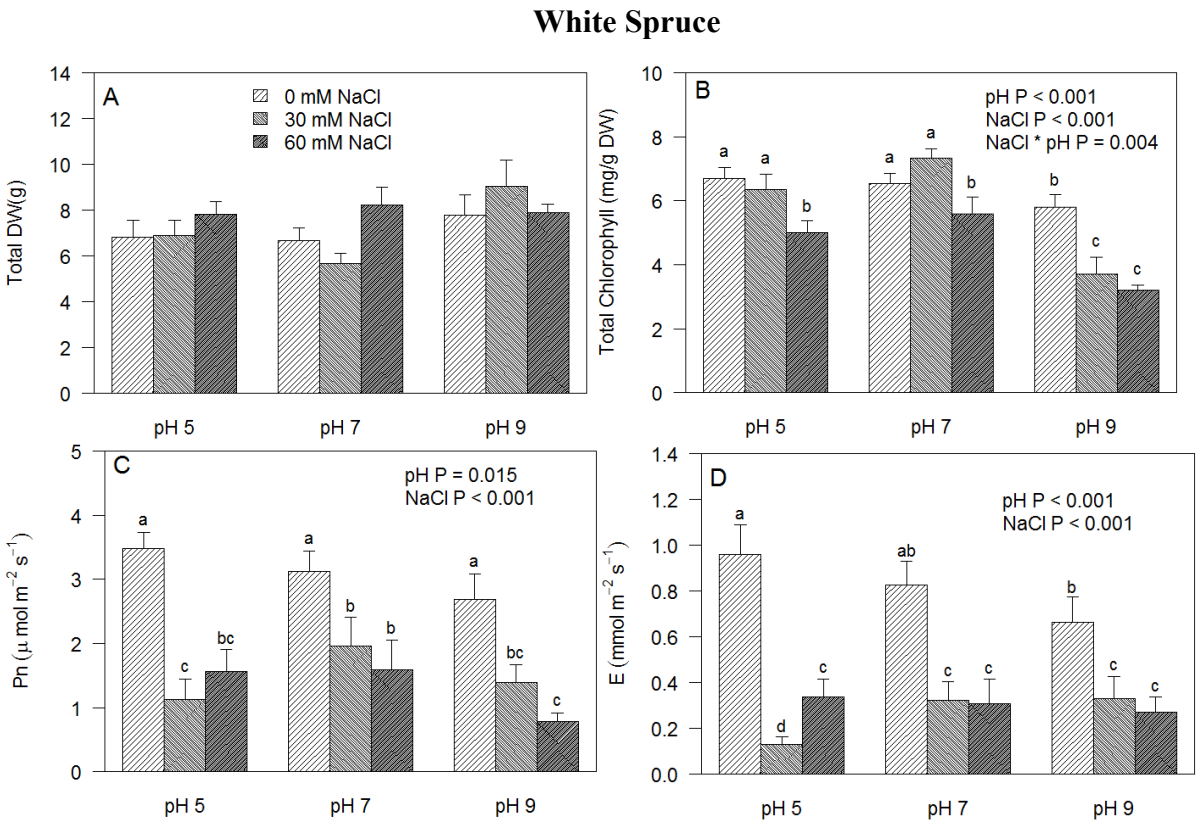


Figure 2.4. Effects of a 50-day treatment with elevated pH and NaCl on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn), and transpiration rate (E) of white spruce. Values represent the mean + SEM (n=6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

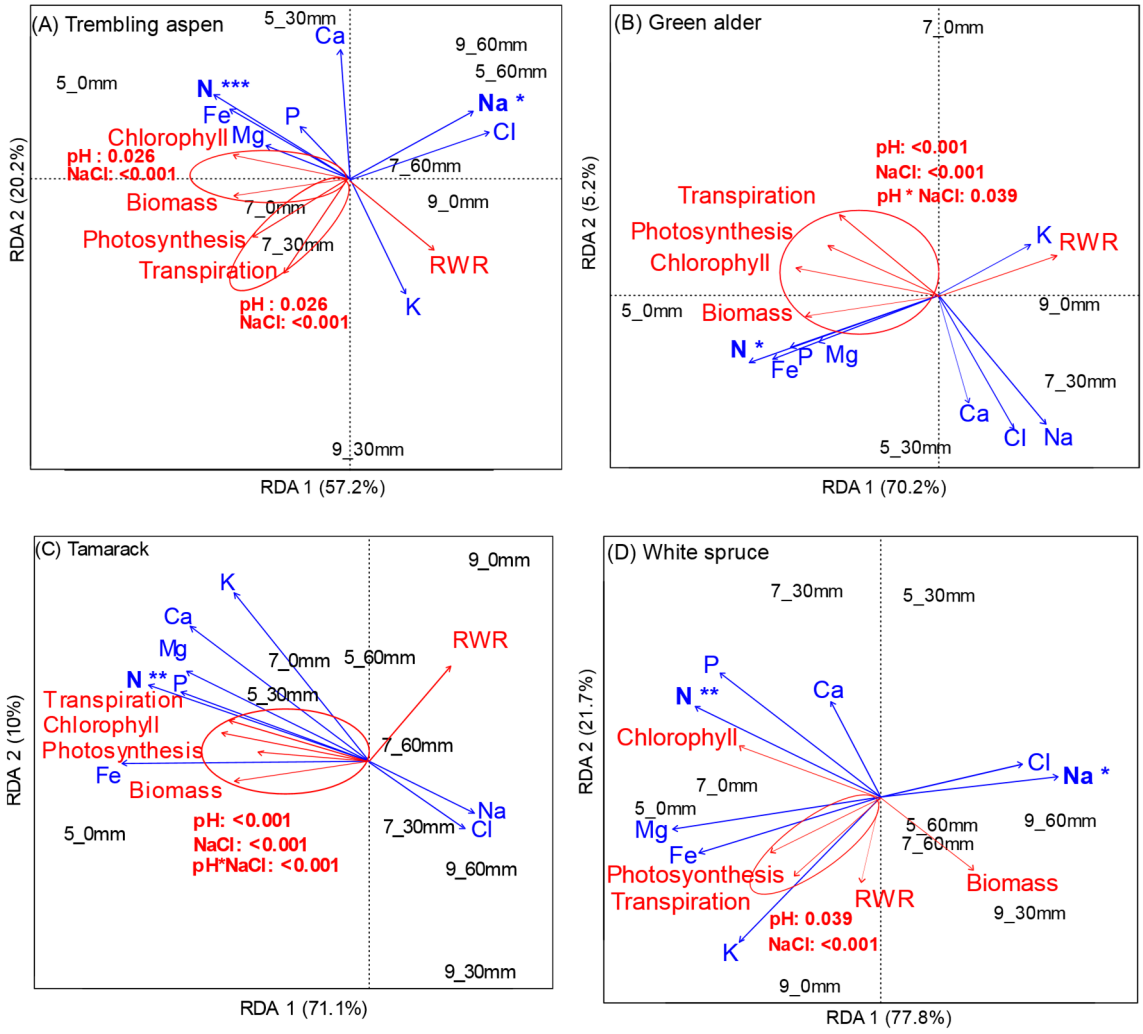


Figure 2.5. Redundancy analysis of foliar elements to physiological parameters for trembling aspen (A), green alder (B), tamarack (C), and white spruce (D). Significant values for blue vectors were designated by asterisks ($P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***). Treatment effect P values for multiple physiological vectors (red ovals) were determined using permutational distance based two-way MANOVAs.

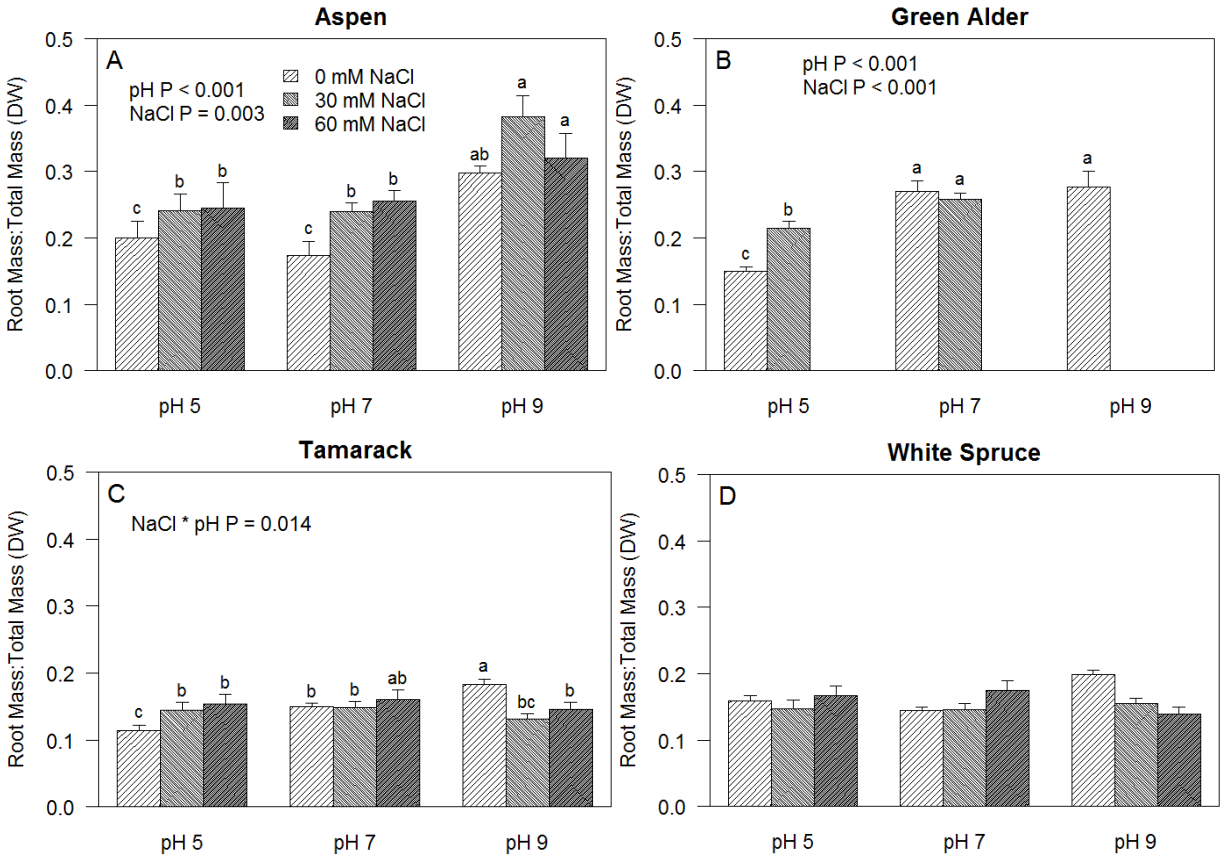


Figure 2.6. Effects of a 50-day treatment with elevated pH and NaCl on the root weight ratio (RWR) of trembling aspen (A), green alder (B), tamarack (C), and white spruce (D). Values represent the mean + SEM (n=3-6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

## 2.8 Supplemental Material

Table 2.S1. $R^2$ values for two-tailed Pearson's correlations comparing foliar elemental concentrations (N, Fe, Na, Cl) to physiological parameters (dry weight, Pn, E, total chlorophyll, RWR) in trembling aspen, green alder, tamarack, and white spruce.

| Species | Physiological parameter | Leaf Elements | | | |
|---|---|---|---|---|---|
| Trembling aspen | | N | Fe | Na | Cl |
| | Total dry weight | **0.575*** ** | **0.678*** ** | **-0.287*** | **-0.478*** ** |
| | Pn | **0.342*** | **0.364**** | **-0.432*** ** | **-0.344*** |
| | E | 0.097 | 0.135 | **-0.406**** | **-0.370**** |
| | Total Chlorophyll | **0.496*** ** | **0.481*** ** | -0.232 | **0.328*** |
| | RWR | **-0.538*** ** | **-0.436*** ** | 0.121 | 0.237 |
| Green Alder | | | | | |
| | Total dry weight | **0.506**** | 0.180 | -0.292 | -0.274 |
| | Pn | **0.655*** ** | **0.396**** | **-0.497*** ** | **-0.419**** |
| | E | -0.013 | 0.039 | -0.119 | -0.056 |
| | Total Chlorophyll | **0.627*** ** | **0.329*** | **-0.553*** ** | **-0.484**** |
| | RWR | **-0.605*** ** | **-0.336*** | 0.157 | 0.162 |
| Tamarack | | | | | |
| | Total dry weight | **0.280*** | 0.104 | -0.045 | -0.014 |
| | Pn | **0.595*** ** | 0.189 | **-0.325**** | -0.209 |
| | E | **0.608**** | 0.168 | **-0.293*** | -0.156 |
| | Total Chlorophyll | **0.662*** ** | **0.365*** ** | **-0.325**** | **-0.259*** |
| | RWR | 0.113 | -0.139 | **0.297*** | **0.287*** |
| White Spruce | | | | | |
| | Total dry weight | **-0.408*** ** | -0.079 | 0.231 | 0.229 |
| | Pn | **0.430*** ** | **0.501*** ** | **-0.489*** ** | **-0.467*** ** |
| | E | **0.327**** | **0.466*** ** | **-0.378**** | **-0.342**** |
| | Total Chlorophyll | **0.621*** ** | **0.283*** | **-0.401**** | **-0.328**** |
| | RWR | 0.068 | 0.065 | 0.066 | 0.074 |

P < 0.05 = * P < 0.01 = ** P < 0.001 = ***

Table 2.S2. Height values prior to experimental treatment for trembling aspen, green alder, tamarack, and white spruce.

| | Initial Height (cm) | | | |
|---|---|---|---|---|
| | Trembling aspen | Green alder | Tamarack | White spruce |
| pH 5 | | | | |
| 0 mM NaCl | 47.28 ± 1.48 | 19.29 ± 1.60 | 21.83 ± 0.93 | 29.23 ± 0.89 |
| 30 mM | 47.11 ± 1.76 | 17.35 ± 0.90 | 20.65 ± 0.93 | 31.53 ± 1.19 |
| 60 mM | 45.73 ± 1.30 | 18.42 ± 1.10 | 20.48 ± 1.18 | 32.08 ± 0.81 |
| pH 7 | | | | |
| 0 mM | 48.13 ± 2.15 | 17.37 ± 1.52 | 20.50 ± 1.34 | 32.80 ± 0.76 |
| 30 mM | 49.96 ± 1.96 | 20.50 ± 1.45 | 20.45 ± 0.84 | 32.80 ± 0.76 |
| 60 mM | 48.23 ± 1.23 | 18.44 ± 1.94 | 22.28 ± 0.99 | 29.43 ± 0.91 |
| pH 9 | | | | |
| 0 mM | 46.03 ± 1.63 | 17.81 ± 1.36 | 20.25 ± 1.15 | 29.35 ± 0.88 |
| 30 mM | 47.86 ± 1.13 | 16.45 ± 1.12 | 21.45 ± 0.90 | 30.95 ± 0.93 |
| 60 mM | 49.10 ± 1.34 | 18.39 ± 0.88 | 21.68 ± 1.20 | 28.25 ± 0.90 |

Table 2.S3. Root collar diameter values prior to experimental treatment for trembling aspen, green alder, tamarack, and white spruce.

| | Initial Root Collar diameter (mm) | | | |
|---|---|---|---|---|
| | Trembling aspen | Green alder | Tamarack | White spruce |
| pH 5 | | | | |
| 0 mM NaCl | 4.28 ± 0.13 | 3.25 ± 0.26 | 3.37 ± 0.09 | 3.43 ± 0.09 |
| 30 mM | 4.15 ± 0.15 | 2.55 ± 0.12 | 3.04 ± 0.14 | 3.26 ± 0.09 |
| 60 mM | 4.10 ± 0.15 | 2.86 ± 0.22 | 3.40 ± 0.15 | 3.29 ± 0.10 |
| pH 7 | | | | |
| 0 mM | 3.92 ± 0.23 | 2.64 ± 0.16 | 3.22 ± 0.11 | 3.25 ± 0.09 |
| 30 mM | 4.57 ± 0.19 | 3.19 ± 0.24 | 3.04 ± 0.15 | 3.30 ± 0.09 |
| 60 mM | 4.40 ± 0.13 | 3.12 ± 0.17 | 3.40 ± 0.15 | 3.16 ± 0.11 |
| pH 9 | | | | |
| 0 mM | 3.85 ± 0.19 | 3.12 ± 0.17 | 3.02 ± 0.12 | 3.22 ± 0.09 |
| 30 mM | 4.36 ± 0.12 | 2.83 ± 0.22 | 3.11 ± 0.13 | 3.23 ± 0.09 |
| 60 mM | 4.11 ± 0.11 | 3.38 ± 0.13 | 3.41 ± 0.16 | 3.44 ± 0.10 |

## Chapter 3: Supplemental nitrogen helps recover NaCl-induced declines in net photosynthesis and transpiration in trembling aspen (*Populus tremuloides*) but has detrimental effects in white spruce (*Picea glauca*).

### 3.1 Introduction:

Plants display different levels of sensitivity to elevated soil pH and NaCl; however, excessive stress causes all species to eventually exhibit characteristic stress responses. It was reported in Chapter two that trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) exhibited different tolerance thresholds to elevated root zone pH and NaCl. Trembling aspen and green alder were more sensitive to higher levels root zone pH and NaCl characterized by decreased physiological performance and growth. Tamarack and white spruce were relatively tolerant and exhibited moderate physiological decline with higher levels of root zone pH and NaCl. Tamarack exhibited growth under moderate levels of root zone pH and NaCl whereas white spruce showed no changes in total dry weight due to elevated root zone pH and NaCl. Interestingly, elevated root zone pH and NaCl caused a nearly identical trend for all tested species, which included decreases in foliar N, leaf chlorophyll concentrations, net photosynthesis, transpiration rates, and growth. It was hypothesized that this trend reflected a downregulation of metabolism as a result of elevated stress (Gilroy et al. 2016). For the current study, I decided to increase N concentration by 4x in liquid culture because elevated NaCl and pH caused a decrease in foliar N for all species. Nitrogen supplementation in plants has been used in many studies with mostly positive results.

Nutrient loading of nursery stock is a common technique used to facilitate luxury uptake of nutrients within tree seedlings. This process can help seedling performance for some species following planting (Timmer, 1997). Seedlings with high levels of internal N have shown greater performance due to increased frost and drought resistance, root hydraulic conductivity, mycorrhization, and root growth (Oliet et al. 2013). Trembling aspen seedlings loaded with nutrients exhibited increased internal N reserves, growth, and better performance on reclamation sites compared to standard seedlings. However, nutrient loading of white spruce did not produce benefits for planting success (Hu et al. 2014; Schott et al. 2015; Pokharel and Chang, 2016). Nutrient loading of nursery stock shows promises in increasing planting success of most species and shows that increasing the N reserves of seedlings may be beneficial for some species.

Fertilization with N in agricultural crops is a common practice to increase yield. It is well known that under ideal soil and weather conditions, agricultural crops are limited by N rather than water (Brueck, 2008). Fertilization with N leads to increased photosynthetic rate presumably due to increased investment in photosynthetic proteins, particularly RuBisCo (Sinclair and Horie, 1989). Chlorophyll concentration is well known to increase from N fertilization and is a reliable indicator of internal N status (Wood et al. 1992; Blackmer and Schepers, 1995; Waskom et al. 1996). Water use efficiency (WUE) is also known to increase from N fertilization; however, the physiological or biochemical processes that cause the increase in WUE are not fully elucidated (Brueck, 2008; Dordas and Sioulas, 2008). Intracellular $CO_2$ concentration (Ci) is known to decrease in some cases, presumably due to decreased mesophyll resistance (Cechin and de Fátima Fumis, 2004; Dordas and Sioulas, 2008). Mature Douglas fir trees with elevated internal N concentration have shown increased photosynthesis, transpiration, and WUE (Mitchell and Hinckley 1993). Fertilization with N caused increased growth, carbon assimilation, and WUE in trembling aspen (DesRochers et al. 2003). Fertilization with NPK resulted in increased trembling aspen seedling establishment and growth on oil sands reclamation soils (Pinno et al. 2012). Fertilization with N is a well-established and successful agricultural practice and shows promise for forestry practices as well.

Limited reports exist on testing the effects of N fertilization to alleviate stress in plants. Saneoka et al. (2004) showed that plants with supplemental soil N were more drought resistant. Supplementation with N caused increased membrane stability, turgor pressure and decreased lipid peroxidation. Papadopoulos and Rending (1983) demonstrated that supplemental N did not alleviate symptoms of NaCl stress in tomato plants. Duan and Chang (2017) demonstrated that N fertilization of white spruce exposed to elevated NaCl would partially recover Pn compared to non-fertilized controls. Studies on and the effects of elevated root zone NaCl on N assimilation in plants are limited. However, NaCl stress has been reported to cause decreases in nitrate uptake as well as leaf and root nitrate reductase activity, which ultimately affects N assimilation (Silveira et al. 2001; Meloni et al. 2004; Debouba et al. 2006). Considering that N fertilization is a common practice and plants are regularly exposed to environmental stress, more research should be focused on the effects of supplemental N on plants under environmental stress.

Trembling aspen and white spruce were chosen for the current study and it should be noted that nitrogen uptake differs between these two species. Multiple reports have shown that trembling aspen can absorb both ammonium and nitrate whereas white spruce favors ammonium uptake both in situ and in liquid culture (McFee et al. 1986; Kronzucker et al. 1997; Hangs et al. 2003; Pritchard and Guy, 2005). Interestingly, it has been shown that in situ trembling aspen stands showed increased nitrification as soil pH increased from 5 to 8 whereas white spruce stands did not. Furthermore, it has been suggested that elevated nitrification caused by trembling aspen stands lead to elevated soil nitrate (Ste-Marie and Paré, 1999). Another study found that soil nitrification in trembling aspen stands was significantly higher compared to white spruce stands (Paré and Bergeron, 1996). Taken together, this suggests that trembling aspen can increase soil nitrification, perhaps to increase nitrate availability. In the current study, ammonium nitrate was used in liquid culture to increase N availability. This provided a nitrogen source for both trembling aspen and white spruce.

From the previous study, elevated root zone pH and NaCl caused decreases in foliar N, leaf chlorophyll concentrations, net photosynthesis, transpiration rates, and growth for all species tested. Considering that nutrient loading of nursery stock and N fertilization are both well accepted practices to increase the health and survival of plants, it was hypothesized that supplemental N would recover the physiological functions of trembling aspen and white spruce exposed to elevated root zone pH and NaCl. To my best knowledge, no previous reports exist for testing the effects of N supplementation on plants exposed to the combined effects of elevated root zone pH and salinity.

## 3.2 Materials and Methods:

### 3.2.1 Plant material and growth conditions

One-year-old dormant trembling aspen and white spruce seedlings were obtained from Smoky Lake Forest Nursery (Smoky Lake, AB, Canada). Plant material was produced from seeds collected from open-pollinated wild tree stands in various locations within Alberta seed zone CM 2.2 by Tree Time Services Inc. (Edmonton, AB, Canada). Both species were transported to the University of Alberta on February 18$^{th}$, 2017 and placed into liquid culture on February 25$^{th}$, 2017. Seedlings were stored in a refrigerated room at 4°C in the dark until the experiment began. Seedling roots were washed of potting medium and placed in aerated solution

culture maintained at pH 5 and with 0 mM NaCl for two weeks before experiment began to break dormancy. Experiments were run in a controlled environmental growth room maintained at 22/18 °C (day/night) temperature, 65 ± 5 % relative humidity, and 16-h photoperiod with 300 μmol $m^{-2}$ $s^{-1}$ PPFD provided by full spectrum fluorescent lights (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). The hydroponics set-up used to run experiments was previously described by Zhang et al. (2013). An individual unit consisted of two 30 L opaque plastic tubs with Styrofoam covers containing 20 holes so seedlings could be placed in the nutrient solution. Each tub was attached to an aerated 120 L opaque plastic reservoir with a circulating pump (Model 9.5 950GPH, Danner MFG Inc., NY, USA). Solution medium consisted of 120 L of 25% Hoagland's solution (Epstein 1972) and was changed once every two weeks throughout the experiment. Solution pH was continuously maintained at the desired level with a pH controller (PHCN-70, Omega Engineering Inc., Laval, QC, Canada) and Orion 9106 BNWP gel-filled combination pH electrode immersed in the nutrient solution (Thermo Scientific, Rochester, NY). The pH was automatically adjusted with small volumes of 5% KOH using an electrode valve (Model 8260G071 120/60 ASCO Valve Inc., Florham Park, NJ, USA) connected to a plastic ball valve (Model R-01377-84, Cole-Parmer Canada Inc., Montreal, QC). The pH fluctuations were approximately ±0.2 from their respective values. A photograph and overhead schematic of the experimental setup can be found in Appendix six (Figure a6.1).

### 3.2.2 Treatments

Trembling aspen and white spruce with no significant difference in height and root collar diameter were selected (Table 3.S1). Each treatment started with 20 replicates per species. Individual seedlings were placed into the Styrofoam cover holes so the roots were submerged in Hoagland solution. Foam plugs were placed in the holes to hold the stems in place. Species were alternated when placed in the Styrofoam cover holes (Figure a6.1 B). Replicates that died during the experiment because of stress were immediately removed. At the onset of the experiment, both pH and NaCl were gradually elevated over seven days to reduce shock to the trees. Seedlings were maintained at two pH levels (5 and 8) with two NaCl levels (0 and 30 mM). Another group of seedlings was also subjected to the same pH and NaCl levels, but N supply was increased 4x to the 100% level of Hoagland's solution. The experiment was run in a factorial

design for a total of eight treatments. Seedlings were then maintained at their respective treatments for 50 days.

### 3.2.3 Gas exchange

After 50 days of treatment, seedlings were randomly taken from each treatment for gas exchange measurements. Typically, six seedlings were taken from each treatment. Net photosynthesis (Pn), transpiration rates (E), and intracellular $CO_2$ (Ci) concentrations were measured using an infrared gas analyzer equipped with a standard 6 $cm^2$ leaf chamber (Li-Cor 6400XT, Li-Cor Inc., Lincoln, NE, USA). Foliar tissue samples were removed from plants and placed in the leaf chamber for measurement. Measurements were conducted in the experimental growth room. Samples were allowed to equilibrate to a steady state for approximately two minutes and measurements were taken no later than five minutes after the foliar tissue was removed from plants. The light intensity for all measurements was 300 μmol $m^{-2}$ $s^{-1}$ PPFD provided by a red-blue light source (6400-02, Li- Cor Inc., Lincoln, NE, USA). The [$CO_2$] was maintained at 400 μmol for all measurements. Light intensity and [$CO_2$] values were chosen to be the same as plant growth conditions in the experimental growth room. Instantaneous water use efficiency was calculated by dividing Pn by E. For white spruce, needle area was calculated using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA, USA).

### 3.2.4 Total height and foliar chlorophyll concentration

After gas exchange measurements, a portion of leaves or needles was collected from live seedlings, lyophilized, ground to a powder, and used to measure chlorophyll concentration. Chlorophyll was extracted from ground tissue (ten mg DW) with eight mL DMSO at 65ºC for 24 h. Extracted chlorophyll was then measured with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden) at 648 nm for chlorophyll-a and 665 nm for chlorophyll-b. Total chlorophyll concentration was calculated using the Arnon's equation (Sestak et al. 1971). Seedlings were then sacrificed and separated into foliar tissue, stems, and roots and then oven-dried at 70ºC for 72 h before weighing. Foliar tissue was ground to a fine powder using a Wiley mill (screen no. 40) and used for elemental analysis.

### 3.2.5 Foliar elemental analysis

Foliar concentrations of Fe, Na, Mg, P, K, and Ca were determined by first digesting dried and ground tissue (200 mg) with ten mL 70% $HNO_3$ and the extracts diluted with DI water to 50 mL. Samples were analyzed by ICP-MS in Radiogenic Isotope facility at the University of Alberta (Zarcinas et al. 1987). For the determination of foliar N concentration, approximately two mg of dried ground samples were analyzed using a CE 440 CHN Elemental Analyzer (Exeter Analytical, MA, USA). Total foliar Cl was analyzed by extracting dried and ground tissue (200 mg) with ten mL of boiling deionized water. Samples were placed in a water bath at 90ºC for one hour. Five mL of liquid extract was combined with five mL of deionized water and 200 µL of ion strength adjuster (Thermo-Fisher Scientific, CA, USA). Total Cl was determined using a Cl electrode (Accumet chloride half cell electrode, Thermo-Fisher Scientific, CA, USA) and reference electrode (Accumet double junction reference electrode, Thermo-Fisher Scientific, CA, USA) attached to a pH meter (Accumet 925 pH/ion meter, Thermo-Fisher Scientific, CA, USA).

### 3.2.6 Statistical analysis

Statistical analyses were carried out using R (https://www.R-project.org). A p value of $P \leq 0.05$ was chose for all analyses. All data were analyzed on all dependent variables using a type III three-way permutational ANOVA linear fixed-effects model with NaCl, pH, and N treatments as fixed independent variables. The model equation is: $Y_{ijkl} = \mu + S_i + P_j + N_k + (S * P)_{ij} + (S * N)_{ik} + (P * N)_{jk} + (S * P * N)_{ijk} + \mathcal{E}_{ijkl}$ where $Y_{ijkl}$ is the $_l$th observation of the $_i$th, $_j$th, and $_k$th treatments, µ is the sample mean, $S_i$ is the $_i$th NaCl treatment, $P_j$ is the $_j$th pH treatment, and $N_k$ is the $_k$th N treatment. The variables in parenthesis are the interactions between the independent variables (ANOVA tables can be found in Appendix 2). The Fisher's LSD post-hoc test was used when significant ($P \leq 0.05$) differences were detected. No data transformations were needed since a permutational ANOVA was used.

