Effects of nickel toxicity on expression of genes associated with nickel resistance in white spruce (*Picea glauca*): Nickel translocation in plant tissues.

By

Meagan Boyd

Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science (MSc) in Biology

Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

© Meagan Boyd, 2020
Title of Thesis
Titre de la thèse
Effects of nickel toxicity on expression of genes associated with nickel resistance in white spruce (Picea glauca): Nickel translocation in plant tissues.

Name of Candidate
Nom du candidat
Boyd, Meagan

Degree
Diplôme
Master of Science

Department/Program
Département/Programme
Biology

Date of Defence
Date de la soutenance
June 25, 2020

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. Kabwe Nkongolo
(Supervisor/Directeur(trice) de thèse)

Dr. Abdelwahab Omri
(Committee member/Membre du comité)

Dr. Frank Mallory
(Committee member/Membre du comité)

Dr. Jong-Hwa Kim
(External Examiner/Examinateur externe)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
Monsieur David Lesbarrères
Dean, Faculty of Graduate Studies
Doyen, Faculté des études supérieures

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

I, Meagan Boyd, hereby grant to Laurentian University and/or its agents the non-exclusive license to archive and make accessible my thesis, dissertation, or project report in whole or in part in all forms of media, now or for the duration of my copyright ownership. I retain all other ownership rights to the copyright of the thesis, dissertation or project report. I also reserve the right to use in future works (such as articles or books) all or part of this thesis, dissertation, or project report. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that this copy is being made available in this form by the authority of the copyright owner solely for the purpose of private study and research and may not be copied or reproduced except as permitted by the copyright laws without written authority from the copyright owner.
Abstract

The main objectives of this study were to determine a) nickel accumulation and translocation within tissues and b) nickel effects on gene expression in white spruce (*Picea glauca*). The results of the study revealed that even at the highest dose of 1,600 mg of Ni/kg of soil, there was no physical evidence of toxicity to *P. glauca* seedlings screened. *P. glauca* was found to be a nickel avoider, as the bioaccumulation factor as well as translocation factors for roots to aerial tissues were less than 1.0. Expression of SAT, GR, ACC, NAS, Nramp, and *AT2G16800* genes in roots and needles were investigated. Expression of ACC and NRAMP were upregulated in the presence of nickel, whereas GR was downregulated at the lowest dose (150 mg/kg) and upregulated at the highest dose (1,600 mg/kg). There were significant differences between ACC expression in roots and needles. The results of the present study also show that that potassium nitrate (a common plant fertilizer) does have an effect on gene expression and can lead to toxicity in *P. glauca* plants at high concentrations. Overall, the findings of this research suggest that the low level of bioavailable nickel in mining sites in Northern Ontario and other mining regions can trigger changes in gene expression.

**Key Words:** White spruce (*Picea glauca*); Nickel toxicity; Avoider; Gene expression; RT-qPCR
Acknowledgements

I would firstly like to thank my supervisor, Dr. Kabwe Nkongolo for the opportunity to work in his lab and providing me with guidance, knowledge, and tools to successfully complete my research. I would also like to thank him for always encouraging me and helping me throughout my degree, it is very much appreciated. I would like also to thank my supervision committee members, Dr. Frank Mallory and Dr. Abdelwahab Omri for their advices.

I would also like to lend a big thank you to Dr. Paul Michael for all of his help within the lab, he was always there to answer any questions and provide knowledge to my project.

Next, I would like to thank my labmate Karolina Czajka for all her assistance and training with lab procedures as well as data analysis. I enjoyed working next to her in the lab, and appreciative for her advice and knowledge that was shared during the process. I would also like to thank the Northern School of Medicine (NOSM) for allowing me to use equipment in their lab, it was much appreciated. As well as, Ramya Narendrula for her guidance and help with data analysis. I am thankful for everyone’s contribution in helping to make this an easier and enjoyable couple of years!

Finally, I would like to thank my family for the words of encouragement and support.
Table of Contents

Abstract.......................................................................................................................... ii

Acknowledgements....................................................................................................... iii

Table of Contents.......................................................................................................... iv

List of Figures.................................................................................................................. vi

List of Tables................................................................................................................... viii

Chapter 1: Literature Review.......................................................................................... 1

1.1 Metal Translocation in plants.................................................................................... 1
1.2 Metal Toxicity in plants............................................................................................ 5
1.3 Nickel Toxicity in plants.......................................................................................... 11
1.4 Gene expression associated with metal contamination............................................ 13
1.5 Species of Interest: Spruce...................................................................................... 17
1.6 Objectives and Rationale......................................................................................... 19

Chapter 2: Nickel uptake and translocation in *Picea glauca*............................................. 20

2.1 Introduction ............................................................................................................. 20
2.2 Materials and Methods............................................................................................ 21
   2.2.1 Metal Analysis.................................................................................................... 21
2.3 Results ...................................................................................................................... 23
2.4 Discussion ............................................................................................................... 27
Chapter 3: Expression of genes associated with nickel resistance induced by different doses of nickel and potassium nitrate in white spruce (Picea glauca) .................................................. 29

3.1 Introduction .............................................................................................................. 29

3.2 Materials and Methods .......................................................................................... 32

3.2.1 Picea glauca treatments with nickel nitrate and potassium nitrate .................... 32

3.2.2 RNA extraction and RT-qPCR ........................................................................... 33

3.2.3 Data analysis ....................................................................................................... 35

3.3 Results .................................................................................................................... 36

3.4 Discussion .............................................................................................................. 48

Chapter 4: General conclusions ..................................................................................... 52

Future studies .............................................................................................................. 54

Literature Cited ............................................................................................................ 55

Appendices ................................................................................................................... 79
List of Figures

Figure 1. Image of white spruce (*Picea glauca*) before and after treatment of highest dose of nickel (1,600 mg/kg)..................................................................................................................24

Figure 2. Metal analysis of soil and *P. glauca* tissues .........................................................25

Figure 3. *ACC* gene expression of *P. glauca* treated with different doses of nickel nitrate and potassium nitrate..................................................................................................................40

Figure 4. *ACC* gene expression of *P. glauca* comparing roots and needles at different doses of nickel nitrate and potassium nitrate..................................................................................................................41

Figure 5. *GR* gene expression of *P. glauca* treated with different doses of nickel nitrate and potassium nitrate..................................................................................................................42

Figure 6. *GR* gene expression of *P. glauca* comparing roots and needles at different doses of nickel nitrate and potassium nitrate..................................................................................................................43

Figure 7. *AT2G16800* gene expression of *P. glauca* treated with different doses of nickel nitrate and potassium nitrate..................................................................................................................44

Figure 8. *AT2G16800* gene expression of *P. glauca* comparing roots and needles at different doses of nickel nitrate and potassium nitrate..................................................................................................................45

Figure 9. *NRAMP* gene expression of *P. glauca* treated with different doses of nickel nitrate and potassium nitrate..................................................................................................................46
Figure 10. NRAMP gene expression of *P. glauca* comparing roots and needles at different doses of nickel nitrate and potassium nitrate.
List of Tables

Table 1. Translocation factors from tissues of white spruce (Picea glauca) ........................................26

Table 2. Candidate genes involved in nickel resistance .................................................................38

Table 3. Sequence of P. glauca primers for RT-qPCR ...............................................................39
Chapter 1: Literature Review

1.1. Metal translocation in plants

Plants uptake water and nutrients to grow and remain viable. To do this, they use their vascular tissues consisting of xylem and phloem which extend throughout the plant (Savage et al. 2016). Phloem and Xylem span from the base of the roots and spreads up into the stem and other aerial tissues (Savage et al. 2016). This well-developed system allows for sap transport, however, in the case of contaminated environments, this can become a problem (Thakur et al. 2016). Heavy metals can lead to phytotoxicity; therefore, plants need to develop strategies to cope with excess amount of these elements (Thakur et al. 2016). There are many factors that may affect the uptake of metal ions, including, root size, metal concentrations, temperature, and nutrient availability (Thakur et al. 2016). Plants are able to uptake metals and store them in their tissues. There are two pathways that metal ions can enter into roots - apoplastic and symplastic (Thakur et al. 2016; Tester and Leigh 2001). The apoplastic pathway occurs between cell walls using passive diffusion, whereas, the symplastic pathway occurs between cytoplasm and vacuoles and uses osmosis (Tester and Leigh 2001). The ions can transport through the apoplastic pathway and reach the endodermis, where it can then either cross into the stele or continue to the symplastic pathways (Tester and Leigh 2001). Ions may also access the symplastic pathway by root hairs, epidermal cells, and through the cortex (Tester and Leigh 2001). Once the ions reach the stele of the plant, they can enter the xylem and travel to the shoot (Saxena and Misra 2010; Tester and Leigh 2001). Within the xylem, metal transport is facilitated by membrane transport proteins (Tester and Leigh 2001). The ions can either move with a concentration gradient or through cation channels (Thakur et al. 2016).
Metal availability in soils can be affected by a number of factors including, the pH of the soil, organic matter, water content, water holding capacity, and the relative surface area of the soil (Harter 1983, Yi et al. 2007, Sharma and Raju 2013, Marques et al. 2009). The absorption of metals in the soil is high when the pH is intermediate (Vellaichamy and Palanivelu 2011).

