Comparative molecular analyses between red maple (*Acer rubrum*) and trembling aspen (*Populus tremuloides*) exposed to soil metal contamination: metal translocation, gene expression, and DNA methylation.

By

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Abstract

The main objectives of the present study were to 1) compare the physiological responses and gene expression in red maple (*Acer rubrum*) and trembling aspen (*Populus tremuloides*) exposed to metal contamination, 2) determine if epigenetic events are associated with metal resistance in *A. rubrum*, and 3) assess global gene expression in *A. rubrum* exposed to different doses of nickel. Metal analyses of soil and plant tissues revealed that *P. tremuloides* is an accumulator of Mg, Zn, and Ni while *A. rubrum* does not accumulate these metals in the leaves as it shows avoidance as the main mechanism of coping with soil metal accumulation. Comparative analysis of gene expression revealed that the four genes tested (Nramps4, Nas3, At2G, and MRP4) were more upregulated in *P. tremuloides* compared to *A. rubrum* in a field study. AT2G and MRP4 genes were significantly down regulated in *A. rubrum* from the targeted metal contaminated sites compared to those from uncontaminated areas but environmental factors driving this differential gene expression couldn't be established. The growth chamber experiment, showed differential gene expression based on p values when the effects of nickel doses were compared. There were more upregulated than down regulated genes in resistant genotypes compared to susceptible genotypes. Most of these genes are associated with coping with abiotic stressors and involves tolerance and detoxification mechanisms. There was a significant variation in the level of cytosine methylation among the metal-contaminated sites, with significant negative correlations between bioavailable nickel / copper content and cytosine methylation being observed.

In Conclusion, the present study reveals that *A. rubrum* exhibits the avoidance strategy as the main mechanism of coping with soil metal accumulation. However, a more informative and in-depth analysis of this mechanism would be very beneficial in deciding whether it is strictly

physiological or genetic. The role of epigenetics can be further understood by determining the distribution of DNA methylation in both *A. rubrum* and *P. tremuloides*.

Declaration

To the best of my knowledge, this dissertation contains no material previously published by any other persons except where acknowledgment was due, and no work that has been accepted for any degree in any university.

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List of Abbreviations

A. rubrum: Acer rubrum

ABC: ATP-binding cassette

ACC: 1-aminocyclopropane-1-carboxylic acid deaminase

Ag: Silver

ANOVA: Analysis of Variance

APX: Ascorbate peroxidase

As: Arsenic

AT2G16800: high affinity nickel transporter family protein

ATP: Adenine Triphosphate

B. papyrifera: Betula papyrifera

BAF: Bioaccumulation Factor

CAT: Catalase

Cd: Cadmium

CDF: Cation Diffusion Facilitator

cDNA: Complementary DNA

CEC: Cation Exchange Capacity

Co: Cobalt

CpGs: Cytosine-Guanine islands

CPM: Counts per million

CRM's: Certified Reference Materials

CTAB: Cetyl trimethylammonium bromide

Cu: Copper

DNA: Deoxyribonucleic acid

DW: Dry weight

EF: Enrichment Factor

FDR: False Discovery Rate

Fe: Iron

GC: Glycine-Cysteine

GCS: γ-glutamyl-Cys synthetase

GOPX: Guaiacol peroxidase

GR: Glutathione Reductase

GRPs: Glycine rich proteins

GS: Glutathione Synthetase

GSH: Glutathione

GSH-Px: Glutathione peroxidase

GSR: Greater Sudbury Region

HCl: Hydrochloric Acid

HG-AAS: Hydride Generation Atomic Emission Spectrometry

HM: Heavy metal

HMAs: Heavy Metal Atpases

HNO3: Nitric Acid

HSD: Honestly Significant Difference

ICP-AES: Inductively-Coupled Plasma-Mass Spectrometry

IREG: Iron-regulated protein

IRM's: Internal Reference Materials

IRT: Iron-Regulated Transporter

LC: Liquid Chromatography

m5C: 5-methylcytosine

m6A: N6-methyladenine

Mg: Magnesium

MG: Methylglyoxal

Mn: Manganese

MRP4: Multidrug Resistance Protein

MRPs: Multidrug Resistance Proteins

MS/MS: Tandem mass spectrometry

MSAP: Methylation sensitive amplified polymorphism

MT: Metallothioneins

NA: Nicotianamine

NAS: Nicotianamine Synthase

NGS: Next-generation Sequencing

Ni: Nickel

NRAMP: Natural Resistance Associated Macrophage Proteins

OAS: O-acetyl-1-serine

P. tremuloides: Populus tremuloides

Pb: Lead

PC: Phytochelatins

PCR: Polymerase Chain Reaction

POD: Peroxidase

PVP: Polyvinyl pyrrolidone

Q. rubra: Quercus rubra

qPCR: Quantative Polymerase Chain Reaction

RG: Resistant genotype

RNA: Ribonucleic acid

RNA-Seq: RNA sequencing

ROS: Reactive Oxygen Species

RPKM: Reads per Kilobase Million mapped

RSEM: Relative Standard Error of Mean

RT-qPCR: Real Time Polymerase Chain Reaction

SAT: Serine Acetyltransferase

SBS: Sequencing-by-synthesis

SG: Susceptible genotype

SOD: Superoxide Dismutase

SOLiD: Sequence by Oligonucleotides Ligation and Detection

SPSS: Statistical Package for the Social Sciences

TFs: Translocation Factors

TMP: Putative transmembrane protein

ZAT11: Zn finger protein of Arabidopsis thaliana

ZIP: ZRT, IRT-like proteins

Zn: Zinc

γ-GC: gamma-glutamylcysteine

List of Original Publications

Publications

Kalubi, K.N., Michael, P., Omri, A. 2018. Analysis of gene expression in red maple (*Acer rubrum)* and trembling aspen (*Populus tremuloides*) populations from a mining region. Genes Genomics 40 (11):1127-1136

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Kalubi, K.N., Mehes-Smith, M., Omri, A., 2016. Comparative analysis of metal translocation in red maple (*Acer rubrum*) and trembling aspen (*Populus tremuloides*) populations from stressed ecosystems contaminated with metals. Chemistry and Ecology. 32 (4): 312-323.

Kalubi, K.N., Mehes-Smith, M, Narendrul, R, P. Michael, A. Omri. 2015. Molecular analysis of red maple (*Acer rubrum*) populations from a reclaimed mining region in Northern Ontario (Canada): soil metal accumulation and translocation in plants. Ecotoxicology. 24: 636–647.

Manuscript for submission

Kalubi, K., Theriault, G., Michael, P., and Omri, A. 2018. Low stringency tests revealed differential gene expression in red maple (Acer rubrum) treated with low doses of nickel: gene regulation in nickel resistant and susceptible genotypes. In review. Cytology and Genetics Journal.

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Chapter 1: Literature Review

1.1 Metal contamination in the City of Greater Sudbury

The Greater Sudbury Region (GSR) in Ontario, Canada, historically recognized for metal deposits of nickel, cobalt, copper, and iron, is considered to be the most ecologically disturbed areas in Canada (Bačkor and Fahselt 2004). The metal deposits derived from anthropogenic activities such as mining have led to a significant increase of metal content in the region (Hutchinson and Whitby 1972; Freedman and Hutchinson 1980; Alloway 1990; Winterhalder 1996; Adamowicz 2014). These activities have had a negative effect on the vegetation by causing metal-induced stress and decreasing plant growth. (Narendrula and Nkongolo 2015; Nkongolo et al. 2016)

Initially, open roast yards used to remove sulfur contained about a quarter of a million tonnes of ores and burnt on beds of corewood in the atmosphere. The use of this method resulted in release of harmful sulfur dioxide into the environment as a result of the oxidation of the metal ores. It also caused soil erosion and increased the number of fire incidents and significantly contributed to the barren landscapes in Sudbury (Amiro and Courtin 1981). In fact, more than 80,000 ha became semi-barren to completely barren and several studies implicated sulfur dioxide emissions and metal particulates in soils as being the main cause of these effects (Wren et al. 2012; Nkongolo et al. 2016).

Smelters were then implemented because they were safer than the open roast yards. However, the use of these smelters resulted also in negative impacts on the landscape and vegetation within the GSR. Both smelters and open roast beds have released more than 100 million tonnes of sulphur dioxide and thousands of tonnes of copper, nickel, and iron for the past 90 years. Forests and plants have been overwhelmed by sulfur dioxide fumigations and heavy metals (Bačkor and Fahselt 2004). Soils were eroded from landscape and hilltops while airborne particulate matter entered

many lakes and rivers (Hutchinson and Symington 1997). Slag and mine tailings have also contributed to Sudbury's landscape, but they have had a much lower impact (Smith 1996).

Several studies conducted over the years reported high concentrations of metals in soil and plants in close proximity to the smelters (Gratton et al. 2000; Wren et al. 2012; Nkongolo et al. 2013). High correlations between distance from the source of pollution and concentrations of particular metals were documented (Narendrula and Nkongolo 2015). Likewise, plant growth and population diversity decrease near the smelters compared to distal sites (Theriault et al. 2013; Narendrula and Nkongolo 2015). Data also revealed that increased levels of bioavailable metals were associated with a decrease in available nutrients and significant changes in the soil chemistry (Winterhalder 1996; Deck 2000).

Several programs have been initiated and successfully completed by the City of the Greater Sudbury, local industries, and Laurentian University to improve the terrestrial landscapes of the region. During the last few decades, production of metals such as copper (Cu) and nickel (Ni) have remained at high levels but the industrial SO_2 emission has been reduced by 90%, mostly because of a combination of industrial technological developments and legislated controls (Wren et al. 2012). This has led to improved air quality and natural recovery of damaged ecosystems. Further, the recovery has been achieved through soil liming, seed distribution and reforestation program (Nkongolo et al. 2013, 2016). To date, over 12 million trees has been planted mostly conifers in the GSR leading to the increase of soil organic matter and microbial biomass/communities (Wren et al. 2012; Nkongolo et al. 2016). In natural stands, *Abies balsamea*, *Acer rubrum, Betula papyrifera, Populus tremuloides,* and *Quercus rubra* are the most predominant tree species in both metal-uncontaminated (reference) and contaminated sites. In fact, for reference sites, the relative proportion of these species were 45% (*A. rubrum*), 36% (*B. papyrifera*), 4% (*P. tremuloides*, and

1.2% (*Q. rubra*). These values were 18 % (*A. rubrum*), 68% (*B. papyrifera*), 0% (*P. tremuloides*, and 12% (*Q. rubra*) in metal-contaminated sites (Theriault et al. 2013; Narendrula and Nkongolo 2015).

1.2 Metal Toxicity and Resistance in Plants

1.2.1 Effects of Metals on plants

Beyond specific threshold levels, metals can become toxic to plants and these effects appears to be concentration - dependent (Burton et al. 1984; Dixon and Buschena 1988; Hohl and Varma 2010). Metal accumulation can impede plant growth and result in compact and shorter root systems. It also causes root browning and foliar necrosis and chlorosis. Increased accumulation of metals in soil is toxic to lower and higher plants. Their roots can absorb metal ions that will be translocated to their aerial biomass affecting important metabolisms. (Foy et al. 1978; Bingham et al. 1986; Kushwaha et al. 2016) Moreover, high concentrations of metals in soil leads to a loss of soil fertility, agricultural yield, as well as a decrease in soil microbial activity (Singh et al., 2011; Sherameti and Varma 2015; Kushwaha et al. 2016).

There are many different factors associated with metal toxicity including plant metal tolerance, bioavailable metal concentration, exposure time, and the type of metal present (Cuypers et al. 2013). For instance, the presence of high concentration of bioavailable nickel in soil can result in reduced plant biomass, leaf necrosis and chlorosis. At the cellular level, enzymatic activity can be impeded, nutrient uptake and translocation can be reduced in the presence of nickel as well as a significant decrease of cell division. Copper and other metals at high concentrations in soil can cause damage to root tissue, reduce photosynthetic activity, increase root cell plasma permeability and damage DNA structure and gene function (Hughes and Poole 1989; Hall 2002; Kamal et al.

2010; (Mehes-Smith and Nkongolo 2015). Some metals affect metal binding sites within cells. This can cause a change in protein structure and a loss of protein activities. Moreover, cellular disruption associated with enzymatic function can displace essential elements leading to nutrient deficiencies (Kushwaha et al. 2016). High concentration of metals will likely result in oxidative stress and an increase of reactive oxygen species (ROS) and free radicals. Examples of ROS include singlet oxygen (O2), hydrogen peroxide (H2O2) and hydroxyl radicals (-OH) (Hall 2002; Emamverdian et al. 2015).

1.2.2. Coping Mechanisms in Plants to Elevated Soil Metal Concentrations

Plants generally have different ways to survive in soil with high metal concentration. As soon as metals access to a cell and its compartments, there are a number of different mechanisms that plants use to prevent phytotoxicity. These coping mechanisms depend on the metal and the plant species. Some of these strategies include binding to macromolecules (DNA, RNA and proteins), disruption of enzymatic activity and radical formation (Winterhalder 1996; Gunn et al. 2007; Kamal et al. 2010; Sherameti and Varma 2015). Other strategies to prevent phytotoxicity in plants include formation of insoluble crystals, vesicles, and retention of elements by cell walls (Mehrotra 2005). Metal tolerance is also very dependent on the accumulation of metals in root cells (Hall 2002; Hohl and Varma 2010). Plants can counteract the effects of oxidative stress from ROS. Cells are protected from damage when their enzymes and other compounds inhibit free radicals and ROS.

1.2.2.1 Physiological Mechanisms

In regards to physiology, plants that are resistant to metals have developed strategies to exclude metals from cells within the roots and also cope with high metal concentrations within the plant (Srivastava and Kowshik 2013). These coping mechanisms allow these plants to colonize metal contaminated soils. Plants have three key coping strategies when dealing with high metal concentrations including restriction of metal uptake and transport, complexation of metals at the cell wall-plasma membrane interface, and complexation and compartmentation of metals with the cells (Das and Jayalekshmy 2015) .

1.2.2.1.1. The Restriction of Metal Uptake and Transport

Some plant species limit access of metals across the cell membrane into the cell while other species permit entry of metals intracellularly. There are different ways that plants restrict entry of metals intracellularly. They either precipitate the excess of bioavailable metals or they form complexes in the root environment. Precipitation of these metals can occur by an increase of pH at the rhizosphere or the excretion of anions (e.g. phosphate) and organic acids (e.g. malate, citrate, oxalate) making them unavailable for uptake (Hall 2002).

1.2.2.1.2. Complexation of Metals at the Cell Wall-Plasma Membrane Interface

The plasma membrane is the first plant structure that metals encounter when they are drawn in from the environment. Its primary role is to act as a border to limit the entry of metals within the cell from the extracellular environment. Metals can enter root tissue by two different structures, the symplast or the apoplast (Das and Jayalekshmy 2015). Not only can high concentrations of metal ions be located outside the cell but they can also be found at the plasma membrane border. This is influenced by the ion binding capacity of the cell wall. The cation exchange capacity (CEC)

varies between metal tolerant and sensitive plants. Cell walls of tolerant plants have a higher CEC which allows the plant to form complexes in that area and prevent entry of metals (Das and Jayalekshmy 2015).

1.2.2.1.3. Complexation and Compartmentation of Metals with the Cells

There have been many studies on cellular mechanisms that are employed by plants during metal uptake (Hall 2002; Emamverdian et al. 2015). Plants are very dynamic when coping with metals to regulate their toxicity. For example, they can remove metals from the cytosol and/or sequester the ions inside a vacuole. Metal toxicity on sensitive organelles are reduced once inside the vacuole through vacuolar pumps (Tong et al. 2004; Yadav 2010). Proteins such as phytochelatins (PC) and metallothioneins (MT) are used to facilitate the transport of metals into cells. At the same time, metal ions compete with other essentials ions of similar ionic radii (Hall 2002; Emamverdian et al. 2015). PCs and MTs both possess an abundance of metal chelating peptides that are capable of binding to many different metals including Cd, Cu, Zn and As (Hall 2002; Saraswat and Rai 2011). When these metals are chelated by these proteins, they are no longer able to move freely within the cell. This results in either their sequestration or compartmentalization.

The occurrence of metals (Cu, Cd, Zn, Ag) in the roots, shoots and leaves can stimulate the production of PCs in tolerant plants such as *Sedum alfredii.* The concentration of Cd in the cytosol decreases as a result of the accumulation of Cd-PC complexes in the vacuoles (Mehes-Smith et al. 2013a; Das and Jayalekshmy 2015). As mentioned earlier, tolerant plants have a variety of ways to reduce the phytotoxic effects of free metal ions within the cell. Metal chelation with specific

ligands is one method. Compartmentalization of metals can also occur in the apoplast and specific cells of the epidermis and trichome (Yruela 2005).

1.3. Classification of Metal Plant Resistance Mechanisms

Plants can cope with metals using either avoidance and/or tolerance strategies.

Avoidance: plants that use this strategy do not allow metals to enter the intracellular environment. Their roots are able to spread further to soils that are less contaminated (Mehes-Smith et al. 2013a). In the root system, plants secrete root exudate in the rhizosphere to immobilize bioavailable metal ions to prevent them from entering the roots. Mycorrhizal fungi play a crucial role in plant metal tolerance as well. They are able to reduce the build-up of metals in the roots of the host plant to prevent metal stress by lengthening their hyphae (Mehes-Smith et al. 2013a). This also allows necessary elements to be transferred to plants. Mycorrhizal fungi can also alter the species of metals and prevent metals from entering the roots. They also have the ability to chelate metals that have gained access inside the cell's environment. Metal ions are chelated with molecules such as organic acids, phytochelatins, and polyphosphates which are subsequently transported to vacuoles (Cuypers et al. 2013; Mehes-Smith et al. 2013a).

Tolerance: Unlike the avoider plants, these metal tolerant species use detoxifying mechanisms to cope with metal ions that have already bypassed the plasma membrane and entered the cell's intracellular environment. Tolerant plants can be further subdivided into three categories; the excluders, indicators and accumulators/hyperaccumulators. The excluder plants regulate metal levels in their aerial tissues by keeping them low while growing on metal contaminated soils. Metals have access to cells in the roots but cannot translocate to the aerial tissues of the plant. Indicators are plants that accumulate metals in their above ground tissues. However, the metal

concentrations in these tissues are similar to those in the soil. Thus, these plants indicate the level of metal contamination in the surrounding environment. Accumulators/hyperaccumulators are plants that amass metals into their roots and then they will be translocated to aerial tissues. The concentration of metals in aerial parts are greater than in the soil (Cuypers et al. 2013; Mehes-Smith et al. 2013a).

1.4. Genes Involved in Metal Resistance in Plants

Proteins encoded by genes that are resistant to metals are directly involved in the detoxification mechanisms of metals or the reactive oxygen species that they produce. There are a number of genes in both model and non-model plant species that have been reported to be associated with Ni and Cu resistance (Djeukam et al. 2016; Theriault et al. 2016a; Makela et al. 2016). A study on the root transcriptome of *Betula papyrifera* reported that a downregulation of genes associated with translation (ribosome), binding, and transporter activities is the key mechanism associated with nickel resistance (Theriault et al. 2016b). Most of the metal resistance genes can be classified as either chelators or transporters.

1.4.1 Chelators

Chelators are molecules that confiscate metals to prevent them from causing any harm by disabling them. Plants do not require a lot of energy to produce these small molecules. In the cytosolic environment, there are increased levels of nicotianamine (Mari et al. 2006), citrate (Ryan et al. 2009), malate (Liu et al. 2009), amino acids such as proline (Sharma and Dietz 2009), histidine (Krämer et al. 1996) and glutathione (GSH) (Freeman et al. 2004). Each of these molecules is a byproduct of the Krebs cycle and plays a key part in metal resistance in plants. Hyperaccumulator plants rely on chelators to cope with metals by storing them properly. For instance, *Sebertia*

acuminate is a hyperaccumulator that only stores nickel in its aerial tissue when it is held by citrate (Lee et al. 1977).

Metal resistant plants possess genes associated with the production of chelators. Studies have reported increased levels of metabolites such as GSH, cysteine, and O-acetyl-l-serine (OAS) in the shoots of *Thlaspi* spp which is a nickel hyperaccumulator (Freeman et al. 2004). It has been shown that there is a relationship between these increased levels of metabolites and high expression levels of serine acetyltransferase (SAT) and glutathione reductase. These enzymes contribute to the production and turnover of GSH (Freeman et al. 2004). There is also a correlation between increased levels of Mn, Fe, Cu, Ni, and Zn and increased expression of nicotianamine synthase (NAS) and nicotianamine production (Kim et al. 2005; van de Mortel et al. 2006). NAS is involved in the production of nicotianamine by the trimerization of S-adenosylmethionine (Shojima et al. 1990).

Other larger molecules which are involved in metal detoxification and resistance have been studied extensively (Hall 2002). One of them is MT, a short peptide rich with cysteines that binds to metals or reactive oxygen species using its thiol groups (Zhu et al. 2009). Although MTs also play a role in homeostasis, they have a significant impact on metal resistance (Hall 2002). MTs are found in all kingdoms of life and are categorized into three different classes. The class 1 MTs are located in vertebrates and their cysteine arrangement is highly conserved. On the other hand, class 2 MTs have a less conserved cysteine arrangement and are found in plants, fungi, and invertebrates. They can be further subdivided in four more groups based on the tissues they are found in (Cobbett and Goldsbrough 2002). The class 3 variety of MTs is found exclusively in plants (Prasad 2004) and its high expression is correlated to Cu (Rauser and Curvetto 1980; Schultz and Hutchinson 1988) and Cd (Hasegawa et al. 1997; Lee et al. 2004) resistance.

Unlike MTs, PCs are also large molecules that do not directly code for genes in plants. They are structurally large oligomers of GSH involved in binding metals and immobilizing them using their thiol groups (Cobbett and Goldsbrough 2002). There are 3 steps involved in the PC pathway; 1) γ-glutamyl-Cys synthetase (GCS) catalyzes the dipeptide gamma-glutamylcysteine (γ-GC) from cysteine and glutamate 2) glutathione synthetase (GS) produces GSH from γ-GC and an additional glutamate and, 3) phythochelatin synthase ligates multiple GSH molecules to form long oligomer chains (Grill et al. 1989; Cobbett and Goldsbrough 2002). To demonstrate the importance and influence of PCs in homeostasis, cad2-1 *Arabidopsis* plant is deficient in GCS (Cobbett et al. 1998). Therefore, they will have low levels of PCs which makes them sensitive to Cd (Cobbett et al. 1998).

1.4.2. Transporters

Transporters are a large group of proteins that are encoded by another class of metal resistant genes. Metals use transporters as a vehicle to move from one area of the plant to another. These transporters are responsible for the compartmentalization of metals in plant tissues of hyperaccumulators, excluders, and indicators. In order to properly carry out their function, they rely on metal chaperons and other ions (Hall 2002). A couple of good examples are antiporters and symporters. Antiporters pump both ions in opposite directions across the membrane and symporters pump both ions in the same direction across the membrane. There are a number of known metal transporters that play an important role in metal resistance in plants. These include NRAMP (natural resistance associated macrophage proteins), Metal ATPase (HMAs), Cation Diffusion Facilitator (CDF), ABC transporter (ATP-binding cassette), and the ZIP (ZRT, IRT-like proteins) family. They help regulate and maintain suitable metal concentration levels in bacteria, animals, and plants (Viehweger 2014). Several transporters have shown low-transport specificity

of metals which allows them to transport a group of metal with similar physical properties. The IRT transporter family transport Fe under normal conditions but they can transport other metals such as Zn, Ni, Cd, Co, and Mg when there is a deficiency (Vert et al. 2002). With regards to low transport specificity, Ni and Zn compete for the AtZIP4/TcZNT1 transporter in *Arabidopsis thaliana* (Hassan and Aarts 2011). Recently, six candidate genes associated with nickel resistance in *B. papyrifera* were identified (Theriault et al. 2016a; Theriault and Nkongolo 2017). They include glutathione S-transferase, thioredoxin family protein, putative transmembrane protein, Nramp transporter, TonB receptor and TonB dependant protein.

1.5. Gene expression associated with metal contamination

Environmental agents such as metals, reactive oxygen species, heat shock and drought are able to cause alterations in gene transcription levels (Matters and Scandalios 1986). Accumulators and hyperaccumulators are metal tolerant plants that often display changes in gene expression levels when exposed to high concentration of metals. In previous studies, A. *thaliana,* was a model plant species that was often used to determine the effects of metal exposure on gene expression levels. Microarrays have been for while the method of choice to assess gene expression. A global gene expression analysis was performed on plants in the presence of cadmium (Cd) and lead (Pb). The results of this study indicated that regulation of the expression of ABC transporters was directly influenced by the presence of Cd (Bovet et al. 2005).

Several gene families associated with nickel resistance in model and non-model plants have been identified. Some of the genes are 1-aminocyclopropane-1-carboxylic acid deaminase (*ACC*), high affinity nickel transporter family protein (*AT2G16800*), iron-regulated protein (*IREG*), glutathione reductase (*GR*), glutathione-s-transferase, Metal transporter (*NRAMP 1,2,3,4*), Nicotianamine synthase (*NAS3*), Putative transmembrane protein (*TMP*), Serine acetyltransferase (*SAT*),

Thioredoxin family protein, Zn finger protein of *Arabidopsis thaliana* (*ZAT11*), and *MRP4* (Freeman *et al*. 2004; Lemaire *et al*. 2004; Stearns *et al.* 2005; Mizuno *et al*. 2005; Schaaf *et al*. 2006; Mari *et al*. 2006; Visioli *et al*. 2012; Liu *et al*. 2014; Theriault et al. 2016b).

NRAMP (Natural resistance associated macrophage protein) is a family of transporter genes that bind to divalent metal ions and subsequent transport (Williams et al. 2000). Plants that showed downregulation of these transporters showed resistance to excess nickel. NAS 3 are a group of enzymes that produce nicotianamine (NA) which is a chelator (Wintz et al. 2003). This is an amino acid that is able to bind and transport transition metals such as Fe, Cu, Zn, Mn, and Ni (Wintz et al. 2003). Studies have shown that increased levels of nicotianamine is correlated with metal tolerance in hyperaccumulator plants which implies that metal translocation is facilitated (Weber et al. 2004; Deinlein et al. 2012). MRPs are pumps that detoxify harmful compounds in the cell and it is also involved in metal vacuolar sequestration (Martinoia et al. 2002).

The expression and activity of antioxidant enzymes is also affected by metal stress. Stimulation of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione s-transferase has already been documented in plants (Gabbrielli et al. 1999; Madhava Rao KV and Sresty 2000; Baccouch et al. 2001; Gajewska and Skłodowska 2008). The activities of these enzymes were reduced in *B. papyrifera,* probably by Ni displacement of other metals in active binding site or by enzyme inactivation from direct binding of Ni2+ to a -SH group (Theriault et al. 2016b). Overall, many plants have displayed changes in gene expression upon metal stress in many studies.

1.6. Assessment of global gene expression using next generation platforms

Global gene expression is usually assessed through transcriptome sequencing, or RNA sequencing (RNA-Seq). This provides fundamental insights into how genomes are organized and regulated—giving us valuable information about the internal state of cells and how altered expression of genetic variants contributes to complex physiological dysfunction. Transcriptome sequencing relies on next-generation sequencing (NGS) methodology and techniques. They are different platforms that can be used for transcriptome analysis (Quince et al. 2009; Rajendhran and Gunasekaran 2011; Li et al. 2014).

The first next generation sequencing method developed was the 454 platform. It was introduced in 2005 by Roche. It is rapid, flexible, cost effective, and produces 25 million base reads in a single run with an accuracy of 99% and does not require a cloning step (Liu et al. 2012).

The Illumina sequencing platform was the second tool that was first established in 2006 and it can produce large quantities of data for each run which makes this technology cost effective. The Illumina utilizes a sequencing-by-synthesis (SBS) approach. The templates are prepared by isothermic PCR. This platform is different from the 454 in two major ways: 1) it does not involve pyrosequencing, but rather dye terminators (approach resembles Sanger sequencing) and 2) it uses a flow cell with a field of oligos attached, instead of a chip containing individual microwells with beads. This platform also produced very short reads. The newest version of this platform (HiSeq 2500) is more rapid than the old one (HiSeq 2000) (Zhou et al. 2015).

Ion Torrent platform was developed by Ion Torrent Systems Inc. and was released in February 2010. This platform sequences DNA by monitoring the addition during DNA synthesis like Illumina. But the two platforms operate on different principles. Unlike Illumina, Ion Torrent

sequencing prepares templates by using emulsion PCR. Details on difference in the operation of these two platforms are discussed in Salipante et al. (2014).

Sequence by Oligonucleotides Ligation and Detection (SOLiD) is another next generation DNA sequencing platform that was also introduced in 2006 by Life Technologies (Zhang et al. 2011; Luo et al. 2012). In contrast to the Illumina platform, SOliD uses sequencing by ligation. SOLiD is also cost effective in terms of the amount of data produced per run and generates the shortest reads. The short reads generated are primarily used for transcriptomics. They are usually not used for *de novo* sequencing projects. SOLid can be used in a variety of different applications such as: whole genome resequencing, targeted resequencing, gene expression profiling, small RNA analysis, whole transcriptome analysis, and epigenome (like ChIPSeq and methylation) (Liu et al. 2012). Since this platform uses the sequencing by ligation approach, the data quality generated is the best (Zhang et al. 2011). Details of this technique are described in (Knief 2014), (Liu et al. 2012), and the Applied Biosystem website.

1.7. Effects of environmental stressors on DNA methylation

DNA methylation is defined as a change in phenotypic expression without altering the genotype of an organism. This change is a heritable epigenetic mark where a methyl group is covalently transferred to the cytosine ring of DNA at the 5' position. This process is modulated by a group of enzymes called DNA methyltransferases. The vast majority of DNA methylation is configured as a cpG dinucleotide context in somatic cells. DNA methylation is primarily required for normal development but is also involved in many processes such as genomic imprinting, X-chromosome inactivation, and suppression of repetitive element transcription and transposition and, when dysregulated, contributes to diseases (Jin et al. 2011).

Plants are generally very dynamic in how they respond to environmental stress in order to compensate and adapt. Abiotic stressors typically decrease DNA methylation levels in plants. Examples include chilling, planting density, rubbing and cutting. Salt stress cause an increase in DNA methylation levels and the effects of metal stress depends on the species. Biotic stressors can cause genome wide hypermethylation and hypomethylation of genes associated with resistance. It has been demonstrated that methylation of GC rich sequences are important epigenetic marks. These marks are also stable so they can be passed on to the next generation (Peng and Zhang 2009).

It should be pointed out that there is a wide range of techniques used to characterize methylcytosine in a genome (Saluz and Jost 1993; Grigg and Clark 1994; Rein et al. 1998; Fraga and Esteller 2002; Laird 2010; Schrey et al. 2013; Alonso et al. 2016; Herrera et al. 2016) . Each technique has its own peculiarities. Tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS) is an established approach to nucleoside quantification specifically to measure global cytosine methylation (Hu et al. 2013; Tsuji et al. 2014). In particular, it is a fast, sensitive, accurate and specific avenue for modified nucleoside quantification at trace (fmol) levels (Alonso et al. 2016). Methylation sensitive amplified polymorphism (MSAP) analysis has been often used to assess methyl-cytosine changes in several studies. It provides several anonymous loci randomly distributed over the genome for which the methylation status can be ascertained (Alonso et al. 2016; Kim et al. 2016). This robust method is highly reproducible. It is a chip-based molecular tool useful to detect variations in the DNA methylation status (Kim et al. 2016). (Aina et al. 2004) reported hypomethlylation of DNA induced by heavy metals. (Greco et al. 2012) observed a hypermethylation induced by cadmium in *Posidonia oceanica* based on MASP. (Cicatelli et al. 2014) showed also a significant level of DNA hypermethylation in leaves when mycorrizal poplar (*Populus alba*) plants were grown in the presence of zinc and copper compared to control. Kim et

al. 2016. reported that Metal contamination affected methylation of cytosine residues in CCGG motifs in the *A. rubrum* populations that were analyzed.

More recent efforts have focused on the characterization of the methylation status of specific DNA sequences (Fraga and Esteller 2002). Optimization of the methods based on bisulfite modification of DNA facilitates the analysis of limited CpGs in restriction enzyme sites and allows very specific patterns of methylation to be revealed (bisulfite DNA sequencing) (Shen and Waterland 2007). New techniques designed to search for new methylcytosine hot spots have significantly contributed to our understanding of DNA modifications without requiring prior knowledge of the genome sequence (Fraga and Esteller 2002; Shen and Waterland 2007; Kim et al. 2016).

1.8. Species of Interest

Two of the four most prevalent tree species in the GSR were of interest because of their population dynamics associated with metal contamination. Trembling Aspen (*Populus tremuloides*) is a plant species in North America that is widely dispersed. This particular species belongs to the *Populus* genus which is part of the larger *Salicaceae* family. There are about 40 species of poplar and the majority of these species including *populus tremuloides*, have important use in the forest industry and manufacture of books and newspaper. It has been suggested that some species could potentially be used as low-cost and low maintenance bioenergy crops in order to produce biofuels (Ye et al. 2011). Due to its resilience and high adaptability to changing climates, it is considered a good model plant species for adaptive responses to climate change (Rai et al. 2013).

This species is potentially very suitable for phytoremediation purposes and land reclamation. For instance, there are separate individuals that develop male and female reproductive organs (Cox 1988). This species propagates primarily by roots suckering, although it can reproduce sexually. This is advantageous for this plant in contaminated and disturbed soil environments because it can

propagate many copies via root suckering once a single tree is established. The effects of this dispersion is the colonization of this plant in the area (Elliott and Baker 2004). Their root system is also widespread which helps them survive in contaminated soils because they can reach areas that are less contaminated (Radwanski et al. 2017). Trembling aspen also produces a large biomass which may facilitate metal tolerance in heavily contaminated soils.

Red maple (*Acer rubrum*) is another tree species that is also widely distributed across North America (Hutnik and Yawney 1961). This species is dominant in several forest types and is associated with 56 cover types (Walters and Yawny, 1990). They grow in a big percentage of the deciduous forest of Eastern North America and the surroundings of the boreal forest (Hosie 1969). Areas such as borders of swamps, lakes, marshes, in hammocks and rich woods are also where red maple is seen to grow (Braun 1961; Radford et al. 1968; Roland and Smith 1969; Clewell 1985; Duncan and Duncan 2000). It grows better than any other forest species in North America in different soil types, textures, and moisture regimes (Walters and Yawny, 1990).