## 3.3 Results:

### 3.3.1 Trembling aspen

Trembling aspen seedlings treated with elevated NaCl and pH exhibited significant decreases in height. Treatment with 4x N resulted in a significant increase in height for the non-

stress treatment but did not affect seedling height for treatments with elevated NaCl and pH (Figure 3.1 A). Supplementation with 4x N caused increased chlorophyll concentration for all treatments. These increases statistically significant at pH 5 0 mM NaCl and pH 8 0 mM NaCl (Figure 3.1 C). A significant disordinal interaction between NaCl and 4x N was detected for Pn. The decreases were greatest with exposure to 30 mM NaCl under normal N levels, regardless of pH level. Supplementation with 4x N caused a partial recovery for 30 mM NaCl treatments, but no changes for seedlings exposed to pH 8 (Figure 3.2 A). A significant disordinal interaction between NaCl and 4x N was found for E. Similar to Pn values, the greatest decreases were observed from exposure to 30 mM NaCl under normal N levels, regardless of pH level. Supplementation with 4x N caused partial a recovery of E from elevated NaCl. Supplementation with 4x N caused significant decreases in E at pH 5 and 8 without NaCl (Figure 3.2 C). Supplementation with 4x N caused significant increases in water use efficiency, but significant decreases in intracellular $CO_2$ regardless of stress treatment (Figure 3.3 A,C). Elevated pH caused significant decreases in foliar N regardless of N treatment (Figure 3.4 A). Regardless of pH, trees exposed to 30 mM NaCl exhibited significant increases in foliar Na and Cl concentrations. Supplementation with 4x N caused decreases in foliar Na for NaCl treatments with significant decreases at pH 8 but significant increases in foliar Cl at pH 5 and 8. A significant interaction between NaCl and 4x N was found for foliar Cl concentration (Figure 3.4 C,E).

### 3.3.2 White spruce

In white spruce, the pH, NaCl, and N treatments had no effect on seedling height (Figure 3.1 B). Significant ordinal interactions between pH and 4x N as well as NaCl and pH were detected. In general, supplementation with 4x N caused increases in chlorophyll concentration in most of the pH and NaCl treatments, except for the pH 8 treatment with 30 mM NaCl, which resulted in a decrease in chlorophyll concentration compared to 1x N (Figure 3.1 D). Both Pn and E decreased in response to elevated NaCl and pH. Supplementation with 4x N caused significant decreases for most treatments (Figure 3.2 B,D). A significant interaction between pH and 4x N was detected for water use efficiency (WUE) because seedlings treated with pH 8 and 4x N exhibited values below zero. The below zero values were attributed to photosynthesis values which were also below zero. Values decreased in seedlings treated with 30 mM NaCl

regardless of pH. Supplementation with 4x N caused further decreases in WUE at pH 8 regardless of NaCl treatment (Figure 3.3 B). A A significant ordinal interaction between pH and 4x N was detected for intracellular [$CO_2$]. At both pH levels, NaCl treatment caused significant increases. Supplementation with 4x N further increased intracellular $CO_2$ concentration at pH 8 regardless of NaCl treatment (Figure 3.3 D). The pH levels and NaCl treatment had no effect on foliar N concentration. Supplementation with 4x N caused significant increases in foliar N concentration at pH 5 but no changes at pH 8 (Figure 3.3 B). Seedlings exposed to 30 mM NaCl exhibited increased foliar Na concentration regardless of pH level. Supplementation with 4x N caused a significant decrease at pH 8 with 30 mM NaCl (Figure 3.3 D). Elevated NaCl caused significant increases in foliar Cl. Supplementation with 4x N caused a significant decrease in foliar Cl concentration at pH 5, but a significant increase at pH 8 (Figure 3.3 F).

**3.4 Discussion:**

The objective of this study was to investigate weather supplementation with 4x N would ameliorate physiological symptoms of elevated root zone pH and NaCl in trembling aspen and white spruce seedlings. Mixed results were obtained because supplementation with 4x N had positive effects in some cases but neutral or detrimental effects in other cases. For example, trembling aspen seedlings exposed to elevated root zone pH and NaCl exhibited decreases in Pn, E, and growth. A significant disordinal interaction between NaCl and 4x N was found for Pn and E because supplementation with 4x N partially recovered Pn and E in trembling aspen seedlings exposed to elevated root zone NaCl. However, 4x N supplementation did not cause changes to Pn but a decrease in E for trembling aspen seedlings exposed to elevated root zone pH. White spruce seedlings exposed to elevated root zone pH and NaCl exhibited decreases in Pn. Supplementation with 4x N had no effects on Pn for white spruce seedlings exposed to elevated root zone NaCl but caused further declines in Pn for seedlings exposed to elevated root zone pH. It has been demonstrated that N fertilization of trembling aspen nursery stock results in increased growth, primarily due to increased internal N remobilization, whereas fertilization of white spruce did not result in new growth (Hu et al. 2014; Pokharel and Chang, 2016; Schott et al. 2016). Thus, further attention should be given to N supplementation in trembling aspen seedlings; however, this approach does not appear to be effective for white spruce seedlings.

There is little information available on the efficacy of N supplementation to alleviate symptoms of NaCl stress; however, plant physiological functions under stress have been recovered by the exogenous application of salicylic acid (SA) and melatonin. The application of 0.5 mM SA to the foliar tissue of mung bean alleviated the NaCl-induced decrease in photosynthesis and nitrate reductase (Nazar et al. 2011). Another study demonstrated that the application of 0.01 mM SA to the root tissue of tomato plants experiencing NaCl stress caused a recovery of growth, photosynthesis, transpiration, and photosynthetic pigments (Mimouni et al. 2016). Interestingly, the exogenous application of melatonin has been also shown to increase plant tolerance to environmental stresses (Zhang et al. 2015). The exogenous application of N and SA or melatonin should be explored as a vegetation management option as it may alleviate symptoms of NaCl stress in tree species such as trembling aspen.

Trembling aspen exhibited increased foliar chlorophyll concentration and WUE, but decreased Ci as a result of 4x N supplementation, regardless of the pH and NaCl treatment. This response has been commonly reported for N fertilization in agricultural species. Intriguingly, net photosynthesis and transpiration rates increased in trembling aspen seedlings supplemented with 4x N when exposed to NaCl. The mechanism of N-induced increases in photosynthesis, transpiration, and WUE and the decrease in Ci under NaCl stress could be attributed to a physiological adaptation by the plant. For example, increases in WUE have been linked to intrinsic factors such as increased chloroplast carbonic anhydrase activity or expression of aquaporins, presumably to increase the fixation or transport of $CO_2$ within mesophyll cells, respectively (Guo et al. 2006; Flexas et al. 2010; Moshelion et al. 2015). Others have suggested that decreased Ci is intrinsically linked to increased mesophyll conductance (Gm) presumably due to higher amounts of $CO_2$ utilized for photosynthesis. Although not measured in the current study, changes in Gm should be considered as a physiological response to both NaCl stress and N supplementation. This is because Gm is known to decrease in response to environmental stressors such as NaCl, drought, and low N availability (Flexas et al. 2008; Moshelion et al. 2015). Furthermore, Gm increases as a result of elevated chloroplast carbonic anhydrase activity and aquaporin expression within mesophyll cells. (Flexas et al. 2008; Flexas et al. 2010; Flexas et al. 2013; Gago et al. 2014; Perez-Martin et al. 2014; Moshelion et al. 2015; Flexas et al. 2016). Interestingly, it has been proposed that improving Gm may increase photosynthesis and WUE

(Flexas et al. 2013). Therefore, future studies should focus on measuring and manipulating Gm to improve photosynthesis and WUE.

This study represents an attempt to recover physiological function under stress conditions by 4x N supplementation. This strategy was not successful for white spruce as 4x N supplementation had negative effects on white spruce because it caused further declines in Pn and E. Supplementation with 4x N showed some success for trembling aspen because Pn and E values were partially recovered for seedlings exposed to elevated root zone NaCl; however, growth was not recovered. Interestingly, trembling aspen supplemented with 4x N exhibited increased WUE but decreased Ci. It is hypothesized that this is a result of increased Gm. Future studies should focus on the mechanisms of increased Pn and E under conditions of NaCl stress and N supplementation. Furthermore, the application of exogenous N, SA, or melatonin to alleviate stress in field conditions should be explored.

**3.5 References:**


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## 3.6 Figures

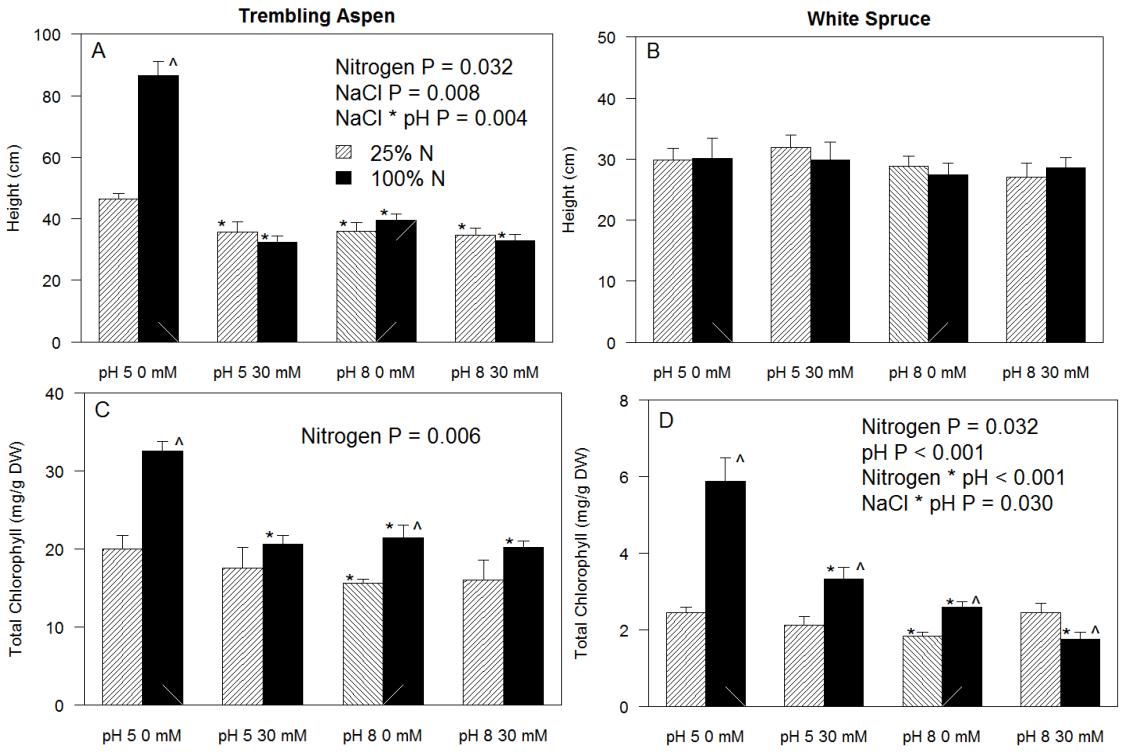


Figure 3.1. Effects of supplemental N on a 50-day treatment with elevated pH and NaCl on height (A & B) and chlorophyll concentration (C & D) of trembling aspen and white spruce seedlings. Values represent the mean (n = 6) + SEM. Asterisks represent a significant difference between elevated root zone pH and NaCl treatments and respective control. Carets represent differences caused by N addition in individual treatments.

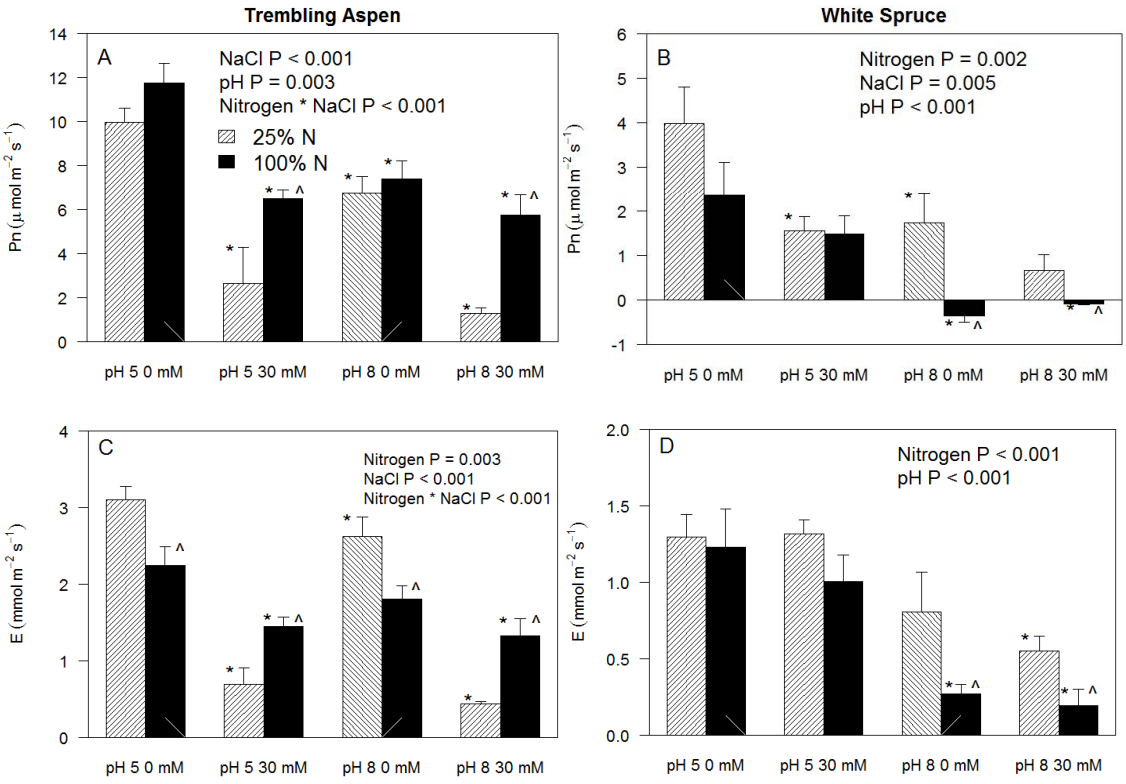


Figure 3.2. Effects of supplemental N on a 50-day treatment with elevated pH and NaCl on net photosynthesis (Pn) (A & B) and transpiration (E) (C & D) for trembling aspen and white spruce seedlings. Values represent the mean (n =6) + SEM. Asterisks represent a significant difference between elevated root zone pH and NaCl treatments and respective control. Carets represent differences caused by N addition in individual treatments.

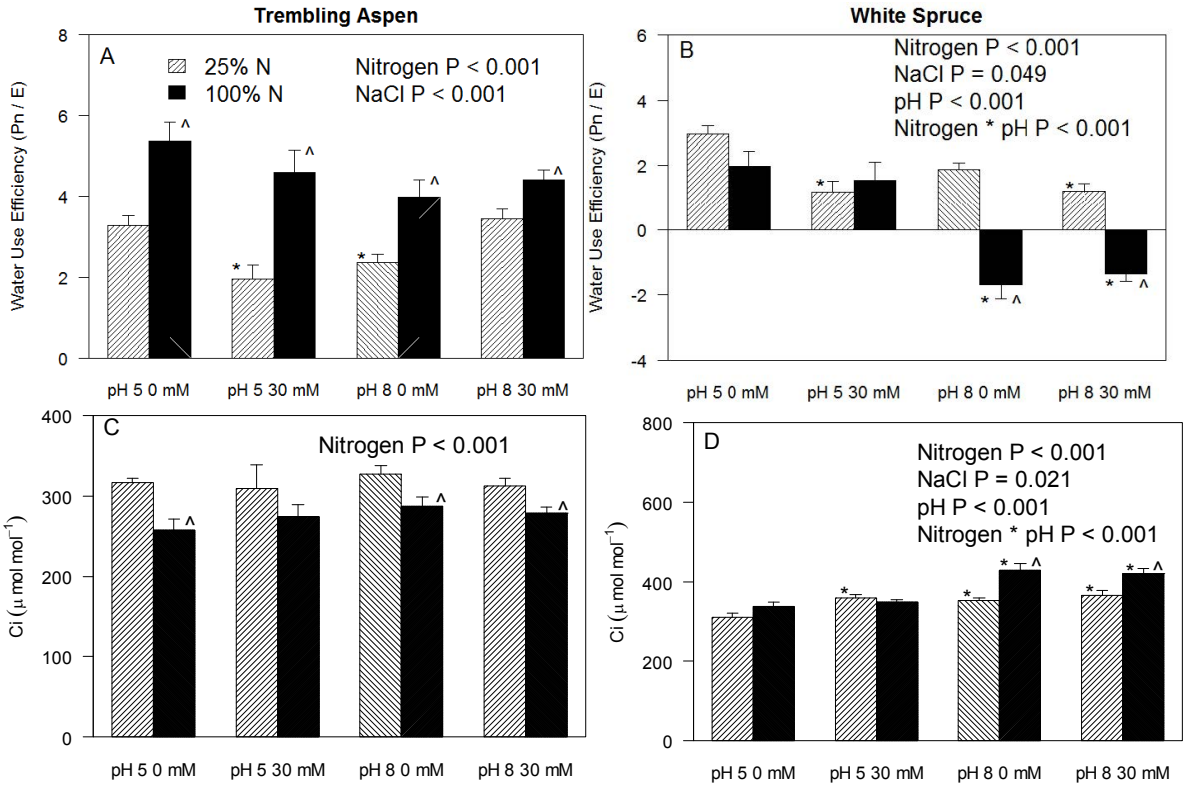


Figure 3.3. Effects of supplemental N on a 50-day treatment with elevated pH and NaCl on water use efficiency (WUE) (A & B) and intracellular $CO_2$ (C & D) for trembling aspen and white spruce seedlings. Values represent the mean (n = 6) + SEM. Asterisks represent a significant difference between elevated root zone pH and NaCl treatments and respective control. Carets represent differences caused by N addition in individual treatments.

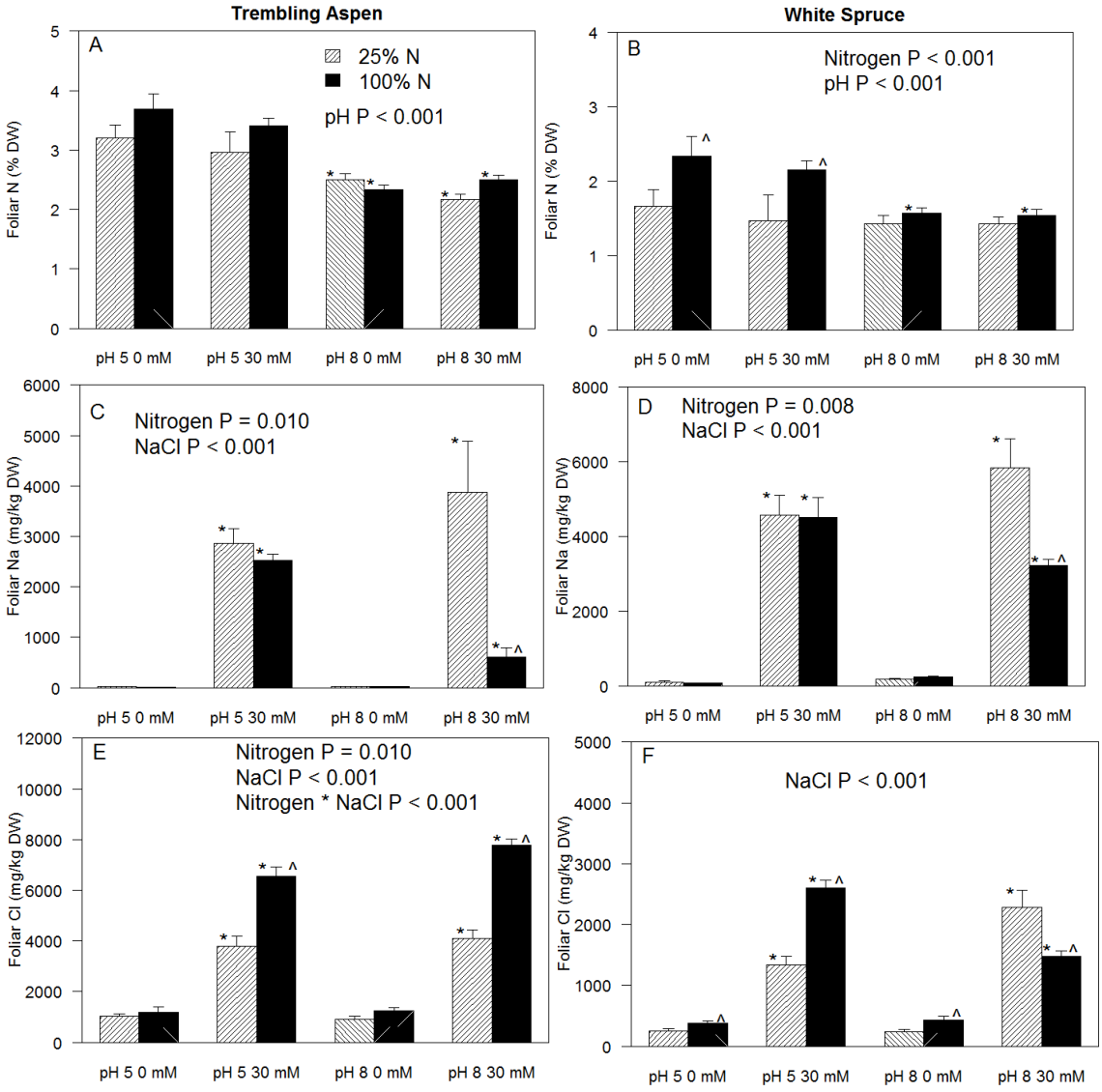


Figure 3.4. Effects of supplemental N on a 50-day treatment with elevated pH and NaCl on foliar N (A & B), Na (C & D), and Cl (E & F) concentrations for trembling aspen and white spruce seedlings. Values represent the mean (n = 6) + SEM. Asterisks represent a significant difference between elevated root zone pH and NaCl treatments and respective control. Carets represent differences caused by N addition in individual treatments.

### 3.7 Supplemental material

Table 3.S1 Initial height and root collar diameter prior to experimental treatment for trembling aspen and white spruce.

| | Initial Height (cm) | | Initial Root Collar diameter (mm) | |
|---|---|---|---|---|
| | Trembling aspen | White spruce | Trembling aspen | White spruce |
| pH 5 | | | | |
| 0 mM NaCl | 46.21 ± 1.35 | 29.15 ± 0.94 | 4.34 ± 0.21 | 3.94 ± 0.75 |
| 30 mM | 48.16 ± 2.46 | 32.54 ± 1.37 | 4.44 ± 0.37 | 3.63 ± 0.16 |
| 0 mM + N | 46.93 ± 1.69 | 29.11 ± 0.98 | 4.08 ± 0.35 | 3.63 ± 0.45 |
| 30 mM + N | 47.43 ± 1.63 | 31.95 ± 1.83 | 3.84 ± 0.43 | 3.25 ± 0.09 |
| pH 8 | | | | |
| 0 mM | 44.84 ± 2.15 | 31.76 ± 1.66 | 4.31 ± 0.33 | 3.44 ± 0.16 |
| 30 mM | 50.03 ± 1.76 | 30.84 ± 0.84 | 4.34 ± 0.14 | 3.11 ± 0.07 |
| 0 mM + N | 49.18 ± 1.74 | 29.16 ± 0.95 | 4.95 ± 0.56 | 3.45 ± 0.31 |
| 30 mM + N | 46.73 ± 1.63 | 29.11 ± 0.95 | 4.63 ± 0.36 | 3.42 ± 0.14 |

## Chapter 4: Recovery of trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) seedlings from NaCl stress: Implications for increased foliar potassium and necrosis as stress resistance mechanisms.

### 4.1 Introduction:

Bitumen deposits in northern Alberta can be extracted via *in situ* methods or surface mining. If surface mining occurs, then boreal forest vegetation and soils are first removed from the site prior to mining (Berkowitz and Speignh, 1975). Operators of bitumen surface mines are required by law to ultimately reclaim boreal forest lands to equivalent land capability (Government of Alberta, 2010). Revegetation of these lands is difficult in part due to heterogeneous and transient levels of NaCl in the soil caused by variations in evapotranspiration, precipitation, water table depth, and upward water flux (Kessler et al. 2010; Carrera-Hernandéz 2012). Thus, it is important to understand the potential of different boreal forest tree species to recover from exposures to periods of NaCl stress. Trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) are commonly used for oil sands revegetation and are among the dominant trees present in the Canadian boreal forests. Seedlings of these tree species have been reported to withstand at least four weeks of 60 mM NaCl stress (Renault et al. 1999; Renault, 2005). However, the processes of recovery from exposure to salt stress in most plant species have not been thoroughly addressed by research.

Salinity stress on glycophytic plants involves a combination of osmotic and ionic factors, which elicit numerous physiological responses. These responses include an almost immediate drop in root hydraulic conductivity that is linked to decreased water transport through aquaporins and a rapid drop in transpiration rates due to stomatal closure (Boursiac et al., 2005; Munns and Tester, 2008; Lee et al., 2010). It is also well established that exposure to salt causes decreases in net photosynthesis, growth, and total biomass production (Kozlowski, 2000, Munns and Tester, 2008; Shabala and Munns, 2012). Leaf senescence is another common characteristic of salt stress and is considered by some to be a tolerance mechanism, which reduces the amount of $Na^+$ that enters younger leaf tissue (van der Moezel et al., 1988; Wolf et al., 1991). Another important factor in NaCl stress tolerance is maintaining a low $Na^+$:$K^+$ ratio in foliar tissue by a combination of $Na^+$ exclusion and $K^+$ accumulation. This process facilitates osmotic adjustment, decreases oxidative damage from reactive oxygen species, and reduces programmed cell death (Wang et al.

2013). It should be noted that plants exposed to salt stress accumulate reactive oxygen species in most cell types (Hernandez et al. 2000; Sairam and Srivastava 2002; Gómez et al. 2004; Fidalgo et al. 2005). Taken together, this suggests that a plant's response to NaCl stress may be linked to complex cell signaling events.

Research on the recovery of plants from NaCl stress is limited. Spinach subjected to 21 days to 100 mM NaCl followed by a 29-day recovery exhibited a recovery of RuBisCO activity, photosynthesis, stomatal conductance, Fv/Fm, and chlorophyll concentration (Delfine et al. 1999). Exogenous jasmonic acid aided the recovery of rice seedlings exposed to 40 and 80 mM NaCl for eight days by restoring growth, water potential, photosynthesis, Fv/Fm, and ion uptake (Kang et al. 2005). In contrast to the limited number of recovery studies from NaCl stress, there are many reports on the recovery of glycophyte plants from drought stress. Recovery from drought is characterized by a return of physiological function, but almost never to full capacity (reviewed by Chaves et al. 2008). It has been demonstrated that plants can recover from both NaCl and drought stress; however, the evidence for recovery of drought stress is far more extensive than recovery from NaCl stress. Therefore, more research should be dedicated to the recovery of plants exposed to NaCl stress.

The objective of this study was to examine the recovery of physiological functions such as photosynthesis, transpiration, chlorophyll, and growth in trembling aspen, tamarack, and white spruce following exposure to NaCl stress. I anticipated that physiological functions such as photosynthesis, transpiration, chlorophyll, and growth would decrease because of NaCl stress, but would increase during the recovery period for all species; however, since little is known about the stress recovery processes in boreal tree species, the degree and timing of recovery was a key question in this study. This information is important to manage vegetation in lands affected by intermittent exposure to salt including the oil sands reclamation areas.

## 4.2 Materials and Methods:

### 4.2.1 Plant material and growth conditions

One-year old dormant trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) seedlings were obtained from Smoky Lake Forest Nursery (Smoky Lake, AB, Canada). Plant material was produced from seeds collected from open-

pollinated wild tree stands in various locations within Alberta seed zone CM 2.2 by Tree Time Services Inc. (Edmonton, AB, Canada). All species were transported to the University of Alberta on March 15th, 2016 and planted on March 16th, 2016. The seedlings had no difference between treatment in height and root collar diameter (Tables 5.S1, 5.S2). The seedlings were planted in four L pots with a mixture of peat moss and sand (1:1 by weight) and grown for six weeks prior to treatments. Seedlings were watered every other day and fertilized with 250 mL of three g/L 20:20:20 fertilizer every two weeks prior to treatments and once every month once the treatments commenced. The experiment was carried out in a controlled-environment growth room maintained at 22/18°C (day/night) temperature, 65 ± 5% relative humidity, and 16-h photoperiod with 300 μmol $m^{-2}$ $s^{-1}$ PPFD using full spectrum fluorescent lights (Philips high output, F96T8/TL835/HO, Markham, ON, Canada).

### 4.2.2 Treatments

After the six-weeks of growth, seedlings were exposed to treatments with 0, 50, and 100 mM NaCl for 60 days by applying 250 mL of the respective NaCl solution once a week. After NaCl treatments, seedlings were thoroughly watered to flush out any remaining NaCl and allowed to recover at 0 mM NaCl for the remainder of the experiment. Selected seedlings (n = 6) were taken for measurements immediately after 60 days of NaCl treatments as well as 30 and 60 days after the termination of NaCl treatments. Some of the trembling aspen seedlings treated with 100 mM NaCl completely defoliated during the NaCl treatment and re-flushed during the recovery period. These seedlings were sampled after 60 days of recovery.

### 4.2.3 Gas exchange

Net photosynthesis (Pn) and transpiration (E) rates were measured using an infrared gas analyzer equipped with a standard 6 $cm^2$ leaf chamber (Li-Cor 6400XT, Li-Cor Inc., Lincoln, NE, USA). Foliar tissue samples were removed from plants and placed in the leaf chamber for measurement. Measurements were conducted in the experimental growth room. Samples were allowed to equilibrate to a steady state for approximately two minutes and measurements were taken no later than five minutes after the foliar tissue was removed from plants. The light intensity for all measurements was 300 μmol $m^{-2}$ $s^{-1}$ PPFD provided by a red-blue light source (6400-02, Li- Cor Inc., Lincoln, NE, USA). The [$CO_2$] was maintained at 400 μmol for all

measurements. Light intensity and [$CO_2$] values were chosen to be the same as plant growth conditions in the experimental growth room. For white spruce and tamarack, needle area was calculated using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA, USA).

### 4.2.3 Dry weight and foliar chlorophyll concentration

After gas exchange measurements, a small amount of leaves or needles was collected from live seedlings, lyophilized, and ground to a powder to determine foliar chlorophyll concentrations. Chlorophyll was extracted from ground tissue (ten mg DW) with eight mL DMSO at 65ºC for 24 h. Extracted chlorophyll was then measured with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden) at 648 nm for chlorophyll-a and 665 nm for chlorophyll-b. Total chlorophyll concentration was calculated using the Arnon's equation (Sestak et al. 1971).

Sampled seedlings were separated into leaves (needles), stems, and roots and then oven-dried at 70ºC for 72 h before weighing. Lyophilized tissue used for chlorophyll analysis was also weighed and added to the total dry weight measurement. For trembling aspen and tamarack, necrotic foliar tissue was first separated from living tissue and weighed separately. Foliar samples were ground to a fine powder using a Wiley mill (screen no. 40) and used for elemental analysis.