Plants used different strategies to deal with soil metal contamination. Avoidance of metals by plants are ways that plants avoid the metals in the soil. They do this by using strategies that allow them to prohibit the entry of metals in their cells. Sometimes, the distribution of metals within soils surrounding plants is heterogeneous (Mehes et al. 2013; Narendrula-Kotha et al. 2019). This allows plants to extend their roots into the soil and explore other soil horizons that are less or not at all contaminated by metals. Sometimes, plants are able to prevent the uptake of bioavailable metals in their roots. They do this by actually immobilizing metal ions by secreting root exudates in their rhizospheres. Root exudates are chemical attractants or repellents to the soil immediately surround plant roots. Plants may also use mycorrhizal fungi to prevent metals from accumulating in plant roots by extending hyphae outside the rooting zone of the plants and transferring necessary elements for the plant’s survival. This extension can be tens meters of length outside the rooting zone to reach appropriate soil. Mycorrhizal fungi provide resistance by alternation metal speciation, prohibiting metal entry in roots, precipitating metals due to secretion of ligands (oxalic acid), and also binding metals to their cell walls. These fungi can chelate metal ions which have entered the cell. They do this by using organic acids, phytochelatins, polyphosphates, and then transporting them into their vacuoles (Mehes et al. 2013; Mehes-Smith et al. 2015; Kalubi et al. 2015; Narendrula-Kotha et al. 2019).
Plant tolerance is a strategy that allows plants to detoxify metal ions that have already entered cells and have already crossed the plasma membrane/bio membranes of their internal organelles. Plants that are tolerant can be grouped into three groups that include metal excluders, indicators and accumulators (Mehes et al. 2013).

In excluders plants, metals enter root cells but the movement of metals from root to aerial parts/tissues is limited. Excluders maintain low metal concentrations in their aerial tissues and can still grow in metal contaminated soil. Indicators are plants that will take up/accumulate metals in their shoot/above ground tissues but the concentrations of metals in their aerial tissues are the same as in the soil. Metal concentration in above tissue is similar to that in the soil. In accumulators/Hyperaccumulators, metals enter the roots and are translocated to the aerial tissues of the plants. The concentration/levels of metals are higher in their harvestable tissues compared to soil around the roots (Mehes et al. 2013; Mehes-Smith et al., 2015; Kalubi et al. 2015).

Hyperaccumulator plants have the ability to accumulate high concentrations of heavy metals in their tissues (Brooks et al. 1977). They are used to restore areas that have been contaminated and experience high levels of heavy metals (Brooks et al. 1977; Rascio and Navari-Izzo 2011). They can use strategies such as, phytovolatilization, phytoextraction, phytostabilization, and rhizofiltration for the remediation of soils (Chaney et al. 1997; Forte and Mutiti 2017; Garbisu and Alkorta 2001).

Metal translocation varies with plant species which have specific transporters (Colangelo and Guerinot 2006). For example, Fe and Zn ions are regulated by families of transporters including Zinc regulated transporters (ZRT) and Iron regulated transporters (IRT) (Colangelo and Guerinot 2006). Heavy metal ATPase such as HMA2 is an enzyme often activated by Zn
and Cd ions; however, 34% to 52% of the time, it can also be activated by other divalent metals such as Pb2+, Ni2+, and Cu2+ (Eren and Arguello 2004). HMA2 transports metals from the cytoplasm out of the cell which helps maintain low metal concentrations and obtain homeostasis within the plant (Eren and Arguello 2004). They have been found to help in metal tolerance when responding to Zn2+, Cd2+, and Pb2+ (Eren and Arguello 2004; Rensing et al. 1999). Another transporter that has been found to regulate metal sequestration into vacuoles, known as cation exchangers (Peng and Gong 2014). The expression of cation exchanger 4 (CAX4) is increased in the primary root apex, lateral root primordium, apex, and primary root elongation zone, when there is an increase of Ni2+ or Mn2+, as well as, when there is a decrease of Ca2+ (Mei et al. 2009; Peng and Gong 2014). Ni2+ is absorbed and often transported from roots to shoots; however, Cataldo et al. (1978) have found that Cu2+, Zn2+, and Fe2+, may become absorbed at the same carrier site. This is due to Ni transport being inhibited with the presence of Cu, Zn, and Fe (Cataldo et al. 1978). More recently, we have seen that Ni in excess amounts will compete with Fe2+ and Zn2+ which prevents these cations from being absorbed by the plant, thus causing deficiency symptoms (Emamverdian et al. 2015). Depending on how Ni is distributed in the roots, it can easily move from the roots to the shoots through phloem and xylem (Emamvredian et al. 2015). However, not all plants will translocate the metal into aerial tissues -- some plants are metal excluders as they store metals in their roots and show little toxicity effects (Ishtiaq and Mahmood 2011; Wang et al. 2020).
1.2. Metal toxicity in plants

Metal toxicity is a problem around the globe; it affects the environment, agriculture, as well as, the health of both humans and animals (Elzwayie et al. 2017; Shen et al. 2020; Singh and Agrawal 2018). The contamination of soils from metals can be caused by several scenarios -- including, certain fertilizers, biosolid applications, material released from smelters, as well as, industrial waste (Yadav 2010). Due to metal toxicity, plants may undergo oxidative stress and thus, experience damage to their cells (Baccouch et al. 2001). The accumulation of metal ions may also cause problems in the homeostasis of cells, providing challenges for cells to function properly and remain viable (Yadav 2010). When a plant is first exposed to high levels of metal ions, reactive oxygen species are produced (Jozefczak et al. 2012; Okamoto et al. 2001). Plants are stationary and cannot avoid unfavourable metal concentrations; however, they can detoxify metals from their tissues (Hall 2002). Such detoxification includes chelation, immobilization, exclusion, and compartmentalization (Hall 2002; Lytle et al. 1998). These processes help protect plants from metals so that the high levels of harmful metals cannot interfere with photosynthesis or respiration (Kupper and Andreson 2016).

The sensitivity of plants depends on several aspects when taking into consideration certain metals; including, how they uptake and accumulate the metals, the efflux of metals from the cytoplasm, the complexation of heavy metals, osmolytes and osmoprotectant accumulation, induction of antioxidative enzymes, as well as, the change in plant metabolism that controls metabolic pathways and repairs of damaged cells (John et al. 2012).

Cadmium (Cd) is a highly toxic to plants and animals (Martelli et al. 2006; Prasad 1995). The use of sewage cake, city waste, and certain fertilizers are responsible for the higher concentrations of Cd in soils (John et al. 2012). Such toxicity may occur due to non-functional
binding to biological ligands that are supposed to be bound to other divalent metals (Moulis 2010). Cd can replace magnesium as the central ion, in which degradation of the pigment occurs, and thus negatively affects photosynthesis (Cakmak and Yazici 2010; Moulis 2010). This creates a problem because plants can no longer create energy for sufficient growth (Cakmak and Yazici 2010; Sun and Payne 1999). Plants exposed to Cd have demonstrated a decrease in growth, chlorophyll content, and carotenoids (John et al. 2012). High levels of Cd results in nutrient deficiencies, oxidative stress, plant growth retardation, and reduced development in crops (Gupta et al. 2017; Nazar et al. 2012; Shafiq et al. 2019). Many transporters within the cells may facilitate Cd uptake into the roots which will then distribute the compound further in the plants (Shenker et al. 2001). This may also initiate the competition for binding sites, which can reduce the uptake of essential ions, such as, copper, iron, magnesium, manganese, and zinc (Kupper and Andreson 2016). This uptake into the root system may result in a deficiency or change of the regulated zinc homeostasis within the plant cells (Sinclair and Kramer 2012). Other issues that Cd brings to an organism includes DNA damage (Hartwig 2018; Lin et al. 2007a). When Cd directly interacts with nucleotides or with the inhibition of DNA repairing enzymes, it may induce damage to the DNA of an organism (Lin et al. 2007b; Tan et al. 2019). Studies show that there is an increase in point mutations at a very low Cd concentration (Ince et al. 1999). Although other metals may show similar mutations, such as copper and nickel, it has been less likely to see DNA damage at low concentrations (Kupper and Andreson 2016). When there are enough nutrients in the soil, these minerals will compete with Cd for transporters within the cells thus reducing the heavy metal accumulation in the plant (Nazar et al. 2012).

Zinc (Zn), an essential micronutrient for plants, is another metal that poses a problem on plants at a high concentration (Yadav 2010). Although much less toxic than other metals, such as
Cd, Zn levels that when too high may lead to deficiencies of other micronutrients such as manganese and copper (Takkar and Walker 1993; Mattiello et al. 2015; Guo et al. 2016). Areas with high concentrations of Zn will cause toxicity in plants which limits their growth (Cheng et al. 2019; Samreen et al. 2017). When the concentration of Zn exceeds the normal concentration that plants require for their nutrient uptake, phytotoxicity usually occurs (Samreen et al. 2017). Zn toxicity occurs due to a replacement of weakly bound divalent metals; often the magnesium is substituted within the chlorophyll (Guo et al. 2016). The change in magnesium levels leads to an inhibition of photosynthesis; thus, the magnesium substitution results in the denaturation of important pigment-protein complexes (Guo et al. 2016; Kupper and Andreson 2016). Therefore, the inhibition of photosynthesis is responsible for chlorosis, which is found to be a visible result of Zn toxicity (Carruthers 2016; Jain et al. 2010). High levels in Zn can lead also to inhibition of metabolic functions in plant (Das et al. 2018; Yadav 2010). The toxic levels of Zn provide not only a decrease in the growth of both the root and the shoot but also causes leaf chlorosis (Barman et al. 2018; Fontes and Cox 1998).