Red maple (*Acer rubrum*) is a resilient forest species to a number of environmental stressors from agents such as metals and air pollution (Krause and Dochinger 1987). These trees dwell in the Greater Sudbury Region (GSR) in Northern Ontario in soils that are heavily contaminated with metals including nickel (James and Courtin 1985).

1.9 Rationale and hypothesis

Resistance to metal of *A. rubrum* exposed to low doses of bioavailable metals (mostly nickel) in in the Greater Sudbury region has been previously reported by (Kirkey et al. 2012). Response of this hardwood species to high levels of nickel has not been characterized in details. Likewise, reaction of *P. tremuloides* to metal contamination is sketchy. Mechanisms of resistance of both

species to nickel, one of the main contaminants in the GSR ecosystems have not been investigated in details. In addition, genes associated with metal resistance have been well identified mostly in model plant species and their regulation in the presence of metals has been characterized in details in these low plants. But their expression in hardwood species and our understanding of the mechanism of metal resistance is still very limited despite the importance of tree species in many ecosystems, especially in the GSR. Moreover, analysis of possible epigenetic contribution to metal resistance in hardwood species is missing. We hypothesise that 1) the physiological mechanisms of metal resistance as well as the expression of genes associated with Ni resistance are different in *A. rubrum* compared to *P. tremuloides* and 2) the level of gene regulation is metal dose dependent.

1.10 Objectives of the research

The mains objectives of this study were to **1)** compare the physiological responses and gene expression in *A. rubrum* and *P. tremuloides* exposed to metal contamination. **2)** Determine if epigenetic events are associated with metal resistance in *A. rubrum* **3)** Assess global gene expression of *A. rubrum* exposed to different doses of nickel. The results of the present research are described in four interconnected chapters. Chapter 2 is a comparative analysis of metal accumulation and translocation in *Populus tremuloides* and *Acer rubrum* growing in the Greater Sudbury Region (GSR). Chapter 3 highlights differences in gene expression in these two species. Associations between metal contamination and differential level of gene expression are also described in details. The man goal of chapter 4 is to establish if nickel, one of the main contaminants in the GSR triggers gene expression at low and high dose corresponding to the bioavailable and total Ni at sites in the GSR with a focus on A. *rubrum*. Finally, chapter 5 aims at determining if metal contamination in the GSR is associated with DNA modification.

Chapter 2: Comparative analysis of metal translocation in red maple (*Acer rubrum*) and trembling aspen (*Populus tremuloides*) populations from stressed ecosystems contaminated with metals.

2.1 Abstract

Uptake of contaminants by plants and their mechanisms have been the subjects of several studies, but reports on the analysis of metal translocation in hardwood species are limited. The main objective of the present study is to compare metal accumulation and translocation in trembling aspen (*Poplar tremuloides*) and red maple (*Acer rubrum*) growing in Northern Ontario. Results show that trembling aspen leaf tissues accumulate more nickel (Ni) and zinc (Zn) than roots. The concentrations of these elements in red maple was low in leaf, branch and roots tissues compared to amount of the bioavailable levels of these metals in soil. The translocation factors (TFs) of metals from roots to leaves were low for copper (Cu) and high for iron (Fe), magnesium (Mg), Ni, and Zn in trembling aspen. They varied from 0.52 to 3.26 for Fe, 3.39 to 5.47 for Mg, 1.41 to 4.1 for Zn, and 2.6 to 16.4 for Ni. For red maple the TF was low for all the elements except Mg. For this species, the TF values from roots to leaves varied from 0.08 to 0.17 for Fe, 0.71 to 0.90 for Zn, 0.26 to 0.81 for Ni, and 2.62 to 4.13 for Mg. Overall, Cu does not accumulate in trembling aspen and red maple tissues, and the two species have different mechanisms in dealing with the other main contaminants in the region, specifically Ni and Zn. Trembling aspen is accumulator for Ni and Zn while red maple is an excluder for Zn and it uses the avoidance strategy to deal with soil Ni contamination.

Key words: *Acer rubrum*; *Populus tremuloides;* Metal translocation; Bioaccumulation; Northern Ontario (Canada).

2.2 Introduction

The Greater Sudbury Region (GSR) is known for an abundance of nickel, copper and various other metal deposits. The discovery and value of these metal deposits in the late 1880s has led to the development of a world renowned mining and smelting industry producing up to 50 000 tonnes of ore per day and staggering total of 1.6 billion tons of base metal rich ores to date (Smith 1996). Metal contamination of soil and water is regarded as one of the most important environmental problems of industrialized countries. Metal contaminated areas can occur naturally or as a result of anthropogenic activities, including mining deposits, aerial fallout from smelters or other industrialized processes (Mengoni et al. 2000). The presence of metal contaminants at elevated concentrations in the soil imposes a severe stress on plants, thus hindering the growth of vegetation (Watmough and Hutchinson 1997).

Several methods have been used to clean the environment. But most of these techniques are too expensive and they are not always efficient. Chemical technologies produce large volumetric sludge and they are very costly (Rakhshaee et al. 2009). Not only can thermal and chemical methods be very technically difficult and expensive but they can degrade essential components of soil (Hinchman et al. 1996). By convention, remediation of heavy metal contaminated soils involves either on site management or diggings and subsequent disposal to a landfill site. The problem with this particular method is that it simply shifts the contamination problem elsewhere along with the hazards associated with transportation of contaminated soil. An alternative method to excavation and disposal to landfill is soil washing. This method is very expensive and generates a rich residue of heavy metals (Gaur and Adholeya 2004).

Recent discussions and concerns regarding the contamination of the environment have led to the development of necessary technologies to assess the presence and movement of metals in the soil, water and wastewater (Shtangeeva and Ayrault 2004). Currently, phytoremediation has become an effective method and an affordable technological solution to remove or extract inactive metals from metal contaminated soil. Phytoremediation is the use of plants to remove contaminants from soils, sediments and water. This method is more effective and favorable because it is more environmentally friendly and potentially cost effective (Cho-Ruk et al. 2006). The method of phytoremediation takes advantage of the unique and selective uptake capabilities by plant root systems. It also takes advantage of translocation, bioaccumulation, and degradation of contaminants from the plant (Hinchman et al. 1996).

Uptake of contaminants by plants and their mechanisms have been studied by several researchers. According to (Sinha et al. 2007) plants can act as both accumulators and excluders. The accumulators are able to survive despite the high concentrations of metals that are contained in their aerial shoots. They biotransform the contaminants into inert forms in their tissue. The excluders limit contaminant uptake into their biomass. 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. 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. These requirements are ranging from 10 to 15 ppm as most trace elements do for most needs. Hyperaccumulator plants are the exceptions, which can take up thousands of ppm of toxic metal ions.

Recent ecological analysis showed that *Acer rubrum* and *Populus tremuloides* are dominants hard wood species found in fragmented populations in the GSR (Narendrula and Nkongolo 2015) .The main objective of the present study is to compare metal accumulation and translocation in *Populus tremuloides* and *Acer rubrum* growing in a mining region in Northern Ontario. We hypothesized that these two hardwood species that have been growing on metal-contaminated sites for half century will have a similar coping mechanism to soil metal pollution.

2.3 Materials and Methods

2.3.1 Sampling

For *Populus tremuloides* soil and plant tissue (root, branch, and leaves) samples from 14 trees per location were collected at four sites contaminated with metals. They included Coniston Bypass, Laurentian, Kelly Lake, and Falconbridge. Two distant reference sites (Low Water and Tamagami) were also sampled (Figure 1). For *Acer rubrum*, nine populations from Northern Ontario were surveyed (Figure 1). They include six metal-contaminated (Daisy Lake, Wahnapitae Hydro-Dam, Airport, Kingsway, Kukagami, and Laurentian) and three reference sites (St. Charles, Onaping Falls and Capreol). The sites were selected based on presence of the two targeted species, similar levels of bioavailable metals based on previous studies (Nkongolo et al. 2013) and proximity to smelters. Only sites with similar levels of bioavailable metals were targeted. Reference sites were > 30 km from the smelters. Soil, branch, and leaf samples from 16 trees of similar size were collected.

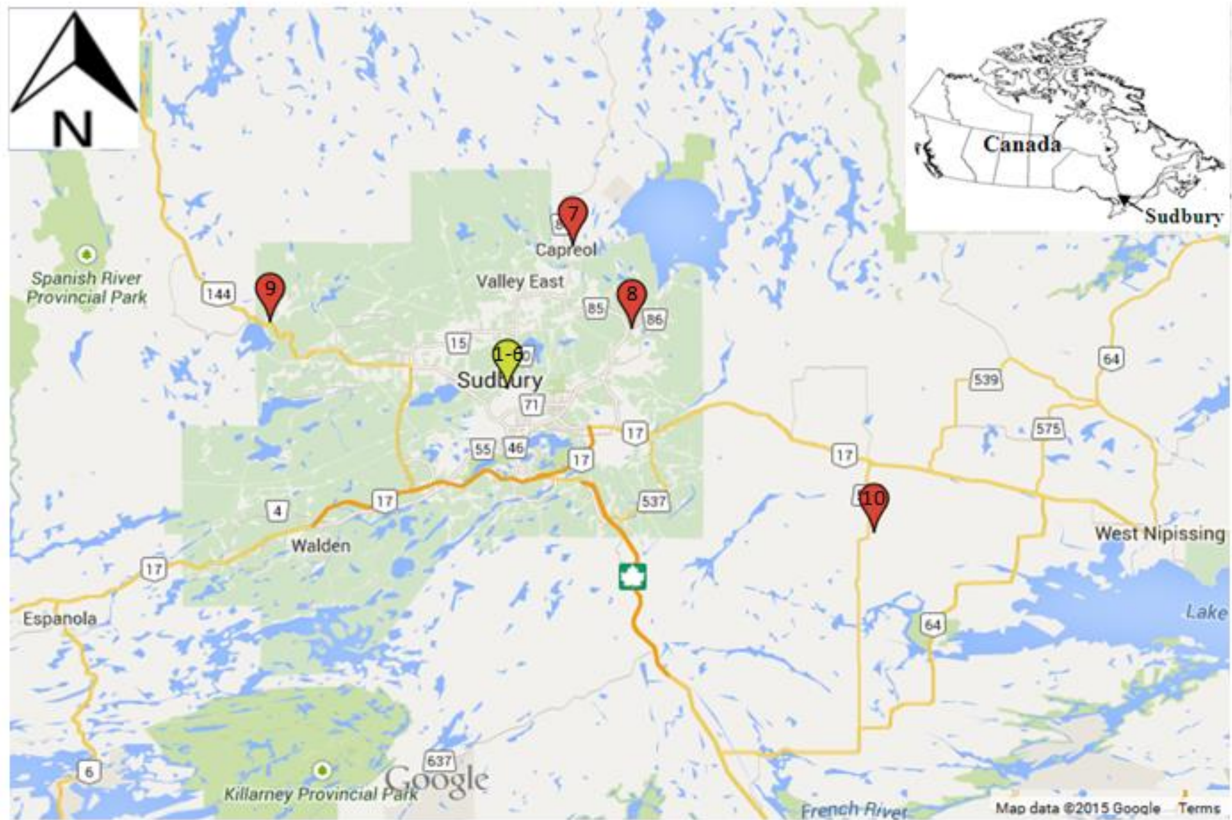

Figure 1. Location of sampling areas from the Greater Sudbury Region.
Site 1: Daisy Lake; Site 2: Wahnapitae Hydro-Dam; Site 3: Laurentian; Site 4: Kukagami; Site 5: Kingsway; Site 6: Falconbridge; Site 7: Capreol (reference); Site 8: St. Charles (reference) and Site 9: Onaping Falls (reference ≈ 90km from Sudbury towards Timmins) and Site 10: Airport.

Sources: Edited from Google Map 2014.

2.3.2 Metal analysis

Only the top soil layer (10 cm depth) was analyzed for the present study as described in (Mehes-Smith et al. 2013b). Five metals (copper, iron, magnesium, nickel and zinc), with a significant level of accumulation in the ecosystems based on previous studies were targeted (Nkongolo et al. 2013). Total and phytoavailable metals were determined as described by (Nkongolo et al. 2013) and (Abedin et al. 2012). For each site, 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 HNO_3 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).

Bioavailable metals were estimated by extracting 5 g of soil with 20 ml of 0.01M $LiNO_3$ in a 50-ml centrifuge tubes in a shaker under ambient lighting conditions for 24 hours at 20 ^{0}C (Abedin et al. 2012). The pH ($LiNO_3$) of the suspension was measured. Subsequently, the mixture was centrifuged at 3000 rpm for 20 minutes followed with filtration of the supernatant through a 0.45 µm filter into a 20 ml polyethylene tube. The filtrate was preserved at approximately 3 ^{0}C for analysis by ICP-MS. All concentrations were calculated in mass/mass dry soil basis. The quality control program completed in an ISO 17025 accredited facility (Elliot Lake Research Field Station of Laurentian University) included analysis of duplicates, Certified Reference Materials (CRM's), Internal Reference Materials (IRM's), procedural and calibration blanks, with continuous calibration verification and use of internal standards (Sc, Y, Bi) to correct for any mass bias. The data obtained for all elements of interest in analyzed CRM soil samples were within ± 12% of the

certified level. Metal contents in leaves were determined according to the protocol described by (Abedin et al. 2012) and (Nkongolo et al. 2008).

To determine the effects of atmospheric deposition of metals on concentration of different elements in plant aerial parts, water-washed and unwashed leaf and branch samples were compared. The translocation factors (TF) were determined according to the equations described by Mehes et al. (2013). Metal translocation factors (TF) were estimated for five metals of interest, copper (Cu), nickel (Ni), iron (Fe), zinc (Zn), and Magnesium (Mg).

2.3.3 Statistical Analysis

The data for the metal levels in soil and tissue samples were analyzed using SPSS 20 for Windows, with all data being transformed using a $\log_{10}$ transformation to achieve a normal distribution. The Student-T test was performed to compare *P. tremuloides* and *A. rubrum* means for accumulation factors and to determine the difference in metal levels between water-washed and unwashed samples ($P \leq 0.05$). Variance-ratio test was done with an assumption of data normality in the underlying population distributions of the data. ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences in metal concentrations ($P \leq 0.05$) among soil, root, branch, and leaf ($P \leq 0.05$) samples.

2.4 Results

2.4.1 Metals content in soil, roots, and leaves

When the metal-contaminated sites were compared to reference sites, the enrichment factors (EF) values were 88.16 (Ni), 53.95 (Cu), 2.47 (Mg), 2.30 (Zn) and 1.62 (Fe) for the bioavailable elements in soil. The EF values for total metals were 34.17, 29.17, 1.68, 1.35 and 0.80 for Cu, Ni, Zn, Fe, and Mg, respectively.

A comparative metal analysis was performed between *Populus tremuloides* and *Acer rubrum* species only for contaminated sites since the amounts of bioavailable elements in reference soils were very small and at lowest detectable levels. Data for the main contaminants (Cu, Ni, and Zn) were analyzed in details. No significant difference was observed between water-washed and unwashed samples for all the metals analyzed based on the student T test. This indicates that metal accumulation in aerial plant parts is internal. For trembling aspen (poplar – *P. tremuloides*), the level of Cu was significantly lower in roots compared to bioavailable portion in soil based on Tukey's multiple comparison test ($P \leq 0.05$). Ni content in roots was also significantly lower than in soil, but the total accumulation of this element in leaves was much higher compared to branches and roots. There was a steady increase of Zn from soil to roots, branches, and leaves (Figure 2).

For red maple (*A. rubrum*), there were similar levels of Cu in roots compared to bioavailable portion in soil. No significant accumulation of Cu in branches and leaves was observed. The amount of Ni was significantly less in roots compared to soil and the accumulation of this element in branches and leaves was limited. The amount of Zn was in general low in soil, roots, branches, and leaves (Figure 3).

The bioaccumulation factors (ratios of metal content in roots over bioavailable metal levels in soil) for *Populus tremuloides* and *A. rubrum* was calculated for key metals. There were significant differences for bioaccumulation factors for Cu and Mg between *P. tremuloides* and *A. rubrum* based on the Student T test (Figure 4). Overall, for *P. tremuloides*, there was no accumulation of metals in roots compared to the bioavailable levels in soil for Cu and Ni (BAF < 1). But the bioaccumulation factor was higher (BAF > 1) for Zn. For red maple, the BAFs followed the same trend.

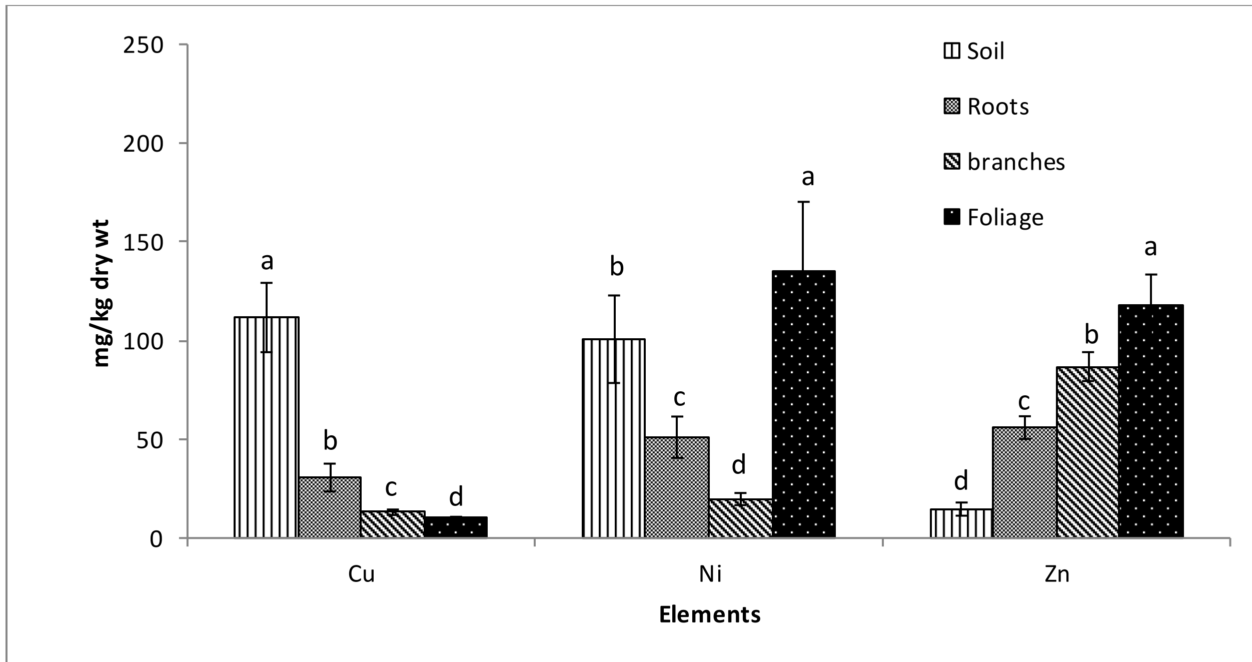

Figure 2. Metal concentration (mg/kg dry wt) in soil and plant tissue for plants found growing on metal contaminated soil for trembling aspen (*Populus tremuloides*).

Mean values for each element followed by the same letter are not significantly different based on Tukey's HSD multiple comparison test ($P \geq 0.05$).

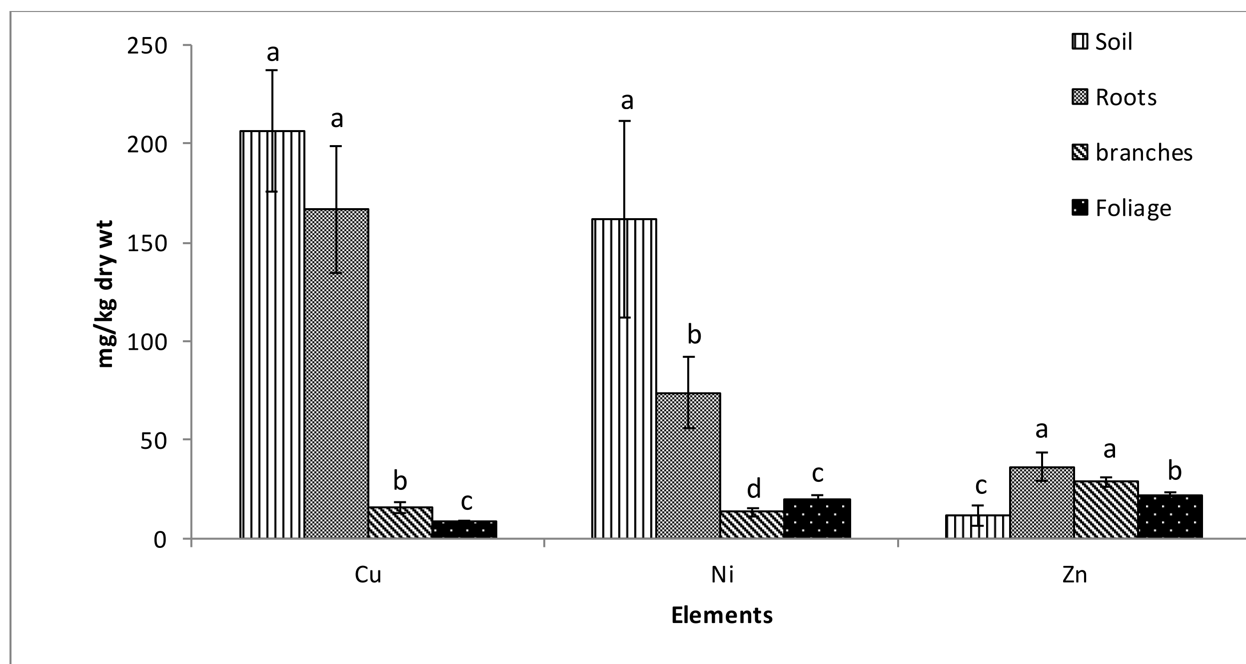

Figure 3. Metal concentration (mg/kg dry wt) in soil and plant tissue for plants found growing on metal contaminated soil for in red maple (*Acer rubrum*).

Mean values for each element followed by the same letter are not significantly different based on Tukey's HSD multiple comparison test ($P \geq 0.05$).

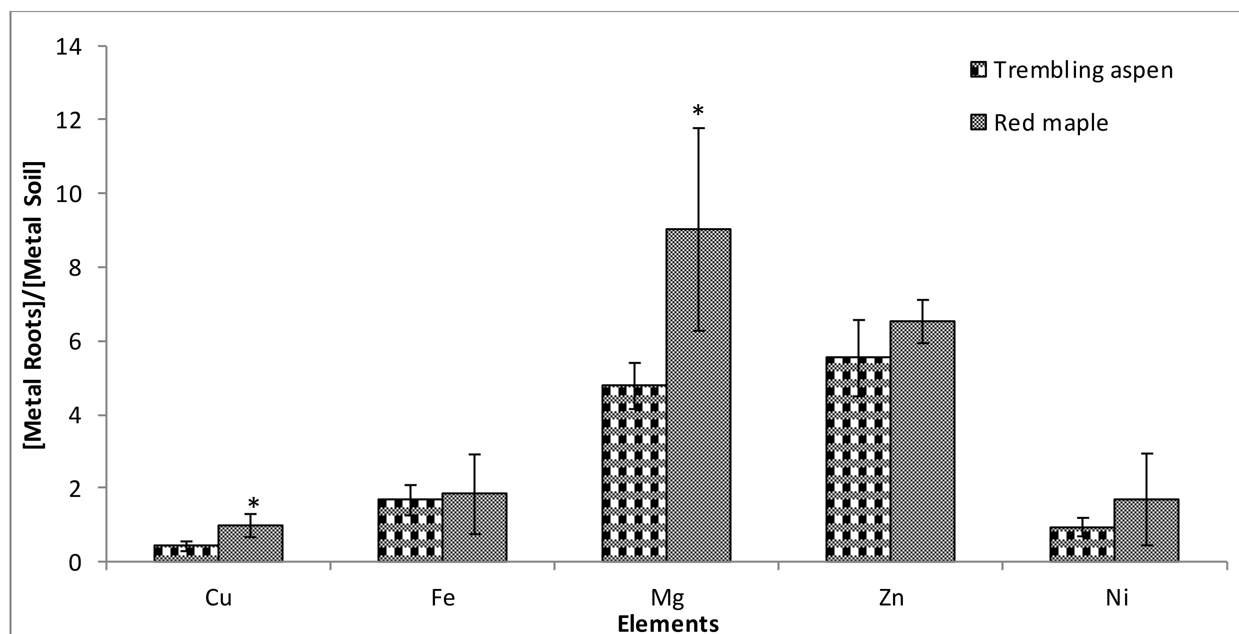

Figure 4. Bioaccumulation factor is the metal concentration ratio of plant roots to soil. BAC for the contaminated sites for Cu, Fe, Mg, Ni and Zn were calculated and recorded for trembling aspen (*Populus tremuloides*) and in red maple (*Acer rubrum*).

*Indicates significant differences between means for each element-based Student T-test ($P \geq 0.05$).

2.4.2 Translocation factors

The translocation factor (TF) was calculated as the ratio of the concentration of total metal in aerial parts of the plants compared to metal content in roots. The TF data are summarized in Tables 1 to 6. The average TF from roots to branches in *P. tremuloides* were 0.86, 0.29, 1.18, 1.20, and 1.76 for Cu, Fe, Mg, Zn, and Ni, respectively. These values were 0.18 (Cu), 0.15 (Fe), 2.05 (Mg), 0.77 (Zn), and 0.86 (Ni) for *A. rubrum* (Table 3 and 4). The TF from branch to leaves for *P. tremuloides* were on average 0.84, 2.69, 2.27, 6.58, and 1.45 for Cu, Fe, Mg, Zn, and Ni, respectively. For Acer rubrum these factors were 0.66 (Cu), 0.69 (Fe), 3.92 (Mg), 0.79 (Zn), and 1.79 (Ni) (Tables 5 and 6). Hence, the translocation factors from roots to leaves for *Populus tremuloides* were generally much greater than *Acer rubrum* for each element. There were significant variations in TF among sites. In general, the TF values were very high for Mg and low for Cu for both species (Tables 1 – 6). The TF from roots to leaves was higher compared to TF from roots to branches. This was translated in more metals accumulations in leaves compared to branches (Tables 1 – 4).

For *P. tremuloides*, the TF values from roots to leaves were low for Cu and high for Fe, Mg, Ni, and Zn. They varied from 0.52 to 3.26 for Fe, 3.39 to 5.47 for Mg, 1.41 to 4.1 for Zn, and 2.6 to 16.4 for Ni. An opposite trend was observed in red maple as the TF was low for all the elements except Mg. It varies from 0.08 to 0.17 for Fe, 0.71 to 0.90 for Zn, and 0.26 to 0.81 for Ni. The TF for Mg varies from 2.62 to 4.13 (Tables 1 – 6).

Table 1. Translocation factor of Cu, Fe, Mg, Ni and Zn in trembling aspen (*Populus tremuloides*) growing on metal contaminated sites (metal concentration ratio of branches to roots).

Sites	Elements				
	Cu	**Fe**	**Mg**	**Zn**	**Ni**
Coniston	0.35±0.17	0.11±0.03	0.83±0.15	0.39±0.26	1.53±0.35
Laurentian	1.15±0.38	0.35±0.10	1.47±0.56	1.16±0.75	1.95±0.36
Kelly Lake	0.81±0.49	0.19±0.07	1.01±0.24	2.45±1.99	1.13±0.26
Falconbridge	1.19±0.29	0.50±0.12	1.39±0.16	0.82±0.13	2.43±0.10

Table 2. Translocation factor of Cu, Fe, Mg, Ni and Zn in red maple (*Acer rubrum*) growing on metal contaminated sites (metal concentration ratio of branches to roots).

	Elements				
Sites	**Cu**	**Fe**	**Mg**	**Zn**	**Ni**
Coniston	0.08±0.02	0.13±0.03	1.39±0.96	1.14±0.17	0.52±0.36
Laurentian	0.42±0.32	0.17±0.12	1.18±1.27	0.27±0.29	1.53±0.35
Kelly Lake	0.04±0.01	0.15±0.04	3.59±4.18	0.89±0.19	0.54±0.56

Table 3. Translocation factor of Cu, Fe, Mg, Ni and Zn in trembling aspen (*Populus tremuloides*) growing on metal contaminated sites (metal concentration ratio of leaves to branches).

Sites	Elements				
	Cu	**Fe**	**Mg**	**Zn**	**Ni**
Coniston	1.08±0.12	3.59±1.05	2.49±0.34	6.34±1.57	1.40±0.38
Laurentian	0.65±0.09	2.57±0.68	1.88±0.28	6.91±1.13	1.57±0.62
Kelly Lake	0.86±0.15	2.27±0.37	1.98±0.16	5.78±0.98	1.16±0.28
Falconbridge	0.78±0.12	2.34±0.48	2.74±0.59	7.28±1.84	1.68±0.58

Table 4. Translocation factor of Cu, Fe, Mg, Ni and Zn in red maple (*Acer rubrum*) growing on metal contaminated sites (metal concentration ratio of leaves to branches).

Sites	Elements				
	Cu	**Fe**	**Mg**	**Zn**	**Ni**
Coniston	0.91±0.13	0.56±0.09	3.81±4.21	0.77±0.08	2.12±2.41
Laurentian	0.61±0.08	0.80±0.11	2.96±0.73	0.76±0.10	1.90±0.44
Kelly Lake	0.45±0.08	0.71±0.05	4.98±0.57	0.83±0.13	1.35±0.05

Table 5. Translocation factor of Cu, Fe, Mg, Ni and Zn in trembling aspen (*Populus tremuloides*) growing on metal contaminated sites (metal concentration ratio of plant leaves to roots).

	Elements				
Sites	**Cu**	**Fe**	**Mg**	**Zn**	**Ni**
Coniston	0.41±0.23	1.55±0.55	5.23±0.77	1.95±0.50	2.16±1.48
Laurentian	0.74±0.25	1.34±0.51	3.39±1.21	2.44±0.46	8.97±6.72
Kelly Lake	0.72±0.43	3.26±1.21	5.00±1.86	1.41±0.65	16.39±13.81
Falconbridge	0.97±0.36	0.52±0.18	5.47±1.20	4.10±1.45	6.01±1.78

Table 6. Translocation factor of Cu, Fe, Mg, Ni and Zn in red maple (*Acer rubrum*) growing on metal contaminated sites (metal concentration ratio of plant leaves to roots).

Sites	Elements				
	Cu	**Fe**	**Mg**	**Zn**	**Ni**
Coniston	0.07±0.02	0.08±0.09	4.13±3.42	0.90±0.92	0.89±0.84
Laurentian	0.32±.027	0.17±0.14	3.12±0.47	0.78±0.35	0.68±0.47
Kelly Lake	0.07±0.03	0.08±0.02	2.62±0.73	0.71±0.13	0.26±0.07

2.5 Discussion

Total concentrations of metals in soils are a poor indicator of metal toxicity since metals exist in different solid-phase forms that can vary greatly in terms of their bioavailability (Krishnamurti and Naidu 2002; Huang and Gobran 2005; Krishnamurti et al. 2007; Violante et al. 2010). In general, the availability of metals for plants depends on soil pH and on organic matter contents (de Matos et al. 2001). The mobility, bioavailability, and potential toxicity of a metal in soil depend on its concentration in soil solution, the nature of its association with other soluble elements, and soil ability to release the metal from the solid phase to replenish that removed from soil solution by the plants (Krishnamurti and Naidu 2002; Huang and Gobran 2005; Violante et al. 2010). Metal uptake by plants depends on the bioavailability of metal in the water phase, which in turn depends on the retention time of the metal, as well as the interaction with other elements and substances in the water (Fritioff and Greger 2003; Tangahu et al. 2011). Because the uptake of metals by plants root system is restricted to the liquid phase, the content of particular element in the soil solution is of primary importance. Plants have evolved specialized mechanisms to increase the concentration of metal ions in soil solution. They can alter the chemical environment of the rhizosphere to stimulate the desorption of ions from soil solids into solution. Some plants can regulate solubility in the rhizosphere by exuding a variety of organic compounds from roots (Romheld and Marschner 1986; Crowley et al. 1991).

The analysis of plant tissues was important in determining the level of metal uptake and the mobility of elements in *Populus tremuloides* and *Acer rubrum*. These two species do not accumulate Cu in their roots and the translocation of this element to leaves is very limited.

Based on plant classification described by Boularbah et al. 2006, Ernst 2006, and Mganga et al. 2011, Mehes-Smith et al. 2013a with reference to metal translocation from roots to leaves, the results of the present study reveal that trembling aspen (*P. tremuloides*) is an accumulator of Mg, Zn, and Ni while red maple (*A. rubrum*) does not accumulate these metals in the leaves. In fact, *A. rubrum* can be considered an excluder for Zn and Mg, and to some extend for Fe but not for Cu and Ni. For these two main contaminants in the region, red maple shows avoidance as the main mechanism of coping with soil metal accumulation. Other studies revealed that in hard wood species such as red oak (*Quercus rubra*) growing in the Greater Sudbury Region, the translocation of metals to leaves is significantly higher (Tran et al. 2014). (Leavitt et al. 1979) also reported accumulations of Ag, Cd, Cu and Zn metals in red oak tissue.

The uptake of metals is affected by plant species characteristics. Phytoextraction or phytoaccumulation is the uptake and translocation of metal contaminants in the soil into the aerial parts of the plants via the roots (Nascimento and Xing 2006; Van Nevel et al. 2007). In general, variation in plants species, the growth stage of the plants and the element characteristics control absorption, accumulation and translocation of metals. The uptake of metals by plants from the soil depends also on the level of pollution, the forms of the elements in soil, its mobility in the soil-plant system, and on plant species (Boruvka and Vacha 2006). The knowledge about the abilities of different plant species or tissues to absorb and transport metals under different conditions is essential for bioremediation studies (Nouri et al. 2009).

Results show that leaf tissues accumulate more metals (Ni and Zn) in trembling aspen (*P. tremuloides*) than roots which indicate greater mobility of these metals. For *A. rubrum*, the

concentrations of Ni and Zn was low in leaf, branch and roots tissues compared to amount of the bioavailable levels of these metals in soil. Zinc is an essential element to all plants. The normal level content in aerial parts is 66 μg/g and the toxic level is above 230 μg/g (Outridge and Noller 1991). Therefore, the amount of zinc (< 100 μg/g) in trembling aspen and red maple tissues is below the toxic level in plants.