### 4.2.4 Foliar elemental analysis

Foliar concentrations of Fe, Na, Mg, P, K, and Ca were determined from dried and ground tissue (200 mg) digested with ten mL 70% $HNO_3$ and diluted with deionized (DI) water up to 50 mL. Samples were then analyzed by ICP-MS (Zarcinas et al. 1987) in Radiogenic Isotope facility at the University of Alberta. Total foliar Cl was analyzed by extracting dried and ground tissue (200 mg) with ten mL of boiling deionized water. Samples were placed in a water bath at 90ºC for one hour. Five mL of liquid extract was combined with five mL of deionized water and 200 µL of ion strength adjuster (Thermo-Fisher Scientific, CA, USA). Total Cl was determined using a chloride electrode (Accumet chloride half cell electrode, Thermo-Fisher Scientific, CA, USA) and reference electrode (Accumet double junction reference electrode,

Thermo-Fisher Scientific, CA, USA) attached to a pH meter (Accumet 925 pH/ion meter, Thermo-Fisher Scientific, CA, USA).

### 4.2.5 Statistical analysis

All data were analyzed using R (https://www.R-project.org). A p value of $P \leq 0.05$ was chose for all analyses. All dependant variables were analyzed using a type III two-way ANOVA linear fixed-effects model with NaCl treatment and day of recovery as fixed independent variables. The model equation is: $Y_{ijk} = \mu + S_i + T_j + (S * T)_{ij} + \mathcal{E}_{ijk}$ where $Y_{ijk}$ is the $_k$th observation of the $_i$th and $_j$th treatments, μ is the sample mean, $S_i$ is the $_i$th NaCl treatment and, $T_j$ is the $_j$th day of recovery. The variable in parenthesis is the interaction between NaCl and day of recovery (ANOVA tables can be found in Appendix 3). A Tukey's HSD test was used when significant differences were detected. To compare the percent of foliar necrotic tissue to living tissue in trembling aspen and tamarack, data was pooled between sampling periods based on the NaCl treatment. The percent of necrotic foliar tissue for trembling aspen and tamarack was analyzed using one-way ANOVA linear fixed-effects model with NaCl as a fixed independent variable. The model equation is: $Y_{ij} = \mu + S_i + \mathcal{E}_{ij}$ where $S_i$ is the $_i$th NaCl treatment. A Tukey's HSD post-hoc test was used when significant differences were detected. Data that did not meet the ANOVA assumptions of normality of distribution and homogeneity of variance were log10 transformed before analysis.

## 4.3 Results:

### 4.3.1 Trembling aspen

In trembling aspen, treatment with 50 and 100 mM NaCl caused a significant and proportional reduction in total dry weight that did not recover over time (Figure 4.1 A). A significant disordinal interaction between NaCl and time was found for foliar chlorophyll concentration. Chlorophyll concentration decreased after treatments with 50 and 100 mM NaCl but recovered after 30 days (Figure 4.1 B). A significant disordinal interaction between NaCl and time was found for Pn. In most cases, Pn showed no significant differences between NaCl treatments for the duration of the experiment. However, seedlings treated with 100 mM NaCl prior to recovery showed 33.6% lower Pn compared to those treated with 0 mM NaCl (Figure 4.1 C). A significant disordinal interaction between NaCl and time was found for transpiration which

decreased after 50 and 100 mM NaCl treatments but returned to 0 mM NaCl levels during the recovery period. No significant differences between NaCl treatments were found after 30 days of recovery (Figure 4.1 D). Interestingly, seedlings treated with 0 and 50 mM NaCl exhibited time-dependent decreases in total chlorophyll concentration and Pn during the recovery period. However, seedlings treated with 100 mM NaCl which re-flushed after 60 days of recovery exhibited the highest values of chlorophyll concentration and Pn compared to other NaCl treatments which led to disordinal interactions being detected.

Foliar Na levels were very low in trembling aspen regardless of NaCl treatment. No significant differences were found between the 50 and 100 mM NaCl treatments; however, Na levels increased for both treatments during the recovery period (Figure 4.2 A). A significant disordinal interaction between NaCl and time was found for foliar Cl concentration. In most cases, foliar Cl concentration increased significantly for seedlings treated with 50 and 100 mM NaCl and remained high for the duration of the experiment. However, seedlings treated with 100 mM NaCl exhibited a significant decrease in foliar Cl after 60 days of recovery because they were new leaves which re-flushed during the recovery period (Figure 4.2 B). Significant increases in foliar K were observed for seedlings treated with 50 and 100 mM NaCl. Foliar K concentrations returned to the control levels after 30 days of recovery for seedlings treated with 50 mM NaCl whereas seedlings treated with 100 mM NaCl had significantly higher levels of foliar K for the remainder of the experiment (Figure 4.2 C). Seedlings treated with 100 mM NaCl had a significantly lower Na:K ratio throughout the experiment (Figure 4.2 D). Seedlings exposed to 100 mM NaCl, which re-flushed after 60 days of recovery showed significantly lower foliar Cl but higher K concentrations compared to the 0 and 50 mM NaCl treatments.

### 4.3.2 Tamarack

In tamarack, both NaCl treatments caused no significant changes in total dry weight and chlorophyll concentration. All seedlings continued to increase in total dry weight during the recovery period; however, seedlings treated with 100 mM NaCl showed significantly lower total dry weight and chlorophyll concentration during the recovery period compared to 0 and 50 mM treatments (Figure 4.3 A,B). Net photosynthesis decreased in seedlings treated with 50 and 100 mM NaCl and remained lower after 30 days of recovery. After 60 days of recovery, Pn returned to control levels in seedlings treated with 50 mM NaCl, but not in those treated with 100 mM

NaCl (Figure 4.3 C). A significant disordinal interaction between NaCl and time was detected for E. This is because E decreased as a result of 50 and 100 mM NaCl treatments; however, after 30 and 60 days of recovery, no differences in E were found between all treatments (Figure 4.3 D).

Foliar Na and Cl increased proportionally as a result of NaCl treatments. Foliar levels of Na and Cl remained unchanged for 0 and 50 mM NaCl treatments during the recovery period; however, seedlings treated with 100 mM NaCl showed increases in foliar Na and Cl. A significant ordinal interaction between NaCl and time was detected for foliar Cl concentration. Seedlings treated with 100 mM NaCl exhibited fluctuations in foliar Cl concentration during the recovery period (Figure 4.4 A,B). Foliar K was elevated in seedlings treated with 50 and 100 mM NaCl compared to 0 mM seedlings. These values returned to control levels after 30 days of recovery (Figure 4.4 C). A significant ordinal interaction between NaCl and time was found for foliar Na:K. These values increased during the recovery period for 0 and 100 mM treatments but did not change for the 50 mM treatment (Figure 4.4 D).

### 4.3.3 White spruce

In white spruce, 50 and 100 mM NaCl treatments resulted in no significant changes in total dry weight and chlorophyll concentration (Figure 4.5 A,B). Net photosynthesis rates were similar in 0 and 50 mM NaCl treatments at all measurement times. Seedlings treated with 100 mM NaCl first showed a significant decrease in Pn, but showed a full recovery of Pn after 60 days (Figure 4.5 C). Transpiration rates showed no differences between NaCl treatments for all timepoints but was lower for all NaCl treatments after 30 days of recovery (Figure 4.5 D). Foliar Na and Cl concentration increased in seedlings exposed to 100 mM NaCl and remained high after 60 days of recovery. A significant ordinal interaction between NaCl and time was found for foliar Cl concentration (Figure 4.6 A,B). Treatment with 100 mM NaCl caused a decrease in foliar K that recovered after 30 days (Figure 4.6 C). Foliar Na:K ratios were higher in seedlings treated with 100 mM NaCl at all time points (Figure 4.6 D).

All species exhibited chlorosis followed by necrosis staring at the foliar tips as a result of NaCl stress. In trembling aspen and tamarack, the percentage of necrotic foliar tissue increased with increasing NaCl treatment concentration. The increase in necrotic tissue was more pronounced in trembling aspen compared to tamarack (Table 4.1).

## 4.4 Discussion:

With the exception of dry weight in trembling aspen, parameters for growth, chlorophyll concentration, Pn, and E returned to control levels in all examined species after 60 days of recovery from 50 mM NaCl treatment. The 50 mM NaCl treatment in this study is comparable to the established threshold of four dS $m^{-2}$ for boreal forest vegetation in natural settings (Lilles et al. 2010). Thus, it is encouraging that the tree species tested in this study can recover chlorophyll concentration, Pn, E, and growth after exposure to 50 mM NaCl.

Recovery for seedlings exposed to the 100 mM NaCl treatment differed by species. Trembling aspen seedlings exhibited an ability to recover primarily through defoliation during the stress period followed by re-flushing of foliar tissue during the recovery period. Leaves newly grown during the recovery period had chlorophyll concentrations and Pn that were higher than control values whereas E returned to values similar to other NaCl treatments and foliar Cl concentrations lower than 50 mM NaCl treatments. This phenomenon likely explains the significant disordinal interaction terms between NaCl and time observed in the aforementioned parameters. Interestingly, foliar Na and Cl concentrations were low, but K was elevated in new leaves. Tamarack seedlings exposed to 100 mM NaCl showed lower levels of chlorophyll concentration, Pn, and total dry weight after 60 days of recovery compared to 0 and 50 mM treatments, suggesting that seedlings were not able to fully recover. White spruce exhibited physiological function similar to control levels after 60 days of recovery from exposure to 100 mM NaCl. These findings are comparable to naturally saline sites in the boreal forest where it has been established that trembling aspen and white spruce exhibit productive growth on sites with soil EC as high as 7.8 dS $m^{-2}$. Trembling aspen exhibited decreased growth as soil EC increased whereas white spruce appeared to be unaffected (Lilles et al. 2012). Long-term exposure of plants to elevated soil Na leads to Na toxicity and causes reduced physiological function (Munns and Tester, 2008). Thus, the management of Na within plant tissue may lead to the recovery of physiological processes such as chlorophyll concentration, Pn, E, and growth. Potential mechanisms of Na management in the species tested include increased foliar K, increased foliar necrosis, Na exclusion at the roots, storage of Na in the cell wall, and vacuole sequestration of Na ions. These topics will be discussed in more detail below.

Trembling aspen and tamarack exhibited increases in foliar K from NaCl treatments. In trembling aspen, the increase in foliar K and low Na concentration caused a decreased Na:K ratio. Foliar increases in cytoplasmic K from NaCl stress is proposed to be a fundamental mechanism of NaCl tolerance in glycophytic plants and maintaining a low foliar Na:K ratio can reduce physiological signs of NaCl stress (Munns and Tester, 2008; Wang et al. 2013). The reason for this strategy has yet to be fully elucidated, but several lines of evidence suggest that elevated foliar K is beneficial by acting as a co-factor for enzymes essential for nearly all growth processes in plants. Furthermore, plants growing in soils with low K availability are more sensitive to NaCl and drought stress. Finally, the exogenous application of KCl to foliar tissue of plants can alleviate symptoms of poor health in soils with low potassium availability (Cakmak, 2005; Chen et al. 2005; Escalante-Pérez et al. 2009; Wang et al. 2013, Zörb et al. 2013). Thus, it is advisable that reclamation sites have adequate levels of available soil K to help plants tolerate periods of elevated environmental stress.

Trembling aspen dry weight remained at post-stress levels throughout the recovery period, perhaps due to accelerated foliar necrosis and senescence. Trembling aspen and tamarack showed increased foliar necrotic tissue with increasing NaCl treatment concentrations. White spruce seedlings treated with NaCl exhibited chlorosis at the needle tips but no necrosis at the time of sampling. Accelerated foliar necrosis is a common response of glycophytic plants to Na toxicity and occurs after prolonged NaCl stress. It is hypothesized that this process aids NaCl tolerance by reducing the amount of toxic Na entering growing tissues (Munns and Tester, 2008). Interestingly, the formation of necrotic foliar tissue was preceded by a chlorosis that began at the foliar apex. The NaCl stress-induced formation of chlorotic foliar tissue has been linked to a cell signaling process that causes the vesiculation and proteolysis of chloroplasts in Arabidopsis (Wang and Blumwald, 2014). Taken together, it appears that Na-induced foliar necrosis is an active process perhaps to extrude toxic Na from the plant.

Foliar Na concentrations in trembling aspen were significantly lower than foliar Cl concentrations within the same leaves as well as foliar Na in other species immediately after NaCl treatment and during the recovery period. The low levels of foliar Na in trembling aspen may in part explain why net photosynthesis was relatively unaffected by NaCl. As discussed previously, accelerated foliar senescence may be one mechanism to maintain relatively low foliar

Na levels. Another possibility for low foliar Na concentration is ion exclusion via suberin deposition in the roots (Franke and Schreiber, 2007; Munns and Tester, 2008). In general, foliar Na and Cl remained at the same level or increased after NaCl treatment and during the recovery period for tamarack and white spruce seedlings. Both tamarack and white spruce had higher foliar Na concentrations compared to Cl for all NaCl treatments. In most cases growth, chlorophyll concentration, Pn, and E were similar to or returned to control values during the recovery period. Considering that cytosolic Na is linked to decreases in growth, chlorophyll concentration, Pn, and E, it is possible that Na was stored in cell walls or vacuoles of foliar tissues to protect the plants from cytosolic Na toxicity (Munns and Tester, 2008; Parihar et al. 2015). Maintaining low levels of cytosolic Na is an important strategy for long term salinity tolerance. Although the cellular and tissue distribution of Na was not examined in this study, it is plausible that Na sequestration was among the principal NaCl tolerance mechanisms in the studied plants.

Trembling aspen, tamarack, and white spruce subjected to a 60-day exposure to 50 mM NaCl fully recovered growth, chlorophyll concentration, Pn, and E after 60 days, which is comparable to the four dS $m^{-2}$ soil threshold for boreal forest vegetation (Lilles et al. 2010). However, trembling aspen and tamarack did not fully recover from the 100 mM NaCl treatment after 60 days of recovery. Trembling aspen and tamarack both exhibited elevated foliar K and accelerated senescence from NaCl stress. Both responses appear to be deliberate tolerance mechanisms designed to reduce the toxic effects of foliar Na. Thus, it would be advisable to maintain high levels of available soil K on site with elevated soil Na. Perhaps fertilizing sites with potash or applying exogenous K sprays could be used to alleviate symptoms of Na toxicity (Zörb et al. 2013). It was noted that foliar Na in trembling aspen was remarkably low. This trend was attributed to accelerated foliar senescence and potentially ion exclusion by the roots. Both tamarack and white spruce had increased levels of foliar Na despite physiological parameters returning to control levels during the recovery period. This was potentially attributed to the storage of Na in the cell wall or vacuole. Future work will focus on the effects of NaCl stress and overwintering on trembling aspen, tamarack, and white spruce and on the accelerated senescence mechanism of trembling aspen.

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## 4.6 Tables

Table 4.1. Percent of necrotic leaf per total leaf dry weight for trembling aspen and tamarack. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

| Days of recovery | 0 | 30 | 60 |
|---|---|---|---|
| Trembling aspen | | | |
| 0 mM NaCl | 0 a | 0 a | 0 a |
| 50 mM | 33.57 ± 7.55 b | 16.22 ± 4.20 b | 25.28 ± 5.15 b |
| 100 mM | 43.02 ± 5.80 b | 50.36 ± 9.24 c | 0 a |
| Tamarack | | | |
| 0 mM | 0 a | 0 a | 0 a |
| 50 mM | 0 a | 9.09 ± 2.28 b | 2.67 ± 0.97 b |
| 100 mM | 15.33 ± 0.54 b | 32.25 ± 6.80 c | 20.41 ± 3.75 c |

## 4.7 Figures

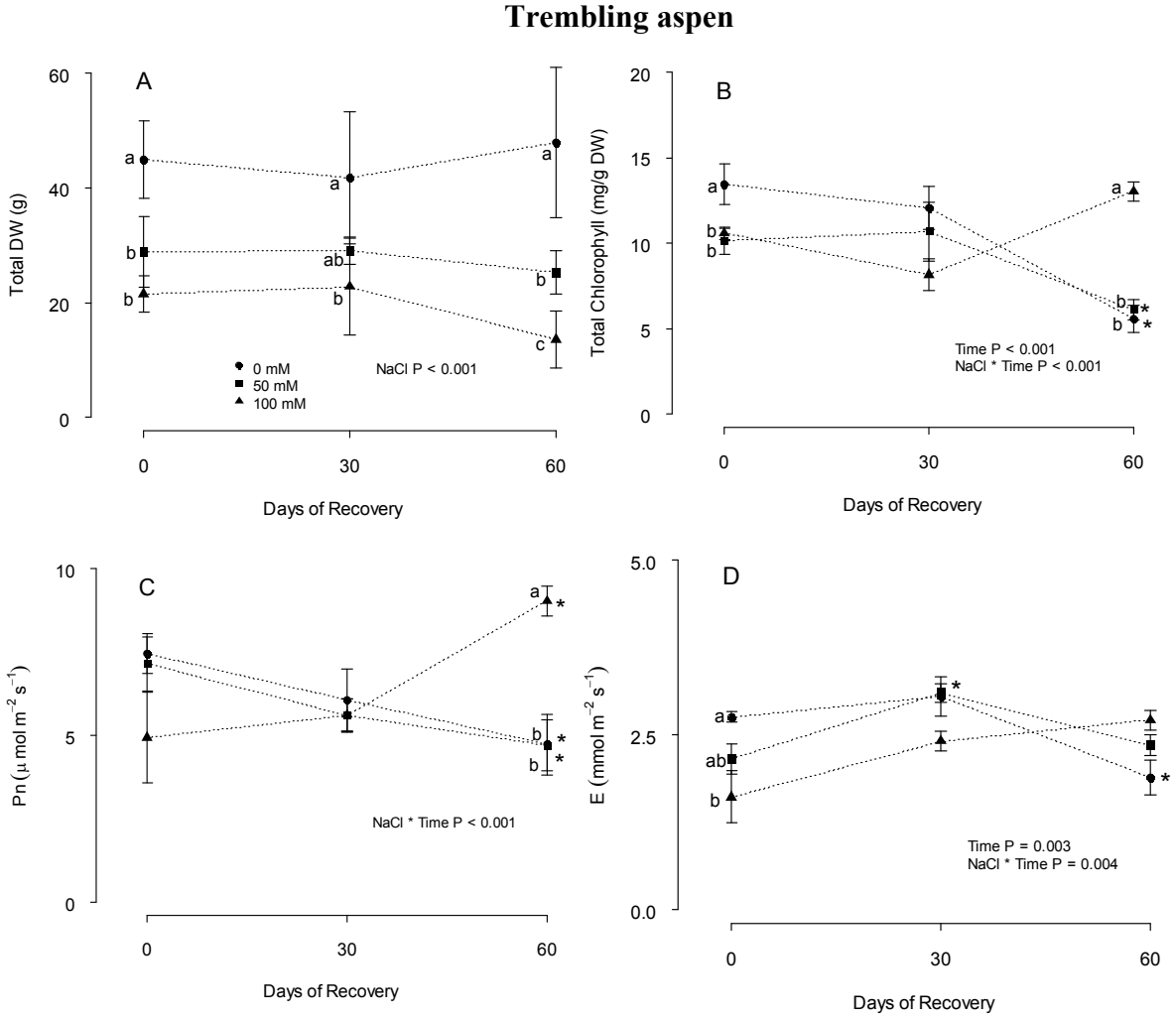


Figure 4.1. Effects of a 60-day NaCl treatment followed by a 60-day recovery period on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rates (E) (D) in trembling aspen seedlings. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test at a specific time point. Asterisks represent a significant difference from day 0 of recovery within the same treatment over time.

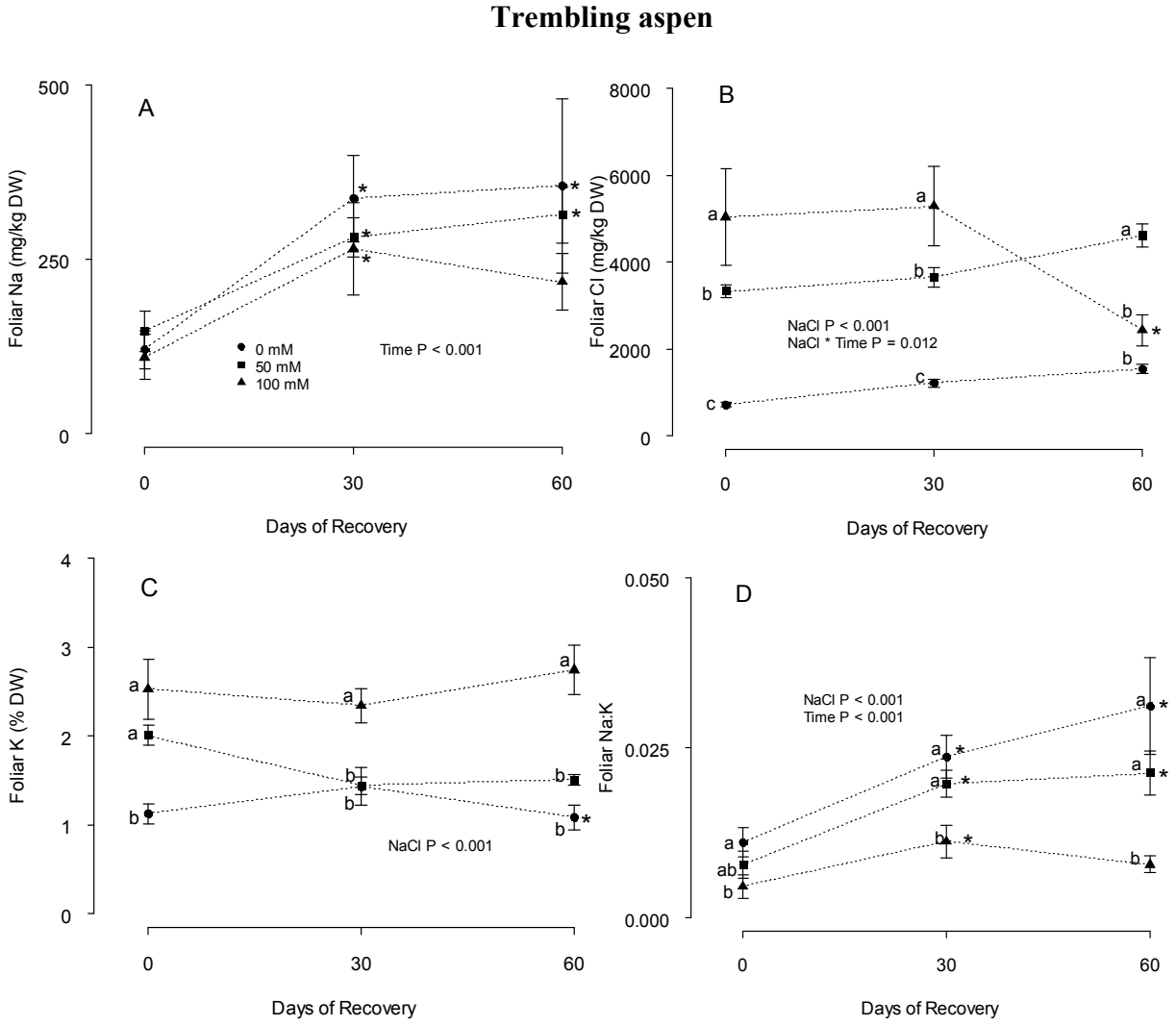


Figure 4.2. Effects of a 60-day NaCl treatment followed by a 60-day recovery period on foliar Na (A), Cl (B), K (C), and Na:K ratio (D) for trembling aspen seedlings. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test at a specific time point. Asterisks represent a significant difference from day 0 of recovery within the same treatment over time.

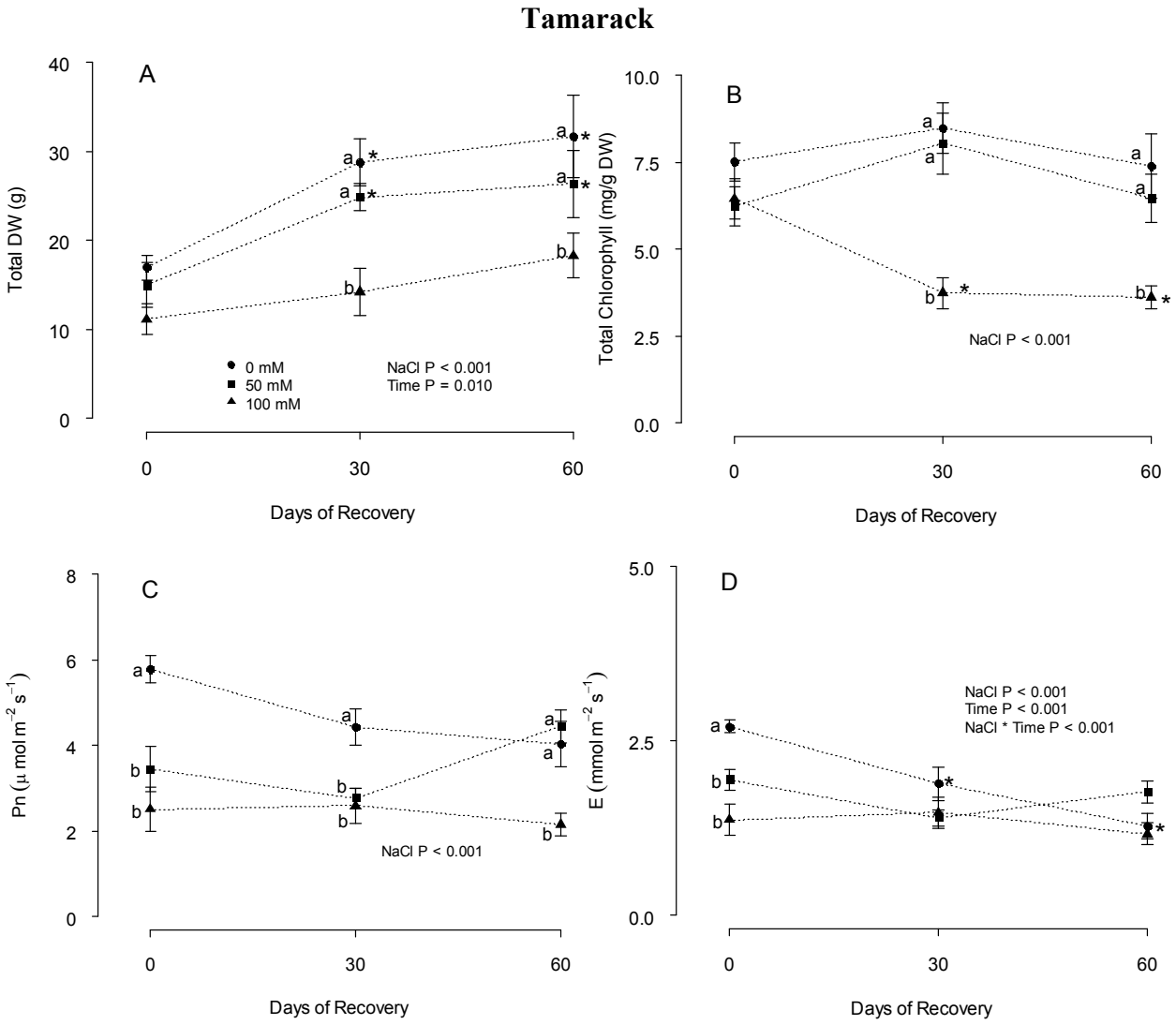


Figure 4.3. Effects of a 60-day NaCl treatment followed by a 60-day recovery period on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rates (E) (D) in tamarack seedlings. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test at a specific time point. Asterisks represent a significant difference from day 0 of recovery within the same treatment over time.

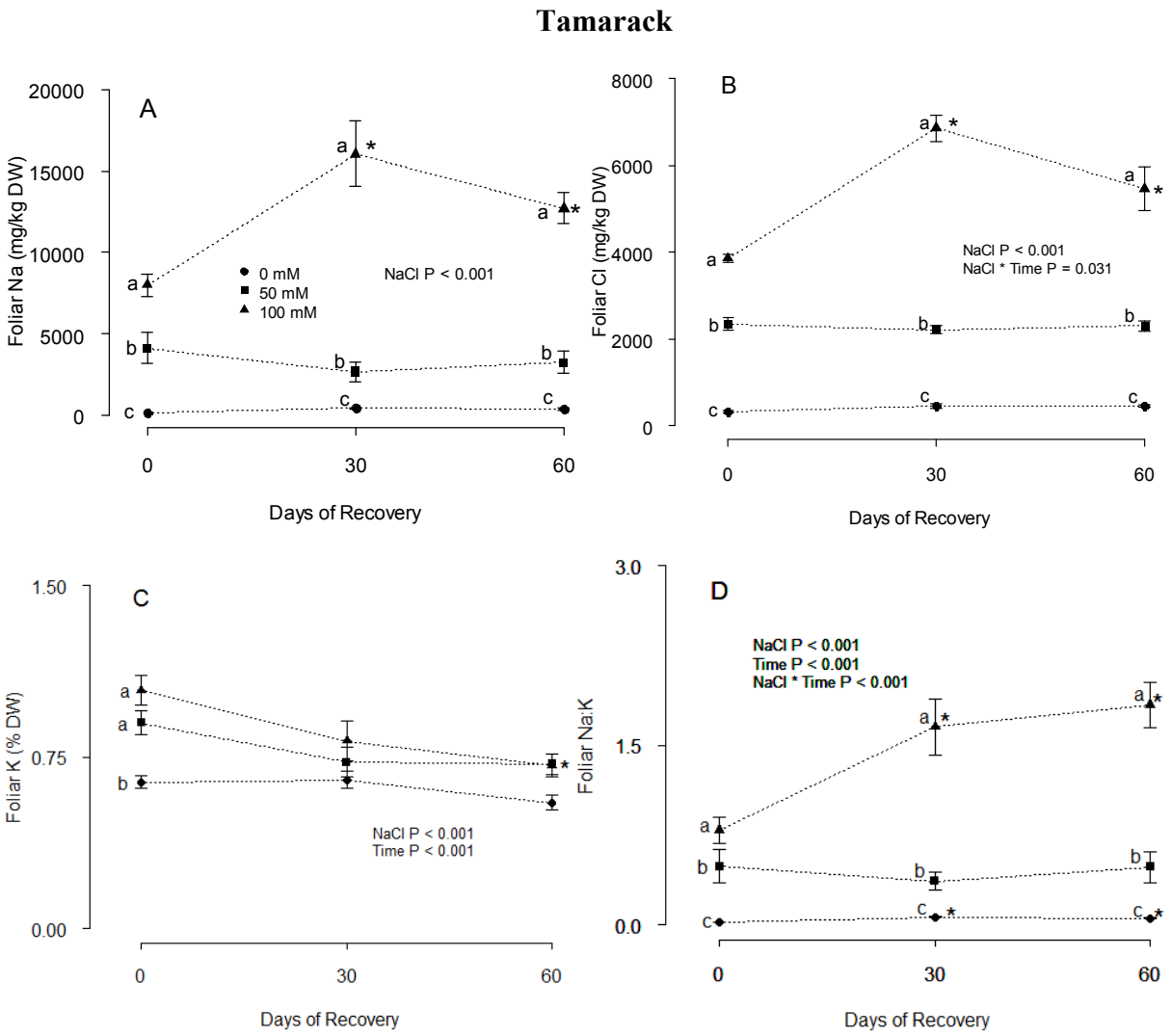


Figure 4.4. Effects of a 60-day NaCl treatment followed by a 60-day recovery period on foliar Na (A), Cl (B), K (C), and Na:K ratio (D) for tamarack seedlings. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test at a specific time point. Asterisks represent a significant difference from day 0 of recovery within the same treatment over time.

