BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF MICROBIAL COMMUNITIES FROM A METAL CONTAMINATED AND RECLAIMED REGION

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ABSTRACT

Metal contamination in the Greater Sudbury Region (GSR) resulted in severe environmental degradation. Soil liming and tree planting have been the main approaches to restoring the damaged ecosystem. The specific objective of the present study was to assess the effects of soil metal contamination and liming on 1) microbial biomass and abundance, 2) bacterial and fungal diversity, and 3) enzymatic activities and soil respiration. Phospholipid fatty acid (PLFA) analysis and 454 pyrosequencing were used to address these research objectives. Total biomasses for bacteria, arbuscular fungi (AM fungi), other fungi and eukaryotes were significantly lower in metal contaminated compared to uncontaminated reference areas. Analysis of bacterial communities revealed Chao1 index values of 232 and 273 for metal contaminated and reference soils, respectively. For fungi, the Chao index values were 23 for metal contaminated and 45 for reference sites. There was a significant increase of total microbial biomass in limed sites (342.15 ng/g) compared to unlimed areas (149.89 ng/g). Chao1 estimates followed the same trend. But the total number of OTUs (Operational Taxonomic Units) in limed (463 OTUs) and unlimed (473 OTUs) soil samples for bacteria were similar. For fungi, OTUs were 96 and 81 for limed and unlimed soil samples, respectively. Bacterial and fungal groups that were specific to either limed or unlimed sites were identified. *Bradyrhizobiaceae* family with 12 genera including the nitrogen fixing *Bradirhizobium* genus was more abundant in limed sites compared to unlimed areas. For fungi, *Ascomycota* was the most predominant phylum in unlimed soils (46.00%) while *Basidiomycota* phylum represented 85.74% of all fungi in the limed areas. Detailed analysis of the data showed that although soil liming increases significantly the amount of microbial biomass, the level of species diversity remained statistically unchanged. Soil respiration rates were higher in limed soils (65 ppm) compared to unlimed soils (35 ppm). They

were significantly lower in metal contaminated sites (55 ppm) compared to reference sites (90 ppm). β-glucosidase (BG), cellobiohydrolase (CBH), β-N-acetylglucosaminidase (NAGase), aryl sulfatase (AS), acid phosphatase (AP), alkaline phosphatase (AlP), glycine aminopeptidase (GAP), and leucine aminopeptidase (LAP) activites were significantly higher in limed compared to unlimed sites. Metal contamination significantly reduced the activities of these enzymes with the exception of LAP. An opposite trend was observed for peroxidase (PER) enzyme activity that was higher in unlimed and metal contaminated sites compared to limed and reference areas.

Keywords: Metals, Soil pH, CEC, PLFA Analysis, Pyrosequencing, Bacterial and Fungal Community, Soil Microbial Diversity and Abundance, Soil Respiration, Microbial Activity.

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CHAPTER 1: LITERATURE REVIEW

1.1 Soil and Ecosystem

Ecosystem serves as the level of biological organization where organisms interact with each other and with their environment [1]. It includes living organisms, dead organic matters and the abiotic environment within which the organisms live and exchange components such as atmosphere, water, and soil [1,2]. An important factor influencing the productivity of various ecosystems is the nature of their soil which is the foundation of entire biosphere. It plays a major role in the development of earth system and it continues to support the needs of contemporary societies [2,3].

Healthy soils function in regulating water flow, cycling nutrients, transforming organic and inorganic materials [4]. They are able to balance a range of functions to meet the needs of both community and agriculture industry [4,5]. This implies that the interactions among soil's internal components are optimal and that soil interactions with its external environment and the production system are sustainable [5]. There is a strong interaction among soil biological activities, organic matter content, pH, and water availability [2,5].

Soils provide an immense array of habitats that contain a vast and still largely unknown biodiversity [2,3]. Several studies on soil have been conducted to understand the distribution, the nature, and specific characteristics of minerals and organic components [2,3,6]. Ecologists and agronomists have detailed how soils provide nutrients and substrates for plant growth [2,3]. However, analysis of how soil functions as a habitat and the way organisms create and live in their habitats has been understudied.

1.1.1 Soil Horizons

Soil scientists have described the vertical gradation of soil horizons using a classification system [2,3,7]. A soil horizon is a layer of mineral or organic material parallel to the soil crust whose characteristics differ from the layers above and beneath [3,7]. It differs from other horizons in properties such as color, structure, texture and consistence, as well as in chemical, biological, and mineralogical composition [2,3,7]. Layers are assigned distinctive alphabetic symbols as a form of shorthand for their characteristic and they are split into two distinct groups: 1) organic horizons – those that contain 17% or more organic carbon (C) and, 2) mineral horizons – with less than 17% organic C (about 30% less organic matter) by weight (Table 1) [2,7].

The major mineral horizons are A, B, and C whereas, O, L, F, and H are the major organic horizons, that are mainly forest litter at various stages of decomposition (Table 1) [2,7]. Plant roots and microorganisms often extend below B horizon in humid regions leading to chemical changes in soil water, biochemical weathering of the regolith (layer of loose, dirt, dust and rocks sitting on top of bedrock), and forming C horizon [2,7]. Horizons are also further divided into subdivisions which are labeled by adding lower case suffixes to some of the major horizon symbols such as Ae or Ah (Table 1) [7]. Other layers are either non-soil layers such as rock and water or layers of unconsolidated material considered to be unaffected by soil forming processes. They are labeled as R, W and IIC, respectively [2,7].

The organically enriched horizon A at the soil surface where most of microorganisms and plant roots are found is also referred to as topsoil [2]. This is a zone that can be altered by enhancing the supply of nutrients, air and water by mixing organic and inorganic materials,

Table 1: Basic description of mineral and organic soil horizons of soil classification.

Soil Horizons	Suffix	Description
Mineral Horizon		
A		Layer nearest to the soil surface dominated by mineral particles
	Ah	Enriched with soil organic matter (SOM)
	Ae	Characterized by the eluviations of clay, Fe (iron), Al (aluminum), SOM
B		Formed by the accumulation of material removed from Ae horizon or by alteration of the parent material
	Bh	Accumulation of SOM
	Bf	Accumulation of Fe and/or Al
	Bn	Strong soil structure and significant amounts of exchangeable Na (sodium)
C		Horizon comparatively unaffected by the pedogenic processes (least weathered part of the soil profile)
	Cca	Accumulation of Ca (calcium) and Mg (magnesium) carbonates
	Cs	Accumulation of soluble salts
	Ck	Original Ca and Mg carbonates
Organic Horizon		
O		Organic horizon derived mainly from wetland vegetation
	Of	Fibrous materials of readily recognizable origin
	Om	Organic material in an intermediate stage of decomposition
	Oh	Highly decomposed organic material
L, F, H		Organic materials that occur from the accumulation of leaves, twigs and woody materials which overlies a mineral soil
	L	Leaf litter
	F	Partially decomposed leaf and twig materials
	H	Decomposed organic materials

loosening the structure, and applying irrigation [2,3]. Subsoils are layers that underlie topsoil. They supply nutrients and much of the water needed by plants [2,3]. Compared to subsoil, topsoil has properties that are far more beneficial for plant growth. Hence productivity is often correlated with the thickness of topsoil [2,3,7]. Studies have shown that subsoil layers that are too dense, acidic or wet can impede root growth [2]. Many of the chemical, biological, and physical processes that take place in the upper soils layers are also observed to some extent in the C horizon [2].

The relative proportions of air, water and inorganic matter greatly influence soil behavior and productivity [2,6]. In addition, soil texture, content, and structure also play an important role in soil fertility [2,3,6].

1.1.2 Soil Organic Matter (SOM) and Rhizosphere

Organic matter consists of vast arrays of carbon compounds in soils that play a variety of roles in nutrient, water and biological cycles. SOMs provide nutrients and habitat to organisms and they bind soil particles into aggregates thereby improving the water holding capacity [2,6]. They include living organisms, carbonaceous remains of organisms as well as organic compounds produced by current and past metabolism in the soil [2,6]. They affect chemical and physical properties and the overall soil health [2,6]. SOM composition and breakdown rate greatly affect: 1) soil structure and porosity, 2) water infiltration rate and moisture holding capacity, 3) diversity and biological activities of soil organisms, and 4) plant nutrient availability [2,6].

The importance of soil structure as a component of soil fertility is evident as documented by several reports. For example, plant roots must spread to access nutrients and they only thrive if

there is an adequate supply of air and water [2,3,6]. Further, soil microorganisms produce organic matters and growth stimulating compounds which play a major role in plant growth and development [2,3,6]. Deterioration of soil affects vegetation as well as soil microorganisms which have a significant influence on soil structure [2,3,6]. Organic matter is a major source of plant nutrients: C, nitrogen (N), phosphorous (P), and sulfur (S) [2,6]. These elements are released as soluble ions and they are taken up by plant roots when SOM decays. In addition, SOM including plant and animal residues is the main food that provides C and energy to soil organisms [2,3,6]. Overall, SOM formation, decomposition and transformation are of great importance for microorganisms, sustainable soil fertility, soil structure, and plant health [2,3,6].

Soil pollution is detrimental to soil health and its function. Rhizosphere is the area that is affected by soil contaminants [2,3,8]. It represents a narrow region of soil that is directly influenced by plant root secretions and associated soil microorganisms [2,3,8]. Enhanced microbial abundance and metabolic activities have been reported in the proximity of plant roots [2,8,9]. Root exudation patterns and associated microbial communities greatly depend on plant species, their developmental stage, and nutritional status [2,8,9]. Different factors including soil type, climate change, soil contamination, pathogen exposure, and agriculture practices significantly affect microbial community composition and its diversity in the rhizosphere [2,3,8,9]. Microorganisms in rhizosphere, in turn, exert strong effects on plant growth, development, and health by nutrient solubilization, N fixation and production of plant hormones [2,3,8,9]. Overall, both bacterial and fungal communities are influenced by plants and vice versa [2,3,8,9].

Soil electrochemical properties are also fundamentally important in understanding physio-chemical phenomena affecting nutrient availability for plants and microorganisms [6,10]. Defined as the capacity of soil to supply plants with essential nutrients, soil fertility is a very complex conservation and management concept and it is closely linked to nutrient bioavailability [6,10]. This refers to the availability of an element in a chemical form that plant is able to absorb rapidly [2,10]. It is affected by soil characteristics such as density of soil layers, nutrient diffusion, and root oxygenation [2,6,10]. In addition, electrochemical properties directly influence the behavior of soil elements [2,6,10]. Cation exchange capacity (CEC) is a soil property that is the most related to the control of nutrient movement and immobilization [2,10,11].

1.1.3 Cation Exchange Capacity (CEC)

CEC is commonly used to evaluate soil fertility as it gives an estimate of soils ability to attract, retain, and exchange cation (positively charged) elements [2,10,11]. It is defined as the total sum of the exchangeable cationic charges that a soil can adsorb [2,12]. CEC is expressed as the number of moles of positive charge adsorbed per unit mass and its values are reported as centimoles of charge per kilogram (cmol/kg) [2,12]. For example, a CEC of 15 cmol/kg, indicates that 1 kg of soil can hold 15 cmol of H^+ ions, and can exchange this amount of charges from H^+ (hydrogen ions/protons) for the same level of charges from any other cation [2,12].

Weil and Brady [2] described the common range of CEC for different soils as well as other organic and inorganic exchange materials. Lower CECs are reported in sandy soils (low in all colloidal material) than in silt loams and clay loams [2,10–12]. In addition, very high CECs were

associated with organic matter and not to inorganic clays (kaolinite, Fe and Al oxides) [2]. CEC increases at higher pH levels which improves soil capacity to supply nutrients to plants [2,10,11].

1.1.4 Soil pH

Soil pH is a logarithmic scale used to measure soil acidity or alkalinity [2,6]. Soil water pH is a measure of pH of the soil solution and it is considered the active pH that affects plant growth [2,6]. The acidity is caused by a dominance of H^+ and alkalinity by a prevalence of OH^- (hydroxyl ions) in soil solution [2,6]. In general, pH scale ranges from 0 to 14, where 7.0 is neutral and values < 7.0 indicate acidic conditions and > 7.0 alkaline [6,13]. The levels of pH for most natural soils vary between 3.0 (extremely acidic) and 8.0 (weakly alkaline) [6]. Plants grow at optimal rate in soils that are slightly acidic to neutral (pH 6.0 to 7.0). Whereas, some plant species such as blueberries (*Vaccinium myrtillus*), cranberries (*Vaccinium macrocarpon*), and blackberries (*Rubus Fruticosus*) and microorganisms require acidic soils (pH 4.5 to 5.5). Other plant species including alfalfa (*Medicago sativa*), thale cress (*Arabidopsis thaliana*), gum tress (*Eucalypts*), and citrus tolerate slightly alkaline soil (pH 7.0 to 7.5) [13–15].

Biochemical reactions in soils are influenced by H^+ activity. The solubilities of various compounds (e.g. metals) as well as microbial activities are influenced by soil pH [2,6]. The optimum pH values for pollutant degrading microorganisms range from 6.5 to 7.5 [2,6,13]. Soil pH is influenced by various factors such as types of organic and inorganic constituents, soil/solution ratio, salt or electrolyte content, and CO_2 (carbon dioxide) [2,6]. Soil pH is the foundation of essentially all soil chemistry and nutrient reactions [2,6].

Overall, soils play a major role in the global ecosystem whether occurring in a farm, forest or a regional watershed. Soils support plant growth, by providing habitat for roots and nutrient

elements. Their properties determine the nature of vegetation and also the number and types of organisms (including animals and humans) that the vegetation can support. Soils regulate water supplies and conditions including water loss, utilization, contamination, and purification. Soils also function as nature's recycling system. In fact, waste products and dead plants, and animals are assimilated within the soil and their basic elements are made available to the next generation of life. Further, soils are home to many creatures from small mammals and reptiles to tiny insects and microorganisms of unimaginable numbers and diversity. In addition, soils influence atmospheric composition by taking up and releasing large quantities of CO_2, O_2 (oxygen), and other gases.

1.2 History of Metal Contamination in the Greater Sudbury Region (GSR)

The discovery and exploitation of nickel (Ni) and copper (Cu) deposits in Greater Sudbury Region (GSR) (Ontario, Canada) in the late 1800s led to intense sulfur dioxide (SO_2) fumigation, soil contamination by aerial fallout and acid precipitation in the region [15–17]. In fact, mining and smelting of sulphide ore, released more than 10^7 tonnes of SO_2 as well as tens of thousands of tonnes of cobalt (Co), Cu, iron (Fe), and Ni biproducts into the atmosphere [15,17,18]. These factors led to soil acidification (pH of 2.0 – 4.0), which resulted in increased aluminum (Al), Ni, and Cu solubility.

Overall, these conditions made the GSR one of the most ecologically disturbed regions in Canada with barren (20,000 ha) and semi barren (80,000ha) lands [15,19–21]. Nieboer *et al*. [22] reported a drastic decrease in lichens populations close to smelters and only a few crustose lichens were observed in highly polluted areas. Gorham and Gordon [23], Gunn *et al*. [19], and Gunn *et al*. [24] reported a decrease in plant growth, natural recolonization, and population

diversity closer to smelters. They also documented the effects of acid precipitation in aquatic systems. Increased soil acidity caused major changes in soil chemistry characterized by an elevated level of bioavailable metals, and a decrease in available nutrients [15,19]. High level of metals in soil and vegetation within short distances of the smelters (24 km radius) in the GSR are still being reported [25,26].

Total concentrations of Cu and Ni were found to be 9700 µg/g and 6960 µg/g in Sudbury soil and the bioavailable levels ranged from 300 to 900 µg/g for Cu and Ni, respectively [15,27]. These values are much higher than those reported in most temperate and boreal soils where total Ni and Cu content range from 20 - 40 µg/g [15]. Recent analysis by Narendula and Nkongolo [28] reported total concentrations of 318 µg/g and 1600 µg/g and the bioavailable levels of 14 µg/g and 53 µg/g for Cu and Ni, respectively [28]. Phytotoxic symptoms appear in plants at bioavailable Cu and Ni levels ranging between 25 and 50 µg/g, and symptoms become extremely severe as pH decreases [15,27]. Detailed analysis revealed that Sudbury's soils are contaminated with Co, Cu, cadmium (Cd), chromium (Cr), Fe, manganese (Mn), Ni, S, and zinc (Zn) [29]. Metals such as Cu and Zn are essential for plant growth and development however, elevated concentrations of essential and non-essential metals in soil lead to toxicity symptoms and inhibition of growth in most plants [15,30].

In addition to metal contamination, SO_2 emissions released into the atmosphere oxidized to SO_4 (sulfate) through acid precipitation [15]. Similar to metal contamination, effect of acid depositions on ecosystem depends on the concentration of SO_4, amount of precipitation, and CEC of soil [9]. Even low levels of acid depositions result in soil acidification, which have adverse effects on plants, animals, and microorganisms [9,31]. Acid deposition leads to soil acidity,

reduced concentration of divalent cations (Ca^{2+}, Mg^{2+}) which results in mobilization and increased bioavailability of metals and other toxic compounds [9,15,19]. Soil acidification and metal availability reduces SOM solubility [9,15]. Increased soil acidity has been also shown to significantly reduce plant and microbial growth rate, activity, community composition, and diversity [9,15,31].

Since the early 1970s, The Ministry of the Environment has been sampling soil and vegetation in the GSR. In September of 2001, the ministry released a report called *Metals in Soil and Vegetation in the Sudbury Area*. Total of 103 samples from various locations were collected to assess environmental impact of mining and smelting operations in the GSR [32]. The study reported that some metals are elevated in the GSR and the highest metal concentrations are typically found in the top soil layers, indicating air emissions as the source [32]. The soil metal levels in GSR are comparable or lower than those found in other Ontario mining communities where elevated soil concentrations are found as a result of historical mining activity [32]. The results confirmed that emissions from over 100 years of mining, smelting, and refining resulted in elevated levels of metals in soil over a large area. Levels of arsenic (As), Co, Cu, Ni, and lead (Pb) were higher in the areas around the three industrial centers of Copper Cliff, Coniston, and Falconbridge compared to other sites. These findings are consistent with periodical ministry reports.

GSR soils were highly acidic which resulted in decreased macronutrients solubility and increased solubility of other micronutrients to toxic levels [15]. This led to a decline in plant and vegetation growth causing soil erosion resulting in severe depletion of topsoil. In fact, chemical analyses revealed significant soil deficiency in available nutrients (P, N, Ca, Mg, and Mn)

[15,19]. In addition, a decrease in number of soil fungi and bacteria has been observed in the vicinity of smelters in the GSR [15,33–35]. It has been shown that revegetation of barren lands is extremely slow due to lack of microorganisms [33,36]. Composition of soil microorganisms is known to affect plant growth, alter species composition, and affect species ability to colonize lands [11,33,36].

In the past 40 years, production of Ni, Cu, and other metals has remained high while SO_2 emissions have been reduced drastically through the combinations of plant closures, major reductions in emissions, greater dispersal through tall stacks, and combination of industrial technological developments and legislated controls [19,24]. Reduced emissions allowed for some degree of recovery to occur such as improved air quality and natural recovery of damaged ecosystems in the GSR [15,19,24]. In 1969 and 1970, barren lands still showed almost total plant mortality indicating that emission controls did little to reduce soil acidity or metal contamination [15,19]. Hence, a regreening program was implemented in late 1970s to improve Sudbury's ecosystem [15,37]. The program involved application of lime (10 tons/ha of dolomitic limestone), fertilizer (400 kg/ha), and tree planting [15,19,24]. Details on specific effects of liming are discussed in the reclamation section (1.4.2).