Plants require also Cu as an essential micronutrient for normal growth and development; when this ion is not available plants develop specific deficiency symptoms, most of which affect young leaves and reproductive organs. The redox properties that make Cu an essential element also contributes to its inherent toxicity. The average content of Cu in plant tissue is 10 μg/g dry weights (Yruela 2005). The concentrations of Cu in *P. tremuloides* and *A. rubrum* tissues in the present study were below 30 μg/g which are also under the toxic level for most plants (Yruela 2005).

The accumulation of Ni and Zn in plants tissues in trembling aspen suggests tolerance mechanisms. The small amount of these elements in red maple suggests avoidance mechanisms. Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification and thus tolerance to heavy metal stress. These mechanisms are involved primarily in avoiding the build-up of toxic concentrations at sensitive sites within the cell and thus preventing the damage effects. There is strong evidence that tolerant plants show homeostatic mechanisms to regulate the concentration of metal ions inside the cell to minimize the potential damage that could result from the exposure to nonessential metal ions (Hall 2002). Tolerance could also involve reduced influx across plasma membrane, active efflux into apoplast, chelation in cytosol by various ligands, repairs and protection of plasma membrane under stress conditions, transport of PC-Cd complex into the vacuole, and transport and accumulation of metals in vacuole (Hall 2002). The

mechanisms used by red maple to exclude potentially toxic elements are unknown. But in general, plant can exclude metal by restricting their entry into root cells. They can also exclude them from active metabolic locations once inside the cell (Leavitt et al. 1979). In fact, metals impose severe stress on plants, especially in the rooting zone, which has led to the evolution of metal-tolerant ecotypes in several herbaceous species like *D. cespitosa* (Cox and Hutchinson 1980).
Selection of metal-tolerant genotypes has been demonstrated to occur rapidly, within one or two generations in populations that contain the necessary genetic information (Wu et al. 1975). Two factors that may affect the plant's ability to tolerate metals are the intensity of the contamination and the amount of time the population has been exposed to the toxic levels (Wu et al. 1975).

Several studies have shown that plants can automatically acquire characteristic resistance against toxicants including heavy metals, depending upon the various ecophysiological factors in times and species (Porter and Peterson 1977). Metals are mobilized and taken by root cells from soil, bound by cell wall and then transported across the plasma membrane driven by ATP dependent proton pumps that catalyzes H+ extrusion across the membrane (Mäser et al. 2001; Singh et al. 2003, 2010). Putative metal transporters have been identified for all transition metals, but in most cases, clear evidence of where and how they operate within the plant is still required.

Translocation of metals from roots to above ground tissues is a crucial physiological process in an effective utilization of plant to remediate polluted sites (Zacchini et al. 2009; Galfati et al. 2010; Majid et al. 2012). Plant uptake-translocation mechanisms are likely to be closely regulated (Tangahu et al. 2011). Higher level of metal accumulation in aerial plant parts of *P. tremuloides* showed biomagnification of metals. Metal hyperaccumulation/accumulation and metal exclusion

or avoidance (identified in red maple) are two opposed physiological strategies used by plants to grow and reproduce on soils containing high, potentially toxic levels of transition metal ions of geogenic or anthropogenic origin (Raskin and Ensley 2000).

2.6 Conclusion

Overall, the two species (*P. tremuloides* and *A. rubrum*) use different mechanisms in dealing with the main contaminant Ni in the region and also with Zn. They can be useful for bioremediation for different purposes. *P. tremuloides* can be used for phytoextraction/phytostabilisation of Ni and Zn. *A. rubrum* on the other hand cannot be recommended for phytoremediation, but will be useful for revegetation or reforestation of Ni contaminated sites because of its avoidance mechanisms.

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Chapter 3: Analysis of gene expression in red maple (*Acer rubrum*) and trembling aspen (*Populus tremuloides*) populations from a mining region

3.1 Abstract

The Greater Sudbury Region has been known as one of the most ecologically disturbed areas in Canada for the past century. Plant adaptation to environmental stressors often results in modifications in gene expression at the transcriptional level. The main objective of the present study was to compare the expression of genes associated with nickel resistance in *Acer rubrum* and *Populus tremuloides* growing in areas contaminated and uncontaminated with metals. Primers targeting Nramps4, Nas 3, At2G, MRP4 and alpha-tubulin genes were used to amplify cDNA of both species. The expression of the At2G gene, was 2x and 9 x higher in *P. tremuloides* than in *A. rubrum* for St. Charles (uncontaminated site) and Kelly Lake (metal contaminated site), respectively. There was a much smaller difference between the two species for the Nramps 4 gene as its expression was 2.5 x and 3 x higher in *P. tremuloides* compared to *A. rubrum* from St. Charles and Kelly Lake, respectively. The same trend was observed for the MRP4 gene whose expression was 2x and 14 x higher in *B. tremuloides* than in *A. rubrum* from St. Charles and Kelly Lake, respectively. For the Nas 3 gene, the expression was similar in both sites. This gene was upregulated 11x and 10 x in *P. tremuloides* compared to *A. rubrum* in samples from St. Charles and Kelly Lake, respectively. In general, no significant difference was observed between the metal contaminated and uncontaminated sites for gene expression. In depth analysis revealed that AT2G and MRP4 genes were significantly down regulated in *A. rubrum* from the metal contaminated sites compared to those from uncontaminated areas, but environmental factors driving this differential gene expression couldn't be established.

Key words: *Acer rubrum; Populus tremuloides*; Gene regulation; Metal contamination; Greater Sudbury Region.

3.2 Introduction

For the past century, the Greater Sudbury Region (GSR) has been heavily affected by logging, mining and sulphide ore smelting. As a result of these activities, more than 100 million tonnes of SO_2 and thousands of tonnes of cobalt, copper (Cu), nickel (Ni), and iron (Fe) ores were released into the atmosphere. These elements came from the open roast beds (1888-1929) and smelters (1888-present) (Freedman and Hutchinson 1980). Terrestrial and aquatic ecosystems within 30 km of the smelters were heavily contaminated with metals and the pH in these areas did decrease significantly(Gratton et al. 2000). Previous studies have reported a strong relationship between the distance from the source of pollution and metal concentration in certain plants (Bagatto et al. 1993).

Cu, Ni, cobalt (Co), and Fe are among the most abundant deposits in GSR. These metals play an important role in plant nutrition. Ni, one of the most abundant metals in the GSR is classified as a micronutrient that is needed in small quantities by higher and lower plants for their growth (Lin and Kao 2005). At higher concentrations ranging from 25 to 246 $\mu g \cdot g^{-1}$ dry weight (DW) of plant tissue, it becomes toxic (Iyaka 2011).

Red maple (*Acer rubrum*) is the most widespread tree in North America. It tolerates environmental stressors and it is resistant to flooding, and to ice damage (Ahlgren and Hansen 1957; Arthur et al. 1981). Even though this species is resistant to land-fill contaminated gases (Arthur et al. 1981), its vegetation can still be damaged by air pollution (Krause and Dochinger 1987). *A. rubrum* is the most abundant tree species after white birch (*Betula papyrifera*) in the GSR even in areas that are heavily contaminated with metals (James and Courtin 1985; Theriault et al. 2013; Kalubi et al. 2015). Trembling aspen (*Populus tremuloides*) is also widely distributed in North America and in

other continents. This plant species is suitable for phytoremediation because of the pumping action of its root system that remove contaminants from soils (Mehes-Smith and Nkongolo 2015) .

Plant adaptation to environmental stressors often result in modifications in gene expression at the transcriptional level. Changes in gene regulation from different stressors have already been reported. Some of these stressors analyzed include metals, water stress, heat shock, anaerobic and aerobic agents (Matters and Scandalios 1986). Most gene expression studies have been conducted in controlled conditions. . A recent review of recent literature revealed that 11 genes have been associated with nickel resistance in model and non-model plants. They include, 1-aminocyclopropane-1-carboxylic acid deaminase (ACC), high affinity nickel transporter family protein (AT2G16800), iron-regulated protein (IREG), glutathione reductase (GR), glutathione-s-transferase, Metal transporter (NRAMP 1,2,3,4), Nicotianamine synthase (NAS3), Putative transmembrane protein (TMP), Serine acetyltransferase (SAT), Thioredoxin family protein, Zn finger protein of Arabidopsis thaliana (ZAT11), MRP4 (Freeman et al. 2004; Lemaire et al. 2004; Stearns et al. 2005; Mizuno et al. 2005; Schaaf et al. 2006; Mari et al. 2006; Visioli et al. 2012; Liu et al. 2014; Theriault et al. 2016). Studies on gene expression in the field are limited.

The main objective of the present study was to compare the expression of metal transporters and chelator gene in *A. rubrum* and *P. tremuloides* growing in field sites contaminated and uncontaminated with metals.

3.3 Materials and Methods

3.3.1 Field Sampling

Roots from *A. rubrum* and *P. tremuloides* were collected at two different sites in the Greater Sudbury Region: St. Charles (metal-uncontaminated site) and Kelly lake (metal-contaminated site) (Fig. 5). Current levels of metals in these sites have been already reported in Nkongolo et al., (2013), Theriault et al. (2013, 2014), and Kalubi et al. (2015). Root samples were collected from 10 trees that were approximately 25 to 35 years of age for each species. All samples were flash frozen in liquid nitrogen on site and then stored at -80°C in the lab until RNA extraction.

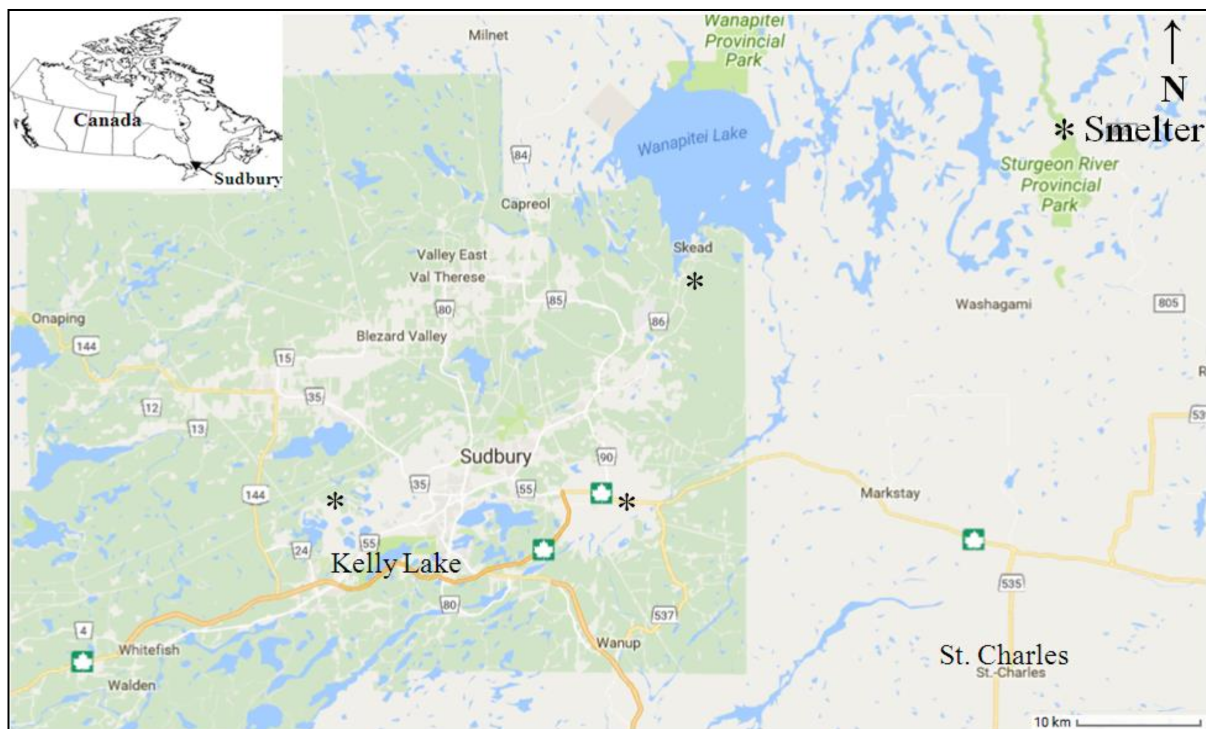

Figure 5: Location of sampling area from the Greater Sudbury region. St. Charles (reference) and Kelly Lake (contaminated site).

Sources: Edited from Google Map 2014.

3.3.2 RNA extraction

Total RNA was extracted from *A. rubrum* and *P. tremuloides* root samples using the procedure previously described by Theriault et al. (2016) and Djeukam et al. (2016). RNA was quantified using the Qubit RNA BR Assay kit from Life Technologies (Carlsbad, United States). A 1% agarose gel was used to verify the quality of the extracted RNA. One microgram of RNA from samples of the same population was pooled together and used for downstream processes.

3.3.3 RT-qPCR

Extracted RNA was treated with DNase 1 (#EN0521) from Life Technologies. There were 11 candidate genes that were chosen based on literature in table 1. The primers for each gene were designed for each species using *A. rubrum* transcriptome and *Populus trichocarpa* genome. The primers were designed to span the exon-exon border of the gene. Genes associated with Ni resistance were matched to the *A. rubrum* transcriptome and *P. tremuloides* genome. For both species, α-tubulin was used as a reference control. Primers were checked for hairpins, self, and hetero-dimers using the OligoAnalyzer 3.1 by IDT (https://www.idtdna.com/calc/analyzer). The cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit by Life Technologies. PCR was performed on the DNA and cDNA of *A. rubrum* and *P. tremuloides*. A 1% agarose gel was made to verify the size of the amplicons which come from the amplification of the primer pairs. Only primers that showed a reproducible single band of the appropriate size were used for RT-qPCR. This was performed using the Dynamo HS SYBR Green Kit by Life Technologies according to the manufacturer's protocol. Each sample was amplified with the MJ Research PTC-200 Thermal Cycler in triplicates. The process included (1) initial denaturing at 95°C for 15 min; 2) denaturing at 94°C for 30 sec; 3) 30 sec at 55 ^{0}C annealing; 4) elongation at 72°C for 30 sec; 5) read 6) repeat step 2 –6 for 41 cycles; 7) final elongation at 72°C for 7 min; 8)

melting curve 72 –95°C, every 1°C, hold for 10 sec; and 9) final elongation period at 72°C for 3 min. The qPCR was ran at two different times with each sample in triplicate. As a result, there were six data points for each sample. The data were analyzed using the MJ Opticon Monitor 3.1 by BioRad and C(t) values were determined using excel. C(t) values were normalized to α-tubulin.

3.3.4 Statistical analysis

The gene expression data were analyzed using SPSS 20 for Windows. All data were transformed to achieve a normal distribution. Student T test was performed to determine significant differences in gene expression levels between *A. rubrum* and *P. tremuloides* and between the two sites. All the differences were determined at $P \leq 0.05$.

3.4 Results

A total of 11 primer pairs targeting 11 different genes (Table 7) were screened for amplification using *A. rubrum* and *P. tremuloides* cDNA. Five of these 11 primers generated consistent amplification bands on agarose gels (Table 8). They include primers targeting Nramps4, Nas 3, At2G, MRP4 and alpha-tubulin (used as housekeeping gene). These selected primers produced a repeatable band with cDNA of both species. The expression of the At2G gene was 2x and 9 x higher in *P. tremuloides* than in *A. rubrum* for St. Charles (uncontaminated site) and Kelly Lake (metal contaminated site), respectively (Fig. 6). There was a much smaller difference between the two species for the Nramps 4 gene as its expression was 2.5 x and 3 x higher in *P. tremuloides* compared to *A. rubrum* from St. Charles and Kelly Lake, respectively (Fig. 7). The same trend was observed for the MRP4 gene whose expression was 2x and 14 x higher in *P. tremuloides* than in *A. rubrum* from St. Charles and Kelly Lake, respectively (Fig. 8). For the Nas 3 gene, the expression was similar in samples from both sites. This gene was upregulated 11x and 10 x in *P. tremuloides* compared to *A. rubrum* in samples from St. Charles and Kelly Lake, respectively (Fig. 9). Overall, the expression level of each gene was significantly higher in *P. tremuloides* compared to *A. rubrum*.

In general, no significant difference was observed between the metal contaminated and uncontaminated sites for gene expression (Fig. 10 and 11). In depth analysis showed that AT2G and MRP4 genes were significantly down regulated in *A. rubrum* from the metal contaminated sites compared to those from the uncontaminated area, but environmental factors driving this differential gene expression couldn't be determined (Fig. 10 and 11).

Table 7. Candidate genes involved in nickel resistance in model and non-model plant species

Gene	Species	Reference
1-aminocyclopropane-1-carboxylic acid deaminase (ACC)	*Brassica napus*	Stearns et al., 2005
high affinity nickel transporter family protein (AT2G16800)	*Arabidopsis thaliana*	Stearns et al., 2005
iron-regulated protein (IREG)	*Arabidopsis thaliana*	Schaaf et al., 2006
glutathione reductase (GR)	*Thlapsi goeisngense*	Freeman et al., 2004
glutathione-s-transferase	*Thlapsi goesingense*	Freeman et al., 2004
	Betula papyrifera	Theriault et al., 2016
metal transporter (NRAMP 1,2,3,4)	*Thlaspi japonicum*	Mizuno et al., 2005
	Noccaea Caerulescens	Visioli et al., 2012
	Thlaspi caerulescens	Wei et al., 2008
	Betula papyrifera	Theriault et al., 2016
nicotianamine synthase (NAS3)	*Noccaea Caerulescens*	Visioli et al., 2012
	Thlapsi goesingense	Mari et al., 2006
putative transmembrane protein (TMP)	*Betula papyrifera*	Theriault et al., 2016
aerine acetyltransferase (SAT)	*Thlapsi goesingense*	Freeman et al., 2004
thioredoxin family protein	*Chlamydomonas reinhardtii*	Lemaire et al., 2004
	Betula papyrifera	Theriault et al., 2016
Zn finger protein of *Arabidopsis thaliana* (ZAT11)	*Arabidopsis thaliana*	Liu et al. 2014

Table 8. Sequences of trembling aspen (*Populus tremuloides*) and red maple (*Acer rubrum)* primers used for RT-qPCR

Target	Melting temp (° C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
Populus tremuloides					
MRP4	F: 60.00	F:TGTTGGCTTAGCGCTCCTAT	158	158	158
	R: 60.01	R:TTGCCTTCATCCTTGAATCC			
NAS3	F: 60.02	F:AAAGTTGCGTTTGTGGGTTC	232	232	232
	R: 59.98	R:CTGCCAAGAAGACGACATCA			
NRAMP4	F: 59.96	F: CCTTGTAAATGCAGGGCAAT	292	292	292
	R: 60.02	R: TGACTGCAGCACATTTAGCC			
AT2G16800	F: 59.97	F:AAAGGACCGACTCCACATTG	239	239	239
	R: 60.21	R:GTAGCCCATGGACAATACCG			
Acer rubrum					
MRP4	F: 60.17	F:ATTCAAAACGGGACCATTGA	146	146	146
	R: 60.26	R:CCACGCTCTCCAATTTCAGT			
NAS3	F: 59.99	F:TGTGGAGAAGCTGAGGGACT	248	248	248
	R: 60.02	R:GCGGTTTTTGTGAGGTGTTT			
NRAMP4	F: 59.96	F: TGGAGTTGTGGGTTGCATTA	240	240	240
	R: 59.96	R: CCTGCATTTACAAGGCCAAT			
AT2G16800	F: 59.99	F:GCTGCTTTGGCTCCACTATC	253	253	253
	R: 60.13	R:CGTTTTCTAGGGCGACACAT			
Housekeeping					
α-tubulin	F: 60.21	F: GGCAAGCAGGGATTCAAGTA	150	752	150
(*Populus tremuloides*)	R: 60.36	R: GGCACATGTTTTCCAGAACC			
α-tubulin	F: 60.01	F: GGTGTTCAATGCTGTTGGTG	213	387	213
(*Acer rubrum*)	R: 59.99	R: TCATTGTCCAAGAGCACAGC			

Primers were designed by matching gene sequences to the *A. rubrum* transcriptome and *Populus trichocarpa* genome. When possible primers were designed to span the exon-exon border of the gene.

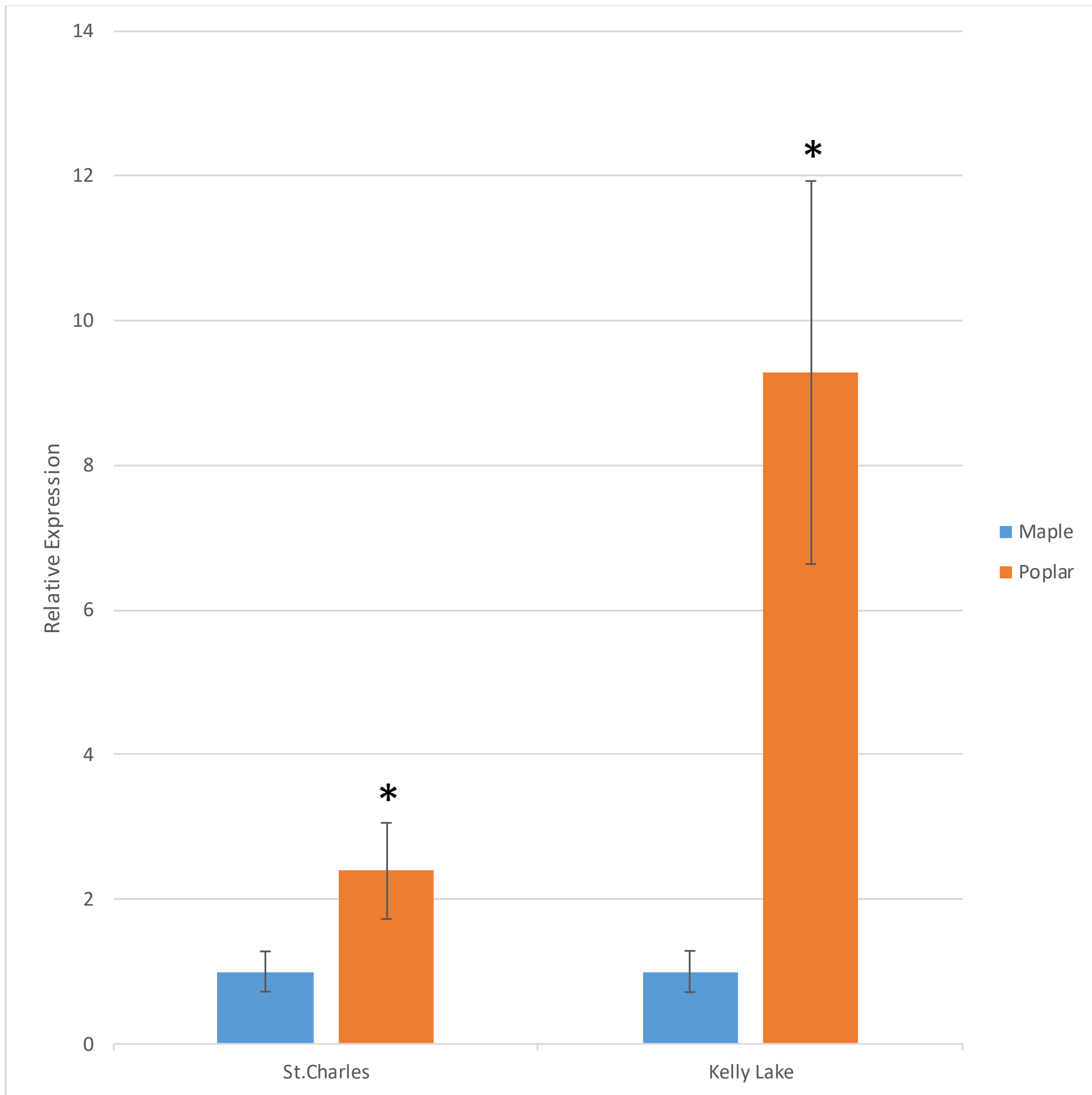

Figure 6: AT2G gene expression of Maple (Acer rubrum) and Poplar (Populus tremuloides) from St.Charles and Kelly Lake. The expression of this gene was normalized based on the α-tubulin housekeeping gene and expressed relative to *A. rubrum* level.

*Represent significant differences between poplar (*Populus tremuloides*) and maple (*Acer rubrum*) ($p \leq 0.05$).

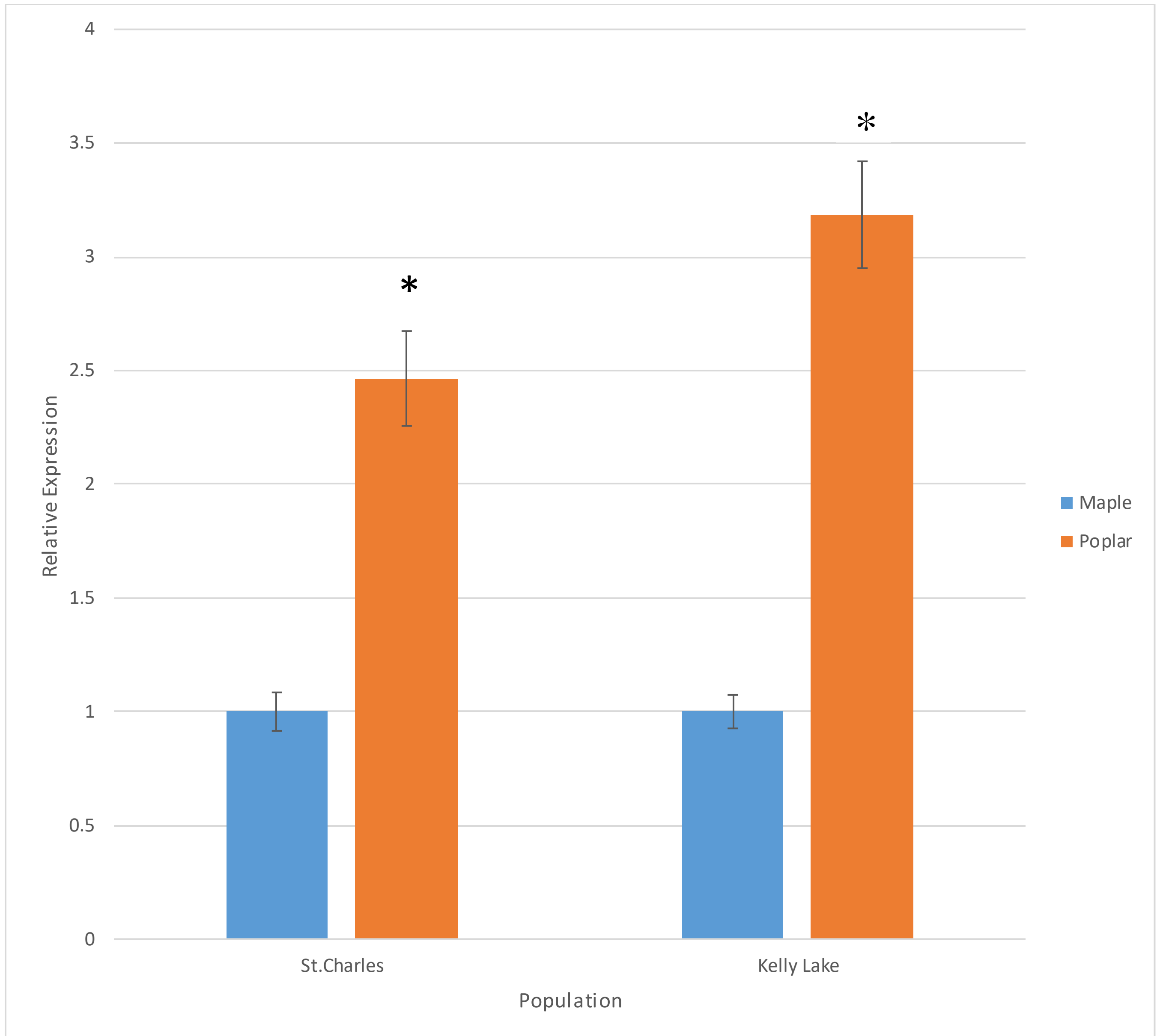

Figure 7: Nramps4 gene expression of Maple (*Acer rubrum*) and Poplar (*Populus tremuloides*) from St. Charles and Kelly Lake. The expression of this gene was normalized based on the α-tubulin housekeeping gene and expressed relative to *A. rubrum* level.

*Represent significant differences between Poplar (*Populus tremuloides*) and maple (*Acer rubrum*) ($p \leq 0.05$).

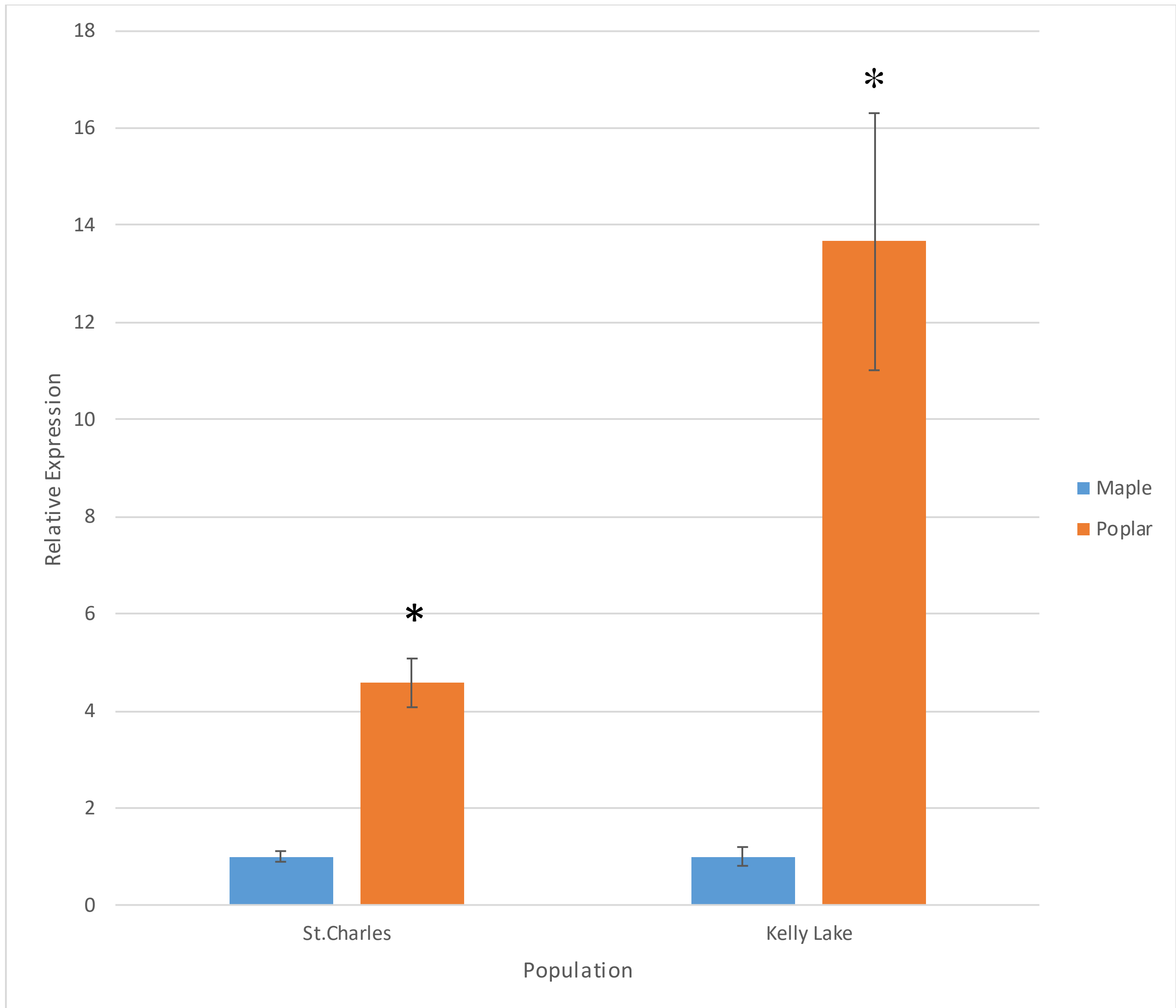

Figure 8: MRP4 gene expression of Maple (Acer rubrum) and Poplar (*Populus tremuloides*) from St. Charles and Kelly Lake. The expression of this gene was normalized based on the α-tubulin housekeeping gene and expressed relative to *A. rubrum* level.

*Represent significant differences between Poplar (*Populus tremuloides*) and maple (*Acer rubrum*) ($p \leq 0.05$).

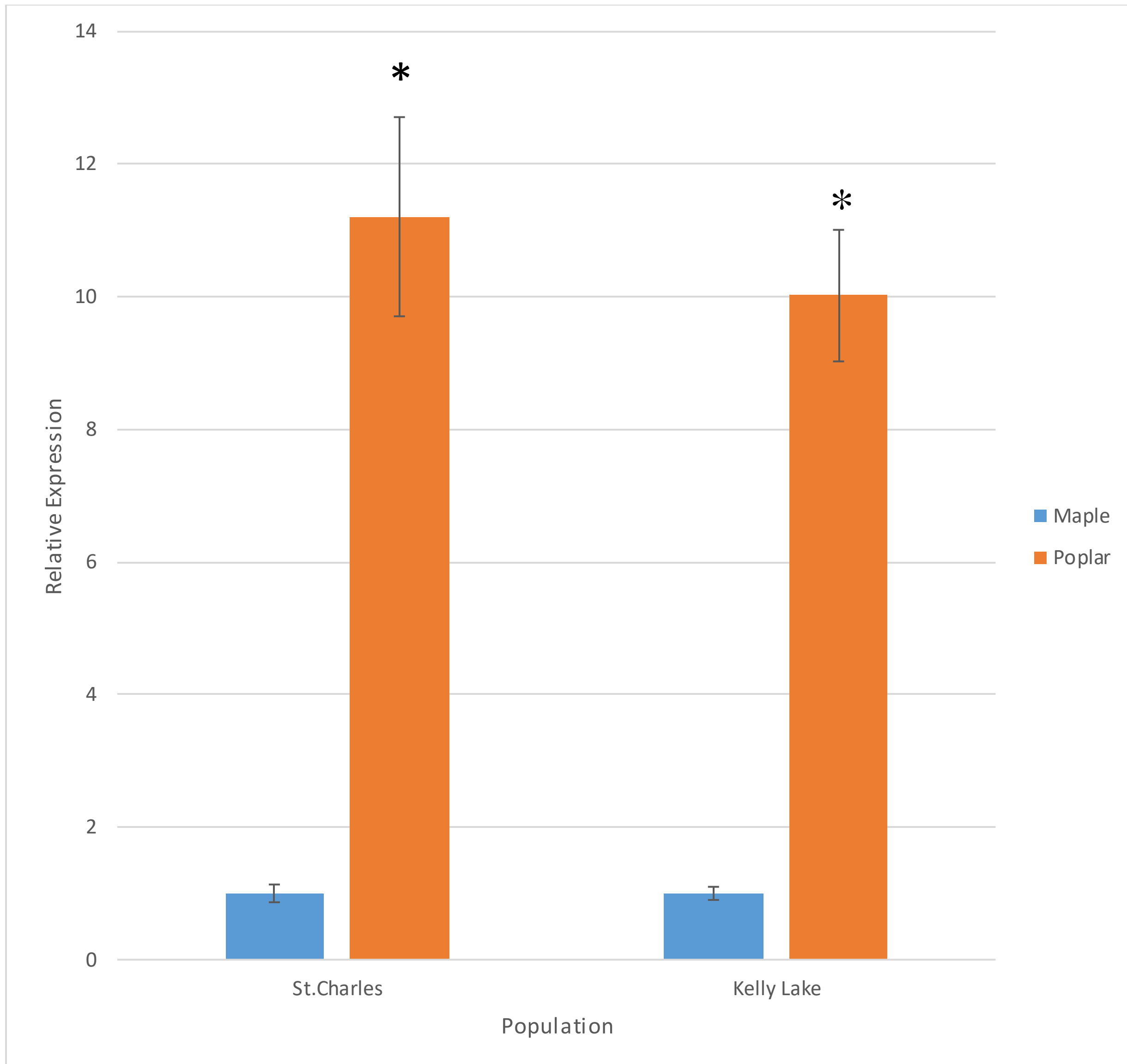

Figure 9: Nas3 gene expression of Maple (Acer rubrum) and Poplar (*Populus tremuloides*) from St. Charles and Kelly Lake. The expression of this gene was normalized based on the α-tubulin housekeeping gene and expressed relative to *A. rubrum* level.

