Molecular Characterization of *Deschampsia cespitosa* populations from metal contaminated areas in Northern Ontario: Nickel and copper toxicity.

By

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Abstract

The main objectives of the present study were to 1) reassess the level of genetic variation in *D. cespitosa* populations from three regions contaminated or uncontaminated with metals in Northern Ontario; 2) determine the degree of toxicity of nickel and copper in *D. cespitosa* genotypes; and 3) investigate cytological damages caused by metals in *D. cespitosa* plants growing in Northern Ontario. An insignificant change in the overall percent of polymorphic loci in populations from Little Current and Cobalt Cart Lake was found. On the other hand, a significant decrease was seen in the Coniston/Wahnapitae and Cobalt Nipissing Tailings sites. The degree of genetic relatedness among the populations has increased, compared to data from the analysis of samples 16 years ago. This strengthens the earlier theory that Sudbury populations of *D. cespitosa* might be from the Cobalt region.

The degree of toxicity of nickel and copper in *D. cespitosa* was investigated under controlled conditions. It was found that copper is more toxic than nickel. Neither copper nor nickel caused damage to plants at low rates of 5.6 and 9.16 mg/kg respectively, corresponding to the bioavailable amounts. Higher dosages (1600 mg/kg) of nickel and (1312 mg/kg) copper equivalent to total elements in site, caused significant damages to plants.

Cytological analysis for *D. cespitosa* revealed significant mitotic disruption from long term exposure of roots to high levels of metal frequently manifested through aneuploidy in metal-contaminated sites when compared to the reference sites. All plants from contaminated areas exhibited varying degrees of mixoploidy compared to the reference sites. These high levels of mitotic abnormalities did not seem to affect *D. cespitosa* survival and growth in highly metal-contaminated sites.

Key words: *Deschampsia cespitosa*; Northern Ontario; Genetic variation; Metal contamination; ISSR markers; Metal toxicity; Cytology.

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Chapter 1: Literature review

1.1 Metal Contamination in Northern Ontario

Metal contamination is the increase in the metal content of an area due to the deposition of metal-containing materials. This deposition is often facilitated by air and water movement, as well as anthropogenic activities (Alloway *et al.*, 1990; Freedman and Hutchinson, 1980; Hutchinson and Whitby, 1972). While these metals are needed in low quantities for life processes (Arnon and Stout, 1939; Alloway *et al.*, 1990; Prohaska, 2011; Vaidyanathan *et al.*, 2013), once their abundance exceeds a threshold, they can cause detrimental effects on living organisms (Lin and Kao, 2005; Chibuike and Obiora, 2014; Freedman and Hutchinson, 1980; Hutchinson and Whitby, 1972). This threshold can change based on a number of factors including pH, organic matter content, and moisture levels of soil along with the plant species being exposed to metals (Alloway *et al.*, 1990; Shabala, 2017).

Both the Greater Sudbury Region and the Cobalt area have been affected greatly by long periods of metal contamination. These regions were home to mines whose activities caused contamination of surrounding environments (Adamowicz, 2014; Dumaresq, 2009; Freedman and Hutchinson, 1980; Hutchinson and Whitby, 1972; Winterhalder, 1996). This resulted in severe metal-induced stress on plant-life, leading to a significant decrease in plant growth and survival rates (Freedman and Hutchinson, 1980; Hutchinson and Whitby, 1972).

In Sudbury, the discovery of the metal deposit occurred in the 1880's and the peak daily production was 50,000 tonnes of ore (Winterhalder, 1996). The main metals of interest were copper and nickel, which had to first be separated from the pentlandite via extreme long-term heating to melt the metals off the rock. Initially, this process was done on huge roast-beds where

several tonnes of ore were placed. The whole pile was then ignited and left to burn for several months. The cooled metal globules were then collected from the ashes for further processing (Winterhalder, 1996; Courtin, 1994).

After 1930, the heating and separation process was performed by centralized smelters which were safer for the environment than the roast-beads (Nriagu *et al.*, 1982; Courtin, 1994). Although the use of a smelter restricts environmental contamination from direct spoil and water contact with the piles, the chimneys from the smelting operations aerosolize sulfur dioxide and metal particulates (Freedman and Hutchinson, 1980; Winterhalder, 1996; Courtin, 1994). These harmful emissions then travel through the air and affect environments at great distances from the central smelting operation (Nriagu *et al.*, 1982; Abedin and Spiers, 2006; Freedman and Hutchinson, 1980; Hutchinson and Whitby, 1972). Atmospheric sulfur dioxide and metal contamination from both procedures have caused the fall of acid rain (Summers and Whelpdale, 1976), lowering the pH and harming plant life. This loss of plant life either directly via clearcutting or through metal contamination and acid rain, led to erosion and direct loss of the top layer of the soil rich in micronutrients (Lautenbach *et al.*, 1995; Courtin, 1994; Winterhalder, 1996; Gunn *et al.*, 1995).

In Cobalt, a large native silver vein deposit was identified in 1903. It had an estimated richness of 12%, with the initial mining operation spanning three decades (Adamowicz, 2014; Dumaresq, 2009). The main ores of interest in the region were the native silver and erythrite, along with small amounts of niccolite (Percival *et al.*, 2007, Dumaresq, 2009). The latter three ores contain either high arsenic or sulfide content bonded with the desired material. Excavation of the surface deposit was done primarily by picks and compressed air drilling, with the ore being processed at first via gravity concentrated milling, with later technologies including

cyanide milling, floatation separation, and the mercury amalgam processing of the Nipissing High Grade Mill (Adamowcz, 2014; Dumaresq, 2009). The tailings from such procedures were deposited in and around the surrounding waterways, further releasing metals and sulfur compounds into the environment. Cobalt's operations greatly increased the rate of arsenic leeching and metal contamination in the surrounding area, and the resulting stress on plant life limited species survival and growth.

As a result of mining-related activities, both the Cobalt and Greater Sudbury regions have high total levels of metal concentrations in soil (Nkongolo *et al.*, 2001, 2008; Nriagu *et al.*, 1998). Nkongolo *et al.* (2001) found that sites in the Cobalt area had high levels of arsenic, lead, zinc, cadmium, cobalt, aluminum and nickel while Sudbury region was predominantly contaminated with nickel, copper, aluminum, lead, and cobalt to varying degrees. In both regions, the metal concentrations measured were much higher and significantly different than the levels observed in the control sites on Manitoulin Island.

1.2 Nickel and Copper Toxicity

Nickel and copper are micronutrients that are essential to certain life processes (Eskew *et al.*, 1984; Arnon and Stout, 1939). However, the essential intake of copper has been shown to be higher relative to nickel, with the incidence of micronutrient deficiency for copper far more common (Alloway, 1990). On contaminated sites, total levels of Cu and Ni can be hundreds of times their normally observed concentrations (Nkongolo *et al.*, 2001; Cox and Hutchison, 1993; Friedlová, 2010).

Nickel is usually found in trace concentrations in soil. However, in regions where the rock formations nearby contain nickel, the concentrations can be many times higher (Yadav,

2010; Nkongolo *et al.*, 2001; Cox and Hutchinson, 1993). At such concentrations, exposure has been recorded to cause chlorosis and necrosis in plants, as well as the disruption of membranes and their proteins through competitively binding sites over the needed metal (Alloway, 1990; Yadav, 2010; Lin and Kao, 2005; Bhalerao *et al.*, 2015).

Copper is an essential micronutrient in living organisms (Alloway, 1990; Prohaska, 2011). It can readily accept and donate electrons in the electron transport chain. Copper also plays a key role as a cofactor in the activation of many enzymes, in addition to being a core component of plastocyanin (Yadav, 2010; Shabala, 2017) in plant photosystems. It plays a key role in iron metabolism and the regulation of protein-synthesis related gene expression by oxidizing the iron and controlling oxidative stress respectively. Zinc is known to limit copper uptake and blinding sites (Shabala, 2017; Alloway, 1990), causing copper deficiency even if ample copper is available in the surroundings. Deficiency symptoms include dieback of shoots and buds, stunting, pale or yellow leaves, and withering (Shabala, 2017). However, the required concentration of copper is fairly low and is not usually a problem unless the zinc concentration of the area increases. At high concentrations, copper will induce symptoms that include chlorosis and growth retardation (Yadav, 2010).

Uptake of contaminants by plants and their mechanisms have been studied by several researchers (Tangahu *et al.*, 2011; Mehes –Smith *et al.*, 2013; Kalubi *et al.*, 2015). 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 within their tissues. The excluders limit contaminant uptake into their aerial 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 *et al.*, 2013). Recent field studies have shown that *D. cespitosa* growing in the GSR does accumulate metals in roots but restricts their translocation to aerial biomass (Mehes-Smith *et al.*, 2013).

1.3 *Deschampsia cespitosa*

Deschampsia cespitosa (Tufted hairgrass) is one of the few species which grows on some of the most metal contaminated sites after the mining operations in Greater Sudbury and Cobalt (Mehes-Smith, 2013; Gunn *et al.*, 1995). Detailed classification of this species can be found in the 2017 USDA NRCS database.

Deschampia cespitosa belongs to the kingdom *Plantae*, subkingdom *Tracheobionta*, supervision *Spermatophyta*, division *Magnoliophyta*, class *Liliopsida*, subclass *Commelinidae*, order *Cyperales*, family, *Poaceae/Gramineae*, genus *Deschampsia* P. Beauv., species *cespitosa*. The species name has many synonyms and common names including blue-green hair-grass, fescue-leaved hairgrass, salt and pepper grass, tussock grass, and canche cespiteuse (St. John *et al.*, 2011).

This grass is a perennial that grows between 20 and 155 cm tall. It has many subspecies and its leaf width, ligule height, and spikelet height can vary quite a bit. The leaf sheath is usually scabrous but can be smooth, with 3-7.5 mm ligules. The leaves themselves are flat or folded and quite fibrous, with toothed nerves on the upperside of the blades giving them a rough texture typical of cespitosa. Its panicle is very open and 5-50 cm long. Spikelets are purpleish and the two glumes are either glabrous or scaberulous and acute (fibrous and individual roots are usually 20 cm in length, with the aforementioned leaf system being usable as forage early in the year when still tender). *D. cespitosa* can be found around the world, usually in temperate regions

(USDA Forest Service Fire Effects Information System). It most frequently inhabits marshy areas, however its habitat range includes marginal and nutrient-rich soils, moist to semi-flooded areas, and coarse to fine textured substrates. It can grow in elevations ranging from sea-level to 14000 feet on soil of pH 3.5 to 7.5, although it is not overly salt- tolerant. There are roughly 20 listed subspecies variants (USDA Forest Service Fire Effects Information System). Some of the variants are cultivars developed for use on golf courses or for landscaping purposes. Other variants are used as pasture in marginal climates and planted for restoration and reclamation purposes (St. John et al. 2011, VSDA Forest Service Fire Effects Information System).

1.4 Effect of metals on genetic variation

Genetic diversity encompasses all genetic characteristics in a species or population's genetic pool (Roy *et al.*, 2015). The higher the level of genetic variation, the better the species can adapt to changing environmental conditions. Selective pressures, such as metal stress, can cause potentially resistant species to adapt and to survive stressors. However, in the process of doing so, a population can inadvertently eliminate other alleles from the gene pool. This loss of diversity can reduce the future adaptation potential of the population (Nei *et al.*, 1975; Gunn *et al.*, 1995). Hence, it is important to monitor the genetic diversity of species or populations. When populations are under anthropogenic influence, knowledge of genetic variation levels allows one to assess the effects of potential stressors and evaluate remediation efforts (Nkongolo *et al.*, 2013).

The most accurate approach to determining genetic variation involves the use of molecular markers (Bornet *et al.*, 2002; Costa *et al.*, 2016; Godwin *et al.*, 1997; Kalubi *et al.*, 2015; Mehes *et al.*, 2007; Nkongolo *et al.*, 2014; Theriault *et al.*, 2013; Williams *et al.*, 1990;

Yeh *et al.*, 1997; Zietkiewicz *et al.*, 1994). Since they are based on the specific innate genetic information of individuals in the population, environmental factors themselves will not modify the results of the screening (Williams *et al.*, 1990) and modern molecular markers do not require meticulous recorded knowledge of the sampled individual's lineage in order to track variation. There are many different molecular markers, each having its pros and cons in terms of specificity, reproducibility, cost, speed, sensitivity, and amount of genetic material necessary to perform the screening (Goodwin *et al.*, 1997). The most common molecular markers include Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplification of Polymorphic DNA (RAPD), Variable Number Tandem Repeat (VNTR), Simple Sequence Repeat (SSR) [also referred to as Short Tandem Repeat (STR)], Single-nucleotide polymorphism (SNP), and Inter Simple Sequence Repeats (ISSR).

1.5 Molecular markers

The first assessments of genetic diversity were done using data from the observation of phenotypic characteristics. Gene expression would be tracked and recorded, sometimes over generations to determine the transmission and heredity of a trait. However, phenotypes can be influenced by the environment, which leads to inconsistent observations and inaccurate results. These environment-based phenotypic changes are referred to as plasticity (DeWitt *et al.*, 1998). This plasticity allows an organism to survive in a greater variety of environments, but in exchange it would not be as competitive as other organisms which are less plastic and better adapted for a specific environment. Due to their inability for mobility to avoid certain unfavourable conditions, plants often have more plastic genomes (DeWitt *et al.*, 1998; Mehes-Smith and Nkongolo, 2015) which made phenotypic tracking of diversity less precise. Development of another tracking system was necessary.

The first molecular markers targeted proteins and used the antigen-antibody reactivity method to measure the specificity of the reaction (Prager and Wilson, 1971). Using this method, the relatedness, also called immunological distance, of proteins with similar function could be measured. However, this technique is limited to expressed proteins. Cases where the recessive genotype was present but completely masked could not be assessed. This changed after the development of DNA-based molecular markers, which used the genetic material itself to check for the presence of a trait or the relatedness of two samples.

The first technique, RFLP, utilized restriction enzymes to cleave the DNA sample into pieces. The solution is loaded onto an agarose gel for primary separation followed by a southern blot for perpendicular separation to observe the banding patterns after exposure to a radioisotope (Jeffreys, 1987). The discovery of this procedure in 1987 was a turning point in genetics, since it facilitated the direct comparison of inheritance and closely related individuals based on their genomic DNA without knowing the specific sequence. However, the long procedure and large amounts of DNA necessary made the procedure less attractive. PCR – based methods were then developed. PCR is the main method for detecting and isolating VNTRs in genomes.

AFLP utilizes restriction enzymes and incorporates the PCR procedure and knowledge of the resulting sequences of the cut end to amplify only a portion of the fragments. Many loci can be analysed with very high accuracy at one time with few artifacts using this technique (Zabeau and Vos, 1993; Meudt and Clarke, 2007). While prior sequence information is not required, a large amount of high-quality DNA from the sample is.