In addition, to date over 12 million trees have been planted including jack pine (*Pinus banksiana*), red pine (*Pinus resinosa*), white pine (*Pinus strobus*), white spruce (*Picea glauca*), larch (*Larix laricina*), northern red oak (*Quercus rubra*), and black locust (*Robinia pseudoacacia*) [15,19,37]. Monitoring studies revealed that survival and growth rate of planted trees have been very good and in addition, pH values in soils had increased to 4.6 - 6.5. After land treatment, metal uptake by plants had declined, insects/bird/small mammal populations

increased at reclaimed sites, natural colonization of herbaceous and woody species has been improved at treated sites, and water quality in some Sudbury lakes has been restored [15,19,37].

Dudka *et al*. [29] revealed a decline in soil Ni and Cu concentrations in the GSR when compared to previous studies. This could be explained by reduction of atmospheric emission, leaching, washing, and erosion processes that have contributed to the decline in metal concentration in the studied soil. Narendrula *et al*. [38], Bakina *et al*. [39], and Narendrula and Nkongolo [40] reported that liming lowered soil acidity, reduced metal toxicity, soil erosion and resulted in an increase in SOM. Nkongolo *et al*. [41] determined metal content in soil and various tissues from *Picea mariana* (black spruce) populations in the Sudbury region. Their results revealed concentrations of Cd, Co, Cu, Fe, Ni, and Zn to be within the limits set by Ontario Ministry of Environment and Energy (OMEE) guidelines in sites within the vicinity of the smelters. The level of these metals in *P. mariana* tissues were far below the toxic levels for vegetation. Other studies showed no correlation between level of genetic diversity and metal contamination in soil for *Deschampsia cespitosa* (tuffed hair grass) populations in Northern Ontario [42]. *Deschampsia* was one of the metal tolerant strains of native grasses that recolonized damaged lands ten years after the initiation of atmospheric improvement [19]. Studies by Vandelight *et al*. [43], and Nkongolo *et al*. [44] revealed that long term exposure of pine (jack pine and red pine) and spruce (red, black, and white spruce) populations to metal is not associated with the level of genetic diversity. Similar results have been observed in other hardwood plant species [45–47].

Ecological studies revealed that plant species diversity and abundance as well as tree species richness were lower in sites close to smelters compared to reference sites in the GSR [40].

Similarly, Nkongolo *et al*. [26] reported a lower tree species diversity and abundance in unlimed sites compared to limed sites. More importantly, genetic analysis of a number of plant populations suggests that the regreening program in the GSR is increasing genetic population variability which is contributing to sustainability of the terrestrial ecosystem in the region.

1.3 Effects of Metal Contamination on Terrestrial Ecosystem

Soils act as a sink for all chemicals generated from various natural and anthropogenic activities. The retention time of many substances in soil ecosystem is longer as contaminants accumulate quickly in soils but deplete at a slow rate [9,48]. Soils can naturally have high concentrations of metals as a result of weathering of parental material with high amounts of metal minerals. In many countries, anthropogenic activties such as mining, smelting, atmospheric deposition, fossil fuel combustion, wastewater irrigation, corrosion, sewage sludge, and agriculture practices have resulted in metal contamination of soils [9,48,49]. Globally, over 10 million sites have been reported to be polluted, with over 50% of them with metals and/or metalloids [49]. Metal pollution of soil and water is one of the most important environmental problems of the industrialized countries, affecting human health, agriculture, and forest ecosystem [6,9]. Worldwide economic impact due to metal pollution is estimated to be over US $10 billion per year [49].

Concentrations of metals in soils should be regularly monitored since they are persistent, cause toxicity, have long half-life, and because of their bioaccumulation potential [9,48,50,51]. Many metals are essential for normal metabolic functions in microorganisms, plants, animals, and humans but they become toxic at high concentrations [9,50]. The toxicity depends on the metal itself, and its total/bioavailable concentrations for different organisms [9,52,50]. Depending

on the metal and organisms, different modes of actions have been identified including, binding to macromolecules (DNA, RNA and proteins), disruption of enzymatic activity, and radical formation [9,15,24,50].

The threshold level of metal toxicity in plants is highly variable [53,54]. Plants prevent phytotoxicity by various processes which include formation of insoluble crystals, vesicles and retention of elements by cell walls [55]. Further, metal tolerance is significantly influenced by accumulation of metals in root cells [6,30]. Presence of excess amounts of metals is also shown to cause acute toxic effects resulting in DNA damage [30,50,56]. Metals can bind to proteins, leading to inhibition of activity or disrupting protein structure and/or metals can displace essential elements resulting in deficiency effects [30]. Elevated levels of metals can stimulate formation of free radicals and reactive oxygen species (ROS) such as singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals (-OH) that cause oxidative stress resulting in cellular damage in organisms [30,57].

Plants have mechanisms to prevent oxidative stress, as well as, enzymes and other compounds to avoid cell damage by inhibiting or quenching free radicals and ROS. Studies have shown that plants have a range of mechanisms at cellular levels that are involved in metal uptake [30,57]. Metal ions enter cells by competing with essential ions of similar ionic radii and with the help of proteins (phytochelatins: PC and metallothioneins: MT) and transporters [30,57]. Both PC and MT are cysteine rich metal chelating peptides capable of binding to various metals including Cd, Cu, Zn, and As [30,58]. In animals and humans the excessive uptake of metals is the result of the successive accumulation of these elements in food chain, with the starting point being soil contamination. Knasmuller *et al*. [59] indicated that plant bioassays (mutagenic assay,

micronucleus assay and cytogenetics study) can effectively detect the genotoxic effects of metals. These systems can be useful for bio-monitoring metals in soils.

Soil microbial community and their activities can be affected for decades from metal contamination. The toxic effects of metals on soil microorganisms highly depend on their bioavailability [9,15,24,33,50]. Significant reduction in a number of microbes, microbial growth rates, biomass, activity, community composition, and diversity have been observed in soils contaminated with metals [9,15,31,33,50]. Metal contamination at low levels has adverse effects on bacteria but not on fungi [31,50,60,61].

Soil microorganisms vary widely in their tolerance to metal contamination. For example, in bacteria, gram-negative cell walls have a lower charge capacity than gram-positive walls. But they have a complex three layered structure that binds and immobilizes many metal ions [mercury (Hg^{+}) and Pb^{2+}] [50,56,62]. Studies have shown that gram-negative bacteria exhibit greater metal tolerance than gram positive [50,56,62]. Various studies have reported a decrease in bacterial activity but an increase in fungal activity in metal contaminated soils [9,50,60,61]. Other studies also reported a delay, reduced, and complete elimination of arbuscular mycorrhizal fungi (AM) and ectomycorrhizal fungi (ECM) colonization and spore germination in metal contaminated soils [50,58,63,64]. In other studies, metal contamination had no significant effect on fungal development, which could be due to presence of different ecotypes exhibiting different degrees of tolerance to metals [50,63,64]. In polluted sites, fungi with higher tolerance to Cu, Zn, Cd, and Pb compared to those from unpolluted soils have been identified. Therefore, relatively high fungal colonization can be observed in plants growing in highly polluted soils [50,63,64]. However, at highest level of soil pollutions, fungal ecotypes and diversity diminish sharply due to

toxic effect of metals causing an inability of certain fungal species to colonize root system and/or multiply in rhizosphere [50,63,64].

Metal resistance in microorganisms is usually due to metal chelation with organic ligands, sequestrations, transportation out of cells, and biotransformation of ions to less bioavailable or less toxic form [50,56,62]. The resistance mechanism varies with microbial species involved. Uranium (Ur) has been shown to rapidly accumulate in cells of *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa* [56,62]. *Bacillus subtilis* cell walls bind to numerous metals (Hg^{2+}, Pb^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, Na^{+} and K^{+}). This binding is a result of the presence of many anionic sites on the cell wall, particularly: 1) phosphodiester groups of teichoic acids, 2) carboxyl group of peptidoglycan, 3) sugar hydroxyl group of wall polymer and 4) amide group of peptide chain (Mitchell; Hughes and Poole 1989). Similaly growing *Saccharomyces cerevisiae* in mercuric chloride ($HgCl_2$) resulted in extensive metal binding to high affinity sites on the cell wall [56]. Hyphal walls in *Neurospora crassa* bind to Cu, Fe, and Zn [56].

Some microorganisms (*Thiobacillus*, *Serratia*, *Pseudomonas*, *Bacillus*, *Penicillium*, *Aspergillus*) produce various organic and inorganic acids that can extract metals from solid substrates [56,62]. In addition to metal solubilization and transport, interaction between bacterial exopolysaccharides and metals has also been identified. Charged functional groups such as phosphate, carboxyl, hydroxyl, sulfate, and amide on the exopolymer are involved in adsorption of metals onto cell wall [56,62]. A number of bacterial and fungal species have the capability to transform metals to non-toxic form through methylation [56,62]. For example, studies have shown that several bacteria (*Clostridium*, *Pseudomonas*, *Bacillus*, *Mycobacterium*, *Escherichia coli*, *Aerobacter aerogenes*, and *Bacillus megaterium*) can methylate mercury (Hg) [56,62].

Fungi species such as *Neurospora crassa* are involved in methylation of Hg by complexing mercuric ions with homocysteine or cysteine residue [56]. This requires enzymatic transfer of methyl groups from alternative methyl donors which occur intracellularly [56,62].

Genes for metal resistance in the microorganisms are often present in plasmids and are easily disseminated through a population or community in response to selection pressure associated with metal exposure [9,50,65]. In bacteria, efflux pumping is the basis of toxic ion resistance, involving transporters such as P-type ATPases or cation/H^+ antiporter [30]. These pumping systems are involved in Cu, Cd, Zn, Co, and Ni resistance. Specific *czc* genes that encode for a cation-proton antiporter (CzcABC) which is responsible for resistance to Cd, Zn, and Co have been indentified in gram-negative bacteria (e.g., *Ralstonia eutropha* and *Cupriavidus metallidurans*) [50,65]. Similarly, *ncc* genes were found in gram-negative bacteria (e.g., *Alcaligenes xylosoxidans* and *Achromobacter xylosoxidans*) resistant to Ni, Cd, and Co. Cd resistance is linked to Cd-efflux ATPase, and *cad* operon which have been identified in gram-positive bacteria (e.g., *Staphylococcus*, *Bacillus* or *Listeria*) [50,65]. Cu resistance has been associated with *cop* system (*Pseudomonas syringae*) and *pco* system (*Escherichia coli*) where, *cop* genes encode for different Cu-binding proteins that allow sequestration of Cu whereas, *pco* system is an ion-dependent Cu antiporter [50].

Complexation of Cd by MT and glutathione (GSH) is a key mechanism for Cd tolerance in ectomycorrhizal fungus (*Paxillus involutus*) [58,66]. Two MTs have been indentified in *S. cerevisiae*: one is induced by Cu (encoded by *CUP1*) and the second is regulated by Cu, Zn, and oxidative stress (encoded by *Crs5*) [66]. MTs have been identified in a number of fungal species such as *Agaricus bisporus*, *Gigaspora margarita*, *N. crassa*, *Pyrenopeziza brassicae*, *Podospora*

anserine, and *P. involutus* [58,63,66]. Two fungal species (*Candida glabrata*, and *Schizosaccharomyces pombe*) produce both MT and PC [58] while, *Candida glabrata* produce metal chelating MT when exposed to toxic concentrations of Cu. But under Cd stress, it strictly produces PCs [58]. In addition to avoidance and compartmentalization, fungi also have other defense mechanisms that reduce oxidative stress generated by metal induced ROS by synthesis of antioxidant enzymes such as superoxide dismutase (SOD), catalases (CATs), ascorbate peroxidases (APX), GSH, glutathione S-transferase (GST), and glutathione reductase (GR) [58,63]. When grown on media with varying concentrations of Zn, *Glomus intraradices* was found to code for GST, SOD, cytochrome P450, and thioredoxin enzymes which are involved in detoxification of ROS [58].

1.4 Remediation Technologies of Soils Contaminated by Metals

Increasing soil metal contamination has important health and economic implications. Metals can be taken up by plants or leached into groundwater leading to food chain contamination. Several cleanup methods have been investigated. They are divided into two groups: 1) those that remove contaminants and 2) those that transform metals into harmless forms [48,51,67]. These cleanup technologies can be applied on or- off-site (in situ or ex situ), using three types of remediation treatments: 1) physical, 2) chemical, and 3) biological techniques [48,51,67]. Some contaminated sites may require a combination of procedures for an efficient remediation. Therefore, physical, chemical, and biological technologies may be used in conjunction with one another to reduce the contamination to a safe and acceptable level.

1.4.1 Physical Remediation

Physical remediation includes soil replacement, thermal desorption, and electrokinetic migration [48,67,68]. Soil replacement consists in using clean soil to replace contaminated soil to dilute the concentration of pollutant [67]. Soil replacement effectively decreases the effect of metals on environment but it is expensive and suitable for severely polluted small areas [67]. Thermal desorption consists in heating soil in a chamber where organic contaminants and certain metals can be vaporized [67,68]. Hence, thermal desorption depends on metals volatility and temperatures (e.g. Hg and As) [67,68]. It is divided into low temperature (90 - 320°C) and high temperature desorption (320 - 560°C) based on temperature in the operating chamber [67,68]. This technique has several advantages. It is simple and less expensive to process, remediated soil is reused, and it is environmentally friendly [67,68]. Its disadvantages include, high cost of device and long desorption time. It is not equally effective for all soil types, and it cannot be used for all metals [67,68].

Electrokinetic process consists in passing a low intensity electric current between a cathode and an anode imbedded in the contaminated soil [48,69]. Anions move towards anode (positive electrode) and cations towards cathode (negative electrode) [69]. Buffer solutions are used to maintain the pH at the electrodes as it is essential in the optimization of the process efficiency [48,69]. Metals are removed by electroplating or precipitation/co-precipitation at the electrodes, using ion exchange resins or they can be recovered by pumping the waste to the surface [48,69]. Metals can be removed from soil since they are bound to it as oxides, hydroxides, and carbonates, [48,69]. In Europe, this technology is used for Cu, Zn, Pb, As, Cd, Cr, and Ni [69]. This method has several advantages: 1) can be used *in situ* or with excavated soil, 2) effective with clay soils of low permeability, 3) accelerated rate of contaminant transport and extraction can be obtained

[48,69]. However, heterogeneities, large metal objects, rocks, amounts of oxides, foundations, rubble, other obstacles, moisture content, temperature, and other contaminants can interfere with the process [48,69]. Electrode duration and spacing is site-specific and may need to be optimized [69].

1.4.2 Chemical Remediation

Chemical remediation technique includes all the methods involving reagents or external compounds [48,68]. This includes chemical leaching and fixation, vitrify technique, and chemical immobilization [48,67,68]. Chemical leaching is a volume reduction and waste minimization treatment done on excavated (*ex situ*) soil or on-site (*in situ*) [68,70]. It involves washing contaminated soil with fresh water and solvents which have the ability to solubilize metals [67,68,70]. Leaching solution and chelators are added to extract metals from soil [48,67,68]. Chemical fixation technique involves addition of reagents to metal contaminated soils. These agents are then used with metals to form insoluble and nontoxic forms. This process decreases the migration of metals into the environment [67].

Vitrify technique involves heating soils to extremely high temperatures between 1,400 °C and 2,000 °C [67]. The mobility of metals is reduced by high temperature treatment which results in the formation of vitreous materials, usually an solid oxide [67,70]. Increased temperature melts contaminated soil, buried wastes or sludges rendering the material nonhazardous [67,70]. This technique can be applied to water, debris, and various soil types. *In situ* vitrifications are preferred due to low energy requirement and cost. *Ex situ* process includes excavation, pretreatment, mixing, feeding, melting and vitrification, gas collection and treatment, and forming or casting of the melted products [67,70].

Chemical immobilization is an *in-situ* technique where inexpensive materials (e.g. lime, fertilizers, fly ash, Fe/Mn oxides, and cement) are added to contaminated soil. They can remove and/or stabilize metals in soils, resulting in a substantial reduction of costs [15,24,48,70,71]. This relies on a fundamental understanding of natural geochemical processes governing the speciation, migration, and bioavailability of a given metal in the environment [48,72]. *In-situ* chemical immobilization technique provides long term remediation solution by decreasing the concentration of contaminants by sorption or precipitation [48,70–72]. Mobility and bioavailability play a huge role in solubility of metals. Increased sorption and decreased solubility reduce pollutant transport and redistribution into the environment [9,71,72]. Chemical immobilization treatments serve also as reactive barrier which prevents metal seepage from recovery pits and other processing areas on active and inactive sites [72]. The redistribution of metals by means of solute transport mechanism can adversely affect ecosystem, water resources, and human populations [70,72].

Chemical immobilization of metals uses phosphate and alkaline based materials to adsorb, chelate or complex metals in soils [70,72]. Metal contaminated soils treated with phosphates (apatite) reduce metal solubility by forming metal phosphate precipitates and minerals [70,72]. Addition of phosphate material and presence of sufficient level of soil P have proven to be extremely effective as chemical immobilization of Pb and Zn [70,72]. In addition to reducing metal solubility, it decrease bioavailability of metals to plants and animals [70,72].

Chemical immobilization using alkaline amendments (lime) reduces metal solubility in soils by increasing pH and metal sorption to soil particles [15,24,39,71]. Increased soil pH and carbonate buffering lead to the formation of metal-carbonate precipitates, complexes, and

secondary minerals [15,24,39,72]. Soils become acidic because basic cations are replaced by H^+ on the soil colloidal complexes [15,24,39,72]. As pH decreases, the availability of P, K, Ca, and Mg is reduced, while the availability of Zn, Mn, Cu, and Fe increases [15,24,39,72]. Various studies have used lime (calcium containing inorganic material) as chemical amendment for remediation of soils contaminated with metals [9,15,48,71].

In addition to increasing pH, lime is used to increase mineralization of soils with Ca and in some cases, Mg [15,24,39,45]. It further, reduces metal solubility, improves soil structure, and stabilizes soil nutrients, which helps reduce soil erosion resulting in an increase in SOM [15,24,39]. Lime can be applied as calcium hydroxide ($Ca(OH)_2$), calcium oxide (CaO) or calcium carbonate ($CaCO_3$) to effectively neutralize soil acidity [72,73]. Addition of lime neutralizes acidity, and increases microbial activity in soils. In GSR, dolomite lime ($CaMg(CO_3)_2$) was applied as it primarily neutralizes acidity but also provides Ca and Mg for plant uptake [15,24]. The role of both Ca and Mg together is crucial to metal toxicity as they create a competitive exclusion of metal ions [15,24,39,45].

The increase in pH when lime is added to soil depends on the CEC [73,74]. Soils with low CEC will show a more marked pH decrease compared to soils with high CEC [73,74]. Also, low CEC is associated with rapid leaching of added basic cations and a quick return of original acidity unless additional liming is applied [73,74]. Ions in liming materials combine with hydrogen ions in the soil to produce water (H_2O) and CO_2 reducing soil acidity as described in following equation:

$$\text{Soil-}4H^+ + CaMg(CO_3)_2 \rightarrow \text{Soil-}Ca^{2+} + \text{Soil-}Mg^{2+} + 2CO_2 + 2H_2O.$$

The reaction neutralizes H^+ and releases Ca^{2+} and Mg^{2+}, resulting in an increase in soil pH and CEC [73,74]. Ca^{2+} and Mg^{2+} ions replace H^+ on the existing pH-dependent CEC whose magnitude changes under the influence of increased alkalinity [73,74]. After lime application, higher exchangeable Ca than Mg is observed due to higher selectivity of the colloidal negative sites for Ca^{2+} than for Mg^{2+} [73,74]. As long as the soil supplies acidity in the form of H^+, the above reaction will continue.