Mercury (Hg) is one of the most toxic metals to any organism and poses serious problems in many areas around the world (Kupper and Andreson 2016). For example, contaminated mining areas found in China result in a health risk to humans, as these areas are used for rice production (Wang et al. 2018). Methylmercury may accumulate in these rice seeds, which acts as a neurotoxin and can cause damage and death to an organism (Wang et al. 2018). Very rarely can a plant reduce Hg; it is found in some phytoplankton species, but this process is unknown (Kupper and Andreson 2016). Naturally, traces of Hg can be found in oceans and soils, where the majority of Hg bioaccumulation is in food webs (Wang et al. 2018). One of the largest sources for methylmercury exposure to humans, is through seafood (Wang et al. 2018). Hg
toxicity is mainly due to the similarity of chemical composition to that of zinc, in which it can substitute in its active sites (Kupper and Andreson 2016; Vieira et al. 2017). Under Hg stress, there is a decrease in chlorophyll content, which ultimately affects plant growth (Ahammed et al. 2018; Pirzadah et al. 2018). High levels of Hg are phytotoxic and can cause physical damages, as well as, physiological damage which slows the water flow in plants (Zhou et al. 2007). High levels of Hg will also cause problems around the mitochondria which activate oxidative stress by the production of reactive oxygen species (Yadav 2010). In most cases, the accumulation of Hg is lower in the shoots compared to roots (Ahammed et al. 2018).

Copper (Cu), another micronutrient required in plants, is an important component of ATP synthesis and carbon dioxide assimilation (Yadav 2010). Compounds of Cu have been used as a pesticide, where it may runoff in agricultural fields and small creeks and Cu in soils is often attributed to mining (Kupper and Andreson 2016). The concentrations that do runoff in areas nearby are damaging to sensitive aquatic plants in a short amount of time, which can ultimately kill the plants (Kupper and Andreson 2016). The toxicity of such metal-containing pesticides may start at very low concentrations (Thomas et al. 2013). Not only will high amounts of Cu result in plant growth inhibition, but it will also affect important cellular processes needed for the healthy survival of the plant (Cota-Ruiz et al. 2018; Yruela 2005). Copper is involved in many important processes in plants including photosynthetic electron transport, mitochondrial respiration, responses to oxidative stress, regulation of cell wall metabolism, as well as the regulation of hormones (Thomas et al. 2013; Yruela 2005). Therefore, it is imperative that copper be available; when the ions are not readily available, the plant will fall into a Cu deficiency which affects leaves and reproductive organs (De Storme and Geelen 2014). A redox cycling occurs between Cu$^{2+}$ and Cu$^{+}$ which can produce toxic radicals that may damage DNA,
lipids, proteins, and other molecules (Yruela 2005). In freshwater, the increase of copper is often due to human activities such as industrial and agricultural practices; these concentrations of Cu become lethal to many plants, including cyanobacteria (Kupper and Andreson 2016; Wu et al. 2017). It has been reported that there is an increase in reactive oxygen species production in response to Cu toxicity, as well as oxidative stress which interferes with some metabolic pathways, resulting in damage to cells and macromolecules (Hegedus et al. 2001). Cu levels may disturb the reactive oxygen species production which can halt the important process of photosynthesis (Miller et al. 2010; Sanchez-Viveros et al. 2010). Roots accumulate much more Cu compared to the shoots, this is attributed to the direct contact with the Cu in the soil that can result in plant biomass reduction (Guo et al. 2010). Other signs of Cu toxicity in plants include a change in root structure, and reduced root hair proliferation (Sheldon and Menzies 2005). The reduction in root tips or organelles within root cells, suggests that there is stress within plant, resulting in a decrease in the production of energy, biomass, and root growth (Lequeux et al. 2010; Sheng et al. 2012).

Another essential nutrient that aids in plant growth, is iron (Fe) (de Oliveira Jucoski et al. 2013). Fe, often noted for its role in plants, may become toxic (de Oliveira Jucoski et al. 2013). This happens when a large amount of Fe III is reduced to Fe II; producing a highly soluble compound that is available to plants (Kupper and Andreson 2016). This occurs naturally when soils that are rich in Fe become flooded and anoxic (Kupper and Andreson 2016). It also occurs in freshwater, wetlands, and salt marshes, where Fe becomes toxic to plants in these ecosystem (Kupper and Andreson 2016). Fe toxicity may also inhibit photosynthesis in plants, which can be a result of oxidative damage and stomatal limitations (Pereira et al. 2013; Pinto et al. 2016). Fe stress has been shown to decrease the activity of photosystem II, which affects plant
photosynthetic capabilities (Nenova 2006; Suh et al. 2002). Oxidation in the rhizosphere can help plants defend themselves against the accumulation of Fe II by creating the insoluble form of Fe (Fe III) (Bartlett 1961). Therefore, plants that can change iron from the ferrous form to the ferric form (Bartlett 1961).

Lead (Pb) is one of the most abundant heavy metals that are toxic in the soils, and this can be attributed to many human activities (Yadav 2010). Soils with high levels of Pb shows significantly lower plant production and growth (Sharma and Dubey 2005). For the most part, plants will accumulate this heavy metal through their roots, and less through their shoots (Sharma and Dubey 2005). These high concentrations that come into the plant lead to hormonal problems which affect the permeability and structure of plant membranes (Sharma and Dubey 2005). Physical changes include deprived growth, chlorosis, and blackening of the root system (Ashraf et al. 2017; Sharma and Dubey 2005).

Although metals often show toxicity to plants, some positive effects have been found when dealing with very low concentrations as reported by Lin et al. (2007). Cd at low concentrations resulted in successful plant growth and had minimum oxidative stress (Lin et al. 2007). This can be due to the hermetic effect, where there is an overcompensation response of plants, thus, creating favourable effects (Calabrese and Blain 2009; Lin et al. 2007). This is not always the case; for example, there is no beneficial effect found in any plant species growing in Hg-contaminated soils (Kupper and Andreson 2016).
1.3. Nickel Toxicity in Plants

Nickel (Ni) is an essential nutrient that plays an important role in plant growth and development (Brown et al. 1987). It is also involved in many redox reactions which provides the proper function to the cells (Sharma and Dietz 2009). But high levels of Ni can cause problems such as, interference in cell functioning, and a change in the metabolism; that may result in cell damage or plant death (Baccouch et al. 1998; Sharma and Dietz 2009). Brown et al. (1989) reported that naturally occurring high levels of Ni found in some fertilizers, and sewage sludge induce nickel toxicity in crops and higher plants. In general, Ni, as well as other heavy metals, cause toxicity in several ways: they displace essential components in the biomolecules, they block the functional group of molecules, and they modify enzymes, proteins, the plasma membrane, and membrane transporters (Yusuf et al. 2011). Enzyme interactions that are interrupted by heavy metals can be due to Ni interaction with SH-groups in proteins, and thus creating a change in its conformation. The enzyme becomes inactivated which has been seen to decrease plant growth significantly (Seregin and Kozhevnikova 2005). However, it is possible that Ni may not only inhibit enzyme activity but also stimulate it. In fact, Seregin and Kozhevnikova (2005) reported an increase in some antioxidant enzyme activity when the level of Ni is high. This is also consistent with Gajewska et al. (2006), who observed a significant increase in peroxidases (POD) and Glutathione S-transferases (GST) activities. On the other hand, activities of enzymes such as superoxide dismutase have been shown to decrease over time when plants are treated with high Ni concentrations, and in some cases chloramphenicol acetyltransferase activities can be completely inhibited (Gajewska et al. 2006). These enzymes play an important role within plants. SOD catalyzes superoxide anion to H$_2$O$_2$ and O$_2$, then CAT
will use H$_2$O$_2$ by converting it to H$_2$O and O$_2$ and POD will reduce it (Gajewska et al. 2006). POD is the key enzyme in reducing GST to glutathione (GSH) (Gajewska et al. 2006).

Physical evidence of Ni toxicity includes chlorosis, necrosis, a decrease in leaf size, and root and shoot growth are reduced or inhibited (Yusuf et al. 2011; Gajewska et al. 2006). Ni levels that become higher than what the plant can tolerate may lead to a physiological and biochemical change in the plant, including, a decrease in leaf chlorophyll content (Yang et al. 1996; Gajewska et al. 2006; Rehman et al. 2016). This decrease in chlorophyll ultimately leads to a decrease in photosynthetic activity (Rehman et al. 2016; Yang et al. 1996). However, it has been found that there is an increase in the chlorophyll a/b ratio, suggesting that chlorophyll b is more sensitive to Ni stress (Gajewska et al. 2006). The decreased amount of chlorophyll and the increase of POD activity results in accelerated senescence (Gajewska et al. 2006). Mohanty et al. (1989) suggests that Ni has an inhibitory site within the electron transport chain. This site is said to be located in photosystem II on the plastoquinone which will create an unfolding of events in which photosynthesis becomes disrupted (Mohanty et al. 1989).

It has also been observed that high levels of nickel will decrease the rate of transpiration, it will accumulate proline and it will fail to effectively supply water to the plant and undergo some osmotic stress (Ain et al. 2016; Gajewska et al. 2006). Proline is a metabolite which is produced under unfavourable conditions, which acts as an osmoprotectant, a membrane stabilizer, and a hunter for reactive oxygen species to help fight off the stress (Szepesi and Szőllősi 2018). Not only did the plants accumulate proline, but the relative water content decreased overtime in individuals that experienced Ni stress (Gajewska et al. 2006). The effects of excess Ni will decrease both the yield of the root and the shoot significantly (Khalid and Tinsley 1980; Yang et al. 2010; Yusuf et al. 2011). Microscopic effects of nickel toxicity include
a change in the root meristem cells and in root cap cells, in which the changes become more pronounced as the concentrations become higher (Liu et al. 1994).

Ni is mostly absorbed through the roots of the plant, due to the direct contact in the soil, in which the metal ions are taken up through passive diffusion or active transport (Seregin and Kozhevnikova 2005). However, studies support the idea that Ni can also be absorbed through plant leaves (Seregin and Kozhevnikova 2005). Ni transport occurs via the apoplast and symplast pathways and it may also be transported through both the xylem and the phloem (Seregin and Kozhevnikova 2005).