*Represent significant differences between Poplar (*Populus tremuloides*) and maple (*Acer rubrum*) ($p \leq 0.05$).

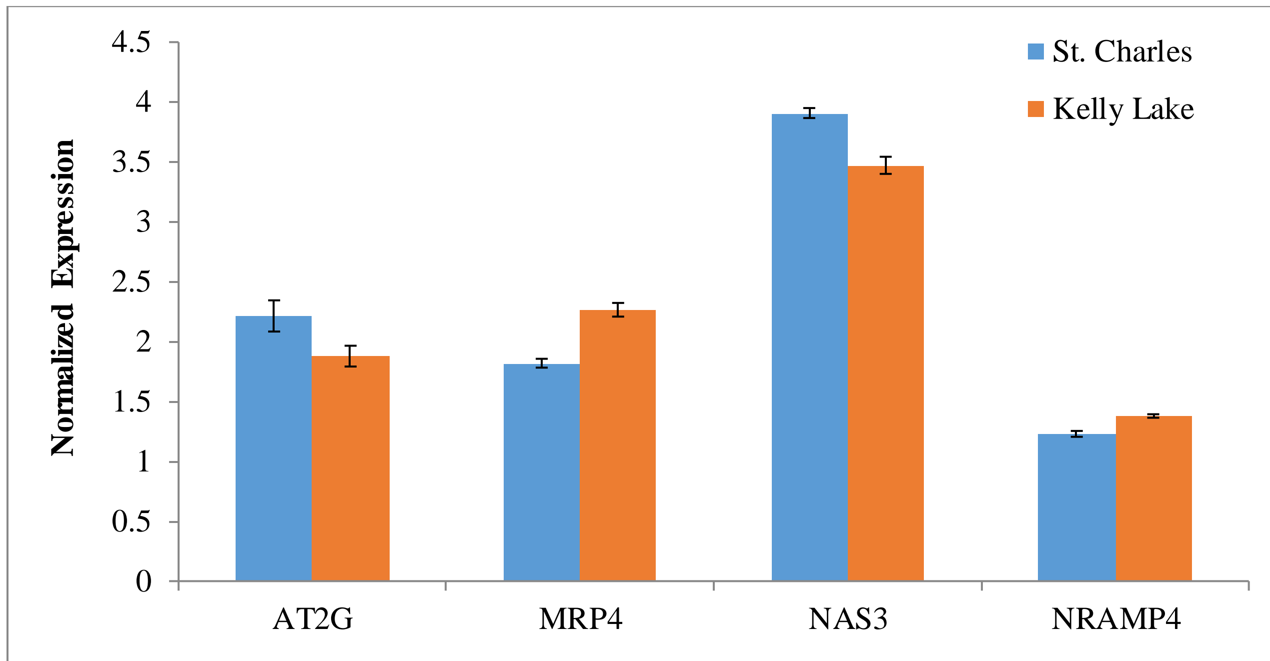

Figure 10: Expression of AT2G, MRP4, NAS3, and NRAMP4 in trembling aspen (*Populus tremuloides*) in metal-contaminated (Kelly Lake) and metal-uncontaminated distal sites (St-Charles) populations of the Greater Sudbury Region. Expression of values were standardized based on the housekeeping gene α-tubulin.

*Represents significant differences between sites using T- test ($p \leq 0.05$).

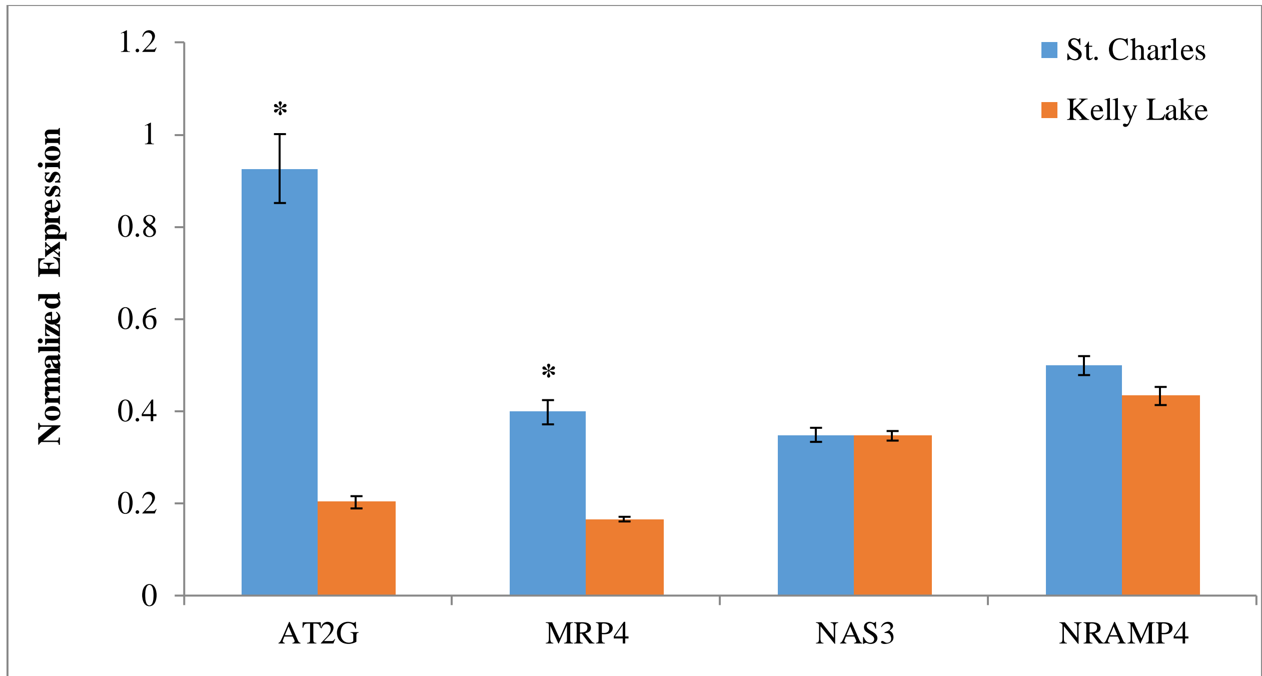

Figure 11: Expression of AT2G, MRP4, NAS3, and NRAMP4 in red maple (Acer rubrum) in metal-contaminated (Kelly Lake) and metal-uncontaminated distal sites (St-Charles) populations of the Greater Sudbury Region. Expression of values were standardized based on the housekeeping gene α-tubulin.

*Represents significant differences between sites using Kolmorgorov - Smirnov ($p \leq 0.05$).

3.5 Discussion

3.5.1 Metal contamination and toxicity

Recent studies have confirmed high levels of Cu, Ni, Zn, and other metals in soil and plant tissues from metal contaminated sites in the Greater Sudbury Region (Nkongolo et al. 2013; Kalubi et al. 2015). In the present studies, a number of genes associated with nickel tolerance were selected and their expression levels in *P. tremuloides* and A. *rubrum* populations from contaminated and uncontaminated sites were compared. Among all the primers that were designed and screened, only Nramps4, Nas 3, At2G, and MRP4 produced differential expression levels in the targeted populations. The lack of amplification with other primer pairs may be caused by the absence of primer binding sites on the template cDNAs. Weak primer bindings caused by a low complementarity between these primers and *P. tremuloides* and A. *rubrum* cDNA may also result in poor amplification products. In this study, each gene was more upregulated in *P. tremuloides* compared to A. *rubrum* in the two sites. The MRP4 and At2G genes showed the greatest difference in gene expression levels between the two species.

Only a certain number of these metals are soluble under appropriate biological conditions. Some plants use metals as micronutrients. Metals can become toxic to plants if their concentrations are too high. This phytotoxicity may be caused by the accumulation of metals within the cytosol and the chloroplast stroma (Brune et al. 1995). Developmental changes in plants induced by heavy metals is usually from compromised metabolisms (Van Assche and Clijsters 1990) or impairment of signaling processes. These would be the key factors that initiate adaptive responses to metal stress (Jonak et al. 2004). Metal detoxification in plants is achieved by their uptake, translocation, and sequestration into the vacuole, metabolization, and oxidation. Metals also go through reduction

and conjugation with glucose, glytanyl cysteine synthase or amino acids (Salt et al. 1998; Meagher 2000; Dietz and Schnoor 2001).

The bioavailability of metals is specific to the plant species and the type of metal in the environment. It also depends on the plant's necessity for metals as micronutrients and the plant's capacity to regulate the mobility of metals in the rhizosphere. Soil chemical properties also play an important role in the regulation of the release of metals in the soil (Hirt & Shinozaki, 2004) (Hirt and Shinozaki 2004). Metal absorption in plants is regulated by its intrinsic characteristics and biotrophic interactions such as metal retention in roots and cell wall binding. Metal exposure often results in a significant decrease in plant growth (Sanità di Toppi and Gabbrielli 1999).

Metal toxicity also induces ROS (reactive oxygen species) in plants which can initiate biological and physiological complications as well as abnormalities. Some of these impairments includes enzymatic imbalance and damage to the cell membrane. Sreekanth et al. (2013) showed that Chl content decreases and electron transport is interrupted due to Ni toxicity. Ghasemi et al. (2012) reported that photosynthetic protein complexes were influenced by high Ni content. Also excess Ni affected the rate of the Hill reaction. Overall, Ni has adverse effects on the photosynthetic system.

3.5.2 Gene expression

Many studies on A. *thaliana,* a model plant species have been conducted to determine which genes are expressed when plants are exposed to metal stress. An analysis was conducted on global gene expression in plants that were exposed to lead (Pb) and cadmium (Cd). There were 65 and 338 up and downregulated genes for Cd and 19 and 76 for Pb in the analysis (Kovalchuk et al. 2005). The study revealed that Cd exposure lead to the ABC transporters being differentially regulated. This would suggest a significant impact on the glutathione-Cd or phytochelatins-Cd complex transport

into and outside of plant cells (Bovet et al. 2005). Other studies on *Arabidopsis* plants used microarray techniques to show that a complex regulatory network that modulates gene expression within the tissues of the plant is present. In the present study, four genes were targeted. All the four genes (Nramps, MRPs, AT2G16800, and NAS3) were present in *P. tremuloides* and *A. rubrum*. They were all more expressed in *P. tremuloides* compared to *A. rubrum*. The expression levels in two sites with different concentration in metals such as Ni, Cu, and Zn were also assessed.

The Natural resistance associated macrophage proteins (Nramps) analyzed in this study function in diverse organisms including bacteria, fungi, plants, and animals. They are a highly conserved family of integral membrane proteins involved in metal ion transport and containing 12 predicted transmembrane domains with a characteristic `consensus transport motif' between TM-8 and TM-9 (Williams et al. 2000; Curie et al. 2001; Hall and Williams 2003). Plant Nramps are clustered into two sub-families: one includes AtNramps 1 and 6 and the other Nramps 2±5 (Thomine et al. 2003). Genes from this Nramps family have now been reported in several plant species, including three (OsNramp 1-3) in rice (Belouchi et al. 1997) and six Nramps in Arabidopsis (Williams et al. 2000; Hall and Williams 2003). This study confirm their presence in *P. tremuloides* and *A. rubrum*. In general, expression of Nramps gene was relatively low compared to other genes tested and slightly higher in *P. tremuloides* samples from the metal contaminated site than in those from the metal-uncontaminated area.

Multidrug resistance proteins (MRPs) are members of the C family of a group of proteins named ATP-binding cassette (ABC) transporters (Sodani et al. 2012). Genes belonging to the multidrug resistance-associated protein (MRP, ABCC) subfamily of ABC transporters have been reported in many plant species. Plant MRPs appear to be involved primarily in the vacuolar sequestration of potentially toxic metabolites. In the present study, there was a significant downregulation of MRP4

in samples from the metal contaminated site compared to the uncontaminated site. But no clear association between metal contamination and the expression of MRP4 can be established because of low number of metal – contaminated site analyzed and the possible effects of other environmental factors involved in MRP regulation that cannot be excluded.

AT2G16800 is a member of the NiCoT protein family that uptakes nickel and/or cobalt using proton motive force (Rodionov et al. 2006; Theriault et al. 2016a). Several studies have been conducted on these transporters in prokaryotes, but knowledge of NiCot regulation in plants is limited. In the present study, a significant down regulation of AT2G16800 gene was observed in *A. rubrum* from the metal contaminated site (Kelly Lake) compared to metal-uncontaminated (St Charles). Like with MRPs, association of this differential gene expression with metal contamination cannot be established. No difference between the two sites was observed in AT268000 expression in *P. tremuloides*.

Nicotianamine (NA), is present in higher plants as a chelator of metals. Inoue et al. (2003) demonstrated that three rice NA synthase (NAS) genes, OsNAS1, OsNAS2, and OsNAS3 are expressed in cells involved in long-distance transport of Fe (Inoue et al. 2003). The present study confirms the presence of this gene in *P. tremuloides* and *A. rubrum*. No significant difference in NA expression was observed between the two targeted sites for both species suggesting that metal contamination is not associated with the regulation of this gene.

Makela et al. (2016) assessed the expression of Ni resistance genes in red oak (*Quercus rubra*) and found no association between gene expression and metal contamination in a field study. Likewise, analysis of gene expression in *Betula papyrifera* by Theriault et al. (2016) showed also no link between gene expression and metal contamination at sites. The two studies concluded that the bioavailable amount of metals in contaminated sites is too low to trigger a differential gene

expression caused by metal toxicity. It is possible that *P. tremuloides* has greater sensitivity to the site abiotic stressors than A. rubrum resulting to high expression of these stress related genes. Global gene expression in *A. rubrum* and *P. tremuloides* exposed to different levels of metals under controlled conditions in the growth chambers will be useful to assess the effects Ni dosage on gene expression in these species.

3.6 Conclusions

The present study reveals that each of the four genes tested (Nramps4, Nas 3, At2G, and MRP4) was more upregulated in *P. tremuloides* than in *A. rubrum*. AT2G and MRP4 genes were significantly down regulated in *A. rubrum* from the metal contaminated sites compared to those from uncontaminated areas, but environmental factors driving this differential gene expression couldn't be established. Analysis of this expression at larger scale and under controlled conditions using different doses of metals will likely establish the link and metal dosage associated with regulation of the four genes studied.

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Chapter 4: Low stringency tests revealed differential gene expression in red maple (*Acer rubrum*) treated with nickel: gene regulation in nickel resistant and susceptible genotypes

4.1 Abstract

Recent field studies have revealed that *Acer rubrum* is a nickel (Ni) avoider and it does not store Ni in its tissues. Analysis of nickel toxicity at low and high doses in this species is limited. The main objectives of this study were to 1) determine the effect of low and high Ni doses on *A. rubrum* seedlings, 2) to determine the effects of low doses of nickel on gene expression using a less stringent test based on *p*-values, and 3) to identify differential gene expression in resistant and susceptible genotypes using this low stringency test. All the Ni toxicity tests were conducted in growth chambers. No morphological damage or change in plant growth were observed when *A. rubrum* where treated with a low dose (150 mg /kg) and high doses (800 mg /kg an1,600 mg /kg) of Ni. Differential gene expression from transcriptome analysis using *p*-values revealed that 464 genes were downregulated and 609 upregulated in samples treated with 150 mg /kg Ni compared to water. These numbers were 994 downregulated and 1569 upregulated genes when the 800 mg /kg dose was compared to water and 3760 downregulated and 7098 when 1,600 mg/kg was compared to the same water control. There were more upregulated genes (1482) than downregulated (623) when Ni-resistant and Ni-susceptible genotypes were compared based on low stringency test (*p*-values). Among the top 50 most upregulated, Serine/threonine-protein kinase, O-methyltransferase, sugar transporter, glutathione-s-transferase and s-adenosylmethioninie-dependent methyltransferase were the only ones that could be relevant for Ni toxicity based on their role in plants. On the other hand, the top 50 most downregulated genes that could be associated with environmental stress include Peroxisomal ascorbate peroxidase, Glycine-rich protein DC7.1, Aquaporin NIP6.1 family protein, Putative membrane protein (Fragment), and Serine/Threonine protein kinase. Overall, the study shows that a less stringent test

such as *p*-Values could be a good tool to study gene expression when the effects of Ni or other metals on global gene transcription are low.

Key words: *Acer rubrum* (red maple); Nickel toxicity; Gene expression; *p*-Values analysis;

4.2 Introduction

Environmental stressors such as metals, drought, heat shock, aerobic and anaerobic agents can induce transcriptional changes in plants (Matters and Scandalios 1986). This can affect metal uptake, translocation, and accumulation. Usually, plants regulate these processes through defense mechanisms (Singh et al. 2002; Saito et al. 2010; Farias et al. 2013; Fidalgo et al. 2013). If these processes are overwhelmed, then oxidative stress defense mechanisms are enabled. This is the result of the formation of free radicals and reactive oxygen species (ROS) when metal concentrations are high in plants causing cellular damage (Emamverdian et al. 2015). Metal tolerance in plants generally includes detoxification, complexation and compartmentalization. Previous studies have reported that glutathione (GSH) and related metabolizing enzymes, proteins, and peptides are heavily involved in heavy metal tolerance by regulating reactive oxygen species (ROS), methylglyoxal (MG) detoxification, heavy metal (HM) uptake, translocation, chelation, and detoxification (Theriault et al. 2016b).

Knowledge on metal, especially nickel tolerance in plants is still in their infancy. Ni is one of the most abundant metals in mining regions in Canada and plays a vital role in plant nutrition. Both higher and lower plants require small amounts of this micronutrient for their growth (Lin and Kao 2005). The level representing nickel toxicity varies from 25 to 246 $\mu g \cdot g^{-1}$ dry weight (DW) of plant tissue, depending on the plant species (Iyaka 2011). Nickel is a complex element in terms of its toxicity exerted on plants (Bhalerao et al. 2015). This is because it has an indirect effect on ROS production in plants. It inhibits the activity and function of many different antioxidant enzymes including ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), guaiacol peroxidase (GOPX), peroxidase (POD), and superoxide dismutase (SOD) (Pandey and Sharma 2002; Freeman et al. 2004; Gomes-Junior et al. 2006;

Bhalerao et al. 2015). Genes associated with nickel resistance have already been reported in non-avoider plant species (Freeman et al. 2004; Mizuno et al. 2005; Stearns et al. 2005; Schaaf et al. 2006; Visioli et al. 2012; Liu et al. 2014; Theriault et al. 2016b). But genetic investigations on Ni toxicity in avoider plant species are lacking.

Red maple (*Acer rubrum*) is fairly resistant to a number of environmental agents and landfill contaminated gases, (Krause and Dochinger 1987). These trees are found in the Greater Sudbury Region (GSR) in Northern Ontario where soils are heavily contaminated with metals including nickel (James and Courtin 1985). Previous studies have shown that this species does not store Ni in its tissues and its translocation to leaves is limited (Kalubi et al. 2016). Therefore, it is classified as a nickel avoider and it is the only hardwood species studied to date that exhibits this mechanism.

Recent studies on global gene expression revealed no significant differences in gene expression between Ni-resistant and susceptible genotypes and between seedlings exposed to doses of Ni < 800 mg / kg based on False Discovery Rate (FDR) analysis (Nkongolo et al. 2018b, a). These studies revealed that no major genes are involved in Ni resistance in *A. rubrum*. Although FDR is very reliable, it might be too stringent and miss the detection of low levels of gene expression that could be associated with Ni toxicity.

The main objectives of this study were to 1) determine the effect of low and high Ni doses on *A. rubrum* seedlings, 2) to determine the effects of low doses of nickel on gene expression using a less stringent test based on *P*-values, and 2) to identify differential gene expression in resistant and susceptible genotypes using this low stringency test.

4.3 Materials and Methods

4.3.1 Nickel toxicity experiment

Assessment of nickel toxicity was carried out as described previously (Theriault and Nkongolo 2016). *A. rubrum* seeds were provided by the Canadian Forest Services (tree seed center, Fredericton, New Brunswick, Canada). Seeds were germinated in "Petawawa" germination boxes and seedlings were grown in a deep tray with soil. 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 six-month old seedlings with an aqueous solution of nickel nitrate salt [$Ni(NO_3)_2$] at the following concentrations: 150 mg, 400 mg, 800 mg, and 1,600 mg of nickel per kg of dry soil. Commercial potassium nitrate (KNO_3) salt was used to control for any possible toxic effect due to an increase in nitrate ions (NO_3) in the plants. 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 kg of dry soil). The 150 mg/kg dose represents the published bioavailable amount of nickel in contaminated sites in the GSR and the 1,600 mg/kg dose is the total amount of nickel in contaminated sites reported by (Kalubi et al. 2016). For this experiment, a randomized block design with 15 replications for each treatment was used.

Damage rating of the treated genotypes was recorded every two days for a week using the 1 to 9 scale where 1 indicates no visible morphological changes and 9 corresponds to dead plants. Plants were rated individually and a genotype with a score of 1 to 3 was considered nickel resistant, 4 to 6, moderately resistant/susceptible, and 7 to 9 susceptible. Height of each plant was also measured

every two days to assess the effects of the nickel treatments on their growth. Genotypes resistant and susceptible to a soil nickel concentration of 1,600 mg/kg were identified. Plant roots and leaves were harvested from seedlings, frozen in liquid nitrogen and stored at -20°C for RNA extraction. For transcriptome analysis, three Ni resistant and three Ni susceptible genotypes were selected along with three genotypes for each of the dose and controls (water and nitrate controls) tested.

4.3.2 RNA extraction

Total RNA was extracted from root samples using methods previously described by Theriault et al. (2016a). Root RNA was extracted from 15 individuals per treatments, and then quantified using the Qubit$^{(R)}$ RNA BR Assay kit by Life Technologies (Carlsbad, United States). The Quality of RNA samples was assessed on a 1% agarose gel. Gene expression of tree genotypes per treatment was analyzed in detail. RNA samples from five seedlings for each treatment were used for transcriptome and global gene expression analysis.

4.3.3 RNA seq analysis

Details of RNA seq analysis have been described in (Theriault et al. 2016b) and (Nkongolo et al. 2018b, a). The RNA libraries were constructed with the Truseq^{TM} RNA Sample Preparation Kit according to the manufacturer's recommendations and quantified using Bioanalyzer 2100. The Illumina HiSeq 2500 sequencing system was the platform used to sequence the RNA libraries at Seq Matic (Fremont California, USA). The transcripts were assembled using the *de novo* method. The RNA-seq data obtained from each sample was used as input for the Trinity software program. The bowtie software program was used to map the raw reads to the transcripts assembled from the

Trinity program. Quantification of these transcripts was assessed with the RSEM program and gene expression was calculated and expressed as Reads per Kilobase per Million mapped (RPKM) (Mortazavi et al. 2008). The average read counts of all the samples (15.9 million) was used to determine the counts per million (CPM) cutoff which was 0.63. Any gene that has a CPM value that is greater than the cutoff in a minimum of 2 samples was used in further analysis. Ten raw reads were nearly equivalent to the cutoff CPM in this experiment. The voom method from the R limma package was used to normalize the raw count data (http://www.bioconductor.org/packages/release/bioc/html/limma.html) (Law et al. 2014). Most of the samples looked the same after normalization.

The R limma package was used to generate multidimensional plots to view sample relationships. Samples were clustered together using the made4 (multivariate analysis of microarrays data using ADE4) program and genes that had variable expression levels across samples were used to draw heatmaps. All the variable genes were selected based on the standard deviation expression values greater than 30% of the average expression value. Any genes with a mean logCPM <1 were removed.

The normalized data for differentially expressed genes were transformed to logCPM values with the voom method. For the analysis, a linear model was constructed for each comparison using the R Limma package and statistics were also calculated. We used log fold change (logFC), p-value for differential gene expression analysis. The ranking of the genes was performed using logFC from the pairwise comparison to determine the top 100 most upregulated and downregulated genes. To verify that the candidate genes responded to nickel and not nitrate, baseline filtering of genes was conducted. This removed any genes that were likely associated with the effect of nitrate.

4.3.4 Statistical analysis

Krustal-Wallis and one-way ANOVA (Post-hoc Dunnett's T3) statistical tests were performed using SPSS 20 for Windows to determine significant differences among means ($p \leq 0.05$) for damage ratings and plant growth

4.4 Results

4.4.1 Nickel toxicity

Effects of nickel toxicity on *A. rubrum* seedlings are described in Table 9. A high level of nickel resistance was observed in most seedlings exposed to doses ranging from 150 mg/kg of soil to 1,600 mg/kg of soil. With the exception of three genotypes that were classified as Ni susceptible to the highest dose used based on damage ratings, no morphological damage was observed after 7 days of treatment (Table 9). Likewise, no change in plant growth caused by Ni was observed when the genotypes treated with different doses of Nickel were compared to controls.

4.4.2 Global gene expression

The transcriptome data for *Acer rubum* has been described by (Nkongolo et al. 2018b, a). They have been deposited in Embl and NCBI under the SRA project number SRP098922. Table 10 described gene expression among the treatments based on *P* Values. In previous studies, no differential expression was observed when 800 mg/kg, 150 mg /kg and water control were compared based on False discover rate (FDR) (Nkongolo et al. 2018a). However, the *P* – values used in the present study revealed that 464 genes were downregulated and 609 upregulated in samples treated with 150 mg /kg compared to water (Table 10). The number of differentially expressed genes increased as the concentration of Ni was increased. In fact, we observed 994 down regulated and 1569 up regulated genes when the 800 mg /kg dose was compared to water and 3760 down regulated and 7098 upregulated genes when the highest dose were compared to water based on P-values. We also observed 1067 down regulated and 1458 upregulated when the 800 mg /kg were compared to 150 mg /kg. Likewise no difference in gene expression was observed when the 1600 mg /kg was compared to 800 mg /kg based on FDR (Nkongolo et al. 2018a). But we identified

in this study 1566 downregulated and 3569 upregulated genes when these high doses were compared based on *P*-values.

Likewise, when resistant genotypes (RG) were compared to susceptible genotypes (SG) in previous analyses (of 1,600 mg of Ni per kg of soil), there were no differences in gene expression based on FDR (Nkongolo et al. 2018b). But the *P* value analysis of the same dataset in the present study revealed that a total of 1482 genes were upregulated and 623 genes were downregulated in RG compared to SG. The transcriptome analysis presents the top 50 up and downregulated genes in RG compared to SG (Tables 11 and 12). Only few genes among these top 50 could play a role in Ni transport, chelating or other activities in plants. The upregulated genes of significance include Serine/threonine-protein kinase, O-methyltransferase, sugar transporter, glutathione-s-transferase and s-adenosylmethioninie-dependent methyltransferase. The downregulated genes that could be involved in Ni activities in plants include Peroxisomal ascorbate peroxidase, Glycine-rich protein DC7.1, Aquaporin NIP6.1 family protein, Putative membrane protein (Fragment), and Serine/threonine protein kinase.

Table 9. Damage rating and plant growth after treatments with nickel nitrate [$Ni(NO_3)_2$] and potassium nitrate KNO_3).

Treatments		**Damage rating * (1 to 9 scale)**	**Plant growth in 7 days (cm)****
$Ni(NO_3)_2$	150 mg/kg	1.0±0.00	0.19±0.23 a
	800 mg/kg	1.07±0.25	0.17±0.18 a
	1600 mg/kg	2.06±2.07	0.14±0.21 a
KNO_3	150 mg/kg	1.0±0.00	0.42±0.42 b
	800 mg/kg	1.0±0.00	0.22±0.21 ab
	1600 mg/kg	1.0±0.00	0.00±0.00 a
Water control	0mg/kg	1.0±0.00	0.24±0.49 a

* Damage rating based on 1 to 9 scale where 1 represents symptomless plants and 9 dead plants.

** Plant growth is calculated as the difference between plant height at day of treatment and 7 days after the treatments

Table 10. Number of differentially expressed genes in red maple (*Acer rubrum*) among the different nickel treatments based on *P* values (0.01) and False discovery rate (in parenthesis) analyses**.**

Treatment	Water Control		150 mg/kg*		800 mg/kg*		1,600 mg/kg*	
	Up	Down	Up	Down	Up	Down	Up	Down
Water Control	-	-	609 (00)	464 (02)	1569 (00)	994 (00)	7098 (6263)	3760 (3142)
150 mg/kg			-	-	1458 (00)	1067 (00)	6707 (5753)	3198 (2382)
800 mg/kg					-	-	3569 (00)	1566 (00)
1,600 mg/kg							-	-

*False discovery rate (FDR) data in parentheses are from Nkongolo et al. (2018a)

Table 11. Top 50 upregulated transcripts in resistant red maple (*A. rubrum*) genotypes compared to susceptible genotypes based on LogFC

Rank	Transcript ID	Resistant	Resistant	Resistant	Susceptible	Susceptible	logFC	P.Value	Description
1	TRINITY_DN419449_c0_g2	24.442	210.81	82.972	0	0	8.223722	0.000727	Serine/threonine-protein kinase
2	TRINITY_DN431953_c5_g1	9.693	18.273	32.088	0	0	8.072386	0.000119	hypothetical protein
3	TRINITY_DN429308_c2_g4	371.25	618.637	207.389	0	0	7.918816	0.000253	F5M15.26
4	TRINITY_DN431953_c4_g1	10.94	13.182	15.839	0	0	7.855089	2.03E-05	Formin-like protein
5	TRINITY_DN423469_c0_g1	2.314	10.32	25.916	0	0	7.775964	0.001156	Short chain dehydrogenase protein
6	TRINITY_DN329922_c0_g1	2.038	16.871	18.246	0	0	7.747927	0.001276	Predicted proteinina
7	TRINITY_DN436216_c0_g1	12.14	8.654	11.565	0	0	7.630548	2.75E-05	putative ribonuclease H protein
8	TRINITY_DN417571_c0_g1	43.914	10.918	53.021	0	0	7.483749	0.000305	NA
9	TRINITY_DN441905_c0_g10	117.326	176.113	130.32	0	0	7.361054	3.66E-05	Predicted protein translation factor
10	TRINITY_DN433607_c0_g1	6.237	6.482	26.475	0.147	0	7.355186	0.003537	O-methyltransferase
11	TRINITY_DN433451_c3_g1	2.457	3.609	55.727	0	0	7.3172	0.00201	ankyrin repeat-containing protein
12	TRINITY_DN437261_c3_g2	66.261	34.765	34.255	0.479	0	7.298592	0.00124	NA
13	TRINITY_DN433899_c1_g1	94.426	6.241	48.057	0	0	7.271905	0.001935	sugar transporter
14	TRINITY_DN427250_c0_g1	16.634	4.229	6.561	0	0	7.2492	0.002758	PREDICTED: F-box protein
15	TRINITY_DN325824_c0_g1	2.409	13.263	8.199	0	0	7.225504	0.000369	Coatomer subunit alpha
16	TRINITY_DN427744_c1_g1	3.171	3.069	15.729	0	0	7.130584	0.000423	Predicted protein
17	TRINITY_DN431493_c1_g1	14.978	9.619	6.781	0.129	0	7.121551	0.000842	Ankyrin repeat-containing protein,
18	TRINITY_DN416544_c0_g1	0.886	4.505	6.362	0	0	7.120704	0.000697	Predicted protein n=4 Tax=Trichoderma
19	TRINITY_DN429719_c0_g3	3.675	5.206	5.832	0	0	7.097734	5.23E-05	NA
20	TRINITY_DN431804_c3_g1	8.712	9.527	10.966	0	0	7.095968	7.04E-05	Caffeic acid 3-O-methyltransferase
21	TRINITY_DN314982_c1_g1	0.676	9.412	4.964	0	0	7.068661	0.001865	Predicted protein n=1 Tax=Hypocrea jecorina
22	TRINITY_DN403025_c0_g2	0.933	9.803	6.541	0	0	7.04241	0.001349	Predicted protein n=2 Tax=Hypocrea jecorina
23	TRINITY_DN377100_c0_g1	2.809	4.815	3.745	0	0	7.026477	6.18E-05	Predicted protein n=3 Tax=Trichoderma
24	TRINITY_DN440122_c7_g4	3.304	17.181	12.484	0	0	7.020816	0.000393	NA
25	TRINITY_DN375089_c0_g1	1.076	4.275	1.668	0	0	7.018614	0.000221	Ornithine monooxygenase
26	TRINITY_DN411075_c0_g1	3.656	6.218	6.072	0	0	6.982789	8.76E-05	Defective in cullin neddylation protein
27	TRINITY_DN362203_c0_g1	1.228	5.229	3.575	0	0	6.974988	0.000264	DUF300-domain-containing protein
28	TRINITY_DN430442_c0_g1	1.742	11.136	5.223	0	0	6.955037	0.000488	Predicted protein n=4 Tax=Trichoderma
29	TRINITY_DN444610_c0_g5	50.17	15.975	17.447	0	0	6.941068	7.86E-05	putative ribosome biogenesis protein slx9-like
30	TRINITY_DN370817_c0_g2	2.342	1.954	9.967	0	0	6.895564	0.000405	Predicted protein n=2 Tax=Hypocrea jecorina
31	TRINITY_DN431526_c3_g1	14.33	4.781	6.971	0	0	6.878483	0.000164	Blue copper protein