In the GSR, the primary factor limiting plant growth was low pH combined with elevated Cu, Ni, and Al concentrations [15,24]. Experiments on germination and early growth of grasses in GSR revealed the inhibitory effects of Cu and Ni on root growth [15]. Synergistic effect was observed where these elements enhanced each other's phytotoxic effect, resulting in total toxicity that is more than the sum of the individual toxicity [15]. On the contrary, interaction between Ni and Al was antagonistic, as Al was protecting the plants from increased Ni concentrations [15]. In Sudbury, liming application to metal contaminated soils created positive feedback loops throughout the system and had an immediate detoxifying effect [15,24,25,45]. Application of limestone to toxic and barren soils triggered immediate colonization of native plant species through germination of existing seed and incoming wind disseminated seeds [15,24]. It also depressed the adverse effects on the early root and mycorrhizal development on planted seedlings [15,24]. Therefore, liming application on metal contaminated soils in GSR became a trigger factor.

Chemical immobilization has its advantages and disadvantages. Advantages include: 1) low cost, 2) simplicity and rapidity, 3) broad spectrum of pollutants can be targeted, and 4) high public acceptability [67,68,70]. The main disadvantages are: 1) difficulty in removing

permanently the contaminants (temporary solutions), 2) change in soil physiochemical properties can activate pollutants, 3) only the surface layer is reclaimed and, 4) permanent monitoring is required [67,68,70]. Therefore, chemical immobilization is often performed along with biological remediation.

1.4.3 Biological Remediation

Biological technique is a sustainable remediation technology to rectify and re-establish natural conditions of the soil [48,75]. This technology consists of *in-situ* remediation using plants (phytoremediation) and microorganisms (bioremediation) to clean up contaminated soil and water [48,67,76]. In many developed and developing nations, phytoremediation has been accepted widely for its potential to clean up polluted sites [48,67,75]. Phytostabilization, phytovolatilization, and phytoextraction are the three main types of phytoremediation [67,75,76].

Phytostabilization consists in immobilization of metals in soil through absorption and accumulation by roots, or adsorption onto roots or precipitation within the root zone. This reduces metal bioavailability and migration into ground water or the food chain [67,75,76]. However, metals that are absorbed by plants get converted into volatile forms. Subsequently, they are released into the atmosphere by the process called phytovolatilization. This has been used for removal of volatile metals like Hg and selenium (Se) from polluted soils [67,76]. This technology is only suitable for volatile contaminants and is limited because it does not remove the metal but rather transfers them from one medium (soil or water) to another (air) from which they can re-enter soil and water [67,75,76].

Phytoextraction refers to the uptake of metals from soil or water by plant roots and their translocation and accumulation into any harvestable plant parts [67,75,76]. Some accumulator

species possess exceptionally high metal accumulating capacity and are known as hyperaccumulators. They can survive and even thrive in heavily contaminated soils [67,76,77]. Recently, removal of metal through phytoremediation, especially by hyperaccumulators has received wide attention due to its efficacy and cost efficiency [67,76]. The main criteria used for hyperaccumulation classification varies according to metals, ranging from 100 mg of metal /kg dry mass of plant for Cd to 1000 mg/kg for Cu, Co, Cr, and Pb [76,77]. According to the US department of energy, to be considered as hyperaccumulators plants should have the following characteristics: 1) have high accumulating efficiency under low contaminants concentration; 2) accumulate high concentrations of contaminants, 3) accumulate different types of metals, 4) grow fast and with large biomass, and 5) have pest and disease resistance ability [67,76,77]. Most of the commonly known metal hyperaccumulators belong to the *Brassicaceae* or *Fabaceae* families [75,77]. However, more than 400 plants species have been reported to be hyperaccumulator plants, and a considerable number of species show the capacity to accumulate two or more elements [75,77].

Phytoremediation technology has certain advantages and disadvantages as well. Advantages include: 1) low-cost, 2) low-energy, 3) being environmentally friendly, 4) far less disruptive to the soil environment, 5) avoids excavation and is socially acceptable, and 6) plants are easy to implement and maintain [76,77]. The disadvantages include: 1) time consuming due to slow growth, 2) affected by changes in climatic conditions, 3) proper disposal of plant biomass after remediation, 4) contaminants may enter soil again due to litter formation, and 5) root exudates may enhance the solubility of pollutants and consequently increase the distribution of metals into soil environment [76,77].

The main drawback of phytoremediation is the storage and accumulation of pollutants in plant materials. The remediation process slows down and often becomes inadequate when the contaminated site has multiple pollutants. The appropriate solution to these problems is to combine microbe-plant symbiosis within the plant rhizosphere or to introduce microbes as endophytes to allow degradation of pollutants within the plant tissues [76,78]. Studies have shown that microbial populations in rhizosphere is much higher than in vegetation-less soil, as plant provide essential nutrients for microorganisms [76,78]. Microorganisms in turn are involved in various processes which benefit plant health, growth, and development [9,76,78]. Therefore, this approach accelerates removal of pollutants and supports high plant biomass production for bioenergy.

In the last decade, attention has been drawn to biological remediation which involves the use of microorganisms to clean up contaminated soil and water. Microorganisms cannot degrade and destroy metals, but affect the migration and transformation by changing their physical and chemical characterization [67,76,78]. Bioremediation is divided into two categories: 1) biosorption and 2) bioaccumulation. Biosorption is a passive adsorption mechanism that is fast and reversible where, metals are retained by means of physicochemical interaction (ion exchange, adsorption, complexation, precipitation, and crystallization) between metals and the functional groups (carboxyl, phosphate, sulfate, phenyl, amide, and hydroxyl groups involved in metal binding) present on the cell surface [76,78,79]. Both living and dead biomass can occur for biosorption because it is independent of cell metabolism. On the other hand, bioaccumulation is an active process of metal removal by living biomass which uses both intra- and extra-cellular processes [76,78,79].

Microbes mobilize metals by leaching, chelating, methylation, and redox transformation [67,76,78]. Leaching is a simple and effective technology for extracting valuable metals and has potential for remediation of mining sites, treatment of mineral industrial water products, detoxification of sewage sludge, and for remediation of soils and sediments contaminated with metals [67,76]. Microorganisms use metals as terminal electron acceptors or reduce them through detoxification mechanism. They then remove them from contaminated environment [76,78]. In addition, they also remove metals through mechanisms that they employ to derive energy from metals redox reaction, to deal with toxic metals through enzymatic and non-enzymatic processes [76,78]. The redox reaction takes place in soil between metal and microorganism where, microorganisms act as oxidizing agents for metal and cause them to lose electrons. In anaerobic conditions, microbes oxidize organic contaminants by reducing electron acceptor, while in aerobic condition, oxygen acts as electron acceptor [76]. For example, microorganisms reduce the state of metals and change their solubility. Dixit *et al*., [76] reported that *Geobacter* species reduce uranium (U) from a soluble state (U^{6+}) to insoluble state (U^{4+}).

Several studies have shown that many microorganisms have a natural capacity to biosorb metals, and use different defense systems (exclusion, compartmentalization, complex formation and synthesis of binding proteins and peptides) to reduce stress developed by toxic metals [76,78,79]. In the presence of metals, some microorganisms produce cysteine-rich metal binding proteins and peptides, such as glutathione GSH, PCs, and MTs, which can bind and sequester metal ions into biologically inactive forms [76,78,79]. In *E.coli*, expression of different metal binding proteins and peptides regulates the accumulation of Cd [76,79]. Dixit *et al*. [76] reported that co-expression of GSH along with PC resulted in two fold increase in Cd accumulation. Coelho [79] on the other hand, demonstrated the ability of certain fungi (e.g., *Aspergillus* and

Penicillium) and some yeasts (e.g., *Saccharomyces cerevisiae*) to remove metals from contaminated sites.

Studies have shown that metal toxicity to plants can be reduced by the use of plant growth promoting microorganisms which exert some beneficial effects on plant development [76,78]. The use of endophytes and rhizospheric microorganisms associated with degradation of pollutants in soil is promising for remediation of metal contaminated sites. *Kluyvera ascorbate*, metal resistant bacterium, has been reported to promote canola (*Brassica campestris*) growth in the presence of high concentrations of Ni [78]. Mycorrhizal fungi reduce metal toxicity to their plant host by binding metals to their cell wall or surrounding polysaccharides [67,78]. Mycorrhizae reduce the concentrations of metals therefore, reducing their hazardous effect to plants [67,78]. Yao *et al*. [67] reported that Cd uptake from hyphal compartment was higher in mycorrhizal than non-mycorrhizal plants and a large proportion of the increased Cd content was sequesterd in roots. It was concluded that AM fungi can transport Cd from soil to plants through extraradical hyphae, but that transfer is restricted due to immobilization of metals.

The application of bioremediation on a large scale can be challenging. Bioremediation can be affected by different factors such as temperature, oxygen, moisture, and pH [67,76,78,79]. There are some limitations to the application of this technology. Some microorganisms can only degrade special contaminants, and native bacterial populations might be affected if new microbes are added to the ecosystem to cleanup contamination. This might incur secondary pollutions [67,76,78,79]. Also, industrial scale application would not be of interest if the microorganism is difficult to cultivate [76,79].

1.5 Soil Microbial Community and its Biodiversity

Microorganisms constitute a small proportion of soil but are vital to the overall functioning, stability and sustainability of ecosystems [50,80]. Soil microbes have body widths of < 100 µm and are the most abundant and diverse groups of organisms, where a single gram can contain tens of thousands of species [3,81]. They have unique ability to adapt to extreme conditions imposed by low nutrients, temperature, pH, and contamination, among others [80]. Soil microbes are considered as the biological engine of the earth, as they perform numerous fundamental processes including nutrient cycling, degrading pollutants, and regulating plant communities [50,80]. Hence, increasing attention is being directed towards microorganisms as the fertility and health of soil depends on their chemical composition and also on the qualitative and quantitative nature of microorganisms inhabiting it [2,3,50].

Microorganisms are divided into two main groups: 1) prokaryotes (simple organisms without a defined nucleus, which include bacteria and archaea) and 2) eukaryotes (complex organism with a true nucleus that include algae, fungi, and protozoa) [50,80]. In soil, all microorganisms are closely associated with soil particles, especially clay-organic matter complexes [50,80]. These microorganisms in soil can be found as single cells or as biofilms embedded in a matrix of polysaccharides and their activities and interactions with other microbes and organisms depend on microhabitat conditions [50,80]. The most primitive microorganisms are bacteria and archaea, which have been observed in diverse environments [50,80]. Fungi appeared comparatively recently and it is thought that terrestrial fungi may have coevolved with plants as they are closely associated with them [50,80]. Although fungi are often thought to be exclusively terrestrial, several species have been found in aquatic ecosystems.

Bacteria are prokaryotic organisms which usually have a rigid cell wall, divide by binary fission, and some are capable of photosynthesis [50,80]. Bacteria are the least structurally complex of the microorganisms and yet have the greatest diversity [80]. They are dominant group of microorganisms in various kinds of soil and play an important role in the ecosystem such as recycle biomass, control atmospheric composition as well as components of phytoplankton and soil microbial populations [50,80]. Bacteria are further divided into: 1) gram-positive bacteria and 2) gram-negative bacteria based on the cell wall [50,80]. Bacterial populations are influenced by temperature and moisture but they can withstand extreme environments [50,80]. For example, bacteria can thrive in arid desert soils, where temperatures are very high and also in arctic regions where temperatures are below freezing [50]. In addition, some bacteria form spores that possess a tough outer covering which facilitates their survival in all adverse environments [50,80]. Bacteria are grouped into three groups based on their temperature tolerance: thermophiles (45-65°C), psychrophiles (below 10°C) and mesophiles (15-45°C) [80]. However, the bulk of bacteria in soils are mesophiles. Bacterial concentrations in soils range from 10^8 to 10^9 per gram of soil and their biomass range between 40 and 500 g/m^2 [82]. It is estimated that there are more than 50 bacterial phyla based on the analysis of the conserved 16S rRNA sequence [80].

Fungi on the other hand, are eukaryotic organisms that have rigid cell wall, single cell forms (yeast), multicellular forms (hyphae, mycelium), reproduce by budding, and have no photosynthetic members [2,50,80]. Although bacteria are the most abundant in terms of number of individuals, fungi are a physically larger group of eukaryotic microorganism with greatest biomass [2,80]. It is reported that fungi concentrations in different soils range from 10^5 to 10^6 per gram of soil and their biomass ranges between 100 and 1500 g/m^2 [2,80,82]. As many as 2500 species have been reported to occur in single location [2]. Fungi have a primary role as

decomposers, and are involved in recycling biomass and stimulate plant growth [50,80]. The quality and quantity of organic matter impact fungal flora and populations as fungi are heterotrophic organisms [80]. In addition, fungi can grow in acidic, neutral, or alkaline soils which give them an advantage over bacterial populations [50]. To date, tens and thousands of species have been identified in soils, representing approximately 170 genera [2].

1.5.1 Biodiversity

Biodiversity is defined as the variability among living organisms on earth from all sources (terrestrial, marine, other aquatic ecosystems) and the ecological complexes of which they are part [83]. Biological diversity is divided into several components: 1) species diversity – number and abundance of different species that occupy a location, 2) species richness – number of different species, 3) relative abundance (evenness) – number of individuals within each species and 4) genetic diversity – variation in genetic material within a species or within a population [40,83]. In addition to biodiversity, there is also functional diversity which describes the biological function of species or group of species in an ecosystem [2,83,84].

1.5.2 Soil Biodiversity

From a biological and physiochemical perspective, soil is the most diverse ecosystem on earth [2,3,85]. Soil harbors a huge variety of organisms, many of which are still unknown. Soil biodiversity denotes all organisms inhabiting soil, and depending on the size, these organisms are divided into micro, meso, and macro-fauna and flora [3,50]. The sizes of soil organisms range from < 100 µm body width for microbes (archaea, bacteria, fungi) and microfauna (protozoa, nematodes) to mesofauna (collembola, mites) with body width between 100 µm and 2mm,

macrofauna (earthworms, insects) with body width > 2 mm and up to megafauna (moles, voles) with body width > 2 cm [3].

Microbial population diversity is significantly affected by various physical (moisture, temperature), biotic factors (predation, competition), and chemical factors (pH, CEC, dissolved nutrients, salinity, organic matter) as well as natural and anthropogenic activites [2,3]. Because of the vast diversity of microorganisms in the soil and as all species cannot be isolated and studied in pure culture, the assessment of microbial community diversity in soil represent one of the most challenging and enthralling aspects of microbial studies.

1.6 Microbial Community Assessment Methods

A wide range of techniques are available that allow various aspects of community structure to be assessed. Traditional approaches are based on culturing microorganisms (bacteria and fungi) on selective media. Although the ecology of individual species can be studied in detail, only 1% of the total microbial community is culturable.

1.6.1 Phospholipid Fatty Acid Analysis (PLFA)

One of the microbial community assessment methods that do not rely on culturing microorganisms is phospholipid fatty acids (PLFA) analysis [3,86,87]. It is widely used for estimation of total microbial biomass and to assess broad changes in the soil microbial community composition [86,87]. PLFA analysis involves using phospholipids which are the primary lipids composing cellular membranes to identify microorganisms [3,88]. Over 200 different fatty acids have been characterized from various prokaryotic and eukaryotic organisms, they vary significantly among different organisms, making them powerful tools in taxonomic

studies [89]. The main advantages of PLFA technique is that, it is quantitative and particular PLFAs are nominally assigned to broad-scale bacterial and fungal groups [3,88]. Also, phospholipids are present only in viable microorganisms as they are associated with the membranes of living cells which break down rapidly when the cells die [88,89].

The technique consists in the extraction of lipids from the soil using organic solvents followed by separation of phospholipids from other lipids according to their polarities using solid phase extraction [86–88]. PLFAs are then converted to fatty acid methyl esters (FAMEs), which are analyzed by gas chromatography (GC) to determine the types and quantities of each microorganism [86–88]. Fatty acids extracts are used to determine bacteria (gram positive, gram negative, actinomycetes, anaerobes), fungi (AM fungi and other fungi), and other eukaryotes biomasses in soil [87]. The 15:0, i15:0, a15:0, c17:0, i17:0, a17:0, c19:0 fatty acids are commonly used as signature fatty acids for bacteria, 16:1ω5, 18:1ω7, 18:1ω9c, 18:2ω6c for fungi, and 10Me16:0, 10Me17:0, 10Me18:0 for actinomycetes

Fatty acids used as specific markers for one group of organisms may occur in other groups in variable concentrations [90,40,91]. For example, gram positive bacteria have relatively more iso-, anteiso- or otherwise-branched fatty acids, gram negative bacteria have more monounsaturated or cyclic fatty acids, actinomycetes often have a methyl group in the tenth carbon atom from the carboxyl end of the chain, and fungi have more long-chain polyunsaturated fatty acids than bacteria [90,88,91]. A number of studies have reported an increase in gram-negative bacteria and a decrease in gram-positive species in different stress conditions (e.g. acidic soils, metal contamination) [90–93]. The presence of cyclo fatty acids in the membranes and outer lipopolysaccharides layer in gram-negative bacteria enhances their survival under stress

conditions [88]. In addition, gram-negative bacteria are fast growing microorganisms that utilize a variety of C sources and adapt quickly to a variety of environmental conditions [88]. Bacterial biomass is estimated by combining several fatty acid markers from both gram-positive and gram-negative bacteria [88,91].

Polyunsaturated fatty acids are associated with fungi. Studies have shown that fungi appear to be more tolerant to metals and acidic soils than bacteria [13,31,90,40,61,93]. However, a decrease in fungal markers in field studies contaminated with metals has been documented [36,88]. Contact of hyphae with metal pollutant affects fungal biomass. PLFA 18:2ω6 and 18:2ω9, indicators of ECM fungi have been linked to damage of fine roots due to pollutions [88,91]. These fatty acids are not exclusive to fungi as they are also present in many eukaryotic organisms, including plants [40,91]. Fatty acids such as 16:1ω5 and 18:1ω7 are good indictors of AM fungi [94,95], and are valuable biomarkers. However, they can be also produced by bacteria. They have been reported to decrease in response to metal pollutions and to increase in soil after restoration [88]. Although studies have reported that fungi prefer acidic soil since their growth is generally optimal in soils with a pH between 2 and 7 (with an optimum growth of 5) [13], a decrease in fungal biomass has been associated with a decrease in soil pH [31,93,96].

In addition to biomass and community structure determinations, PLFA analysis has been used to get an insight into physiological status of microbes. For example, fungal/bacterial ratio is used as an index of relative abundances of these two main groups of microbial decomposers in polluted soils [88,91]. Fungi:bacteria ratios are reduced in systems impacted by disturbances [13,31,90,40,61,93]. In addition, an increase in the trans/cis and the saturated/unsaturation ratio in soil indicate stressed environment [92]. An increase in trans/cis ratio in the presence of metals

has been observed. This is due to the interaction of metals with microbial membrane fatty acids, disturbing their conformation [88,97]. The increase in cy17:0/16:1ω7c ratio is also associated with stressed conditions. A relative increase in cyclopropyl fatty acids compared to their monoenoic precursors has been observed during prolonged stationary growth phase, growth under low C and O_2 concentrations, low pH and high temperatures [88]. An increase in cy17:0 and cy19:0 relative to 16:1ω7c and 18:1ω7c (metabolic precursors) has been linked to physiological stress due to metal pollution [88,97]. In addition, transformation of cis double bonds into a cyclopropane ring restricts overall mobility, reducing the impact of environmental stress in membrane fluidity. An increase in the abundance of 16:1ω5, monounsaturated fatty acids, and cy19:0 with a decrease in pH are general indicators of direct pH effect [40,92].