Traditionally, RAPD was the marker of choice when estimating polymorphism in species with unexplored genomes due to its use of randomly generated primer sequences and low cost (Williams *et al.,* 1990) with the main prerequisites being the GC content and not being a

palindrome of itself. However, the short primer length leads to specificity issues in cases where the experimental conditions are not stringently kept consistent (Costa *et al.*, 2016; Williams *et al.*, 1990). Artifact bands are often produced in cases where conditions change between runs.

VNTR stands for Variable Number of Tandem Repeats, and it falls under the subcategory of markers composed of repeated units just like SSR (Short Sequence Repeat) markers. SSRs are microsatellites which are 2-5bp in length, while VNTRs are 10-100bp long. Both VNTR and SSR sequences are highly conserved, with the polymorphic marker being the number of repeats of the base bp sequence at a particular locus that a sample contains in comparison to another. Both techniques are extremely useful in fingerprinting individuals with high accuracy using their DNA, but prior sequence knowledge and a large bank of the markers with their loci is required for their use (Costa *et al.*, 2016).

SNP or Single Nucleotide Polymorphism is a single base pair change in the sequence at a locus. They are quite rare, but the changes are heritable and could be used for fingerprinting. However, there is a reasonable possibility of the convergent evolution of the same SNP in multiple individuals. For accuracy of use in fingerprinting, many SNPs would need to be screened individually. The technique is used when screening for diseases where a SNP would be a causality, to aid in diagnosis.

ISSR technique combines the speed and low cost of RAPD with the increased specificity of SSR due to ISSR primer design incorporating inverted microsatellite sequences for enhanced binding specificity (Zietkiewicz *et al.*, 1994). ISSR markers are primer sequences of 16-25 bp long that flank the sequences they amplify (Zietkiewicz *et al.*, 1994). Their relatively long primer length enables higher annealing temperatures, which increases primer specificity and reproducibility of the results (Costa *et al.*, 2016) in comparison to other molecular techniques. In

addition, the specific genome sequence of the screened individual does not need to be known (Bornet *et al.*, 2002; Costa *et al.*, 2016; Mehes *et al.*, 2007; Nkongolo *et al.*, 2014) and more than one loci can be screened simultaneously. Such a primer design restricts amplification to the coding region of the genome (Bornet *et al.*, 2002; Costa *et al.*, 2016), compared to the former method (RAPD) which can amplify both regions with mutually exclusivity if another site is nearby. RAPD primers also do not always amplify the most polymorphic sites due to the aforementioned reason (Costa *et al.*, 2016).

Hence, due to its high reproducibility, cost efficiency, and speed of testing (Bornet *et al.*, 2002; Costa *et al.*, 2016; Zietkiewicz *et al.*, 1994), ISSR is usually the marker of choice in studies involving species whose sequence is not known, allowing one to fingerprint and differentiate (Bornet *et al.*, 2002; Kalubi *et al.*, 2015; Nkongolo *et al.*, 2014) population banding trends of otherwise closely related individuals. ISSR was therefore used to assess genetic variability in the present study.

1.6. Effects of metals on plant cytology

The effects of metal on mitotic and meiotic behaviors are not well documented in plants. Patel and Patel (2010) reported an increase in aberrant cells in *Trigonella foenum-garecum* as the dose of zinc increased. Other studies have demonstrated a decrease in mitotic frequency and an increase in the number of chromosomal abnormalities when some plant species are exposed to acute doses of metals or a chronic exposure to subtoxic doses. There are no published reports on the effects of long term exposure of plants to metals in natural stands on cytological features.

1.7. Objectives

The main objectives of this study are to 1) reassess the level of genetic variation in *D. cespitosa* from three regions contaminated or uncontaminated with metals in Northern Ontario; 2) determine the degree of toxicity of nickel and copper on *D. cespitosa*; and 3) investigate cytological damages caused by metals in *D. cespitosa* growing in Northern Ontario.

Chapter 2: Reassessment of molecular variation in isolated populations of *Deschampsia cespitosa* from metal contaminated regions in Northern Ontario (Canada) after 16 years of potential genetic recombination

2.1 Abstract

The effects of ore extraction and processing procedures in the Greater Sudbury and Cobalt regions have been long-lasting. The objective of this study is to determine the current level of genetic variation in *Deschampsia cespitosa* populations from metal contaminated and uncontaminated sites in samples collected in Northern Ontario in 2015 after 16 years of potential genetic recombination since the last study in 1999. *D. cespitosa* leaf samples collected from the City of Greater Sudbury (CGS), Cobalt, and Little Current were analyzed using ISSR primers. The levels of genetic variation were moderate to high within targeted populations. No significant difference was observed in the overall percent of polymorphic loci in metal – uncontaminated site of Little Current (from 70% in 1999 to 77% in 2015) and in a Cobalt Cart Lake site (from 48% in 1999 to 55% in 2015). But a significant decrease in genetic variation was observed in CGS Wahnapitae site (from 72% in 1999 to 54% in 2015). On the other hand, a significant increase was observed in Cobalt Nipissing (from 46% in 1999 to 64% in 2015). The Kelly Lake site in the CGS with the lowest level of polymorphic loci (42.5%) in 2015 was not surveyed in 1999. The degree of genetic relatedness among sites has increased since the populations are more genetically closely related than 16 years ago. No population-specific ISSR marker was identified. The clustering of Cobalt and Sudbury populations strengthens the earlier theory that Sudbury populations of *D. cespitosa* might be from the Cobalt region.

2.2 Introduction

The effects of ore extraction and processing procedures on the aquatic and terrestrial ecosystems in the Greater Sudbury and Cobalt regions have been long-lasting. In Sudbury, the roasting and smelting of sulfur-rich pentlandite and niccoline ores reduced the soil pH through acidic precipitation and spread airborne metal particulates in the region (Amiro and Courtin, 1981; Dudka *et al.*, 1995; Gratton *et al.*, 2000; Nkongolo *et al.*, 2013). The predominant ore was erythrite in the Cobalt region's operations with the environmental impact tied primarily to the deposition on the tailings that has contaminated the surrounding soils with metals (Percical *et al.*, 2007; Adamowicz 2014; Dumaresq, 1993). Changes in pH and elevated metal concentrations induce stress and limit plant growth, leading to erosion and the leaching of nutrients in the soils of the area (Winterhalder, 1996; Spiers *et al.*, 2012; Nkongolo *et al.*, 2013). While both regions have undertaken restoration efforts which include liming, seeding, and capping, total levels of metals on-site are still high (Percical *et al.*, 2007; Adamowicz 2014; Nkongolo *et al.*, 2013, 2016).

Selective pressures such as metal stress can cause potentially resistant species to adapt to these challenging conditions. Over time, a population can inadvertently eliminate other alleles from its gene pool. This loss of diversity can reduce its adaptation potential in the future (Mejnartowicz, 1983; Mengoni *et al.*, 2001; Deng *et al.*, 2007; Gervais and Nkongolo, 2011). It is therefore important to monitor the level of genetic variation within and among populations. This will be useful in assessing the effects of potential stressors and the sustainability of reclaimed ecosystems.

ISSR is a reproducible nuclear marker that can be used when the sequence of the species being tested is not known (Nagaoka and Ogihara, 1997; Nkongolo *et al.*, 2005, 2016), allowing one to fingerprint and differentiate closely related populations (Raina *et al.*, 2001; Nkongolo *et al.*, 2003, 2005). It can be used to study polymorphism and assess genetic variability which can shift over generations.

Dechampsia cespitosa is a cosmopolitan grass and is found in damp habitats and has colonized disturbed microsites in Cobalt and Sudbury. Nkongolo *et al.* (2001) and Gervais and Nkongolo (2011) assessed genetic variation within and among *D. cespitosa* populations from Northern Ontario. They found a low level of polymorphic loci in samples collected in 1999 from cobalt compared to samples from Sudbury. The highest level of genetic variation was observed in samples from metal uncontaminated sites (Gervais and Nkongolo, 2011).

The objective of this study is to determine the current level of genetic variation in *D. cespitosa* populations from metal contaminated and uncontaminated sites in samples Northern Ontario in 2015 after 16 years of genetic recombination.

2.3 Materials and Methods

2.3.1 Sampling

D. cespitosa leaves were collected from five populations in Northern Ontario (Cobalt, Manitoulin Island, and Sudbury). The sampling site locations are illustrated in Figure 1. Two sites were in the town of Cobalt in the district of Timiskaming. They include Cobalt 1 and Cobalt 3, which refer to the Nipissing Tailings (CNT) and Cart Lake Tailings respectively (CLT). The Sudbury area samples were from Wahnapitae (Wah) and Kelly Lake (KL) sites, while the

uncontaminated control site was from Little Current (LC) on Manitoulin Island. With the exception of Kelly Lake, all the sites were surveyed in 1999. For each site, about 10 % of the populations were collected. Leaf materials from each mature individual were wrapped in aluminum foil, flash-frozen and stored at -20 ^{o}C until DNA extraction.

Figure 1. Map showing study sites where D. cespitosa samples were collected. 1: Cobalt-1 (Nipissing Tailings); 2: Cobalt-3 (Cart Lake Tailings); 3: Wahnapitae; 4: Kelly Lake; 5: Little Current.

2.3.2 DNA Extraction

Genomic DNA extraction was performed using the CTAB extraction protocol as described by Mehes *et al.* (2007). This protocol is a modified version of the procedure outlined in Doyle and Doyle (1987). The following modifications were applied: the addition of 1% polyvinyl pyrrolidone (PVP) and 2% β-mercaptoethanol to the cetyl triethylammonium bromide (CTAB) buffer solution, the addition of two chloroform centrifugation steps of ten minutes each prior to the isopropanol spin, and the removal of the RNAse step. The extracted DNA was then stored in the freezer at -20^{o}C until further analysis.

2.3.3 Degradation analysis and DNA Quantitation

The condition and intactness of the DNA samples was tested via gel electrophoresis on 1% agarose gels in 0.5x Tris-Borate-EDTA (TBE) that was pre-stained with 1 μL of ethidium bromide. All wells were loaded with 6 μL of solution (1 μL of 6x loading buffer combined with 5 μL of stock DNA). The two flanking wells of each gel were loaded with 1Kb+ ladder, and then the gel was then run at 64 V for 90 minutes. Gels were visualized under a ultra-violet light source, documented with Bio-Rad Chemidoc XRSTM system, and analyzed with Image Lab Software version 4.1.

All samples that did not demonstrate clear banding were subjected to an additional chloroform centrifugation step, and then re-run on agarose gels for a reassessment of the DNA quality.

DNA quantification was performed based on the Bio-RadTM quantitation kit's fluorochrome Hoechst procedure (catalog # 170-2480). The dye mixture was comprised of 4 μL of 10 mg/mL of Hoechst dye, 2.0 mL of 10xTEN Assay Buffer, and 17.996 mL of ddH_2O per well. A standard curve was produced using known concentration stock solutions of calf thymus

DNA of 100 ng/μL and 10 ng/μL. From the 100 ng/μL standard, the following volumes were used: 10 μL and 5 μL to make a 1000 ng and 500 ng point on the curve. Four more points were added to the curve using 10 μL (100 ng), 5 μL (50 ng), 2 μL (20 ng), and 1 μL (10 ng) of the 10 ng/μL stock calf thymus solution. All extracted DNA samples (2 μL) and standards were incubated in duplicate to check consistency. DNA fluorescence intensity was measured using the fluorescence detection setting of the BMG LABTECH FLUOstar OPTIMA microplate multi-detection reader. Using the quantification data, 500 μL of standardized 5 ng/μL DNA solution was prepared for each individual of each population from the stock extracted DNA.

2.3.4 ISSR Analysis

The six primers that were used in the analysis of the 1999 samples were selected for this ISSR analysis (Gervais and Nkongolo, 2011). They include: ISSR 17898B, UBC818, UBC 827, UBC 835, and UBC 841 (Table 1). PCR amplification was carried out as described in Mehes *et al.* (2007) with modifications. A solution of 25 μL total volume containing a master mix of 8.9 μL distiller water, 5 μL MgSO4, 2.1 μL 10x buffer, 0.5 μL dNTP mix (equal parts dTTP, dATP, dCTP, and dGTP), 0.5 μL of the ISSR primer being screened, a Taq mix of 3.475 μL distilled water, 0.4 μL 10xbuffer and 0.125 μL Taq polymerase (Bio Basic Inc.) and 4 μL standardized DNA. A negative control of master mix and Taq mix without any DNA were used. All samples were covered with one drop of mineral oil to prevent evaporation and amplified with the PERKIN ELMER DNA Thermal Cycler. The program was set to a hot start of 5 minutes at 95^{o}C followed by 2 minutes at 85^{o}C at which time the Taq mix was added. In total, 42 cycles of 1.5 minutes at 95^{o}C, 2 minutes at 55^{o}C, and 1 minute at 72^{o}C were performed. A final extension of 7 minutes at 72^{o}C completed the reaction, after which samples were removed from the thermocycler, cooled in ice then kept at -20^{o}C in a freezer until further analysis.

Amplified DNA products were separated on a 2% agarose gel in 0.5 x TBE containing ethidium bromide. This was followed by an addition of 5 μL of 6x loading buffer to each PCR product tube; and 10 μL of this solution was loaded into wells in a gel. The gel was run at 64 V for 120 minutes and then documented with a Bio-Rad ChemiDoc XRS system and analyzed using Image Lab (ver, 4.1) SoftwareTM.

The ISSR bands on each gel were scored as either present (1) or absent (0). Popgene software version 1.32 was used to determine percentage of polymorphic loci, observed and effective number of alleles, Nei's gene diversity and Shannon's information index. The genetic distances were calculated using Jaccard's similarity matrix with FreeTree version 0.1.9.50. A dendrogram was produced using the Jaccard similarity coefficients using TreeViewX, with patristic distance to scale branch lengths based on the neighbor-joining analysis from FreeTree version 0.1.9.50.

2.4 Results

2.4.1 ISSR Analysis

Detailed characteristics of the six selected primers are described in Error! **Switch argument not specified.**. Figure 2 to Figure 5 exemplify the amplified products. Total number of bands varied from 73 to 83, with band sizes ranging from 200 to 1700 bp.

2.4.2 Genetic Diversity

Using Popgene Software version 1.32 (Yeh et al. 1997), Table 2 describes the percentage of polymorphic loci (%), the observed number of alleles (Na), the expected number of alleles (Ne), Nei's gene diversity (h), and Shannon's information index (I). The mean of total gene diversity (HT) value was 0.35 and the gene diversity between populations (HS) was 0.22. The

mean population differentiation (Gst) value was 0.37 and the estimated gene flow (Nm) was found to be 0.86. The mean of inter-population polymorphism was 98.8%.

Within each population, the levels of genetic variation were moderate to high. In the present study, the percentage of polymorphic loci varied between 42.5% (Kelly Lake) and 77.0% (Little Current). Specifically, the highest level of genetic variation was observed in samples from metal uncontaminated site (Little Current). The two metal contaminated sites from the CGS had a low to moderate level of genetic variation with the level of polymorphic loci being 42.5% for Kelly Lake and 54 % for Wahnapitae site. Surprisingly, for Cobalt, the genetic variation was moderate in Cobalt Cart lake (55%) compared to a high genetic variation (69%) observed in samples collected in Cobalt Nipissing.