32	TRINITY_DN440779_c1_g3	4.237	4.551	33.736	0	0	6.86415	0.001021	Anthocyanidin reductase
33	TRINITY_DN431440_c0_g1	4.056	1.299	9.867	0	0	6.857507	0.000957	Major facilitator superfamily protein
34	TRINITY_DN402230_c1_g1	2.837	4.632	5.822	0	0	6.836948	9.26E-05	Carbon catabolite repressor protein
35	TRINITY_DN450475_c1_g1	4.742	11.435	14.631	0	0	6.822476	0.000175	F-box/FBD/LRR-repeat protein
36	TRINITY_DN16505_c0_g2	13.349	15.262	9.238	0.47	0	6.821249	0.004356	Beta-xylanase
37	TRINITY_DN445296_c1_g4	18.596	44.304	18.346	0.101	0	6.808683	0.000808	60S ribosomal protein
38	TRINITY_DN306065_c1_g1	1.781	11.7	4.284	0	0.045	6.802065	0.002981	Predicted protein n=4 Tax=Trichoderma
39	TRINITY_DN449745_c1_g2	11.626	2.046	44.562	0	0	6.737006	0.000667	Tetratricopeptide repeat-like superfamily protein,
40	TRINITY_DN435333_c4_g1	77.621	17.204	43.064	1.079	0	6.736188	0.006049	Beta-galactosidase
41	TRINITY_DN445835_c1_g2	2.409	3.816	10.117	0	0	6.72691	0.000285	cytochrome P450 71D9-like
42	TRINITY_DN397950_c0_g1	34.069	14.458	14.371	0	0	6.710341	0.000178	NA
43	TRINITY_DN330872_c0_g2	1.819	5.735	8.858	0	0	6.708986	0.000384	Fusarium graminearum chromosome 3,
44	TRINITY_DN416264_c0_g1	50.655	324.426	117.766	0	0	6.683836	0.000545	NA
45	TRINITY_DN418021_c0_g1	8.198	7.574	5.303	0	0	6.679146	9.93E-05	Glutathione S-transferase
46	TRINITY_DN431447_c1_g1	3.352	1.712	10.127	0	0	6.675859	0.000627	probable caffeine synthase
47	TRINITY_DN446020_c0_g1	2.399	13.32	1.268	0	0	6.673442	0.001283	S-adenosylmethionine-dependent methyltransferase
48	TRINITY_DN409999_c0_g2	67.947	43.063	99.56	0	0	6.664398	9.69E-05	NA
49	TRINITY_DN421435_c0_g1	52.569	18.043	100.589	0	0	6.651225	0.000421	cytochrome P450 76M5
50	TRINITY_DN438325_c0_g2	3.894	3.873	5.133	0	0	6.6321	8.72E-05	NADH-ubiquinone oxidoreductase chain 5-like

Table 12. Top 50 downregulated transcripts in resistant red maple (*A. rubrum*) genotypes compared to susceptible genotypes based on LogFC

Rank	Transcript ID	Susceptible	Susceptible	resistant	Resistant	Resistant	logFC	P.Value	Description
1	TRINITY_DN439029_c0_g7	318.849	353.072	1.171	0	0	-8.48	1.63E-05	NA
2	TRINITY_DN429635_c5_g1	13.321	8.72	0	0	0	-7.93	9.67E-06	Peroxisomal ascorbate peroxidase
3	TRINITY_DN15726_c0_g1	3.466	11.842	0	0	0	-7.38	0.000162	membrane protein
4	TRINITY_DN446102_c0_g1	376.843	172.255	0	24.928	0	-7.08	0.005509	Glycine-rich protein DC7.1
5	TRINITY_DN66483_c0_g2	31.26	12.206	0	0	0	-6.72	8.79E-05	Translation initiation factor eIF-1A,
6	TRINITY_DN438106_c0_g2	58.74	44.338	0	3.839	0	-6.68	0.003171	Ferritin
7	TRINITY_DN434380_c0_g1	20.502	13.148	0	0	0	-6.68	4.20E-05	Aquaporin NIP6.1 family protein
8	TRINITY_DN428352_c1_g1	30.605	31.815	0.638	4.666	0	-6.65	0.007078	hypothetical protein
9	TRINITY_DN436284_c1_g4	7.052	13.523	0	0	0	-6.61	0.000138	NA
10	TRINITY_DN298964_c0_g1	2.36	13.568	0	0	0	-6.59	0.000699	Mixed-linked glucanase (Fragment)
11	TRINITY_DN422742_c0_g2	42.055	34.574	0	0	0	-6.54	4.33E-05	Putative membrane protein (Fragment)
12	TRINITY_DN458483_c0_g2	17.847	27.886	0	0	0	-6.42	7.39E-05	Ribosomal protein
13	TRINITY_DN450351_c0_g5	41.64	26.841	0	0	0	-6.39	6.31E-05	Putative aminotransferase class III
14	TRINITY_DN451708_c0_g1	4.471	7.8	0	0.115	0	-6.23	0.000382	Fusarium graminearum chromosome 3.
15	TRINITY_DN183860_c0_g2	15.33	4.19	0	0	0	-6.19	0.000294	Major egg antigen
16	TRINITY_DN445543_c1_g3	6.84	19.461	0	0	0	-6.18	0.000256	Histidine kinase cytokinin receptor,
17	TRINITY_DN62808_c0_g2	4.738	9.98	0	0	0	-6.15	0.000162	hydroxyproline N-acetylglucosaminyltransferase
18	TRINITY_DN396769_c0_g1	6.942	14.568	0.838	0	0	-6.14	0.003062	Endoglucanase-4
19	TRINITY_DN434155_c2_g3	20.705	17.588	0	0	0	-6.13	8.19E-05	Serine/threonine protein kinase
20	TRINITY_DN181103_c1_g1	35.722	70.851	0	0	0	-6.11	0.000158	Histone lysine demethylase PHF8-like isoform 1
21	TRINITY_DN451676_c0_g1	42.931	13.829	0	0	0	-5.96	0.000288	Guanine nucleotide-binding protein, subunit alpha
22	TRINITY_DN331066_c1_g1	67.581	41.999	0	0	0	-5.94	0.000131	TolAlike protein
23	TRINITY_DN381543_c0_g1	17.054	20.824	0	0	0	-5.89	0.000183	Zinc finger protein 652-like
24	TRINITY_DN434705_c1_g3	6.886	7.233	0	0	0	-5.88	0.000127	Px domain containing protein
25	TRINITY_DN450742_c1_g2	11.145	8.448	0	0	0	-5.88	0.00013	NA
26	TRINITY_DN411865_c0_g1	252.209	14.556	0	0	0	-5.85	0.000397	TMV resistance protein N-like
27	TRINITY_DN432292_c0_g3	13.21	24.9	0	0	0	-5.83	0.000691	CBS domain-containing protein CBSX3,
28	TRINITY_DN329358_c0_g1	1.825	8.629	0	0	0	-5.82	0.000849	Ribosomal protein L37
29	TRINITY_DN428515_c0_g1	10.288	18.269	0	0	0	-5.80	0.000145	NA

30	TRINITY_DN376653_c0_g1	0.986	3.066	0	0	0	-5.78	0.000481	Cellobiose dehydrogenase
31	TRINITY_DN451195_c1_g7	191.044	145.278	0	0	0	-5.78	0.000153	26S protease regulatory subunit 6A homolog A
32	TRINITY_DN416896_c0_g2	271.153	197.314	0	0	0	-5.77	0.000158	NA
33	TRINITY_DN307386_c0_g1	29.287	8.164	0	0	0	-5.76	0.000458	Ras family GTPase
34	TRINITY_DN388376_c0_g1	5.374	2.873	0	0	0	-5.75	0.000207	Predicted protein (Fragment)
35	TRINITY_DN449585_c0_g1	2.047	10.094	0	0.207	0	-5.75	0.004642	Acetylxylan esterase
36	TRINITY_DN443381_c0_g9	21.212	30.963	0	0	0	-5.73	0.000202	NA
37	TRINITY_DN444006_c1_g8	12.758	7.278	0	0	0	-5.71	0.000209	Non-expressor of PR1
38	TRINITY_DN446626_c2_g1	17.681	17.792	0	0	0	-5.71	0.000172	Calreticulin
39	TRINITY_DN434607_c0_g2	30.522	33.892	0	0.931	0	-5.68	0.00053	Phosphoprotein phosphatase
40	TRINITY_DN446779_c3_g4	15.376	31.712	0	0.736	0	-5.68	0.001042	Branched-chain-amino-acid aminotransferase
41	TRINITY_DN437399_c0_g1	24.549	18.099	0	0	0	-5.67	0.00019	Calcium-dependent protein kinase
42	TRINITY_DN444707_c1_g1	9.043	17.622	0	0	0	-5.67	0.000291	Cysteine-rich RLK 29,
43	TRINITY_DN167373_c0_g1	5.089	23.185	0	0	0	-5.66	0.000942	Component of SCAR regulatory complex train SH3
44	TRINITY_DN396280_c0_g1	2.563	3.985	0	0.264	0	-5.66	0.002579	Fusarium graminearum chromosome 4, genome
45	TRINITY_DN335488_c0_g1	55.772	48.687	0	0	0	-5.65	0.00019	Factin-capping protein subunit alpha-2, putative
46	TRINITY_DN439684_c0_g1	186.813	107.604	0	0	0	-5.63	0.000235	Iron-sulfur cluster biosynthesis family
47	TRINITY_DN354938_c0_g2	3.641	5.212	0	0	0	-5.63	0.000233	Pectate lyase C
48	TRINITY_DN458773_c0_g1	4.738	14.726	0	0	0	-5.62	0.000566	Surfactant protein B
49	TRINITY_DN432830_c2_g1	1.327	9.765	0	0	0	-5.62	0.001952	Disease resistance family protein
50	TRINITY_DN431535_c10_g1	2.36	7.846	0	0.494	0	-5.62	0.009279405	Strain CBS 40295 unplaced genomic scaffold

4.5 Discussion

Plants are continually exposed to abiotic stress in the environment due to their immobile nature. This forces them to adapt to their environment and develop a variety of different morphological, physiological and biochemical responses to cope with abiotic stressors. Previous field studies have shown that *A. rubrum*, a nickel avoider, is resistant to the bioavailable amount of nickel in soils in GSR (Kalubi et al. 2015) The present study revealed that under controlled conditions, *A. rubrum* did not show any signs of morphological damage in most genotypes treated with high dose of nickel at 1600 mg/kg which represents the total concentration of nickel in the field.

In previous studies, (Nkongolo et al. 2018b) observed upregulation of genes associated with transport in cytosol was prevalent in resistant genotypes compared to water in *A. rubrum*. (Theriault et al. 2016b), on the other hand showed that the main nickel resistance mechanism in *B. papyrifera* is a downregulation of genes associated with translation (in ribosome), binding, and transporter activities. The results of this study showed that unlike the previous data where no significant differences in gene expression levels among treatments were observed based on (FDR) (Nkongolo et al. 2018a), there were differential gene expressions when the different treatments were compared to each other. In addition, the *P*-values revealed difference between RG and SG in gene expression. In fact, there were more upregulated than downregulated genes when RG were compared to SG.

(Benjamini and Hochberg 1995), introduced the false discovery rate analysis to manage multiple testing problems. It is defined as a method that controls the expected quantity of false positive discoveries in an experiment. Due to its statistical power, this method has become very popular since it identifies more true positive discoveries (Capanu and Seshan 2015). Over the years, a number of amendments have been made to the original FDR approach that was proposed (Genovese and Wasserman 2002; Storey 2002; Benjamini et al. 2006; Genovese et al. 2006; Yekutieli 2008; Zeisel et al. 2011). In hypothesis testing, the p value is calculated based on the probability that the null hypothesis (H0) is true. Although it cannot show whether the null hypothesis is true or not, it offers a measure of the strength of evidence against the null hypothesis (Dorey 2010). Because of high stringency of FDR, differences that might exist among treatments could have been missed in previous analyses

reported by Nkongolo et al. (2018a, b). Significant differences between RG and SG were detected in the present study based on the P-values. This was expected, because no major genes are associated with resistance to Ni toxicity in *A. rubrum* (Nkongolo et al. 2018b). Hence, identification of genes of significance using FDR was unlikely. The *P*-values appears to provide some relevant information when the level of differential genes expression is low despite the probability of false positive detections, especially for global gene expression investigations.

In this study, a number of notable genes have been identified among the top 50 upregulated genes when RG and SG were compared. They include glutathione S transferases which are a group of multifunctional enzymes that are present in each part of the plant. These enzymes play a major role in detoxifying a wide range of environmental chemicals and secondary products from oxidative stress (Clemens 2001; Espinoza et al. 2012). Sugar transporters on the other hand are known to regulate the distribution of compounds in plants at different concentrations. They have been reported to be used as checkpoints to monitor and regulate the dissemination of carbon fixed compounds produced from photosynthesis (Antony and Borland 2009). O-methyltransferases are a group of enzymes found in plants that methylate the oxygen atom from secondary metabolites such as phenylpropanoids, flavonoids and alkaloids. They are involved in a number of physiological processes which include, lignin biosynthesis, stress tolerance, and disease resistance in plants (Lam et al. 2007). S-adenosylmethionine-dependent methyltransferases are a group of enzymes found in all divisions of life. They transfer methyl groups to a number of different biomolecules such as DNA and proteins. The methylation reaction of theses biomolecules infers processes associated with diseases (Struck et al. 2012).

The top 50 most downregulated genes identified when RG were compared to SG include genes involved in different pathways in plants. Peroxisomal ascorbate peroxidase which is an enzyme that is heavily involved in defense against oxidative stress and is able to protect plants from abiotic stressors (Singh et al. 2014). Glycine rich proteins (GRPs) are found in plants and their structure is very diverse. This diversity permits them to have very dynamic roles in plants. Some studies have reported that GRPs play an important role in cell wall integrity and plant defense from transgenic and biochemical analysis studies (Mangeon et al. 2010). Aquaporins are a

family of proteins that play a major role in facilitated transport of water and nutrients across biological membranes. Plants depend on this transport mechanism mediated by aquaporins for their development and growth (Park et al. 2010).

4.6 Conclusion

In this study, low and high doses of nickel (150 mg/kg, 800 mg/kg, and 1600 mg/kg) did not induce morphological damage in the majority of the *A. rubrum* genotypes. Only a few plants showed damage and were classified as the susceptible genotypes when seedlings were treated with 1600 mg/kg). The less stringent test (p value) showed differential gene expression when the effects of nickel doses were compared. There were more upregulated than down regulated genes in RG compared to SG. Among these genes, a few of them are associated with molecules that serve a specific function in plants and other organisms. Most of these genes are associated with coping with abiotic stressors and involves tolerance and detoxification mechanisms.

Data and materials used

Repository/DataBank Accession: EmbL
SRA project number: SRP098922.
Databank URL: http://www.ebi.ac.uk/ena

Repository/DataBank Accession: NCBI gene bank
SRA project number: SRP098922.
Databank URL: http://www.ncbi.nlm.nih.gov/genbank

4.8 Acknowledgements

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Chapter 5: Variation in whole DNA methylation in red maple (*Acer rubrum*) populations from a mining region: association with metal contamination and cation exchange capacity (CEC) in Podzolic Soils

5.1 Abstract

Although a number of publications have provided convincing evidence that abiotic stresses such as drought and high salinity are involved in DNA methylation reports on the effects of metal contamination, pH, and cation exchange on DNA modifications are limited. The main objective of the present study is to determine the relationship between metal contamination and Cation Exchange Capacity (CEC) on whole DNA modifications. Metal analysis confirms that nickel and copper are the main contaminants in sampled sites within the Greater Sudbury Region (Ontario, Canada) and liming has increased soil pH significantly even after 30 years following dolomitic limestone applications. The estimated CEC values varied significantly among sites, ranging between 1.8 and 10.5 cmol(+) kg^{-1}, with a strong relationship being observed between CEC and pH ($r = 0.96^{**}$). Cation exchange capacity, significantly lower in highly metal contaminated sites compared to both reference and less contaminated sites, was higher in the higher organic matter limed compared to unlimed sites. There was a significant variation in the level of cytosine methylation among the metal-contaminated sites. Significant and strong negative correlations between [5mdC]/[dG] and bioavailable nickel (- 0.71**) or copper ($r = -0.72^{**}$) contents were observed. The analysis of genomic DNA for adenine methylation in this study showed a very low level of [6N-mdA]/dT] in *A. rubrum* plants analyzed ranging from 0% to 0.08%. Significant and very strong positive correlation was observed between [6N-mdA]/dT] and soil bioavailable nickel ($r = 0.78^{**}$) and copper ($r = 0.88^{**}$) content. This suggests that the increased bioavailable metal levels associated with contamination by nickel and copper particulates are associated with cytosine and adenine methylation.

Keywords: Bioavailable metal contamination levels; Cation exchange capacity (CEC); DNA methylation; *Acer rubrum*; Northern Ontario.

5.2 Introduction

Plants that are exposed to hostile environments such as drought, high temperature, high salt and toxicity will make adjustments and modify their growth and development to minimize damage caused by environmental stress. Such physiognomic modifications are usually reversible, temporary, and occur at the epigenetic level in plants. The exposure time will also influence the induction level of the plant or organism (Steward et al. 2002; To et al. 2011; Iwasaki and Paszkowski 2014). Epigenetics refers to the heritable changes that occur in the organism without changing DNA sequence or genotype (Bonasio et al. 2010).

DNA methylation, one of the most common epigenetic changes that can occur in organisms, is specific and is present in three different sequence contexts, CG, CHG, and CHH (where H= A, C, or T) (Law and Jacobsen 2010). In *Mesembryanthemum crystallinum* L, DNA methylation at the satellite sequences plays a key role in salt adaptability and ability to switch from C3 to CAM photosynthesis (Bloom 1979; Vernon and Bohnert 1992). The salt adaptation mechanism was attributed to the observed change in DNA methylation status in the satellite sequence, although DNA methylation was not detected within the promotor region of key photosynthesis genes (Dyachenko et al. 2006). As a result of this development, a change in chromatin structure will lead to overall changes in global gene expression. In another example, drought stress increases DNA methylation in some rice genotypes. However, only 70% of the total DNA methylation changes are returned back to normal levels even after recovery in non-drought conditions (Wang et al. 2011). A loss of DNA methylation in the genome is often associated with the inability of a plant to tolerate environmental stresses. For instance, a loss of methylation decreases the ability of the *Arabidopsis* species to cope with salt stress. Met1-3 mutants of this species are hypersensitive to salt because the sodium transporter gene (AtHKT1) is not expressed in *Arabidopsis,* which is

essential for salt tolerance (Baek et al. 2011). The level of DNA methylation also helps distinguish stress-tolerant varieties. For example, after 10 days of salt exposure, the salt tolerant wheat variety had a higher level of methylation than the salt-sensitive variety (Zhong and Wang 2007). Environmental stress produced by heavy metals such as cadmium, nickel and chromium will have diverse effects on plants in terms of Global DNA methylation. (Peng and Zhang 2009) looked at this matter more carefully and found that the level of DNA methylation depends on the plant species and the heavy metal exposure.

The Greater Sudbury Region (GSR) in Northern Ontario was one of the most ecologically disturbed regions in Canada, with numerous studies documenting the effects of SO_2 and metals in soils in the region for > 100 years (Cox and Hutchinson 1980; Amiro and Courtin 1981; Hutchinson and Symington 1997; Gratton et al. 2000; Nkongolo et al. 2008; Spiers et al. 2012). Elevated concentrations of metal accumulation have been reported in both soils and vegetation up to 100 km distant from the smelters compared to reference sites and regional soil parent materials (Freedman and Hutchinson 1980; Gratton et al. 2000; Nkongolo et al. 2008; Dobrzeniecka et al. 2011; Vandeligt et al. 2011; Spiers et al. 2012). The presence of metal contaminants at elevated concentrations in the soil imposes a severe stress on plants, thus hindering the growth of vegetation (Wren et al. 2012). Effects of metals on physiological and genetic processes in hardwood species such as *A. rubrum* and *Betula papyrifera* have been reported. Epigenetic analysis of these populations is lacking (Theriault et al. 2013, 2014, Kalubi et al. 2015, 2016).

The main objective of the present study is to determine the relationship between metal contamination and Cation Exchange Capacity (CEC) on whole DNA modifications.

5.3 Materials and Methods

5.3.1 Field study

5.3.2 Sampling

Soil and leaf samples were collected from seven locations throughout the GSR as described in (Kalubi et al. 2015). The sampling sites include three metal-contaminated sites based on previous studies (Wahnapitae-Dam; Laurentian; Falconbridge). Four distal sites were used as reference (Kukagami, Capreol, St. Charles, and Onaping Fall) (Fig. 12 and Table 13). To avoid variation caused by biological samplings, all the trees selected for this study were at the same developmental stage and between 25 and 30 years. They were from the second generation of *A. rubrum* populations in GSR. For each site, 20 soil and leaf samples were collected. All the collected leaves were flash frozen and stored at -80°C for DNA extraction.

Table 13. Coordinates of sampling sites

Sites	GPS Coordinates
Capreol	46’45’28’N/80’55’21’W
St.Charles	46’25’21’N/80’25’19’W
Onaping Falls	46’35’32’N/81’23’3’W
Kingsway	46’29’54’N/80’58’14’W
Laurentian	46’28’5’N/80’58’35’W
Falconbridge	46’34’49’N/80'48’39’W
Kukagami Road	46’32’11’N/80’38’35’W

Figure 12. Location of sampling areas from the Greater Sudbury Region. These locations include: Wahnapitae-Dam, Laurentian, Falconbridge, Kukagami, Capreol (reference), St. Charles (reference), and Onaping Falls (reference).

Sources: Edited from Google Map 2015.

5.3.3 Soil chemistry

Soil pH was measured in water and a neutral salt solution pH (0.1 M $CaCl_2$) (Carter and Gregorich 2007). The exchangeable cations (Al^{3+}, Ca^{2+}, Fe^{3+}, K^+, Mg^{2+}, Mn^{2+}, and Na^+) were quantified by ICP-MS analysis of ammonium acetate (pH 7) extracts of soil samples, with the total exchange capacity (CEC) being estimated by summation of the exchangeable cations (Hendershot et al. 2007).

Soil and leaf metal analysis was performed as described in (Kalubi et al. 2015). For the estimation of total metal concentrations, a 0.5 g soil sample was treated with 10 mL of 10:1 ratio HF:HCl, heated to 110°C for 3.5 hours in open 50 mL Teflon™ tube in a programmable digestion block to dry down samples, followed by addition of 7.5 mL of HCl and 7.5 mL of HNO_3 and heating to 110°C for another 4 hours to dry gently. The samples are then heated to 110°C for 1 hour following addition of 0.5 mL of HF, 2 mL of HCl and 10mL of HNO_3 to reduce sample volume to 8-10 mL. On cooling, the samples are made to 50 mL with ultrapure water for subsequent analysis by plasma spectrometry. Bioavailable metals were estimated by extracting 5 g of soil with 20 ml of 0.01M $LiNO_3$ in a 50 ml centrifuge tubes in a shaker under ambient lighting conditions for 24 hours at 20°C (Abedin et al. 2012; Nkongolo et al. 2013). The pH ($LiNO_3$) of the suspension was measured prior to centrifugation at 3000 rpm for 20 minutes, with filtration of the supernatant through a 0.45 µm filter into a 20 ml polyethylene tube and made to volume with deionized water. The filtrate was preserved at approximately 3°C for analysis by ICP-MS. The quality control program completed in an ISO 17025 accredited facility (Elliot Lake Research Field Station of Laurentian University) included analysis of duplicates, Certified Reference Materials (CRM's), Internal Reference Materials (IRM's), procedural and calibration blanks, with continuous calibration verification and use of internal standards (Sc, Y, Bi) to correct for any mass bias. All

concentrations were calculated in mass/mass dry soil basis. The data obtained for all elements of interest in analyzed CRM soil samples were within ± 12% of the certified level.

5.3.4 Whole DNA methylation

Genomic DNA was extracted from fresh frozen leaf materials using the CTAB extraction protocol as described by (Nkongolo 2004; Mehes et al. 2007). For each site, 20 leaf sample were analyzed. The DNA exctraction protocol is a modification of the Doyle and Doyle (1987) procedure. The modifications included the addition of 1% polyvinyl pyrrolidone (PVP) and 0.2% beta mercaptanol to the cetyl trimethylammonium bromide (CTAB) buffer solution, two additional chloroform spins prior to the isopropanol spin and no addition of RNAse. After extraction, DNA was stored in a freezer at -20°C.

The general protocol for whole cytosine methylation is described in (Tsuji et al. 2014). Nucleoside quantification was determined using Tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS). Genomic DNA was digested with DNA Degradase Plus (ZYMO RESEARCH) per the manufacturer protocol. LC separation was performed on a dC18 2.1x100 mm column at flow rate of 0.2 ml/min. The mobile phase was 15% CH3OH, 85% H2O with 1% formic acid and 10 mM ammonium formate. The injection volume was 15 µL. A Waters/Micromass Quattro Micro mass spectrometer was used for the detection of nucleosides. Electrospray ionization in positive ion mode was used to generate ions. Cytosine and adenine methylation levels are reported as [5mdC]/[dG], and [6N-mdA]/dT] ratios, respectively.

5.3.5 Statistical analysis

The data for the CEC, total and bioavailable metals, and cytosine and adenine methylation levels were analyzed using SPSS 20 for Windows, with all data being transformed using a $\log_{10}$ transformation to achieve a normal distribution. ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences in metal concentrations among soil and leaf samples ($P \leq 0.05$). Correlation coefficients among pH, CEC, metal content, cytosine, and adenine methylation were determined.

5.4 Results

5.4.1 Cation exchanges, pH and soil metal contamination

Soil metal contamination levels are described in Table 14. The levels of copper and nickel were higher in metal – contaminated compared to reference sites. The amounts of Cu and Ni in leaves were too small and similar among and within sites to be considered in further analyses. This is consistent with previous studies that indicate that *A. rubrum* does not accumulate metals in their leaves (Kalubi et al. 2016). Soil pH in metal contaminated and reference sites were similar and consistent with the Canadian shields soil acidity, ranging from 4.4 to 5.3 in all the sites with exception of the limed sites with a high pH of 6.6.

The total cation exchange capacity CEC values on the other hand varied significantly among sites (Table 3). No association between the levels of metals and CEC was observed. The Cation exchange capacity (CEC) of soil is a sum of exchangeable cations that it can adsorb at a specific pH, thus representing the capability of soil to attract, retain and hold exchangeable cations. Exchangeable cations include both basic cations (K^+, Ca^{2+}, Mg^{2+} and Na^+) and acidic cations (H^+, Al^{3+} and Fe^{2+}). The highest total effective CEC (10 cmol/) was found in Falconbridge, a limed site, reflective of higher exchangeable Ca^{2+} and Mg^{2+} on the soil organic and mineral colloid surfaces released by the dissolution of the dolomitic milestone applications up to 30 years ago (Table 15). The dolostone dissolution led to an increase in soil pH which favoured the development of surface humus forms higher in stabilized soil organic matter (Table 15). The lowest CEC value (1.80 cmol/kg) was recorded on highly contaminated sites that were not treated with the dolostone application. The CEC value for the reference sites ranged from 2.10 at a coarse textured, low

organic matter site to 6.00 cmol/kg at a site with a well-developed humus form. The correlation between these pH and CEC was very strong and highly significant (r = 0.96**) (Table 16).

Table 14. Total and bioavailable (in parenthesis) metal concentrations in soil samples from the Greater Sudbury Region. Metal concentration in mg/kg)

Sites	Copper (Cu)	Nickel (Ni)	Zinc (Zn)
Laurentian	2020.00 a (19.20)	3010.00 a (12.90)	109.00 a (2.19)
Wahnapitae Dam	1890.00 a (14.20)	2030.00 b (10.40)	147.00 a (1.46)
Falconbridge (limed)	888.50 b (6.67)	838.00 c (4.93)	62.90 b (0.25)
Kukagami	162.00 c (3.17)	188.00 d (2.62)	43.80 bc (1.30)
Capreol	188.00 c (0.00)	259.00 d (0.00)	96.80 ab (0.19)
Onaping-Falls	110.00 c (2.09)	165.00 d (2.47)	85.80 ab (3.37)
St.- Charles	115.00 c (0.71)	217.00 d (5.61)	60.50 b (3.55)

Mean values for Cu, Ni, and Zn with the same letter are not significantly different based on Tukey's HSD multiple comparison test ($P \geq 0.05$).

Table 15. Cation Exchange Capacity (cmol/Kg) and pH in soil samples from meta-contaminated and reference sites in the Greater Sudbury Region. .

Sites	Al	Ca	Fe	K	Mg	Mn	Na	Effective CEC*	pH
Metal-contaminated									
Laurentian	0.050	1.038	0.141	0.253	0.267	0.084	0.038	1.90 e	4.51
Wahnapitae Dam	0.080	1.123	0.071	0.174	0.402	0.021	0.016	1.80 e	4.75
Falconbridge (limed)	0.014	6.188	1.348	0.188	2.732	0.013	< DL	10.5 a	6.57
Kukagami	0.071	1.307	0.189	0.165	0.304	0.030	< DL	2.10 de	4.44
References (control)									
Capreol	0.070	1.787	0.263	0.254	0.237	0.028	< DL	2.60 d	5.1
Onaping-Falls	0.095	4.142	0.843	0.192	0.641	0.048	0.012	6.00 b	5.3
St-Charles	0.082	2.256	0.392	0.366	0.430	0.143	0.006	3.70 c	4.9

< DL represents below detection level

*The effective cation exchange capacity (ECEC) is defined as the total amount of exchangeable cations.

Mean ECEC values with the same letter are not significantly different based on Tukey's HSD multiple comparison test ($P \geq 0.05$).

Table 16. Correlations between cytosine methylation, adenine methylation, CEC, pH, bioavailable nickel and copper content in soil

	Cytosine Methylation	Adenine Methylation	CEC	pH	Nickel	Copper
Cytosine Methylation	-	- 0.50	- 0.11	- 0.26	- 0.71**	- 0.72**
Adenine Methylation		-	- 0.53	- 0.52	0.78**	0.88**
CEC			-	0.96**	- 0.36	-0.30
pH				-	- 0.28	- 0.20
Nickel					-	0.93**
Copper						-

** represents significant correlation coefficients ($P \leq 0.01$)

5.4.2 Whole DNA methylation

The extent of cytosine methylation in DNA was assessed by the ratio of 5-methyldeoxycytidine (5 mdC) to deoxyguanosine (dG). There was a significant variation in the level of cytosine methylation among the metal-contaminated sites (Fig. 13). The mean levels of [(5-mdC)/dG] were 0.002 (0.02%) for Wahnapitae Hydro Dam (contaminated), 0.088 (8.8%) for Laurentian (contaminated), 0.05 (5.0%) for Falconbridge, 0.113 (11.3%) for Kukagami, 0.088 (8.8%) for Capreol (uncontaminated), 0.091 (9.1%) for Onaping Falls (uncontaminated), and 0.081 (8.1%) for St. Charles.

Strong and significant negative correlations between the bioavailable nickel / copper content and cytosine methylation were observed. The correlation values for bioavailable nickel and [(5-mdC)/dG] and copper and [(5-mdC)/dG] were – 0.71** and - 0.72**, respectively (Table 16). Very weak and insignificant correlation was observed, however, between cytosine methylation levels and CEC (Table 16). Genotypes from the Laurentian site appears to be recalcitrant to cytosine methylation as the levels of [(5-mdC)/dG] was comparable to those observed in reference sites despite a high level of both total and bioavailable Ni and copper in soils. The highest decrease in cytosine methylation was observed in samples from Wahnapitae Hydro Dam (Fig. 13).

The analysis of genomic DNA for adenine methylation in this study showed a very low level of [6N-mdA]/dT] in *A. rubrum* plants analyzed, ranging from 0% to 0.08%. There was a significant difference in adenine methylation between the highly metal-contaminated sites and the uncontaminated or less contaminated sites. In fact, [6N-mdA]/dT] level was higher in Laurentian and Wahnapitae – Dam samples compared to the reference sites (Capreol, Onapings, and St Charles) (Fig. 14). Strong and significant positive correlation was observed between [6N-mdA]/dT] and soil bioavailable nickel and copper content (Table 16). Detailed analysis of the chromatogram revealed two peaks that were resolved for the N6-mdA, one within the expected range at 5.65 and a second at 4.82 in 25% of the samples from both contaminated and uncontaminated sites (Appendix 1 – Supplementary materials). This unknown compound close to adenine methylation peak has not been reported in any prokaryotes or mammalian species that have been analyzed extensively for DNA modifications to date.

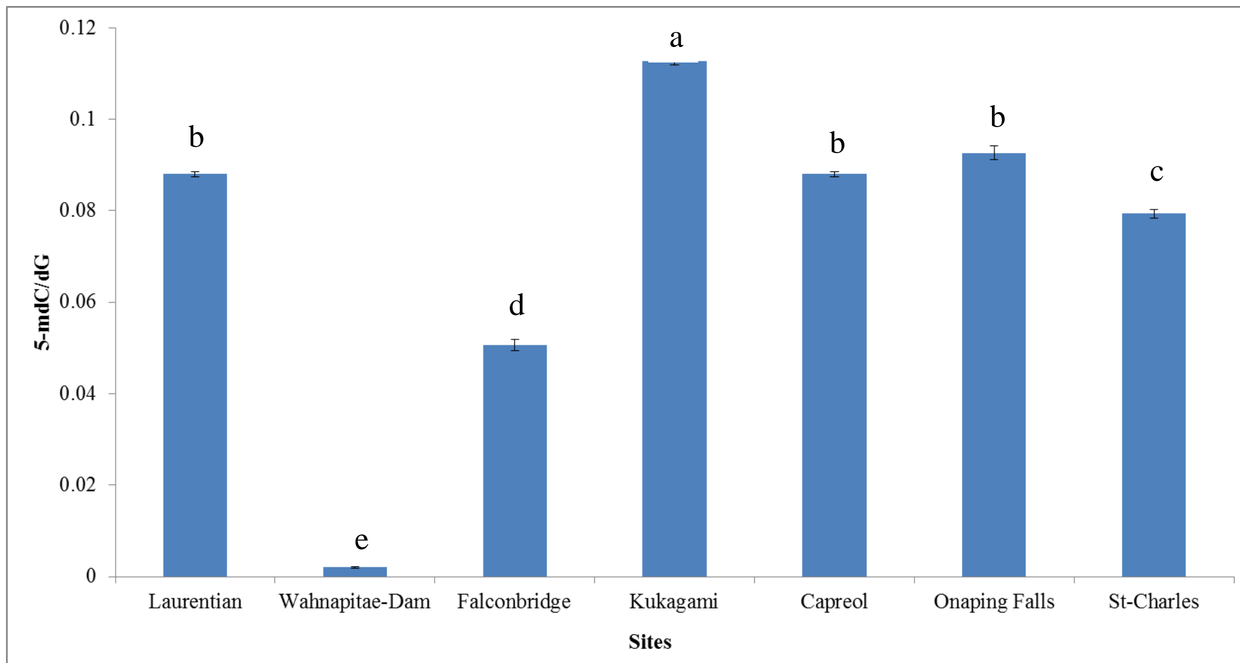

Figure 13. Whole DNA methylation levels [(5-mdC)/dG] in DNA from *Acer rubrum* trees from Laurentian (metal-contaminated), Wahnapitae-Dam (metal-contaminated), Onaping Falls (reference), and Capreol (reference).