Phospholipids make up a relatively constant fraction of cell biomass, thus, a change in fatty acid profile signifies an alteration in the microbial community [88,89]. Although, soil analysis using PLFA does not result in detection at species level, it gives an overall picture of the community structure and provides an estimate of overall change [88,89]. In fact, since the early 90's, this technique has been employed to study soil microbial community composition and population density, changes to environmental conditions, soil type, contamination, and alterations in plant community and agricultural practices [35,36,40,97]. PLFA analysis has shown that microbial community can be altered by changes in management practices, substrate availability and composition, soil type, seasonal variation in soil nutrient, and plant characteristics. When compared to molecular analysis, PLFA has an advantage as it has the ability to determine fungi to bacteria ratios for a given soil system [98].

1.6.2 454 Pyrosequencing

Quantifying community structure at a higher resolution requires the use of molecular techniques. Among various methods used to estimate microbial community compositions and diversity in habitats, the most useful involves sequencing of ribosomal genes [99–101]. This technique is well suited for different types of community analysis studies as: 1) they are found universally in archaea, bacteria, and eukarya, 2) these genes are composed of highly conserved regions and regions with considerable sequence variation and 3) they are easily amplified using polymerase chain reaction (PCR), and 4) can be sequenced rapidly [99–101].

Next generation sequencing such as 454 pyrosequencing facilitates identification of microorganisms at the species level and their relative abundance in soils [100,102]. The 454 pyrosequencing platform is a new type of second generation sequencing technology that is rapid, flexible, cost effective, produces 25 million base reads in a single run with an accuracy of 99% and does not require a cloning step [102]. The capabilities of 454 pyrosequencing have led to its use for microbial whole-genome sequencing and improvements in sequence quality and read length have enabled applications to high-resolution analysis of microbial populations [99–102].

Pyrosequencing analysis of soil microbiome consists in determining the sequences of 16S ribosomal RNA (rRNA) genes (i.e. encoded by rDNA) in prokaryotes, 5S or 18S rRNA genes or internal transcribed spacer (ITS) region sequence variation in eukaryotes [100,103]. The 16S rRNA is found in the smaller subunit of the ribosome and is highly conserved between organisms [104]. Related organisms have fewer differences in the gene sequence than less related ones and those with a 98% match are considered to be isolated from the genome of the same bacterial species [101,104]. As sequencing of the 16S rDNA genes has been carried out frequently in

microbial ecology, there is a considerable database of known sequences [101]. The ITS regions includes ITS1 and ITS2 regions, separated by the 5.8S gene, and situated between the 18S and 28S genes in the rDNA repeat unit [105]. Because of its higher degree of variation than other regions of rDNA, variability among individual rDNA repeats can sometimes be observed within the ITS regions [106]. This region is the most frequent and widely sequenced DNA region in fungi and it is routinely used to address research questions related to systematic, phylogeny and identification of strains and specimens at and below the species level [103]. Currently ~172,000 full length fungal ITS sequences are deposited in GenBank [103].

In general, 454 pyrosequencing generates a large number of reads through a massively parallel sequencing by synthesis approach [100,107]. It is capable of generating up to one millions reads of ~ 500 bp in a single run on the genome sequencer FLX [107]. Metagenomics refers to the analysis of genetic material sampled directy from the environment. In environmental genomics application of pyrosequencing, DNA is extracted from an entire microbial community and a particular target region is flanked by conserved primers which are amplified by PCR before sequencing [3,88]. In 454 pyrosequencing, a unique sample-specific identifier (barcode) sequence is added to the DNA that is to be sequenced [81,107,108]. Barcoding of pyrosequencing templates allows for multiple samples to be sequenced in parallel [3,81,107,108]. After sequencing, reads are sorted into sample libraries via detection of the appropriate barcode. DNA barcodes have been used to detect undescribed and cryptic species, allowing complex and ecological interactions to be investigated and to determine accuracy of species content of commercial products [107].

In spite of the large sequence output and dropping costs, substantial sampling efforts may still be required to ensure sequence representativeness [3,81,108]. While relatively low sequencing effort (as few as 100 sequences) is needed to compare very different environments according to their microbial diversity, thousands of reads might be necessary for comparing closely related communities [3,107]. Pyrosequencing delivers a fast and substantial sequencing output which provides between 4500 and 52,000 unique operational taxanomic units (OTUs) [81,109].

Acosta-Martinez *et al*. [109] reported differences in bacterial diversity in soil under different vegetation and management. They observed difference not only in the richness (presence or absence) but also in evenness (distribution) of bacteria. Golebiewski *et al*. [110] looked at the bacterial community in metal polluted soils and reported that Zn decreased both diversity and species richness at species and family levels and that plant species richness did not correlate with bacterial diversity. They also found that in spite of differences between samples, they shared many OTUs. Li *et al*. [99] characterized bacterial diversity in a reclamation site undergoing fertilization practices and an adjacent coal-excavated site using 454 high-throughput 16S rDNA sequencing. They identified dominant taxonomic groups that include *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Actinobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Chloroflexi*, and *Firmicutes* in both sites [99]. However, the bacterial communities' abundance, diversity, and composition differed significantly between the two sites [99]. Lauber *et al*. [111] and Rousk *et al*. [93] analyzed bacterial community in soils with different pH. Bacterial community composition, as measured by pairwise UniFrac distances, was significantly correlated with differences in soil pH which was largely driven by changes in relative abundance of *Acidobacteria*, *Actinobacteria*, and

Bacteroidetes across the range of soil pHs [111]. Similarly, Rousk *et al*. [93] reported that both relative abundance and diversity of bacteria were positively related to pH. The composition of bacterial communities in both studies was closely defined by soil pH indicating the dominance of pH in structuring bacterial communities.

Although pyrosequencing has revolutionized our understanding of bacterial communities, very few studies have investigated fungal diversity and community structure. Lim *et al*. [112] looked at fungal communities in soils from Korea and China. A total of 372 tentative taxa were identified and the majority of fungal sequences recovered in the study belonged to the *Ascomycota* (182 taxa in 2,708 reads) and *Basidiomycota* (172 taxa in 6,837 reads). The predominant species of *Ascomycota* detected have been described as lichen-forming fungi, litter/wood decomposers, plant parasites, endophytes, and saprotrophs. Most sequences derived from *Basidiomycota* matched ECM and wood rotting fungi. In addition, a high number of sequences in the *Thelephorales*, *Boletales*, *Stereales*, *Hymenochaetales*, and *Ceratobasidiomycetes* were also found. Rousk *et al*. [93] looked at the fungal community across a pH gradient in soils and reported that relative abundance and diversity of fungi were unaffected by soil pH. This was consistent with results from pure culture studies that showed that fungi exhibit wider pH ranges for optimal growth [13,35].

The effectiveness of 454 pyrosequencing in analyzing soil microbial diversity has been validated and an association between land ecological system restoration, mostly mediated by microbial communities, and an improvement in soil properties in reclaimed areas has been established. In addition, studies suggest that structure of soil microbial communities is

predictable, to some degree, across large spatial scales, and the effect of soil pH and metal pollution on microbial community composition is evident.

1.7 Microbial Activity and Functionality Measurement

Species diversity is correlated with a high degree of functional diversity, which is the capacity to utilize a wide variety of substrates and carry out a wide array of processes [2,3,9]. Soil microorganisms perform a large number of functions including soil nutrient biochemical cycling, decomposition of both above and below-ground litter, and formation of organic matter [2,3,9,80]. Functional diversity is an important mechanism which allows for soil microbial communities to successfully respond to anthropogenically induced changes to the soil environment [9,80].

A number of methods have been developed to investigate soil functional diversity which involves understanding communities and ecosystem [113]. Soil biological activities are the most common approaches used to assess microbial function and is determined by measuring the overall rate of entire metabolic processes (soil respiration), and soil enzyme activity [9,34,114,115]. These properties are most useful in detecting the deterioration of soil quality and are closely related to nutrient cycles. Soil respiration has great potential as an indicator of ecosystem metabolism and fine scale processes [116]. Enzyme activities are especially significant because of their major contribution to the ability of the soil to degrade organic matter [117].

1.7.1 Soil Respiration

Soils are the largest carbon pool in the terrestrial ecosystems. The carbon cycle in an ecosystem is usually initiated when plants fix CO_2 from air and converts it to organic carbon

compounds through photosynthesis [118]. Some of the CO_2 is released back into the atmosphere through plant respiration (aboveground respiration). During this process some organic carbon compounds are broken down to provide plants with nutrients. Similarly, belowground dead plant materials (litter) are decomposed by microorganisms to provide energy for microbial growth and other activities [119]. Microbial biomass is mixed with organic residuals of dead plants and microbes to form soil organic matter (SOM) [120]. SOM can store carbon in soil for many years before it is broken down to CO_2 through respiration.

Soil respiration is defined as the process of gas exchange between organisms and environment. It is the major pathway of carbon transfer from soil to atmosphere [116]. It is assessed by measuring total soil CO_2 efflux at the soil surface and it depends on many biotic and abiotic factors [121]. Biotic process comprises of rhizosphere, microbial, and faunal respiration [118,121]. Abiotic factors are divided into chemical and physical processes. The chemical process consist of oxidation of soil minerals [118,121]. Physical process involves soil CO_2 degassing and its transport through soil to the surface [118,121]. Fluctuations in soil respiration rate have profound impact on SOM, soil microbial community, nutrient availability, plant health, and the atmosphere [119]. Research on soil and its metabolism has been conducted since the 1800's and major factors that influence soil fertility and respiration have been identified. Some of the factors include: substrate quantity and quality, soil temperature, soil moisture, O_2 and N concentrations, soil texture, and pH [118]. Soil respiration decreases under saturated or dry conditions and the biological activity doubles for every 7 °C rise in temperature until the optimum temperature is reached (varies between organisms) [122]. The most efficient soil organic matter decomposers are aerobic; thus, soil respiration rates decline as soil oxygen concentrations decrease [118,122].

Stres *et al*. [123] looked at the relationship between soil water content and microbial activity and reported that microbial communities are stable and do not respond significantly to seasonal changes in soil conditions. Stres *et al*. [123] and Yiqi and Zhou [118] reported that air drying increased soil fertility (nutrients), and decreased the number of microorganisms. They found that that CO_2 efflux is curvilinear to soil moisture. Soil microbial communities can adapt to stringent environment conditions of the terrestrial life, where stress from repeated cycles of precipitation and drought have created highly unpredictable environments [124]. Microbial activity is reduced under critical levels of moisture, resulting in desiccation-resistant dormant stages such as spores or cysts in some species [118].

Anderson [125] stated that soil fungi, with extensive multicellular networks of hyphae, produce hyphal strands that bridge across air-filled pores and can tolerate greater water stress compared to bacteria. The study also demonstrated that amoebae, and common protists in soils, encyst at low levels of soil moisture, but rapidly excyst under favorable conditions when sufficient moisture is present. Raich and Schlesinger [126] showed that soil CO_2 efflux is usually low under dry conditions due to low root and microbial activities, it increases with soil moisture. In very high soil moisture condition, CO_2 efflux is reduced due to limitation of diffusion of oxygen and suppression of CO_2 emissions [118,125,126]. Overall, a linear relation between global soil respiration and mean annual precipitation was observed. Review of different studies suggests that patterns of precipitation and soil moisture are likely to have a significant effect on soil microbial community and their respiratory rate.

Yiqi and Zhou [118] showed that roots contribute 7 to 17% of total respiration, litter 6 to 16%, and soil microbes 67 to 80% in grasslands. Likewise, other studies on forest ecosystems

revealed that 35% of soil respiration was from roots, 48% from litter, and 17% from soil [118,127]. Yiqi and Zhou [118] estimated annual soil CO_2 efflux to be 452 gC/m^2yr^1 (grams of carbon per unit of area per unit of time) in tall grass prairies by applying a temperature-respiration regression to continuous temperature records [118]. This was consistent with other analyses of samples from grassland that estimated annual CO_2 efflux to be between 357 and 421 gC/m^2yr^1 based on monthly averages of soil respiration [118,127]. In tropical forests, the estimated annual release of CO_2 was about 1000 gC/m^2yr^1 [128,129]. CO_2 released at the soil surface depends on CO_2 gradient and is strongly affected by wind gusts, turbulences, and fluctuations in atmospheric pressure [118,127–129].

Knowledge of sampling sites and characteristics of nearby soils also become very important when evaluating respiration [130]. Soil color provides some help when interpreting the amount of organic matter and respiration rates [2,118]. For example, a very black soil generally contains more organic matter (dead plants and animal matter) than a brown soil [2,6]. Lighter color soil with high respiration rate is indicative of soil being depleted of organic matter [131]. A relatively darker soil with the same respiration rate is considered healthy. Water and air move through reddish soils easily and this red color is from the oxidation of Fe [2,131]. In grey or blue-grey soils, water and air movement is slow [131]. This color also reflects that soil is old and tired out, and has very little organic matter [2,131].

Addition of organic materials generally increases soil respiration [118]. Organic matter provides food or substrate on which heterotrophic soil microbes feed [2,6]. Organic materials with low C/N ratio (high nitrogen content) decompose quickly, thus the addition of these materials to soil will increase soil respiration [132]. Materials with a high C/N ratio decompose

more slowly but provide a more stable, long term supply of organic material than legumes, biosolids, and manures [122]. Further, soil microbes compete with plants for N when soil is amended with compounds having C/N ratios higher than 25/1 [122]. Stable soil aggregates increase active organic matters and protect from rapid microbial decomposition [2,6]. Agroecological practices that increase soil moisture, soil temperature, and optimal aeration accelerate soil organic matter decomposition [118,122,132].

Overall, soil respiration is generally positively correlated with soil organic matter content, microbial biomass and activity. However, this correlation is not always significant when soils are polluted with metals [133]. The reasoning is that metals can reduce soil respiration by killing microorganism or forming complexes with the substrates [134]. Shermati and Varma [9] found a significant decrease in CO_2 release in metal contaminated soil. Other studies reported an increase of CO_2 production in metal polluted soils [135,136]. These differences among studies may be due to variations in the levels of metal contamination, source of contamination, period over which the responses were monitored, and soil characteristics.

There are several methods used to measure soil respiration. Solvita soil test is the most cost efficient method for estimating soil respiration [137,138]. Soil is analyzed using a CO_2-burst (Haney-Brinton) test [138]. This procedure is a standard soil protocol which is listed by the ALP (Agricultural Lab Proficiency) program and the NAPT (North American Proficiency Testing) program. The test is easy to perform and accurately measures CO_2 respiration. The test further helps to 1) determine how well a soil is provided with active SOM, 2) estimate potential N and P mineralization potential, 3) measure or evaluate the C flux from surface soils, and 4) estimate soil microbial biomass [138].

1.7.2 Soil Enzymatic Activity

Enzymes are vital activators that play a substantial role in maintaining soil health and its environment [117]. Soil enzymes are synthesized by microorganisms, and act as biological catalysts that facilitate different reactions and metabolic processes [139,140]. They play a key biochemical function in the overall process of organic matter decomposition in the soil system [117,141]. Soil enzymatic activities vary because of the quality and quantity of SOM content, composition, and activity of its microbial community as well as intensity of biological processes [117,141]. They are often used as indices for microbial growth and activity [117]. Major soil enzymes include arylsulfatases, chitinase, cellulose, dehydrogenase, glucosidase, phosphatase, protease, and urease released from plants, animals, and microorganisms [117].

Research has demonstrated that terrestrial ecosystems, plants and microorganisms compete for resources but are mutually dependent on each other [142]. Soil microbes need labile organic compounds from plants to mineralize nutrients from organic to inorganic forms [143]. Plants lack biochemical pathways and enzymes necessary to breakdown/convert organic compounds to obtain nutrients [143]. Therefore, they rely on nutrient supply mediated by soil microorganisms. For example, for N cycling, plants are only capable of using inorganic N (ammonia: NH^{+4} and nitrate: NO^{-3}) [142]. They need ammonia to build amino acids, but most N in the atmosphere is in the form of N_2. Plants lack nitrogenase enzyme to convert gaseous N to ammonia. Some microorganisms (N_2 fixing bacteria) are capable of converting organic N into inorganic N [142–144].

Sulfur is found in two forms in soil: 1) sulfate-esters and 2) sulfonates [145,146]. These forms of oragno-S are not directly available to plants and many bacteria and fungi are capable of

mineralizing S from sulfate-esters [146]. Sulfonates are mobilized exclusively by bacterial multicomponent mono-oxygenase enzyme [145,146]. Soil S cycling involves complex interactions between several free living and symbiotic root associated microbial populations [146]. Like S and N, soils contain a large amount of total P but only a small portion is readily available for plant uptake. Plants obtain P from soils in the form of orthophosphate anions (HPO_4^{-2} and $H_2PO_4^{-1}$) [144]. Phosphatases are required for the mineralization of organic P [144]. Therefore, microorganisms are essential to plant growth as they enhance their ability to acquire nutrients from soils.

Enzymatic activities are frequently used to determine the influence of various pollutants on soil microbial quality [139,141]. Reports have shown that soil enzymes are inhibited by metals to different extent depending on soil structure and chemistry [117,141]. In addition, soil enzyme inhibition also depends on the nature and concentration of metals and its extent varies from one enzyme to another. Some metals stimulate enzymatic activities [139,141,147]. Reduction in soil microbes and the inhibition of soil enzyme activities caused by metals contamination negatively affect soil fertility [139,141].

Metals inhibit enzyme activities by interacting with substrate complexes, denaturing enzyme protein and interacting with its active site [139,141]. Cu inhibits β-glucosidase activity more than it does with cellulase activity [141]. Belyaeva *et al*. [148] showed that activities of urease, catalase, and acid phosphatase were significantly decreased in soil due to Pb contamination. Lorenz *et al*. [149] reported that As contamination significantly affected arylsulfatases activity whereas Cd contamination had a negative effect on the activities of protease, urease, alkaline phosphatase, and arylsulfatases. Overall, studies have reported a drastic

decrease in enzyme activities as the concentration of metals increases [117,141,150,151]. The relative toxicities of metals towards enzymes were found to be: Cd≈Cu>Pb [151].

It is established that extracellular enzymes are inactivated by metals. This mechanism involves binding of metals to amino acids in the enzymes which indirectly reduces the amount of microorganisms involved in enzyme production [141]. For example, cellulose binds to cellulase in the region of cellulose binding domain (CBD). Cu forms complexes with tryptophan in the CBD, resulting in the inhibition of cellulase [141]. Also, it is well documented that metals react with sulfhydryl groups of enzymes and thereby inhibit and/or inactivate enzymatic activities [139]. Lorenz *et al*. [149] reported that binding of Cd to sulfhydryl group decreased enzymatic activities. Karac *et al*. [141] showed that amidase activity was not strongly inhibited by Zn and Cu, whereas, these metals inhibited urease and nitrate reductase activities [141].

Enzyme activities are sensitive to changes in pH [117,141]. Significant increase in soil pH by lime application stimulates microbial population and diversity, resulting in an increase in soil enzyme activities and thus, affecting nutrient cycling [109]. The exception is acid phosphatase which is sensitive in alkaline soils [109,141].

1.8 Rationale

Physical, chemical, and biological properties of soils are indicators of their quality. Soil fertility on the other hand is determined by its biological activity. Soil provides natural habitat for the survival of microorganisms which require favorable physical and chemical conditions for their optimal function. Imbalance of soil microorganisms, nutrient deficiency and changes in physiochemical properties (e.g. contamination, decrease in pH) result in decreased soil quality and fertility.