The observed number of alleles ranged from 1.42 (Kelly Lake) to 1.77 (Little Current) with a mean of 1.595. The expected number of alleles ranged from 1.28 (Kelly Lake) to 1.51 (Little Current) with a mean of 1.387. Nei's gene diversity, h, ranged from 0.16 (Kelly Lake) to 0.28 (Little Current) with a mean of 0.22. Shannon's information index revealed a range between 0.23 (Kelly Lake) and 0.42 (Little Current with a mean of 0.325) (Table 2).

Genetic distance among sites were estimated based on 0 (identical populations) to 1 (completely different populations) scale. Overall, all the five populations are genetically closely related since the genetic distance values vary between 0.048 and 0.264 (Error! **Switch argument not specified.**). The lowest value of 0.048 was observed between the Cobalt-Cart Lake and Wahnapitae sites, suggesting that they are the most closely related of the sampled sites. The genetic distance value (0.048) between Cobalt-Cart Lake and Wahnapitae was the lowest which suggests that they are the closest genetically related. Hence, the population clustering based on ISSR data was not associated with geographic distances. The dendrogram generated shows that

all the metal contaminated sites cluster together with the uncontaminated site (Little Current) being separate from the cluster with 100% degree of confidence (Figure 6). No population diagnostic markers were found between metal contaminated and uncontaminated sites.

Table 1. Primer nucleotide sequences used to produce ISSR profiles by genomic DNA amplification from five populations of *Deschampsia cespitosa.*

Primer Identification	Nucleotide sequence (5' → 3')	Amplification	Fragment size range (bp)
ISSR 17898B	CACACACACACAGT	Good	260-1200
UBC 818	CACACACACACACACAG	Good	250-1560
UBC 827	ACACACACACACACACG	Good	290-1330
UBC 835	AGAGAGAGAGAGAGAGYC	Good	200-1400
UBC 841	GAAGGAGAGAGAGAGAYC	Good	220-1700

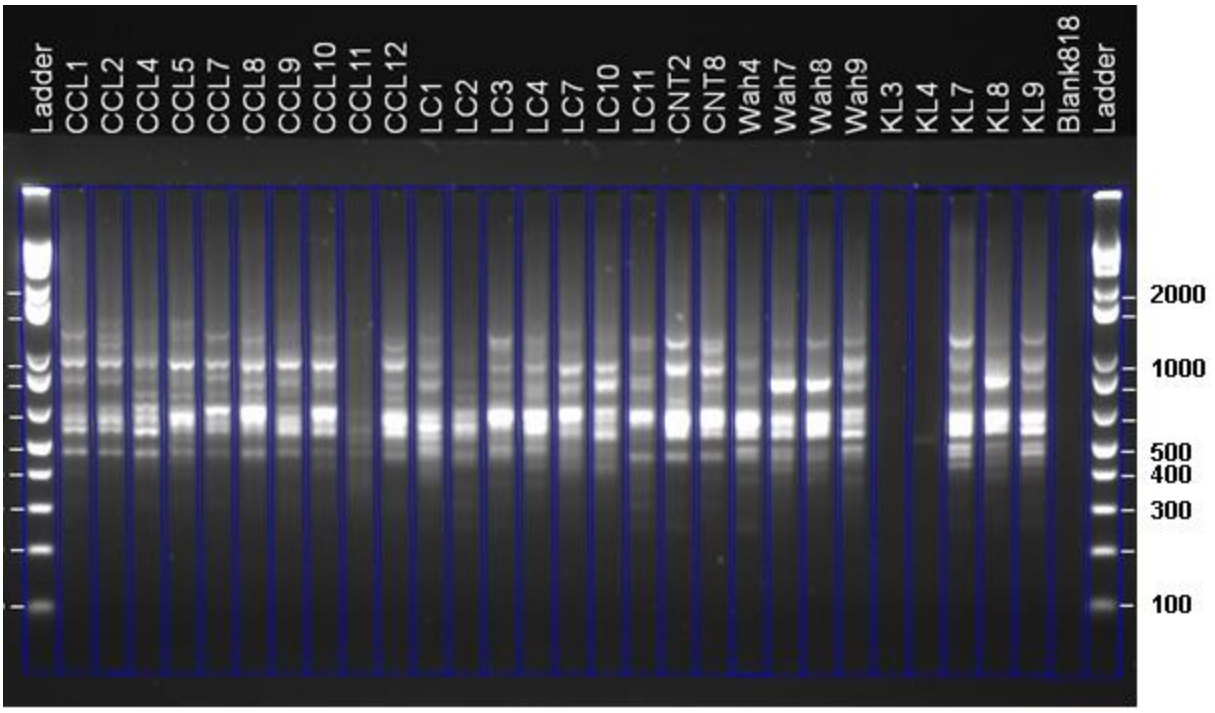

Figure 2. Sample ISSR amplification of *D. cespitosa* with primer UBC 818. The flanking lanes (1 and 31) contain 1Kb+ ladder, lanes 2 to 30 samples from Cobalt 3 (Cart Lake), Little Current, Cobalt 1 (Nipissing Tailings), Wahnapitae, and Kelly Lake.

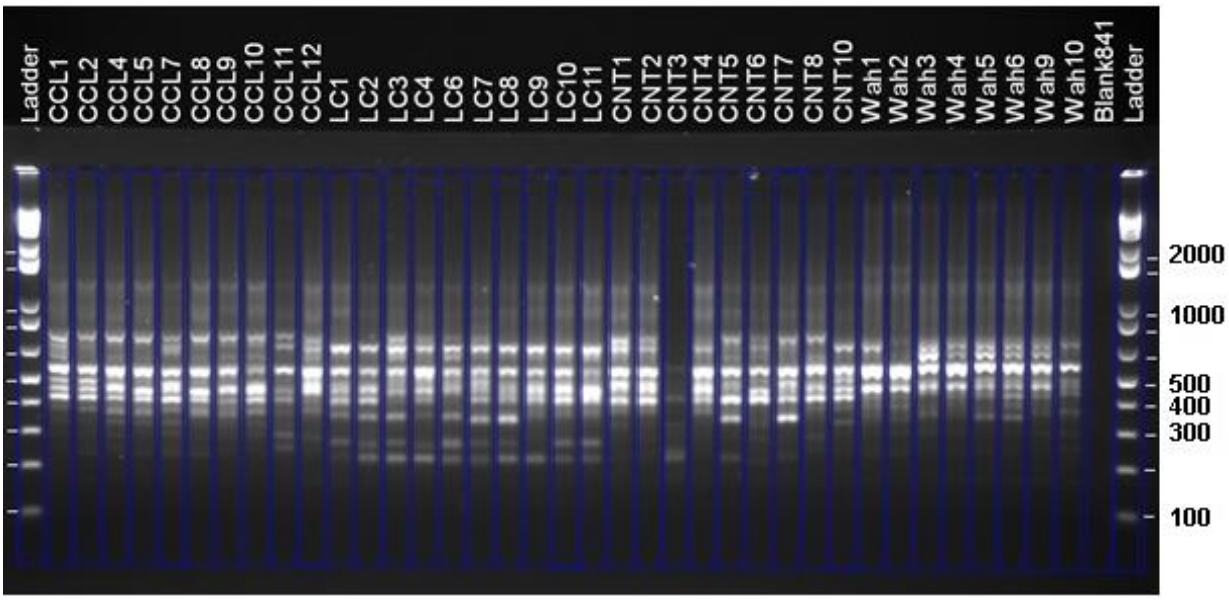

Figure 3. Sample ISSR amplification of *D. cespitosa* with primer UBC 841. The flanking lanes (1 and 40) contain 1Kb+ ladder, lanes 2 to 39 samples from Cobalt 3 (Cart Lake), Little Current, Cobalt 1 (Nipissing Tailings), and Wahnapitae.

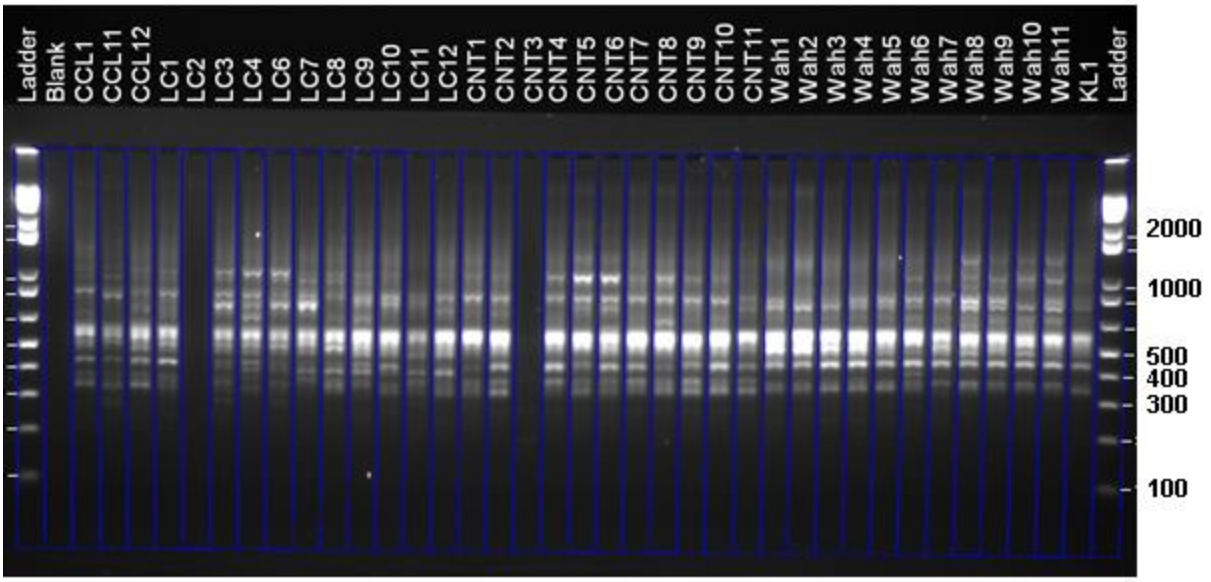

Figure 4. Sample ISSR amplification of *D. cespitosa* with primer UBC 835. The flanking lanes (1 and 40) contain 1Kb+ ladder, lanes 2 to 39 samples from Cobalt 3 (Cart Lake), Little Current, Cobalt 1 (Nipissing Tailings), Wahnapitae, and Kelly Lake.

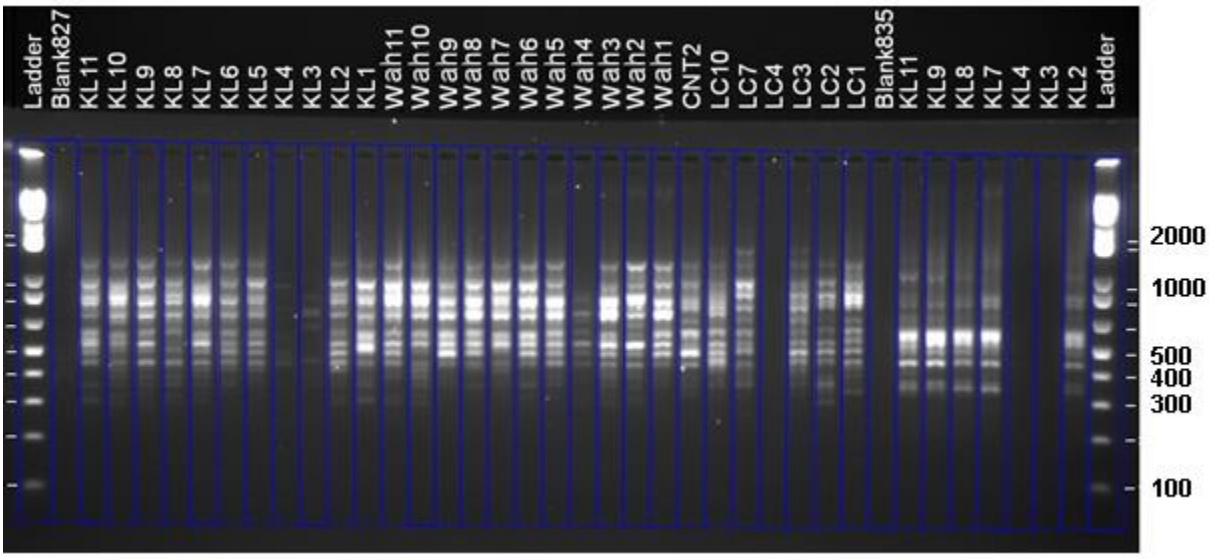

Figure 5. Sample ISSR amplification of *D. cespitosa* with primer UBC 827. The flanking lanes (1 and 40) contain 1Kb+ ladder, lanes 2 to 31 samples from Kelly Lake, Wahnapitae, Cobalt 1 (Nipissing Tailings), and Little Current.

Table 2. Genetic Diversity values for five *D. cespitosa* populations from Northern Ontario.

	Na	Ne	H	I	P (%)
Cobalt Nipissing Tailings (Cob1)	1.6897	1.4108	0.2371	0.3543	68.97
Cobalt Cart Lake (Cob3)	1.5517	1.4112	0.2257	0.3270	55.17
Wahnapitae	1.5402	1.3249	0.1892	0.2826	54.02
Kelly Lake	1.4253	1.2830	0.1607	0.2366	42.53
Little Current	1.7701	1.5095	0.2878	0.4241	77.01
Mean	1.5954	1.3879	0.2201	0.3249	59.54

Genetic diversity descriptive statistics. Na: observed number of alleles; Ne: expected number of alleles; H: gene diversity; I: Shannon's information index; P: percentage of polymorphic loci.

Table 3. Distance matrix generated using the neighbor-joining analysis using *Deschampsia cespitosa* ISSR data (FreeTree version 0.1.9.50).

	1	2	3	4	5
1	0.000	0.149	0.172	0.264**	0.169
2		0.000	0.048*	0.186	0.174
3			0.000	0.167	0.176
4				0.000	0.207
5					0.000

1 represents *D. cespitosa* Cobalt-1 population (Nipissing Tailings); **2** *D. cespitosa* Cobalt-3 population (Cart Lake); **3** *D. cespitosa* Wahnapitae population; **4** *D. cespitosa* Kelly Lake population; **5** *D. cespitosa* Little Current population.

* Most genetically similar (Cobalt-3 and Wahnapitae).
** Most genetically different (Cobalt-1 and Kelly Lake).