Mean values with the same letter are not significantly different based on Tukey's HSD multiple comparison test ($P \geq 0.05$).

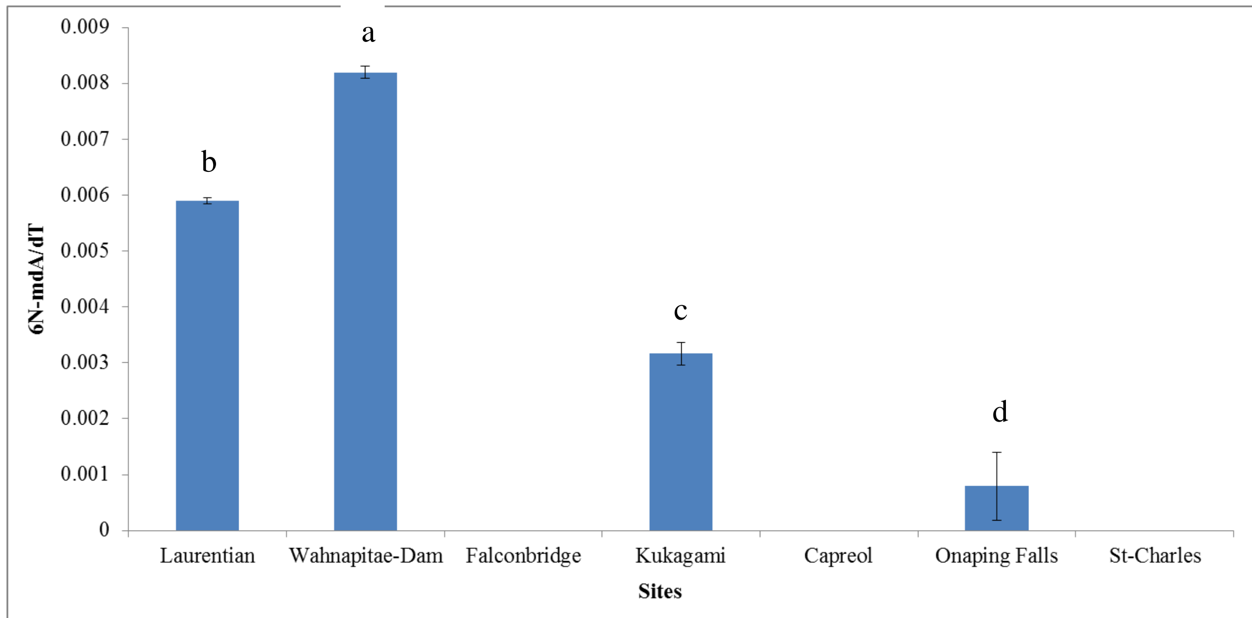

Figure 14. Whole DNA methylation levels [(6N-mdA)/dT] in DNA from *Acer rubrum* trees from Laurentian (metal-contaminated), Wahnapitae-Dam (metal-contaminated), Onaping Falls (reference), and Capreol (reference).

Mean values with different letters are significantly different based on Tukey's HSD multiple comparison test ($P \geq 0.05$).

5.5 Discussion

5.5.1 Cation exchanges, pH and soil metal contamination

In the present study, with the exception of the limed site, the soil samples were acidic. The reference sites show significantly lower levels of metals than contaminated sites located close to smelters. Proton toxicity (low-pH stress) is considered to be one of the major stresses limiting plant growth in acid soils (Kochian et al. 2004). Low pH levels directly inhibited plant growth via high H^+ activity (Schubert et al. 1990; Koyama et al. 2001). A high concentration of H^+ triggers typical oxidative stress on plants by inducing the accumulation of excess reactive oxygen species (ROS), such as superoxide radicals ($O_2^{-}\bullet$) and hydrogen peroxide (H_2O_2) in plant tissues (Shi et al. 2006; Liu et al. 2011). Reactive oxygen species (ROS) is the main toxicity mechanism involved in plant stresses including metal contamination. This has been confirmed in a number of studies (Schützendübel and Polle 2002; Keunen et al. 2011; Das and Roychoudhury 2014; Zhang et al. 2015). Toxic metal-induced oxidative stress is usually greater in sensitive plants than in tolerant ones.

CEC is also a very important soil chemical property which reflects soil structure stability, nutrient availability, soil pH and the soil reaction to the application of fertilizers and other ameliorants (Adeniyan et al. 2011; Tomašić et al. 2013). Many soil physical, chemical and mineralogical parameters influence soil exchange capacity, especially soil pH, soil texture, specifically secondary mineral content and organic matter content. Various studies have also reported a strong correlation between soil pH and CEC (Tomašić et al. 2013) as observed in the present study.

Overall, with the exception of Falconbridge and Onaping Falls reference site, the effective CEC for all the sites were below the expected values of CEC > 3 for podzolic soil (Evans 1982). CEC in weathered soils can be improved by adding lime which dissolves and raises the pH, encouraging the formation of stable surface humus forms on both forested and marginal lands. In fact, the dissolution of the applied dolostone decreases soil acidity, reduces Al availability to the plant roots, and also improves the Ca and Mg nutrient status of the soils. This fertility

improvement supports increased vegetation productivity which, in turn, provides a source of additional litter for decomposition in the well-developed Ha layers of the thicker, stable soil humus forms (Rizvi et al. 2012).

The most effective way of improving the CEC in agricultural soils is to increase organic matter content with the increased root mass under permanent pasture, regular slashing, growth of green manure crops, retention and degradation of crop stubble, rotation of crops or pasture, and the addition of mulch and manure materials to the soil surface.

For metal analysis, there is a variety of methods of varying ionic strength and acidity levels used to assess metal bioavailability from soils (Cooper and Morse 1998; Lambrechts et al. 2011; Wren et al. 2012). In the present study, the bioavailable elemental concentrations were estimated using a dilute neutral electrolyte, 0.01M lithium nitrate solution, at a 1:10 soil:extractant ratio as described in (Abedin et al. 2012) and (Nkongolo et al. 2013). This neutral dilute salt solution extracts lower concentrations closer to that expected in the actual soil solution at the soil-root interface. The measured concentrations are lower than routinely obtained using the popular agronomic acidic Mehlich III extracting solution (Gavlak et al. 2005) adopted by (Mehes-Smith and Nkongolo 2015) to estimate the potentially bioavailable Ni, Cu, and Zn levels from the same sites. As the bioavailable elements levels are highly correlated to the total content of metals in the soil samples, the application of a variety of different and complementary approaches is useful as it provides researchers with a larger comparative database (Abedin et al. 2012).

5.5.2 DNA methylation

Analysis of DNA modifications caused by abiotic stresses have shown increased methylations (hypermethylation), but few studies conducted to examine the effects of heavy metals reveal hypomethylation associated with a high levels of metal contamination (Aina et al. 2004). Both phenomena may contribute to the adaptation of plants to stress (Peng and Zhang 2009).

The lowest level of cytosine methylation was observed in samples from Wahnapitae Hydro Dam and the highest in Kukagami. These two populations are not genetically related based on previous analyses (Kalubi et al. 2015). However, close genetic relationships (genetic distances from 0.2 to 0.3) has been confirmed among populations from Wahnapitae Dam, Falconbridge, Capreol, and St. Charles) (Kalubi et al. 2015). The genetic distances for Laurentian, Onaping, and Kukagami with other populations were moderate ranging from 0.3 to 0.6 (Kalubi et al. 2015). Overall, these distance values indicated that the targeted populations are distinct. Hence the methylation differences between Wahnapitae Hydro Dam and other populations for cytosine methylation might be from site differences.

Although adenine methylation is mostly common in prokaryotes, several studies have reported highly methylated plant DNA, containing 5-methylcytosine (m5C) and N6-methyladenine (m6A) (Vanyushin 2006). In fact, m6A was found also in total DNA of various organs, plastids and mtDNA of wheat, and in rice plant DNA (Vanyushin et al. 1988; Ngernprasirtsiri et al. 1988; Kirnos et al. 1992). However unlike in bacteria, little is known about the mechanisms, enzymes, and biological significance of adenine methylation (Vanyushin 2006). Eukaryotic adenine DNA methyl-transferases appear to originate from bacterial ancestors (Charles et al. 2004).

A number of publications have provided convincing evidence that suggest abiotic stresses such as drought and high salinity, are involved in DNA methylation (Choi and Sano 2007; Chinnusamy and Zhu 2009; Peng and Zhang 2009; Kimatu et al. 2011; Wang et al. 2011). The present study suggests that metal contamination of the root zone of soils might be impacting the extent of DNA methylation within growing plant species. Considering that all the *A. rubrum* populations from the GSR are derived from the same natural gene pool, the variations in both cytosine and adenine modifications are likely site-induced.

DNA methylation is considered to be a protective mechanism against endonuclease digestion and undesired transposition (Bender 1998). Global DNA methylation generally increases and resistance-related gene

methylation decreases after viral infection (Kovalchuk et al. 2003; Boyko et al. 2007). When plants are exposed to biotic or abiotic stress, there is an increase in homologue recombination frequency (Boyko and Kovalchuk 2010; Boyko et al. 2010; Bilichak et al. 2012). An increase in global DNA methylation in the plant induces a reduction in global transcription, with an associated decrease in the rate of energy consumption of the cells. On the contrary, the extent of hypomethylation resistance gene expression will increase and support the cells when faced with a short-term challenge. Overall, DNA methylation pattern change is an important regulatory mechanism for sensing and responding to stress conditions. The variability for stress tolerance in plants should depend on the extent and patterns of DNA methylation. In other words, DNA methylation and stress adaptation should be directly related to each other (Garg et al. 2015).

It is established that two different systems of the genome modification based on methylation of adenines and cytosines coexist in higher plants. Plant gene may be methylated at both adenine and cytosine residues. Adenine DNA methylation may influence cytosine modification and vice versa (Vanyushin and Ashapkin 2011). In the present study, it is unclear why cytosine methylation and Ni and Cu content were negatively correlated while the adenine and Ni and Cu correlation values were positive.

This is because plant DNA methylation is more complex and sophisticated than in microorganisms and animals (Vanyushin 2006; Law and Jacobsen 2010; Vanyushin and Ashapkin 2011). DNA methylation also varies among species, tissues, organelles and developmental stages. It is involved in the control of DNA replication, transcription, repair, gene transposition and cell differentiation. It plays an important role in gene silencing and parental imprinting, and it controls foreign DNA including transgenes. Unlike animals, plants have also their plastids with their own unique cytosine and adenine modifications system that control plastids differentiation and functioning (Vanyushin et al. 1988; Vanyushin and Ashapkin 2011).

The tandem mass spectrometry (MS/MS) coupled with LC (LC-MS/MS) used to measure overall levels of DNA methylation in the present study is an established approach to nucleoside quantification specifically developed to measure global cytosine methylation (Hu et al. 2013; Tsuji et al. 2014), being a rapid, sensitive, accurate and

specific avenue for modified nucleoside quantification at trace (fmol) levels. Other methods such as methylation-sensitive amplified polymorphism (MSAP), as well as based on bisulfite modifications of DNA that analyze the methylation status of specific sequences, have been also used in many studies. Each of these methods has its own peculiarities. Methylation sensitive amplified polymorphism (MSAP), recently used to assess the effect of heavy metals on cytosine methylation in *Acer rubrum* (red maple), was not sensitive enough to detect quantitative differences in DNA methylation between metal-contaminated and non-contaminated populations in *Acer rubrum* populations growing in the GSR (Kim et al. 2016). Although bisulfite sequencing for cytosine methylation would be more informative in mapping the distribution of DNA modifications, the widespread application in plant epigenetic studies is cost prohibitive, especially for species such as *A. rubrum* whose genome has not been completely sequenced.

5.6 Conclusion

The present study confirms that nickel and copper are major metallic contaminants in anthropogenically acidified soils in targeted sites within the GSR, with the spreading of dolomitic lime maintaining an increased soil pH 30 years after application. The measured CEC values, on the other hand, varied significantly among sites, being significantly lower in highly metal contaminated sites than in both reference and less contaminated sites, and higher in limed sites than in unlimed. A strong association was observed between CEC and $pH_{(H2O)}$, with soils on the limed sites also having improved development surface humus forms. There was a significant variation in the level of cytosine methylation among the metal-contaminated sites, with significant negative correlations between bioavailable nickel / copper content and cytosine methylation being observed. The analysis of genomic DNA for adenine methylation in this study showed a very low level of [6N-mdA]/dT] in *A. rubrum* plants analyzed, ranging from 0% to 0.08%. A significant positive correlation was observed between [6N-mdA]/dT] and bioavailable soil nickel and copper content. The results of this study suggest that the nickel and copper metal contamination in the regional soils are associated with cytosine and adenine methylation.

Future Research Directions, Limitations and Implications.

Control experiments with different dosages of these metals are being conducted to confirm the role of these metals in DNA methylation. In addition, bisulfite sequencing will be used to assess the distribution of methylation in the *B. papyrifera* genome.

5.7 Acknowledgement

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Chapter 6: Perspectives and General Conclusions

6.1. General Conclusions

The specific objectives of the present study were to 1) compare the physiological responses and gene expression in *A. rubrum* and *P. tremuloides* exposed to metal contamination, 2) determine if epigenetic events are associated with metal resistance in *A. rubrum*, and 3) assess global gene expression of *A. rubrum* exposed to different doses of nickel. We hypothesised that 1) the physiological mechanisms of metal resistance as well as the expression of genes associated with Ni resistance are different in *A. rubrum* compared to *P. tremuloides* and 2) the level of gene regulation is metal dose - dependent.

To address the first objective, a comparative metal analysis between *Populus tremuloides* and *Acer rubrum* was performed. The analysis was exclusive to contaminated sites because the bioavailable levels of contaminants in reference sites were negligible. The student t-test revealed no significant differences between washed and unwashed samples for all metals analyzed (Cu, Ni, Zn). This indicates that metal accumulation in aerial tissues is internal. In *P. tremuloides*, the Cu levels were significantly lower in roots compared to bioavailable portion in soil based on Tukey's multiple comparison test ($P \geq 0.05$). Ni content in roots was also significantly lower than in soil, but the total accumulation of this element in leaves was much higher compared to branches and roots. There was a steady increase of Zn from soil to roots, branches, and leaves. In *A. rubrum*, there were similar levels of Cu in roots compared to bioavailable portion in soil. No significant accumulation of Cu in branches and leaves was observed. The amount of Ni was significantly less in roots compared to soil and the accumulation of this element in branches and leaves was limited. The amount of Zn was in general low in soil, roots, branches, and leaves.

The bioaccumulation factors (ratios of metal content in roots over bioavailable metal levels in soil) for *P. tremuloides* and *A. rubrum* were calculated for key metals. The student t test revealed that there were no significant differences for BAF between *P. tremuloides* and *A. rubrum* for Cu and Mg. There was no accumulation of metals in roots compared to the bioavailable levels in soil for Cu and Ni ($BAF < 1$) for P. *tremuloides*. However, the bioaccumulation factor was higher for zinc ($BAF > 1$). The same results were seen for *A. rubrum*. The translocation factor (TF) was calculated as the ratio of the concentration of total metal in

aerial parts of the plants compared to metal content in roots. Overall, the translocation factors from roots to leaves for *P. tremuloides* were generally much greater than A. *rubrum* for each element. There were significant variations in TF among sites.

Four genes were targeted for the gene expression analysis including Nramps4, Nas 3, At2G, MRP4. Alpha-tubulin was used as the housekeeping gene. The results of this study revealed that the expression level of each gene was significantly higher in *P. tremuloides* compared to A. *rubrum*. In general, no significant difference was observed between the metal contaminated and uncontaminated sites for gene expression. In depth analysis showed that AT2G and MRP4 genes were significantly down regulated in *A. rubrum* from the metal contaminated sites compared to those from the uncontaminated area, but environmental factors driving this differential gene expression couldn't be determined.

To further understand the effect of nickel on gene expression in *A. rubrum*, a growth chamber experiment was conducted where low and high doses of nickel were administered to *A. rubrum* seedlings. The goal was to identify differential gene expression at different doses of nickel treatments. In fact, Ni toxicity was assessed by treating six-month old seedlings with an aqueous solution of nickel nitrate salt [$Ni(NO_3)_2$] at the following concentrations: 150 mg, 400 mg, 800 mg, and 1,600 mg of nickel per 1 kg of dry soil. Most of the seedlings displayed a high level of nickel resistance for the Ni treatments. There were no signs of morphological damage or change in plant growth.

Gene expression among the treatments were compared based on P Values. These statistical analyses revealed that 464 genes were downregulated and 609 upregulated in samples treated with 150 mg /kg compared to water. The number of differentially expressed genes increased as the concentration of Ni was increased. We observed 994 down regulated and 1569 up regulated genes when the 800 mg /kg dose was compared to water and 3760 down regulated and 7098 upregulated genes when the highest dose were compared to water based on *P*-values. We also observed 1067 down regulated and 1458 upregulated when the 800 mg /kg were compared to 150 mg /kg.

Likewise, we identified in this study 1566 downregulated and 3569 upregulated genes when the 1600 mg /kg was compared to 800 mg /kg based on P-values.

The *P* value analysis in the present study revealed that a total of 1482 genes were upregulated and 623 genes were downregulated in resistant genotypes (RG) compared to susceptible genotypes (SG). The top 50 up and downregulated genes when RG was compared to SG was presented. Only a few genes among the top 50 could play a role in Ni transport, chelating or other activities in plants.

The final epigenetic analysis aimed at determining the relationship between metal contamination and Cation Exchange Capacity (CECT) on whole DNA modifications. The levels of copper and nickel were higher in metal – contaminated compared to reference sites. Soil pH in metal contaminated and reference sites were similar and consistent with the Canadian shields soil acidity, ranging from 4.4 to 5.3 in all the sites with exception of the limed sites with a high pH of 6.6. The total cation exchange capacity CEC values on the other hand varied significantly among sites. No association between the levels of metals and CEC was observed. The highest total effective CEC (10 cmol/) was found in Falconbridge, a limed site, reflective of higher exchangeable Ca^{2+} and Mg^{2+} on the soil organic and mineral colloid surfaces released by the dissolution of the dolomitic milestone applications up to 30 years ago. The dolostone dissolution led to an increase in soil pH which favored the development of surface humus forms higher in stabilized soil organic matter. The lowest CEC value (1.80 cmol/kg) was recorded on highly contaminated sites that were not treated with the dolostone application. The CEC value for the reference sites ranged from 2.10 at a coarse textured, low organic matter site to 6.00 cmol/kg at a site with a well-developed humus form. The correlation between these pH and CEC was very strong and highly significant (r = 0.96**).

When the extent of cytosine DNA methylation was assessed, there was a significant variation in the level of cytosine methylation among the metal-contaminated sites. Strong and significant negative correlations between

the bioavailable nickel / copper content and cytosine methylation were observed. Very weak and insignificant correlation was observed, however, between cytosine methylation levels and CEC. Genotypes from the Laurentian site appears to be persistent to cytosine methylation as the levels of [(5-mdC)/dG] was comparable to those observed in reference sites despite a high level of both total and bioavailable Ni and copper in soils. The highest decrease in cytosine methylation was observed in samples from Wahnapitae Hydro Dam. Adenine methylation in *A. rubrum* plants were generally very low. There was a significant difference in adenine methylation between the highly metal-contaminated sites and the uncontaminated or less contaminated sites. Strong and significant positive correlation was observed between [6N-mdA]/dT] and soil bioavailable nickel and copper content. Detailed analysis of the chromatogram revealed two peaks that were resolved for the N6-mdA, one within the expected range at 5.65 and a second at 4.82 in 25% of the samples from both contaminated and uncontaminated sites. Overall, low dose of nickel does not induce change in gene expression but might be associated with global DNA methylation in *A. rubrum*.

6.2. Further direction

A detailed analysis of the metal avoidance mechanism in *A. rubrum* would be useful in determining if this phenomenon is strictly physiological or genetic. Identification of putative genes associated with this mechanism would be very informative. The same approach would be recommended to understand the metal accumulation in *B. tremuloides*. The role of epigenetics can be extended by determining the distribution of DNA methylation throughout the genome of both species. But the most cost-effective approach would be to analysis DNA methylation in CpG islands (or CG islands) and targeted genes of interest.

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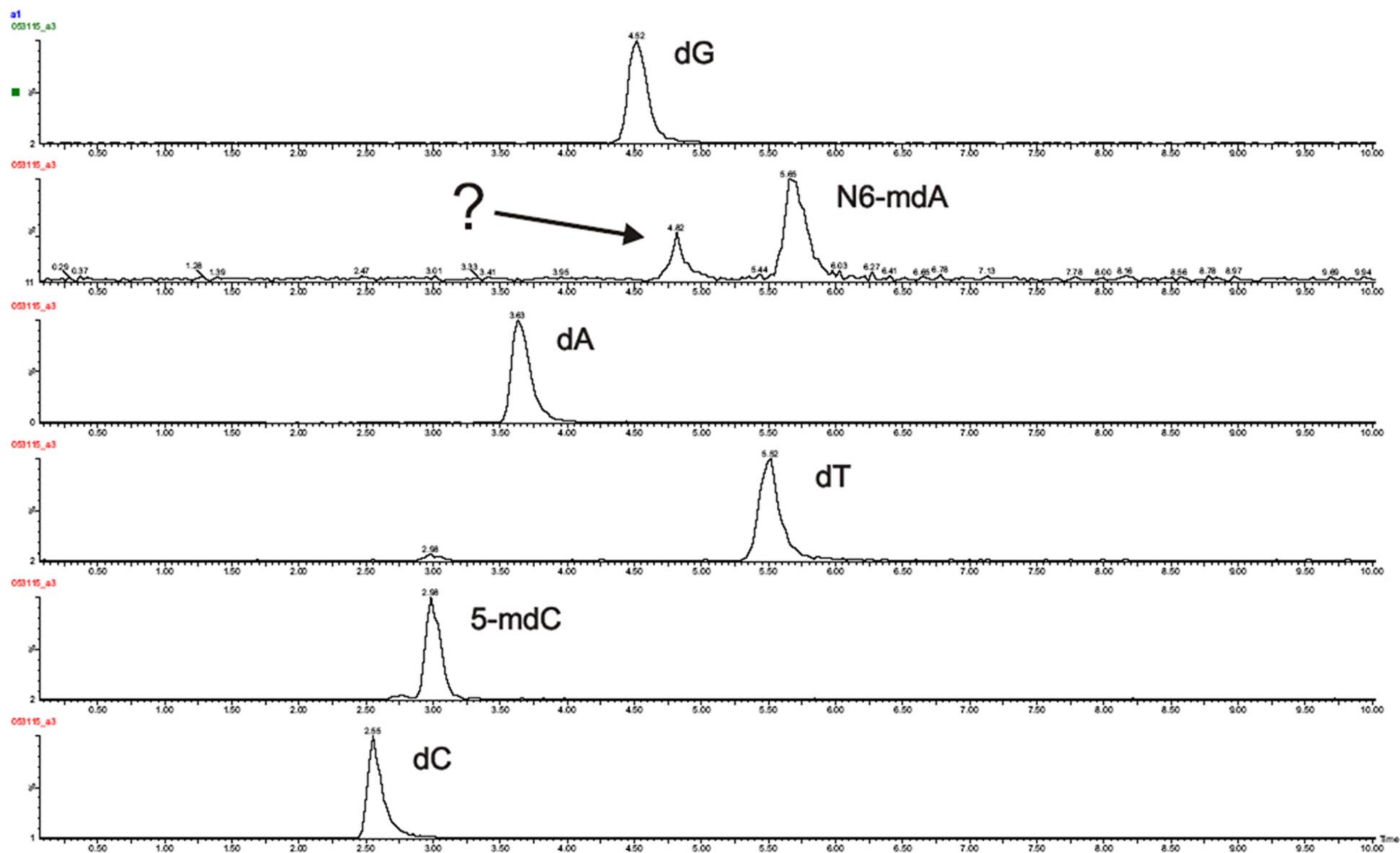
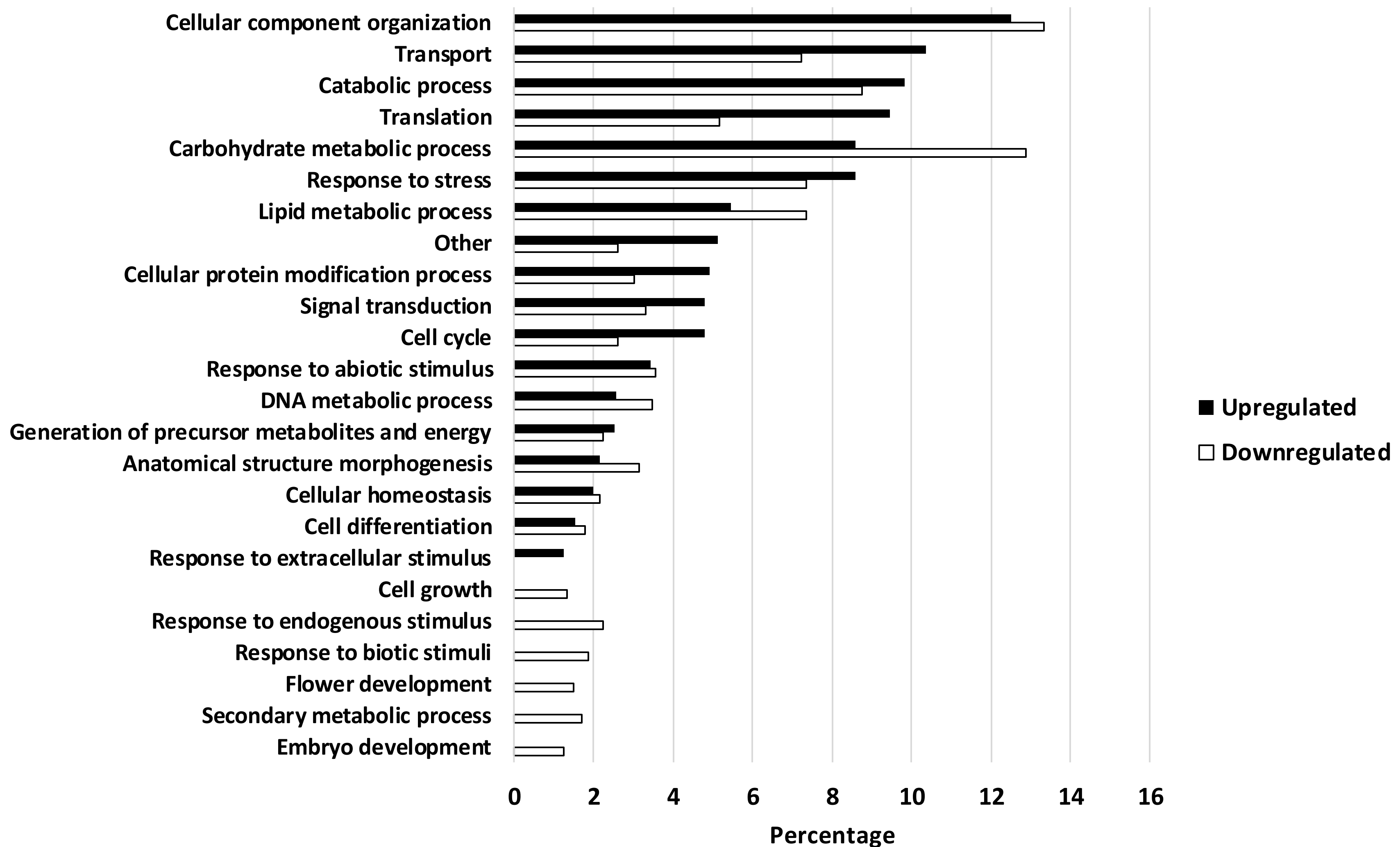
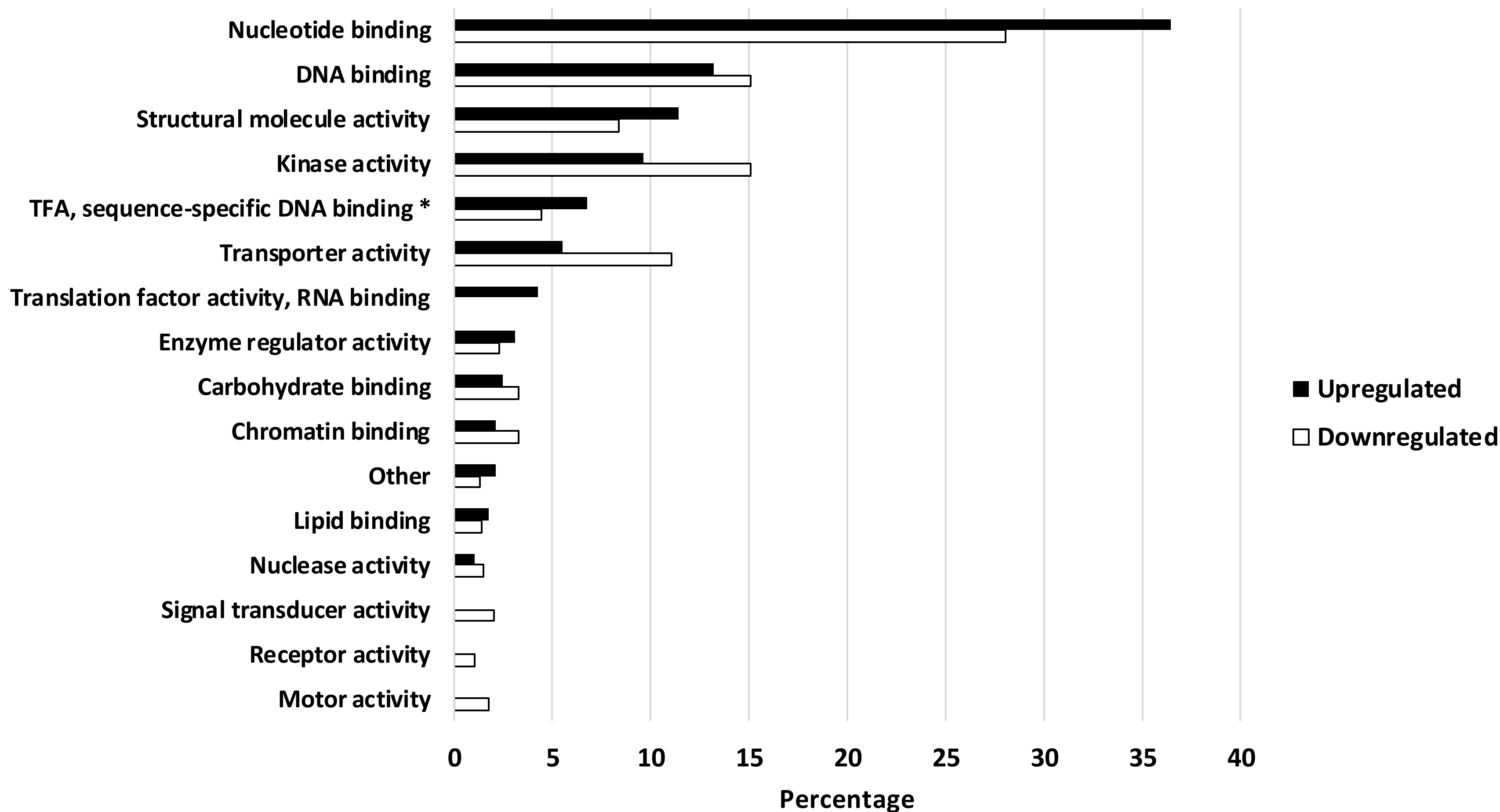
Appendice



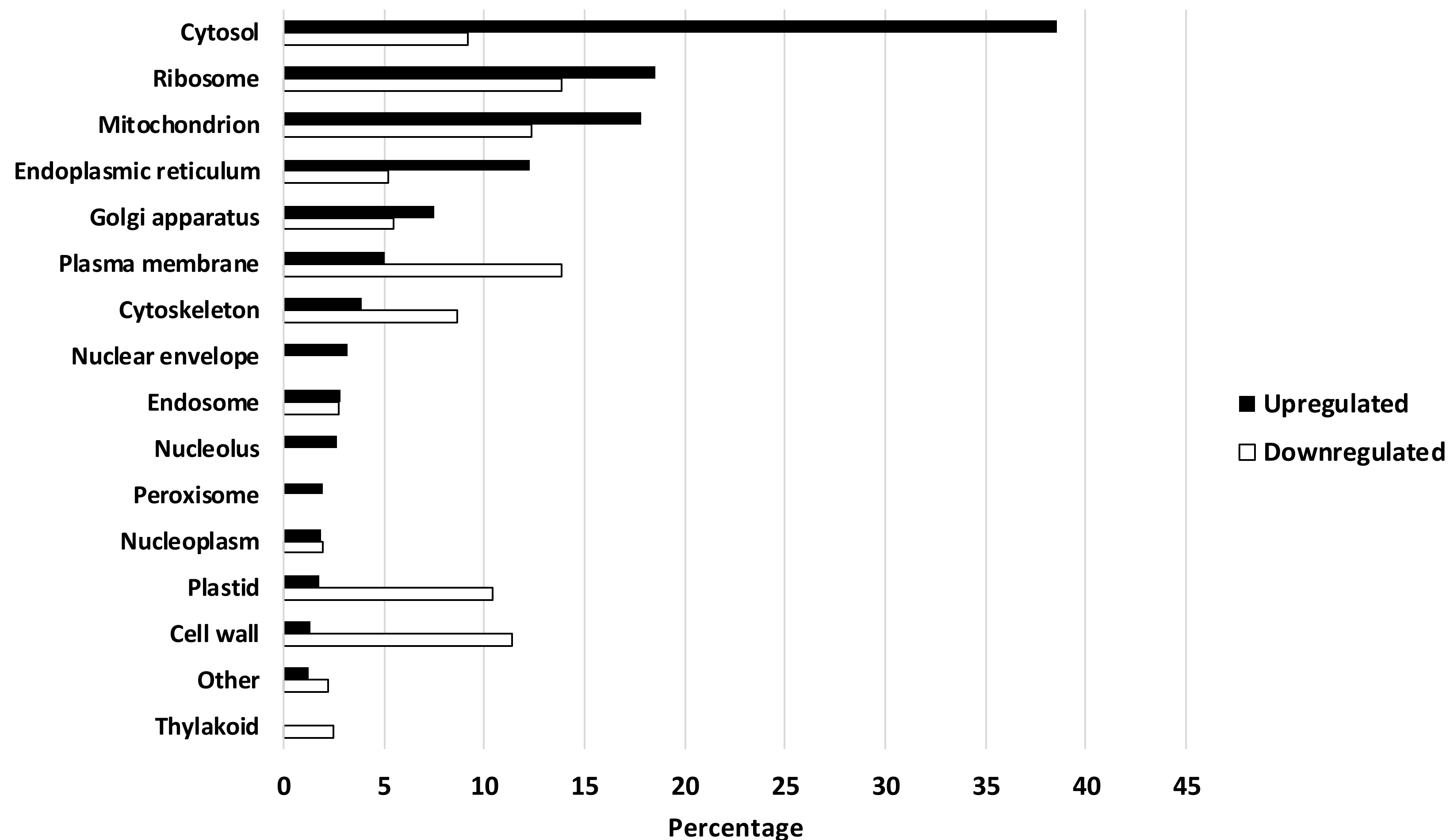
Appendix 1. Supplementary material of chapter 5. Integrated LC-MS/MS chromatograms for dG, N6-mdA, dA, dT, 5-mdC, and C. The arrow indicates unusual second peak for N6-mDA in *Acer rubrum* DNA samples.