Because Ni is only required for the functioning of the urease enzyme in plants, only small amounts are needed; therefore, deficiencies are hard to come by and toxicity is easily attainable (Eskew et al. 1994). Rascio and Navari-Izzo (2011) described traits that determine the uptake of heavy metals which can lead to toxicity. Plants are more susceptible to Ni toxicity if they have a better mechanism to take in metals from the soil, if their transport processes are quick and effective bringing up the metal from the root to the shoot, as well as, depending on their ability to detoxify (Rascio and Navari-Izzo 2011).

1.4. Gene expression associated with metal contamination

Gene expression may occur during the development of an organism, but it may also change due to environmental factors (Jaenisch and Bird 2003; Kim et al. 2015). Certain environmental factors that could affect gene expression include, drought, salinity, and temperature (Kim et al. 2015). However, changes to soil content such as the presence of metals can also affect gene expression (Shabani et al. 2016; Shahabivand et al. 2016). Mechanisms, such as that of epigenetics, may allow the response to environmental factors change the
expression of genes (Jaenisch and Bird 2003; Andrews 2000). There are proteins that are involved in environmental factors that cause stress, including metallothioneins (Andrews 2000; Benhamed et al. 2016). Metallothioneins are small metal-binding proteins which are rich in cysteine and try to protect organisms enduring stressful scenarios (Liu et al. 2016). These small proteins are capable of regulating certain metals and protect against them, as well as protecting possible oxidative stress (Hassinen et al. 2011). Metallothioneins genes are quickly transcribed and increased when under zinc and cadmium stress (Andrews 2000). In the presence of Ni, metallothionein accumulates in plants to reduce stress of the nickel-induced environment (Shabani et al. 2016). Another protein that responds to metal contamination is the ATP binding cassette (ABC); the transporters of these proteins control many transport processes, including, elimination of toxic metals (Shabani et al. 2016). An up-regulation of the ABC gene in plants that were in contact with higher Ni concentrations has been reported (Shabani et al. 2016). The expression of this gene was increased in the presence of Ni in roots and shoots of non-mycorrhizal plants (Shabani et al. 2016). However, in mycorrhizal plants, there were no changes in the expression of the ABC gene in the shoots (Shabani et al. 2016). There was a dramatic decrease in the expression of the ABC in the same study, when the highest concentrations of Ni were used, (Shabani et al. 2016). Therefore, by changing gene expression, mycorrhizal can reduce Ni stress and decrease the Ni transport into the shoots (Shabani et al. 2016).

In regard to gene expression, enzymes may also show responses to heavy metal contamination (Shahabivand et al. 2016). These enzymes can include catalase (CAT), ascorbate peroxidase (APX) and glutathione S-transferase (GST). In the study of Shahabivand et al. (2016), cadmium was investigated, where they found that exposure to cadmium resulted in the transcription of CAT being decreased, whereas, the transcription of APX and GST was up-
regulated. Certain plants such that of, *Funelliformis mosseae* are able to induce the expression of antioxidant genes, which could help in the reduction of cadmium stress (Shahabivand *et al.* 2016; Sharma *et al.* 2016). The steroid hormone, 24-epibrassinolide (EBL) found in plants, improves metal tolerance in *Oryza sativa* by up-regulating genes for antioxidative enzymes (Sharma *et al.* 2016).

Often hyperaccumulators will demonstrate changes in gene expression as they take up an unusual amount of heavy metals (Assuncao *et al.* 2001; Van der Mortel *et al.* 2008). These plants will accumulate the metals in their shoots at levels that are exceptionally higher compared to the usual plant (Assuncao *et al.* 2001). There are differences in species, as well as in populations, in regard to the level of hyperaccumulation within plants (Rascio and Narai-Izzo 2011; Van der Mortel *et al.* 2008). The ability for plants to hyperaccumulate compared to non-hyperaccumulating plants is associated with the expression of genes, rather than a difference in genes (Assuncao *et al.* 2001; Rascio and Narai-Izzo 2011). Depending on the metals that they are hyperaccumulating, the expression of certain genes is targeted (Assuncao *et al.* 2004). Assuncao *et al.* (2001), found that the three Zn transporter genes in *Thlaspi caerulescens* increase in their gene expressions when plants are hyperaccumulating zinc. *T. caerulescens* showed a higher gene expression in roots and shoots (Assuncao *et al.* 2001). The genes of the Zn-regulated transporter (ZIP), including, *ZTN1* and *ZTN2* found in *T. caerulescens* are responsible for the hyperaccumulation and it is Zn regulated (Assuncao *et al.* 2001).

Other metals also demonstrate a difference in gene expression. Van de Mortel *et al.* (2008) found that there is also a change in gene expression when plants are in the presence of Cd. Several genes were responsive to Cd, including, uroporphyrin III (UPM1), a protein that responds to phosphate, a nodulin-like protein, and an MA3 protein (Van der Martel *et al.* 2008).
These genes were studied in *Arabidopsis* and they were under expressed in the presence of Cd (Van der Martel *et al.* 2008). Whereas, 26 genes from *Arabidopsis* were found to be overexpressed due to the heavy metal; including genes that are involved in transport (CAX3, MATE efflux), transcription (*MYB107*), stress responses (peroxidases), as well as, proteins that are related to pathogenesis, and genes that play a role in the cell wall biosynthesis (Van der Martel *et al.* 2008). Genetic responses to heavy metals, like Cd, demonstrate the role that genes play in processes such as the metabolism of lignin, glutathione, and sulfate (Van der Martel *et al.* 2008).

Natural resistance associated macrophage protein (*Nramp*) genes encodes for proteins which acts as a divalent metal ion transporter found in plants (Thomine *et al.* 2000; Theriault 2016). These genes have been often studied in *Arabidopsis*, where their *Nramp* genes play a role in metal sensitivity (Thomine *et al.* 2000). Gene expression of *Nramp* in *Saccharomyces cerevisiae* results in Cd sensitivity within the plants cells (Thomine *et al.* 2000). Levels of the At*Nramp3* mRNA become higher when there is an underwhelming amount of Fe. This overexpression of the gene contributes to Fe transport as well as Cd sensitivity (Thomine *et al.* 2000). *Nramp* also plays a role in metal homeostasis, which is an essential mechanism performed in plants (Mizuno *et al.* 2005). *Nramp* is also important in Ni transport where Ni sensitivity and abundance are increased when there is expression from the *TjNramp4* in *S. cerevisiae* (Mizuno *et al.* 2005). The expression of *TjNramp4* also shows a decrease in yeast growth. Theriault *et al.* (2016), using *Betula papyrifera*, found that *Nramp* is associated with Ni resistance.

Nicotianamine (NA) chelates metals and plays an important role in metal homeostasis; nicotianamine synthase (*NAS*) genes synthesizes NA (Lee *et al.* 2009). An overexpression of these genes has been found to increase metal concentrations in plants; also, the
expression of this gene results in an increase in the amount of NA (Lee et al. 2009; Takahashi et al. 2003). The higher amounts of NA have been found to help plants tolerate high levels of nickel (Douchkov et al. 2005). However, in some cases, these NAS genes use NA as a substrate, thus leading to an increase in iron concentration (Cheng et al. 2007). Genes associated with NAS, including OsNAS genes; OsNAS1 and OsNAS2 show an increase in their expression when there is iron deficiency (Inoue et al. 2003). In rice, OsNAS3 was overexpressed in iron deficient roots, however, the gene was downregulated in the shoots; whereas, in a deficient zinc environment, there was an overexpression of the gene in both the roots and the shoots (Lee et al. 2009). In the study of Lee et al. (2009), they concluded that if NAS expression is increased within rice grain, then there is an increase in the bioavailable mineral content within the plant.

1.5. Species of Interest: Spruce

Spruce trees are classified under the genus *Picea* where there are about 35 species. They are large coniferous evergreen trees that are commonly found in northern forests, and all across Canada (Weng and Jackson 2000). These trees create not only an important ecosystem, but also a microenvironment for many species to thrive in (Bisbee et al. 2001). Their foliage consists of needles that cover the twig. Most species of spruce needles are four-sided where they attach to the twig by a peg-like structure. Spruce trees can often be confused and difficult to distinguish between other coniferous trees, such as pines, and firs (Weng and Jackson 2000). Not only is it difficult to distinguish between different families, but it is also quite challenging to distinguish among the spruce (*Picea*) species (Weng and Jackson 2000). However, it is possible to distinguish the spruce species based on needle and cone morphology (Weng and Jackson 2000). For example, *Picea mariana* have needles that contain two resin ducts extending from the base to
the tip; whereas, *Picea engelmannii* have needles that consist of a number of short, intermittent resin ducts or sacs (Weng and Jackson 2000). Other ways species may be identified other than DNA barcoding, is to use plant macrofossils and species may be characterized due to the different ecological adaptations of each species (Weng and Jackson 2000). Also, according to Webb *et al.* (1987), spruce trees are often important in quaternary pollen and macrofossil assemblages, and therefore, helps gain knowledge on previous types of vegetation as well as, previous climates in the area. It is important to note that not all spruce species grow in the same environment; for example; *Picea mariana* often grows in nutrient-poor environments; where as *Picea glauca* often grows in areas that are nutrient-rich and well-drained soils (Shugart *et al.* 1992). These species also have evolutionary trends in which they provide distinctive functions in succession, and they have adapted to fires differently (Foster 1985).