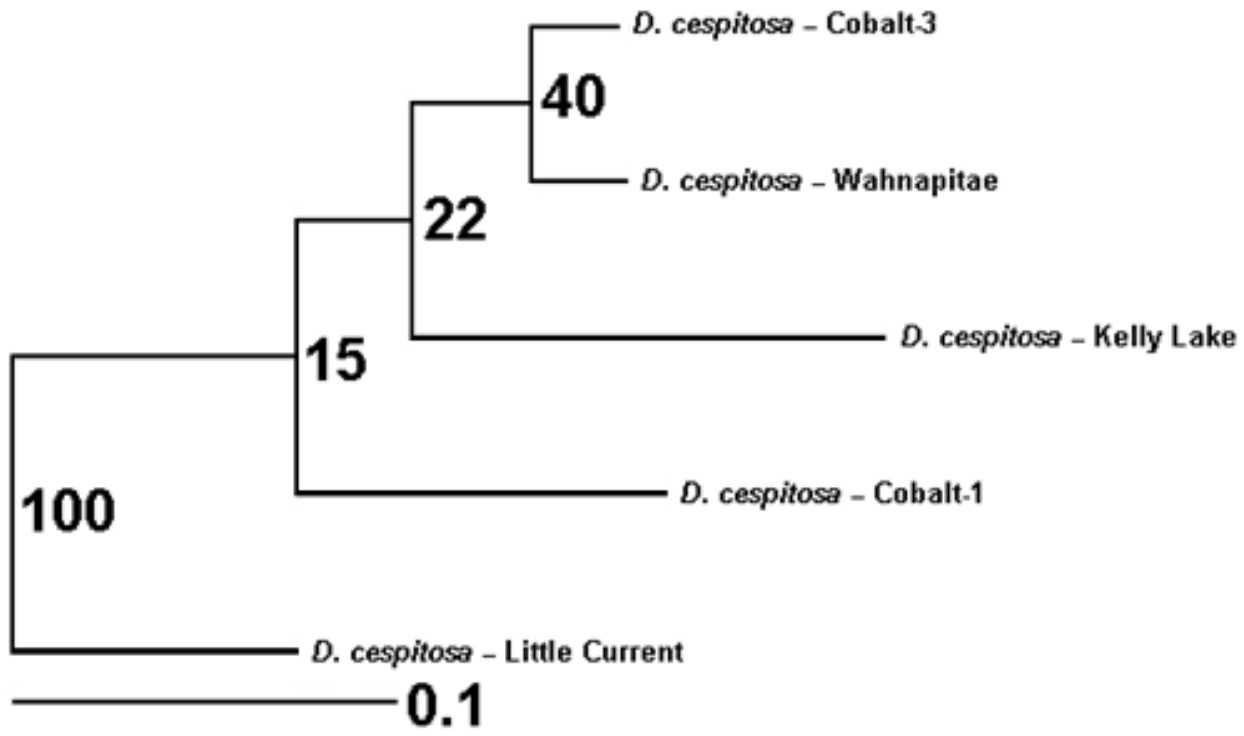

Figure 6. Dendrogram of the genetic relationships between the five *D. cespitosa* populations from Northern Ontario using the Jaccard neighbor-joining similarity matrix from ISSR profiles. Branch lengths are to scale and show the patristic distance based on the neighbor-joining analysis. Values within branches indicate bootstrapping percentages.

2.5 Discussion

Data related to metal content for the five targeted sites have been discussed in previous reports (Nkongolo *et al.*, 2001; Nkongolo *et al.*, 2015; Kalubi *et al.*, 2016). Nickel and copper are the main contaminants of soil in the Greater Sudbury region including the Wahnapitae and Kelly Lake areas while cadmium, cobalt, copper, lead, zinc and to some extent nickel, are found in high concentration in Cobalt sites. Little Current is among the least metal-contaminated and was used as reference for this study (Nkongolo *et al.*, 2001).

It is expected that genetic variance will increase when the population is challenged with novel environmental conditions (Service and Rose, 1985; Holloway *et al.*, 1990). If resistance to

metal is a polygenic trait as suggested by Von Frenkell-Insam and Hutchinson (1993) and MacNair (1993), it is very likely that allelic frequency in an outcrossing and perennial species like *D. cespitosa* will be maintained over time resulting in a neutral genetic variation (Gervais and Nkongolo, 2011).

Resistant plants can develop if the toxic stress continues at a sublethal level for many generations (Nkongolo et al., 2001; Gervais and Nkongolo, 2011). A decrease in genetic variation can result from selection. Plants regulate the concentration of metal ions inside the cell through different mechanisms to minimize the potential damage that could result from the exposure to nonessential metal ions (Benavides et al., 2005). These mechanisms serve to control the update, accumulation and detoxification of metals (Foy *et al.*, 1978). Selection of metal-resistant genotypes has been demonstrated to occur rapidly, within one or two generations, in populations that contain the necessary genetic information (Wu *et al.*, 1975). The intensity of the contamination and the amount of time the population has been exposed to the toxic levels are the main factors that may affect plant's ability to tolerate metals.

In the present study, we report the application of ISSR markers to the study of plant species with populations adapted to metals. In general, the appropriate timeframe for sampling genetic diversity to detect meaningful changes will vary according to species and to the environment. Most of the Northern Ontario sites were mining areas well known for the severity of environmental degradation. The populations of *D. cespitosa* in Cobalt are believed to have been there for a much greater amount of time than the populations in Sudbury. The Cobalt tailings are more contaminated than the Sudbury soil. But the two sites selected in Sudbury (Kelly Lake and Wahnapitae wet lands) are the most metal-contaminated within the CGS. This has resulted in a decrease and possibly a loss of alleles at some loci and many rare alleles that

has lead ultimately to a lower genetic variation *D. cespitosa* populations sampled in 1999 (Nkongolo *et al.,* 2016). Evidence of loss of genetic variation at the population level caused by pollution has been demonstrated in other species (van Straalen and Timmermans, 2002; Lopes *et al.,* 2004). Mengoni *et al.* (2001) using chloroplast microsatellite analysis showed a large reduction in genetic diversity associated with *Silene paradoxa* populations growing in copper-contaminated soils.

In the present study, the levels of genetic variation were moderate to high within targeted populations from Sudbury, Cobalt and Little Current. When the polymorphism levels between the 1999 and the 2015 samples were compared, there was an insignificant change in the overall percent of polymorphic loci in metal – uncontaminated site of Little Current (from 70% in 1999 to 77% in 2015) and in a Cobalt Cart Lake site (from 48% in 1999 to 55% in 2015). But a significant decrease in genetic variation was observed in Wahnapitae site (from 72% in 1999 to 54% in 2015). On the other hand, a significant increase was observed in Cobalt Nipissing (from 46% in 1999 to 64% in 2015). The Kelly Lake site with the lowest level of polymorphic loci of 42.5% in 2015 was not surveyed in 1999. The levels of observed number of alleles, Nei's gene diversity, Shannon's information index followed the same trend.

The increase in genetic variation in Cobalt Nipissing population is surprising. It might be due to new genetic recombination involving de novo or outside sources of genetic materials that did change the genetic profile of this population. Since *D. cespitosa* is an open pollinated species, pollens from other areas transported by wind could be involved in reshuffling the genetic makeup of this population. This scenario is unlikely considering the distance between the two sites. Since it is a short-lived species, it is expected that the samples collected are from at least three generations of genetic recombination. Hence de novo recombination without external

sources might be the most acceptable explanation especially if the alleles in the population were not fixed. The contribution of seeds carried by birds and anthropogenic activities must also not be discounted. The sharp decrease of genetic variation in Wahnapitae site might be due to population changes. It is known that gene flow into a population can counteract gene frequency changes because of selection, imposing a limit on local adaptation (Lenormand, 2002). It is possible that population surveyed in 2015 in this site was composed of survivors of the 1999 population that was growing in soils with very high level of nickel leading to a decrease of genetic variation.

The degree of genetic relatedness between sites has increased since the populations are more closely related than 16 years ago **Error! Switch argument not specified.** and **Error! Switch argument not specified.**). A significant change in genetic distance is in part caused by a high gene flow. But, in the present study, the gene flow was low (< 1). Usually gene flow between 1 and 0.5, is weak but possibly effective for the transfer of favorable genes and values below 0.5 suggest that groups are almost or fully isolated (Wolf and Soltis, 1992; Lenormand, 2002). Indeed, the level of difference in genetic variation observed between the two Sudbury sites suggests that gene exchange between them is limited. The same phenomenon was observed for the two Cobalt sites. Analysis of samples collected in 1999 also revealed a low level of gene flow among the targeted populations based on ISSR and microsatellite markers (Gervais and Nkongolo, 2011). The low level of gene flow can be explained by the geographic distance between the targeted populations as the closest sites are about 2 km away from each other and the most distant sites are > 300 km apart. The clustering of Cobalt and Sudbury populations strengthens the earlier theory that Sudbury populations of *D. cespitosa* might be from the Cobalt region (Frenckell-Insam and Hutchinson, 1993; Gervais and Nkongolo, 2011).

2.6 Conclusion

The present study showed that in two of the populations studied, the level of genetic variation is unchanged after 16 years of genetic recombination, in one population, polymorphic loci degree has increased while in another population it has decreased. The metal uncontaminated site showed the highest level of genetic variation compared to metal contaminated sites. The targeted populations are more genetically closely related than they were 16 years ago. No population diagnostic ISSR marker was identified.

Chapter 3. Toxicity of nickel and copper in *Deschampsia cespitosa*

3.1 Abstract

The objective of the current study is to determine the toxicity of nickel and copper at different doses in *D. cespitosa* under controlled conditions. It was found that overall, copper was more toxic than nickel in the assays. For nickel, no damage to plants was observed during the seven days of treatment with 5.6 mg/kg dose. However, significant differences for damage rating, root, and leaf biomass were observed between the 1,600 mg/kg dose (representing the total Ni level found in contaminated Greater Sudbury soil) and the control (0.0 mg/kg). The 4,800 mg/kg treatment was extremely toxic as all the plants were dying (damage rating of 8 on a scale of 1 to 10) within 48 hours after the treatment. For copper, no damage to plants was observed during the seven days of treatment with the 9.16 mg/kg dose. Significant differences for damage rating, root and leaf biomass were observed between the 1,312 mg/kg dose (representing the total Cu level found in contaminated Greater Sudbury soil) and the control (0.0 mg/kg). The 3,936 mg/kg treatment was extremely toxic as all the plants were dying (damage rating of 8 on a scale of 1 to 10) within 48 hours after the treatment.

3.2 Introduction

While nickel and copper are essential for plant growth at low levels (Aubert and Pinta, 1977; Shabala, 2017), exposure to higher concentrations can impede metabolic processes and results in high toxicity (Mishra and Kar, 1974; Cambrolle *et al.*, 2015). This often manifests in the form of oxidative stress and lipid peroxidation in plant tissues, limiting plant growth and leading to mortality or a severely reduced viable seed-bearing rate in susceptible species (Shabala, 2017). In resistant species, heavy metal exposure has been shown to trigger resistance mechanisms (Bush and Barrett, 1993) which most often either reduce the metal's reactivity

through complexing, or restricting the metals' access to the plant's tissues using selective membrane proteins (Theriault and Nkongolo, 2016). Some species do the latter by adopting an exclusion strategy to avoid excessive uptake and transport of metal ions (Taylor, 1987; Mehes-Smith and Nkongolo, 2015; Kalubi *et al.*, 2015). Avoiders restrict metals from entering the plant. Excluders accumulate metals in roots, but restrict their translocation to aerial parts (Baker, 1981; Taylor, 1987). In accumulator species, metals are concentrated in the above ground parts and, if harmful, are complexed and kept where they will not affect plant processes. Recent field studies showed that *Betula papyrifera* accumulates nickel in leaves, but does not store copper in its tissues (Theriault *et al.*, 2013, 2014). Edaphic ecotypes tolerant to high levels of metal contamination have been reported around old mines and smelters (Kirkey *et al.*, 2012).

D. cespitosa, is also known for its ability to survive, adapt, and populate under marginal and contaminated conditions. *D. cespitosa* inhabits many environment types (St. John *et al.*, 2011, VSDA Forest Service Fire Effects Information System) and has been shown to be resistant to metal contamination (Bush and Barrett, 1993; Cox and Hutchinson, 1980; Mehes-Smith and Nkongolo, 2015; Nkongolo *et al.*, 2014). It is categorized as an excluder by Mehes-Smith *et al.* (2013) since it limits metal translocation to its aboveground biomass, keeping the metals in its root mass. However, the specific genetic mechanisms involved in responding to metal contamination are not well defined.

The main objective of the present study was to determine the toxicity of nickel and copper at different doses in *D. cespitosa* under controlled conditions.

3.3 Materials and Methods

3.3.1 Seed Germination and Plant Growth

D. cespitosa seeds were collected from two metal-contaminated sites (Wahnapitae and Coniston) in the Greater Sudbury Region (GSR), Ontario (Canada), and stored at 4°C. The seeds were then germinated at 27°C within wet filter paper-lined Petawawa boxes. Once germinated with a 1 cm leaf and 1cm root, they were transplanted into a peat moss/pearlite/vermiculite mixture and grown for four months at 27°C. The plants were watered and fertilized as needed. After the four months elapsed, the plants were transplanted into a 50:50 mix of washed quartz sand and the peatmoss/pearlite/vermiculite mixture (Figure 7). Plants were then left to grow for an additional month, until an average height of 20 cm was reached.

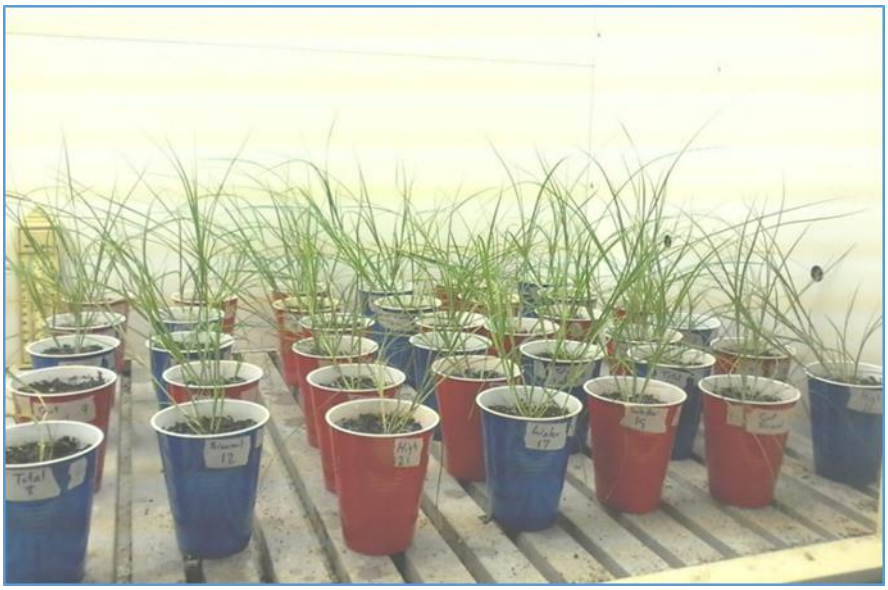

Figure 7. *D. cespitosa* plants growing in a growth chamber.

3.3.2 Assessment of metal toxicity

The present study focuses only on toxicity of Ni and Cu since they were the most preponderant elements in contaminated sites compared to uncontaminated areas in the RGS (Nkongolo *et al.,* 2013). To assess the toxicity of these two elements on *D. cespitosa,* plants were treated under controlled conditions in growth chambers. Commercial $Ni(NO_3)_2$ and $Cu(SO_4)$ salts were used for Ni and Cu treatments respectively. The experimental design was a completely randomized block with seven to ten replications per treatment.