To date, studies have provided information on landscape degradation, soil toxicity, acidification, plant metal accumulation, plant genetic diversity, and forest composition in Northern Ontario but knowledge on soil health and microbial community is lacking. In addition, the effect of metal contamination and liming on microbial (bacterial and fungal) biomass, relative abundance and diversity in the GSR needs to be investigated. Continued monitoring of soils are essential in understanding ecosystem recovery following the reduction of emissions from smelters and the establishment of a reforestation program.

1.9 Objectives

The main objective of this study was to assess the long term effect of liming and metal contamination on the belowground components of the ecosystem. The specific objectives were:

1) To determine the effects of long-term soil metal contamination on bacterial and fungal diversity and abundance using PFLA and pyrosequencing analyses

2) To assess the long term effects of liming on: a) soil microbial biomass and composition (bacteria, fungi, and actinomycetes), and b) bacterial and fungal population dynamic and diversity in reclaimed ecosystems.

3) To assess the effect of liming and metal contamination on soil enzymatic activities and respiration.

We hypothesize that metal contamination will decrease microbial biomass, abudance, diversity and their enzymatic activity. For liming, an increase in these parameters will be observed.

CHAPTER 2: BACTERIAL AND FUNGAL COMMUNITY STRUCTURE AND DIVERSITY IN THE GREATER SUDBURY REGION UNDER LONG-TERM METAL EXPOSURE REVEALED BY PHOSPHOLIPID FATTY ACID AND PYROSEQUENCING ANALYSIS

2.1 Abstract

Metals are known to alter soil ecosystem diversity, structure and function. The main objective of the present study is to determine the effects of soil metal contamination on bacterial and fungal biomass, relative abundance, and diversity based on phospholipid fatty acid (PLFA) and pyrosequencing analyses. Soil samples from six sites from Northern Ontario (Canada) were analyzed. Chemical analysis showed significant difference between metal contaminated and reference sites for pH, cations exchange capacity (CEC), soil organic carbon (C) and nitrogen (N). Significant differences between metal contaminated and uncontaminated reference sites were observed for total bacteria, arbuscular fungi (AM fungi), other fungi and eukaryotes. *Acidobacteria* and *Proteobacteria* were the most dominant bacterial taxonomic groups in all the sites. For fungi, *Ascomycota* were more prevalent in metal contaminated soils (35.07%) while *Basidiomycota* represented 59.26% of all fungi in reference areas. Site-specific bacterial and fungal families and genera were identified and characterized. Analysis of bacterial communities revealed Chao1 index values of 232 and 273 for metal contaminated and reference soils, respectively. For fungi, the Chao index values were 23 for metal contaminated and 45 for reference sites. OTUs followed the same trend for both bacteria and fungi. No significant differences were observed for Simpson index, Shannon index and species evenness between two soil groups for bacteria and fungi. Overall, PLFA and pyrosequencing analyses revealed significant reductions of microbial biomass and relative abundance in contaminated sites compared to reference soil type. No significant variations in microbial diversity were observed when all the sites were compared.

Keywords: Microbial biomass and diversity, Phospholipid fatty acid (PLFA), Pyrosequencing, Metal contamination, Northern Ontario

2.2 Introduction

While the effects of many factors such as invasive species, habitat diversification, climate change, pollution and temperature on the biodiversity of plants and animals are well established and studied, little attention has been paid to soil microbial communities. Soil contains enormous microbial diversity, with an estimated 10^7–10^9 distinct bacterial species [152] and 1.5 million fungi taxa [153] worldwide. Nowadays, an ever increasing rate of species extinction is resulting in destructive consequences for ecosystem functions and will also limit the potential economic benefits of biodiversity [35,40,154,155].

Mining related activities have resulted in severe land disturbances throughout the world. For nearly a century, logging, smelting and mining activities have caused severe negative effects to the environment in the Greater Sudbury Region (GSR) in Northern Ontario, Canada [15,27,29,38,40,156]. This region was reported to be the greatest single source for sulfur dioxide (SO_2) emissions in Canada [15,27,29,156]. In addition, Sudbury smelters released into the atmosphere large quantities of metals including iron (Fe), nickel (Ni), and copper (Cu). In fact, Falkowski [18] reported that, over 14,000 tonnes of Fe, 2000 tonnes of Ni, and 1800 tonnes of Cu were released annually into the atmosphere.

These metals and SO_2 emissions have caused damage to plants, animals, and soil microorganisms. Severe contamination and acidification of soils and water at sites around smelters have been documented [15,27,38,40]. In the last 40 years, SO_2 and metal emissions have been reduced drastically through a combination of developments in industrial technologies (safer extraction methods and better filtration) and legislated controls in the GSR [15,38,40]. This has resulted in an improvement in atmospheric quality and natural recovery of damaged ecosystems.

In addition, land reclamation is being implemented by a regional regreening program. It consists of limestone application to soils, seed distribution and tree planting (with over 12 million trees planted so far in many areas of the GSR) [15,40]. This has led to an increase of plant species diversity, soil organic matter, and microbial biomass [15,33,40].

Although some metals are required for life's physiological processes, excessive accumulation in living organisms is always detrimental. A sustainable ecosystem depends on functional microbial communities, which play a significant role in organic matter decomposition, degradation of toxic substances, nitrogen fixation, nutrient cycling, production of phytohormones as well as plant health and growth [35,40,99,155]. Knowledge of the interaction between metals and soil microorganisms is very limited.

There is no consensus on the effects of metals on microbial diversity and abundance. Guo *et al*. [157] reported no impact on bacterial diversity in soils from two abandoned copper mines contaminated with metals using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. Other studies have shown that both short-term and long-term exposure to metals results in the reductions of soil microbial biomass, diversity (based on PCR-DGGE) and its activity [158–160]. It is also established that metals at high concentrations cause enzyme inactivation and damage cells by acting as antimetabolites or by forming precipitates or chelates with essential metabolites in microorganisms [161]. Analyses of microbial diversity in metal contaminated samples based on a sensitive and detailed high throughout sequencing analysis are sketchy. Chodak *et al*. [162] analyzed bacterial diversity in forest soil with different degrees of heavy metal pollutions and found no effects of heavy metals on the soil bacteria structure measured by pyrosequencing. Golebiewski *et al*. [110] found that

zinc (Zn) decreased bacterial diversity and species richness based on 16S rDNA pyrosequencing. Data on damages caused by nickel (Ni) and copper (Cu) to soil microbial communities are sketchy. Likewise, studies on the effects of metals on fungi diversity and structure are lacking.

We hypothesized that metal contamination (as due to mining activities such in the GSR) imposes distinct impacts on the microenvironment in which microorganisms (specifically bacteria and fungi) exist and that variation in the microbial community structures would be associated with the changes in the physiochemical properties of the soils. The main objective of the present study is to determine the effects of long-term soil metal contamination on bacterial and fungal diversity and relative abundance using PFLA and pyrosequencing analyses.

2.3 Materials and Methods

2.3.1 Study Site and Soil Sample Collection

The study was carried out at six locations close to mining sites in Sudbury, Northern Ontario, Canada. This region has been exposed to abundant deposition of metals, notably Ni and Cu, emitted through active smelting for over a century. The soils of the Sudbury area are mostly tills, podzols and brunisols and formed in sandy fluvial and glaciofluvial deposits [163]. Six sites were selected from Northern Ontario (Fig. 1). Sites were classified as metal contaminated and reference based on data from previous soil physico-chemical analyses [25,38,40]. Metal contaminated sites include Laurentian, Kelly Lake, and Kingsway. Reference sites include Onaping Falls, Capreol, and Killarney. The GPS coordinates of these sites are given in table S1. No specific permissions were required for soil sampling at these locations since the sites are part of Laurentian University research areas and crown (public) lands that are not within a park or a conservation reserve.

Major environmental conditions such as temperature, and rainfall were presumed similar among the sites based on literature [163–165] while metal, organic matter contents, and cation exchange capacity (CEC) may vary [25,38,40]. Metal contaminated sites were characterized as sandy/clay soil rich in total Ni and Cu. Each site was approximately 5 km^2 in size. At each site, 20 samples were collected randomly from the organic layer (0–5 cm in depth). Plant material, stones and residues were removed; and soil samples from each site were bulked and mixed. They were sieved using a 2 mm mesh and stored for a short period prior to analyses (in an incubator for soil chemical analyses and in a freezer for PLFA analysis). DNA from the soil samples were extracted the same day (within hours) after sampling.

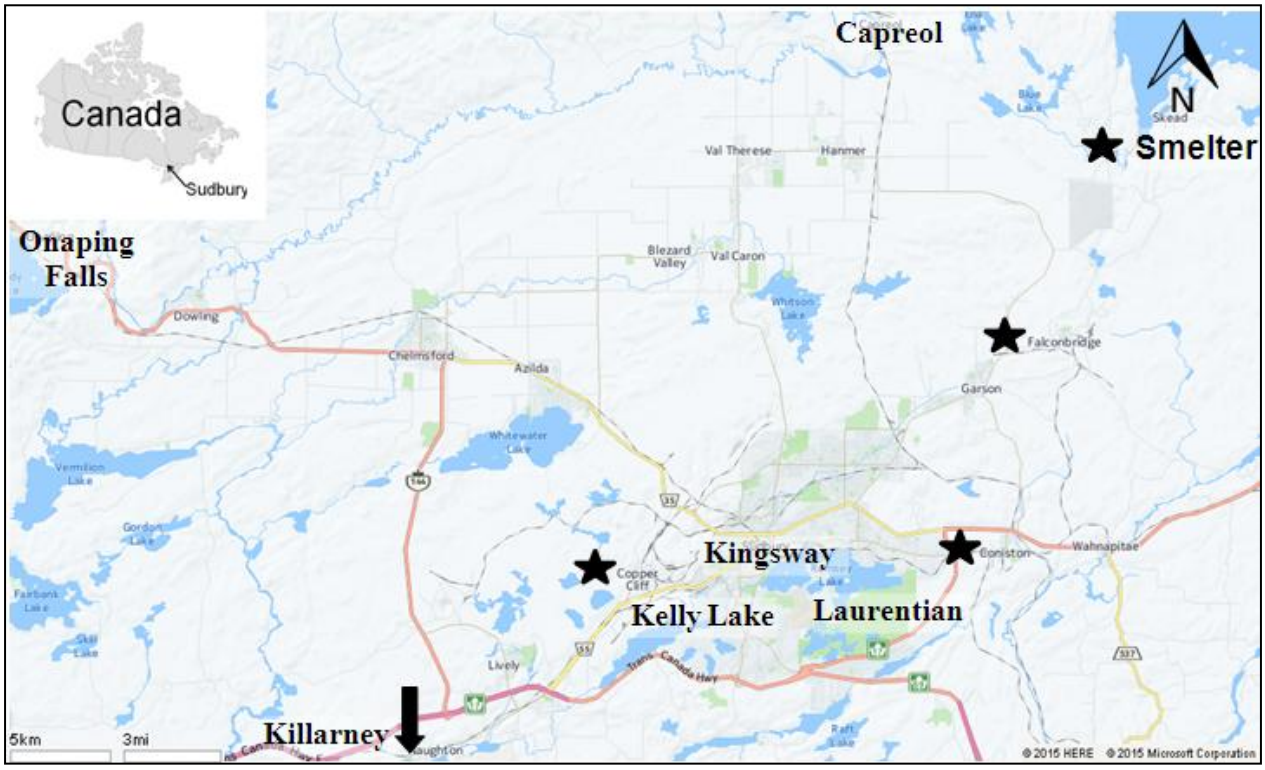


Figure 1: Geographical locations of the sampling area from the Greater Sudbury Region (GSR) in Northern Ontario.

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney

2.3.2 Soil Chemical Analysis

Soil pH was determined on air dried subsamples in deionized water and in neutral salt solution (0.01 M $CaCl_2$) [166]. CEC was measured using an ammonium acetate extraction method at pH 7 developed by Lavkulich [167]. CEC is a measure of the quantity of readily exchangeable cations neutralizing negative charge in the soil. The exchangeable cations aluminum, Al^{3+}; calcium, Ca^{2+}; iron, Fe^{3+}, potassium, K^{+}; magnesium, Mg^{2+}, manganese, Mn^{2+} and sodium, Na^{+} were quantified by inductively coupled plasma mass spectrometry (ICP-MS). The total exchange capacity was estimated as the sum of the exchangeable cations [168]. Total concentration of metals were measured after digestion of 0.5 g of soil samples with 10 ml of 10:1 ratio of HF/HCl at 150 °C [25]. Bioavailable metals were assessed after shaking 5 g of soil samples with 20 ml of 0.01 M $LiNO_3$ for 24 h at 20 °C followed by filtration of extracts [25]. Total and bioavailable metals were detected using ICP-MS.

2.3.3 Phospholipid Fatty Acid Analysis

PLFA analysis was performed at FAME Lab, Microbial ID. Inc, Newark, Delaware (USA) as described by Buyer and Sasser [86]. Mole percentage of each PLFA was used to indicate the relative abundance of bacteria (gram positive and gram negative bacteria), arbuscular mycorrhizal (AM) fungi and other fungi in soil. Total PLFA extracted from soil was used as an index of living microbial biomass [40,86].

2.3.4 Microbial DNA Extraction and Purification

Microbial DNA was extracted from 10 g of fresh soil per site by using the PowerMax® Soil DNA Isolation Kit for soil (MO BIO Laboratories, Inc., Carlsbad, CA, USA, cat # 12,988–10), according to the manufacturer's instructions. The concentration and purity of the extracted

DNA were determined with a fluorescent DNA quantification kit (Bio-Rad Laboratories, Hercules, CA, USA, cat # 170-2480) following the procedure described by the manufacturer. Finally, extracted DNAs were diluted to concentration of 2 ng/µl for all samples prior to pyrosequencing analysis that was performed at Molecular Research DNA laboratory (MR DNA, Shallowater, Texas, USA).

2.3.5 PCR Amplification and Pyrosequencing

Bacterial and fungal microbiotas were assessed using high throughout sequencing of 16S and internal transcribed spacer (ITS) genes. Tag-encoded FLX-titanium 16S rDNA gene amplicon pyrosequencing (bTEFAP) was performed using 16S universal eubacterial primers 530F (5′ GTG CCA GCM GCN GCG G) and 1100R (5′ GGG TTN CGN TCG TTR) for amplifying the 600 bp region of 16S rDNA genes [108]. Fungal tag-encoded FLX amplicon pyrosequence (fTEFAP) was performed using ITS specific primers ITSF (5′ TCC GTA GGT GAA CCT GCG G) and ITSR (5′ TCC TCC GCT TAT TGA TAT GC) to amplify 600 bp fragments of the ITS region [103]. Generation of the sequencing library utilized a one-step PCR with a total of 30 cycles, a mixture of hot start and Hotstar high fidelity Taq polymerases as described by Dowd *et al*. [108]. Tag-encoded FLX amplicon pyrosequencing analyses utilized a Roche 454 FLX instrument with titanium reagents. This bTEFAP and fTEFAP process was performed at the MR DNA Laboratory based on established and validated protocols.

2.3.6 Data Processing

Sequences were processed using a proprietary analysis pipeline (MR DNA) for analyzing 16S rDNA data. Barcodes and primers were removed from the sequences. Additionally, sequences less than 200 bp, sequences with ambiguous base calls and homopolymer runs

exceeding 6 bp were removed. Also, sequences were denoised, and chimeras and singleton sequences were deleted. Filtered sequences were clustered into OTUs (Operational Taxonomic Units) at 97% sequence similarity cut-off. These OTUs were taxonomically classified using BLASTN (Basic Local Alignment Search Tool for Nucleotides) against a curated GreenGenes database and compiled into each taxonomic level.

2.3.7 Statistical Analysis

Data was tested for normality using Shapiro-Wilk test. All statistical analyses were performed with SPSS version 20 statistical software (SPSS, Chicago, IL, USA) for windows. Metal content, CEC and pH data were subjected to a t-test. A Pearson r correlation coefficient was used to determine association between soil pH and CEC as well as between pH and exchangeable cations ($p \leq 0.05$). Microbial biomass (total PLFA), abundance and composition (bacterial and fungal PLFAs) were also analyzed by t-test.

t-test was also used to determine differences between bacterial and fungal species relative abundance. Alpha diversity (within each site) and beta diversity (between sites) were analyzed. Alpha diversity includes community diversity (Shannon and Simpson diversity indices), evenness (Shannon equitability index), and richness (abundance based coverage estimator, Chao1). Differences in the frequency distribution for bacterial and fungal taxonomies were determined between metal contaminated and reference soils based on a non-parametric (Kolmogorov-Smirnov test).

Beta diversity (pair wise comparisons) among sites was analyzed using Jaccard index (similarity index, based on the presence/absence data). In addition, Whittaker index that is a dissimilarity index determined based on abundance or relative abundance. Beta diversity was also

estimated by computing weighted UniFrac distances among sites. Weighted UniFrac is a qualitative variant that is widely used in various microbial studies as it accounts for relative abundance of observed organisms.

2.4 Results

2.4.1 Characteristics of the Soil Samples

Results of metal analysis are described in Figs. 2 and 3. As expected, there were significant differences ($p \leq 0.05$) between metal contaminated and reference sites for arsenic (As), Cu, Ni, and zinc (Zn). The amounts of total metals in reference sites were small and consistent with the levels found in other uncontaminated soils in Canada (Fig. 2). The highest level of metal content was observed in metal contaminated sites. Soil pH ranged from 4.50 to 5.30 in the top organic layer. In addition, differences in pH were observed between all the metal contaminated sites and the reference sites (Table 2). The highest level of CEC was observed in the reference soil and lowest in metal contaminated soils (Table 2). This was a reflection of the amount of exchangeable Ca^{2+}, Mg^{2+}, and K^{+} in the two soil types. The values of exchangeable Na^{+} were low in both the metal contaminated and reference sites (Table 2).

The sum of exchangeable cations was high in reference sites and low in metal contaminated sites (Table 2). Overall, positive correlations between soil pH and CEC ($r = 0.78$; $p \leq 0.05$) and between soil pH and exchangeable cations ($r = 0.67$; $p \leq 0.05$) were observed. Organic matter content was significantly lower ($p \leq 0.05$) in metal contaminated compared to reference sites (Table 2).