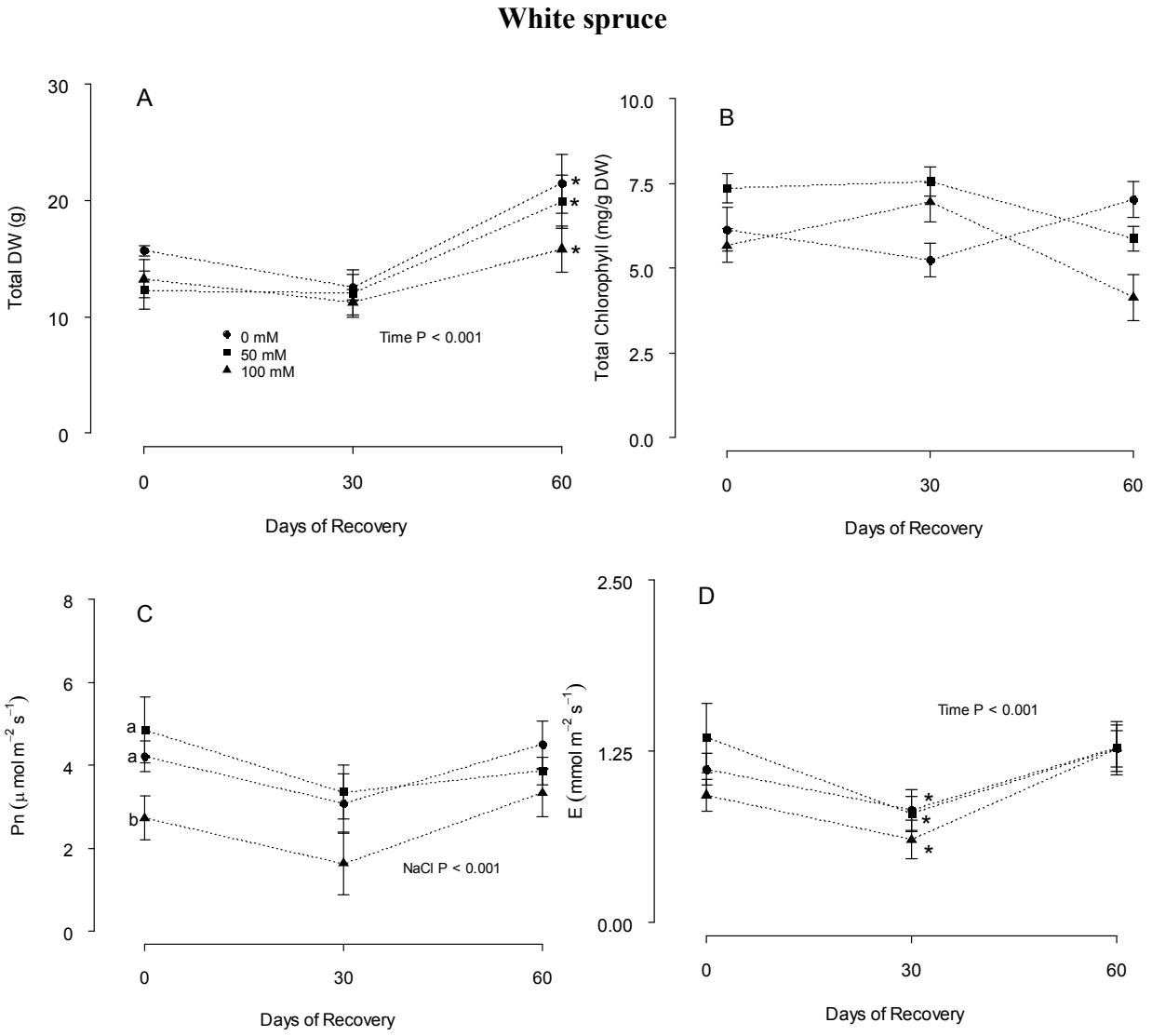


Figure 4.5. Effects of a 60-day NaCl treatment followed by a 60-day recovery period on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rates (E) (D) in white spruce seedlings. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test at a specific time point. Asterisks represent a significant difference from day 0 of recovery within the same treatment over time.

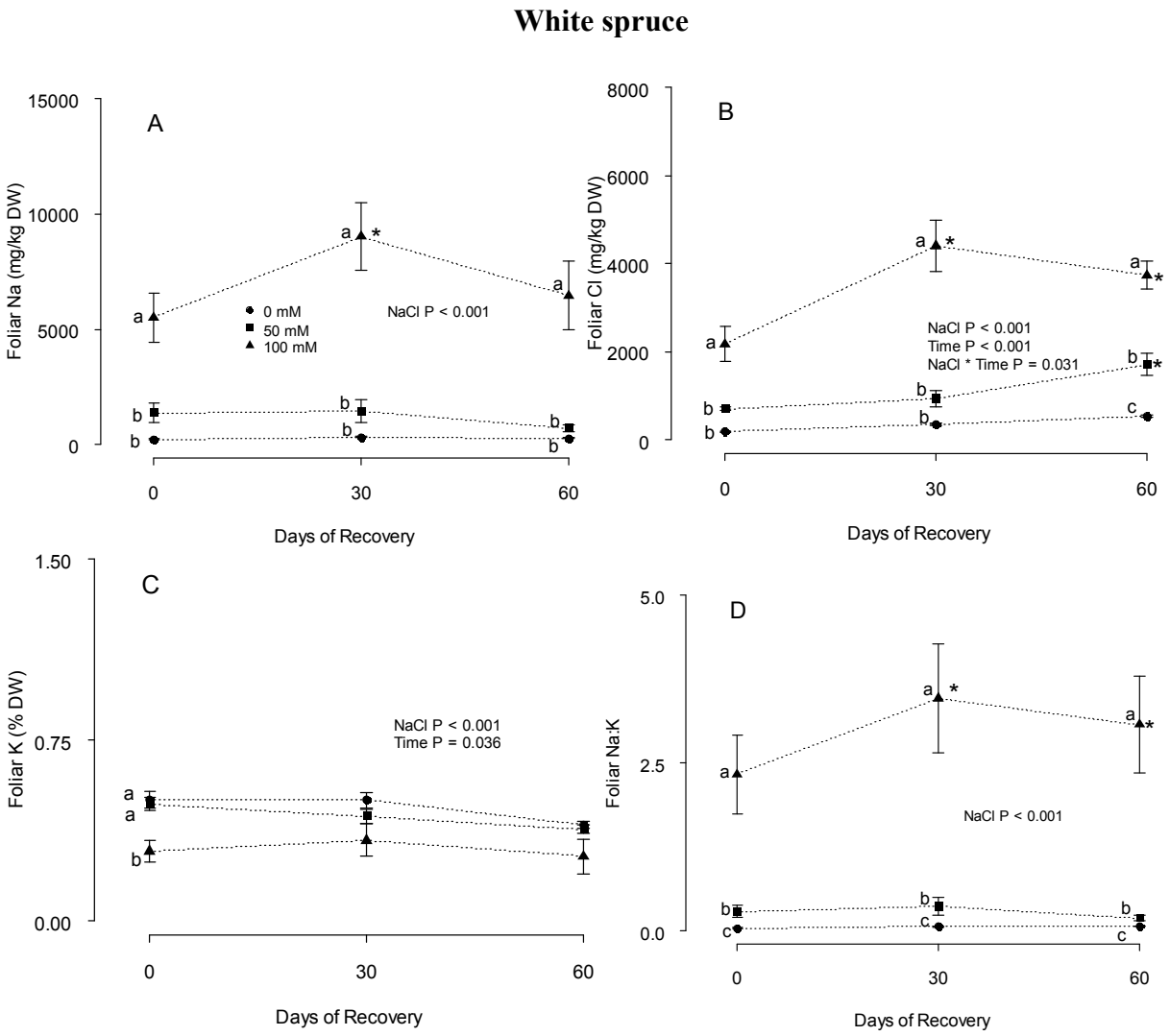


Figure 4.6. Effects of a 60-day NaCl treatment followed by a 60-day recovery period on foliar Na (A), Cl (B), K (C), and Na:K ratio (D) for white spruce seedlings. Values represent the mean ± SEM (n = 6) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test at a specific time point. Asterisks represent a significant difference from day 0 of recovery within the same treatment over time.

## 4.8 Supplemental material

Table 4.S1 Initial height prior to experimental treatment for trembling aspen, tamarack, and white spruce.

| | Initial Height (cm) | | |
|---|---|---|---|
| | Trembling aspen | Tamarack | White spruce |
| 0 mM NaCl | | | |
| 0 days | 45.34 ± 1.27 | 21.37 ± 0.39 | 29.34 ± 0.94 |
| 30 day | 46.15 ± 1.65 | 20.53 ± 0.32 | 30.36 ± 1.34 |
| 60 days | 45.87 ± 1.95 | 20.86 ± 1.81 | 32.82 ± 0.18 |
| 50 mM NaCl | | | |
| 0 days | 47.34 ± 2.55 | 20.56 ± 1.47 | 32.08 ± 0.67 |
| 30 days | 49.47 ± 1.67 | 20.43 ± 1.46 | 31.08 ± 1.54 |
| 60 days | 48.39 ± 1.32 | 22.85 ± 1.39 | 29.38 ± 0.94 |
| 100 mM NaCl | | | |
| 0 days | 46.45 ± 1.37 | 21.54 ± 1.58 | 28.55 ± 0.68 |
| 30 days | 47.62 ± 1.35 | 20.57 ± 0.68 | 31.09 ± 0.34 |
| 60 days | 49.15 ± 1.43 | 21.83 ± 1.80 | 29.54 ± 0.97 |

Table 4.S2 Initial root collar diameter prior to experimental treatment for trembling aspen, tamarack, and white spruce.

| | Initial Root Collar diameter (mm) | | |
|---|---|---|---|
| | Trembling aspen | Tamarack | White spruce |
| 0 mM NaCl | | | |
| 0 days | 3.84 ± 0.35 | 3.75 ± 0.43 | 3.37 ± 0.31 |
| 30 days | 4.55 ± 0.58 | 3.44 ± 0.16 | 3.72 ± 0.43 |
| 60 days | 4.63 ± 0.56 | 3.38 ± 0.54 | 3.74 ± 0.24 |
| 50 mM NaCl | | | |
| 0 days | 4.92 ± 0.64 | 3.54 ± 0.29 | 3.54 ± 0.79 |
| 30 days | 4.78 ± 0.95 | 3.45 ± 0.39 | 3.36 ± 0.38 |
| 60 days | 4.65 ± 0.37 | 3.65 ± 0.35 | 3.48 ± 0.41 |
| 100 mM NaCl | | | |
| 0 days | 3.76 ± 0.49 | 3.24 ± 0.24 | 3.73 ± 0.83 |
| 30 days | 4.43 ± 0.37 | 3.39 ± 0.25 | 3.18 ± 0.48 |
| 60 days | 4.45 ± 0.15 | 3.28 ± 0.36 | 3.47 ± 0.41 |

## Chapter 5: Recovery of trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) seedlings from NaCl stress following winter dormancy.

### 5.1 Introduction:

Reclamation of bitumen surface mining sites in northern Alberta may contain heterogeneous levels of NaCl in the soil caused by variations in evapotranspiration, precipitation, water table depth, and upward water flux (Kessler et al. 2010; Carrera-Hernandéz 2012). This is problematic for reclamation efforts because elevated soil NaCl causes declines in root water flux, photosynthesis, transpiration, biomass, and ultimately death for nearly all plant species (Munns and Tester, 2008). Since soil NaCl levels can be transient on reclamation sites, the ability of plants to recover following exposure to NaCl may be essential to their survival in these sites. Seedlings planted on reclamation sites in northern Alberta experience harsh winters with temperatures reaching -40°C for prolonged periods of time. The effects of NaCl or overwintering have been well studied on perennial plants. However, the combined effects of these stressors have not been studied.

In the previous study, NaCl treatment for 60 days caused declines in total chlorophyll concentration, Pn, and E for trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*); however, all species showed a recovery of physiological parameters after 30 days of recovery from 50 mM NaCl stress. Trembling aspen and tamarack exhibited higher levels of foliar K and accelerated foliar senescence in response to NaCl treatment. Interestingly, all species exhibited foliar yellowing starting at the shoot apex. It was concluded that elevated foliar K and accelerated foliar senescence are deliberate survival strategies of trembling aspen, tamarack, and white spruce exposed to NaCl stress.

In the previous study, increased foliar K concentration was observed in trembling aspen and tamarack. Increased foliar cytoplasmic K is recognized as universal mechanisms of NaCl tolerance in glycophytic plants (Munns and Tester, 2008). Elevated foliar Na negatively affects metabolism by interfering with the subcellular role of potassium as an enzymatic co-factor necessary for nearly all growth processes in plants. Elevated NaCl triggers the release of K from root cortex cells to be deposited in the surrounding soil or translocated to foliar tissues. Plants growing in soils deficient in K are more sensitive to NaCl stress but the exogenous application of potassium can alleviate symptoms of NaCl and drought stresses (Cakmak, 2005; Chen et al.

2005; Escalante-Pérez et al. 2009; Wang et al. 2013). In my previous study, both trembling aspen and tamarack exhibited elevated foliar K concentrations in response to NaCl stress. In the current study, I investigated weather NaCl stress and overwintering influenced this trend.

Both trembling aspen and tamarack exhibited increased foliar necrosis as a result of NaCl stress. Long-term NaCl stress causes Na toxicity in foliar tissue causing leaves to undergo a yellowing (chlorosis) at the margins followed by premature senescence. Along with elevated foliar K, foliar necrosis sis also recognized as a universal response of glycophyte plants to NaCl stress (Munns and Tester, 2008). Foliar chlorosis is caused by a cell signaling event and can be induced by aging, high light, or environmental stress such as soil Na and helps plants to complete their life cycle under stressful conditions by reducing water loss through transpiration as well as by remobilizing nutrients to younger tissues, flowers, or fruits. On the cellular level, chlorosis is characterized by the degradation of chlorophyll and proteins followed by programmed cell death (Munné-Bosch and Alegre, 2004; Wang and Blumwald 2014). For the current study, increased foliar senescence was studied in more detail for trembling aspen. In particular, I investigated the interface between green, yellowing, and necrotic tissue to see if foliar necrosis had a positive effect for trembling aspen exposed to NaCl stress.

In the present study, non-lethal levels of NaCl were applied to trembling aspen, tamarack, and white spruce seedlings in the first growing season. Seedlings were overwintered and again subjected to NaCl stress in the second growing season. The primary objective of this study was to investigate whether seedlings treated with sub-lethal levels of NaCl during the first year of growth would exhibit acclimation or cumulative salt injury when exposed to overwintering followed by NaCl treatment in the second year. It was hypothesized that NaCl stress and overwintering in year one would hinder the ability of seedlings to recover from NaCl stress in year two. The question of the importance of chlorosis and necrosis of foliar tissue for plant survival was especially interesting since both trembling aspen and tamarack are deciduous plants and lose their foliage before winter whereas white spruce can maintain needles for many years. It was hypothesized that accelerated foliar yellowing acts as a mechanism to remove toxic Na from the seedlings. The process of chlorosis was studied in more detail in trembling aspen leaves. The results generated from this study are important to manage boreal forest lands affected salinity including the oil sands reclamation areas.

## 5.2 Materials and Methods:

### 5.2.1 Plant material and growth conditions

One-year old dormant trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) seedlings were obtained from Smoky Lake Forest Nursery (Smoky Lake, AB, Canada). Plant material was produced from seeds collected from open-pollinated wild tree stands in various locations within Alberta seed zone CM 2.2 by Tree Time Services Inc. (Edmonton, AB, Canada). All species were transported to the University of Alberta on March 8$^{th}$, 2017 and planted on March 14$^{th}$, 2017. Seedlings were stored in a refrigerated room at 4°C in the dark before planting. Seedlings were planted in four L pots with a mixture of peat moss and sand (1:1 by weight) and placing outside for one year. During the growing season, all seedlings were watered daily and half of the seedlings for each species were treated with 250 mL of 50 mM NaCl every two weeks. Trees were fertilized with 250 mL of 3 g/L 20:20:20 (N:P:K) fertilizer every two weeks from May to July of the growing season and every 30 days from August to October. Before the onset of winter, seedlings were thoroughly watered to flush out remaining NaCl from the soil and left outside over winter. Pots were covered with soil and hay to prevent root freezing. Seedlings were transported into a controlled-environment growth room several weeks before bud break in early May. They were then allowed to grow for six weeks before the NaCl treatments. Immediately prior to the treatments, seedlings from each species that were provided with 50 mM NaCl and water (control) from year one (n=3) were taken for analysis prior to NaCl treatment during year two. No significant difference existed between treatments for height and root collar diameter for all species (Tables 6.S1,6.S2). Control and NaCl-treated seedlings were then subjected to treatments with 0, 50, and 100 mM NaCl for a total of six treatments. The NaCl treatment was administered by applying 250 mL of the respective NaCl concentration once a week for eight weeks. The controlled-environment growth room was maintained at 22/18°C (day/night) temperature, 65 ± 5 % relative humidity, and 16-h photoperiod with 300 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) using full spectrum fluorescent lights (Philips high output, F96T8/TL835/HO, Markham, ON, Canada). Trees were watered every other day and fertilized with 250 mL of three g/L 20:20:20 (N:P:K) fertilizer every two weeks.

### 5.2.2 Gas exchange

After eight weeks of treatments, gas exchange measurements were carried out (n=6). Net photosynthesis (Pn) and transpiration (E) rates were measured using an infrared gas analyzer equipped with a standard 6 $cm^2$ leaf chamber (Li-Cor 6400XT, Li-Cor Inc., Lincoln, NE, USA). Foliar tissue samples were removed from plants and placed in the leaf chamber for measurement. Measurements were conducted in the experimental growth room. Samples were allowed to equilibrate to a steady state for approximately two minutes and measurements were taken no later than 5 minutes after the foliar tissue was removed from plants. The light intensity for all measurements was 300 μmol $m^{-2}$ $s^{-1}$ PPFD provided by a red-blue light source (6400-02, Li- Cor Inc., Lincoln, NE, USA). The [$CO_2$] was maintained at 400 μmol for all measurements. Light intensity and [$CO_2$] values were chosen to be the same as plant growth conditions in the experimental growth room. For white spruce and tamarack, needle area was calculated using the Sigmascan Pro 5.0 computer software (Systat Software, San Jose, CA, USA).

### 5.2.3 Dry weight and foliar chlorophyll concentration

After gas exchange measurements, a small amount of leaves or needles was collected from seedlings, lyophilized, and ground to a powder for chlorophyll extraction. Chlorophyll was extracted from ground tissue (ten mg DW) with eight mL DMSO at 65ºC for 24 h. Chlorophyll concentrations were measured with a spectrophotometer (Ultrospec, Pharmacia LKB, Uppsala, Sweden) at 648 nm for chlorophyll-a and 665 nm for chlorophyll-b. Total chlorophyll was calculated using the Arnon's equation (Sestak et al. 1971).

For dry weight determination, seedlings were separated into foliar tissue, stems, and roots and oven-dried at 70ºC for 72 h before weighing. Lyophilized foliar tissue used for chlorophyll analysis was also weighed and added to the total dry weight measurement. For aspen, necrotic foliar tissue was separated from living tissue prior to oven drying and weighed separately. Living and necrotic foliar tissue was ground to a fine powder using a Wiley mill (screen no. 40) and used for elemental analysis.

### 5.2.4 Foliar elemental analysis

For the determination of foliar concentrations of Fe, Na, Mg, P, K, and Ca, foliar tissue from all species as well as necrotic aspen leaf tissue (200 mg) were digested with ten mL 70% $HNO_3$ and diluted with deionized (DI) water up to 50 mL. Samples were analyzed by ICP-MS in Radiogenic Isotope facility at the University of Alberta (Zarcinas et al. 1987). For the determination of foliar N concentration for all species as well as necrotic aspen leaf tissue, approximately two mg of dried ground samples were analyzed using a CE 440 CHN Elemental Analyzer (Exeter Analytical, MA, USA). Total foliar Cl for all species and necrotic aspen leaf tissue was analyzed by extracting dried and ground tissue (200 mg) with ten mL of boiling DI water. Samples were placed in a water bath at 90ºC for one hour. Five mL of liquid extract was combined with 5 mL of DI water and 200 µL of ion strength adjuster (Thermo-Fisher Scientific, CA, USA). Total chloride was determined using a chloride electrode (Accumet chloride half cell electrode, Thermo-Fisher Scientific, CA, USA) and reference electrode (Accumet double junction reference electrode, Thermo-Fisher Scientific, CA, USA) attached to a pH meter (Accumet 925 pH/ion meter, Thermo-Fisher Scientific, CA, USA).

### 5.2.5 Foliar chlorosis in trembling aspen

The process of accelerated foliar chlorosis was studied in further detail in trembling aspen by analyzing both green and chlorotic tissue for the presence of ROS, relative chlorophyll:carotenoid ratio, and night-time respiration. The presence of ROS in green and yellow foliar tissue was tested using the ROS-specific fluorescent probe 2,7-dichlorofluorescein-diacetate (DCFH-DA; Invitrogen, Carlsbad, CA, USA). Excised leaf segments were incubated in 5 µM DCFH-DA in DI water for 15 min and then washed three times with DI water to remove unbound probe. Brightfield and fluorescent imaging (ex 488 nm/em 525 nm) was conducted on a Leica DMRXA compound light microscope with a QI click camera (Leica Microsystems, Buffalo Grove, IL). Hyperspectral imaging of green and yellow foliar tissue was taken using a Colorflow XR1 camera (Stream Technologies Inc., Edmonton, AB, Canada). The relative chlorophyll:carotenoid ratio was calculated an equation simplified from Sims and Gamon (2002) where relative chlorophyll:carotenoid ratio = 645 nm/531 nm. Respiration of green and yellow foliar tissue was measured at night using a Li-Cor 6400XT as described above; however, the light source was not illuminated.

### 5.2.6 Statistical analysis

All data were analyzed using R (https://www.R-project.org). A P value of $P \leq 0.05$ was chose for all analyses. All dependant variables were analyzed using a type III two-way ANOVA linear fixed-effects model with NaCl treatment in year one and NaCl treatment in year two as fixed independent variables. The model equation is: $Y_{ijk} = \mu + O_i + T_j + (O * T)_{ij} + \mathcal{E}_{ijk}$ where $Y_{ijk}$ is the $_k$th observation of the $_i$th and $_j$th treatments, μ is the sample mean, $O_i$ is the $_i$th NaCl treatment in year one and $T_j$ is the $_j$th NaCl treatment in year two. The variable in parenthesis is the interaction between NaCl treatment in year one and NaCl treatment in year two (ANOVA tables can be found in Appendix 4). A Fisher's LSD post-hoc test was used when significant differences were detected. To compare the elemental concentrations of green and necrotic foliar tissue in trembling aspen, data from NaCl treatments from year one was pooled based on the NaCl treatment from year two. To compare the elemental concentrations of green and necrotic foliar tissue in trembling aspen, data from NaCl treatments from year one was pooled based on the NaCl treatment from year two. The data were then analyzed using a one-way ANOVA linear fixed-effects model with NaCl as a fixed independent variable. The model equation is: $Y_{ij} = \mu + S_i + \mathcal{E}_{ij}$ where $Y_{ij}$ is the $_j$th observation of the $_i$th treatment, μ is the sample mean, $S_i$ is the $_i$th NaCl treatment from year two. Significant differences between treatments were analyzed using a Tukey's HSD test and represented by letters. Data collected prior to the experiment, relative chlorophyll:carotenoid ratio, and night-time respiration were analyzed using a student's t-test. Data that did not meet the assumptions of normality of distribution and homogeneity of variance were log10 transformed before statistical analysis.

## 5.3 Results:

### 5.3.1 Preliminary analyses

Prior to the NaCl treatment in year two, seedling height and root collar diameter were measured, and no differences were found between treatments (data not shown). Immediately before the year two NaCl treatments, seedlings from control and 50 mM NaCl treatments for each species (n=3) were measured for physiological and elemental parameters. Trembling aspen seedlings treated with NaCl in year one showed no changes in physiological parameters (Table 5.1). Trembling aspen seedlings exhibited increases in foliar Cl and soil Na concentration compared to trees watered with no NaCl in year one; however, the soil Na concentration was

much lower than treatments administered in year two (Table 5.2). Tamarack seedlings treated with NaCl in the previous year had higher Pn, foliar Na and Cl concentrations compared to control plants (Tables 6.1 and 6.2). White spruce seedlings treated with NaCl in year one showed significant decreases in Pn and chlorophyll concentration but had higher foliar Na and Cl concentrations compared to control plants (Tables 6.1 and 6.2).

### 5.3.2 Trembling aspen

A significant disordinal interaction between NaCl treatment in year one and year two was detected in total DW for trembling aspen seedlings. Seedlings exhibited lower total dry weight after 50 and 100 mM NaCl treatment in year two regardless of NaCl treatment in year one. However, seedlings treated with NaCl in year one had lower total dry weight at 0 and 50 mM NaCl compared to trees not treated with NaCl in year one (Figure 5.1 A). Seedlings treated with 100 mM NaCl in year two had lower chlorophyll concentrations regardless of NaCl treatment in year one (Figure 5.1 B). Seedlings treated with 100 mM NaCl exhibited lower Pn regardless of NaCl treatment in year one; however, seedlings treated with NaCl in year one had lower Pn at 0 mM NaCl compared to those not treated with NaCl in year one (Figure 5.1 C). Seedlings watered with freshwater in year one exhibited lower E when treated with NaCl in year two, however; seedlings treated with NaCl in year one had no changes in E when treated with NaCl in year two (Figure 5.1 D).

Foliar Na was remarkably low in trembling aspen for all treatments. Seedlings treated with freshwater in year one had the same concentrations of foliar Na regardless of NaCl treatment in year two. Seedlings treated with NaCl in year one had significantly lower concentrations of foliar Na when treated with 0 and 50 mM NaCl, but when treated with 100 mM, Na concentrations were similar to those in the seedlings that were watered with no NaCl in year one (Figure 5.2 A). Foliar Cl increased at 50 and 100 mM NaCl treatments in all seedlings regardless of NaCl treatment in year one. Seedlings treated with NaCl in year one had a significant increase of Cl concentrations in 0 mM NaCl treatment in year two but significant decrease in 50 mM NaCl treatment in year two compared to control seedlings treated with freshwater in year one (Figure 5.2 B). Seedlings exhibited a significant increase in foliar K concentrations in 50 and 100 mM NaCl treatments in year two treatments regardless of NaCl treatment in year one (Figure 5.2 C). A significant ordinal interaction between NaCl treatment in

year one and year two was detected for foliar N:K ratio which was lower in all treatments compared to seedlings treated with only freshwater (Figure 5.2 D).

### 5.3.3 Tamarack

Tamarack seedlings not treated with NaCl in year one had significantly lower dry weight when subjected to 50 and 100 mM NaCl treatments in year two. Seedlings treated with 0 mM NaCl in year one and 100 mM NaCl in year two showed a significantly higher dry weight compared to seedlings treated with NaCl in year one and 100 mM NaCl in year two. Seedlings treated with 50 mM NaCl in year one exhibited a significantly lower dry weight in 0 and 100 mM NaCl treatments in year two compared to seedlings that were treated with 0 mM NaCl in year one (Figure 5.3 A). No significant changes in foliar chlorophyll concentrations were observed between the treatments (Figure 5.3 B). Seedlings treated with 0 mM NaCl in year one showed significant declines in 50 and 100 mM NaCl treatment in year two, whereas seedlings treated with NaCl in year one showed a decline only in the 100 mM NaCl treatment in year two. Interestingly, seedlings treated with NaCl in year one had higher Pn values in the 50 and 100 mM NaCl treatments in year two compared with the seedlings that were treated 0 mM NaCl in year one (Figure 5.3 C). Seedlings treated with 0 mM NaCl in year one showed a decline in E when subjected to 100 mM NaCl treatment in year two, whereas seedlings treated with NaCl in year one showed no changes in 50 and 100 mM NaCl treatments in year two (Figure 5.3 D).

Tamarack seedlings treated with NaCl exhibited higher concentrations of foliar Na and Cl. Significant ordinal interactions between NaCl treatment in year one and year two were detected for both foliar Na and Cl concentrations primarily because seedlings treated with NaCl in year one had significantly higher foliar sodium and chloride concentrations in the 100 mM NaCl treatment in year two compared to seedlings treated with freshwater in year one (Figure 5.4 A,B). Foliar K and Na:K increased as a result of NaCl treatment, regardless of treatment in year one (Figure 5.4 C,D).

### 5.3.4 White spruce

NaCl treatment had relatively no changes on total dry weight, foliar chlorophyll concentrations, Pn and E in white spruce. However, dry weight was significantly lower in seedlings treated with NaCl in year one and 100 mM NaCl in year two (Figure 5.5 A-D).

Seedlings treated with NaCl showed increases in foliar Na and Cl concentrations with increasing NaCl treatment concentration. Seedlings treated with NaCl in year one showed significant increases in both Na and Cl foliar concentrations compared to seedlings treated with 0 mM NaCl in year one. A significant ordinal interaction between NaCl in year one and year two was detected for foliar Cl concentration (Figure 5.6 A,B). No changes in foliar K were observed (Figure 5.6 C). Treatment with NaCl in year two resulted in increases in foliar Na:K due to increased foliar Na concentration. This trend was enhanced when seedlings were treated with NaCl in year one (Figure 5.6 D).