For nickel, there were three treatment groups of seven plants each which were treated with three doses of $Ni(NO_3)_2$ equivalent to 5.6 mg/kg (5.6 mg of Ni per 1 kg of dry soil), 1,600 mg/kg (1,600 mg of Ni per 1 kg of dry soil), and 4,800 mg/kg (4,800 mg of Ni per 1 kg of dry soil) and a seven-plant water control group (0 mg/kg). The dosage of 5.6 mg/kg Ni corresponds to the published bioavailable amount of nickel in contaminated sites while 1,600 mg/kg Ni is the level of total nickel found in contaminated sites in the RGS as described in Nkongolo *et al.* (2013).

For copper, the dosages included 9.16 mg/kg (9.16 mg of Cu per 1 kg of dry soil), 1,312 mg/kg (1,312 mg of Cu per 1 kg of dry soil), and 3,936 mg/kg (3,936 mg of Cu per 1 kg of dry soil) which are respectively equivalent to bioavailable, total at site, and three times the amount of total Cu in contaminated sites.

A water treatment (0 mg of Ni or Cu per 1 kg of dry soil) was used as a control. Potassium nitrate (KNO_3) and potassium sulfate (K_2SO_4) treatments were used for three control concentrations for the nitrate and sulfate concentrations of the respective metal salts. The 4,800 mg/kg Ni (18 mmol of Ni), 1,600 mg/kg Ni (6 mmol of Ni), and 5.56 mg/kg Ni (0.021 mmol of Ni) contained 36 mmol, 12 mmol, and 0.042 mmol of nitrate, respectively. The 3,936 mg/kg,

1,312 mg/kg, and 9.16 mg/kg of Cu corresponded to 30 mmol, 10 mmol, and 0.063 mmol of Cu, respectively. The same molarity was used of the KNO_3 and K_2SO_4 salts.

All treatments were administrated in water. All dosages were based on dry soil weight and the measured total amount of nickel found in contaminated mining-affected soils of Northern Ontario. Damage was assessed every two days on a scale of 1 to 9 described in Table 4. Genotypes with damage ratings of 1 to 3 were considered resistant genotypes (RG), 4 to 6, moderately susceptible (MSG), and 7 to 9 susceptible (SG). Table 4 depicts RG, MSG, and SG.

3.3.3 Statistical analysis

SPSS version 20 for Windows was used to analyze the growth rate and damage rating data for both metal experiments. In order to achieve a normal distribution, the data was log transformed. Data sets were analyzed via ANOVA followed by Tukey's HSD multiple comparison analysis, to determine the significant differences between the means ($P < 0.05$).

Table 4. Damage rating scale and plant classification based on reaction to nickel and copper treatments.

% of Leaf area with chlorosis/necrosis	Damage Rating	Genotype Classification
0-10	1	Resistant (RG)
10-20	2	
20-30	3	
30-40	4	Moderately Susceptible (MSG)
40-50	5	
50-60	6	
60-70	7	Susceptible (SG)
70-80	8	
> 80	9-10	

a)

b)

Figure 8. *Deschampsia cespitosa* treated with 1,600 mg/kg a) at day 1 after treatment and b) day 7 after treatment.

a)

b)

Figure 9. *Deschampsia cespitosa* treated with 1,312 mg/kg of copper a) at day 1 after treatment and b) day 7 after treatment.

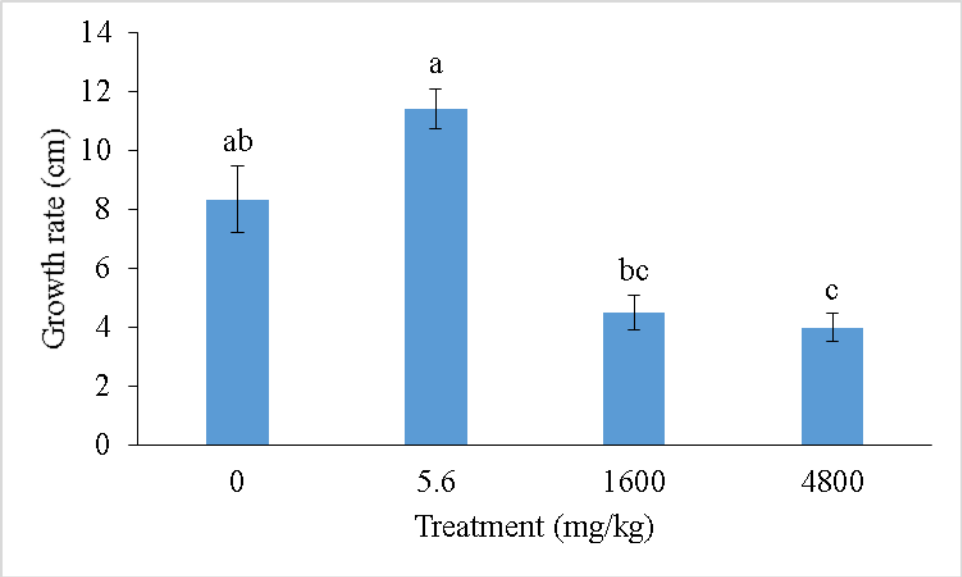

Figure 10. Growth rate by of *Deschampsia cespitosa* treated with different doses of nickel. Six days elapsed.

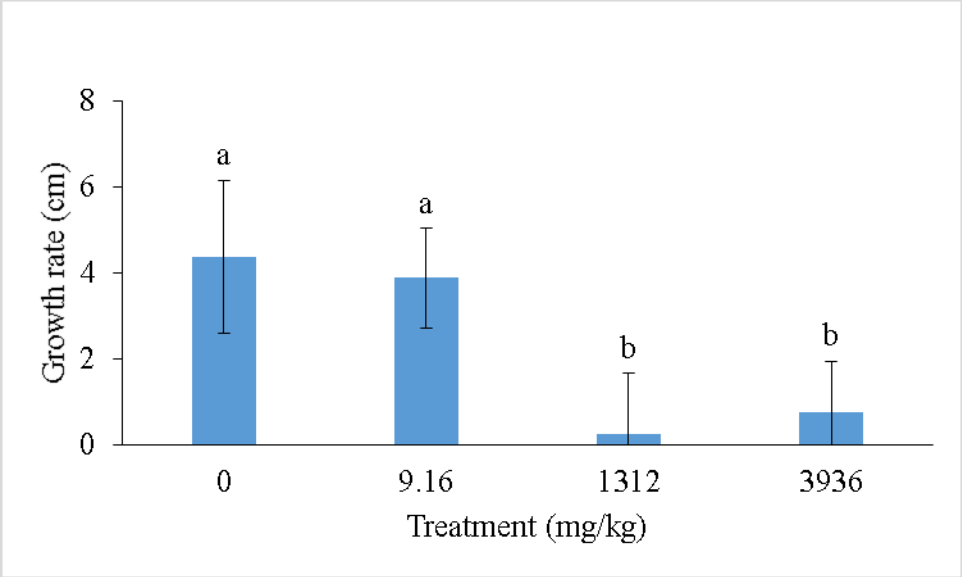

Figure 11. Growth rate by of *Deschampsia cespitosa* treated with different doses of copper. Six days elapsed.

Table 5. Damage rating of *Deschampsia cespitosa* treated with different doses of nickel and copper.

Treatment	Soil concentration (mg/kg)	Damage rating		
		Days after treatment		
		Day 2	Day 4	Day 6
Ni	5.56	1a	1a	1a
	1,600	3.3b	6.6b	8.6b
	4,800	7.6c	9.3c	9.8c
	9.16	1a	1a	1a
Copper	1,312	4.8b	8.1b	9b
	3,936	5.5b	8.9b	9b

ANOVA, followed by Tukey's HSD multiple comparison analysis, were performed to determine significant differences among means. Means followed by different letters for each element (nickel, copper) are significantly different ($P < 0.05$; $n = 15$).

3.4 Results

In the present study, we focused on testing the toxicity of bioavailable and total Ni and Cu in soil. Bioavailable metals are the proportion of total metals in soil that are available for incorporation into biota (Abedin *et al.*, 2012; Narendrula *et al.*, 2013; Nkongolo *et al.*, 2013). Significant differences for damage rating and growth rate were observed among nickel dosages after seven days of treatment based on ANOVA and Tukey mean comparison tests. Plant reactions to Ni and Cu treatments are summarized in Table 5. No significant damage was observed during the seven days of treatment with the concentration of nickel at 5.6 mg/kg dosage corresponding to bioavailable amount at site. At day 6 after nickel treatments, the mean damage ratings for 1,600 mg/kg and 4,800 mg/kg dosages were 8.6 and 9.8, respectively (Figure 8 and Table 5). The 4,800 mg/kg dose was extremely toxic as all the plants died within 48 hours of the treatment.

In general, the reaction of *D. cespitosa* to copper treatment follows the same pattern as nickel. No toxicity was observed for the 9.16 mg/kg (amount of bioavailable Cu in the natural site) throughout the experiment (Table 5). Significant differences in plant damage and growth rate were observed between the 9.16 mg/kg and 1,312 mg/kg dosages throughout the sixth day of the trial (**Error! Switch argument not specified.**, Error**! Switch argument not specified.** and Table 5). In fact, the average rating at day 6 for this latter dose was 9, compared to the rating of 1 for the former dosage (9.16 mg/kg). This 1,312 mg/kg treatment represents the total Cu level in the Greater Sudbury soil or 143 fold the amount of bioavailable Cu. The highest dose of 3,929 mg/kg resulted in severe damage on plants two days after the treatment and almost all the plants were dead within four days of Cu application.

3.5 Discussion

In the present study, the effects of three nickel and copper bioavailable concentrations were examined. They include 5.56 mg/kg, 1,600 mg/kg, and 4,800 mg/kg for nickel and 9.16 mg/kg, 1,312 mg/kg, and 3,936 mg/kg for copper. For reference purposes, a mean total Ni content of 26.8 mg/kg is considered to be representative of typical Ni concentration in Canadian background soil. This excludes areas of Ni enriched rocks and Ni bearing mineral occurrences (Sheppard *et al.*, 2007). The average total copper concentration in Canada soil is estimated to be 20 mg/kg (McKeague and Wolynetz, 1980).

Overall, this study indicates that *D. cespitosa* is tolerant to low doses of Ni and Cu similar to the bioavailable levels in contaminated sites. The treatments at high doses of 1,600 mg/kg of Ni and 1,912 mg/kg of Cu revealed that this species is susceptible to high doses of both nickel and copper. Theriault *et al.* (2016) treated *Betula papyrifera* with nickel salts and revealed no significant damage caused by nickel at 5.6 mg/kg dosage (Theriault *et al.*, 2016). At day 8 after nickel treatments, the mean damage ratings for 1,600 mg/kg and 4,800 mg/kg dosages were 5.7 and 9, respectively. The 4,800 mg/kg dose was extremely toxic as all the plants died within 48 hours of the treatment. Djeukam *et al.* (2016) also reported no significant damage caused by Cu at 9.16 mg/kg dosage on *Betula papyrifera*. However, in the present study significant differences in plant damage ratings were found between the 9.16 mg/kg and 1,312 mg/kg dosages. The highest dose of 3,929 mg/kg resulted in severe damage on plants two days after the treatment and almost all the plants were dead within four days of Cu application. There was a trend of reduced plant growth as the dosage increased. No significant difference in plant

growth was observed between 9.16 mg/kg and the control and 1,312 mg/kg and 3,936 mg/kg dosages (Figure 11).

Several studies have demonstrated that an oxidative stress is involved in metal toxicity in plants, either by inducing oxygen free radical production or by decreasing enzymatic and non-enzymatic anti-oxidants (Fornazier *et al.*, 2002; Cho *et al.*, 2003; Cho and Seo, 2004; Theriault *et al.*, 2016a). The sensibility or resistance of plants to metals depends on physiological and molecular mechanisms that include uptake and accumulation of metals usually by binding to extracellular exudates and cell wall complexation of ions inside the cells. Some plants prevent metal ions from entering the cytosol through the action of plasma membrane (Benavides *et al.*, 2005; Kalubi *et al.* 2015; Theriault *et al.*, 2014). Overall the scarcity of knowledge on mechanisms of Ni and Cu homeostasis, uptake, transport, and accumulation in *D. cespitosa* limit our understanding of relationship between metal toxicity and plant genetic response.

3.6 Conclusion

Significant differences between low and high dose of nickel and copper were observed for damage rating and plant growth in the growth chamber screening tests.

Chapter 4. Cytogenetic analysis of *Deschampsia cespitosa* genotypes from metal contaminated and uncontaminated sites in Northern Ontario

4.1 Abstract

The objective of this study is to investigate cytological damages caused by metals in *Deschampsia cespitosa* growing in Northern Ontario. It was found that metal-contaminated sites significantly increased the level of aneuploidy compared to reference sites. However, the cytological instability did not result in poor plant health. The benefits of the outcrossing and large seed counts of *D. cespitosa* are thought to negate the long-term effects of aneuploidy.

4.2 Introduction

Soil metal contamination is found in various parts of the world. These toxic sites result from either natural processes such as acidic, saline, and serpentine soils or by anthropogenic effects such as mining activities, aerial fallout and the run-off from galvanized sources of electricity pylons or motorway verges polluted by vehicle exhaust fumes (Bradshaw, 1984).

Northern Ontario (Canada), especially the Greater Sudbury Region, has a long history of mining activities dating back to 1888. Soil and ground water contamination with Co, Cu, and Ni came from deposition of aerial pollutants from smelting operations. Elevated Al and Fe levels are also found in this region. These elevated metal levels resulted in barren and semi-barren landscapes. The natural recovery of this area has been impeded by various factors such as the elevated levels of total and available metal in surface soils. Nkongolo *et al.* (2008), Dobrzeniecka *et al.* (2011) and Nkongolo *et al.* (2013) reported high concentrations of Cu and Ni

close to the smelter. This excessive concentration can have deleterious effects on the surrounding ecosystems (Kopittke *et al.*, 2010).

Excess Ni can reduce plant biomass and induce leaf deformities, chlorosis, and necrosis. Ni phytotoxicity can inhibit the activity of some enzymes, reduce the uptake and translocation of some nutrients, and decrease cell division (Nishida *et al.*, 2011; Solanki and Dhankhar, 2011). The precise mechanism(s) by which Ni toxicity limits the growth of plants are not well understood (Seregin and Kozhevnikova, 2006). Similarly, an elevated Cu concentration in the soil can also lead to symptoms of toxicity, including root tissue damage, increased permeability of the root cell plasma membrane, inhibition of photosynthesis, and DNA damage (Paschke and Redente, 2002).

Phytoremediation can improve nutrient soil conditions leading to the establishment of a self-sustaining vegetative cover, which in turn can prevent wind and water erosion of the toxic soil (Wei *et al.*, 2005). This technique can be used to remove, stabilize and detoxify organic and inorganic pollutants including heavy metals from soil and liquid substrates as well as in air (Salt *et al.*, 1998). Plant species selected for this purpose should grow and spread quickly, as well as be able to establish an effective soil cover. They should also have adapted to the polluted soil in addition to the local climate (Wei *et al.*, 2005). *D. cespitosa* has naturally colonized metal contaminated soils, an indication of its metal tolerance characteristics.