There exists a close association between environmental variables and morphology in spruce, including the influence of altitude (Roche 1969). As transpiration and the use of stored water from wood increases, the water potential in the foliage decreases (Schulze *et al.* 1985). This water potential in spruce is due to an unequal efflux of transpiration and influx of soil water during the depletion of the stored water (Schulze *et al.* 1985). Using the trees stored water, allows high rates of transpiration, and CO2 can be taken up when there is low influx from the roots (Schulze *et al.* 1985). The water potential in the leaves may recover when transpiration is decreased when the stomata is closed; however, the xylem water flux is high (Schulze *et al.* 1985). This water potential gradient is important for refilling the water storage when transpiration is lowered in the later hours of the day (Schulze *et al.* 1985). For the present study, white spruce (*Picea glauca*) is being investigated for gene expression and metal analysis because
it is one of the spruce species along with black spruce growing in metal contaminated areas in Northern Ontario

1.6. Objectives and Rationale

A number of studies have been conducted recently on metal resistance mechanisms in hardwood species such as birch (*Betula papyrifera*), maple (*Acer rubrum* and *A. saccharinum*), and poplar (*Populus tremuloides*). Investigations on metal resistance in conifers are lacking. This group of tree species represents more than 70% of planted trees in the City of Greater Sudbury. The main objectives of this study are to 1) determine the coping mechanism of Ni contamination in *P. glauca*, 2) investigate main effects of nickel nitrate and potassium nitrate on gene expression.
Chapter 2. Nickel uptake and translocation in *Picea glauca*

2.1 Introduction

Uptake of contaminants by plants and their mechanisms have been studied by several researchers. Plants have developed specific mechanisms to translocate and store micronutrients. These mechanisms are involved in the uptake, translocation and storage of toxic elements (Mehes-Smith et al. 2013a, 2013b). It is very likely that plant uptake-translocation mechanisms are closely regulated. In general, plants do not accumulate contaminants beyond near term metabolic needs (Mehes et al. 2013 a, Kalubi et al. 2015). With the exception of hyperaccumulators which can take up thousands of ppm of toxic metal ions), most plant species need 10 to 15 ppm of trace elements. Recent studies of hard wood species showed that *Betula papyrifera, Quercus rubra, Populus tremuloids* are nickel accumulator while *Acer sacharinum* and *A. rubens* are Ni excluder and avoider, respectively (Theriault et al, 2014; Narendrula et al., 2014; Kalubi et al. 2016). In general, analysis of Ni translocation in conifer is lacking. The objective of this specific study was to determine the mobility of Ni from soil to needles in *P. glauca*. 
2.2 Materials and Methods

2.2.1 Metal Analysis

*P. glauca* seedlings were grown in a climate-controlled greenhouse at College Boreal (Sudbury, Ontario). In total 44 seedlings were transplanted to a sand/soil mixture and grown in a growth chamber. They were watered periodically. After two weeks, half the trees (22) were treated with 50 mLs of water (0 mg/kg), and the other half (22) were treated with 50 mLs of nickel nitrate (1,600 mg/kg). A week after treatment, roots, stems, and needles were harvested separately and placed in a growth chamber to dry. Soil and plant tissues were dried and weighed (approximately 5 grams of sample) and they were separated by treatment. Each treatment was replicated three times. Each treatment and element of the plants were replicated.

Metal analysis was performed at TESMARK laboratories. The total metals in soil, branches and leaf samples were first digested separately in aqua regia. Roughly 0.05 - 0.5 g of the sample was digested with 5 ml of concentrated HNO3 and HCl using a MARS 5 microwave oven. The supernatant was transferred and brought up to 50 ml with deionized water. Metal levels were measured using an Inductively-Coupled Plasma-Optical Emission Spectrometry (ICP-AES), Inductively - Coupled Plasma-Mass Spectrometry (ICP-MS) and Hydride Generation Atomic Emission Spectrometry (HG-AAS).
The bioaccumulation/bioconcentration factor was calculated as the ratio of metal concentration in roots over the metal content in soil. The translocation factor was the ratio of metal concentration in leaves over the metal content in roots.

SPSS 20 was used to analysis metal data. An ANOVA was performed to determine any significance among means of metal content in soil, roots, stems, and needles. Tukey Post Hoc Test was used to determine significance (P<0.05). An independent t-test was also used to compare the means between water and total Ni treatments for each element (soil/stem/needles/roots).
2.3 Results

There was no significant damage caused by Ni in plants two weeks after the treatment with 1,600 mg of Ni/kg of soil (Figure 1). Metal analysis revealed negligible amount of Ni in soil, roots, branches, and needles from control samples treated with water (Figure 2). There were significant differences in Ni levels in samples treated with 160 mg/kg in soil, and tissues compared to water control. For Ni treated samples, there were seven times more Ni in soil compared to roots, branches, and needles. The levels of Ni in roots, branches, and needles were statistically similar.

The bioaccumulation factor (Ni ratio root/soil) is found to be only 0.11. The translocation factors from roots to branches; and roots to needles were also significantly low (Table 1). A detectable movement of Ni from branches to needles was observed but it was found to be not significant.
Figure 1: *Picea glauca* treated with highest dose of Ni (1,600 mg/kg) a) before b) after treatment.
**Figure 2:** Metal analysis of water samples (0 mg/kg) and 1,600 mg/kg of nickel in all elements of the plant; soil, roots, stems, and needles. The lowercase letter represents a significant difference between the plant elements. Significant differences between treatments, for each element of the plant, are represented with an asterix (*)
Table 1: Translocation factors found in *Picea glauca*

<table>
<thead>
<tr>
<th>Translocation pathway</th>
<th>Translocation factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots to stems</td>
<td>0.196</td>
</tr>
<tr>
<td>Stems to needles</td>
<td>3.08</td>
</tr>
<tr>
<td>Roots to needles</td>
<td>0.604</td>
</tr>
</tbody>
</table>
2.4 Discussion

The analysis of plant tissues was important in determining the level of metal uptake and the mobility of elements in *Picea glauca*. This study also aimed at determining how nickel is translocated and accumulated in *P. glauca*. We found that the bioaccumulation factor (BF) was < 1 which suggests that *P. glauca* is not a metal excluder (Mehes et al., 2013a). Translocation factor (TF) can be helpful in investigating the phytoremediation potential (Usman et al. 2019). TF values that are <1 suggest that plants accumulate metals in roots more than shoots or aerial tissues (Usman et al. 2019; Yoon et al. 2006). However, metal analysis in the present study shows that *P. glauca* did not have a high concentration of Ni in their roots, rather a significantly higher concentration in the soil, thus suggesting an avoidance mechanism (Mehes-Smith and Nkongolo 2015).

Translocation factors in our study were <1 from roots to stems because the concentrations in roots were initially low and therefore, not translocated readily throughout the plant. Similar results were reported by Gratton et al. (2000) who showed that Ni concentration in jack pine (*P. banksiana*) needles of trees in the mining region of the Greater Sudbury continues to be elevated (Concentrations ranged from 28.9 to 50.8 mg/kg) compared to control sites. But the levels of metal in soil was much higher compared to needles suggesting that *P. banksiana* species is not a Ni accumulator. Measures of Ni concentrations in other plant parts (roots and branches) were not measured to determine the coping mechanisms in *P. banksiana*.

The metal content found within plant tissues are the main source for determining whether the plant is considered an accumulator or an avoider (Mirecki et al. 2015; Usman et al. 2019). Based
on plant classification described by (Boularbah et al. 2006), (Ernst 2006), and (Mganga et al. 2011), Mehes-Smith et al. (2013) with reference to metal translocation from roots to leaves, the results of the present study reveal that white spruce (P. glauca) is a Nickel avoider. Although the exact avoidance mechanism is unknown, these mechanisms can be complex and may include, immobilization by mycorrhizal fungi, by root exudates, changing the pH of the rhizosphere pH, as well as, restrict the transfer of metals (Dalvi and Bhalerao 2013; Mehes-Smith et al. 2013).

In conclusion, P. glauca does not accumulate Ni in their roots and the translocation of this element to branches and needles is limited. This species is classified as Ni avoider.
Chapter 3: Expression of genes associated with nickel resistance induced by different doses of nickel and potassium nitrate in white spruce (*Picea glauca*)

### 3.1 Introduction

There are several heavy metals that have negative effects on plant growth and development when they are in excess in soil (Mustafa and Komatsu 2016). These metals may include, aluminum, iron, zinc, and cadmium. A major source of the increase of metals in our environment are due to anthropogenic activities (Nrigau and Pacyna 1988; Jozefczak *et al.* 2012). Nickel (Ni), a heavy metal, is an important micronutrient that is essential in all higher plants and was first discovered by Dixon *et al.* (1975). It is an important element in urease, which is responsible for nitrogen metabolism, the hydrolysis reaction of urea in carbon dioxide (CO2) and ammonium (NH4) within the tissues of plants (Dixon *et al.* 1975). For example, researchers have been unable to produce viable barley grains when Ni is limited in soil due to a disruption of the maturation process that occurs after the formation of the embryo (Brown *et al.* 1987). Although Ni is known to be an important element for plants, it can also be toxic in excess amounts (Czajka *et al.* 2018; Djeukam and Nkongolo 2018; Khalid and Tinsley 1980). Ni at high concentrations will compete with cations, including, Fe$^{2+}$ and Zn$^{2+}$, which leads to these molecules not being able to be absorbed by the plant (Callahan *et al.* 2007). Therefore, this mechanism causes a deficiency of Fe and Zn, thus, ultimately resulting in chlorosis of the plant (Callahan *et al.* 2007; Khan and Khan 2010). Ni toxicity may also lead to other problems within the plant, including, seed germination, plant growth, and total biomass (Czajka *et al.* 2018; Ma *et al.* 2011). In fact, Ni toxicity affects plants germination process and seedling growth by disrupting the activity of enzymes and interfering with the hydrolyzation of food storage within
germinating seeds (Khan and Khan 2010; Sethy and Gosh 2013; Zhi et al. 2015). Ni from soil is able to move through the phloem and xylem, thus, can easily translocate these ions from the root to the shoots; therefore, affecting plants by inhibiting the growth of their tissues (Ishtiaq and Mahmood 2012). This growth reduction is due to this movement of Ni throughout the plant, especially in the roots, which results in a decrease of the mitotic cell division (L’Huillier et al. 1996). L’Huillier et al. (1996), found that the haltering of mitotic cell division was probably due to contact with the meristem. The decrease in mitotic activity may also lead to further problems such as, an accumulation of starch in leaves (L’Huillier et al. 1996). In high stress environments, such as Ni toxicity, activity of enzymes including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) are affected (Yan et al. 2008). The increase of SOD is thought to arise because of the need for the organelles to fight the unfavourable conditions (Yan et al. 2008). POD, SOD and CAT, are based on redox metalloenzymes that play a role in cell defense against oxidative stressors, which becomes a good indicator of the condition of the environment (Yan et al. 2008). During high Ni stress, the increase of POD activity builds a physical barrier to halt the incoming of these metals which in turn, helps the metabolic activity defend against nickel toxicity (Yan et al. 2008). Many other genes have been associated with nickel resistance in plants.