### 5.3.5 Visible injury

All seedlings treated with NaCl had a similar visible injury pattern which included yellowing at the foliar margins or tips and eventual death of older leaves or needles. Trembling aspen grew new leaves near the top of the seedling, and the new leaves had higher levels of Pn compared with the existing leaves (data not shown). Tamarack showed a legacy of NaCl treatment from year one as some seedlings exhibited mortality of the terminal buds. White spruce showed significant needle chlorosis and death in the previous year's growth. The current year's needles in white spruce showed slight chlorosis at the needle apex.

### 5.3.6 Measurements in green, chlorotic, and necrotic foliar tissue in trembling aspen

In trembling aspen, the percentage of necrotic leaf tissue increased with increasing NaCl treatment concentrations in year two. Necrotic tissue had higher concentrations of Na, Cl, N, Ca, and Mg compared to the green parts of the leaves. Foliar K concentrations increased in the green leaf tissues following NaCl treatments but were lower in the necrotic tissue (Table 5.3). Compared to green tissue, chlorotic tissue showed an accumulation of reactive oxygen species, likely near the chloroplasts (Figure 5.7 A). Chlorotic tissue also had a lower chlorophyll:carotenoid ratio, but higher night-time respiration rate compared to green tissue (Figure 5.7 B,C).

**5.4 Discussion:**

The current study examined the responses of trembling aspen, tamarack, and white spruce seedlings to NaCl stress applied over two growing seasons. Elevated soil NaCl is generally known to causes decreases in photosynthesis, transpiration, and growth as well as accelerated foliar senescence (Munns and Tester, 2008). Perennial plants exposed to extreme cold are vulnerable to cellular desiccation caused by the formation of extracellular ice (Bertrand and Castonguay 2003). The current study was an attempt to understand the recovery of seedlings experiencing NaCl stress and overwintering from year one and NaCl stress in year two. It was hypothesized that NaCl stress and overwintering in year one would hinder the ability of seedlings to recover from NaCl stress in year two. Therefore, visible signs of injury were combined with physiological and elemental data to provide insight into potential mechanisms of acclimation and cumulative salt injury.

In most cases, treatment with NaCl in year two decreased total dry weight, chlorophyll, Pn, and E in trembling aspen and tamarack seedlings. For white spruce seedlings, NaCl treatment had no effect on total dry weight, chlorophyll, Pn, and E. These results were congruent with findings found in Chapter four. Although not tested in the current study, the lowered total dry weight, chlorophyll, Pn, and E in trembling aspen and tamarack seedlings may be the result of an acclimation to NaCl stress. Current literature reviews show that NaCl stress leads to rapid and systemic long-distance electrical signals within the vascular tissue of plants resulting in decreased Pn, E, $CO_2$ assimilation, and growth. Furthermore, NaCl stress is known to induce an immediate halt of plant growth followed by a slower, more conservative growth form with enhanced lateral root formation (Julkowska and Testerink, 2015; Gilroy et al. 2016). Considering that NaCl induced decreases total dry weight, chlorophyll, Pn, and E in trembling aspen and tamarack from the current study, it appears possible that the observed trend was a result of the aforementioned proceses. However, extensive testing is needed to confirm this hypothesis.

Evidence of acclimation or cumulative salt injury were evident in all species exposed to two years of NaCl stress and overwintering. Trembling aspen seedlings treated with NaCl in year one exhibited lower total dry weight and Pn when treated with 0 and 50 mM NaCl in year two compared to seedlings treated with freshwater in year one. Seedlings treated with NaCl in year one exhibited E rates slightly lower at 0 mM but remained unchanged from NaCl stress in year

two whereas seedlings treated with freshwater in year one exhibited lower E rates when exposed to NaCl in year two. Interestingly, older leaves exhibited accelerated senescence whereas new growth appeared healthy. Considering that growth and physiological response rates of trembling aspen seedlings were lowered by multiple years of NaCl stress, it is plausible that these seedlings exhibited a mechanism of acclimation, perhaps to increase survival under stress conditions.

Tamarack seedlings exhibited changes in physiology and increased salt injury from multiple years of NaCl stress. Seedlings exposed to NaCl in year one exhibited lower total dry weight for all treatments compared to seedlings treated with 0 mM NaCl in year one. Second, seedlings exposed to NaCl in year one and 0 mM NaCl in year two had lower total dry weight, Pn, and E compared to seedlings treated with 0 mM NaCl in year one and two. Third, seedlings exposed to NaCl in year one had higher levels of Pn compared to those treated with 0 mM NaCl in year one when treated with 50 and 100 mM NaCl in year two. Interestingly, seedlings with higher levels of Pn exposed to NaCl in year two also had higher WUE values compared to seedlings treated with freshwater in year one (data not shown). Higher levels of WUE have been linked to factors at the cellular level such as increased chloroplast carbonic anhydrase activity or expression of aquaporins (Guo et al. 2006; Flexas et al. 2010; Moshelion et al. 2015). It is hypothesized that higher levels of Pn and WUE in seedlings exposed to NaCl in two consecutive years are attributed to a physiological acclimation. Many tamarack seedlings exposed to NaCl in year one exhibited terminal bud dieback in year two which lead to multiple lateral branches forking. Considering that the phenomenon of terminal bud dieback only occurred in seedlings exposed to NaCl stress in year one, this may be attributed to the combined stresses of NaCl and overwintering. The predominant cause of frost injury in perineal plants is cellular desiccation caused by the formation of extracellular ice. Cellular supercooling is a response observed in woody plants to prevent ice nucleation within the cell. The process involves changes in cell membrane lipid content as well as increases in cytosolic sugars and hydrophilic polypeptides (Bertrand and Castonguay, 2003). This process has been observed in many tissue types, including buds (George and Burkey, 1984). It may be possible that the combined effects of NaCl stress and overwintering interfered with cellular supercooling mechanisms, resulting in terminal bud dieback.

NaCl stress did not alter any of the measured physiological parameters and growth in white spruce. However, seedlings exposed to two years of NaCl stress showed significant foliar chlorosis and mortality of older needles suggesting that the consequences of NaCl would eventually manifest themselves over time. Younger needles were green with slight yellowing at the needle tips, but the seedlings appeared to have healthy buds. It has been established that foliar yellowing is attributed to a cell signaling process suggesting that it is actively induced by the plant (Wang and Blumwald 2014). Considering that older tissue appeared to be dead or dying whereas the young growth and new buds appeared healthy, it is plausible that toxic Na ions accumulated in older tissue to protect younger, growing tissue (Munns and Tester, 2008).

Treatment of seedlings with NaCl in year two induced changes in foliar elemental concentration similar to those found in the previous chapter. For example, foliar Na in trembling aspen was remarkably low. This may be attributed to accelerated foliar senescence or ion exclusion via suberin deposition at the roots (Franke and Schreiber, 2007; Munns and Tester, 2008). Tamarack and white spruce seedlings exhibited significant increases in foliar Na concentration when treated with NaCl in year two. Elevated cytosolic Na causes disruptions in cellular function and is linked to physiological decline. Therefore, mechanisms such as vacuole sequestration of Na or storage within cell walls may be occurring (Munns and Tester, 2008; Parihar et al. 2015). Tamarack exhibited increases in foliar K from NaCl stress in year two. Elevated foliar K may have resulted in increased Pn and E from NaCl treatment in year two by out competing Na as a co-factor for enzymatic reactions. It should be noted that plants grown in soils with low K availability are more sensitive to NaCl and drought stress which leads to programmed cell death. The exogenous application of KCl to foliar tissue of plants is known to alleviate stress sensitivity in soils with low potassium availability (Cakmak, 2005; Chen et al. 2005; Escalante-Pérez et al. 2009; Wang et al. 2013, Zörb et al. 2013). Again, it is advisable that reclamation sites have adequate levels of available soil K in to increase plant health during periods of environmental stress.

Seedlings treated with NaCl in year one showed some differences in foliar elemental concentrations in year two compared to those treated with freshwater in year one. Trembling aspen seedlings treated with NaCl in year one had significantly lower foliar Na concentration compared to seedlings treated with freshwater in year one when treated 0 and 50 mM NaCl in

year two whereas foliar chloride was relatively unaffected by NaCl treatment in year one. This again suggests that physiological processes such as accelerated foliar senescence or ion exclusion at the roots were employed to keep foliar Na levels low (Franke and Schreiber, 2007; Munns and Tester, 2008). Foliar K concentrations were unaffected by NaCl treatment in year one for trembling aspen and tamarack seedlings treated with NaCl in year two. Contrary to trembling aspen and tamarack, white spruce is an evergreen species and maintains needles for a number of years. Therefore, it was anticipated that NaCl resistance strategies would vary between the deciduous and evergreen plants. Tamarack and white spruce seedlings both exhibited higher levels of foliar Na and Cl when treated with NaCl in both years compared to seedlings treated with 0 mM NaCl in year one. With the exception of foliar sodium concentration in white spruce, the Na and Cl values exhibited significant ordinal interactions between NaCl treatment in year one and NaCl treatment in year two. This suggests that both species translocated these elements to foliar tissue from other organs such as roots or stems. Considering that accelerated foliar senescence was observed in both tamarack and white spruce seedlings, the elevated levels of foliar Na and Cl could be a mechanism to extrude these ions from living tissue.

Treatment with NaCl caused the foliar chlorosis in all species, which ultimately lead to fully necrotic leaves or needles. It was hypothesized that accelerated foliar chlorosis acted as a mechanism to remove toxic Na ions from the seedlings. The foliar yellowing process was studied in more detail in trembling aspen leaves. It was found that yellowing foliar tissue exhibited common signs of leaf senescence and had elevated respiration but decreased chlorophyll:carotenoid ratios compared to green tissue. Yellowing tissue also showed an accumulation of ROS in the cells, possibly surrounding the chloroplasts. This is intriguing because foliar yellowing can be induced by NaCl which leads to a cell signaling event to trigger the deliberate degradation of chloroplasts (Wang and Blumwald 2014). A thorough review on this topic has yet to be conducted; however, similarities can be drawn to drought-induced foliar chlorosis. In a review of drought-induced leaf senescence, Munné-Bosch and Alegre (2004) stated that foliar yellowing is characterized by elevated levels of ABA and ROS as well as an upregulation of senescence-associated genes and an organ-wide triggering of programmed cell death (PCD). The process is initiated by a cell signaling cascade which leads to decreased photosynthesis, chlorophyll degradation, and a loss of cell integrity linked to nutrient remobilization to younger tissue. Interestingly, the chloroplasts, which contain the majority of

proteins and lipids within the cell, are targeted for degradation. The current study did not directly show that PCD was occurring in yellowing region of trembling aspen leaves, but the evidence suggests that it is likely the case.

Green and necrotic tissue were separated in individual leaves on trembling aspen seedlings exposed to NaCl in year two to measure differences in elemental concentrations. Compared to living tissue, the necrotic tissue had higher levels of most elements except for K. This is intriguing because foliar K concentrations increased from NaCl stress in the current study as well as the study in the previous chapter. This suggests that the yellowing region exhibited selective retention of K but the extrusion of Na and Cl to necrotic tissue. Elevated respiration may indicate the active transport of K to green tissue or NaCl to necrotic tissue. Parihar et al. (2015) states that foliar yellowing is a caused by ion toxicity; however, it may be an actively induced process by the plant as a tolerance mechanism.

All species tested in the current study appear to be good candidates for reclamation of areas disturbed by surface mining in northern Alberta. Seedlings exposed to NaCl stress and overwintering exhibited mechanisms of recovery such as elevated foliar K and accelerated foliar senescence. Growth processes in white spruce appeared relatively unaffected by two years of NaCl stress whereas trembling aspen and tamarack showed lower total dry weights. Furthermore, overwintering after NaCl stress caused the death of terminal buds in some tamarack seedlings. This did not appear to negatively affect the health of the seedlings but may change the form of trees in a longer term. Improving the potassium status of plants by soil amendments such as potash could make plants more resilient to environmental stress (Cakmak, 2005; Neid and Biesboer, 2005). Taken together, trembling aspen, tamarack, and white spruce make may be suitable for reclamation of sites with moderate levels of soil Na; however, adequate levels of available soil potassium could potentially increase the health and survivability of seedlings.

**5.5 References:**


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## 5.6 Tables

Table 5.1. Plant dry weights, leaf chlorophyll concentrations, net photosynthesis, and transpiration rates in control and 50 mM NaCl-treated trembling aspen, tamarack, and white spruce in year one. Values represent the mean ± SEM (n =3) and an asterisk represents a significant difference at $P \leq 0.05$ using a student's T-test.

| | Trembling aspen | Tamarack | White Spruce |
|---|---|---|---|
| Dry weight (g) | | | |
| 0 mM | 33.74 ± 0.51 | 48.1 ± 10.33 | 39.83 ± 6.72 |
| 50 mM | 28.29 ± 2.99 | 38.74 ± 5.30 | 38.39 ± 7.37 |
| Net photosynthesis (μmol $m^{-2}$ $s^{-1}$) | | | |
| 0 mM | 8.79 ± 0.26 | 3.33 ± 0.16 | 5.90 ± 0.46 |
| 50 mM | 9.87 ± 0.85 | 5.03 ± 0.21* | 4.31 ± 0.33* |
| Chlorophyll (mg $g^{-1}$ DW) | | | |
| 0 mM | 10.40 ± 1.02 | 8.67 ± 1.89 | 7.35 ± 0.77 |
| 50 mM | 10.05 ± 0.30 | 7.65 ± 0.96 | 4.29 ± 0.29* |
| Transpiration (mmol $m^{-2}$ $s^{-1}$) | | | |
| 0 mM | 2.97 ± 0.16 | 0.73 ± 0.03 | 2.72 ± 0.46 |
| 50 mM | 2.75 ± 0.07 | 1.28 ± 0.03 * | 1.98 ± 0.11 |

Table 5.2. Foliar elemental concentrations in control and 50 mM NaCl-treated trembling aspen, tamarack, and white spruce in year one. Values represent the mean ± SEM (n=3) and asterisks represent a significant difference at $P < 0.05$ using a student's T-test.

| | Trembling aspen | Tamarack | White Spruce |
|---|---|---|---|
| Foliar K (% DW) | | | |
| 0 mM | 0.97 ± 0.05 | 1.28 ± 0.10 | 0.62 ± 0.01 |
| 50 mM | 1.15 ± 0.13 | 1.33 ± 0.14 | 0.60 ± 0.01 |
| Foliar Na (mg/kg DW) | | | |
| 0 mM | 15.44 ± 0.80 | 67.29 ± 24.46 | 85.62 ± 0.94 |
| 50 mM | 16.90 ± 1.11 | 167.9 ± 20.91* | 330.97 ± 160.99* |
| Foliar Cl (mg/kg DW) | | | |
| 0 mM | 279.53 ± 22.59 | 677.42 ± 159.49 | 421.70 ± 55.03 |
| 50 mM | 623.71 ± 42.45* | 1371.43 ± 131.40* | 1796.56 ± 375.66* |
| Foliar Na:K | | | |
| 0 mM | 0.015 ± 0.001 | 0.067 ± 0.024 | 0.086 ± 0.001 |
| 50 mM | 0.017 ± 0.001 | 0.168 ± 0.021* | 0.331 ± 0.161* |
| Soil Na (mg/kg DW) | | | |
| 0 mM | 175.99 ± 27.01 | 191.39 ± 63.87 | 338.64 ± 32.26 |
| 50 mM | 451.94 ± 34.24* | 252.37 ± 42.46 | 369.42 ± 176.43 |
| Soil Cl (mg/kg DW) | | | |
| 0 mM | 142.68 ± 16.40 | 118.21 ± 7.10 | 17.21 ± 14.24 |
| 50 mM | 206.43 ± 20.94 | 139.44 ± 18.03 | 208.35 ± 50.50 |

Table 5.3. Percentage of necrotic tissue and foliar elemental concentrations of green and necrotic tissue in trembling aspen. Values represent the mean ± SEM (n=12) and letters represent a significant difference at $P < 0.05$ using Tukey's HSD test.

| | 0 mM NaCl | 50 mM | 100 mM | 50 mM Necrotic | 100 mM Necrotic |
|---|---|---|---|---|---|
| % Necrotic | N.A. | N.A. | N.A. | 34.86 ± 6.30 b | 53.17 ± 7.32 a |
| N (% DW) | 1.79 ± 0.06 b | 2.08 ± 0.08 b | 1.98 ± 0.08 b | 2.23 ± 0.09 a | 2.13 ± 0.08 a |
| K (% DW) | 1.12 ± 0.07 e | 1.52 ± 0.08 c | 2.05 ± 0.11 a | 1.22 ± 0.06 d | 1.76 ± 0.09 b |
| Ca (% DW) | 1.20 ± 0.06 b | 1.22 ± 0.06 b | 1.18 ± 0.08 b | 1.64 ± 0.05 a | 1.70 ± 0.09 a |
| Mg (% DW) | 0.47 ± 0.03 b | 0.45 ± 0.01 b | 0.42 ± 0.02 b | 0.61 ± 0.03 a | 0.68 ± 0.03 a |
| P (% DW) | 0.19 ± 0.01 b | 0.23 ± 0.02 a | 0.27 ± 0.01 a | 0.25 ± 0.02 a | 0.24 ± 0.01 a |
| Na (ppm DW) | 54.88 ± 6.96 c | 54.69 ± 5.36 c | 68.93 ± 4.71 b | 101.49 ± 12.90 a | 97.71 ± 10.08 a |
| Cl (‰ DW) | 0.29 ± 0.03 c | 1.53 ± .02 b | 2.01 ± 0.02 b | 3.15 ± 0.21 a | 3.89 ± 0.23 a |
| Fe (ppm DW) | 69.67 ± 6.22 | 80.99 ± 7.81 | 53.11 ± 4.02 | 94.46 ± 9.21 | 64.77 ± 4.07 |

## 5.7 Figures

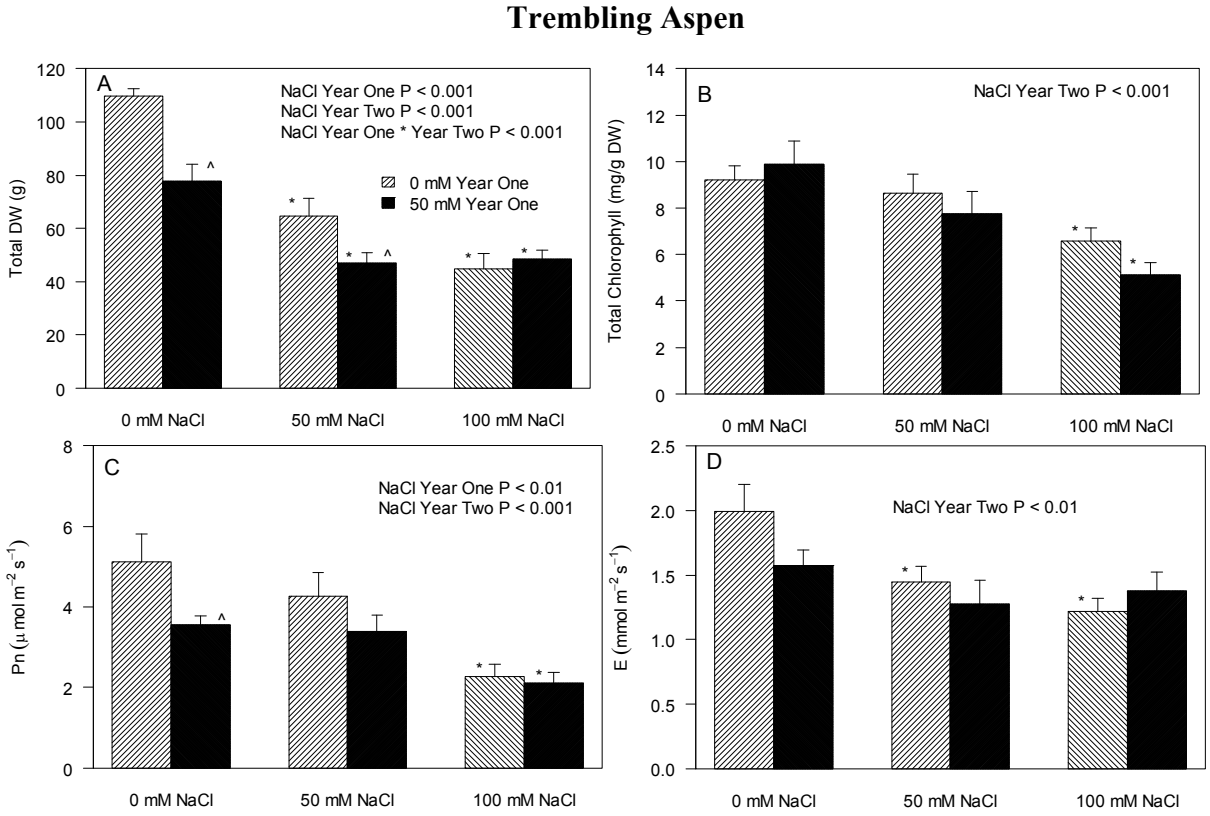


Figure 5.1. Effects of a 60-day NaCl treatment in year two on trembling aspen seedlings treated with 0 or 50 mM NaCl in year one on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rate (E) (D). Values represent the mean + SEM (n=6). Asterisks represent differences between control and NaCl stress treatments from year two for seedlings with the same watering regime from year one. Carets represent differences between NaCl treatments in year one within respective NaCl treatments from year two.

## Trembling Aspen

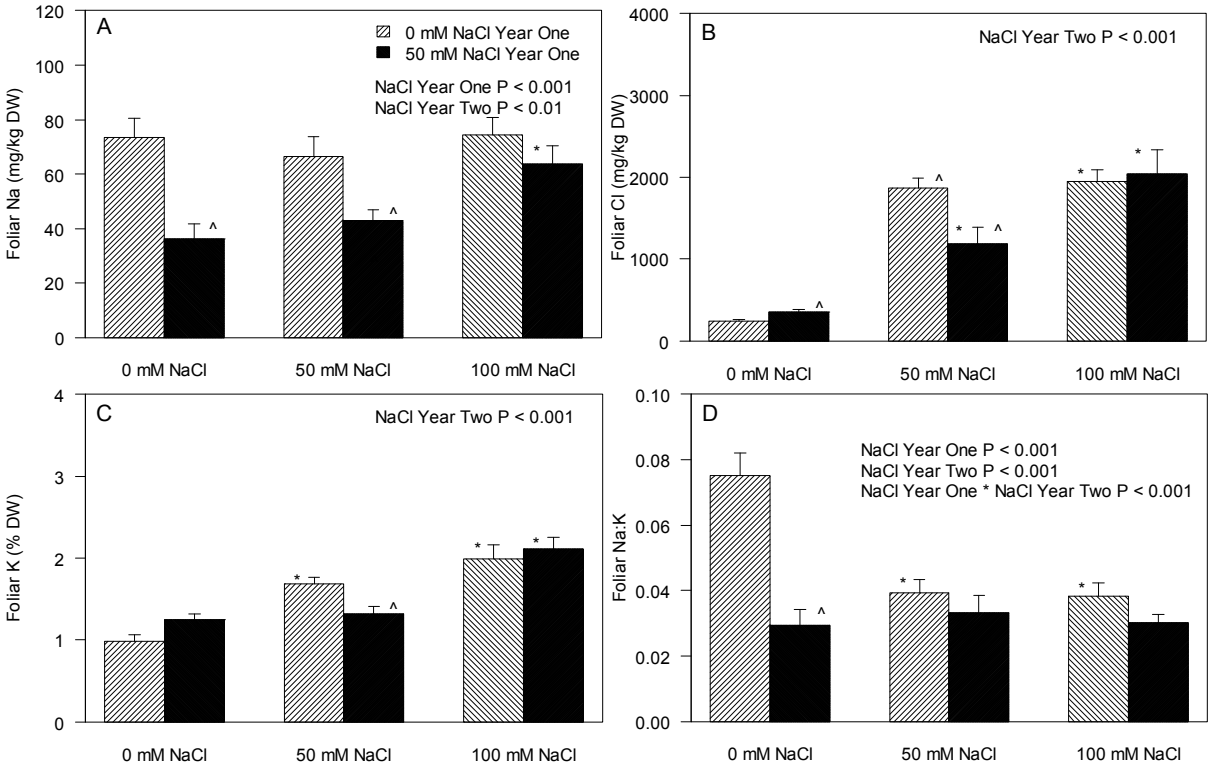


Figure 5.2. Effects of a 60-day NaCl treatment in year two on trembling aspen seedlings treated with 0 or 50 mM NaCl in year one on foliar Na (A), Cl (B), K(C), and Na:K ratio (D). Values represent the mean + SEM (n=6). Asterisks represent differences between control and NaCl stress treatments from year two for seedlings with the same watering regime from year one. Carets represent differences between NaCl treatments in year one within respective NaCl treatments from year two.

**Tamarack**

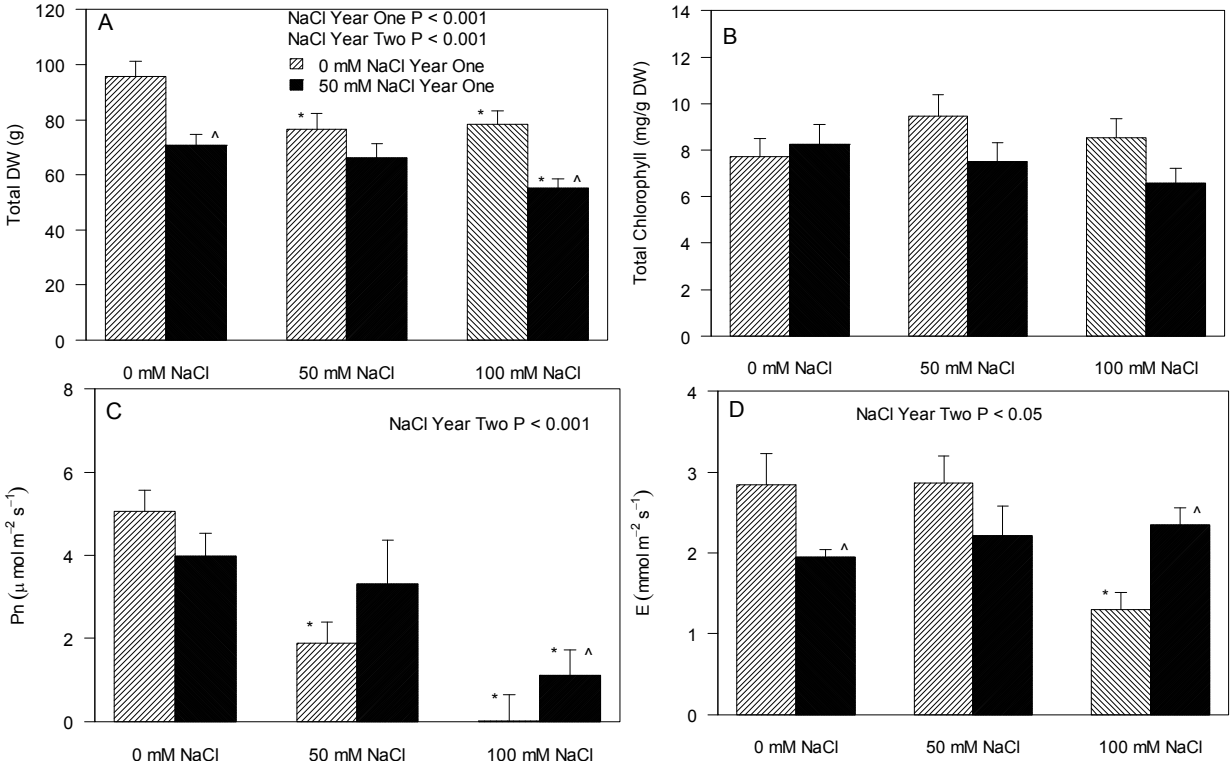


Figure 5.3. Effects of a 60-day NaCl treatment in year two on tamarack seedlings treated with 0 or 50 mM NaCl in year one on total dry weight (A), chlorophyll concentration (B), net photosynthesis (C), and transpiration rate (E) (D). Values represent the mean + SEM (n=6). Asterisks represent differences between control and NaCl stress treatments from year two for seedlings with the same watering regime from year one. Carets represent differences between NaCl treatments in year one within respective NaCl treatments from year two.

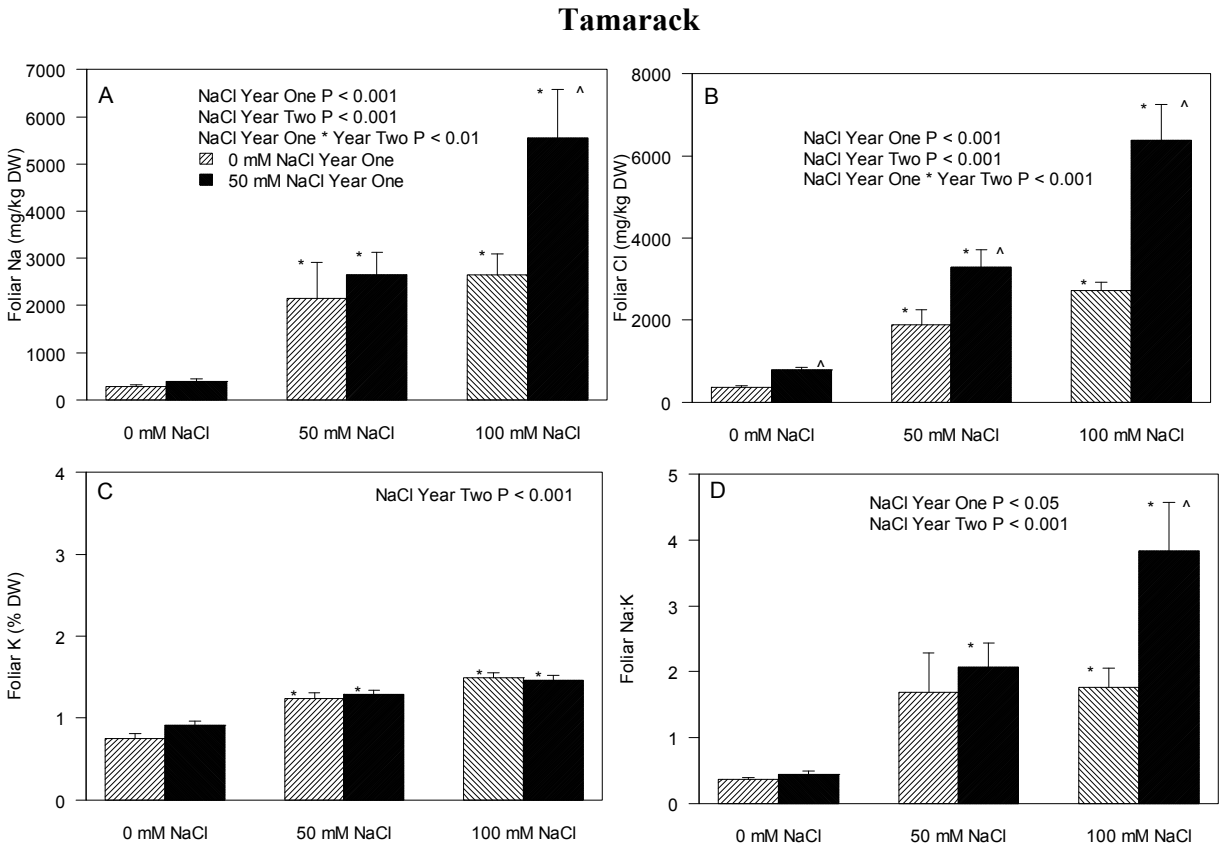


Figure 5.4. Effects of a 60-day NaCl treatment in year two on tamarack seedlings treated with 0 or 50 mM NaCl in year one on foliar Na (A), Cl (B), K (C), and Na:K ratio (D). Values represent the mean + SEM (n=6). Asterisks represent differences between control and NaCl stress treatments from year two for seedlings with the same watering regime from year one. Carets represent differences between NaCl treatments in year one within respective NaCl treatments from year two.