Until now, most of the genetic studies on species with metalliferous populations have dealt with the genetic determinism of metal tolerance (Bert *et al.*, 2002; Zha *et al.*, 2004; Willems *et al.*, 2007; Roosens *et al.*, 2008). Such studies have yielded valuable insight into the mechanisms that might underlie plant adaptation to metal availability in lower plants. No study has been conducted to determine the tolerance mechanism and tissue allocation of Cu and Ni (the

main contaminants in Northern Ontario) in *D. cespitosa.* Tolerance, metal uptake and compartmentalization are variable among species. The phytotoxic threshold is unknown, as are the internal toxic effects.

Dechampsia cespitosa has shown a remarkable ability to colonize and dominate metal contaminated lands. They have colonized several thousands of hectares of barren lands in the Greater Sudbury Region, following the construction of the Super Stack in 1972 (Nkongolo *et al*., 2001). However, knowledge of physiological mechanisms involved in *D. cespitosa* metal tolerance as well as the cytological effects of metals on these ecotypes is limited. Despite the recent exploitation of high-throughput methodologies such as cDNA microarrays, the overall picture of plant responses to metal contamination is far from complete. Likewise, the effects of long term exposure to high levels of metal on cytological stability in *D. cespitosa* population are not fully understood.

The main objective of the present study is to investigate the effects of metals, specifically Cu and Ni, on cytological stability of *D. cespitosa* plants.

4.3 Material and Methods

4.3.1 Sampling

Deschampsia cespitosa populations from three wet land sites in Northern Ontario (Wahnapitae Hill, Coniston Hydro, Kelly Lake), and two reference sites (over 100 km distant) at Low Water and Mississagi Lighthouse were selected for metal analysis (Figure 12). Soil, root, shoot, and foliage samples were collected from 16 plants in each of the metal contaminated and the reference sites. For each plant, 10 small soil samples from the rhizosphere were collected. Around 10 roots of the same size were harvested from each plant. The roots from each plant

were pulled and cleaned with water to remove any external debris and soil. Overall, 5 to 10 shoot and foliage samples from different tillers of the same plant were collected.

4.3.2 Cytological analysis

Root samples for cytological analysis were collected only from *D. cespitosa* plants growing in wetlands for over ten years. These sites included two metal contaminated areas: Wahnapitae Hill and Coniston Hydro, as well as two reference sites: Low Water and Mississagi Lighthouse. The site conditions were optimal for root meristematic cell division. A 2-4 cm terminal portion of each root was pretreated in ice water for 48 h. They were then fixed in 3:1 ethanol: glacial acetic acid for at least 1 h. The samples were stained with acetocarmine 30 minutes prior to examination. The squash preparations were made with glacial acetic acid. Cells at different mitotic stages were analyzed in all preparations derived from root as previously described in Nkongolo and Klimaszewska (1995), Mehes-Smith *et al.* (2009) and Mehes-Smith *et al.* (2011). For each plant, chromosomes were counted in 40 metaphases and pro-metaphase cells. The presence of bridges, lagging chromosomes and chromosome fragments was determined for anaphase and early-telophase cells.

4.4 Results

Cytological analysis was conducted only for *D. cespitosa*. This analysis revealed a significant increase of the level of aneuploidy in metal-contaminated sites compared to the reference sites. Figure 13 depicts examples of normal and abnormal mitotic cells in samples that were analyzed. Overall, 100% of plants from contaminated areas exhibited varying degrees of mixoploidy compared to 17% for the reference sites. The most frequent abnormality observed in *D. cespitosa* plants was the changes in chromosome number at metaphase. This was followed by

the presence of lagging chromosomes during anaphase. None of the plants from the metal contaminated sites were 100% euploid since they all exhibited varying levels of aneuploidy (Table 6). Only hypoploids (n < 26) cells were observed in all the mixoploid plants. These high levels of mitotic abnormalities did not seem to affect *D. cespitosa* survival and growth in highly metal-contaminated sites.

4.5 Discussion

4.5.1 Mitotic Instability

Tufted hairgrass *(D. cespitosa)* exist in two different cytotypes that include diploids (2n = 26) and tetraploid (2n = 52) (Brilman and Watkins, 2003). A previous survey of Northern Ontario (Canada) ecotypes revealed only the presence of diploid form in all the targeted areas (Nkongolo *et al.*, 2001). In the present study, a high level of aneuploidy and mixoploidy was observed in genotypes growing on metal contaminated soils. Theoretically, it is expected that over few generations, the mitotic abnormalities should result in meiotic instability that will eventually affect the long term sustainability of these populations. Since *D. cespitosa* is an outcrossing species and produces a large number of seeds every year, these characteristics should negate the long term effects of aneuploidy by maintaining a sufficient portion of euploid genotypes over time.

The practical biological significance of the mixoploidy in *D. cespitosa* populations is minimal since a significant level of seeds with complete euploid cells was recovered in the next generation. This is probability due to the open pollination between aneuploidy and euploid genotype. This high level of mitotic instability contrasts however with cytological stability

observed in *Picea mariana* (black spruce) trees growing in the same areas (Dobrzeniecka *et al.*, 2011).

Aneuploidy is a condition in which cells acquire a karyotype that is not a whole-number multiple of the haploid complement. This deviation from the normal chromosome number can involve the loss (monosomy) or gain (trisomy) of one or more individual chromosome(s) or large chromosomal segments (segmental aneuploidy). In the present study, all the aneuploid cells were hypoploid. Within species, different aneuploidies induced similar changes in gene expression, independent of the specific chromosomal aberrations. The imbalance of genes on the affected chromosomes can cause severe phenotypic syndromes in both plants and animals (Birchler and Veitia, 2007; Dierssen *et al.*, 2009). Although plants are generally more tolerant to aneuploidy than animals (Matzke et al. 2003), aneuploid plants exhibit a variety of phenotypic syndromes, including developmental delays, partial sterility, and alterations in plant architecture (Birchler and Veitia, 2007; Birchler *et al.*, 2001; Makarevitch *et al.*, 2008; Makarevitch and Harris 2010).

There is substantial variation in the plant ability to tolerate gene dosage imbalance caused by aneuploidy, both among different plant species and among varieties of the same species (Henry *et al.*, 2007; Henry *et al.*, 2005). In the present study, the level of aneuploid cells was very high, but most of the plants exhibit mixoploidy. Considering that the balance in gene dosage is essential for normal function, the absence of abnormal phenoptype for all the mixoploid plants observed in metal contaminated sites can be attributed to the compensation in genes expressions by euploid cells within each genotype. Studies of dosage regulations showed that there is an equal gene expression regardless of the copy number of the respective gene in aneuploids involving significant cytological length. This indicates that gene expression is often

dosage compensated *(Birchler et al.*, 2001; Cooper and Birchler, 2001). There is still only a limited understanding in the literature of the molecular mechanisms that lead to phenotypic alterations in aneuploid organisms as well as gene interactions involved in coping with gene dosage imbalance caused by aneuploidy on the global genomic level.

Previous analysis of conifer seedlings from trees growing in metal – contaminated sites in the targeted areas showed cytological stability of all the progenies that were analyzed (Dobrzeniecka *et al.*, 2011). This suggests that the cytological disturbance might not be as severe in tree genotypes as it is in grass such as *D. cespitosa*.

4.6 Conclusion

Cytological analysis showed clearly that long exposure of roots to high levels of metal lead to significant mitotic disruption. However, this overall cytological instability did not result in poor plant health or significant morphological changes compared to genotypes from reference sites. This suggests dosage compensation for gene expression in the mixoploid genotypes analyzed.

Table 6. Chromosome number, mixoploidy levels, and anaphase abnormalities in *D. cespitosa* plants from Northern Ontario.

Location	No. of chromosomes (range)	Mixoploid plants (%)	Cells with anaphase lagging chromosomes (%)
Wahnapitae Hill	19-26	100	28
Coniston Hydro	21-26	100	25
Low Water (Reference site)	18-26	17	0
Mississagi Lighthouse (Reference site)	18-26	17	0

Figure 12. Map of Northern Ontario showing *Deschampsia cespitosa* sampling sites (Sites 1 to 5: *Deschampsia cespitosa*)

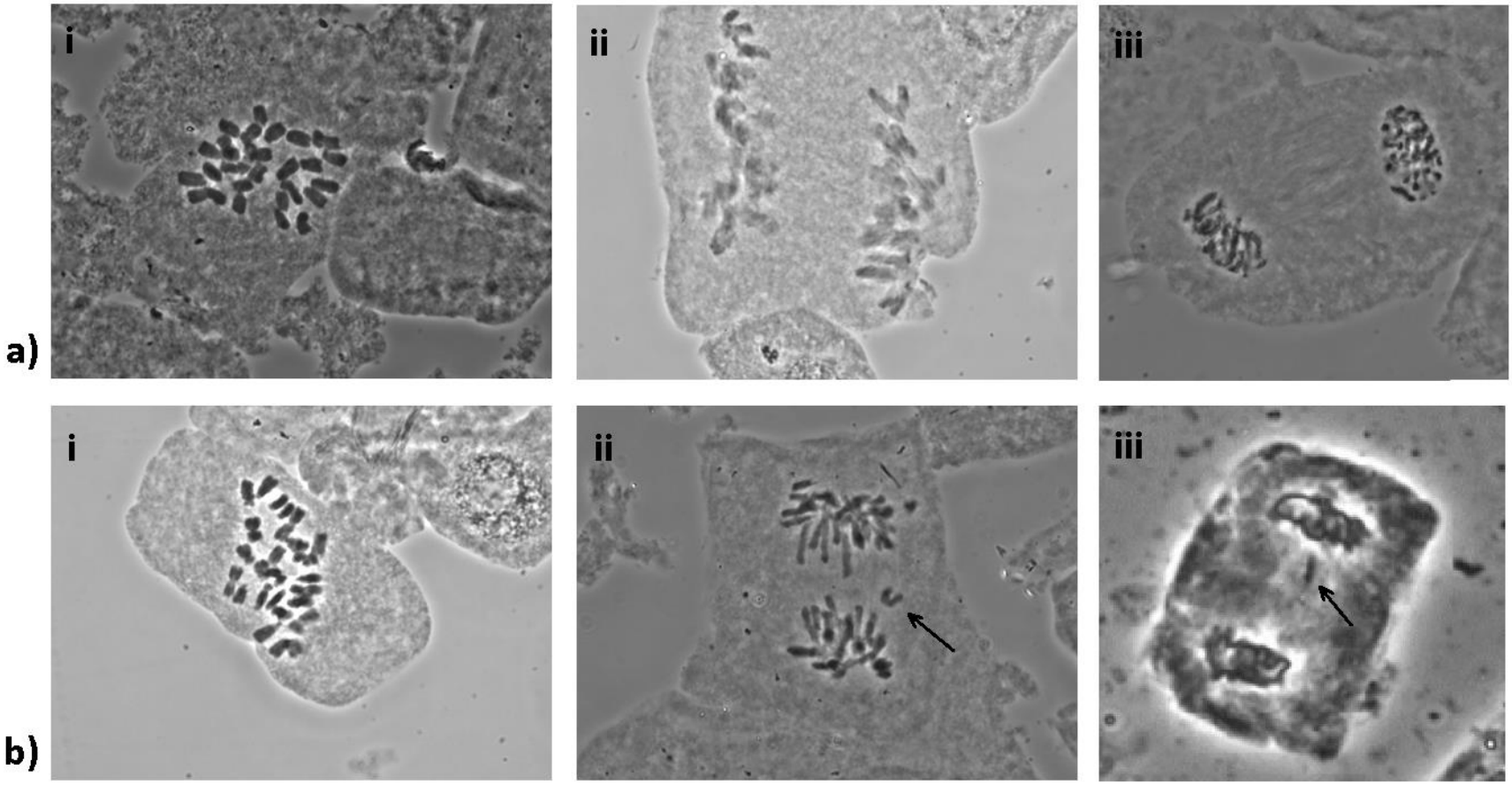

Figure 13. Major stages in the mitotic sequence in *Deschampsia cespitosa*.

a) All the photos show regular mitotic events (i-metaphase (n = 26; ii-anaphase; iii-telophase)**;** b) All the photos show irregular mitotic events {i-metaphase (n = 24); ii-anaphase with a lagging chromosome; iii-late telophase with a lagging chromosome}. Arrows indicate lagging chromosomes.

Chapter 5. General Discussion and Conclusions

Deschampsia cespitosa (Tufted hairgrass) has a very large growth range, while also being one of the few species that can grow on some of the most metal contaminated sites in Greater Sudbury and Cobalt. It has been shown to be a species that accumulates metals in its roots while also excluding those elements from translocating to its aerial tissues. However, neither the toxicity levels of the metals nor the specific resistance mechanism are known. The long-term effects of metal stress on the mitotic and meiotic behaviours, as well as genetic variation levels in *Deschampsia cespitosa* also require investigation. The main objectives of this study are to 1) reassess the level of genetic variation in *D. cespitosa* from three regions contaminated or uncontaminated with metals in Northern Ontario; 2) determine the degree of toxicity of nickel and copper on *D. cespitosa*; and 3) investigate cytological damages caused by metals in *D. cespitosa* growing in Northern Ontario.

As a result of mining-related activities, the Cobalt and Greater Sudbury regions have high total metal concentrations. *Deschampsia cespitosa* has remained a predominant species on sites in the affected areas, both before and throughout the remediation efforts. Due to the selective pressure of metal stress, it is expected that a loss of alleles would indirectly occur while selecting for metal-resistance traits. Monitoring genetic diversity over generations of the same population can give a reading on the gene pool health of a population, and can be used to assess the effect of not only the stressor, but also the impact of any remediation efforts in the area.

The current study utilized ISSR to reassess the genetic diversity of five Norther Ontario populations of *Deschampsia cespitosa*. The sites and the primers used on the 1999 samples were matched in this study of the 2015 populations. There was an insignificant change in the overall

percent of polymorphic loci in the metal – uncontaminated site of Little Current (from 70% in 1999 to 77% in 2015) and in a Cobalt Cart Lake site (from 48% in 1999 to 55% in 2015). However, a significant decrease in genetic variation was observed in the Coniston/Wahnapitae site (from 72% in 1999 to 54% in 2015), while an increase was observed in Cobalt Nipissing (from 46% in 1999 to 64% in 2015). The Kelly Lake site with the lowest level of polymorphic loci of 42.5% in 2015 was not surveyed in 1999.

The degree of genetic relatedness between sites has increased since the populations are more closely related than 16 years ago. The clustering of Cobalt and Sudbury populations strengthens the earlier theory that Sudbury populations of *D. cespitosa* might be from the Cobalt region.