The objective of this study is to determine the level of gene expression in root and needle of *P. glauca* treated with nickel nitrate and potassium nitrate. The genes that have been investigated in this study include, serine acetyltransferase (SAT), glutathione reductase (GR), nicotianamine synthase (NAS), Natural resistance-associated macrophage proteins (NRAMP), 1-aminocyclopropane-1-carboxylic acid deaminase (ACC), and, a high affinity Ni transporter
family protein, AT2G16800. It is hypothesized that if these genes play a role in metal resistance, they will either become underexpressed or overexpressed to help cope with the stressor. It is predicted that ACC, GR, and NRAMP will be overexpressed, and AT2G16800 will be underexpressed in response to Ni toxicity.
3.2 Materials and Methods

3.2.1 *Picea glauca* treatments with nickel nitrate and potassium nitrate

Six-month-old seedlings were transplanted into pots containing a 50:50 sand/soil mixture and left to grow for an additional month and a half in a growth chamber. Plants were watered as needed and fertilized twice a week with equal amounts of nitrogen, phosphorus and potassium (20-20-20).

Ni toxicity was assessed by treating seedlings with an aqueous solution of nickel nitrate salt [Ni(NO3)2] at the following concentrations: 150 mg, 800 mg, and 1,600 mg of nickel per 1 kg of dry soil. These doses represent the bioavailable fraction of total nickel available to biota, half total, and total nickel, respectively found in metal-contaminated soils in the GSR. (Nkongolo et al. 2013; Kalubi et al. 2016). These levels correspond to 301.69 μmol, 150.85 μmol, 75.42 μmol, and 56.54 μmol of Ni, respectively. To control for any possible toxic effects due to the increase in nitrate ions (NO3) in the plants, an aqueous solution of commercial potassium nitrate (KNO3) salts was used for controls in equal molar amounts to each dose of the nickel salts. The nitrate controls for 1,600 mg/kg, 800 mg/kg, and 150 mg/kg corresponds to 603.38 μmol, 301.69 μmol, and 113.08 μmol of nitrate respectively. Salt-free water was used as a negative control (0 mg Ni per 1 kg of dry soil). The experimental design was a completely randomized block design with 12 replications per each nickel treatment, 11 for the water control, and 5 per nitrate control.

Roots and needles were harvested from seedlings seven days after treatments, frozen in liquid nitrogen and stored at -20°C.

Before the harvest, pictures were taken to document Ni damage on each plant. All needles were picked off from the branches, and quickly put into its respectively labeled tin foil, which was folded, and dipped into liquid nitrogen, before storage in a 20°C freezer. The roots were pulled
out from the soil containers, rinsed well under water, and then dried well with a paper towel. Each root sample was also placed into an individually wrapped tin foil, dipped into liquid nitrogen, and then stored at -20 °C until RNA extraction.

### 3.2.2 RNA extraction and RT-qPCR

RNA was extracted from the root and needle samples using the protocol previously described by Theriault *et al.* (2016). However, some modifications were made including, using only 0.3g of tissue per sample throughout the extraction process. Also, the chloroform phase separation steps were completed using 1 mL of CTAB solution: 1 mL phenol chloroform. The precipitation of RNA was done using 100 μl of SDS buffer, in which the chloroform steps were properly scaled down. The 53 samples of extracted RNA underwent a quality check using a 1% agarose gel. Using the Qubit® RNA BR assay kit by Life Technologies (Carlsbad, United States), all samples were quantified. Each RNA sample from the same treatment was pooled together using equal amounts with a total of 10 μg of tissue. The pooled RNA samples were then treated with DNase 1 from Life Technologies (#EN0521). For each RNA sample, 1μL DNase, 1 μL buffer, and water were added for a final volume of 10 μL. Each sample of DNase treated RNA was incubated at 37°C for 1 hour. A 3-step PCR was performed with all pooled samples, followed by a 1% agarose gel to confirm that there was no DNA contamination within the samples before inactivating the DNase 1. The pooled samples with no DNA contamination (where bands did not appear on the gel), were used for the gene expression analysis.

Primers were designed based on the *P. glauca* genome, using the BLAST program. Based on previous literature, six genes were chosen that had been associated with nickel resistance in higher plants (SAT, GR, ACC, NAS, Nramp, AT2G16800) (Table 2) (Narendrula-Khota and
Nkongolo, 2019). Also, four housekeeping genes were designed including alpha tubulin, cyclophilin, ELF1, and 18S RNA. Alpha tubulin provided the most consistent and reproducible results therefore, it was used as the housekeeping gene used for the experiment.

Finally, cDNA was produced from the pooled RNA samples to test primers to ensure they would amplify by PCR reaction. This cDNA was generated by directly adding 1 μL of 50 mM EDTA to each pooled sample which was then incubated at 65°C for 10 minutes to denature the strand. Then, 2X Reverse Transcriptase (RT) Master Mix (without RNase inhibitor) mixture composed of 2.0 μL 10X RT Buffer, 0.8 μL 25X dNTP Mix (100 mM), 2.0 μL 10X RT Random Primers, 1.0 μL MultiScribe™ Reverse Transcriptase, and 4.2 μL of Nuclease-free H2O per 10 μL of RNA was made. This mix was added to the thermal cycler set at the following conditions. Step 1 consisted of 10 minutes at 25°C, step 2 continued for 120 minutes at 37°C, and step 3 was 5 minutes at 85°C. The synthesized cDNA was checked using a 1% agarose gel to ensure each sample amplified at the proper size.

Quantitative PCR (qPCR) was performed to measure the expression of each gene for each treatment and each tissue for the species. The qPCR was performed according to the Dynamo HS SYBR Green Kit (Life Technologies) protocol. Mineral oil (10 μL) was added to the top of each sample. Each reaction was completed in triplicates using the MJ Research PTC-200 Chromo 4 Thermal Cycler. The set program consisted in, 1) initial denaturation at 95°C for 10 minutes, 2) denaturation at 95°C for 15 seconds, 3) annealing temperature which depended on the primer (between 55-64°C) for 60 seconds, 4) read, 5) repeat step 2-4 for 41 cycles, 7) final elongation at 72°C for 7 minutes 8) melting curve 72–95°C, every 1°C, hold for 10 seconds, and 9) final elongation at 72°C for 3 minutes. The RT-qPCR was performed two separate times for
each targeted gene, and samples were loaded in triplicates. This resulted in six data points per pooled sample. Outliers among the replicates were excluded from further analysis.

3.2.3 Data Analysis

For gene expression, the CFX Connect was used to analyze the data. The data was exported to Excel where the C(q) values were quantified using the equation for the standard curve and then normalized to the housekeeping gene α-tubulin. SPSS 20 was used to determine statistical significance among means (P<0.05). The Shapiro Wilk test was performed to verify normal distribution of data. Analysis of variance (ANOVA) and Dunnett's T3 as well as Tukey (if variances were equal) Post Hoc Tests were used to determine any significant differences among means for different treatments and controls. The difference between needles and roots was also analyzed using an independent t-test to determine significance (P<0.05) among groups for each gene target and treatment. The Levene’s test was used to determine if means were equal (P>0.05) or unequal (P<0.05), in which a 2-tailed test determined any significance.
3.3 Results

*P. glauca* was classified as nickel resistant as there was no physical evidence of toxicity when comparing the tree before and after treatment. No Ni susceptible genotype was identified since all the samples scored between 1 or 2 on the 1 to 9 scale.

Primer pairs used to amplify the housekeeping and the target genes are listed in Table 3. Based on the PCR amplification data, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC), glutathione reductase (GR), high affinity Ni transporter family protein AT2G16800, and Natural resistance-associated macrophage protein (NRAMP), along with α-tubulin were selected for RT-qPCR and further gene expression analysis.

An increase of ACC expression was observed in needle tissues of samples treated with the 1,600 mg/kg of nickel nitrate (Figure 3a). An increase of ACC expression was also observed in root tissues for all concentrations of nickel nitrate, 150 mg/kg, 800 mg/kg, and 1,600 mg/kg; whereas there was an overexpression of ACC at 800 mg/kg and 1,600 mg/kg of potassium nitrate (Figure 3b). The expression of needles compared to roots was significantly different at 800 mg/kg and 1,600 mg/kg of nickel nitrate (Figure 4a). In regard to potassium nitrate, there was a significant difference between needles and root tissues at 150 mg/kg and 1,600 mg/kg (Figure 4b).

A decrease of GR expression was observed in needle tissue at 150 mg/kg, and an increase of expression was observed in samples treated with 1,600 mg/kg nickel nitrate. As for potassium nitrate, there was an overexpression at 150 mg/kg (Figure 5a). For root tissue samples treated with nickel nitrate, all concentrations increased GR expression, and for samples treated with potassium nitrate there was an increase in expression at 800 mg/kg and 1,600 mg/kg (Figure 5b).
When comparing needles and roots, there was no significant difference between nickel treatments (Figure 6a). However, samples treated with potassium nitrate showed a significant difference at 150 mg/kg between the two tissues (Figure 6b).