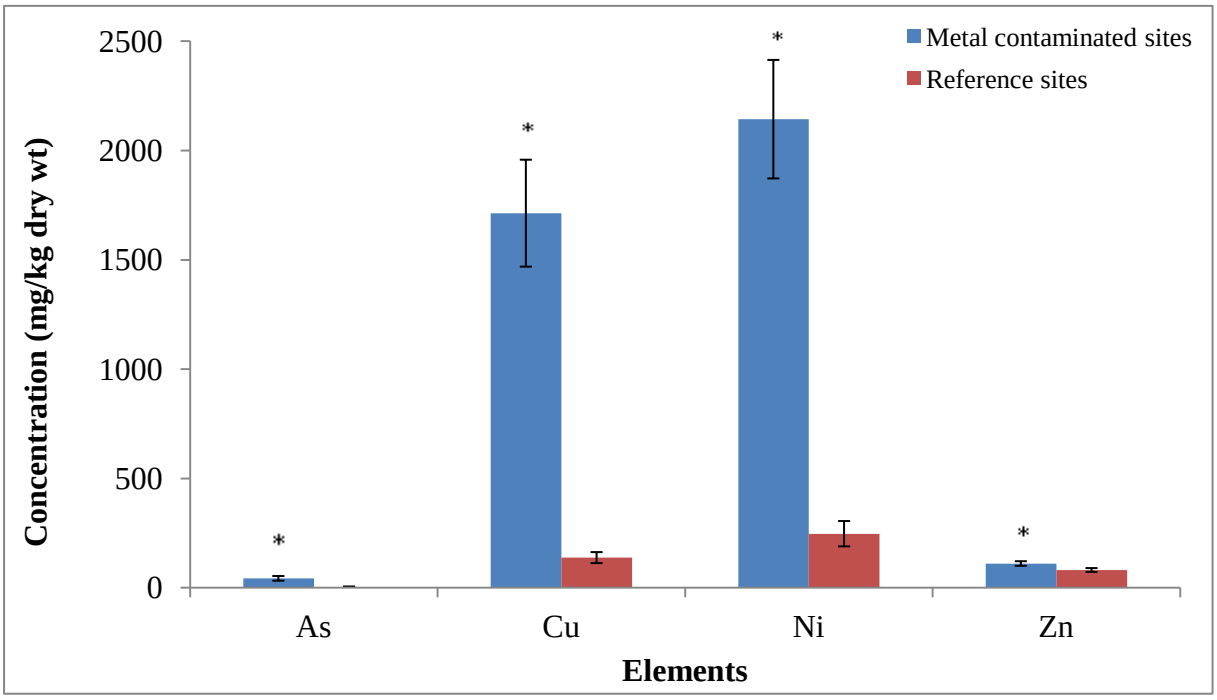


Figure 2: Total metal concentration (mg/kg dry wt) of elements in soil from the GSR in Northern Ontario.

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

Sites are grouped based on metal content: 1) Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, 2) Reference sites: Onaping Falls, Capreol and Killarney

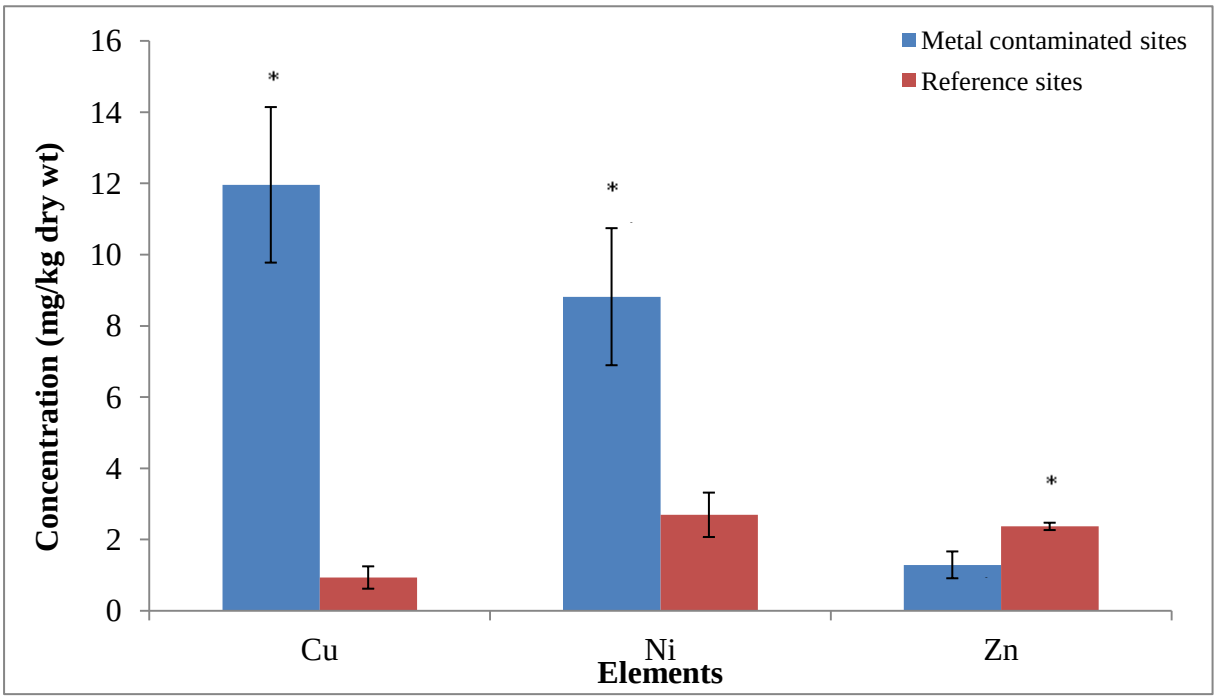


Figure 3: Bioavailable metal concentration (mg/kg dry wt) of elements in soil from the GSR in Northern Ontario.

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

Sites are grouped based on metal content: 1) Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, 2) Reference sites: Onaping Falls, Capreol and Killarney

Bioavailable Arsenic (As) concentration was below detection limit for all the sites

Table 2: Mean values of basic cations (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}), sum of cations, CEC, pH and total C and N in the Greater Sudbury Region.

Characteristics	Metal contaminated sites	Reference sites
Ca^{2+} (cmol/kg)*	0.94 ± 0.36	4.38 ± 0.30
Mg^{2+} (cmol/kg)	0.29 ± 0.15	0.71 ± 0.22
K^{+} (cmol/kg)	0.15 ± 0.05	0.25 ± 0.04
Na^{+} (cmol/kg)	0.02 ± 0.01	0.01 ± 0.00
Sum of cations (cmol/kg)	1.39	5.35
CEC (cmol/kg)*	2.20 ± 1.06	6.20 ± 2.01
pH (H_20)*	4.50 ± 0.16	5.30 ± 0.12
pH (0.01M $CaCl_2$)*	3.90 ± 0.08	4.80 ± 0.08
Total organic C (mg/kg)*	129750.00 ± 7932	257333.33 ± 3975
Total organic N (mg/kg)*	5688.33 ± 1277	9490.00 ± 1344
Total organic C/N	22.17	27.12

Results are expressed as mean values ± standard error

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

CEC: cation exchange capacity; C: carbon and N: nitrogen

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney

2.4.2 Phospholipid Fatty Acid Analysis

Results of the PLFA analysis are described in Table 3. The analysis revealed significantly high ($p \leq 0.05$) total microbial biomass in reference soil samples compared to metal contaminated soil (Table 3). The same trend was observed for the abundance of bacteria, AM fungi, other fungi and other eukaryotes (Table 3).

There were twice more gram negative than gram positive bacteria in reference sites. Similar trend was observed in metal contaminated sites. Overall, there were more bacteria than fungi in the soil samples analyzed (Table 3). In fact, bacteria represent 64.62% and 69.68% in metal contaminated and reference sites, respectively. These values were only 13.16% (metal contaminated) and 16.15% (reference sites) for fungi biomass.

Palmitic acid (16:0) was the most prevalent (13.96%) of all the fatty acids identified in all the soil samples. In addition to palmitic acid, other common and abundant fatty acids were 15:0 (2.42%), i15:0 (7.18%), i16:0 (2.10%), 16:1ω5c (2.63%), 16:1ω7c (6.65%), 10Me16:0 (3.97%), cy17:0ω7c (2.87%), 18:0 (2.42%), 18:1ω7c (8.66%), 18:1ω9c (10.83%), 18:2ω6c (8.07%) and cy19:ω7c (9.82%). These fatty acids were present in all the samples and made up 79.93% and 81.93% of total fatty acid content in the metal contaminated and reference soil samples, respectively. Also, several fatty acids representing bacteria (15:0, i15:0, a15:0, c17:0, i17:0, a17:0, c19:0), fungi (16:1ω5, 18:1ω7, 18:1ω9c, 18:2ω6c) and actinomycetes (10Me16:0, 10Me17:0, 10Me18:0) were present in all soil samples. Gram positive bacteria include many branched PLFAs such as 17:0 and 18:0, or iso- and anteiso-branched PLFAs, like i15:0, i16:0, i16:1 and i17:0. Fatty acids 16:1w5, 16:1w9, 17:1w9, cy17:0, 18:1w7and cy19:0 are used as indictors of gram negative bacteria.

Table 3: Microbial organisms identified using phospholipid fatty acid (PLFA) analysis in soil samples from the GSR. Data in ng/g.

Sites	Total Microbial Biomass*	AM Fungi*	Other Fungi*	Gram Negative*	Gram Positive*	Other Eukaryote*	Anaerobe	Actinomycetes
Metal contaminated sites	160.28	6.10	18.34	64.09	42.45	5.09	2.13	31.62
	±51.19	±1.67	±9.92	±7.42	±6.30	±1.82	±1.05	±5.29
Reference sites	422.89	16.37	52.24	200.08	93.98	17.44	4.99	37.79
	±50.71	±1.81	±10.04	±12.66	±11.03	±4.35	±0.84	±4.16

Results are expressed as mean values ± standard error

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney

Monounsaturated 18 carbon fatty acids (18:1ω7c and 18:1ω9c) were mostly enriched in reference soil samples compared to metal contaminated soil. Reference soils had higher abundances for the majority of monounsaturated fatty acids (16:1ω5c, 17:1ω9c, 18:1ω7c and 18:1ω9c), cy19, branched fatty acids (i14:0, i15:0, a15:0, i17:0 and a17:0), straight chain fatty acids (14:0, 15:0, 16:0, 17:0 and 20:0), cy17, and other 10Me fatty acids compared to metal contaminated sites.

2.4.3 Pyrosequencing Analysis

Gene based analysis identified various bacterial and fungal taxa illustrated in Tables S3 and S6. Statistical analysis revealed significant differences in relative abundance of bacterial and fungal species between the two soil types (Tables S3 and S6). The reads generated in this project have been deposited in the NCBI Short Read Archive database (Accession number: SRP071853).

2.4.3.1 Bacterial Community Composition and Diversity Analysis

A total of 131 bacterial species were identified from all the soil samples analyzed (Table S3). Further analysis revealed some species that were found only in certain soil group. Overall, 18 species were identified only in metal contaminated soils and 35 in reference soils (Table S2). All the bacteria identified belong to 15 phyla and four *Proteobacterial* classes. The most dominant phyla across all samples were *Acidobacteria* (39.10%), *Proteobacteria* (44.06%), *Actinobacteria* (5.43%), *Chloroflexi* (4.94%) and *Firmicutes* (3.55%). *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were the most abundant *Proteobacteria* in both soil types. *Chloroflexi* and *Firmicutes* were the least abundant of the five phyla in the soil samples analyzed (Table 4). *Armatimonadetes*, *Bacteroidetes*, *Candidate division*,

Cyanobacteria, *Gemmatimonadetes*, *Lentisphaerae*, *Planctomycetes*, *TM6*, *TM7* and *Verrucomicrobia* were rare phyla identified representing globally <1% of all identified phyla.

At the genus level, comparison of the relative abundance revealed significant differences ($p \leq 0.05$) between metal contaminated and reference soils. *Acidobacterium* was the most abundant genus across all soil samples, representing 18.96% in metal contaminated and 15.08% in reference soil. The distribution of other dominant genera *Afipia*, *Aquicella*, *Candidatus koribacter*, *Candidatus solibacter*, *Geobacillus*, *Granulicella*, *Nitrosococcus*, *Rhodoplanes*, *Skermanella*, *Thermosporothrix* and *Thioalkalispira* varied significantly between the metal contaminated and reference soils ($p \leq 0.05$). *Afipia*, *Candidatus solibacter*, *Granulicella*, *Nitrosococcus* and *Rhodoplanes* showed a higher relative abundance ($p \leq 0.05$) in reference soils compared to metal contaminated soil samples. The opposite trend was observed for the relative abundance of *Aquicella*, *Candidatus koribacter*, *Geobacillus*, *Skermanella*, *Thermosporothrix* and *Thioalkalispira* that was higher ($p \leq 0.05$) in metal contaminated soil than in reference sites. Several bacterial genera known for their roles as human and animal pathogens were also identified, including *Escherichia*, *Mycobacterium*, *Roseomonas* and *Sphingomonas*. Figs. 4–6 describe specific classes, families and genera identified in the two site groups. No differences in the frequency distribution for bacterial taxonomies (class: $p = 0.83$; family: $p = 0.31$ and genera: $p = 0.10$) were observed when metal contaminated and reference soil samples were compared using Kolmogorov-Smirnov test (non-parametric test).

At the species level, 96 bacterial species were identified in metal contaminated soil samples and 113 bacterial groups in reference soil samples (Table S3). Differences in relative abundance among the two soil types were observed for some bacterial groups (Table S3). *Achromobacter*

spp., *Acidicaldus* spp., *Acidisphaera* spp., *Acidobacterium* spp., *Acidocella* spp., *Acidothermus* spp., *Azospira* spp., *Caldanaerobacter thermoanaerobacter tengcongensis*, *Caulobacter* spp., *Conexibacter* spp., *Ferruginibacter* spp., *Filomicrobium* spp., *Labrys* spp., *Methylosinus trichosporium*, *Mycobacterium riyadhense*, *Planctomyces* spp., *Sorangiineae* spp., *Thermosporothrix* spp., *Thioalkalispira* spp. and *TM7* were more abundant in the metal contaminated sites ($p \leq 0.05$) (Table S3) compared to reference sites. Likewise, there were more *Acidimicrobiales* spp., *Afipia felis*, *Arenimonas* spp., *Blastochloris* spp., *Bradyrhizobium* spp., *Candidatus chloracidobacterium* spp., *Candidatus entotheonella* spp., *Candidatus koribacter* spp., *Edaphobacter modestum*, *Inquilinus* spp., *Methylocystis* spp., *Metyhlovirgula* spp., *Phenylobacterium* spp., *Pilimelia* spp., and *Rhodoplanes* spp., in reference soil samples compared to metal contaminated sites ($p \leq 0.05$) (Table S3).

Results of the diversity indices used to compare sites are summarized in Table 5. Chao 1 index values for reference sites were statistically higher than metal contaminated sites (Table 5). Data yielded a total of 543 OTUs of which 399 were identified in metal contaminated sites and 413 in reference sites (Table 5). No differences between the soil types were observed for Simpson index, Shannon index and species evenness (Table 5). Whittaker dissimilarity and Jaccard's similarity pair wise comparisons between soil types were calculated. The average Whittaker dissimilarity between microbial communities was 56.00% and the mean Jaccard's similarity index was 28.00%. In addition, sites were compared with one another using clustering based on weighted UniFrac distances. Low values were observed between individual sites based on distance matrix (Table S4).

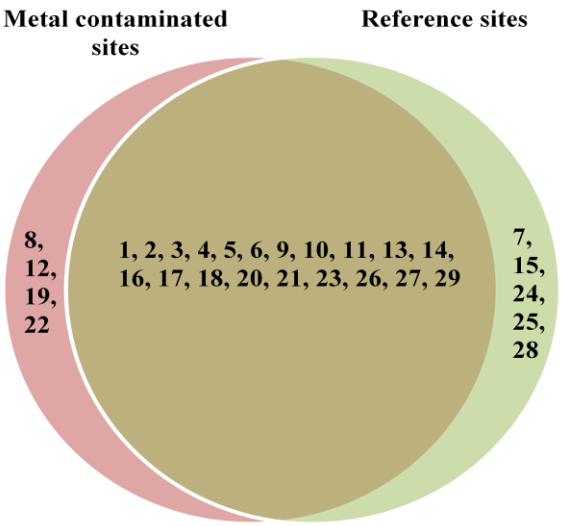
Table 4: Main bacterial and fungal phyla and their relative abundance identified in soil samples from the GSR. Data in percentage.

Phylum	Metal contaminated sites	Reference sites
Bacteria		
Actinobacteria	5.26 ± 1.43	5.60 ± 1.58
*Acidobacteria**	35.08 ± 2.60	43.11 ± 3.08
*Chloroflexi**	9.78 ± 3.20	0.09 ± 0.01
Firmicutes	4.91 ± 2.30	2.18 ± 1.33
Proteobacteria	41.63 ± 3.41	46.49 ± 2.49
Fungi		
*Ascomycota**	35.07 ± 4.69	27.70 ± 4.01
*Basidiomycota**	59.26 ± 4.16	63.88 ± 2.48
Glomeromycota	0.00 ± 0.00	6.06 ± 6.06
*Zygomycota**	6.44 ± 3.81	2.35 ± 1.04

Results are expressed as mean values ± standard error

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

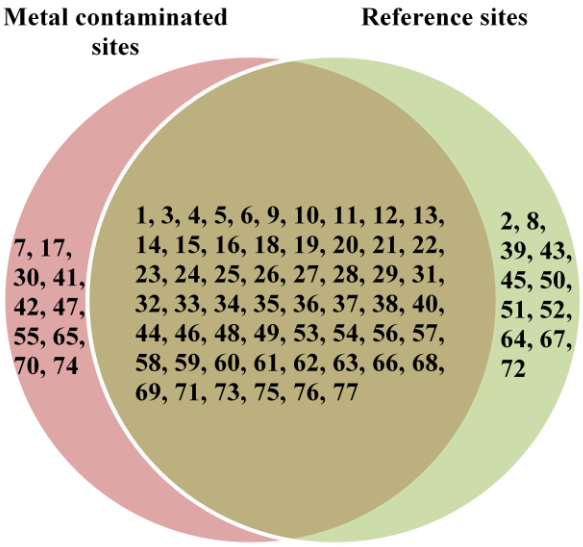
Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney



1. *Acidimicrobiia*
2. *Acidobacteria* (unclassified)
3. *Acidobacteria* (class)
4. *Actinobacteria* (subclass)
5. *Actinobacteria* (class)
6. *Alphaproteobacteria*
7. *Anaerolineae*
8. *Armatimonadia*
9. *Bacilli*
10. *Betaproteobacteria*
11. *Bryopsida*
12. *Caldilineae*
13. Class unspecified
14. *Clostridia*
15. *Cytophagia*
16. *Deltaproteobacteria*
17. *Gammaproteobacteria*
18. *Gemmatimonadetes*
19. *Jungermanniopsida*
20. *Ktedonobacteria*
21. *Opitutae*
22. *Oscillatoriophycideae*
23. *Planctomycetacia*
24. *Spam* (*Candidate division*)
25. *Spartobacteria*
26. *Sphingobacteria*
27. *Thermoleophilia*
28. *TM6* classes
29. *TM7* classes

Figure 4: Venn diagram showing distribution of bacterial classes identified in metal contaminated and reference soil samples from the GSR.

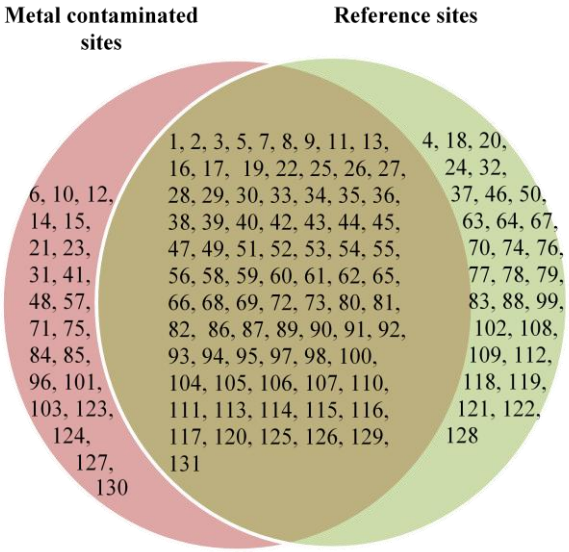
Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.