## White Spruce

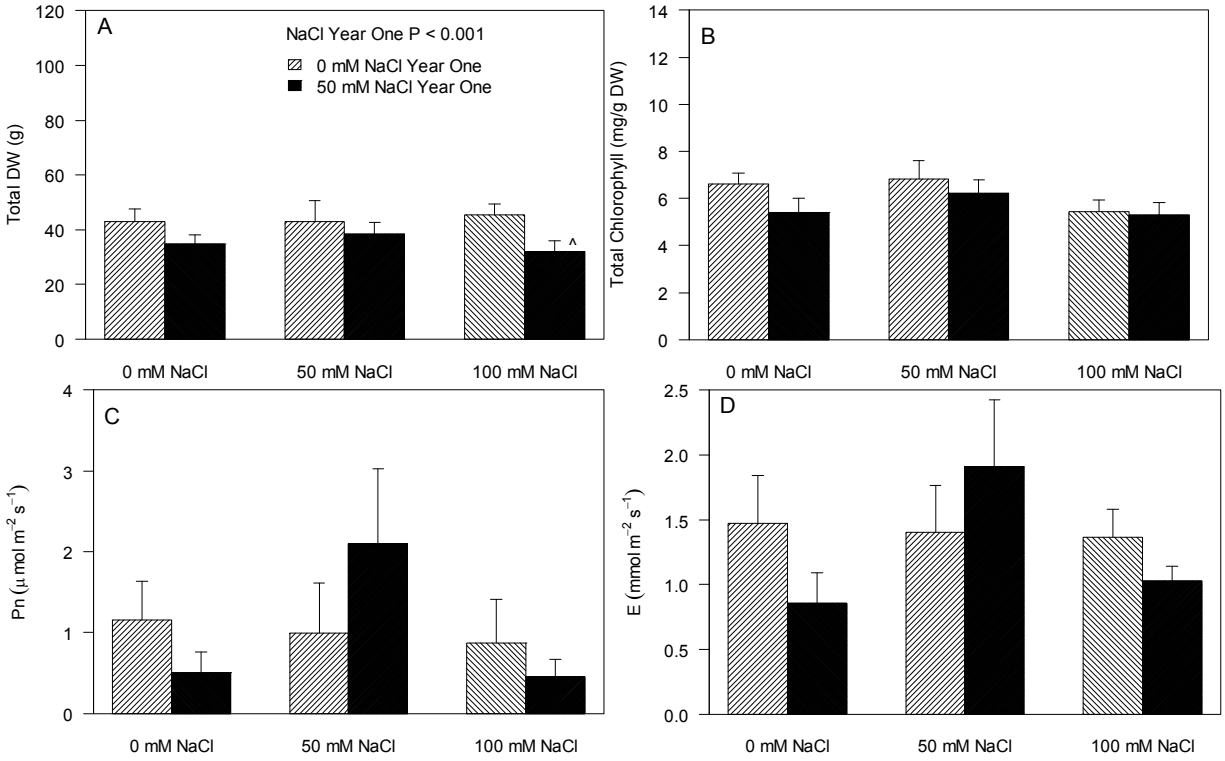


Figure 5.5. Effects of a 60-day NaCl treatment in year two on white spruce seedlings treated with 0 or 50 mM NaCl in year one on total dry weight (A), chlorophyll concentration (B), net photosynthesis (Pn) (C), and transpiration rate (E) (D). Values represent the mean + SEM (n=6). Asterisks represent differences between control and NaCl stress treatments from year two for seedlings with the same watering regime from year one. Carets represent differences between NaCl treatments in year one within respective NaCl treatments from year two.

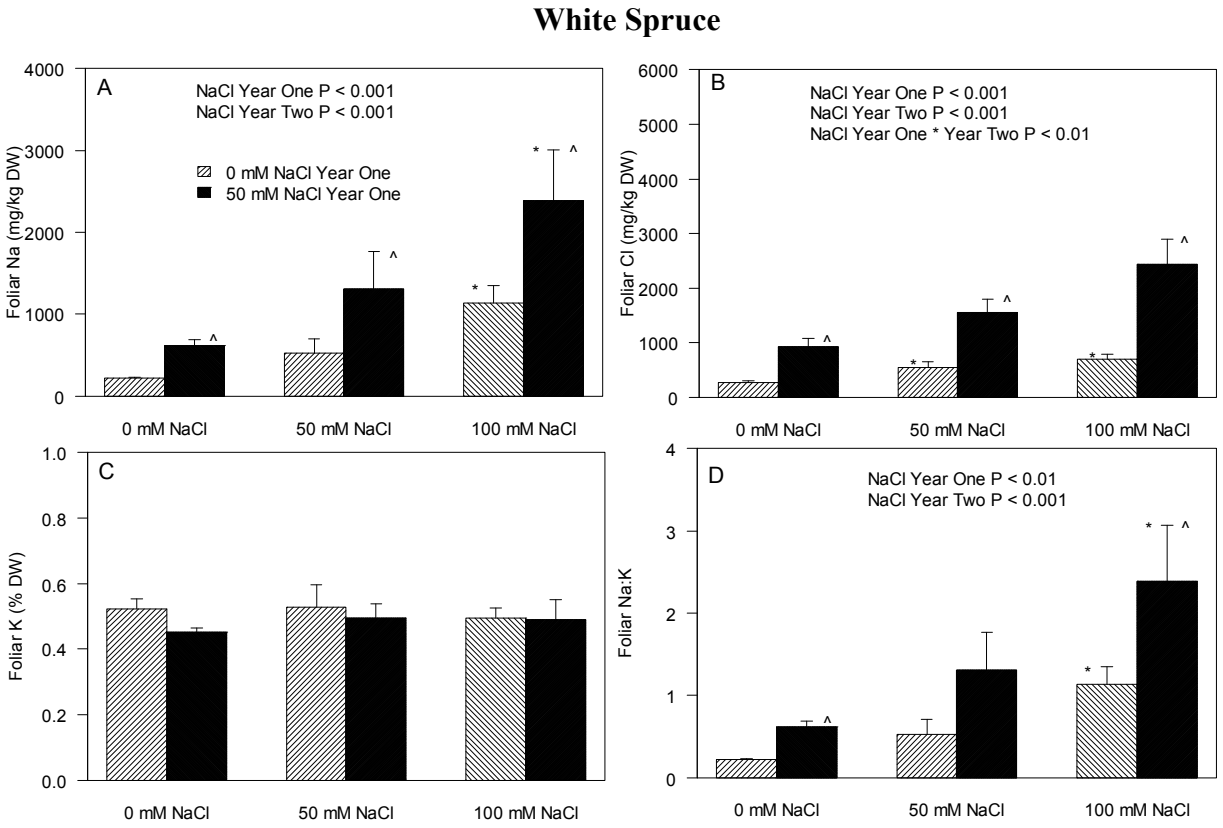


Figure 5.6. Effects of a 60-day NaCl treatment in year two on white spruce seedlings treated with 0 or 50 mM NaCl in year one on foliar Na (A), Cl (B), K (C), and Na:K ratio (D). Values represent the mean + SEM (n=6). Asterisks represent differences between control and NaCl stress treatments from year two for seedlings with the same watering regime from year one. Carets represent differences between NaCl treatments in year one within respective NaCl treatments from year two.

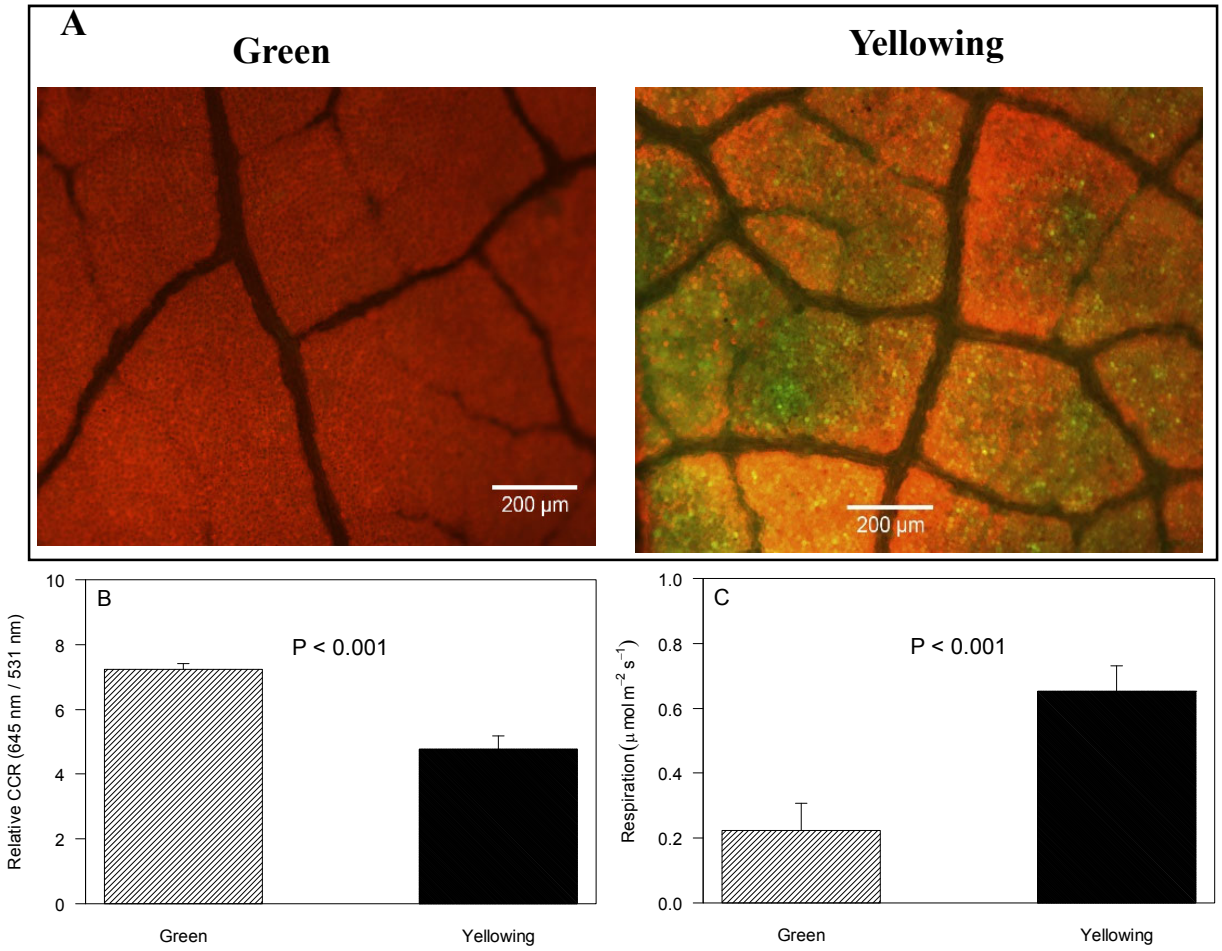


Figure 5.7. Comparison of green and NaCl-induced foliar yellowing in trembling aspen for the presence of ROS (A), relative chlorophyll:carotenoid ratio (B), and night-time respiration (C). Tissue was stained for the presence of ROS using the fluorescent probe DCFH-DA. The red color represents the autofluorescence of chlorophyll whereas the green color represents the presence of ROS. Values represent the mean + SEM (n=6). Significant differences were tested using a student's T-test.

## 5.8 Supplemental material

Table 5.S1 Initial height prior to experimental treatment for trembling aspen, tamarack, and white spruce.

| | Initial Height (cm) | | |
|---|---|---|---|
| | Trembling aspen | Tamarack | White spruce |
| 0 mM NaCl Year 1 | | | |
| 0 mM NaCl | 44.45 ± 1.43 | 20.97 ± 0.38 | 30.44 ± 0.74 |
| 50 mM | 47.54 ± 1.53 | 21.64 ± 1.43 | 31.22 ± 1.40 |
| 100 mM | 46.64 ± 1.45 | 20.48 ± 1.11 | 31.44 ± 0.72 |
| 50 mM NaCl Year 2 | | | |
| 0 mM NaCl | 45.53 ± 1.76 | 21.32 ± 1.84 | 29.95 ± 0.93 |
| 50 mM | 47.23 ± 1.45 | 21.22 ± 0.84 | 30.94 ± 0.83 |
| 100 mM | 49.29 ± 1.72 | 21.04 ± 1.70 | 29.92 ± 1.78 |

Table 5.S2 Initial root collar diameter prior to experimental treatment for trembling aspen, tamarack, and white spruce.

| | Initial Root Collar diameter (mm) | | |
|---|---|---|---|
| | Trembling aspen | Tamarack | White spruce |
| 0 mM NaCl Year 1 | | | |
| 0 mM NaCl | 4.54 ± 0.63 | 3.93 ± 0.29 | 3.59 ± 0.28 |
| 50 mM | 4.32 ± 0.27 | 3.34 ± 0.49 | 3.84 ± 0.42 |
| 100 mM | 4.38 ± 0.73 | 4.29 ± 0.54 | 3.83 ± 0.73 |
| 50 mM NaCl Year 2 | | | |
| 0 mM NaCl | 4.34 ± 0.86 | 3.94 ± 0.67 | 3.85 ± 0.36 |
| 50 mM | 4.83 ± 0.20 | 3.87 ± 0.53 | 3.34 ± 0.73 |
| 100 mM | 4.27 ± 0.92 | 3.64 ± 0.27 | 3.32 ± 0.96 |

# Chapter 6: General Conclusions and Synthesis

## 6.1 Summary of Findings & Future Direction

In the first research study, I subjected trembling aspen (*Populus tremuloides*), green alder (*Alnus viridis*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) to three levels of pH (5, 7, 9) and three levels of NaCl treatment (0, 30, 60 mM) in a factorial design for a total of nine treatments. Trembling aspen exhibited tolerance at pH 5 & 7 with 30 mM NaCl. but showed significant decreases in total dry weight, chlorophyll concentration, photosynthesis, and transpiration at pH 5 & 7 60 mM and all pH 9 treatments. Green alder was sensitive to elevated root zone pH and NaCl where moderate increases caused significant physiological decline. Tamarack exhibited declines in total dry weight, chlorophyll concentration, photosynthesis, and transpiration as root zone pH and NaCl increased. White spruce showed no changes in total dry weight from elevated root zone pH and NaCl; however, elevated NaCl caused significant declines in photosynthesis and transpiration rates.

Each species exhibited different tolerance thresholds to elevated stress; however, all species exhibited a similar underlying pattern. All species showed decreases in foliar nitrogen, total dry weight, chlorophyll concentrations, net photosynthesis, and transpiration rates as a result of increased stress. This trend was compared to contemporary literature reviews which state that the introduction of root zone NaCl induces the propagation of long-distance electrical signals within the phloem. These signals cause an immediate stunting of growth followed by a more conservative growth form. It was proposed that all species may have experienced a similar response to elevated pH and NaCl. However, conclusive testing to verify this hypothesis was not conducted in this study. Future research should be directed to studying the potential of long distance electrical signaling of these species as well as potential mechanisms to stunt growth under elevated root zone pH and NaCl.

From the first study, it was found that tamarack and white spruce could tolerate root zone pH levels as high as 9 and NaCl levels as high as 60 mM. Trembling aspen could tolerate root zone pH up to 7 and NaCl levels of 30 mM. Green alder was very sensitive to any increases of pH and NaCl. However, it should be noted that the experiments were conducted in liquid culture

in a controlled environment with pseudo-replicated experimental units. Thus, great caution must be used when comparing the results generated in this study to seedlings planted in situ.

The second research study was designed to be a follow up on the results of the previous study. All species tested in the previous experiment exhibited a nearly identical trend in response to elevated root zone pH and NaCl. The trend included decreases in foliar N, total chlorophyll concentrations, net photosynthesis, transpiration rates, and growth. For the follow up experiment, it was decided to test weather supplementation with 4x N would help recover physiological functions under elevated pH and NaCl stress in trembling aspen and white spruce. Treatments of elevated root zone pH to 8 and NaCl to 30 mM had a negative effect on net photosynthesis and transpiration rates for both trembling aspen and white spruce. Supplementation with 4x N caused a partial recovery of net photosynthesis and transpiration rates in trembling aspen exposed to elevated root zone NaCl but did not cause a recovery in growth. Interestingly, trembling aspen seedlings treated with 4x N caused increased water use efficiency but decreased intracellular [$CO_2$]. It was proposed that this trend was caused by increased mesophyll conductance. White spruce seedlings exposed to elevated root zone pH and supplemented with 4x N had no effects on photosynthesis. However, seedlings exposed to elevate root zone NaCl and supplemented with 4x N caused further declines in photosynthesis.

From the second study, it was found that trembling aspen had a positive response from 4x N supplementation under elevated root zone NaCl whereas white spruce had a negative response. The positive response of trembling aspen to supplementation with 4x N under elevated root zone NaCl warrants further investigation. In particular, fundamental knowledge on nitrogen metabolism under NaCl stress should be generated. Additionally, the linkage between supplemental nitrogen, increased WUE, and a potential link with mesophyll conductance and plasma membrane aquaporins should be investigated. Finally, the use of exogenous sprays made with salicylic acid and melatonin to alleviate symptoms of NaCl stress should be investigated.

Studies on the physiological effects of NaCl on glycophytic plants are prevalent whereas studies on the physiological recovery from NaCl stress are very limited. Therefore, the third study was focused on studying the recovery of trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*) from NaCl stress. Seedlings were grown in soil within environmental chambers and treated with 0, 50, or 100 mM NaCl for 60 days and then

allowed to recover from stress for another 60 days. Seedlings were sacrificed and sampled for physiological and elemental parameters at day 0, 30, and 60 of recovery. The majority of the seedlings treated with 50 mM NaCl showed a return to control levels of total dry weight, foliar chlorophyll concentration, photosynthesis, and transpiration rates after 30 days of recovery. This is encouraging because the 50 mM NaCl concentration is comparable to the established soil threshold of four dS $m^{-2}$ for boreal forest vegetation. Recovery from 100 mM NaCl varied by species. Trembling aspen exhibited an interesting trend where some seedlings completely defoliated during the stress period and partially re-flushed during the recovery period. After 60 days of recovery, the new leaves exhibited higher levels of chlorophyll concentration, net photosynthesis, and transpiration rates compared to untreated controls. Tamarack seedlings exposed to 100 mM NaCl exhibited lower levels of dry weight, chlorophyll concentration, and net photosynthesis compared to controls after 60 days of recovery. White spruce showed no changes in any of the measured parameters after 60 days of recovery. For all species, increased NaCl caused foliar yellowing. For trembling aspen and tamarack, an increase in foliar necrotic tissue was also associated with increased NaCl. It was hypothesized that accelerated foliar yellowing acted as a tolerance mechanism to reduce the amount of NaCl entering younger growing tissue. Elevated NaCl caused an increase in foliar K for all species. It was hypothesized that accelerated senescence and elevated K in foliar tissue were tolerance mechanisms for all species. Considering that elevated foliar K is a common stress response to elevated root zone NaCl, future research should focus on supplementing soils with potash or using exogenous K sprays on foliar tissue.

The fourth study was designed to be a follow up to the third experiment where overwintering was incorporated to NaCl stress and recovery on trembling aspen (*Populus tremuloides*), tamarack (*Larix laricina*), and white spruce (*Picea glauca*). Seedlings were first subjected non-lethal NaCl stress followed by overwintering. Seedlings were then subjected to 0, 50, or 100 mM NaCl for eight weeks. It was hypothesized that NaCl stress and overwintering in year one would hinder the ability of seedlings to recover from NaCl stress in year two. Plants of all three species exhibited some form of salt injury from NaCl treatment in the first year. Aspen and tamarack seedlings treated with 50 mM NaCl in year one exhibited lower total dry weights compared to non-treated control in year one. For white spruce, NaCl treatment in year two had virtually no effect on total dry weight, chlorophyll concentration, photosynthesis, or transpiration

of seedlings. However, seedlings exposed to two years of NaCl stress showed significant foliar chlorosis and mortality of older needles. For trembling aspen, growth and physiological response rates were lowered by multiple years of NaCl stress, suggesting that seedlings exhibited a mechanism of acclimation, perhaps to increase survival under stress conditions. Tamarack seedlings exposed to NaCl in year one exhibited increases in photosynthesis and water use efficiency when exposed to NaCl in year two compared to control seedlings that were not treated with NaCl in year one. Many tamarack seedlings treated with NaCl in year one exhibited dieback of the terminal bud, which led to forked branching. It was hypothesized that the combined effects of NaCl stress and overwintering interfered with cellular supercooling mechanisms, resulting in terminal bud dieback. Both tamarack and white spruce treated with NaCl in year one exhibited higher foliar Na and Cl concentration compared to seedlings that were not treated with NaCl in year one. It was hypothesized that vacuole sequestration of Na or storage within cell walls may be potential mechanisms of Na storage. Treatment with NaCl caused accelerated foliar senescence in all species. In trembling aspen, the percentage of necrotic leaf tissue increased as salinity levels increased from 50 mM to 100 mM NaCl. Considering that this was a consistent trend from previous experiments, it was decided to study this phenomenon in more detail. Compared to green tissue, yellowing tissue showed an accumulation of ROS and decreased chlorophyll:carotenoid ratio, but higher dark respiration rates. In other studies, foliar yellowing was induced by NaCl which leads to a cell signaling event to trigger the deliberate degradation of chloroplasts and eventually PCD. It was not shown directly that PCD was occurring in yellowing region of trembling aspen leaves, but it was hypothesized that this was occurring. Taken together, it appears that the three species tested can withstand 50 mM NaCl and overwintering stress but some physiological and morphological deficiencies arise. Future research should be directed to studying the effects of overwintering and NaCl stress. In particular, the effects of NaCl on cellular supercooling mechanisms should be investigated. Secondly, the potential of hyperspectral imaging to detect foliar senescence as an indicator of stress should be explored.

A literature review was written to generate a model on how glycophyte plants respond to NaCl stress. The novelty of this work was focused on combining the responses of whole plant physiology to cell physiology as well as identifying gaps in current knowledge. The majority of glycophytes exhibit a response to NaCl stress that includes rapid decreases in root water flux, transpiration, photosynthesis, and growth, but increased compatible solute concentration,

root:shoot ratio, and NPQ. Recent evidence suggests that plants send stress signals using a primitive nervous system which initiates a secondary cell signaling cascade to elicit a physiological response on the cellular level. It was proposed that the response differs depending on the cell type. However, the general trend appears to be a decrease in water transport and carbon fixation as well as changes in gene expression which leads to an overall downregulation of metabolism and a more conservative growth pattern. The signaling cascade appears to be similar in many cell types and is characterized by increased cytoplasmic $Ca^{+2}$, ROS, and NO. The induction of autophagy may also be an important component to the stress response, perhaps to recycle damaged proteins. The proliferation of peroxisomes may be another important component of the cell signaling cascade in order to produce $H_2O_2$ and NO. It should be noted that secondary cell signaling processes at the root tips, root cortex, guard cells, and yellowing leaf are well studied whereas signaling processes at the mesophyll cells, vascular cambium, and root epidermis are mostly unexplored. Future work should focus on studying the adaptive behavior of plants in response to NaCl stress. In particular, studying the presence and potential effects of long-distance electrical signals on the cellular responses of different tissue types. By fully understanding the cellular response of plants for each cell type, it may be possible to improve plant performance under NaCl stress.

Taken collectively, this work shed light on how selected boreal forest tree species responded to elevated pH and NaCl as well as their ability to recover from NaCl stress. Trembling aspen, tamarack, and white spruce should be considered for reclamation for their ability to withstand elevated pH, NaCl, and overwintering stress. In addition to the research ideas mentioned previously, more works should be dedicated to family level differences within species. This is because stress tolerance thresholds can vary greatly between families. Also, land reclamation may be improved by copying nature and utilizing aspen as a pioneer species and underplanting spruce. Finally, seedling performance on reclamation sites may be improved by incorporating pyrogenic carbon as well as fostering bacterial and fungal associations in the soil. Successful land reclamation of boreal forest disturbed by surface mining in northern Alberta is a complex issue. This work attempted to address several main issues which hinder revegetation efforts. From this work, fundamental knowledge was generated as well as potential solutions were presented to improve the revegetation of boreal forest land.

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**Appendix 1**

(ANOVA tables for Chapter 2)

Table a1.1 Two-way ANOVA tables showing the effects of pH and NaCl on measured parameters for trembling aspen.

| Trembling Aspen Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Foliar N | pH | 2 | 45.34 | **<0.001** |
| | NaCl | 2 | 5.26 | **<0.001** |
| | pH * NaCl | 4 | 3.61 | **0.012** |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar Fe | pH | 2 | 42.68 | **<0.001** |
| | NaCl | 2 | 3.90 | **0.028** |
| | pH * NaCl | 4 | 9.08 | **<0.001** |
| | Error | 40 | | |
| | Total | 48 | | |
| Foliar Na | pH | 2 | 5.05 | **0.011** |
| | NaCl | 2 | 27.34 | **<0.001** |
| | pH * NaCl | 4 | 5.12 | **0.002** |
| | Error | 42 | | |
| | Total | 50 | | |
| Foliar Cl | pH | 2 | 4.60 | **0.015** |
| | NaCl | 2 | 162.91 | **<0.001** |
| | pH * NaCl | 4 | 3.78 | **0.010** |
| | Error | 46 | | |
| | Total | 54 | | |
| Total DW | pH | 2 | 7.65 | **0.001** |
| | NaCl | 2 | 10.14 | **<0.001** |
| | pH * NaCl | 4 | 4.89 | **0.003** |
| | Error | 48 | | |
| | Total | 56 | | |
| Total Chlorophyll | pH | 2 | 11.71 | **0.023** |
| | NaCl | 2 | 6.33 | **0.007** |
| | pH * NaCl | 4 | 2.92 | **0.031** |
| | Error | 48 | | |
| | Total | 56 | | |

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|---|---|---|---|---|
| Net Photosynthesis | pH | 2 | 0.80 | **<0.001** |
| | NaCl | 2 | 6.16 | **<0.001** |
| | pH * NaCl | 4 | 3.63 | **<0.001** |
| | Error | 45 | | |
| | Total | 53 | | |
| Transpiration | pH | 2 | 2.18 | 0.125 |
| | NaCl | 2 | 6.51 | **<0.001** |
| | pH * NaCl | 4 | 1.63 | 0.184 |
| | Error | 45 | | |
| | Total | 53 | | |
| RWR | pH | 2 | 17.83 | **<0.001** |
| | NaCl | 2 | 6.68 | **0.003** |
| | pH * NaCl | 4 | 0.80 | 0.530 |
| | Error | 45 | | |
| | Total | 53 | | |

Table a1.2 Two-way ANOVA tables showing the effects of pH and NaCl on measured parameters for green alder.

| Green Alder Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Foliar N | pH | 2 | 66.01 | **<0.001** |
| | NaCl | 1 | 0.80 | 0.378 |
| | pH * NaCl | 1 | 4.08 | 0.051 |
| | Error | 35 | | |
| | Total | 39 | | |
| Foliar Fe | pH | 2 | 6.49 | **0.004** |
| | NaCl | 1 | 0.01 | 0.994 |
| | pH * NaCl | 1 | 0.71 | 0.406 |
| | Error | 35 | | |
| | Total | 40 | | |
| Foliar Na | pH | 2 | 4.99 | **0.012** |
| | NaCl | 1 | 60.61 | **<0.001** |
| | pH * NaCl | 1 | 9.75 | **0.004** |
| | Error | 35 | | |
| | Total | 39 | | |
| Foliar Cl | pH | 2 | 3.09 | 0.058 |
| | NaCl | 1 | 93.74 | **<0.001** |
| | pH * NaCl | 1 | 7.73 | **0.007** |
| | Error | 35 | | |
| | Total | 39 | | |

| | | | | |
|---|---|---|---|---|
| Total DW | pH | 2 | 19.84 | **0.001** |
| | NaCl | 1 | 9.51 | **<0.001** |
| | pH * NaCl | 1 | 4.86 | 0.084 |
| | Error | 39 | | |
| | Total | 43 | | |
| Total Chlorophyll | pH | 2 | 36.75 | **<0.001** |
| | NaCl | 1 | 32.43 | **<0.001** |
| | pH * NaCl | 1 | 1.22 | 0.276 |
| | Error | 35 | | |
| | Total | 40 | | |
| Net Photosynthesis | pH | 2 | 53.18 | **<0.001** |
| | NaCl | 1 | 59.36 | **<0.001** |
| | pH * NaCl | 1 | 0.02 | 0.878 |
| | Error | 35 | | |
| | Total | 39 | | |
| Transpiration | pH | 2 | 35.07 | **<0.001** |
| | NaCl | 1 | 65.09 | **<0.001** |
| | pH * NaCl | 1 | 4.94 | 0.674 |
| | Error | 35 | | |
| | Total | 39 | | |
| RWR | pH | 2 | 22.99 | **<0.001** |
| | NaCl | 1 | 13.19 | **<0.001** |
| | pH * NaCl | 1 | 5.09 | 0.062 |
| | Error | 35 | | |
| | Total | 39 | | |