The degree of toxicity of nickel and copper on *D. cespitosa* was investigated under controlled conditions, with the metal concentrations decided upon based off of previous soil analyses of the sites. In the assays, it was found that copper is more toxic than nickel. Neither copper nor nickel caused damage to plants while present at a normal bioavailable dosage. At higher dosages (1600 mg/kg and 1312 mg/kg) for nickel and copper respectively, there were significant differences between the plant response at those dosages and the control and bioavailable dosages. The highest doses (4800 mg/kg and 3936 mg/kg) caused severe damage due to the metal toxicity within 48 hours of treatment with nickel and copper respectively. A similar trend was found regarding growth rate.

Cytological analysis for *D. cespitosa* revealed significant mitotic disruption from long term exposure of roots to high levels of metal frequently manifested through aneuploidy in metal-contaminated sites when compared to the reference sites. All plants from contaminated

areas exhibited varying degrees of mixoploidy compared to 17% for the reference sites. The most frequent abnormalities observed in *D. cespitosa* plants was a change in chromosome number at metaphase followed by the presence of lagging chromosomes during anaphase. None of the plants from the metal contaminated sites were 100% euploid; they all exhibited varying levels of aneuploidy and only hypoploids ($n < 26$) cells were observed in all the mixoploid plants. It is expected that theoretically over a few generations, the mitotic abnormalities should result in meiotic instability that eventually affect the long term sustainability of these populations. However, since *D. cespitosa* is an outcrossing species and produces a large number of seeds every year, these characteristics should negate the long term effects of aneuploidy by maintaining a sufficient portion of euploid genotypes. These high levels of mitotic abnormalities did not seem to affect *D. cespitosa* survival and growth in highly metal-contaminated sites.

Further studies

Exposure to high metal concentrations of nickel and/or copper have been found to trigger the activation of specific gene response mechanisms in model species (Visioli *et al.*, 2014; Mari *et al.*, 2006; Kobayashi *et al.*, 2008; Keinanen *et al.*, 2007; Guo *et al.*, 2008).

A study on differential gene expression levels would give further insight on the coping mechanisms used by this species to deal with metals. In recent years, similar studies on the expression of Ni and Cu-related genes have been performed on white birch (Theriault *et al.*, 2016; Djeukam *et al.*, 2016), and red oak (Makela *et al.*, 2016). Transcriptome analysis can also help identify genes that are differentially expressed at different levels of nickel and copper.

References

Abedin J, Spiers G. (2006) Metal bioavailability in smelter-impacted land systems. In Proceedings, 31st Annual Meeting and Conference of the Canadian Land Reclamation Association, August 20–23, 2006, Ottawa, ON (pp. 1–17), 2006.

Adamowicz, M. (2014). Exploring Cobalt: The Historic Silver Capital of Canada, http://www.mindat.org/article.php/1398/Exploring+Cobalt+The+Historic+Silver+Capital+of+Canada

Alloway, B. J. "Heavy Metal in Soils," John Wiley & Sons, New York, NY, USA, 1990.

Amiro, B.D., and G.M. Courtin. (1981). Patterns of Vegetation in the Vicinity of an Industrially Disturbed Ecosystem Sudbury Ontario Canada. Can. J. Bot. 59:1623–1639. DOI:10.1139/b81-221.

Armstrong, J.S., Gibbs, A.J., Peakall, R. and Weiller, G. (1994). The RAPDistance package. Available via http://life.anu.edu.au/molecular.software/rapd.html.

Arnon, D. I. and Stout, P. R. (1939). The Essentiality of Certain Elements in Minute Quantity for Plants with Special Reference to copper. Plant Physiol. 14: 371-375. DOI:10.1104/pp.14.2.371.

Bert, V., Bonnin, I., Saumitou-Laprade, P., de Laguerie, P., & Petit, D. (2002). Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? New Phytologist, 155, 47-57.

Berta, M., Giovannelli, A., Camussi, A., & Racchi, M. L. (2010). Transcriptome changes in the cambial region of poplar (*Populus alba* L.) in response to water deficit. Plant Biology, 12, 341-354.

Bhalerao, SA, AS Sharma, and AC Poojari. (2015). Toxicity of Nickel in Plants. International Journal of Pure & Applied Biosience 3(2):345–55.

Birchler, J.A., & Veitia, R. A. (2007). The gene balance hypothesis: from classical genetics to modern genomics. Plant Cell, 19, 395-402.

Birchler, J.A., Bhadra, U., Bhadra, M. P., & Auger, D.L. (2001). Dosage-dependent gene regulation in multicellular eukaryotes: implications for dosage compensation, aneuploid syndromes, and quantitative traits. Developmental Biology, 234, 275-288.

Borghi, M., Tognetti, R., Monteforti, G., & Sebastiani, L. (2008). Responses of two poplar species (*Populus alba* and *Populus x canadensis*) to high copper concentrations. Evironmental and Experimental Biology, 62, 290-299.

Bornet, B., Muller, C., Paulus, F., Branchard, M. (2002). High informative nature of inter simple sequence repeat (ISSR) sequences amplified with tri- and tetra-nucleotide primers from cauliflower (*Brassica oleracea* var. botrytis L.) DNA. Genome 45:890–896.

Bradshaw A. D. (1984). Adaptation of plants to soils containing toxic metals - a test for conceit. 4-19.

Brilman L. A., Watkins E. (2003). Hairgrasses *(Deschampsia spp.*). In Casler, Duncan (Eds.), Turfgrass biology, genetics and breeding. John Wiley & Sons, Hoboken, New Jersey, 225-231.

Carter M. R. (1993). Soil sampling and methods of analysis. Lewis, Boca Raton, FL.

Chang, S., Puryear, J., Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116.

Charles Dumaresq. (2009). "Cobalt Mining Legacy" http://www.cobaltmininglegacy.ca/index.php

Chibuike, G. U. and Obiora, S. C. (2014). Heavy Metal Polluted Soils: Effect on Plants and Bioremediation Methods. Applied and Environmental Soil Science, vol. 2014, Article ID 752708, 12 pages.

Cooper, J. L., & Birchler, J. A. (2001). Developmental impact on trans-acting dosage effects in maize aneuploids. Genesis 31, 64-71.

Costa, R., G. Pereira., I. Garrido, M. M. Tavaresde-Sousa and F. Espinosa (2016). Comparison of RAPD, ISSR and AFLP molecular markers to reveal and classify orchardgrass (*Dactylis glomerata* L.) germplasm variations. PloS one, 11: 1-15.

Courtin GM. (1994). The last 150 years: a history of environment degradation in Sudbury. Sci Total Environ 1994;148:99-102.

Cox, R. M., & Hutchinson, T. C. (1980). Multiple metal tolerances in the grass *Deschampsia cespitosa* (L.) Beauv. from the Sudbury smelting area. New Phytologist, 84, 631-647.

Deng, J., B. Liao, M. Ye, D. Deng, C. Lan and W. Shu, (2007). The effects of heavy metal pollution on the genetic diversity in zinc/cadmium hyperaccumulator *Sedum alfredii* populations. Plant Soil. 297: 83-92.

DeWitt, Thomas J., Andrew Sih, and David Sloan Wilson. (1998). Costs and limits of phenotypic plasticity. Trends in ecology & evolution 13.2: 77-81.

Di Baccio, D., Kopriva, S., Sebastiani, L., & Rennenberg, H. (2005). Does glutathione metabolism have a role in the defence of poplar against zinc excess? New Phytologist, 167, 73-80.

Di Baccio, D., Galla, G., Bracci, T., Andreucci, A., Barcaccia, G., Tognetti R., Sebastiani, L., & Moshelion, M. (2011). Transcriptome analyses of *Populus* × *euramericana* clone I-214 leaves exposed to excess zinc. Tree Physiology, 31:1293-1308.

Dierssen, M., Herault, Y., & Estivill, X. (2009). Aneuploidy: from a physiological mechanism of variance to Down syndrome. Physiol Rev 89:887-920.

Djeukam, C. L., Therialt, G., Michael, P., Nkongolo, K. K. (2016). Analysis of Gene Expression Associated with Copper Toxicity in White Birch (*Betula papyrifera*) Populations from a Mining Region. British Biotechnology Journal, 15(3): 1-10, 2016, Article no.BBJ.28425.

Dobrzeniecka, S., Nkongolo, K. K., Michael, P., Mehes-Smith, M., & Beckett, P. (2011). Genetic Analysis of Black Spruce (*Picea mariana*) Populations from Dry and Wet Areas of a Metal-Contaminated Region in Ontario (Canada). Water Air Soil Poll 215:117-125.

Doyle, J. J. and J. L. Doyle. (1990). Isolation of plant DNA from fresh tissue. Focus, 12: 13-15.

Dudka, S., Ponce-Hernandez, R. and Hutchinson, T.C. (1995). Current levels of total element concentrations in the surface layers of Sudbury's soils. Sci. Total Environ. 162: 161-171.

Eskew, D.L., Welch, R.M. and Norvell, W.A. (1984). Nickel in higher plants: further evidence for an essential role. Plant Physiol. 76: 691-693.

Fischerova, Z., Tlustos, P., Szakova, J., & Sichorova, K. (2006). A comparision of phytoremediation capability of selected plant species for given trace elements. Environmental Pollut 144:93-100.

Foy, C. D., Chaney, R. L., & White, M. C. (1978). The Physiology of Metal Toxicity in Plants. Ann Rev Plant Physio 29:511-566.

Freedman B, Hutchison TC (1980). Pollutants inputs from atmosphere and accumulations in soils and vegetation near a nickel-copper smelter at Sudbury, Ontario, Canada. Can J Bot 58(1):108–132.

Friedlová, M. (2010). The influence of heavy metals on soil biological and chemical properties. Soil and Water Research, vol. 5, no. 1, pp. 21–27.

Gavlak, R. G., Horneck, D. A., & Miller, R.O. (2005). Plant, soil and water reference methods for the Western Region. Western Rural Development Center (WREP), Corvallis, Oregon USA. pp. 198.

Gervais, S. and K. Nkongolo, (2011). Effect of Metal Contamination on the Genetic Diversity of *Deschampsia cespitosa* Populations from Northern Ontario: An Application of ISSR and Microsatellite Markers. In: Relevant Perspectives in Global Environmental Change, Agboola J. (Ed.), pp: 115-138.

Godwin, I.D., Aitken, E.A.B. and Smith, L.W. (1997). Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis, 18: 1524-1528. doi:10.1002/elps.1150180906.

Gratton, W.S., Nkongolo, K.K., and Spiers, G.A. (2000) Heavy metal accumulation in soil and Jack Pine (*Pinus banksiana*) needles in Sudbury, Ontario, Canada. Bull. Environ. Contam. Toxicol. 64: 550-557.

Gunn LM, Keller W, Negusanti L, Potvin R, Beckett P, Winterhalder K. (1995). Ecosystem recovery after emission reductions: Sudbury, Canada. Water Air Soil Pollut 85:1783-1788.

Guo WJ, Meetam M, Goldsbrough PB. (2008). Examining the specific contributions of individual arabidopsis metallothioneins to copper distribution and metal tolerance. Plant Physiol. 146:1697-1706.

Hall, J.L., & Williams, L.E. (2003). Transition metal transporters in plants. J Exp Bot 54:2601-2613.

Henry, I. M., Dilkes, B.P., & Comai, L. (2007). Genetic basis for dosage sensitivity in *Arabidopsis thaliana*. PLoS Genetics 3:e70.

Henry, I. M., Dilkes, B. P., Young, K., Watson, B., Wu, H., & Comai, L. (2005). Aneuploidy and genetic variation in the *Arabidopsis thaliana* triploid response. Genetics 170:1979-1988.

Holloway, G.J., Povey, S.R., and Sibly, R.M. (1990). The effect of new environment on adapted genetic architecture. Heredity 64: 323-330.

Hutchinson, TC and LM Whitby. (1972). Study of Airborne Contamination of Vegetation and Soils by Heavy Metals from the Sudbury, Ontario, Copper-Nickel Smelters. Trace Substances in Environmental Health 7:1–18.

Hutchinson, T.C. and Whitby, L.M. (1974). Heavy – metal pollution in the Sudbury mining and smelting region of Canada, I. Soil and vegetation contamination by nickel, copper, and other metals. Environ. Conserv. 1 (2): 123 – 132.

Jaccard P (1908). Nouvelle recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44: 223–270.

Jeffreys, A. J. (1987). Highly variable minisatellites and DNA fingerprints. Biochemical Society transactions 15 (3): 309–17. PMID 2887471.

Kalubi KN, Mehes-Smith M, Narendrula R, Michael P, Omri A. (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(3):636-47.

Keinänen SI, Hassinen VH, Kärenlampi SO, Tervahauta AI. (2007). Isolation of genes upregulated by copper in a copper-tolerant birch (*Betula pendula*) clone. Tree Physiol. 27(9):1243–52.

Kerkeb, L., & Krämer, U. (2003). The role of free histidine in xylem loading of nickel in *Alyssum lesbiacum* and *Brassica juncea*. Plant Physiol 131:716-724.

Kobayashi Y, Kuroda K, Kimura K, Southron-Francis JL, Furuzawa A, Kimura K, *et al.* (2008). Amino acid polymorphisms in strictly conserved domains of a P-type ATPase HMA5 are involved in the mechanism of copper tolerance variation in arabidopsis. Plant Physiol. 148(2): 969-80.

Kohler, A., Blaudez, D., Chalot, M., & Martin, F. (2004). Cloning and expression of multiple metallothioneins from hybrid poplar. New Phytologist, 164:83-93.

Kopittke, P., Blamey, F., Asher, C., & Menzies, N. (2010). Trace metal phytotoxicity in solution culture: a review. J Exp Bot 61:945-954.

Krämer, U., Talke, I. N., & Hanikenne, M. (2007). Transition metal transport. FEBS letters 581:2263-2272.

Lautenbach W, Miller J, Beckett P, Negusanti J, Winterhalder K (1995). Municipal land restoration program: the regreening process. In: Gunn J (ed) Restoration and recovery of an industrial region. Springer, New York, pp 109–122.

Leavitt, S. W., Dueser, R. D., & Goodell, H. G. (1979). Plant Regulation of Essential and Non-Essential Heavy Metals. J Appl Ecol 16:203-212.

Lin, Y.C. and Kao, C.H. (2005). Nickel toxicity of rice seedlings: Cell wall peroxidase, lignin, and NiSO4-inhibited root growth. Crop, Environment Bioinformatics, vol. 2, pp. 131–136.

Lonardo, S., Capuana, M., Arnetoli, M., Gabbrielli, R., & Gonnelli, C. (2011). Exploring the metal phytoremediation potential of three *Populus alba* L. clones using an *in vitro* screening. Environ Sci Pollut Res 18:82-90.

Lopes, I., Baird, D.J., and Ribeiro, R. (2004). Genetic determination of tolerance to lethal and sublethal copper concentrations in field populations of *Daphnia longispina*. Arch. Environ. Contam. Toxicol. 46: 43-51.

MacNair, M.R. (1993). The genetics of metal tolerance in vascular plants. New Phytol., 124: 541-559. DOI: 10.1111/j.1469-8137.1993.tb03846.x.

Makarevitch, I., & Harris, C. (2010). Aneuploidy causes tissue-specific qualitative changes in global gene expression patterns in maize. Plant Physiol 152:927-938.