There were no changes in AT2G16800 expression for needle samples treated with nickel nitrate or potassium nitrate treatments (Figure 7a). Similar to needles, the root samples did not show any change in AT2G16800 expression when treated with nickel nitrate; however, there was an increase in gene expression at 800 mg/kg and 1,600 mg/kg for samples treated with potassium nitrate (Figure 7b). Needles compared to roots showed no significant difference for nickel nitrate treatments (Figure 8a). There were significant differences observed between needles and roots at 150 mg/kg and 1,600 mg/kg of potassium nitrate (Figure 8b).

An increase in NRAMP expression for needles was observed only for potassium nitrate at a concentration of 150 mg/kg (Figure 9a). As for root samples, there was an overexpression of NRAMP observed at all nickel nitrate concentrations, 150 mg/kg, 800 mg/kg, and 1,600 mg/kg only (Figure 9b). There were no significant differences between nickel nitrate treatments when the two tissues were compared (Figure 10a). Needles compared to roots showed significant difference in samples treated with potassium nitrate at 150 mg/kg and 800 mg/kg (Figure 10b).

No data were generated with SAT or NAS primers because the amplifications were not consistent with the expected amplification and qPCR showed more than one melting point -- therefore these target genes were excluded from the analysis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-aminocyclopropane-1-carboxylic acid deaminase(ACC)</td>
<td><em>Brassica napus</em></td>
<td>Stearns et al. 2005</td>
</tr>
<tr>
<td>High affinity nickel transporter family protein (AT2G16800)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Stearns et al. 2005</td>
</tr>
<tr>
<td>The family of iron-regulated proteins (IREG)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Schaaf et al. 2006</td>
</tr>
<tr>
<td>Glutathione reductase (GR)</td>
<td><em>Thlaspi goesingense</em></td>
<td>Freeman et al. 2004</td>
</tr>
<tr>
<td>Glutathione-s-transferases (GST)</td>
<td><em>Thlaspi goesingense</em></td>
<td>Freeman et al., 2004</td>
</tr>
<tr>
<td>Metal transporter NRAMP (1-4)</td>
<td><em>Thlaspi japonicum</em></td>
<td>Mizuno et al. 2005</td>
</tr>
<tr>
<td></td>
<td><em>Noccaea Caerulescens</em></td>
<td>Visioli et al. 2012</td>
</tr>
<tr>
<td></td>
<td><em>Thlaspi caerulescens</em></td>
<td>Wang et al. 2008</td>
</tr>
<tr>
<td></td>
<td><em>Betula papyrifera</em></td>
<td>Theriault et al. 2016a</td>
</tr>
<tr>
<td>Nicotianamine synthase (NAS3)</td>
<td><em>Noccaea Caerulescens</em></td>
<td>Visioli et al. 2012</td>
</tr>
<tr>
<td></td>
<td><em>Thlaspi goesingense</em></td>
<td>Mari et al. 2006</td>
</tr>
<tr>
<td>Putative transporter protein (TMP)</td>
<td><em>Betula papyrifera</em></td>
<td>Theriault et al. 2016a</td>
</tr>
<tr>
<td>Serine acetyltransferase (SAT)</td>
<td><em>Thlaspi goesingense</em></td>
<td>Freeman et al. 2004</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>Lemaire et al. 2004</td>
</tr>
<tr>
<td></td>
<td><em>Betula papyrifera</em></td>
<td>Theriault et al. 2016b</td>
</tr>
<tr>
<td>TonB-dependent receptor/protein</td>
<td><em>Betula papyrifera</em></td>
<td>Theriault and Nkongolo 2017</td>
</tr>
<tr>
<td>Zinc finger protein (ZAT11)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>van der Zaal et al. 1999</td>
</tr>
</tbody>
</table>
Table 3. Sequences of white spruce (*Picea glauca*) primers used for RT-qPCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Melting temp (°C)</th>
<th>Primer (5’ TO 3’)</th>
<th>Expected amplification</th>
<th>PCR product in cDNA (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAT</td>
<td>F:68 R:69</td>
<td>F: TGGTGGTGAGGAGAGACTGCT R: CAGCCGTCGGTAAAGGGGGC</td>
<td>142</td>
<td>200-142</td>
</tr>
<tr>
<td>GR</td>
<td>F:70 R:70</td>
<td>F: GATCGGAGCTGGAGGATGGCG R: GCCACCAACACCCCCAGCAT</td>
<td>124</td>
<td>124</td>
</tr>
<tr>
<td>NAS</td>
<td>F:69 R:70</td>
<td>F: GACCCCCGTTGCAACTCCGT R: CGCCGCCAGAAACACGACCT</td>
<td>138</td>
<td>200-130</td>
</tr>
<tr>
<td>ACC</td>
<td>F:64 R:68</td>
<td>F: AGTAGCTGCCATTGAGATCTGT R: TGCCTTCCATCTCTTCGC</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>16800</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-tubulin</td>
<td>F:72 R:70</td>
<td>F: GGGCGATGAGGATGAGGGCG R: GCAAGCCCAGTTCGCCAAAACCA</td>
<td>134</td>
<td>134</td>
</tr>
</tbody>
</table>
Figure 3: ACC gene expression in white spruce (*Picea glauca*) treated with different doses of nickel nitrate and potassium nitrate. The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression of all the treatments combined for a) needles b) roots. Bars with different lowercase indices represent significant differences (p ≤ 0.05) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences (p ≤ 0.05) among the means of the nitrate treatments with reference to water. Significant differences (p ≤ 0.05) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk. (*)
Figure 4: ACC gene expression in white spruce (*Picea glauca*). The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression observed for the comparison between needles and roots; a) nickel nitrate treatments b) potassium nitrate treatments. Significant differences (*p ≤ 0.05*) between tissues are represented with an asterisk. (*)
Figure 5: GR gene expression in white spruce (*Picea glauca*) treated with different doses of nickel nitrate and potassium nitrate. The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression of all the treatments combined for a) needles b) roots. Bars with different lowercase indices represent significant differences ($p \leq 0.05$) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences ($p \leq 0.05$) among the means of the nitrate treatments with reference to water. Significant differences ($p \leq 0.05$) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk. (*)
Figure 6: GR gene expression in white spruce (Picea glauca). The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression observed for the comparison between needles and roots; a) nickel nitrate treatments b) potassium nitrate treatments. Significant differences (p ≤ 0.05) between tissues are represented with an asterisk. (*)
Figure 7: AT2G16800 gene expression in white spruce (*Picea glauca*) treated with different doses of nickel nitrate and potassium nitrate. The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression of all the treatments combined for a) needles b) roots. Bars with different lowercase indices represent significant differences (p ≤ 0.05) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences (p ≤ 0.05) among the means of the nitrate treatments with reference to water. Significant differences (p ≤ 0.05) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk. (*)
Figure 8: AT2G16800 gene expression in white spruce (Picea glauca). The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression was observed for the comparison between needles and roots; a) nickel nitrate treatments b) potassium nitrate treatments. Significant differences (p ≤ 0.05) between tissues are represented with an asterisk. (*)
Figure 9: NRAMP gene expression in white spruce (Picea glauca) treated with different doses of nickel nitrate and potassium nitrate. The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression of all the treatments combined for a) needles b) roots. Bars with different lowercase indices represent significant differences (p ≤ 0.05) among the means of the nickel treatments with reference to water. Bars with different uppercase indices represent significant differences (p ≤ 0.05) among the means of the nitrate treatments with reference to water. Significant differences (p ≤ 0.05) between a nickel concentration and its corresponding nitrate control dose are represented with an asterisk. (*)
**Figure 10:** NRAMP gene expression in white spruce (*Picea glauca*). The gene expression was normalized to the housekeeping gene (α-tubulin) and water was used as the negative control. Gene expression was observed for the comparison between needles and roots; a) nickel nitrate treatments b) potassium nitrate treatments. Significant differences (p ≤ 0.05) between tissues are represented with an asterisk. (*)
3.4 Discussion

Nickel toxicity in *Picea glauca* shows that this species is resistant to Ni based on lack of plant damage at all nickel concentrations. This study aimed to determine if genes associated with nickel tolerance are involved in the *P. glauca* response to nickel.

ACC plays an important role in ethylene response where it regulates signals involved in stressors of a plant (Fernandez-Moreno and Stepanova 2019; Matilla-Vazquez and Matilla 2014). Because of this role, ACC confer metal tolerance in plants (Glick et al. 1994). In the present study, ACC becomes overexpressed in roots at low and high doses and in needles at the highest Ni dose used. This may be because roots are exposed to the metals first and therefore, having a greater effect on expression (Shahid et al. 2017). This was also reported in the study of Stearns et al. (2005) where canola plants quickly express ACC in response to nickel stress. Similarly, to our present study, tissues do not noticeably become damaged by Ni (Stearns et al. 2005). Zhang et al. (2019) concluded that an increase in ACC helped regulate ethylene production and thus enhanced the stress tolerance in wheat. The increase of ACC helped minimize toxicity by initiating molecular responses and interfering with intracellular homeostasis (Zhang et al. 2019). Our study demonstrates a significant difference in expression compared to water when treated with nickel as well as the potassium nitrate. However, the nitrate controls only show significant difference from water in roots at 800 mg/kg and 1,600 mg/kg Ni doses. This suggests that nickel has a greater effect on gene expression compared to the controls, however, at concentrations that are above the bioavailable amount (150 mg/kg), nitrates can also induce genetic changes within the roots.