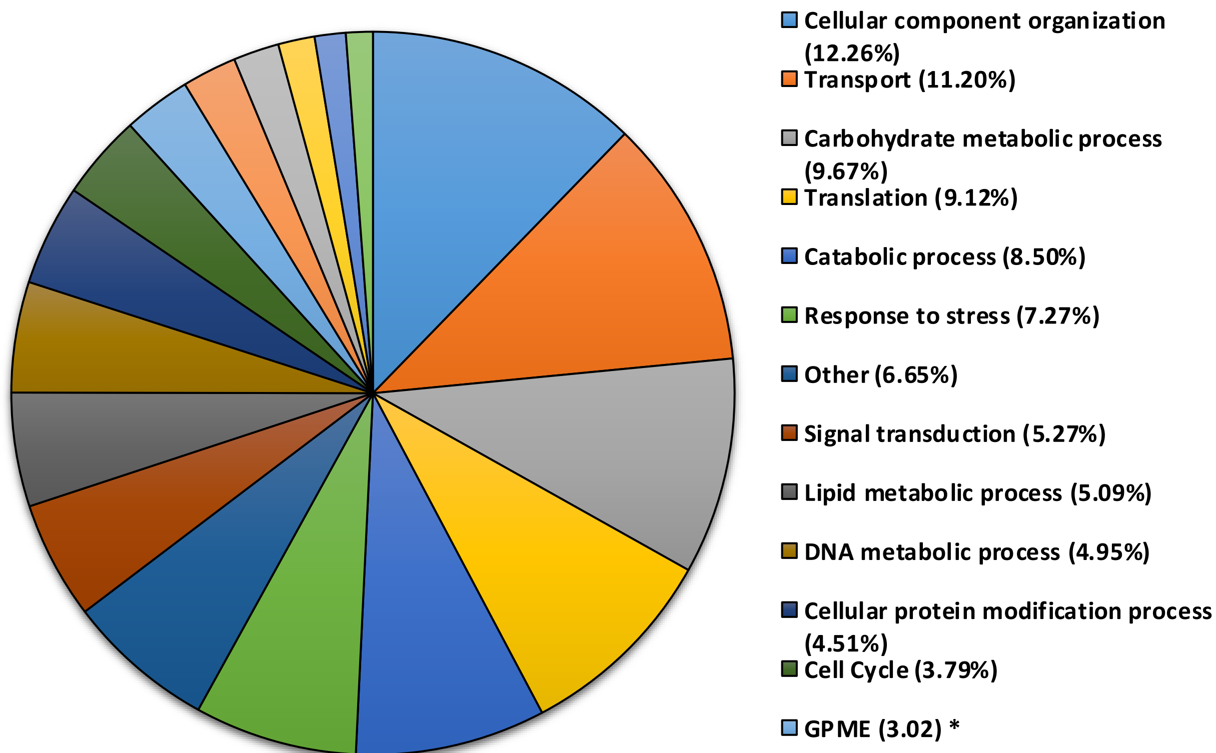
Appendix 2. Supplementary material of chapter 4. Percentage of upregulated and downregulated differentially expressed transcripts when red maple (*Acer rubrum*) treated with 1,600 mg of Ni /kg were compared to plants treated with 150 mg/kg nickel. For upregulated and downregulated transcripts, 1,985 and 1,117 ontologies were identified and classified by biological process using the BLAST2GO software.



Appendix 3. Supplementary material of chapter 4. Percentage of upregulated and downregulated differentially expressed transcripts when red maple (*Acer rubrum*) treated with 1,600 mg of nickel per kg of soil were compared to plants treated with 150 mg of nickel. For upregulated and downregulated transcripts, 1,396 and 788 ontologies were identified and classified by molecular function using the BLAST2GO software.

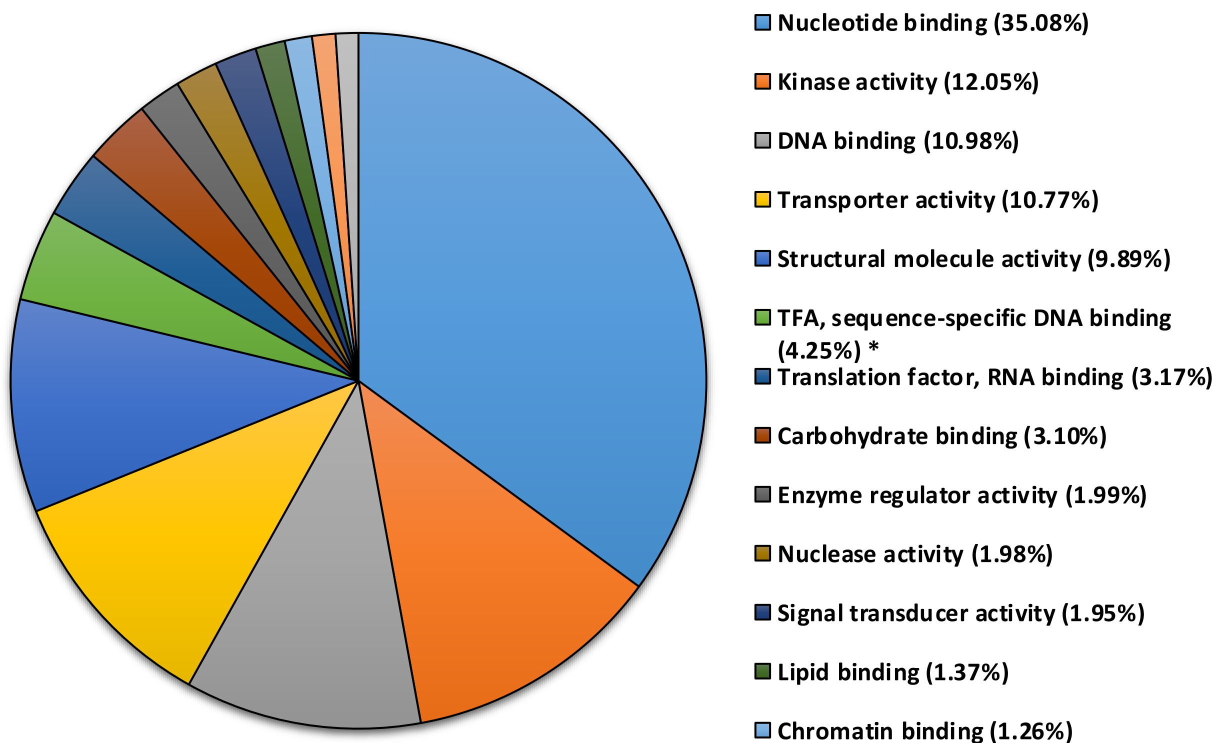


Appendix 4. Supplementary material of chapter 4. Percentage of upregulated and downregulated differentially expressed transcripts when red maple (*Acer rubrum*) treated with 1,600 mg of nickel per kg of soil were compared to plants treated with 150 mg of nickel. For upregulated and downregulated transcripts, 1,171 and 403 ontologies were identified and classified by cellular compartment using the BLAST2GO software.



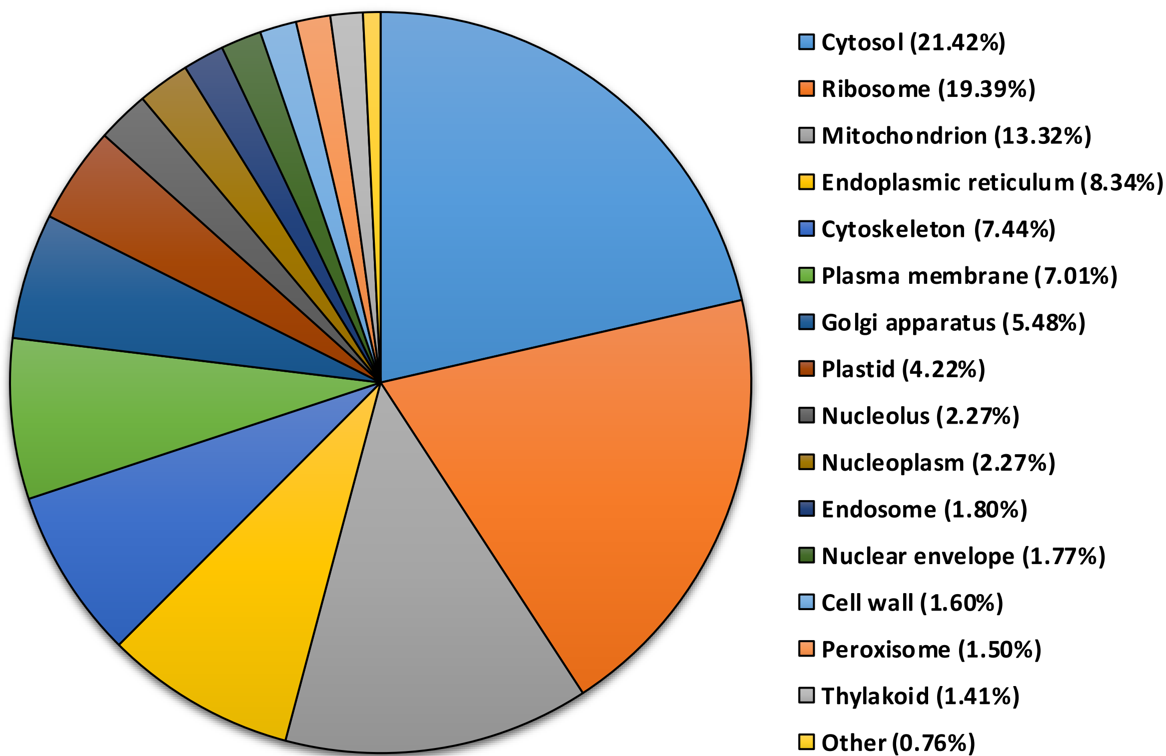
Appendix 5. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) control plants (water only). A total of 15,078 ontologies were grouped under biological function using the BLAST2GO software.

* GPME stand for Generation of precursor metabolites and energy

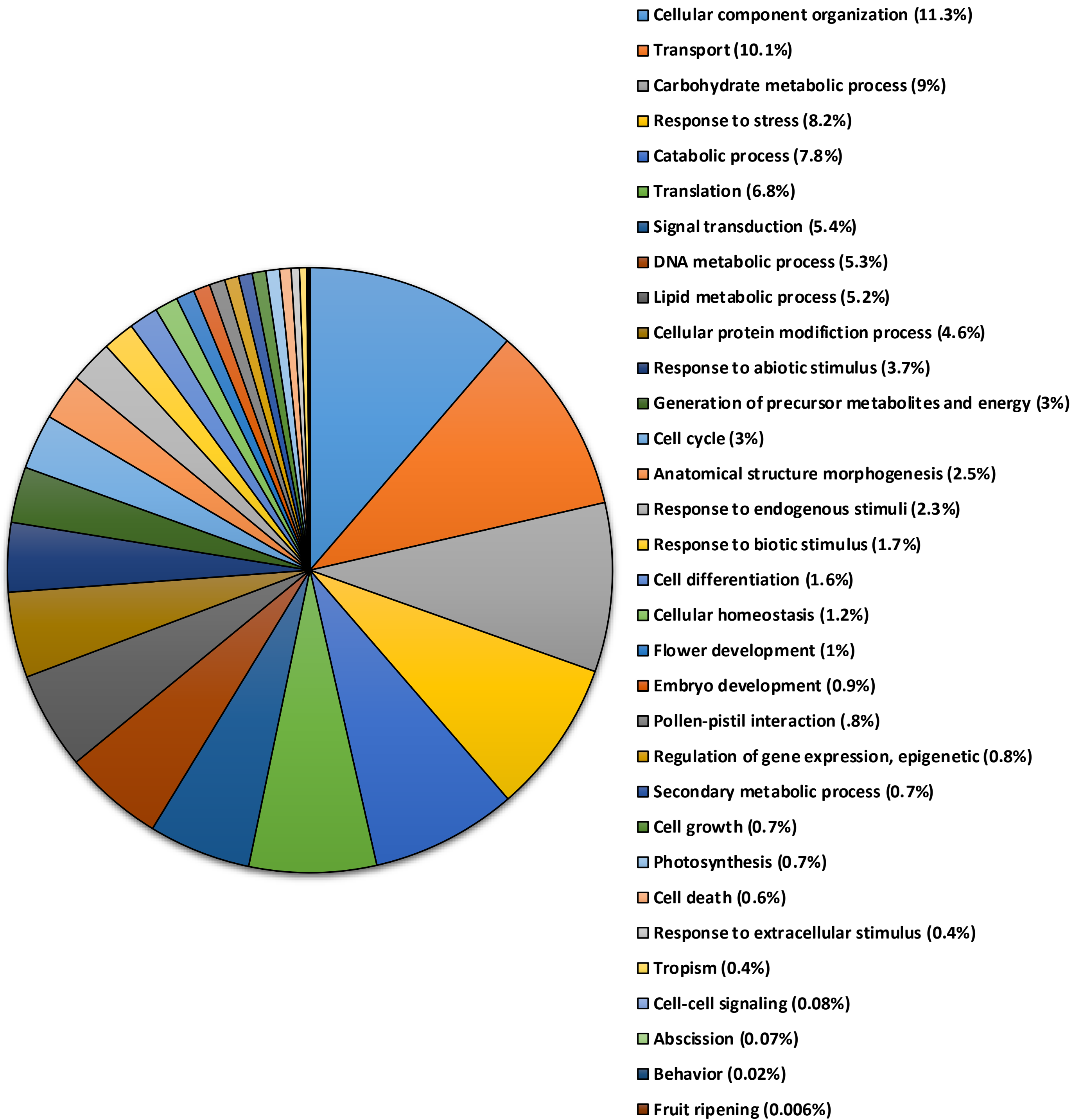


Appendix 6. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) control plants (water only). A total of 13,676 ontologies were grouped under molecular function using the BLAST2GO software.

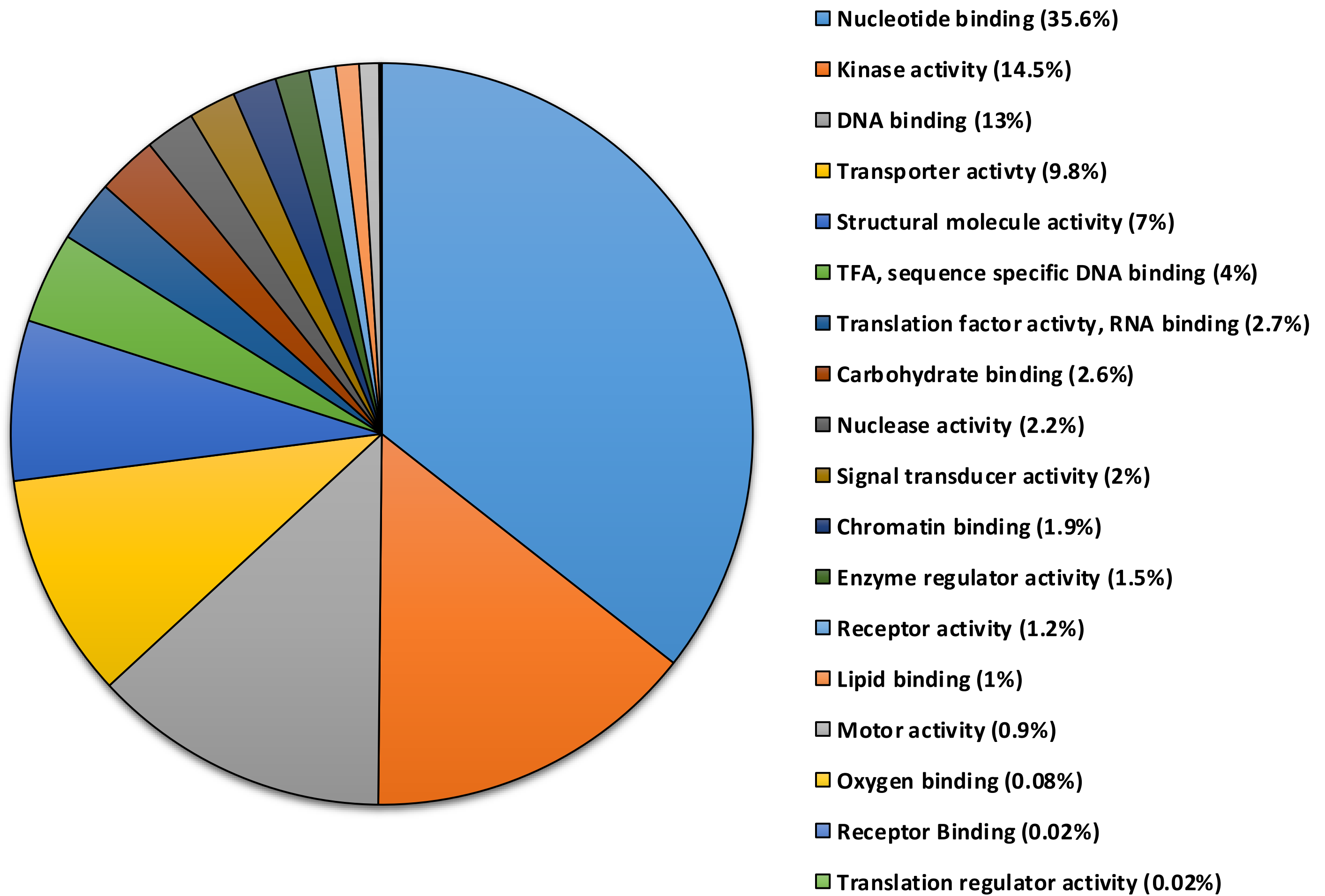
* TFA stands for Transcription factor activity



Appendix 7**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) control plants (water only). A total of 6,951 ontologies were grouped under cellular compartment using the BLAST2GO software.

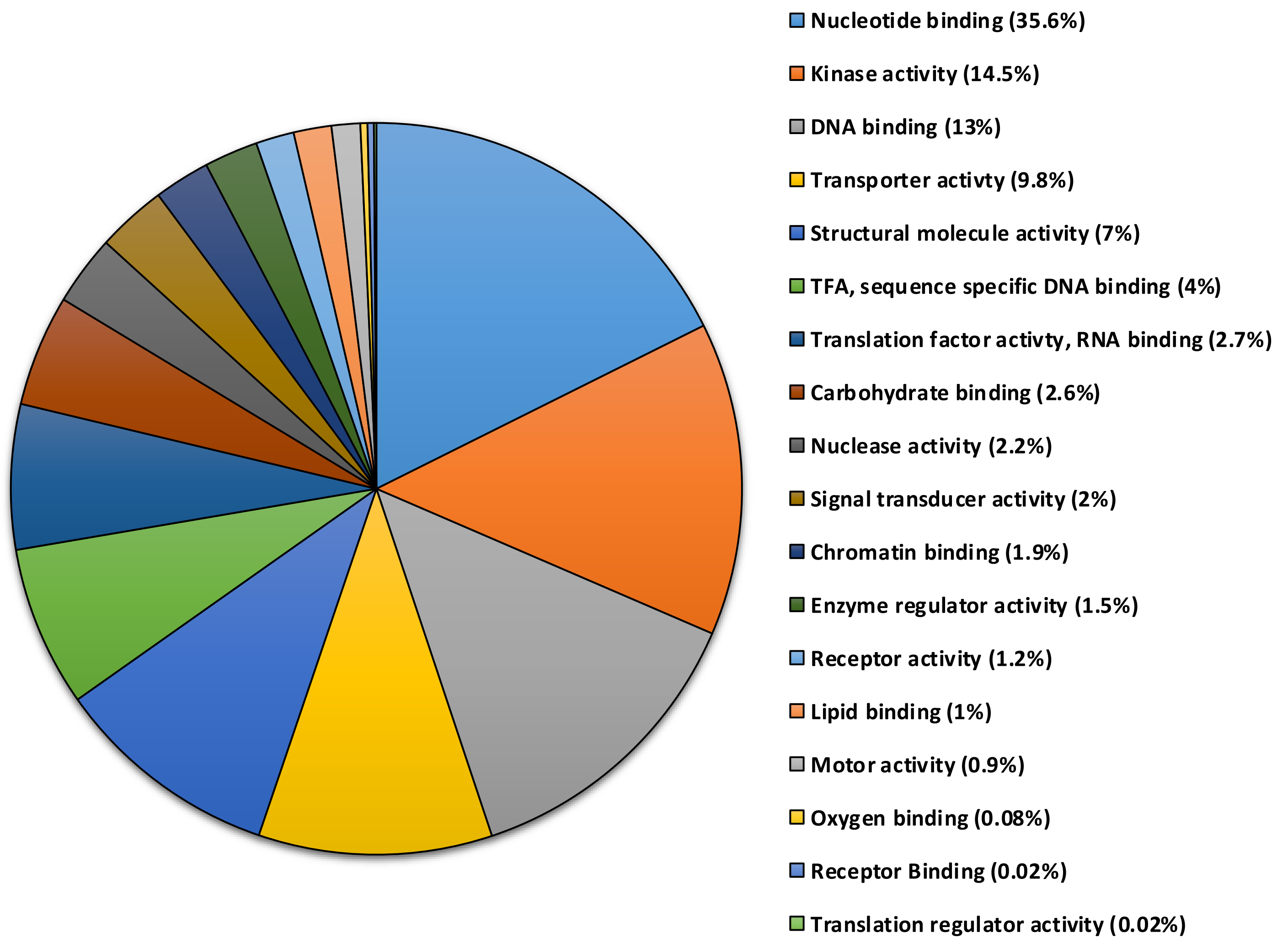


Appendix 8**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) plants treated with the bioavailable dose of nickel only. A total of 17,821 ontologies were grouped under cellular compartment using the BLAST2GO software.

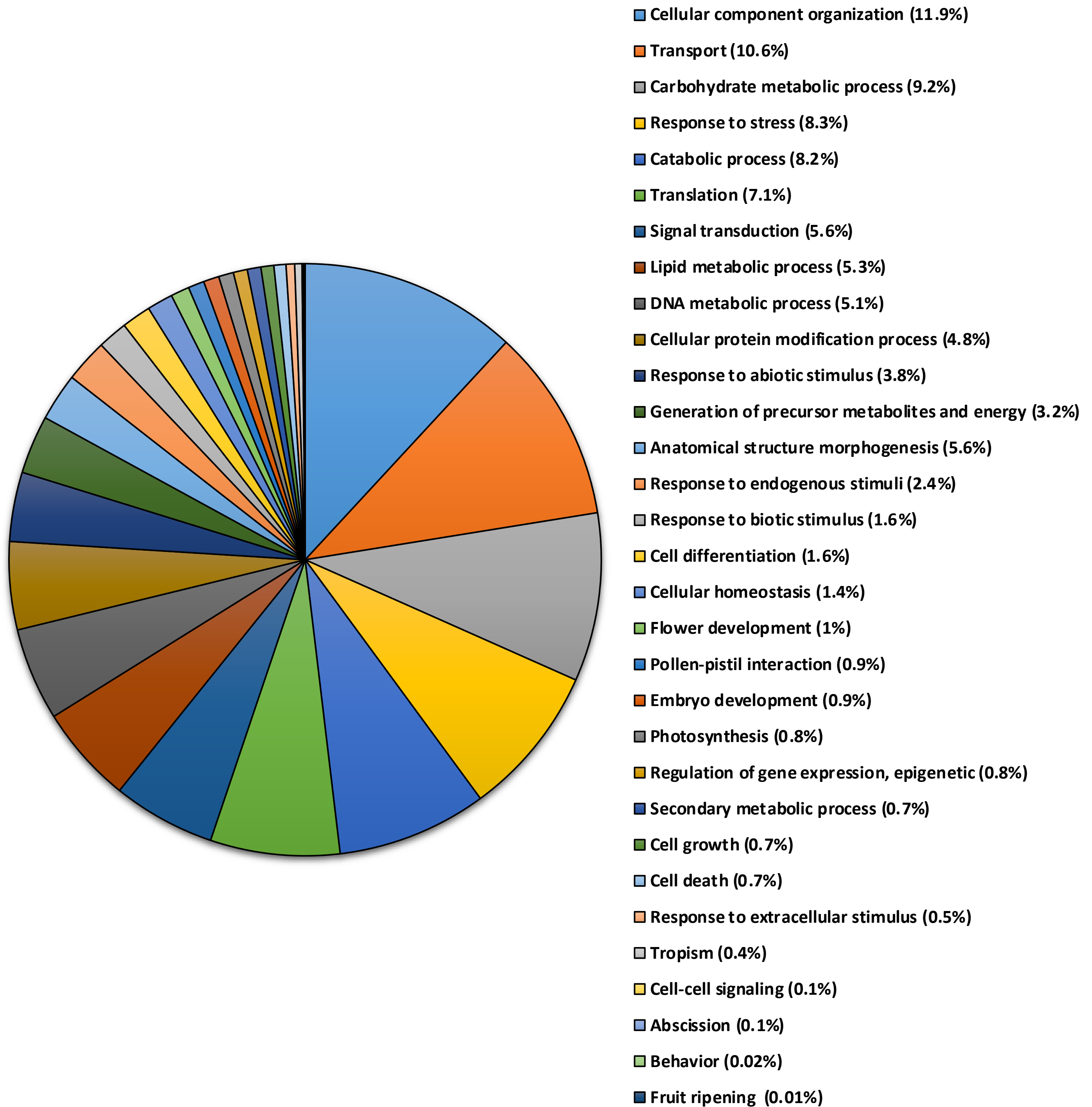


Appendix 9. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) plants treated with the bioavailable dose of nickel only. A total of 16,450 ontologies were grouped under molecular function using the BLAST2GO software.

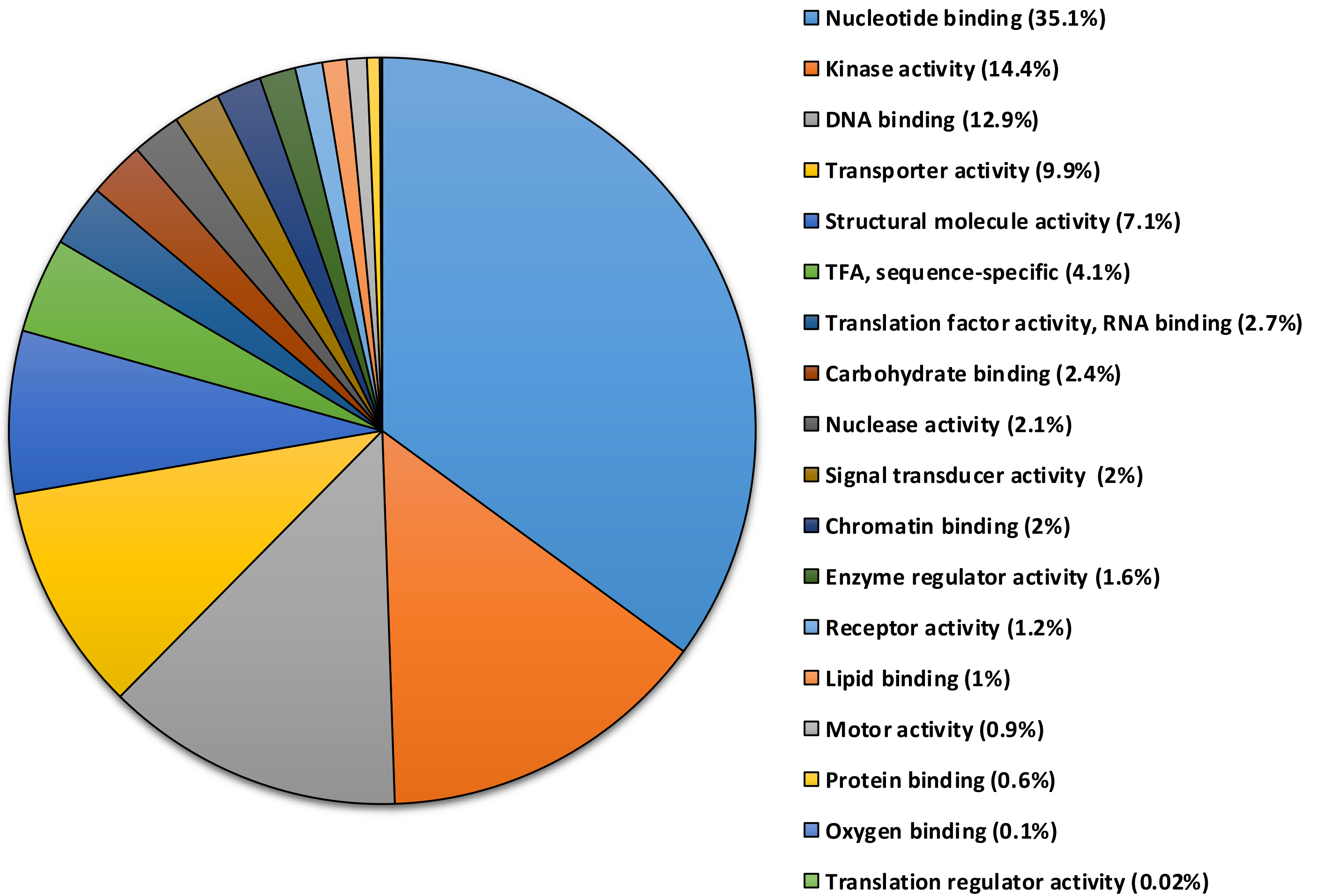
* TFA stands for transcription factor activity



Appendix 10**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) plants treated with the bioavailable dose of nickel only. A total of 6,399 ontologies were grouped under cellular compartment using the BLAST2GO software.

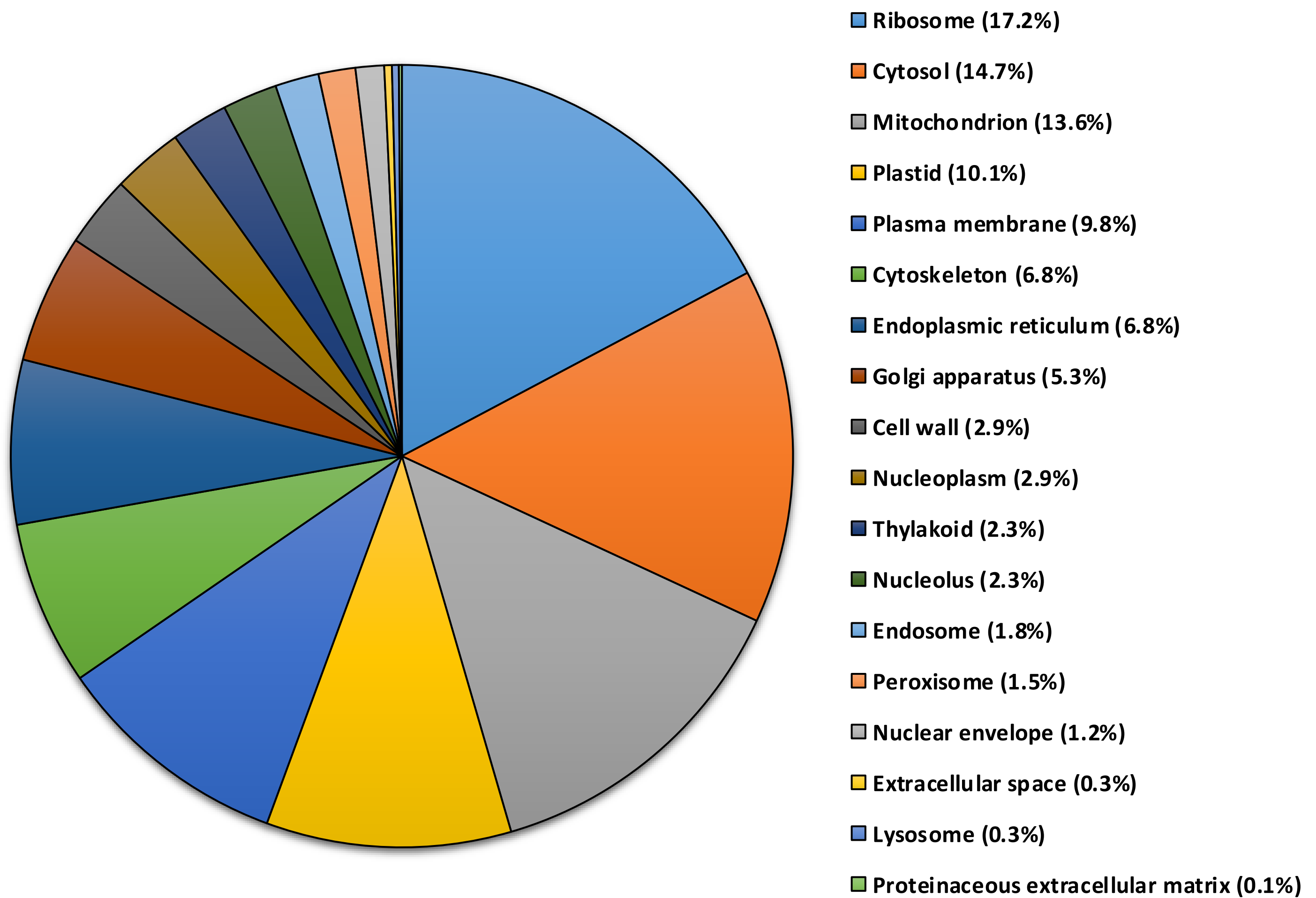


Appendix 11. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) plants treated with the half-total dose of nickel only. A total of 16,462 ontologies were grouped under cellular compartment using the BLAST2GO software.