Table a1.3 Two-way ANOVA tables showing the effects of pH and NaCl on measured parameters for tamarack.

| Tamarack Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Foliar N | pH | 2 | 21.35 | **<0.001** |
| | NaCl | 2 | 2.77 | 0.071 |
| | pH * NaCl | 4 | 3.51 | **0.012** |
| | Error | 63 | | |
| | Total | 71 | | |

| | | | | |
|---|---|---|---|---|
| Foliar Fe | pH | 2 | 2.79 | 0.069 |
| | NaCl | 2 | 1.33 | 0.271 |
| | pH * NaCl | 4 | 1.53 | 0.206 |
| | Error | 63 | | |
| | Total | 71 | | |
| Foliar Na | pH | 2 | 5.49 | **0.006** |
| | NaCl | 2 | 92.72 | **<0.001** |
| | pH * NaCl | 4 | 5.39 | **0.001** |
| | Error | 63 | | |
| | Total | 71 | | |
| Foliar Cl | pH | 2 | 4.76 | **0.012** |
| | NaCl | 2 | 101.37 | **<0.001** |
| | pH * NaCl | 4 | 5.01 | **0.001** |
| | Error | 60 | | |
| | Total | 68 | | |
| Total DW | pH | 2 | 16.43 | **0.001** |
| | NaCl | 2 | 7.38 | **<0.001** |
| | pH * NaCl | 4 | 8.65 | **<0.001** |
| | Error | 62 | | |
| | Total | 71 | | |
| Total Chlorophyll | pH | 2 | 75.59 | **<0.001** |
| | NaCl | 2 | 28.73 | **<0.001** |
| | pH * NaCl | 4 | 13.96 | **<0.001** |
| | Error | 57 | | |
| | Total | 65 | | |
| Net Photosynthesis | pH | 2 | 59.25 | **<0.001** |
| | NaCl | 2 | 16.19 | **0.004** |
| | pH * NaCl | 4 | 0.34 | 0.847 |
| | Error | 61 | | |
| | Total | 69 | | |
| Transpiration | pH | 2 | 32.36 | **<0.001** |
| | NaCl | 2 | 19.97 | **<0.001** |
| | pH * NaCl | 4 | 1.65 | 0.172 |
| | Error | 61 | | |
| | Total | 69 | | |

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|---|---|---|---|---|
| RWR | pH | 2 | 2.46 | 0.095 |
| | NaCl | 2 | 1.06 | 0.354 |
| | pH * NaCl | 4 | 5.42 | **0.014** |
| | Error | 61 | | |
| | Total | 69 | | |

Table a1.4 Two-way ANOVA tables showing the effects of pH and NaCl on measured parameters for white spruce.

| White Spruce Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Foliar N | pH | 2 | 32.80 | **<0.001** |
| | NaCl | 2 | 4.34 | **0.018** |
| | pH * NaCl | 4 | 3.60 | **0.011** |
| | Error | 55 | | |
| | Total | 63 | | |
| Foliar Fe | pH | 2 | 21.24 | **<0.001** |
| | NaCl | 2 | 26.42 | **<0.001** |
| | pH * NaCl | 4 | 6.30 | **<0.001** |
| | Error | 52 | | |
| | Total | 60 | | |
| Foliar Na | pH | 2 | 1.47 | 0.239 |
| | NaCl | 2 | 135.25 | **<0.001** |
| | pH * NaCl | 4 | 0.98 | 0.425 |
| | Error | 54 | | |
| | Total | 62 | | |
| Foliar Cl | pH | 2 | 1.22 | 0.302 |
| | NaCl | 2 | 126.17 | **<0.001** |
| | pH * NaCl | 4 | 0.920 | 0.459 |
| | Error | 53 | | |
| | Total | 61 | | |
| Total DW | pH | 2 | 3.13 | 0.061 |
| | NaCl | 2 | 1.52 | 0.227 |
| | pH * NaCl | 4 | 1.79 | 0.151 |
| | Error | 53 | | |
| | Total | 61 | | |
| Total Chlorophyll | pH | 2 | 24.47 | **<0.001** |
| | NaCl | 2 | 14.84 | **<0.001** |
| | pH * NaCl | 4 | 3.24 | **0.004** |
| | Error | 55 | | |
| | Total | 64 | | |

| | | | | |
|---|---|---|---|---|
| Net Photosynthesis | pH | 2 | 2.83 | **0.015** |
| | NaCl | 2 | 31.06 | **<0.001** |
| | pH * NaCl | 4 | 1.49 | 0.219 |
| | Error | 54 | | |
| | Total | 63 | | |
| Transpiration | pH | 2 | 4.45 | **<0.001** |
| | NaCl | 2 | 40.35 | **<0.001** |
| | pH * NaCl | 4 | 2.29 | 0.710 |
| | Error | 55 | | |
| | Total | 64 | | |
| RWR | pH | 2 | 0.41 | 0.667 |
| | NaCl | 2 | 1.34 | 0.272 |
| | pH * NaCl | 4 | 1.87 | 0.351 |
| | Error | 55 | | |
| | Total | 64 | | |

## Appendix 2

(ANOVA tables for Chapter 3)

Table a2.1 Three-way ANOVA tables showing the effects of supplemental N, pH, and NaCl on measured parameters for trembling aspen.

| Trembling Aspen Parameters | Source of Variation | DF | Iterations | P |
|---|---|---|---|---|
| Height | N | 1 | 295644 | **0.032** |
| | pH | 1 | 181858 | **0.004** |
| | NaCl | 1 | 884315 | **0.008** |
| | N * pH | 1 | 729549 | 0.154 |
| | N * NaCl | 1 | 996737 | 0.554 |
| | pH * NaCl | 1 | 993304 | 0.794 |
| | N * pH * NaCl | 1 | 318478 | 0.387 |
| | Error | 28 | | |
| | Total | 35 | | |
| Total Chlorophyll | N | 1 | 510430 | **0.006** |
| | pH | 1 | 953396 | 0.514 |
| | NaCl | 1 | 751646 | 0.523 |
| | N * pH | 1 | 151020 | 0.461 |
| | N * NaCl | 1 | 377952 | 0.832 |
| | pH * NaCl | 1 | 951715 | 0.293 |
| | N * pH * NaCl | 1 | 76007 | 0.418 |
| | Error | 28 | | |
| | Total | 35 | | |
| Net Photosynthesis | N | 1 | 965747 | 0.577 |
| | pH | 1 | 234542 | **0.003** |
| | NaCl | 1 | 572662 | **<0.001** |
| | N * pH | 1 | 90740 | 0.982 |
| | N * NaCl | 1 | 133618 | **<0.001** |
| | pH * NaCl | 1 | 267261 | 0.072 |
| | N * pH * NaCl | 1 | 378359 | 0.341 |
| | Error | 28 | | |
| | Total | 35 | | |

| | | | | |
|---|---|---|---|---|
| Transpiration | N | 1 | 729318 | **0.003** |
| | pH | 1 | 137231 | 0.183 |
| | NaCl | 1 | 729193 | **<0.001** |
| | N * pH | 1 | 286173 | 0.946 |
| | N * NaCl | 1 | 174784 | **<0.001** |
| | pH * NaCl | 1 | 417675 | 0.515 |
| | N * pH * NaCl | 1 | 427233 | 0.765 |
| | Error | 28 | | |
| | Total | 35 | | |
| WUE | N | 1 | 543174 | **<0.001** |
| | pH | 1 | 283144 | **<0.001** |
| | NaCl | 1 | 325583 | 0.069 |
| | N * pH | 1 | 327466 | 0.072 |
| | N * NaCl | 1 | 545069 | 0.884 |
| | pH * NaCl | 1 | 896608 | 0.381 |
| | N * pH * NaCl | 1 | 875493 | 0.206 |
| | Error | 28 | | |
| | Total | 35 | | |
| Ci | N | 1 | 205102 | **<0.001** |
| | pH | 1 | 598158 | 0.088 |
| | NaCl | 1 | 357494 | 0.144 |
| | N * pH | 1 | 215540 | 0. 132 |
| | N * NaCl | 1 | 321273 | 0.709 |
| | pH * NaCl | 1 | 343427 | 0.801 |
| | N * pH * NaCl | 1 | 540997 | 0.438 |
| | Error | 28 | | |
| | Total | 35 | | |
| Foliar N | N | 1 | 434349 | 0.521 |
| | pH | 1 | 445156 | **<0.001** |
| | NaCl | 1 | 241651 | 0.553 |
| | N * pH | 1 | 420030 | 0.649 |
| | N * NaCl | 1 | 897987 | 0.632 |
| | pH * NaCl | 1 | 229561 | 0.854 |
| | N * pH * NaCl | 1 | 897881 | 0.453 |
| | Error | 28 | | |
| | Total | 35 | | |

| | | | | |
|---|---|---|---|---|
| Foliar Na | N | 1 | 82020 | **0.010** |
| | pH | 1 | 988044 | 0.918 |
| | NaCl | 1 | 760053 | **<0.001** |
| | N * pH | 1 | 963242 | 0.473 |
| | N * NaCl | 1 | 488180 | 0.056 |
| | pH * NaCl | 1 | 38430 | 0.414 |
| | N * pH * NaCl | 1 | 652408 | 0.356 |
| | Error | 28 | | |
| | Total | 35 | | |
| Foliar Cl | N | 1 | 195638 | **0.010** |
| | pH | 1 | 301357 | 0.539 |
| | NaCl | 1 | 364773 | **<0.001** |
| | N * pH | 1 | 808379 | 0.391 |
| | N * NaCl | 1 | 822624 | **<0.001** |
| | pH * NaCl | 1 | 592568 | 0.876 |
| | N * pH * NaCl | 1 | 504001 | 0.539 |
| | Error | 28 | | |
| | Total | 35 | | |

Table a2.2 Three-way ANOVA tables showing the effects of supplemental N, pH, and NaCl on measured parameters for white spruce.

| White Spruce Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Height | N | 1 | 882683 | 0.758 |
| | pH | 1 | 484383 | 0.641 |
| | NaCl | 1 | 834657 | 0.861 |
| | N * pH | 1 | 743179 | 0.812 |
| | N * NaCl | 1 | 171022 | 0.784 |
| | pH * NaCl | 1 | 793475 | 0.799 |
| | N * pH * NaCl | 1 | 271064 | 0.958 |
| | Error | 39 | | |
| | Total | 46 | | |
| Total Chlorophyll | N | 1 | 860822 | **0.032** |
| | pH | 1 | 563997 | **<0.001** |
| | NaCl | 1 | 800218 | 0.191 |
| | N * pH | 1 | 397510 | **<0.001** |
| | N * NaCl | 1 | 345158 | 0.081 |
| | pH * NaCl | 1 | 223269 | **0.030** |
| | N * pH * NaCl | 1 | 497781 | 0.489 |
| | Error | 39 | | |
| | Total | 46 | | |

| | | | | |
|---|---|---|---|---|
| Net Photosynthesis | N | 1 | 166557 | **0.002** |
| | pH | 1 | 946882 | **<0.001** |
| | NaCl | 1 | 867283 | **0.005** |
| | N * pH | 1 | 542253 | 0.949 |
| | N * NaCl | 1 | 94294 | 0.078 |
| | pH * NaCl | 1 | 364470 | 0.159 |
| | N * pH * NaCl | 1 | 12281 | 0.357 |
| | Error | 39 | | |
| | Total | 46 | | |
| Transpiration | N | 1 | 242801 | **<0.001** |
| | pH | 1 | 288453 | **<0.001** |
| | NaCl | 1 | 343701 | 0.464 |
| | N * pH | 1 | 220796 | 0.247 |
| | N * NaCl | 1 | 385525 | 0.946 |
| | pH * NaCl | 1 | 146278 | 0.811 |
| | N * pH * NaCl | 1 | 993885 | 0.259 |
| | Error | 39 | | |
| | Total | 46 | | |
| WUE | N | 1 | 383228 | **<0.001** |
| | pH | 1 | 488215 | **<0.001** |
| | NaCl | 1 | 339396 | **0.049** |
| | N * pH | 1 | 95621 | **<0.001** |
| | N * NaCl | 1 | 939370 | 0.817 |
| | pH * NaCl | 1 | 871391 | 0.610 |
| | N * pH * NaCl | 1 | 451851 | 0.648 |
| | Error | 39 | | |
| | Total | 46 | | |
| Ci | N | 1 | 599282 | **<0.001** |
| | pH | 1 | 118499 | **<0.001** |
| | NaCl | 1 | 896561 | **0.021** |
| | N * pH | 1 | 912936 | **<0.001** |
| | N * NaCl | 1 | 983975 | 0.241 |
| | pH * NaCl | 1 | 820416 | 0.178 |
| | N * pH * NaCl | 1 | 616057 | 0.234 |
| | Error | 39 | | |
| | Total | 46 | | |

| | | | | |
|---|---|---|---|---|
| Foliar N | N | 1 | 406107 | **<0.001** |
| | pH | 1 | 839497 | **<0.001** |
| | NaCl | 1 | 213264 | 0.268 |
| | N * pH | 1 | 351145 | 0.071 |
| | N * NaCl | 1 | 751637 | 0.252 |
| | pH * NaCl | 1 | 957836 | 0.783 |
| | N * pH * NaCl | 1 | 166960 | 0.673 |
| | Error | 39 | | |
| | Total | 46 | | |
| Foliar Na | N | 1 | 435065 | **0.0010** |
| | pH | 1 | 704482 | 0.326 |
| | NaCl | 1 | 907312 | **<0.001** |
| | N * pH | 1 | 648046 | 0.103 |
| | N * NaCl | 1 | 369064 | 0.061 |
| | pH * NaCl | 1 | 798501 | 0.322 |
| | N * pH * NaCl | 1 | 72838 | 0.059 |
| | Error | 39 | | |
| | Total | 46 | | |
| Foliar Cl | N | 1 | 112098 | 0.615 |
| | pH | 1 | 159448 | 0.802 |
| | NaCl | 1 | 642644 | **<0.001** |
| | N * pH | 1 | 331484 | 0.081 |
| | N * NaCl | 1 | 770794 | 0.676 |
| | pH * NaCl | 1 | 131826 | 0.518 |
| | N * pH * NaCl | 1 | 527313 | 0.064 |
| | Error | 39 | | |
| | Total | 46 | | |

**Appendix 3**

(ANOVA tables for Chapter 4)

Table a3.1 Two-way ANOVA tables showing the effects of NaCl and recovery time on measured parameters for trembling aspen.

| Trembling Aspen Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Total DW | NaCl | 2 | 8.60 | **<0.001** |
| | Time | 2 | 0.12 | 0.892 |
| | NaCl * Time | 4 | 0.26 | 0.903 |
| | Error | 34 | | |
| | Total | 42 | | |
| Total Chlorophyll | NaCl | 2 | 1.93 | 0.161 |
| | Time | 2 | 5.61 | **<0.001** |
| | NaCl * Time | 4 | 6.87 | **<0.001** |
| | Error | 34 | | |
| | Total | 42 | | |
| Net Photosynthesis | NaCl | 2 | 0.47 | 0.627 |
| | Time | 2 | 0.58 | 0.563 |
| | NaCl * Time | 4 | 4.24 | **<0.001** |
| | Error | 34 | | |
| | Total | 42 | | |
| Transpiration | NaCl | 2 | 1.39 | 0.264 |
| | Time | 2 | 6.96 | **0.003** |
| | NaCl * Time | 4 | 4.62 | **0.004** |
| | Error | 34 | | |
| | Total | 42 | | |
| Foliar Na | NaCl | 2 | 0.96 | 0.392 |
| | Time | 2 | 7.79 | **<0.001** |
| | NaCl * Time | 4 | 0.346 | 0.845 |
| | Error | 34 | | |
| | Total | 42 | | |
| Foliar Cl | NaCl | 2 | 76.85 | **<0.001** |
| | Time | 2 | 1.71 | 0.195 |
| | NaCl * Time | 4 | 8.96 | **0.012** |
| | Error | 34 | | |
| | Total | 42 | | |

| | | | | |
|---|---|---|---|---|
| Foliar K | NaCl | 2 | 45.45 | **<0.001** |
| | Time | 2 | 0.70 | 0.502 |
| | NaCl * Time | 4 | 3.37 | 0.061 |
| | Error | 34 | | |
| | Total | 42 | | |
| Foliar Na:K | NaCl | 2 | 9.86 | **<0.001** |
| | Time | 2 | 10.01 | **<0.001** |
| | NaCl * Time | 4 | 1.23 | 0.316 |
| | Error | 34 | | |
| | Total | 42 | | |

Table a3.2 Two-way ANOVA tables showing the effects of NaCl and recovery time on measured parameters for tamarack.

| Tamarack Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Total DW | NaCl | 2 | 12.44 | **<0.001** |
| | Time | 2 | 10.72 | **0.010** |
| | NaCl * Time | 4 | 1.47 | 0.227 |
| | Error | 45 | | |
| | Total | 53 | | |
| Total Chlorophyll | NaCl | 2 | 19.45 | **<0.001** |
| | Time | 2 | 2.01 | 0.146 |
| | NaCl * Time | 4 | 1.62 | 0.137 |
| | Error | 45 | | |
| | Total | 53 | | |
| Net Photosynthesis | NaCl | 2 | 24.36 | **<0.001** |
| | Time | 2 | 1.92 | 0.159 |
| | NaCl * Time | 4 | 1.925 | 0.164 |
| | Error | 45 | | |
| | Total | 53 | | |
| Transpiration | NaCl | 2 | 10.16 | **<0.001** |
| | Time | 2 | 9.93 | **<0.001** |
| | NaCl * Time | 4 | 5.79 | **<0.001** |
| | Error | 45 | | |
| | Total | 53 | | |

| | | | | |
|---|---|---|---|---|
| Foliar Na | NaCl | 2 | 143.45 | **<0.001** |
| | Time | 2 | 2.25 | 0.189 |
| | NaCl * Time | 4 | 1.77 | 0.231 |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar Cl | NaCl | 2 | 482.72 | **<0.001** |
| | Time | 2 | 4.56 | 0.065 |
| | NaCl * Time | 4 | 8.17 | **0.031** |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar K | NaCl | 2 | 16.78 | **<0.001** |
| | Time | 2 | 10.96 | **<0.001** |
| | NaCl * Time | 4 | 2.95 | 0.090 |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar Na:K | NaCl | 2 | 110.73 | **<0.001** |
| | Time | 2 | 7.57 | **<0.001** |
| | NaCl * Time | 4 | 7.85 | **<0.001** |
| | Error | 45 | | |
| | Total | 53 | | |

Table a3.3 Two-way ANOVA tables showing the effects of NaCl and recovery time on measured parameters for white spruce.

| White Spruce Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Total DW | NaCl | 2 | 2.38 | 0.104 |
| | Time | 2 | 13.42 | **<0.001** |
| | NaCl * Time | 4 | 0.75 | 0.563 |
| | Error | 45 | | |
| | Total | 53 | | |
| Total Chlorophyll | NaCl | 2 | 1.47 | 0.227 |
| | Time | 2 | 1.80 | 0.146 |
| | NaCl * Time | 4 | 1.77 | 0.231 |
| | Error | 45 | | |
| | Total | 53 | | |

| | | | | |
|---|---|---|---|---|
| Net Photosynthesis | NaCl | 2 | 6.74 | **<0.001** |
| | Time | 2 | 3.31 | 0.119 |
| | NaCl * Time | 4 | 0.55 | 0.703 |
| | Error | 45 | | |
| | Total | 53 | | |
| Transpiration | NaCl | 2 | 1.28 | 0.288 |
| | Time | 2 | 9.05 | **<0.001** |
| | NaCl * Time | 4 | 0.51 | 0.732 |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar Na | NaCl | 2 | 68.58 | **<0.001** |
| | Time | 2 | 2.35 | 0.107 |
| | NaCl * Time | 4 | 1.80 | 0.146 |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar Cl | NaCl | 2 | 123.40 | **<0.001** |
| | Time | 2 | 13.68 | **<0.001** |
| | NaCl * Time | 4 | 6.08 | **0.031** |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar K | NaCl | 2 | 13.60 | **<0.001** |
| | Time | 2 | 3.26 | **0.036** |
| | NaCl * Time | 4 | 0.49 | 0.746 |
| | Error | 45 | | |
| | Total | 53 | | |
| Foliar Na:K | NaCl | 2 | 68.21 | **<0.001** |
| | Time | 2 | 1.10 | 0.343 |
| | NaCl * Time | 4 | 0.93 | 0.453 |
| | Error | 45 | | |
| | Total | 53 | | |

## Appendix 4

(ANOVA tables for Chapter 5)

Table a4.1 One-way ANOVA tables for trembling aspen necrotic leaf tissue.

| Trembling aspen necrotic leaf parameter | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Foliar N | Treatment | 4 | 4.66 | **0.003** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar K | Treatment | 4 | 23.51 | **<0.001** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar Ca | Treatment | 4 | 14.21 | **<0.001** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar Mg | Treatment | 4 | 17.78 | **<0.001** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar P | Treatment | 4 | 5.10 | **0.001** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar Na | Treatment | 4 | 7.04 | **<0.001** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar Cl | Treatment | 4 | 67.63 | **<0.001** |
| | Error | 55 | | |
| | Total | 59 | | |
| Foliar Fe | Treatment | 4 | 1.54 | 0.147 |
| | Error | 55 | | |
| | Total | 59 | | |

Table a4.2 Two-way ANOVA tables showing the effects of NaCl treatment in year one and year two on measured parameters for trembling aspen.

| Trembling Aspen Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Total DW | NaCl Year One | 1 | 13.31 | **<0.001** |
| | NaCl Year Two | 2 | 46.49 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 12.01 | **<0.001** |
| | Error | 30 | | |
| | Total | 35 | | |
| Total Chlorophyll | NaCl Year One | 1 | 1.04 | 0.317 |
| | NaCl Year Two | 2 | 15.87 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 1.36 | 0.271 |
| | Error | 30 | | |
| | Total | 35 | | |
| Net Photosynthesis | NaCl Year One | 1 | 6.18 | **0.009** |
| | NaCl Year Two | 2 | 13.65 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 1.27 | 0.297 |
| | Error | 30 | | |
| | Total | 35 | | |
| Transpiration | NaCl Year One | 1 | 3.05 | 0.076 |
| | NaCl Year Two | 2 | 5.93 | **0.007** |
| | NaCl Year One *Year Two | 2 | 2.34 | 0.113 |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Na | NaCl Year One | 1 | 21.90 | **<0.001** |
| | NaCl Year Two | 2 | 3.14 | **0.005** |
| | NaCl Year One *Year Two | 2 | 2.22 | 0.127 |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Cl | NaCl Year One | 1 | 1.38 | 0.250 |
| | NaCl Year Two | 2 | 53.31 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 3.10 | 0.061 |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar K | NaCl Year One | 1 | 0.01 | 0.935 |
| | NaCl Year Two | 2 | 36.23 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 3.47 | 0.120 |
| | Error | 30 | | |
| | Total | 35 | | |

| | | | | |
|---|---|---|---|---|
| Foliar Na:K | NaCl Year One | 1 | 28.67 | **<0.001** |
| | NaCl Year Two | 2 | 9.30 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 11.85 | **<0.001** |
| | Error | 30 | | |
| | Total | 35 | | |

Table a4.3 Two-way ANOVA tables showing the effects of NaCl treatment in year one and year two on measured parameters for tamarack.

| Tamarack Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Total DW | NaCl Year One | 1 | 24.86 | **<0.001** |
| | NaCl Year Two | 2 | 6.16 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 1.39 | 0.266 |
| | Error | 30 | | |
| | Total | 35 | | |
| Total Chlorophyll | NaCl Year One | 1 | 3.43 | 0.074 |
| | NaCl Year Two | 2 | 0.78 | 0.466 |
| | NaCl Year One *Year Two | 2 | 1.83 | 0.178 |
| | Error | 30 | | |
| | Total | 35 | | |
| Net Photosynthesis | NaCl Year One | 1 | 0.95 | 0.338 |
| | NaCl Year Two | 2 | 21.21 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 2.53 | 0.096 |
| | Error | 30 | | |
| | Total | 35 | | |
| Transpiration | NaCl Year One | 1 | 0.52 | 0.477 |
| | NaCl Year Two | 2 | 3.46 | **0.044** |
| | NaCl Year One *Year Two | 2 | 6.75 | **0.004** |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Na | NaCl Year One | 1 | 8.05 | **<0.001** |
| | NaCl Year Two | 2 | 20.67 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 5.33 | **0.009** |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Cl | NaCl Year One | 1 | 47.13 | **<0.001** |
| | NaCl Year Two | 2 | 74.65 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 13.08 | **<0.001** |
| | Error | 30 | | |
| | Total | 35 | | |

| | | | | |
|---|---|---|---|---|
| Foliar K | NaCl Year One | 1 | 1.57 | 0.220 |
| | NaCl Year Two | 2 | 62.49 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 1.38 | 0.267 |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Na:K | NaCl Year One | 1 | 5.68 | **0.024** |
| | NaCl Year Two | 2 | 15.61 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 3.10 | 0.060 |
| | Error | 30 | | |
| | Total | 35 | | |

Table a4.4 Two-way ANOVA tables showing the effects of NaCl treatment in year one and year two on measured parameters for white spruce.

| White Spruce Parameters | Source of Variation | DF | F | P |
|---|---|---|---|---|
| Total DW | NaCl Year One | 1 | 7.64 | **<0.001** |
| | NaCl Year Two | 2 | 0.11 | 0.895 |
| | NaCl Year One *Year Two | 2 | 0.51 | 0.607 |
| | Error | 30 | | |
| | Total | 35 | | |
| Total Chlorophyll | NaCl Year One | 1 | 2.07 | 0.161 |
| | NaCl Year Two | 2 | 2.24 | 0.124 |
| | NaCl Year One *Year Two | 2 | 0.46 | 0.637 |
| | Error | 30 | | |
| | Total | 35 | | |
| Net Photosynthesis | NaCl Year One | 1 | 0.01 | 0.959 |
| | NaCl Year Two | 2 | 2.64 | 0.088 |
| | NaCl Year One *Year Two | 2 | 2.83 | 0.075 |
| | Error | 30 | | |
| | Total | 35 | | |
| Transpiration | NaCl Year One | 1 | 0.38 | 0.543 |
| | NaCl Year Two | 2 | 1.74 | 0.192 |
| | NaCl Year One *Year Two | 2 | 1.94 | 0.161 |
| | Error | 30 | | |
| | Total | 35 | | |

| | | | | |
|---|---|---|---|---|
| Foliar Na | NaCl Year One | 1 | 10.96 | **<0.001** |
| | NaCl Year Two | 2 | 9.35 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 0.92 | 0.412 |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Cl | NaCl Year One | 1 | 48.20 | **<0.001** |
| | NaCl Year Two | 2 | 11.72 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 4.83 | **0.009** |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar K | NaCl Year One | 1 | 1.14 | 0.294 |
| | NaCl Year Two | 2 | 0.20 | 0.821 |
| | NaCl Year One *Year Two | 2 | 0.33 | 0.719 |
| | Error | 30 | | |
| | Total | 35 | | |
| Foliar Na:K | NaCl Year One | 1 | 9.96 | **0.004** |
| | NaCl Year Two | 2 | 9.35 | **<0.001** |
| | NaCl Year One *Year Two | 2 | 0.92 | 0.412 |
| | Error | 30 | | |
| | Total | 35 | | |

## Appendix 5: Linking cell physiology to whole plant responses to NaCl stress in glycophytes.

### A5.1 Abstract

Responses of glycophytic plants to NaCl stress have been well documented. They include rapid decreases in root water flux, transpiration, photosynthesis, and growth, and increased concentrations of compatible solutes, root:shoot ratios, and non-photochemical quenching. Prolonged periods of stress decreases chlorophyll concentration but increases necrosis and senescence of older leaves. Once acclimated, plants exhibit a more conservative growth form characterized by increased lateral root formation. The secondary cell signaling responses to NaCl stress also show a similar pattern and includes elevated cytosolic $Ca^{+2}$, the enzymatic production of reactive oxygen species and NO, the induction of autophagy, and the proliferation of peroxisomes. The responses on the cellular level lead to fundamental changes in the cell's behavior, which ultimately leads to physiological changes to the whole plant. The purpose of this review is to link physiological responses at the whole plant level to changes in the cellular level. The cell signaling processes in the root tips, root cortex, guard cells, and chlorotic leaves are well studied whereas signaling processes in the mesophyll cells, vascular cambium, and root epidermis are mostly unexplored. By fully understanding the cellular responses in different cells, it may be possible to better understand the coordination of plant responses between the various tissues and unravel complex NaCl tolerance mechanisms.

### A5.2 Traditional Approaches to NaCl Stress Tolerance vs. Contemporary Views

Plants are well known to have rapid and deliberate responses to biotic and abiotic stimuli. For example, numerous reports exist on rapid thigmonastic movement of carnivorous plants and some flowers in response to touch (Braam, 2005). In response to biological threats such as insect attack, pathogens, or necrotrophic fungi; plants release volatile compounds through the air or electrical signals through mycorrhizal root networks to warn neighboring plants of pending danger (Johnson and Gilbert, 2015). Interestingly, it has been reported that leaves produce defense chemicals in response to the auditory stimulus of chewing (Appel and Cocroft, 2014). These examples have led some scientists to suggest that plants have a level of intelligence or minimal cognition. Charles Darwin first proposed the concept of plant intelligence and postulated that root tips act like the brain of a primitive animal to direct growth and decision making, perhaps to dictate the various plant tropisms observed (Darwin, 1897). In a review by Calvo Garzón and Keijzer (2011), the authors revisited this concept and discussed the current evidence and controversy on plant intelligence, adaptive behavior, and neurobiology. The authors reviewed empirical studies showing that auxin is transported in vesicles to stimulate long distance electrical signals which propagate within the vascular tissue, causing adaptive behaviors in plants. Congruent with Darwin's hypothesis, they propose that plants exhibit a minimal cognition with decision making at the roots as well as learning through trial and error which is controlled by electrical signals initiated at the root tip. The majority of glycophyte plants respond to NaCl stress in a similar manor. The emerging evidence of long-distance electrical signals linked to adaptive behavior in plants is now being considered as a mechanism to elicit this response. The purpose of this review is to link physiological responses at the whole plant level to changes in the cellular level. I used established literature reviews and empirical studies to propose that the physiological response of glycophyte plants to NaCl stress is elicited by long distance electrical signals which trigger cell signalling cascades to alter the cell physiology and ultimately whole plant physiology.