GR helps protect plant cells by controlling the levels of reactive oxygen species present in the plant (Gill et al. 2013). In fact, GR plays an important role in controlling the number of
reactive oxygen species within the plant by maintaining the supply of the reduced form of GR (Couto et al. 2016). Many studies show an overexpression of GR to help cope with stressors; however, our study shows a decrease in GR expression in needles in samples treated with the 150 mg/kg dose, which can increase hydrogen peroxide (H2O2) in shoot tissues (Ding et al. 2016). The accumulation of H2O2 plays an important role in homeostasis, and therefore, at low levels may not require all ROS to become elevated but require some effort to help tolerate the metal (Ding et al. 2016; Mehla et al. 2017). At 800 mg/kg, GR expression was not affected, but then became overexpressed at 1,600 mg/kg. An increase in GR expression is commonly seen in many plant species in metal resistance mechanisms (Mudalkar et al. 2017; Sharma et al. 2016). Studies demonstrate that GR in plants may increase due to Fe-deficiency (Bashir et al. 2007). Excess amount of Ni competes with Fe, and thus creates an Fe-deficiency response; therefore, Fe-deficiency can play a role in the changes in expression demonstrated in our study (Lešková et al. 2017; Merlot 2020). In root tissues, GR expression was increased at all Ni concentrations, however, the expression was also increased at 800 mg/kg and 1,600 mg/kg of potassium nitrate. Therefore, nitrates may induce signals for gene expression. Nitrate has been found to regulate gene expression in Arabidopsis thaliana in which nitrate treatments created a response by changing expression of many genes when studying roots (Alvarez et al. 2019). The correlation between production of ROS and enzymatic activity has been found to play a role in plant defense responses, and nitrate treatments have been found to increase peroxidase activity; thus, ROS and peroxidase activity can increase the expression of GR (MIÝčkovß et al. 2004; Vidal et al. 2018).

The expression of AT2G16800 was not affected by the Ni treatments in roots or in needles. Potassium nitrate also did not induce a change in expression for needles; however, there was an increase in expression at 800 mg/kg and 1,600 mg/kg for roots. AT2G16800 is known to
be a nickel ion transport protein in *Arabidopsis thaliana* (Wang *et al.* 2007). Needles may not have shown any changes in expression as they were not directly affected since Ni was not readily transported to the needles, as translocation and bioaccumulation factors suggested an avoidance mechanism. Unexpectedly, we saw a significant increase in AT2G16800 expression due to potassium nitrates. Other studies have found that too much nitrate can increase the expression of cadmium transporter, OsIRT1, as the ions compete with one another (Yang *et al.* (2016). The same idea has been seen for iron transporters in which Ni is taken up through the Fe transport system, and thus provoking a response in an Fe transporter AtIRT1 (Nishida *et al.* 2011). When plants became Fe-deficient and the level of Ni increased suggesting Ni and Fe compete and can interchange transport systems since Ni is transported via the Fe system (Nishida *et al.* 2011; Tang *et al.* 2019). In the present study, potassium nitrate may have initiated the increase in expression of AT2G16800, where Fe levels may have been in excess compared to Ni and used the Ni transport system. Initially we expected Ni toxicity to significantly reduce the expression of AT2G16800 to reduce the amount of Ni entering the plant cells; however, if Fe is using this transport system, the plant may increase expression as Fe is a micronutrient and not highly toxic for plants (Kobayashi *et al.* 2019).

NRAMP is a known metal ion transporter and helps maintain homeostasis in plants (Campo *et al.* 2013). Theriault *et al.* (2016) demonstrated the role NRAMP plays in Ni resistance in *Betula papyrifera*. In the present study, significant differences were observed only in a low dose of potassium nitrate treatments for needles. Similarly, Czajka (2018) reported an increase of NRAMP in *P. tremuloides* leaf tissues caused by potassium nitrate groups rather than Ni. Significantly overexpression of NRAMP was observed in roots for all concentration levels. An increase in NRAMP expression has been studied in response to Cd stress (Shaheen *et al.* 2018).
NRAMP genes are found to become overexpressed during Fe-deficiency (Thomine and Schroeder 2004). Roots have been found to be more sensitive to Fe-deficiency and thus NRAMP genes in these tissues are more easily disrupted (Ullah et al. 2018).

In all genes that were studied, only ACC provided a significant difference in expression when comparing roots and needles for Ni treatments. Significant differences in the expression of ACC between the tissues may be the result of ACC being synthesized in roots first, then transported to the shoots of the plant (Calvo-Polanco et al. 2017; Else and Jackson 1998). However, our study has found a significant difference between expression of all genes in roots and needles for the potassium nitrate treatments – except for AT2G16800 needle treatments. This expression may result from the interaction between potassium and nitrate. Nitrate can induce toxicity in plants, thus altering the expression of certain genes (Goyal and Huffaker 1984). Alternatively, there are arguments that other than vegetables, nitrate helps the supply of nitrogen for future growing and is not found to have any toxic effects (Li et al. 2013). However, research also suggests plants may favor a nitrogen-reduced form more than another; for example, preference of ammonium rather than nitrate (Li et al. 2013). Horchani et al. (2010) describes how nitrates can alter chlorophyll content, photosynthetic activity, and levels of carbohydrates.
Chapter 4: General Conclusions

The main objectives of this study are to 1) determine the coping mechanism of Ni contamination in *Picea glauca*, 2) investigate main effects of nickel nitrate and potassium nitrate on gene expression. The results of the present investigation revealed that *P. glauca* is a metal avoider because of low bioaccumulation factors. We also determined that there are significant differences when comparing water and nickel nitrate concentration of 1,600 mg/kg in soil and each plant element: stems, roots, and needles. Soil had significantly greater metal concentration compared to the plant tissues, thus, concluding *p. glauca* avoids taking up metal ions that are in excess.

Nickel is a micronutrient needed for plant growth and function. However, Ni in excess results in Ni toxicity that affects plants physically, as well as physiologically. Therefore, it is important to understand how increasing concentrations of Ni can affect plants.

Changes in expression of ACC, *GR*, *AT2G16800*, and *NRAMP* were investigated. ACC was significantly different from water at 1,600 mg/kg of nickel nitrate in needles; this suggests higher concentrations of Ni will induce an upregulation of the gene to help cope with the metal stress. When the expression in roots was observed we found that at all concentrations of nickel nitrate there was an overexpression. This overexpression was observed only at 800 mg/kg and 1,600 mg/kg in samples treated with potassium nitrate. Hence, there was only a significant difference between the salts at the 150 mg/kg dose. This suggests that potassium nitrates may be playing a role in the expression of ACC. When the tissues were compared, there was a significant difference between the needles and roots at 800 mg/kg and 1,600 mg/kg of nickel nitrate, and at 150 mg/kg and 1,600 mg/kg of potassium nitrate.
GR expression was underexpressed at 150 mg/kg and overexpressed at 1,600 mg/kg of nickel nitrate in needles. Whereas potassium nitrate was significantly different from water at 150 mg/kg. The two salts were significantly different from one another at both 150 mg/kg and 1,600 mg/kg treatments. For roots, GR was overexpressed at all concentrations of nickel nitrate and at 800 mg/kg and 1,600 mg/kg for potassium nitrate. There was only a significant difference between the salts at 150 mg/kg, suggesting that potassium nitrates are influencing gene expression.

When comparing the two tissues for GR, AT2G16800, and NRAMP expression, there were no significant differences among treatments of nickel nitrate, however, there was a significant difference between needles and roots at 150 mg/kg. There was also a significant difference at 1,600 mg/kg for AT2G, and at 800 mg/kg for NRAMP. This suggests that the P. glauca plants were taking up nickel and potassium differently.

AT2G16800 expression did not change for any needle treatments. As for the roots, there was only an increase in expression at 800 mg/kg and 1,600 mg/kg of potassium nitrate. This again confirms that nitrates are inducing a change in expression.

NRAMP expression is only increased at 150 mg/kg of potassium nitrate for needles. However, in roots there is no change in expression for potassium nitrate treatments, but all concentrations of nickel nitrate are significantly different than water. Significant differences between nickel nitrate and potassium nitrate were observed at 150 mg/kg, and 1,600 mg/kg suggesting that the differential gene expression is likely due to nickel.

Overall, the results of the present study show that that potassium nitrate (a common plant fertilizer) does have an effect on gene expression and can lead to toxicity in P. glauca plants at high concentrations.
**Future Studies**

To better understand the mechanism underlying the expression of genes in *Picea glauca*, a global transcriptome analysis can be performed to validate if the expression of genes is dose dependent. It would also be important in future studies to dive deeper into nitrate toxicity and to learn more on how they are affecting *P. glauca* at the cellular level. A deeper investigation into the avoidance mechanism used by *P. glauca* to cope with metal stress would be beneficial, as well as, studying older trees in the Greater Sudbury area would help to confirm our findings on the avoidance mechanism in *P. glauca*. 


and distribution in Malus halliana seedlings under zinc toxicity stress. *Arid Land Research and Management*, 1-17.


antioxidant Systems in Plants: role and regulation under abiotic stress (pp. 1-23).

Springer, Singapore.


*Environmental and experimental botany, 134,* 141-150.


Webb III, T. (1987). Climatic change in eastern North America during the past 18,000 years; comparisons of pollen data with model results. *North America and adjacent oceans during the last deglaciation*.


Appendices

Appendix 1: White spruce (*Picea glauca*) in growth chamber randomly associated a treatment (blue=water, green=150 mg/kg, yellow=800 mg/kg, red=1,600 mg/kg)
Appendix 2: Treatment of trees using pipette for accurate addition of 50 mLs per pot
Appendix 3: RNA quality check using gel electrophoresis. Lane 1 contains 1-kb ladder, lane 2-11 contains extracted RNA samples of *Picea glauca*