Makarevitch, I., Phillips, R. L., & Springer, N. M. (2008). Profiling expression changes caused by segmental aneuploidy in maize. BMC Genomics 9:7.

Makela, M., *et al*. (2016). High genetic variation among closely related red oak (*Quercus rubra*) populations in an ecosystem under metal stress: analysis of gene regulation. Genes & Genomics 38.10: 967-976.

Mari S, Gendre D, Pianelli K, Ouerdane L, Lobinski R, Briat JF, Lebun M, Czernic P (2006). Root-to-shoot long-distance circulation of nicotianamine and nicotianamine–nickel chelates in the metal hyperaccumulator *Thlaspi caerulescens.* J Exp Bot 57:4111–4122

Matzke, M. A., Mette, M. F., Kanno, T., & Matzke, A. J. (2003). Does the intrinsic instability of aneuploid genomes have a causal role in cancer? Trends Genet 19:253-256.

Mejnartowicz, L. (1983). Changes in genetic structure of Scotch pine (*Pinus sylvestris L*.) population affected by industrial emission of fluoride and sulphur dioxide. Genet. Polonica 24: 41-50.

Mehes-Smith, M., Nkongolo, K. K., & Kim, N. S. (2011). A comparative cytogenetic analysis of five pine species from North America, *Pinus banksiana*, *P. contorta*, *P. monticola*, *P. resinosa* and *P. strobus*. Plant Syst Evol 292:153-164.

Mehes MS, Nkongolo KK, Michael P (2007). Genetic analysis of Pinus strobus and Pinus monticola populations from Canada using ISSR and RAPD markers: development of genomespecific SCAR markers. Plant Syst Evol 267(1–4):47–63.

Mehes-Smith M, Nkongolo KK, Narendrula R, Cholewa E (2013). Mobility of heavy metals in plants and soil: a case study from a mining region in Canada. Am J Environ Sci 9(6):483–493.

Mehes-Smith, M. and KK Nkongolo. (2015). "Physiological and Cytological Responses of *Deschampsia Cespitosa* and *Populus Tremuloides* to Soil Metal Contamination." Water, Air, & Soil Pollution 226(4):125.

Mengoni, A., Barabesi, C, Gonnelli, C., Galardi, F., Gabbrielli, R., and Bazzicalupo, M. (2001). Genetic diversity of heavy metal-tolerant populations in *Silene paradoxa* (Caryophyllaceae): a chloroplast microsatellite analysis. Mol. Ecology 10: 1909-1916.

Meudt HM, Clarke AC (March 2007). Almost forgotten or latest practice? AFLP applications, analyses and advances. Trends Plant Sci. 12 (3): 106–17. doi:10.1016/j.tplants.2007.02.001. PMID 17303467.

Nagaoka T. and Ogihara, Y. (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Appl. Genet. 94: 597-602.

Nei, M. (1973). Analysis of Gene Diversity in Subdivided Populations. Proceedings of the 7 3 National Academy of Sciences of the United States of America 70(12):3321–23.

Nei M, Maruyama T, Chakraborty R (1975). The bottleneck effect and genetic variability in populations. Evolution 29:1–10.

Nei M (1978). Estimation of average heterozigosity and genetic distance from a small number of individuals. Genetics, 89:583–590. pmid:17248844.

Nishida, S., Tsuzuki, C., Kato, A., Aisu, A., Yoshida, J., & Mizuno, T. (2011). AtIRT1, the primary iron uptake transporter in the root, mediates excess nickel accumulation in *Arabidopsis thaliana*. Plant Cell, Physiol 52:1433-1442.

Nkongolo, K. K., & Klimaszewska, K. (1995). Cytological and molecular characterization of *Larix decidua*, *L. leptolepis* and *L. eurolepis*: Identification of species specific chromosomes and enhancement of mitotic index. Theor Appl Genet 90:827-834.

Nkongolo, K. K., Deck, A., & Michael, P. (2001). Molecular and cytological analyses of *Deschampsia cespitosa* populations from Northern Ontario (Canada). Genome 44:818-825.

Nkongolo, K.K., Michael, P., and Demers, T. (2005). Application of ISSR, RAPD, and cytological markers to the certification of *Picea mariana, P. glauca,* and *P. engelmannii* trees, and their putative hybrids. Genome 48: 302-311.

Nkongolo, K. K., Vaillancourt, A., Dobrzeniecka, S., Mehes, M., & Beckett, P. (2008). Metal content in soil and black spruce (*Picea mariana*) trees in the Sudbury region (Ontario, Canada): Low concentration of nickel, cadmium and arsenic detected within smelter vicinity. Bull Environ Contam Toxicol 80:107-111.

Nkongolo, K. K., Spiers, G., Beckett, P., Narendrula, R., Theriault, G., Tran, A., & Kalubi, K. (2013). Long-term effects of liming on soil chemistry in stable and eroded upland areas in a mining region. Water Air Soil Poll 224:1-14.

Nkongolo KK, Gervais S, Michael P, Zhou Y (2014). Comparative analysis of inter simple sequence repeats and simple sequence repeats markers: genetic analysis of *Deschampsia cespitosa* populations growing in metal contaminated regions in Canada. Am J Biochem Biotechnol 10:69–80.

Nriagu JO, Wong HKT, Coker RD. (1982). Deposition and chemistry of pollutant metals in lakes around the smelters at Sudbury, Ontario. Environ Sci Technol 16:551-560.

Paschke, M. W., & Redente, E. F. (2002). Copper toxicity thresholds for important restoration grass species of the western United States. Environ Toxicol Chem 21:2692-2697.

Patel, Kalpesh M., and Kailash P. Patel. (2010). Cytological changes in *Trigonella foenum-graecum* (L.) under the zinc stress, Industrial Pollution Control 26 (2), 231-234.

Percival J., Kwong Y.T.J., Dumaresq C. and Michel F. (2007). Distribution of As, Ni and Co in Tailings and Surface Waters in the Cobalt Area, Ontario. Paper was presented at Mining and the Environment IV Conference, Sudbury, Ontario, Canada, October 19-27.

Porter, E. K., & Peterson, P. L. (1977). Arsenic tolerance in grasses growing on mine waste. Environmental Pollution, 14:255-265.

Prager, Ellen M., and Allan C. Wilson. (1971). The dependence of immunological cross-reactivity upon sequence resemblance among lysozymes I. Micro-complement fixation studies. Journal of Biological Chemistry 246.19: 5978-5989.

Prager, Ellen M., and Allan C. Wilson. (1971). The Dependence of Immunological Cross-Reactivity upon Sequence Resemblance among Lysozymes II. Comparison of Precipitin and Micro-complement fixation results." Journal of Biological Chemistry 246.22: 7010-7017.

Prohaska JR. (2011). Impact of copper limitation on expression and function of multicopper oxidases (ferroxidases). Adv Nutr. 2(2):89-95.

Pulford, I. D., & Watson, C. (2003). Phytoremediation of heavy metal-contaminated land by trees—a review. Environment International, 29:529-540.

Qiu, Q., Ma, T., Hu, Q., Liu, B., Wu, Y., Zhou, H., Wang, Q., Wang, J., & Liu, J. (2011). Genome-scale transcriptome analysis of the desert poplar, *Populus euphratica*. Tree Physiology, 31, 452-461.

Rai, H. S., Mock, K. E., Richardson, B. A., Cronn, R. C., Hayden, K. J., Wright, J. W., Knaus, B. J., & Wolf, P. G. (2013). Transcriptome characterization and detection of gene expression differences in aspen (*Populus tremuloides*). Tree Genetics & Genomes, 9, 1031-1041.

Rascio, N., & Navari-Izzo, F. (2011). Heavy Metal hyperaccumulating plants: How and why they do it? And what makes them so interesting? Plant Science, 180, 169-180.

Reeves, R. D., & Baker A. J. M. (2000). Metal-accumulating plants. In Raskin, & Ensley (Eds.), Phytoremediation of toxic metals: Using plants to clean up the environment. John Wiley & Sons, Inc., New York, 193-229.

Roosens, N. H. C. J., Willems, G., & Saumitou-Laprade, P. (2008). Using Arabidopsis to explore zinc tolerance and hyperaccumulation. Trends Plant Science, 13, 208-215.

Roy S., Banerjee A., Mawkhlieng B., Misra A.K., Pattanayak A., Harish G.D., Singh S.K., (...), Bansal K.C. (2015). Genetic diversity and population structure in aromatic and quality rice (Oryza sativa L.) landraces from North-eastern India. PLoS ONE, 10 (6), art. no.e0129607.

Salt, D. E., Smith, R. D., & Raskin, I. (1998). Phytoremediation. Annual Review of Plant Physiology and Plant Molecular Biology, 49, 643-668.

Seregin, I. V., & Kozhevnikova, A. D. (2006). Physiological role of nickel and its toxic effects on higher plants. Russian Journal of Plant Physiology, *53*, 257-277.

Service, P.M. and Rose, M.R. (1985). Genetic covariance among life-history components: the effect of novel environments. Evolution 39: 943-945.

Shabala, Sergey. Plant stress physiology. 2nd edition. Wallingford, Oxfordshire: CABI, 2017. Print.

Shannon, C. E. (1949). Communication theory of secrecy systems. Bell System Technical Journal, 28: 656-715.

Singh, R., Singh, D., Kumar, N., Bhargava, S., & Barman, S. (2010). Accumulation and translocation of heavy metals in soil and plants from fly ash contamianted area. Journal of Environmental Biology, 31, 421-430.

Solanki, R., & Dhankhar, R. (2011). Biochemical changes and adaptive strategies of plants under heavy metal stress. Biologia, 66, 195-204.

Spiers, G. A., Wren, C. D., & McLaughlin, D. (2012). Risk assessment and environmental management: A case study in Sudbury, Ontario, Canada. In C. D. Wren (Ed.), Distribution of chemicals of concern. The Netherlands: Maralte.

St. John, L., D.G. Ogle, D. Darris, S. Parr. (2011). Plant Guide for Tufted Hairgrass (*Deschampsia caespitosa*). USDA-Natural Resources Conservation Service, Aberdeen, ID Plant Materials Center. 83210-0296.

Summers, PW and DM Whelpdale. (1976). Acid Precipitation in Canada. Water, Air, and Soil Pollution 6(2-4):447–55.

Tangahu BV, Abdullah SR, Basri H, Idri M, Anuar N, Mukhlisin M. (2013). A review on heavy metals (AS, Pb, and Hg) uptake by plants through phytoremediation. International Journal of Chemical Engineering. Volume 2011. 31 pages. Hindawi Publishing Corporation. DOI: 10.1155/2011/939161.

Theriault, Gabriel, and Kabwe Nkongolo. (2016). Nickel and Copper Toxicity and Plant Response Mechanisms in White Birch (*Betula papyrifera*). Bulletin of environmental contamination and toxicology 97.2: 171-176.

Unterbrunner, R., Puschenreiter, M., Sommer, P., Wieshammer, G., Tlustoš, P., Zupan, M., & Wenzel, W. W. (2007). Heavy metal accumulation in trees growing on contaminated sites in Central Europe. Environmental Pollution, 148, 107-114.

USDA, NRCS. (2017). The PLANTS Database (http://plants.usda.gov, 2017). National Plant Data Team, Greensboro, NC 27401-4901 USA.

Vaidyanathan VG, Asthana Y, Nair BU. (2013). Importance of ligand structure in DNA/protein binding, mutagenicity, excision repair and nutritional aspects of chromium(III) complexes. Dalton Trans. 42(7):2337-2346.

Van Straalen, N.M., and Timmermans, M.J.T.N. (2002). Genetic variation in toxicant-stressed populations: An evaluation of the "genetic erosion" hypothesis. Human Ecol. Risk Assess 8: 983-1002.

Visioli G, Vincenzi S, Marmiroli M, Marmiroli N (2012). Correlation between phenotype and proteome in the Ni hyperaccumulator *Noccaea caerulescens* subsp. caerulescens. Environ Expl Bot 77:156–164.

Von Frenckell-Insam, B.A.K. and Hutchinson, T.C. (1993). Occurance of heavy metal tolerance and co-tolerance in *Deschampsia cespitosa* (L.) Beauv. from European and Canadian populations. New Phytology, 125: 555 – 564.

Walsh, Roberta A. 1995. *Deschampsia cespitosa*. In: Fire Effects Information System, [Online]. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory (Producer). Available: http://www.fs.fed.us/database/feis/ [2017].

Wei, S., Zhou, Q., & Wang, X. (2005). Identification of weed plants excluding the uptake of heavy metals. Environment International, 31, 829-834.

Willems, G., Dräger, D. B., Courbot, M., Godé, C., Verbruggen, N., & Saumitou-Laprade, P. (2007). The Genetic Basis of Zinc Tolerance in the Metallophyte *Arabidopsis halleri* ssp. *halleri* (Brassicaceae): An Analysis of Quantitative Trait Loci. Genetics, 176, 659-674.

Williams, C. G., Elsik, C.G., & Barnes, R. D. (2000). Microsatellite analysis of *Pinus taeda* L. in Zimbabwe. Heredity, 84, 261-268.

Winterhalder, K. (1995). Early history of human activities in the Sudbury area and ecological damage to the landscape. In Restoration and recovery of an industrial region . In J.M. Gunn (ed.), pp 17 -32. Springer Verlag. New York.

Winterhalder K (1996). Environmental degradation and rehabilitation of the landscape around Sudbury, a major mining and smelting area. Environ Rev 4(3):185–224.

Wu, L. Brashaw, A.D., and Thurman, D.A. (1975). The potential for evolution of heavy metal tolerance in plants: III. The rapid evolution of copper tolerance in *Agrostis stolonifera*. Heredity 34: 165-187.

Yadav, S.K., (2010). Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S. Afr. J. Bot. 76, 167–179.

Yeh, FC *et al.* (1997). Population Genetic Analysis of Co-Dominant and Dominant Markers and Quantitative Traits. Belgian Journal of Botany (129):157.

Yeh FC, Young RC, Boyle T (1997). Microsoft Window-based freeware for population genetic analysis (POPGENE, ver. 1.31).ftp://ftp.microsoft.com/softlib/mslfiles/hpgl.exe, 1999.

Zabeau, M and P. Vos. (1993). Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office, publication 0 534 858 A1, bulletin 93/13.

Zietkiewicz, E., Rafalski, A., and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics, 20: 176–183.

Zha, H., Jiang, R., Zhao, F., Vooijs, R., Schat, H., Barker, J., & McGrath, S. (2004). Co-segregation analysis of cadmium and zinc accumulation in *Thlaspi caerulescens* interecotypic crosses. New Phytologist, 163, 299-312.

Appendices

Appendix 1. *Deschampsia cespitosa*

Appendix 2. Cobalt 3 (Cart Lake Tailings) sampling site July 2015.

Appendix 3. Cobalt 1 (Nipissing Tailings) sampling site July 2015.

a) 

b) 

Appendix 4. a) Roast beds of wood with ore piled on top, b) set aflame to smoulder for months.

Appendix 5. Copper Cliff smelter