Studies investigating the effects of NaCl stress on plants are prevalent and common themes have emerged which are highlighted within review articles. In general, elevated NaCl causes rapid decreases in root water flux, transpiration, photosynthesis, and growth but increased compatible solute concentration, root:shoot ratio, and non-photochemical quenching (NPQ).

Prolonged periods of stress cause decreased chlorophyll concentration, but increased necrosis and senescence of older leaves. Once acclimated, plants exhibit a more conservative growth form characterized by increased lateral root formation. Previous reviews have focused on the cellular responses to NaCl stress only in a limited capacity; however, they have noted that stress causes an immediate decrease in cell elongation, rapid changes in gene expression, the upregulation of cytosolic $Ca^{+2}$, reactive oxygen species (ROS), ABA, and long distance signaling from the roots to the leaves in order to stunt growth (Volkmar et al. 1998; Munns, 2002; Parida and Das, 2005; Munns and Tester, 2008; Julkowska and Testerinc, 2015; Parihar et al. 2015). Despite evidence to support the concept of plant adaptive behavior, previous reviews do not directly consider the possibility that the physiological response to NaCl stress may be a deliberate protective mechanism governed by changes on the cellular level.

The application of NaCl to the roots of glycophytes causes a rapid decrease in root water uptake and elevated suberin deposition. The overall decrease in water uptake is marked by lowered symplastic and transcellular water transport but increased apoplastic water flow through root cortex cells. The overall decrease in root water uptake may be caused by osmotic shock or decreased water transport through aquaporins. Root water uptake is known to partially or totally recover in some species after several days of NaCl stress. During the recovery period, root cortex cells undergo osmotic adjustment and see an enhancement of cell-to-cell water transport through aquaporins. The recovery is often associated with increased suberin deposition in the epidermis and endodermis, presumably to lower the amount of $Na^{+}$ that enters the transpiration stream (Aroca et al. 2011). Suberin is a hydrophobic polymer that forms in epidermal and endodermal cells in response to environmental stimuli. It acts as a physical barrier between the plant and environment to keep toxins out and prevent desiccation and oxygen loss from roots. The amount of suberin deposition is highly species dependent and appears to regulate the apoplastic transport of water and solutes (Franke & Schreiber, 2007). Decreased root water uptake and the deposition of suberin to root tissue are common responses of glycophyte plants to NaCl stress; however, the nature of the cellular signals that govern the changes in root water uptake remains unresolved.

Plant growth is rapidly stunted in response to NaCl application and is a result of decreased water uptake. This is primarily attributed to lower water potential compared to freshwater and forces the plant to obtain water against an unfavorable osmotic gradient. Plants respond by

lowering transpiration and upregulating compatible solutes in foliar tissue (Volkmar et al. 1998; Munns, 2002; Parida and Das, 2005; Munns and Tester, 2008; Parihar et al. 2015). Compatible solutes are low molecular weight, highly soluble compounds that are non-toxic at high concentrations but are not often used in normal metabolism. They are well known to accumulate in plant tissue under many stress conditions, including NaCl stress, and play several beneficial roles. Traditionally, they were thought to help maintain osmotic balance and cell turgor under periods of stress. However, more recent evidence highlights additional benefits under stress such as free radical scavenging, protection of biomolecules from oxidative stress, and potentially acting as storage compounds to be used upon relief from stress. Proline and glycine are examples of compatible solutes that are found in elevated levels in plant tissue under stress (Hare et al. 1998; Chen and Murata, 2002). Interestingly, the exogenous application of these compounds has been shown to enhance NaCl tolerance in some cases (Ashraf and Fooland, 2007). Thus, the application of NaCl to glycophytes is well known to cause an immediate stunting of growth associated with stomatal closure followed by the upregulation of compatible solutes. The cell signaling pathways behind stomatal closure have been well studied. In contrast, little is known about the pathways involving compatible solute accumulation (Szabados and Savoure, 2010).

Decreases in photosynthesis and chlorophyll concentration as well as increased leaf necrosis are commonly reported for NaCl stress in glycophytes. The decrease in photosynthesis is believed to be caused by decreased water potential, reduced $CO_2$ supply from lowered stomatal conductance, rapid changes in gene expression and enzyme activity associated with photosynthesis, and Na toxicity around the chloroplasts (Parida and Das, 2005; Munns and Tester, 2008; Chaves et al. 2009; Parihar et al. 2015). A clear mechanism for the decrease in chlorophyll has not been elucidated, but some studies have noted a concomitant deterioration of the thylakoid membrane and an upregulation of the chlorophyll degrading enzyme, chlorophyllase (Svitsev, et al. 1973; Reddy, 1986; Santos, 2004; Parida and Das, 2005; Parihar et al. 2015). Prolonged periods of NaCl stress causes Na toxicity to develop in foliar tissue and is characterized by an initial yellowing at the leaf margin. This is followed by the development of necrotic tissue where the entire leaf will eventually die and fall off the plant. It is believed that as Na ions enter the transpiration stream, they are stored in the cell walls and vacuoles of leaf cells to avoid accumulation within the cytoplasm. Cells will die once the storage capacity of the cell walls and vacuoles is reached. Typically, older leaves fall off first, acting as sacrificial leaves to

protect younger tissue from Na toxicity (Volkmar et al. 1998; Munns, 2002; Parida and Das, 2005; Munns and Tester, 2008; Parihar et al. 2015). Decreases in photosynthesis and chlorophyll concentration with increased leaf necrosis appear to be universal responses to elevated NaCl in glycophytes. However, the cellular mechanisms for these processes are not fully considered in current literature reviews.

Increased non-photochemical quenching (NPQ) in foliar tissue is a commonly reported response to NaCl stress and is used by plants to dissipate excess light energy as heat. This form of photoprotection is used to shield foliar tissue from photodamage and occurs under conditions of excess light and other environmental stresses, such as NaCl stress (Müller et al. 2001). Considering that plants typically exhibit a lower photosynthetic rate under NaCl stress, they must dissipate excess light energy to avoid photodamage. Two major, yet often overlooked, mechanisms of NPQ are cyclic electron flow and photorespiration. Cyclic electron flow is a process where electrons in the light reactions are recycled through PS I which produces no NADPH or $O_2$ but does produce ATP (Munekage et al. 2004). Photorespiration occurs when RuBisCO uses $O_2$ as a substrate instead of $CO_2$. This causes a major shift in the cell's metabolism and consumes NADPH and ATP but produces $H_2O_2$ and $NH_3$, thus making it a wasteful metabolic process, but also an effective electron sink when Calvin cycle activity is lowered (Wingler et al. 2000). There is evidence that both cyclic electron flow and photorespiration increase under NaCl stress; however, their physiological importance for photoprotection under stress is not always considered from a wholistic prospective. These processes are discussed in more detail below.

A major component of NPQ is energy dependent quenching (qE) when a light-absorbing buffer is generated on the thylakoid membrane. This phenomenon is associated with an initial lowering lumen pH followed by the activation of the xanthophyll cycle as well as a conformational change of the thylakoid membrane (Müller et al. 2001). Interestingly, the initial decrease in lumen pH has been linked to elevated cyclic electron flow (Munekage et al. 2002). Cyclic electron flow is an integral portion of normal photosynthetic function in order to generate ATP without hydrolysis and the subsequent accumulation of NADPH (Munekage et al. 2004). It also plays a critical role in photoprotection under stress by stabilizing the light reactions and dissipating excess energy as heat when Calvin cycle activity is reduced (Munekage et al. 2002;

Munekage et al. 2004; Huang et al. 2012). This process is well known to increase in response to NaCl stress within cyanobacteria (Joset et al. 1996; Sudhir and Murthy, 2004); however, reports for this change in plants are very limited (Yang et al, 2006). Cyclic electron flow may be an integral component to increased qE, NPQ, and photoprotection in response to NaCl stress; however, a clear link has yet to be elucidated in plants.

Photorespiration is known to increase because of environmental stress due to lower [$CO_2$] relative to [$O_2$] in foliar tissue. Under these conditions, RuBisCO favorably fixes $O_2$ instead of $CO_2$ (Kangasjärvi et al. 2012). Photorespiration is traditionally seen as a wasteful process under most conditions because it produces less photosynthate compared to traditional photosynthesis and leads to N loss in the form of ammonia gas from foliar tissue (Lea et al. 1990; Osmond et al. 1997; Betti et al. 2016). Bypassing the photorespiratory pathway by genetic modification has been an area of significant research in agriculture (Betti et al. 2016). It has been estimated that a 5% reduction of photorespiration in US soy and wheat production would decrease losses by approximately $500 million per year (Walker et al. 2016). However, bypassing the photorespiratory pathway has not led positive results because it makes plants more sensitive to environmental stress (Betti et al. 2016). Therefore, photorespiration appears to have a positive role under stress, primarily by dissipating excess light energy to protect the photosynthetic reaction centers (Osmond et al. 1997; Wingler et al. 2000; Silva et al. 2015). The number of reports on increased photorespiration in response to NaCl stress is limited; however, its protective role under NaCl stress has been noted (Di Martino et al. 1999; Hoshida et al. 2000; Chaves et al. 2009). Thus, photorespiration may be a key component for photoprotection during NaCl stress but is generally overlooked as a component of the NaCl response in plants.

In a recent review by Gilroy et al. (2016), the authors discuss the emerging concept of a systemic acquired acclimation (SAA) in response to abiotic stress, including NaCl stress. They state that in response to abiotic stress, plants generate a self-propagating wave of $Ca^{2+}$ and ROS which induces a rapid and systemic electrical signal in the vascular tissue. The systemic signal is associated with many physiological effects that are commonly observed in other studies such as decreases in photosynthesis, quantum yield of PSII, and $CO_2$ assimilation but increased NPQ. The above review showcases empirical evidence for the propagation electrical signals to elicit a physiological response to NaCl stress. This is congruent with the concepts of plant adaptive

behavior and neurobiology discussed previously (Calvo Garzón and Keijzer 2011). However, the aforementioned studies do not consider secondary cell signalling cascades. This concept should be included in the discussion of this emerging topic because an overwhelming amount of evidence suggests that physiological changes are often associated with changes at the subcellular level. Interestingly, patterns on the subcellular level exist which suggest that a plant's physiological response to NaCl stress is governed by cell signaling cascades.

### A5.3 Secondary Cell Signaling and Intracellular Responses to NaCl Stress

It has been established that environmental stress triggers a general signal transduction pathway within plant cells. One of the first steps of the signaling cascade is the activation of a phospholipase enzyme to cleave phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into inositol triphosphate ($IP_3$) and diacylglycerol (DAG). The two products trigger $Ca^{2+}$ release and the activation of protein kinase C respectively. The signaling cascade also includes the activation of $Ca^{2+}$ dependent protein kinases, increased ABA synthesis as well as the enzymatic production of ROS and NO (Xiong et al. 2002; Arasimowicz and Floryszak-Wieczorek, 2007; Nakamura, 2014; Julkowska and Testerinc, 2015). The secondary cell messengers commonly upregulated within the cytosol in response to NaCl stress are $Ca^{2+}$, ROS, and NO. Each compound exhibits specific roles which alter the cell's behavior. For example, elevated cytosolic $Ca^{2+}$ can cause increased calmodulin dependent protein kinase activity as well as the enzymatic production of ROS. This leads to ion homeostasis via cytoplasmic extrusion as well as enhanced cellular protection and damage repair (Xiong et al. 2002, Julkowska and Testerinc, 2015). Intracellular ROS and NO are rapidly and enzymatically produced in response to NaCl stress. They have a dual role within the cell as they induce conformational changes to proteins and transcription or cause oxidative damage to all biomolecules. Interestingly, many salt tolerant varieties of plants exhibit an increased antioxidant capacity compared to sensitive varieties, perhaps as a mechanism to reduce oxidative damage within the cell (Neill et al. 2002; Arasimowicz and Floryszak-Wieczorek, 2007; Sharma et al. 2012; Julkowska and Testerinc, 2015). The signaling cascade alters the cell's behavior to allow plants to withstand periods of NaCl stress. This includes the induction of autophagy, proliferation of peroxisomes, and changes in organelle activity.

Autophagy is a cellular process where cytosolic components are transported to a double membrane autophagosome and then transported to the vacuole or lysosome for degradation, recycling, and remobilization of nutrients. The primary purpose of autophagy is to degrade oxidized proteins; thus, the process may play a housekeeping role under stress. Autophagy is induced rapidly and systemically in response to NaCl stress and is essential for survival as autophagy deficient mutants are hypersensitive to NaCl stress (Liu et al. 2009; Liu and Bassham, 2012; Michaeli et al. 2016). Interestingly, NaCl stress-induced autophagy in *Arabidopsis thaliana* causes AtPIP2;7 aquaporin to be relocated from the plasma membrane to the vacuole for degradation, thus decreasing the cell surface quantity of AtPIP2;7 and water permeability of the cell (Hachez et al. 2014). Programed cell death (PCD) is also initiated by the plant in response to most environmental stressors (Danon et al. 2000; Petrov et al. 2015). Early stage PCD is characterized by the loss of cell membrane asymmetry and the translocation of phosphatidylserine proteins to the cell outer membrane whereas late stage PCD is characterized by DNA degradation (Danon et al. 2000). Interestingly, the early stages of PCD can be chemically reversed in plant cells (O'Brien et al. 1998). It should be noted that autophagy and PCD share many similarities and a clear distinction between the two processes have yet to be made in plants (Liu and Bassham, 2012; Michaeli et al. 2016). Both autophagy and PCD are common and essential components to a plant's survival of environmental stress.

The proliferation of intracellular peroxisomes is another common response to plant cells experiencing environmental stress. Under stress conditions, peroxisomes generate ROS and NO enzymatically but reduce the activity of peroxisomal antioxidant enzymes. The subsequent increase in ROS and NO have profound impacts on the cell physiology by altering the behavior of proteins and transcription factors. A large amount of $H_2O_2$ is produced non-enzymatically as a by-product of photorespiration which has been shown to alter gene expression under stress conditions. Furthermore, peroxisomal by-products of photorespiration are subsequently utilized by the mitochondria and chloroplasts. Specifically, peroxisomes produce glycine for the mitochondria as well as glycerate and glutamate for the chloroplasts. (Del Rio et al. 1996; Del Rio et al. 1998; Corpas et al. 2001; Del Rio et al. 2003; Del Rio and López-Huertas, 2016). Interestingly, Leshem et al. (2007) demonstrated that NaCl induced the proliferation of endosomes which produce ROS enzymatically. It seems possible that the proliferation of peroxisomes may be initiated by endocytosis. Interestingly, intracellular vesical trafficking and

endocytosis are important components of normal cellular function as well as stress signaling cascades (Levine, 2002; Sorkin and von Zastrow, 2002; Piddini and Vincent, 2003; Julkowska and Testerinc, 2015; Del Rio and López-Huertas, 2016). The proliferation of peroxisomes appears to create an oxidizing environment within the cell to elicit conformational changes of proteins and subsequent cellular responses under stress conditions.

The aforementioned signaling cascade leads to major changes on the subcellular level in order to withstand periods of NaCl stress. For example, rapid changes in gene expression is a common response of many plants to NaCl stress (Kreps et al. 2002; Maathuis et al. 2003; Sreenivasulu et al. 2004; Yao et al. 2011; Bazakos et al. 2012). Secondly, the chloroplasts exhibit a drastic decrease in PSII efficiency and carbon fixation but increased NPQ (Parihar et al. 2015; Sukhov, 2016). Thirdly, the central vacuole aids in autophagy and in the sequestration of $Na^+$ but releases $K^+$ into the cytosol (Zhu, 2003; Michaeli et al. 2016).

Changes to the mitochondria and respiratory processes are more complex but can be strongly linked to NaCl tolerance in many plant tissues in a variety of contexts. Carbon fixation is limited under NaCl stress and causes a transition from growth to maintenance respiration by the plant. Theoretically, mitochondrial activity must become more efficient to survive periods of NaCl stress. However, studies on respiration have produced contradictory results where a roughly equal number of reports show increases, decreases, or no changes in respiration rates within various tissue types. Interestingly, approximately 60% of salt-sensitive species exhibited an increase in respiration from NaCl stress (Jacoby et al. 2011). An increase in the mitochondrial alternative oxidase pathway may be a common response to NaCl stress. This process decouples the linear relationship between electron transport and ATP production to dissipate energy as well as control levels of ROS and NO (Vanlerberghe, 2013). The perception of NaCl stress causes changes to gene expression as well as the activity within the chloroplasts, central vacuoles, and mitochondria. These changes significantly alter the cell physiology and thus, the physiology of the plant (See Figure a5.1).

### A5.4 Cell Physiology Response and Summary

The concept of a systemic acquired acclimation (SAA) and plant adaptive behavior must be revisited but should include changes at the subcellular level. It is hypothesized that NaCl stress elicits an elaborate stress perception and response in plants, which ultimately affects its physiology, growth, and survival. The perception of NaCl stress likely begins at the root tips. Under normal conditions the root tip is in a reduced state but after the application of NaCl, it becomes rapidly oxidized by the enzymatic production of intracellular ROS within endosomes. A self-propagating electrical wave is produced within the vascular tissue, particularly phloem, by elevated $Ca^{+2}$ and ROS which then travels throughout the plant (Leshem et al. 2007; Choi et al. 2014; Jiang et al. 2016). It appears plausible that the electrical signal then triggers secondary cell signaling cascades to alter cellular behavior, and ultimately the whole plant physiology. Within the root cortex cells, a rapid decrease in aquaporin-mediated water transport and root water flux is often observed. Treatment of root cortex cells with NaCl causes the dephosphorylation and internalization of aquaporins and decreased aquaporin gene expression (Boursiac et al. 2005; Prak et al. 2008; Horie et al. 2011). The cellular mechanism of suberin deposition from NaCl stress is relatively unexplored; however, it is known that it is dependent on the enzymatic production and degradation of ROS to cross-link polymers and stiffen cell walls (Tenhaken, 2015). Xylem growth under NaCl stress is another relatively unexplored area of research. It has been shown that NaCl stress results in vessels to become smaller, more numerous, and with thicker cell walls. This phenomenon may occur to reduce the risk of cavitation under limited water supply (Junghans et al. 2006; Escalante-Pérez et al. 2009; Janz et al. 2012). Within the mesophyll cells, NaCl stress induces the degradation of the photosynthetic oxygen evolving complex and decreases in PSII efficiency, electron transport through the light reactions but increased NPQ (Parihar et al. 2015). The observed changes in mesophyll cells are known to be induced by electrical signals produced by the plant (Sukhov, 2016). In guard cells, NaCl stress is well known to induce stomatal closure which causes decreased transpiration. Stomatal closure is preceded by increased xylem pH, which causes the activation and accumulation of ABA around guard cells, which then triggers a signaling cascade (Wilkinson, 2002; Pei et al. 2000). Long-term NaCl stress is known to cause a yellowing at the leaf margin followed by senescence. This is an active process involving the degradation of thylakoid and stromal proteins within the

chloroplasts presumably to recycle nutrients, particularly N. The yellowing process is associated with ion toxicity and is independent of autophagy (Wang and Blumwald 2014). It appears plausible that the overall downregulation of metabolism and growth associated with NaCl stress is a deliberate action by the plant as a survival mechanism (See Figure a5.2).

The physiological response of glycophytic plants has been well studied from the perspective of the whole plant. A common trend of rapid decreases in root water flux, transpiration, photosynthesis, and growth, but increased compatible solute concentration, root:shoot ratio, and NPQ is often observed. This review represents an attempt to bridge the gap between whole plant and cellular physiology to identify gaps in our knowledge. For example, it appears that plants produce long distance electrical signals to initiate a secondary cell signaling cascade which elicits a physiological response at the cellular level. The response differs depending on the cell type. However, the general trend appears to be a decrease in water transport and carbon fixation as well as changes in gene expression which leads to an overall downregulation of metabolism and a more conservative growth pattern. Interestingly, the signaling cascade appears to be similar in many cell types and is characterized by increased cytoplasmic $Ca^{+2}$, ROS, and NO. The induction of autophagy plays an important role in the stress response, perhaps to recycle damaged proteins. Increased peroxisome proliferation appears to be important for the cell signaling cascade as they are known to be the primary intracellular source of ROS and NO. It should be noted that secondary cell signaling processes at the root tips, root cortex, guard cells, and yellowing leaf are well studied whereas signaling processes at the mesophyll cells, vascular cambium, and root epidermis are mostly unexplored. By fully understanding the cellular response of plants for each cell type, it may be possible to improve plant performance under NaCl stress.

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## A5.6 Figures

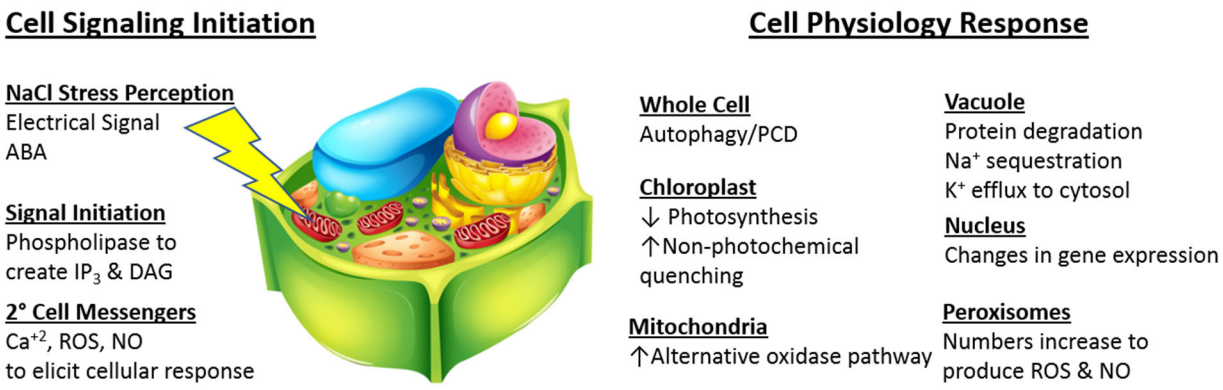


Figure a5.1. Proposed single cell response to NaCl stress. After the perception of stress, a cell signaling cascade is induced and leads to changes in cellular activity. These changes will ultimately alter the behavior of the cell and thus, the physiology of the plant. (Image modified from: https://www.freepik.com/free-vector/different-biology-cells-white-background_1169191.htm.)

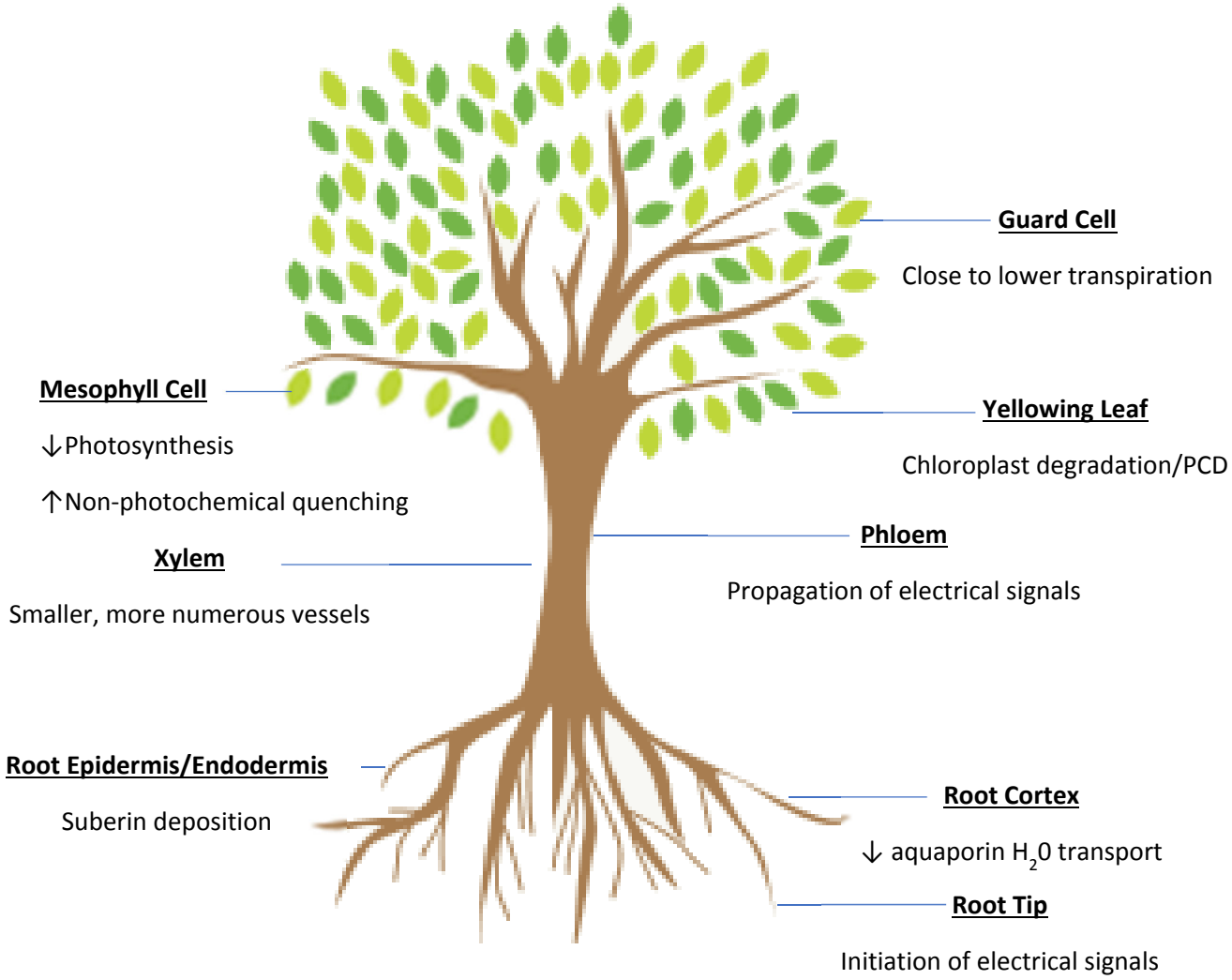


Figure a5.2. Proposed whole plant response to NaCl stress at different cell types. It is hypothesized that the changes on the cellular level are induced by a signaling cascade to induce physiological changes and tolerance in plants. (Image modified from: https://www.vecteezy.com/free-vector/tree.)

**Appendix 6**

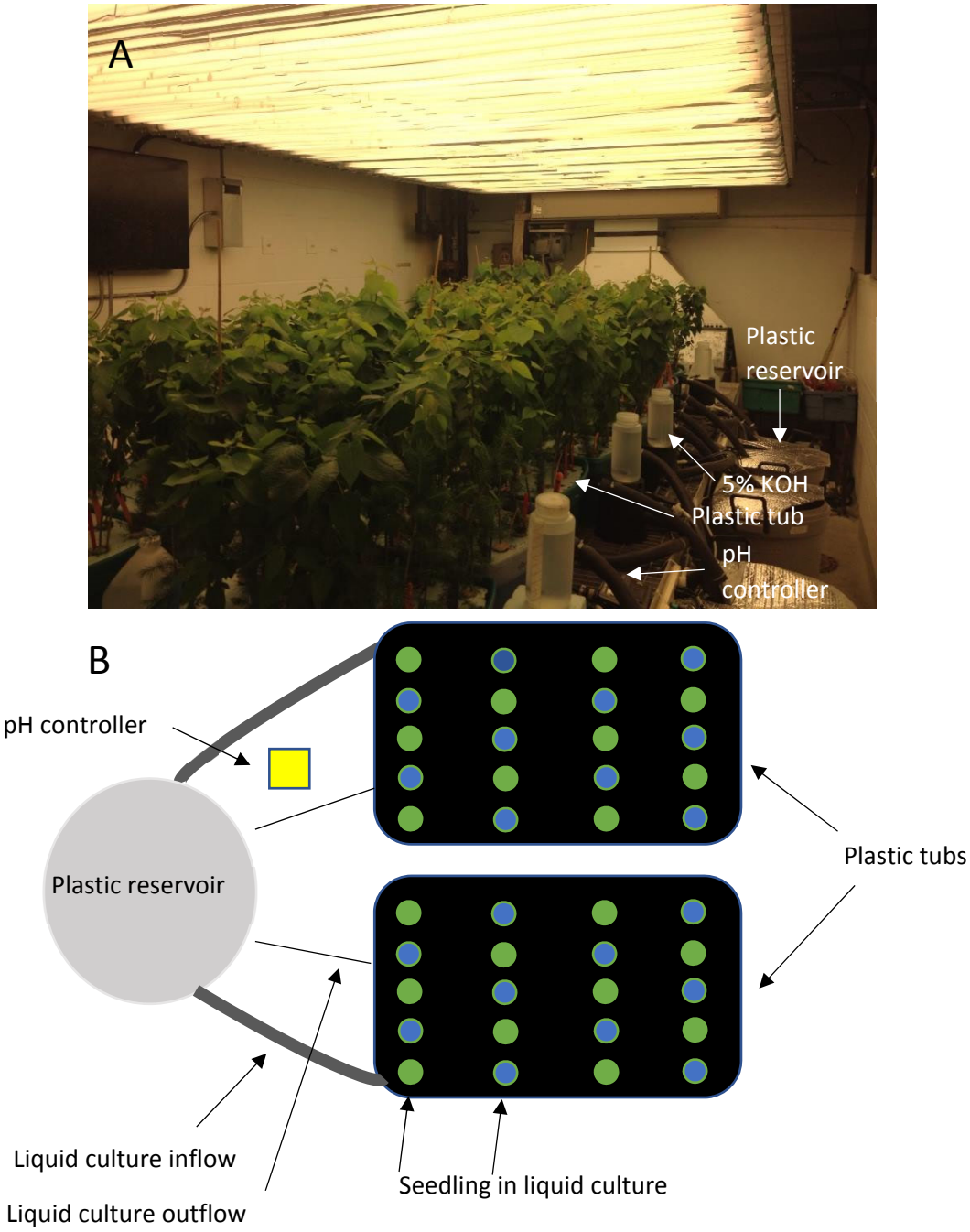


Figure a6.1 Photograph (A) and overhead schematic (B) of liquid culture experimental setup for Chapters 2 & 3. Green and blue circles represent individual seedlings of different species. The first experiment of chapter three and the experiment in chapter four was performed on trembling aspen and white spruce whereas the second experiment of chapter three was performed on trembling aspen and green alder.