1. *Acetobacteraceae*
2. *Acidimicrobiaceae*
3. *Acidimicrobiales* families
4. *Acidobacteriaceae*
5. *Acidothermaceae*
6. *Alcaligenaceae*
7. *Anaerolineaceae*
8. *Armatimonadaceae*
9. *Bacillaceae*
10. *Beijerinckiaceae*
11. *Bradyrhizobiaceae*
12. *Brucellaceae*
13. *Burkholderiaceae*
14. *Caldilineaceae*
15. *Candidatus alysiosphaera*
16. *Candidatus chloracidobacterium*
17. *Candidatus chlorothrix*
18. *Candidatus solibacter*
19. *Caulobacteraceae*
20. *Chitinophagaceae*
21. *Chromatiaceae*
22. *Climaciaceae*
23. *Colwelliaceae*
24. *Comamonadaceae*
25. *Conexibacteraceae*
26. *Corynebacteriales* families
27. *Coxiellaceae*
28. *Cryomorphaceae*
29. *Cytophagaceae*
30. *Dermatophilaceae*
31. *Ectothiorhodospiraceae*
32. *Enterobacteriaceae*
33. *Frankiaceae*
34. *Gallionellaceae*
35. *Gemmatimonadaceae*
36. *Hyphomicrobiaceae*
37. *Hyphomonadaceae*
38. *Iamiaceae*
39. *Kineosporiaceae*
40. *Koribacteraceae*
41. *Ktedonobacteraceae*
42. *Lepidoziaceae*
43. *Methylobacteriaceae*
44. *Methylocystaceae*
45. *Microbacteriaceae*
46. *Micromonosporaceae*
47. *Mitochondria*
48. *Mycobacteriaceae*
49. *Nannocystineae* families
50. *Nitrosomonadaceae*
51. *Nitrospinaceae*
52. *Nocardioidaceae*
53. *Opitutaceae*
54. *Oxalobacteraceae*
55. *Phormidiaceae*
56. *Planctomycetaceae*
57. *Pseudonocardiaceae*
58. *Rhizobiaceae*
59. *Rhodobiaceae*
60. *Rhodocyclaceae*
61. *Rhodospirillaceae*
62. *Solirubrobacteriaceae*
63. *Sorangiineae* families
64. *Spam* (*Candidate division*)
65. *Sphingobacteriaceae*
66. *Sphingomonadaceae*
67. *Sporichthyaceae*
68. *Thermoanaerobacteraceae*
69. *Thermodesulfobiaceae*
70. *Thermogemmatisporaceae*
71. *Thermosporotrichaceae*
72. *TM6* families
73. *TM7* families
74. *Victivallaceae*
75. *Xanthobacteraceae*
76. *Xanthomonadaceae*
77. *Xiphinematobacteraceae*

Figure 5: Venn diagram showing distribution of bacterial families identified in metal contaminated and reference soil samples from the GSR.

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.



1. *Achromobacter*
2. *Acidicaldus*
3. *Acidimicrobiales* generas
4. *Acidimicrobium*
5. *Acidiphilium*
6. *Acidisoma*
7. *Acidisphaera*
8. *Acidobacterium*
9. *Acidocella*
10. *Acidomonas*
11. *Acidothermus*
12. *Acidovorax*
13. *Afipia*
14. *Anaerolinea*
15. *Anderseniella*
16. *Aquicella*
17. *Arenimonas*
18. *Armatimonas*
19. *Azospira*
20. *Azovibrio*
21. *Bazzania*
22. *Blastochloris*
23. *Blastomonas*
24. *Bosea*
25. *Bradyrhizobium*
26. *Burkholderia*
45. *Crossiella*
46. *Cupriavidus*
47. *Defluviicoccus*
48. *Dermatophilus*
49. *Dongia*
50. *Dyella*
51. *Edaphobacter*
52. *Escherichia shigella*
53. *Ferruginibacter*
54. *Filomicrobium*
55. *Fluviicola*
56. *Frankia*
57. *Geitlerinema*
58. *Gemmata*
59. *Gemmatimonas*
60. *Geobacillus*
61. *Granulicella*
62. *Haliangium*
63. *Herbiconiux*
64. *Hgci clade*
65. *Hirschia*
66. *Hymenobacter*
67. *Hyphomicrobium*
68. *Iamia*
69. *Inquilinus*
70. *Kineosporia*
89. *Novosphingobium*
90. *Opitutus*
91. *Pedomicrobium*
92. *Phenylobacterium*
93. *Pilimelia*
94. *Planctomyces*
95. *Pleomorphomonas*
96. *Polaromonas*
97. *Pseudochrobactrum*
98. *Pseudolabrys*
99. *Pseudonocardia*
100. *Rhizobium*
101. *Rhodobium*
102. *Rhodomicrobium*
103. *Rhodopirellula*
104. *Rhodoplanes*
105. *Rhodovastum*
106. *Rhodovibrio*
107. *Roseomonas*
108. *Sandarakinorhabdus*
109. *Schlesneria*
110. *Sciscionella*
111. *Sideroxydans*
112. *Simplicispira*
113. *Singulisphaera*
114. *Skermanella*

27. *Caldanaerobacter*
28. *Caldilinea*
29. *Candidatus alysiosphaera*
30. *Candidatus chloracidobacterium*
31. *Candidatus chlorothrix*
32. *Candidatus entotheonella*
33. *Candidatus koribacter*
34. *Candidatus solibacter*
35. *Candidates xiphinematobacter*
36. *Caulobacter*
37. *Chelatococcus*
38. *Climaciaceae*
39. *Collimonas*
40. *Colwellia*
41. *Comamonas*
42. *Conexibacter*
43. *Coprothermobacter*
44. *Corynebacteriales*
71. *Ktedonobacter*
72. *Labrys*
73. *Leptothrix*
74. *Limnobacter*
75. *Limnohabitans*
76. *Magnetospirillum*
77. *Marmoricola*
78. *Massilia*
79. *Meganema*
80. *Methylocystis*
81. *Methylosinus*
82. *Metyhlovirgula*
83. *Microvirga*
84. *Mitochondria*
85. *Mucilaginibacter*
86. *Mycobacterium*
87. *Mitrosococcus*
88. *Mitrosovibrio*
115. *Solirubrobacter*
116. *Sorangiineae*
117. *Sorangium*
118. *Spam* (*Candidate division*) generas
119. *Sphingomonas*
120. *Telmatospirillum*
121. *Tepidamorphus*
122. *Thalassospira*
123. *Thermogemmatispora*
124. *Thermomonas*
125. *Thermosporothrix*
126. *Thioalkalispira*
127. *Tistlia*
128. *TM6* generas
129. *TM7* generas
130. *Victivallis*
131. *Zavarzinella*

Figure 6: Venn diagram showing distribution of bacterial genera/species identified in metal contaminated and reference soil samples from the GSR.

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.

Table 5: Bacterial and fungal diversity parameters and indices in different soil types in the GSR.

	Chao 1*	# of OTUs	Simpson Index	Shannon Index (H')	Species Evenness
Bacteria					
Metal contaminated sites	232 ± 10.99	399	0.82 ± 0.05	5.88 ± 0.25	0.54 ± 0.02
Reference sites	273 ± 10.91	413	0.88 ± 0.09	6.10 ± 0.19	0.56 ± 0.02
Fungi					
Metal contaminated sites	23 ± 3.09	60	0.85 ± 0.13	2.96 ± 0.13	0.59 ± 0.01
Reference sites	45 ± 3.36	100	0.87 ± 0.03	3.76 ± 0.12	0.62 ± 0.05

Results are expressed as mean values ± standard error

* represents significant differences between metal contaminated and reference sites for both bacteria and fungi based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney

2.4.3.2 Fungal Community Composition and Diversity Analysis

A total of 68 fungi species belonging to 59 genera were identified from all the soil samples analyzed (Table S6). The mean abundance of the fungi species identified in metal contaminated and references soil samples are illustrated in table S6. Detailed analysis revealed site-specific fungi. In fact, nine fungal groups were identified only in metal contaminated soil and 22 in reference soil (Table S5).

All the fungi identified in this study belong to four distinct phyla which include *Ascomycota*, *Basidiomycota*, *Glomeromycota* and *Zygomycota* (Table 4). The majority of them are members of the *Ascomycota* and *Basidiomycota* phyla. *Ascomycota* was more abundant in metal contaminated soil compared to reference sites (Table 4). For *Basidiomycota*, a high relative abundance was observed in reference soil samples compared to metal contaminated soil. The overall relative abundance of *Zygomycota* was low compared to *Ascomycota* and *Basidiomycota* (Table 4). *Glomeromycota* phylum was only identified in reference soil samples (Table 4).

At the genus level, there were significant differences ($p \leq 0.05$) between metal contaminated and reference soil for relative abundance. *Russula* was the most abundant genus across all soil samples, representing 18.26% in metal contaminated and 11.02% in reference soil. The distribution of other dominant genera that include *Agaricomycotina*, *Ascomycota*, *Basidiomycota*, *Cenococcum*, *Helotiaceae*, *Laccaria*, *Piloderma*, *Sebacinaceae* and *Thelephoraceae* varied significantly between metal contaminated and reference soils. A higher relative abundance of *Ascomycota*, *Helotiaceae*, *Herpotrichiellaceae*, *Laccaria* and *Sebacinaceae* was observed in metal contaminated compared to reference soil ($p \leq 0.05$). *Agaricomycotina*, *Basidiomycota*, *Cenococcum*, *Piloderma*, *Russula* and *Thelephoraceae* relative abundance were higher in

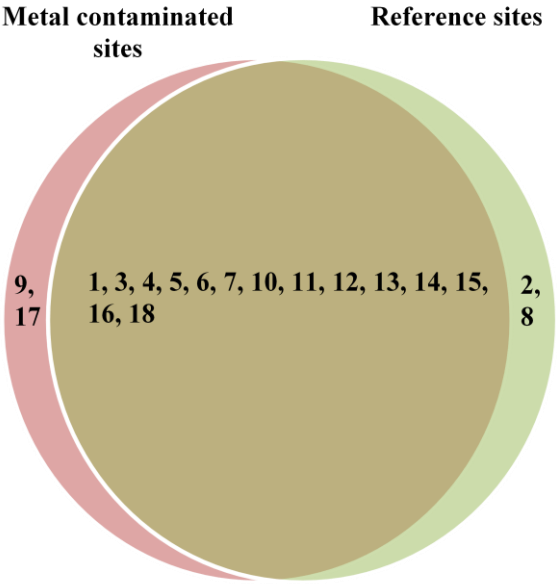
reference soil samples. In addition, several fungi genera classified as human, plant and animal pathogens were identified. They include *Dermateaceae*, *Dothideomycetes*, *Herpotrichiellaceae*, *Leotiomycetes*, *Magnaporthales*, *Mortierella*, *Pezizales*, *Scleroderma* and *Sordariomycetes*.

Overall, a total of 18 fungal classes, 44 families and 59 genera were identified. Figs. 7–9 illustrate the classes, families and genera of fungi that were identified in each soil type. No differences in the frequency distribution for fungal taxonomies (class: $p = 0.77$; family: $p = 0.99$ and genera: $p = 0.67$) were observed between metal contaminated and reference soil samples based on Kolmogorov-Smirnov test. At the species level, 46 fungal species were identified in metal contaminated soil samples and 58 fungal groups in reference soil samples (Table S6).

Several fungal groups were identified with a high relative abundance in metal contaminated soils. They include *Basidiomycota* sp., *Cryptococcus podzolicus*, *Dermateaceae* sp., *Helotiaceae* sp., *Laccaria proxima*, *Laccaria* sp., *Mortierellales* sp., *Scleroderma citrinum* and *Sebacinaceae* sp. (Table S6). In reference soils, *Agaricomycotina* sp., *Lactarius camphorates*, *Pyronemataceae* sp. and *Tylospora asterophora* were more abundant compared to metal contaminated soil samples ($p \leq 0.05$) (Table S6). Overall, these results indicate that the two types of sites (metal contaminated disturbed and reference sites) share the majority of fungal groups while showing difference in fungal community composition. In fact, 5–10% of fungal families and genera identified were site-specific.

On the other hand, we found that fungal microbial diversity was not affected by metal contamination. Overall, Chao 1 index values for fungi were significantly lower compared to bacteria ($p \leq 0.05$) (Table 5). For fungi, Chao 1 index values for reference sites were statistically higher compared to metal contaminated disturbed soils ($p \leq 0.05$) (Table 5). A total of 120 OTUs

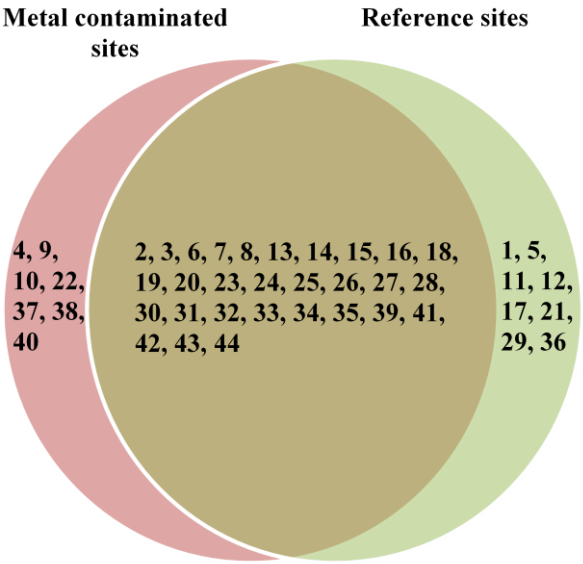
were identified of which 60 were found in metal contaminated and 100 in reference sites (Table 5). No differences between the soil types were observed for Simpson index, Shannon index and species evenness (Table 5). In addition, pair wise comparisons between soil samples revealed low values for Jaccard's similarity index (average 26%) and high values for Whittaker dissimilarity index (average 59%). Distance matrix values were high among soil samples (Table S7).



1. *Agaricomycetes*
2. *Archaeosporomycetes*
3. *Ascomycota* classes
4. *Basidiomycota* classes
5. *Dothideomycetes*
6. *Eurotiomycetes*
7. *Fungi* classes
8. *Geoglossomycetes*
9. *Lecanoromycetes*
10. *Leotiomycetes*
11. *Mortierellomycotina* classes
12. *Mucoromycotina* classes
13. *Pezizomycetes*
14. *Pezizomycotina* classes
15. *Saccharomycetes*
16. *Sordariomycetes*
17. *Taphrinomycotina* classes
18. *Tremellomycetes*

Figure 7: Venn diagram showing distribution of fungal classes identified in metal contaminated and reference soil samples from the GSR.

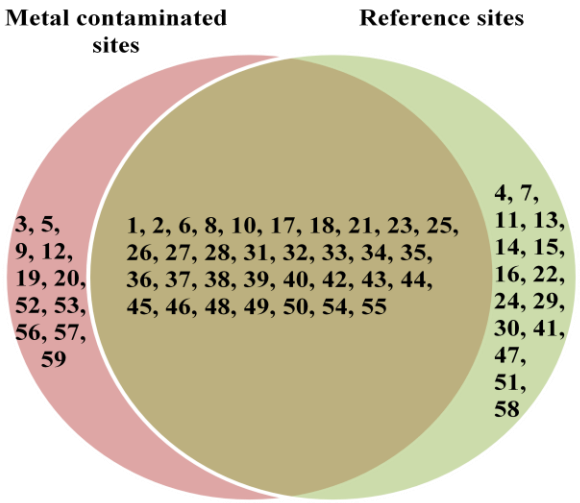
Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.



1. *Agaricales* families
2. *Agaricomycotina* families
3. *Amanitaceae*
4. *Archaeorhizomycetaceae*
5. *Archaeosporales* families
6. *Ascomycota* families
7. *Atheliaceae*
8. *Basidiomycota* families
9. *Caliciaceae*
10. *Cladoniaceae*
11. *Clavulinaceae*
12. *Cortinariaceae*
13. *Dermateaceae*
14. *Dothideomycetes* families
15. *Elaphomycetaceae*
16. *Fungi* families
17. *Geoglossaceae*
18. *Helotiaceae*
19. *Helotiales* families
20. *Herpotrichiellaceae*
21. *Hygrophoraceae*
22. *Icmadophilaceae*
23. *Leotiomycetes* families
24. *Magnaporthales* families
25. *Mortierellaceae*
26. *Mortierellales* families
27. *Mucoromycotina* families
28. *Myxotrichaceae*
29. *Pezizales* families
30. *Pezizomycotina* families
31. *Pyronemataceae*
32. *Russulaceae*
33. *Saccharomycetes* families
34. *Sclerodermataceae*
35. *Sebacinaceae*
36. *Sordariales* families
37. *Sordariomycetes* families
38. *Suillaceae*
39. *Thelephoraceae*
40. *Tremellaceae*
41. *Tremellales* families
42. *Trichocomaceae*
43. *Tricholomataceae*
44. *Venturiales* families

Figure 8: Venn diagram showing distribution of fungal families identified in metal contaminated and reference soil samples from the GSR.

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.



1. *Agaricomycotina*
2. *Amanita*
3. *Archaeorhizomyces*
4. *Archaeosporales*
5. *Ascomycete*
6. *Ascomycota*
7. *Atheliaceae*
8. *Basidiomycota*
9. *Calicium*
10. *Cenococcum*
11. *Chaetomella*
12. *Cladonia*
13. *Clavulina*
14. *Clavulinaceae*
15. *Cortinariaceae*
16. *Cortinarius*
17. *Cryptococcus*
18. *Dermateaceae*
19. *Dibaeis*
20. *Dothideomycetes*
21. *Elaphomyces*
22. *Ericoid*
23. *Fungi*
24. *Geoglossum*
25. *Gyoerffyella*
26. *Helotiaceae*
27. *Helotiales*
28. *Herpotrichiellaceae*
29. *Hygrocybe*
30. *Inocybe*
31. *Laccaria*
32. *Lactarius*
33. *Leotiomycetes*
34. *Magnaporthales*
35. *Mortierella*
36. *Mortierellales*
37. *Mucoromycotina*
38. *Myxotrichaceae*
39. *Oidiodendron*
40. *Penicillium*
41. *Pezizales*
42. *Pezizomycotina*
43. *Phialocephala*
44. *Piloderma*
45. *Pyronemataceae*
46. *Russula*
47. *Russulaceae*
48. *Saccharomycetes*
49. *Scleroderma*
50. *Sebacinaceae*
51. *Sordariales*
52. *Sordariomycetes*
53. *Suillus*
54. *Thelephoraceae*
55. *Tomentella*
56. *Tremella*
57. *Tricholoma*
58. *Tylospora*
59. *Venturiales*

Figure 9: Venn diagram showing distribution of fungal genera identified in metal contaminated and reference soil samples from the GSR.

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.

2.5 Discussion

2.5.1 Soil Chemistry

Metal analysis showed higher levels of As, Cu, Ni and Zn in samples from contaminated sites compared to reference soil samples [25,40,47]. However, results indicate only a fraction (approximately 1%) of the total metal concentration is in the bioavailable form [25,40,47]. This is consistent with other recent reports [25,45,46]. The pH values were low for metal contaminated soils compared to reference soil. This low pH found in various sites in the GSR is consistent with the acidity levels documented for soils from the Canadian Shield [25,40,47]. Winterhalder [15] reported that Sudbury soils near smelters were acidified to pH levels ranging from 3.2 to 3.9. At low pH (<4.5) many metals remain in soluble form thereby providing a good potential for transport in the leachate soil system. In acidic soils, various metals such as Ca, Mg and P (phosphorous) become deficient whereas Al and Mn are abundant and sometimes reach toxic levels [40]. In addition, low pH and metal deficiency/abundance can be detrimental to plants and affect soil microbial composition and diversity [13,35,40]. Our current study and other recent reports [25,33,38,40] show a significant increase in soil pH due in part to various legislated controls and soil rehabilitation programs which improved soil chemistry in the GSR from acidic to slightly acidic.