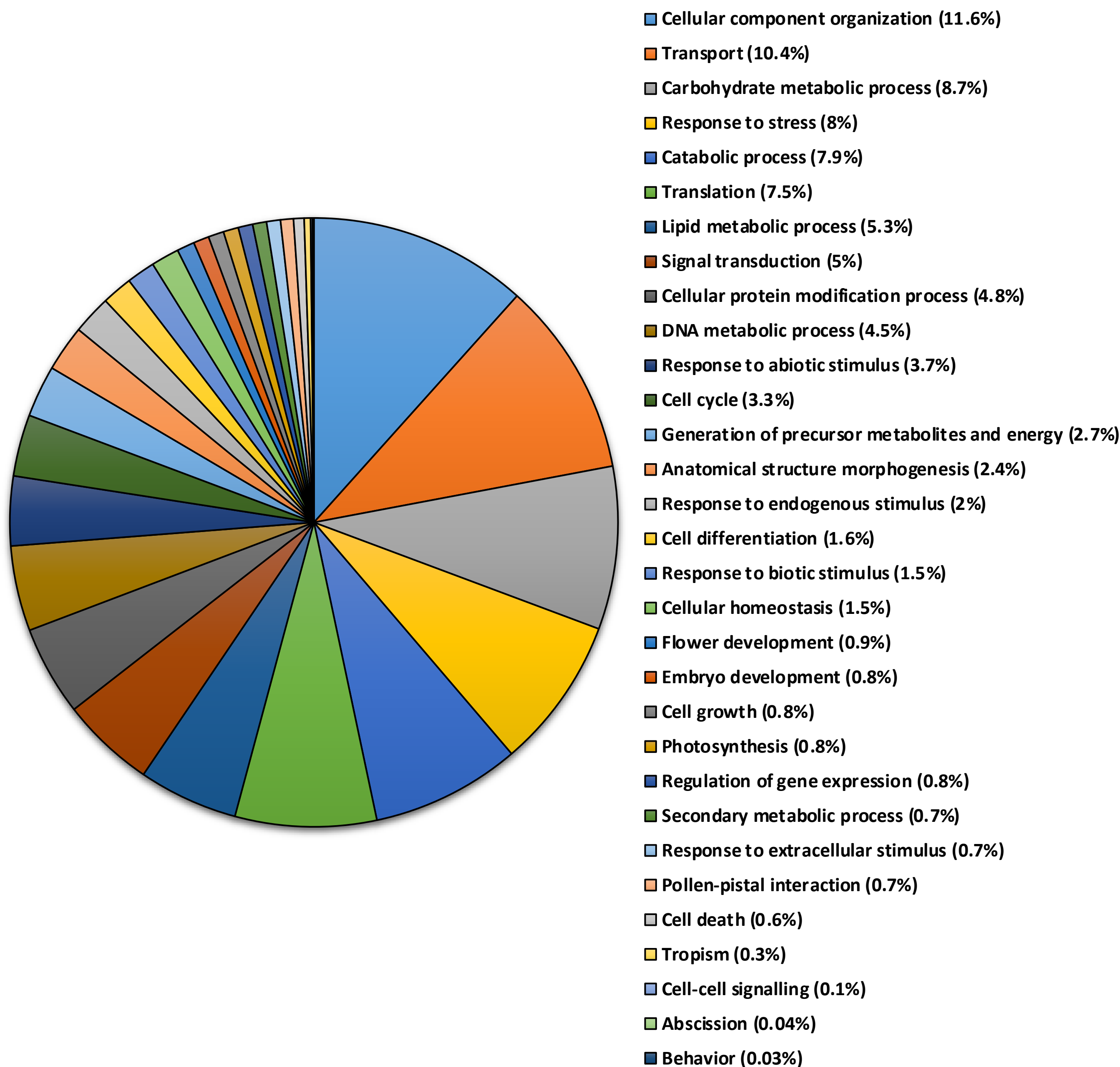


Appendix 12**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) plants treated with the half-total of nickel only. A total of 10,216 ontologies were grouped under molecular function using the BLAST2GO software.

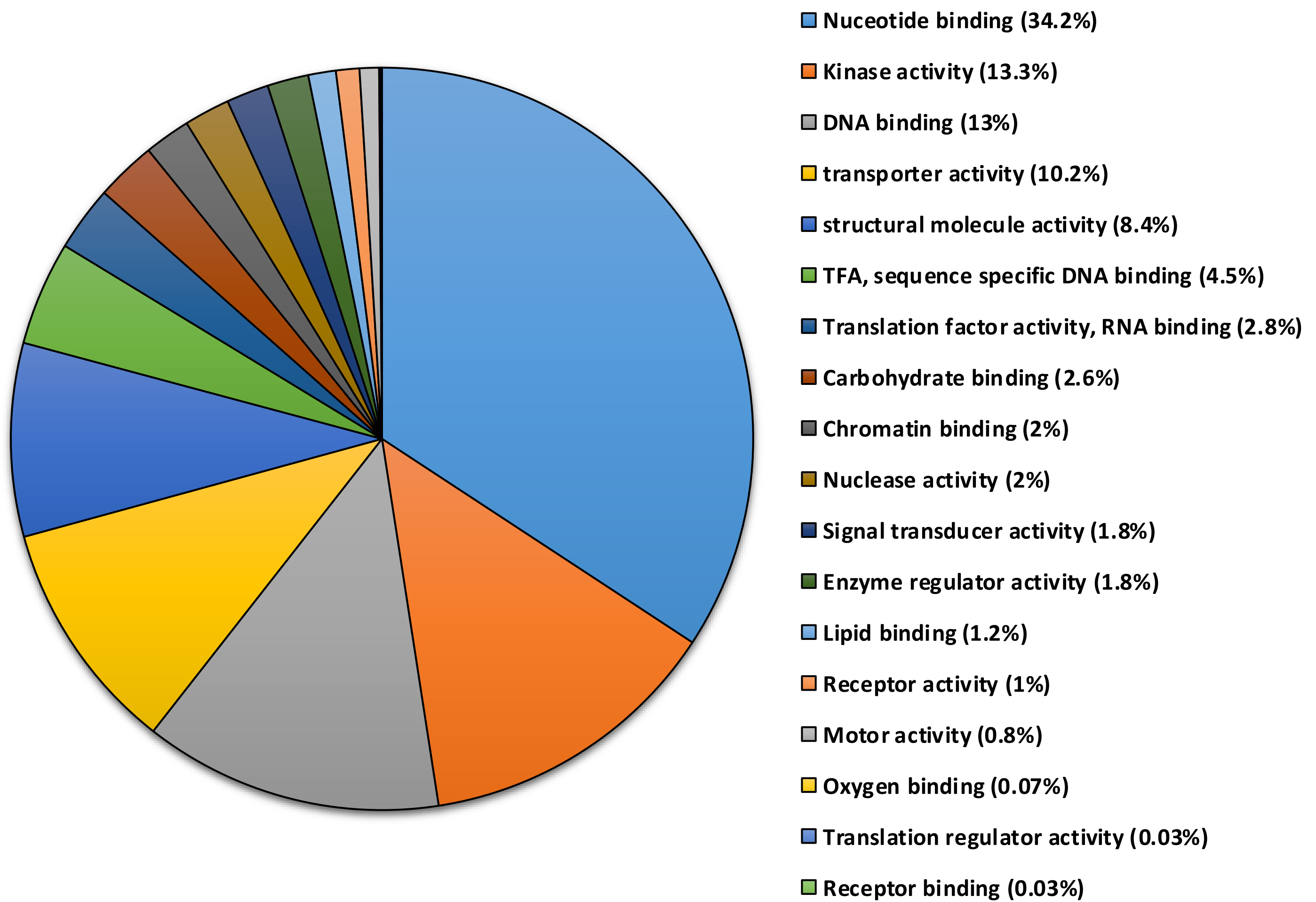
* TFA stands for transcription factor activity



Appendix 13**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) plants treated with the half-total dose of nickel only. A total of 6,364 ontologies were grouped under cellular compartment using the BLAST2GO software.

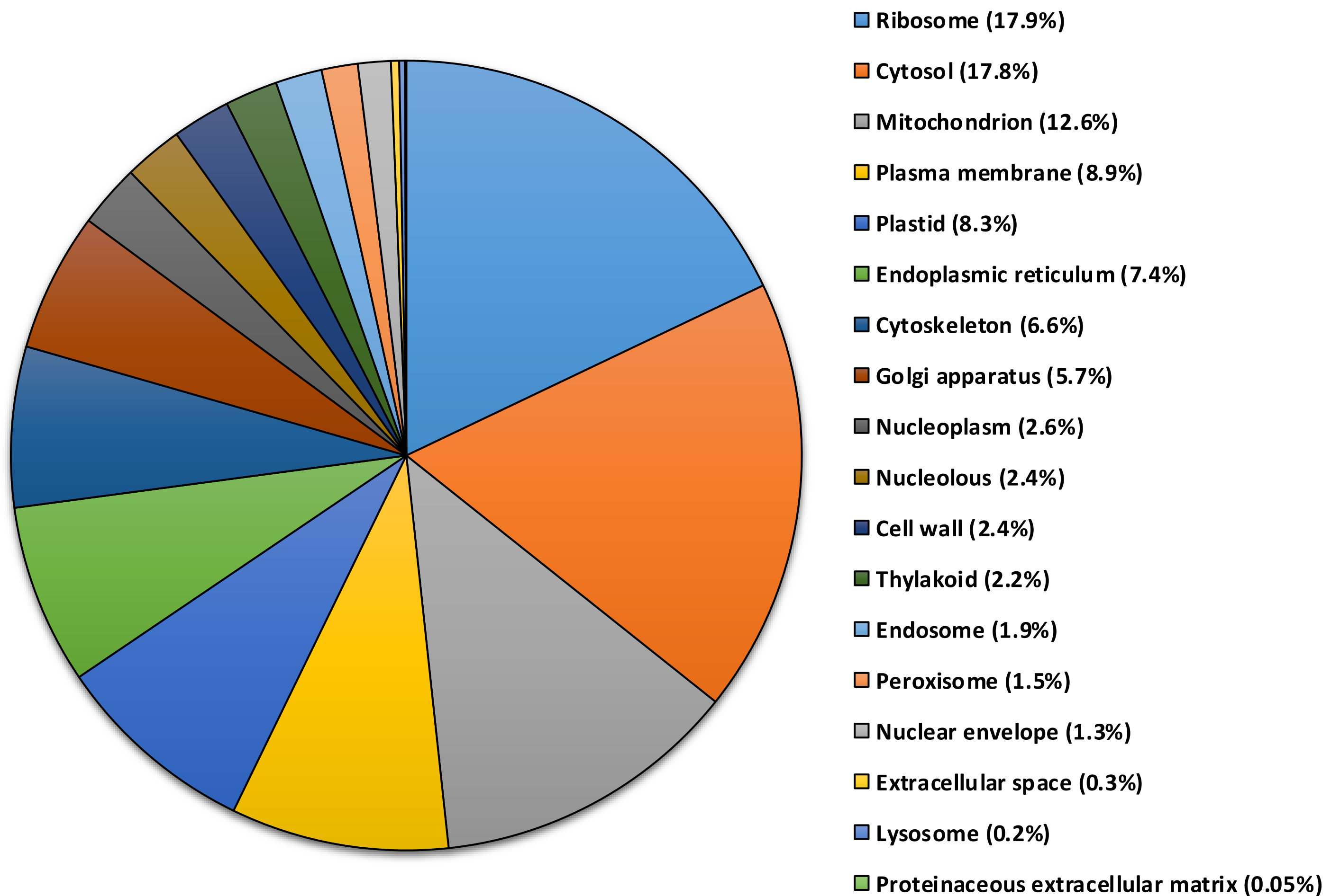


Appendix 14**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) resistant plants treated with the total dose of nickel only. A total of 27,490 ontologies were grouped under biological function using the BLAST2GO software.

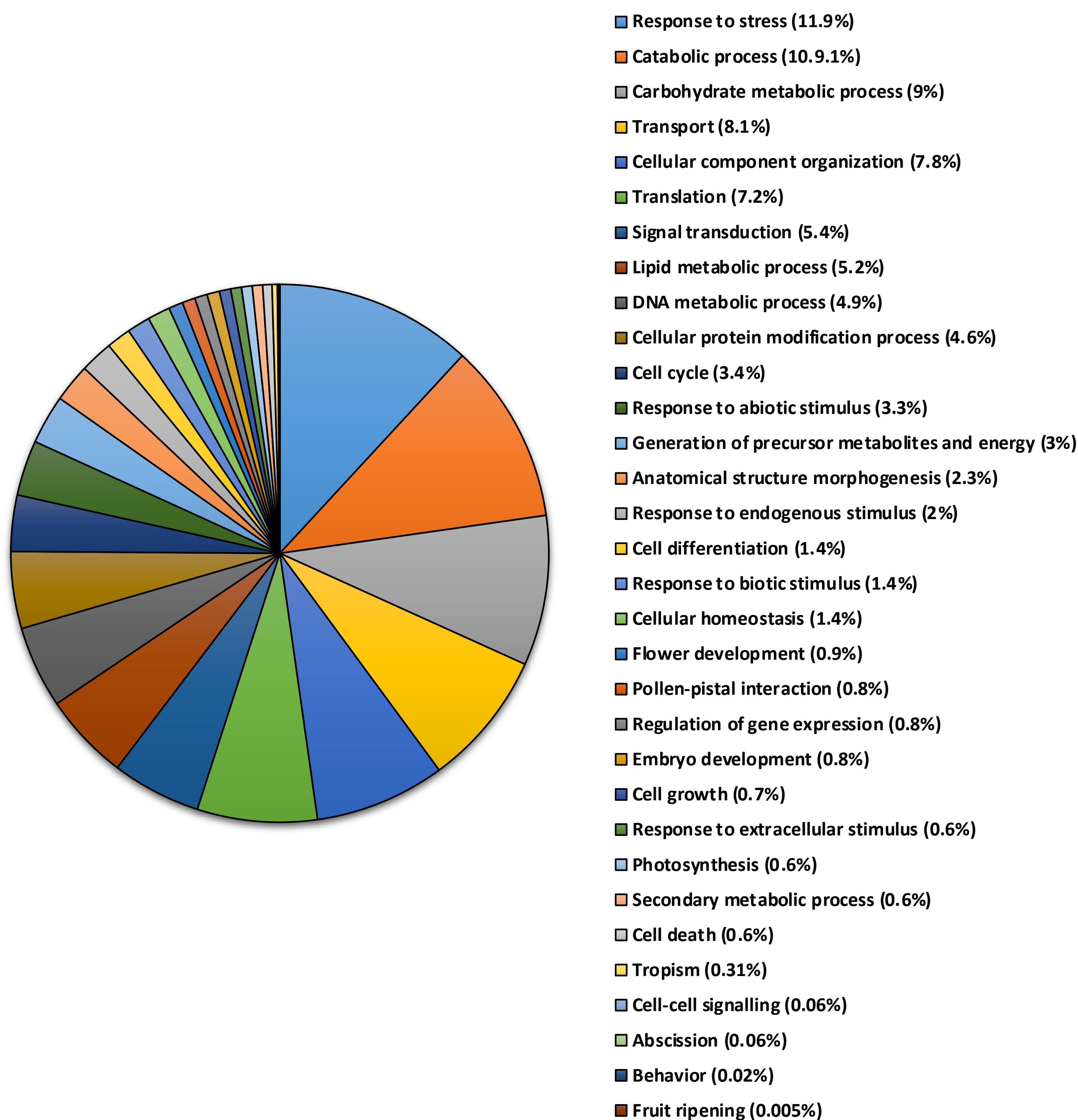


Appendix 15. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) resistant plants treated with the total dose of nickel only. A total of 23,432 ontologies were grouped under molecular function using the BLAST2GO software.

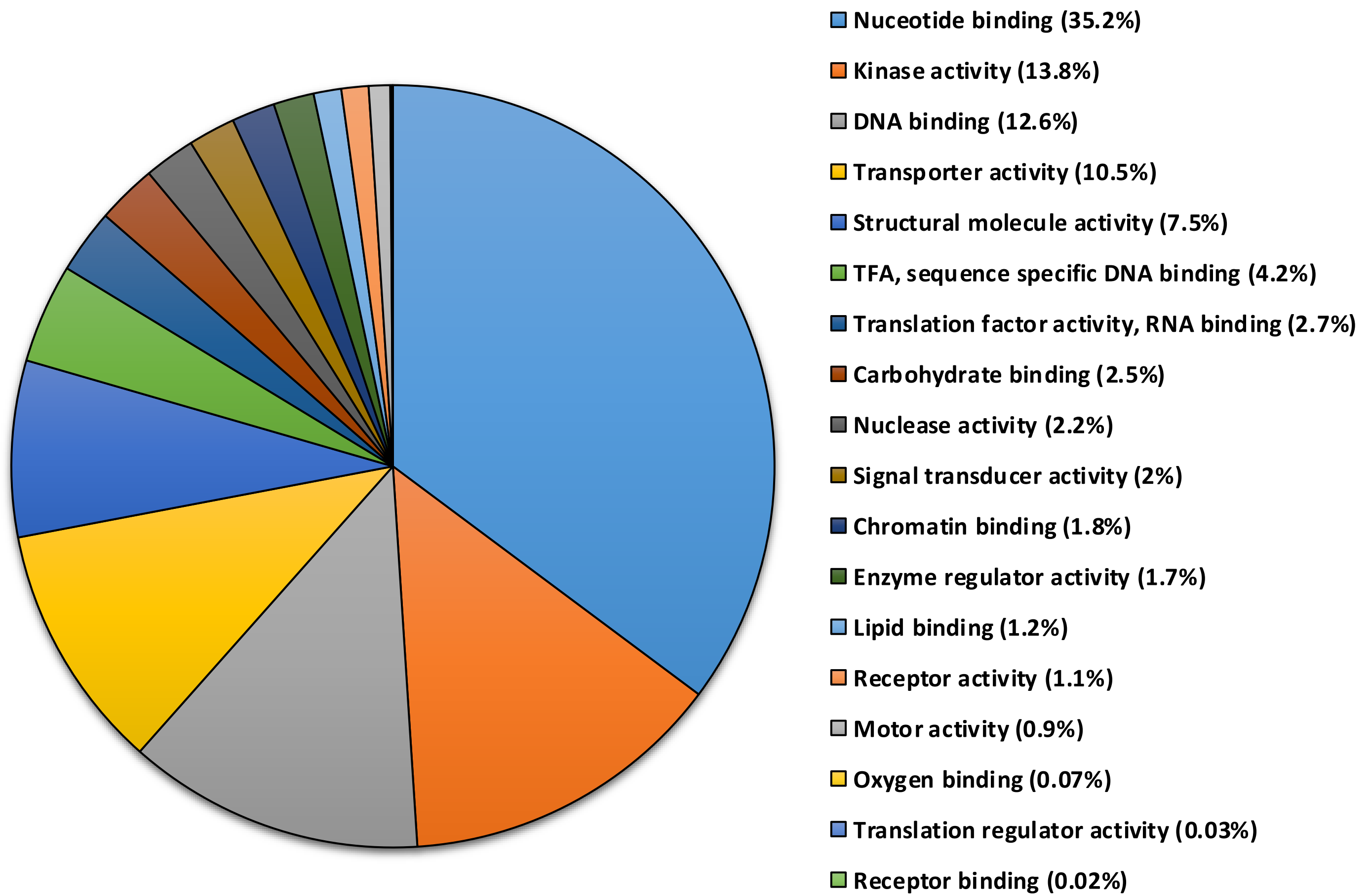
* TFA stands for transcription factor activity



Appendix 16. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) resistant plants treated with the total dose of nickel only. A total of 11,093 ontologies were grouped under cellular compartment using the BLAST2GO software.

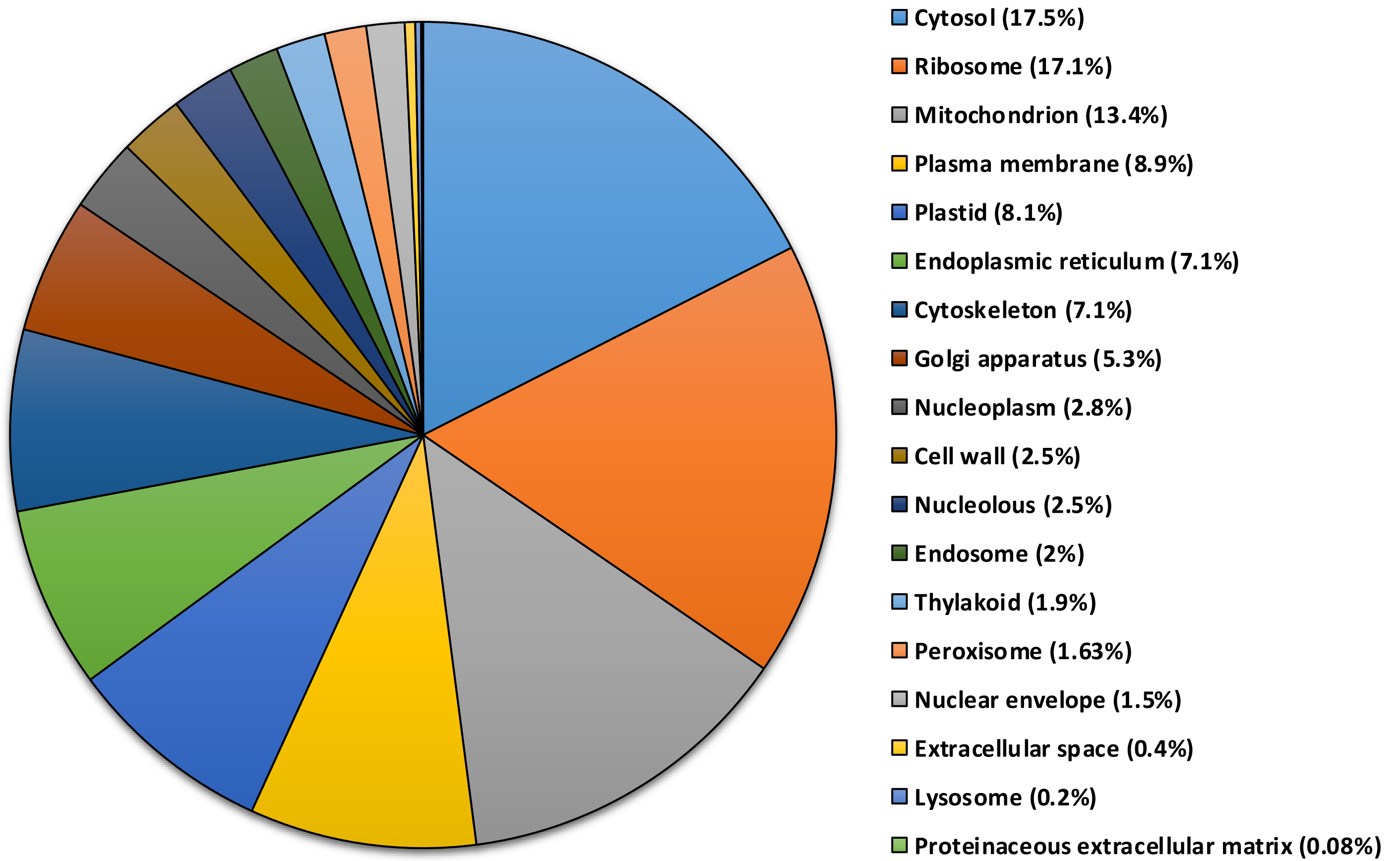


Appendix 17**.** Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) susceptible plants treated with the total dose of nickel only. A total of 19,685 ontologies were grouped under biological function using the BLAST2GO software.



Appendix 18. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) susceptible plants treated with the total dose of nickel only. A total of 18,181 ontologies were grouped under molecular function using the BLAST2GO software.

* TFA stands for transcription factor activity



Appendix 19. Supplementary material of chapter 4. Gene ontology of transcripts from red maple (*Acer rubrum*) susceptible plants treated with the total dose of nickel only. A total of 7,835 ontologies were grouped under cellular compartment using the BLAST2GO software.

Appendix 20. **Supplementary material of chapter 4. Number of differentially expressed genes in red maple (*Acer rubrum*) among the different nickel treatments.**

Treatment	Water Control		150 mg/kg*		800 mg/kg*		1,600 mg/kg*	
	Up	Down	Up	Down	Up	Down	Up	Down
Water Control	-	-	0	2	0	0	6263	3142
150 mg/kg			-	-	0	0	5753	2382
800 mg/kg					-	-	0	0
1,600 mg/kg							-	-

A standard cut-off of two fold and a FDR of 0.05 were used.
*Comparative gene regulation in *Acer rubrum* plants treated with 150 mg /kg of nickel compared to water, 800 mg /kg of nickel compared to 150 mg /kg, 1,600 mg /kg of nickel compared to 150 mg / kg and 800 mg /kg.

Appendix 21. **Supplementary material of chapter 4. Top 25 (LogFC) upregulated differentially expressed transcripts from red maple (*Acer rubrum*) treated with 1,600 mg of nickel per kg of soil compared to 150 mg of nickel per kg of soil.**

Rank	Transcript ID	Plants (RPKM)*						LogFC	Adj. P.Value	Description
		Low Ni	Low Ni	Low Ni	High Ni	High Ni	High Ni			
1	TRINITY_DN371175_c0_g1	0.0	0.0	0.0	320.5	931.6	693.2	13.8	0.00227	NA
2	TRINITY_DN425283_c1_g1	0.0	0.0	0.0	538.4	925.4	985.4	13.3	0.00152	CFEM-domain-containing protein
3	TRINITY_DN428415_c1_g2	0.0	0.0	0.0	37.0	372.7	111.4	12.6	0.01440	Exoglucanase 1
4	TRINITY_DN442833_c3_g1	0.0	0.0	0.0	65.8	307.7	185.8	12.4	0.00291	Neutral protease 2
5	TRINITY_DN420778_c2_g4	0.0	0.0	0.0	78.4	142.5	169.9	12.4	0.00152	Proteinase T
6	TRINITY_DN417864_c0_g1	0.0	0.0	0.0	30.5	319.0	76.3	12.2	0.01109	Glucanase
7	TRINITY_DN428052_c1_g1	0.0	0.0	0.0	43.3	245.0	173.2	12.0	0.00351	Kelch repeat protein
8	TRINITY_DN426651_c2_g1	0.0	0.0	0.0	159.0	366.6	365.8	11.9	0.00152	Alkaline proteinase
9	TRINITY_DN425996_c0_g1	0.0	0.0	0.0	205.4	635.9	335.1	11.9	0.00152	Hypothetical protein AOR_1_1366184
10	TRINITY_DN424308_c2_g2	0.0	0.0	0.0	11.1	96.3	74.3	11.9	0.00929	Predicted protein
11	TRINITY_DN430490_c3_g1	0.0	0.0	0.0	55.6	378.2	94.7	11.8	0.00409	Endo-1,4-beta-xylanase 2
12	TRINITY_DN432999_c0_g3	0.0	0.0	0.0	50.5	207.3	115.9	11.7	0.00174	Woronin body major protein-like protein
13	TRINITY_DN432608_c1_g1	0.0	0.0	0.0	53.5	249.6	177.3	11.7	0.00232	CFEM-domain-containing protein
14	TRINITY_DN416426_c0_g1	0.0	0.0	0.0	49.4	194.7	109.6	11.7	0.00422	RTM1
15	TRINITY_DN43011_c0_g1	0.0	0.0	0.0	39.2	142.1	30.3	11.6	0.00220	Alpha-L-arabinofuranosidase
16	TRINITY_DN425833_c0_g1	0.0	0.0	0.0	26.8	58.5	56.3	11.6	0.00152	Predicted protein n
17	TRINITY_DN437738_c0_g1	0.0	0.0	0.0	48.9	279.6	156.1	11.4	0.00244	Predicted protein
18	TRINITY_DN436811_c1_g3	0.0	0.0	0.0	14.7	99.2	78.2	11.4	0.00371	Sugar transporter
19	TRINITY_DN435092_c1_g1	0.0	0.0	0.0	14.7	94.1	20.1	11.4	0.00339	WGS project CABT00000000 data, contig 2.29
20	TRINITY_DN432373_c0_g1	0.0	0.0	0.0	15.9	77.8	42.8	11.4	0.00189	Putative alcohol oxidase protein
21	TRINITY_DN414752_c1_g1	0.0	0.0	0.0	21.1	200.3	568.9	11.2	0.02649	Predicted protein
22	TRINITY_DN422569_c0_g1	0.0	0.0	0.0	28.3	228.3	92.5	11.2	0.00363	Predicted protein (Fragment)
23	TRINITY_DN423947_c0_g1	0.0	0.0	0.0	21.5	348.1	29.1	11.2	0.01560	Endopolygalacturonase
24	TRINITY_DN436416_c3_g1	0.0	0.0	0.0	42.0	290.5	182.2	11.1	0.00315	Superoxide dismutase [Cu-Zn]
25	TRINITY_DN284529_c0_g1	0.0	0.0	0.0	162.9	637.4	1096.6	11.0	0.00281	Metallothionein

*Low and high represent plants treated with 150 mg or 1,600 mg of Ni per kg of soil

Appendix 22. **Supplementary material of chapter 4. Top 25 (LogFC) downregulated differentially expressed transcripts from red maple (*Acer rubrum*) treated with 1,600 mg of nickel per kg of soil compared to 150 mg of nickel per kg of soil.**

Rank	Transcript ID	Plants (RPKM)*						LogFC	Adj. P.Value	Description
		Low Ni	Low Ni	Low Ni	High Ni	High Ni	High Ni			
1	TRINITY_DN438303_c0_g1	10.3	54.2	31.2	0.0	0.0	0.0	-9.89	0.00243	Purple acid phosphatase
2	TRINITY_DN430711_c1_g1	62.8	37.0	91.6	0.0	0.0	0.0	-9.16	0.00152	Pectinesterase
3	TRINITY_DN431193_c1_g1	51.9	74.2	88.2	0.0	0.0	0.0	-8.58	0.00152	Putative 14 kDa proline-rich protein DC2.15
4	TRINITY_DN438114_c1_g1	67.9	102.4	114.8	0.0	0.0	8.2	-8.43	0.01128	Glutathione S-transferase family protein
5	TRINITY_DN428010_c1_g1	28.3	42.8	2.1	0.0	0.0	0.0	-8.35	0.02624	Lipoxygenase
6	TRINITY_DN435667_c2_g2	13.6	9.9	45.6	0.0	0.0	0.0	-8.33	0.00152	Pectinesterase
7	TRINITY_DN440736_c1_g2	18.6	21.9	20.7	0.1	0.0	0.0	-8.25	0.00152	Expansin B3, BETA 1.6 isoform 1
8	TRINITY_DN434405_c0_g2	7.2	22.1	21.4	0.0	0.0	0.0	-8.13	0.00152	Membrane associated protein
9	TRINITY_DN429524_c0_g2	8.0	12.0	36.0	0.0	0.0	0.0	-8.06	0.00187	Putative protein LURP-one-related 10-like
10	TRINITY_DN413182_c0_g1	13.8	37.8	56.3	0.0	0.0	0.0	-8.03	0.00173	Putative remorin-like
11	TRINITY_DN380434_c0_g1	2.2	10.4	5.1	0.0	0.0	0.0	-7.95	0.00188	Transcription factor PIF1
12	TRINITY_DN444293_c3_g4	51.9	50.5	46.9	0.0	0.0	0.0	-7.90	0.00152	Putative polygalacturonase At1g48100 isoform X2
13	TRINITY_DN442826_c0_g1	5.4	7.6	7.1	0.0	0.0	0.0	-7.84	0.00152	Receptor-type protein tyrosine kinase (Fragment)
14	TRINITY_DN433736_c0_g1	162.9	22.9	30.5	0.0	0.0	0.0	-7.84	0.00445	Putative squalene synthase 1
15	TRINITY_DN437970_c0_g1	11.9	38.0	18.9	0.0	0.0	0.6	-7.82	0.00920	Putative cyclin B1
16	TRINITY_DN438742_c0_g1	4.2	17.0	12.4	0.0	0.0	0.0	-7.82	0.00160	PHD finger family protein
17	TRINITY_DN435667_c3_g1	182.0	615.5	180.1	0.1	3.9	3.9	-7.73	0.02821	Histone H2A
18	TRINITY_DN427242_c0_g1	760.7	823.4	521.2	0.2	5.1	20.6	-7.65	0.00933	Putative cytochrome P450 71D10
19	TRINITY_DN432222_c0_g2	85.4	186.8	93.6	0.0	0.5	10.7	-7.62	0.03097	4-coumarate:CoA ligase 3
20	TRINITY_DN439656_c0_g3	5.5	32.8	10.7	0.0	0.2	0.0	-7.61	0.00257	Pectin lyase-like superfamily protein isoform 1
21	TRINITY_DN427429_c3_g1	80.5	185.3	160.7	0.0	0.9	5.1	-7.55	0.01589	Putative arabinogalactan peptide 13-like
22	TRINITY_DN441216_c1_g2	73.6	87.2	68.6	0.0	1.1	1.6	-7.54	0.00613	Leucoanthocyanidin reductase
23	TRINITY_DN434996_c3_g3	3.0	15.5	9.2	0.0	0.1	0.0	-7.52	0.00468	Coffea canephora DH200=94 genomic scaffold, scaffold_91
24	TRINITY_DN438161_c2_g3	6.1	15.0	14.1	0.0	0.0	0.1	-7.52	0.00214	Putative sphingolipid delta 4 desaturase/C-4 hydroxylase protein des2
25	TRINITY_DN440779_c1_g2	318.1	854.0	755.1	0.0	4.9	0.0	-7.45	0.00336	Anthocyanidin reductase

*Low and high represent plants treated with 150 mg or 1,600 mg of Ni per kg of soil, respectively