Cation exchange capacity (CEC) of the soil was low in metal contaminated compared to reference soil. Positive correlations between CEC and other factors including pH, soil organic C, organic matter and soil clay have been reported [169–171]. In this study, a low level of organic C and organic matter in metal contaminated soil resulted in low CEC values. Among all the parameters, soil pH is considered an important factor affecting CEC [172].

2.5.2 Phospholipid Fatty Acid Analysis

PLFA profiles revealed significant differences between metal contaminated and reference sites for total microbial biomass, AM fungi, other fungi, gram negative bacteria, gram positive bacteria and other eukaryotes. A significant decrease of these microbial elements observed in metal contaminated soil appears to be related to low organic matter and CEC rather than to soil metal content. This is consistent with other studies at different locations around the world [36,40,97].

Studies have reported that fungi appear to be more tolerant to metals than bacteria and actinomycetes [36,173,174]. In agreement to this, a study showed increased proportions of the fungal 18:2w6 in metal polluted soils [36]. However, in the present study, PLFAs 16:0, 18:1ω9, and 18:2ω6 considered as reliable indicators of fungi biomass were lower in metal contaminated soils compared to reference samples. This decrease in the amount of fungal PLFAs could also be due to a decrease in ectomycorrhizal fungi (EM fungi), which in turn could be attributed to poor vegetation in these sites [40]. PLFAs 16:1ω5 and 18:1ω7 considered as good indicators of AM fungi, are also found in bacteria in less amount. These fatty acids were in lower concentrations in metal contaminated soil compared to reference soil. This is another indication that changes in PLFA profile are not caused by metal levels in the disturbed sites.

PLFAs such as methyl branched 10Me16:0, 10Me17:0 and 10Me18:0 are found almost exclusively in actinomycetes [97]. Previous studies have reported that actinomycetes either decreased or were unaffected in response to metals [97]. Hiroki [175] reported that actinomycetes were generally less tolerant to metals compared to bacteria and fungi. Various studies reported that the abundance of actinomycetes in soils is affected by cadmium (Cd), Cu and Zn

contamination [97,174]. Our results show no significant difference in actinomycetes biomass between metal contaminated and references soil samples.

It is usually thought that gram negative bacteria dominate in metal contaminated and stressed soils compared to gram positive bacteria. Studies have shown that survival of gram negative bacteria under stress conditions could be attributed to the presence of cyclo fatty acids in their membrane and the outer lipopolysaccharide layer [40]. Decrease in relative abundance of both gram positive and gram negative bacteria in the metal contaminated disturbed soils could also be due to reduction in vegetation and plant root biomass. Overall, a predominance of gram negative bacteria over gram positive bacteria was observed in both types of soil analyzed. It is unclear which factors drive the relative dominance of bacteria over fungi in the soil types. It has been speculated that soil texture and changes in soil moisture affects PLFA profiles. It is very likely that soil conditions in the GSR favor bacteria growth over fungi.

2.5.3 Pyrosequencing Derived Data

High throughput next generation sequencing has dramatically increased the resolution and detectable spectrum of diverse microbial phylotypes from environmental samples and it plays a significant role in microbial ecology studies [99–101]. Next generation sequencing such as 454 pyrosequencing method employed in this study facilitates identification of unculturable microorganisms and their relative abundance in soils [99–101]. This platform is a new type of second generation sequencing technology as it is rapid, flexible, inexpensive and produces reads with an accuracy of 99% [102]. Long term metal contamination has been shown to have a marked decrease in numbers of different microbial groups and their composition. Sequence analyses in

this study revealed that the levels of bacterial and fungal diversities are comparable to findings of similar studies related to metal contamination [81,176].

2.5.3.1 Bacterial Communities' Analysis

Actinobacteria relative abundance was similar in metal contaminated and reference soils. It is an important phylum for metal remediation due to its metabolic power and ability for fast colonization of selective substrates. Despite these properties, data on *Actinobacterial* resistance to metals are still scarce compared to gram negative bacteria. *Acidobacteria* (acidophilic heterotrophs) was one of the most prevalent phyla in all the soil samples analyzed. This phylum has been consistently detected in many habitats around the world. More than 30% of the sequences from 16S rDNA analyses of soil belong to this phylum based on survey of many studies [177,178]. This phylum has been detected in freshwater habitats, hot spring microbial mats, sewage sludges, wastewater treatment bioreactors, rhizospheres and other habitats [177]. *Acidobacteria* phylum members have been shown to possess ability to withstand metal contamination, acidic and other extreme environments [178]. *Proteobacteria* was another phylum with high relative abundance in both soil types characterized compared to other phyla. It was largely composed of subphyla *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*. This phylum has been found to be predominant in many mine environments and is known to exhibit complex lifestyle with degrading ability of various complex organic molecules, enabling it to adapt to many different environments [179]. Studies have also reported that most *Proteobacteria* belong to members of nitrogen fixing and photosynthetic bacteria [180]. Bacterial organisms belonging to this phylum play an important role in carbon, and nitrogen cycles and in maintaining integrity of the ecosystem [99,161,180].

Therefore, low plant diversity, respiration rates and organic matter content in the metal contaminated sites may also explain the lower abundance of bacterial phyla such as *Acidobacteria* and *Proteobacteria*. The analysis at the phylum level shows that *Proteobacteria*, *Acidobacteria* and *Actinobacteria* phyla were prevalent in all the soil samples whereas *Bacteriodetes*, *Verrucomicrobia* and *Planctomycetes* phyla were less abundant. These trends have been observed in other studies [179]. Other phyla present at lower proportion include *Chloroflexi* and *Firmicutes* that have been previously reported to be abundant in soils with higher concentrations of C, Fe and Mn [181]. Studies have shown that although these groups are dominant across soil types, the relative proportion of the bacterial community belonging to these phyla are influenced by factors such as pH, depth within the soil profiles, degree of saturations and anaerobiosis [181].

Overall, *Acidobacterium*, *Afipia*, *Aquicella*, *Geobacillus*, *Granulicella*, *Koribacter*, *Nitrosococcus*, *Rhodoplanes*, *Skermanella*, *Solibacter*, *Thermosporothrix* and *Thioalkalispira* were the predominant genera in the soils. The composition and distribution of these 12 common bacterial genera varied between metal contaminated and reference soil samples. *Geobacillus* was found at high relative abundance in metal contaminated soils. It denitrifies nitrate to nitrogen and species belonging to this genus are known for their metal binding capacity and are reported to biosorb and remove toxic metals [182]. *Rhodoplanes* known as complete denitrifiers are present in areas with high plant diversity [183]. They were more abundant in reference soils which had high plant species diversity compared to metal contaminated soils. *Thioalkalispira* were present in all the sites, but they were more abundant in metal contaminated soils compared to reference soil. These bacteria are known as sulfur oxidizing microorganisms and they play an important role in element cycling in nature that is rich in reduced sulfur compounds [184,185].

Many of the bacterial isolates known to catalyze dissimilatory redox transformations of iron and/or sulfur in acidic environments were identified. They include ferric iron reducers (*Acidobacteriaceae* isolates; *Geobacillus* sp., and *Acidocella* sp.) and sulfur oxidizers (*Thioalkalispira* sp. and *Candidate* isolates). Bacteria involved in nitrogen fixation such as *Azovibrio* spp., *Azospira* spp., *Rhizobium* spp. and *Fluviicola* spp. were also present in the targeted sites. Nitrifying bacteria (*Nitrosococcus* spp.) were more abundant in reference soils samples compared to metal contaminated soils. Ammonia oxidizing bacterium (*Nitrosovibrio tenuis*) was found only in reference soils. In addition, various bacterial species that are classified as decomposers (*Acidiphilium* spp., *Comamonas* spp.), mutualists (*Blastochloris* spp., *Frankia* spp., *Rhizobium* spp.), pathogens (*Acidovorax* spp., *Corynebacteriales* spp., *Escherichia shigella* spp., *Mycobacterium insubricum*), autotrophs (*Thioalkalispira* spp.) and chemotrophs (*Hirschia* spp., *Inquilinus* spp., *Microvirga bosea thiooxidans*) were found in all the samples. Bacterial groups with anti-fungal (*Collimonas fungivorans*, *Haliangium* spp., *Pseudonocardia* spp.) and anti-bacterial (*Acidovorax* spp., *Haliangium* spp., *Pseudonocardia* spp.) activity were also identified. In addition, bacteria involved in nitrate reduction (*Afipia felis* and *Hymenobacter* spp.), carbon cycles (*Acidobacterium* spp.), tolerance to metals (*Acidocella* spp., *Caulobacter* spp., *Sphingomonas* spp. and *Rhizobium* spp.), and xenobiotics degradation (*Comamonas* spp., *Limnobacter* spp., *Polaromonas* spp.) were present in the targeted sites. Likewise bacteria that are common in acidic soil (*Collimonas fungivorans* and *Acidisphaera* spp.), stress conditions and environment (*Arthrobacter* spp.), sludge and waste of water treatments (*Candidates microthrix* spp.) were also found. Overall, a higher percentage of gram negative bacterial species were identified in all the sites compared to gram positive. These data are consistent with PLFA results.

2.5.3.2 Fungi Communities' Analysis

Most of the current knowledge on microbial diversity in soil is related to bacteria and information on fungal communities in different ecosystems is sketchy. Studying and understanding fungal communities is of paramount importance since fungi comprise a major portion of the biodiversity and biomass in soils and they play crucial roles in maintaining soil processes which affect the functioning of the ecosystem. Few studies have reported the negative effects of metals on fungal growth and reproduction [35,186]. A decrease in fungal radial growth after exposure to Cd, Cu, Zn and Ni has been observed [186]. In addition, metals such as Cd and Zn have been shown to inhibit the conidial production in some fungal species [187,188]. Reports from previous studies have pointed out that higher toxicity of metals affect conidial production than mycelia growth [188]. Overall, studies have shown that metal toxicity varies with fungal species, metal type, metal concentration, environmental factors such as soil pH, nutrient availability and plant species diversity, which are all expected to affect fungal activity [35,153,186–188].

Among the four fungal phyla identified in this study *Basidiomycota* and *Ascomycota* showed the highest relative abundance in both soil types. *Basidiomycota* was the most prevalent in reference soils and *Ascomycota* in metal contaminated soils. *Ascomycota* (sac fungi) is monophyletic and accounts for approximately 75% of all described fungi. Several *Ascomycota* classified as lichen forming fungi, litter/wood decomposers, plant parasites, human pathogens, animal pathogens, endophytes, saprotrophs and edible mushrooms were identified. *Basidiomycota* includes some of the most familiar fungi known for the production of large fruit bodies such as mushrooms, puffballs and shelf fungi as well as rust and smut fungi that are plant

and yeast parasites [189,190]. *Basidiomycota* which includes EM fungi are important agents of wood decay/decomposers of plant litter and animal dung [189,190]. EM fungi are economically important as they form a symbiotic relationship with plant roots and help plants take up water, mineral salts and metabolites and in turn gain carbon and essential organic substances from plants [189,190]. Various decomposers identified in this study are involved in the decomposition of organic matter and decaying matter, and these actions release elements such as nitrogen and phosphorous making them available to other living organisms. *Zygomycota* was another phylum identified in both soil samples but their relative abundance was significantly lower compared to *Ascomycota* and *Basidiomycota*. *Zygomycota* represents approximately 1% of all the fungal groups and the most familiar representatives include fast growing molds often found on fruits with high sugar content [191]. Fungi from the *Zygomycota* phylum are commonly found in terrestrial ecosystems and they live close to plants, usually in soil, decaying plant matter as they decompose soil and play a major role in carbon cycle [191]. *Zygomycota* are also pathogens for animals, plants, other fungi and are known to cause serious infections in humans [191]. *Mortierella* sp., *Mortierellales* sp., and *Mucoromycotina* sp. belonging to the *Zygomycota* phylum have been reported as common fungi in soils. Many species from these genera that were abundant in metal contaminated sites are known as plant pathogens causing abortions and pulmonary infections in cattle [191]. *Archaeosporales* sp. belonging to *Glomeromycota* phylum was present only in reference soil. *Glomeromycota* are symbionts found only in terrestrial habitats and form AM with plants (herbaceous and tropical) [189]. The fungi benefit their host by functioning as an extension of the root system and the fungi benefit as they have access to constant source of organic nutrients supplied by their host. *Archaeosporales* sp. is a mycorrhizal

fungus forming arbuscules and vesicles in roots. They can enhance phosphorous and nitrogen supply to host and they are known to have a widespread distribution [192].

Agaricomycotina, *Ascomycota*, *Basidiomycota*, *Cenococcum*, *Helotiaceae*, *Laccaria*, *Lactarius*, *Leotiomycetes*, *Pezizomycotina*, *Russula* and *Sebacinaceae* were the most abundant microbial groups. In this study, the composition and distribution of these fungi varied from one soil type to another. *Agaricomycotina* a diverse clade of the *Basidiomycota* phylum includes mushrooms, jelly fungi and basidiomycetous yeasts. Large concentrations of wood decayers, litter decomposers, and EM fungi, along with small numbers of pathogens of timber, vegetable crops and humans [193] are also part of this group. *Cenococcum* is a genus of ectomycorrhizal *Ascomycota* and has been documented to be associated with a broad diversity of host plants (angiosperms and gymnosperms) in various habitats, environments and geographic regions [194]. *Leotiomycetes* are ecologically diverse and include plant pathogens, saprobes of leaves and wood, endophytes, mycorrhizas and aquatic hyphomycetes [195]. Similarly, other fungi groups identified in this study have been shown to be involved in different functions in the terrestrial environment such as decomposers of organic matter, natural enemies of pests and symbionts. Some are involved in elemental cycles and maintaining soil structure. Other groups are plant and animal (including human) pathogens.

Among the 68 fungal groups identified, only one species *Oidiodendron maius* has been recognized to have metal tolerance. Hence, all the fungal species growing in sites contaminated with metals for century show resilience to Cu and Ni, the main contaminants in the region. *Oidiodendron maius* belongs to phylum *Ascomycota* and has been identified as an endomycorrhizal fungus that prefers acidic conditions [187,196]. Other studies have described

Aspergillus niger, *Penicillium simplicissimum*, *Aspergillus foetidus* and *Aspergillus carbonarius* as species tolerant to metals such as Ni, cobalt (Co), molybdenum (Mo), vanadium (V), Fe, Mg and Mn at high concentrations [197].

2.5.4 Microbial Diversity

Advances in sequencing technologies have enabled researchers to characterize more accurately microbial diversity compared to traditional methods. In the present study, the number of operational taxonomic units (OTUs), Chao1, Shannon and Simpson index were used to determine bacterial and fungal diversities. OTU is defined as a cluster of reads which correspond to microbial species and are widely used to estimate bacterial and fungal species. OTUs are observations of organisms and the numbers of observations correlate well with the total number of individuals present in the community [198]. Roesch *et al*. [81] reported that these approaches provide estimates of diversity greater than 10,000 species (OTUs) per gram of soil. Another study looked at the bacterial diversity across reclaimed and natural boreal forest in British Columbia and reported 577 OTUs for bacteria. Results from this study indicated similar microbial diversity in both soil types. Chao1 index is commonly used species richness estimator which estimates total number of species present in a community based on the number of OTUs found in a sample [199]. Chao1 index revealed higher bacterial species richness compared to fungi species richness in soils. Shannon and Simpson index are most widely used indices to determine community diversity and it takes into account both species richness and abundance [200]. For Shannon index, fungal diversity was low compared to bacterial diversity. In both bacteria and fungi, metal contaminated and reference soils show similar levels of diversity based on all the diversity parameters tested. This is an indication of adaptation of these microorganisms to metal exposure.

The low level of Chao 1 values observed in metal contaminated sites appears to be associated with other conditions than the metal content. No difference between soil types was observed for species evenness. Gotelli [200] reported that communities dominated by one or two species exhibit low evenness whereas communities where abundance is distributed equally amongst species exhibit high evenness.

Beta diversity indices were calculated to compare community composition between the sites. Low values for Jaccard's similarity index between soil types for both bacterial and fungal communities indicate low level of similarity (high beta diversity). High values for Whittaker dissimilarity index indicates that there are several species that are different among sites (high beta diversity). Overall, both similarity and dissimilarity indices reveal that community compositions between sites are different from each other. This confirms the Venn diagram characterization. Daniel [201] and Grundmann [202] reported that the vast microbial diversity is closely associated to variety of characteristics of soil environment including soil physical structures and the complex chemical and biological properties. Overall, our microbial diversity data are consistent with these previous observations.

In addition, weighted UniFrac distances which take into account differences in abundance of taxa between metal contaminated and reference sites indicated that for bacterial communities, metal contaminated sites are closely related to each other. Whereas for fungal communities, high distance values indicated that the sites are different from each other.

The relative abundance of bacterial and fungal communities was different in the soil types analyzed. But metal contamination did not have any effect on the number of OTUs and the level of microbial diversity. Studies have shown that increased metal content had a negative effect on

soil microbial population [52]. The negative impact on soil microbial population have a direct negative effect on soil fertility [52]. Rathnayake *et al*. [203] reported that soil microorganisms may adapt to the increased, even toxic metal and other xenobiotics concentrations in soil by developing various mechanisms to resist contamination. There are several factors that can contribute to microbial resilience to metals such as Cu, Ni, Zn, and As. They include transfer of metal tolerance genes in microbial communities through substitution of metal-sensitive strains by tolerant or resistant ones or transposons/retrotransposon elements; and low proportion of bioavailable metals [204,205]. Generally, development of resistant bacteria or fungi is indicative of deterioration of ecosystems. Indeed, in the present study, the ratios of fungi over bacteria were very low and the proportion of gram-neagtive bacteria was very high suggesting that the targeted region is under severe environmental stress. Moreover, the level of bioavailable As, Cu, Ni, and Zn in soil was low and the microbial communities have been exposed to metals for >100 years. This long term exposure to small amounts of metals might explain the development of metal resilience within the context of microbial diversity in the targeted sites based on the theory described by Azarbad *et al*. [176] and Berg *et al*. [206].

2.5.5 PLFA vs Pyrosequencing Analysis

In general, PLFA based methods are rapid, inexpensive, sensitive and reproducible. There are a number of scientific publications based on PFLA analysis that have increased our understanding of the soil ecosystem [98,207–210]. Unlike pyrosequencing, PLFA has limitations such as overlap in the composition of microorganisms and the specificity of signature PLFAs. In the present study, PFLA data revealed significant differences in microbial biomass between the metal contaminated and reference sites. But these differences were not always detected by

pyrosequencing. For example, PFLA analysis shows that both soil types contain substantial amounts of AM fungi, but pyrosequencing shows that AM fungi (*Glomeromycota*) are only detected in the reference sites. This could be attributed to the fact that pyrosequencing identification is based on comparative analysis with existing sequences in databases. In fact, many difficulties existed in pyrosequencing analysis due to variety of genes, complexity in computations and more specifically absence of appropriate reference gene sets [211]. The use of both, PFLA and pyrosequencing analyses is the most appropriate approach in assessing microbial abundance, structure and diversity.

2.6 Conclusions

The results of the present study demonstrate that long-term exposure to metals for almost 100 years reduce microbial biomass and abundance but had no effect on microbial diversity. Metal contaminated areas with low CEC and organic matter (OM) showed significantly lower levels of microbial biomass and abundance compared to metal uncontaminated sites with high CEC and OM. The effects of metal were also assessed at the community composition and phyla levels. Overall, 62.00% of bacterial families identified were common to metal contaminated sites and the reference sites. But 11.50% were specific to the contaminated sites and 10.00% to the reference sites. This trend was also observed at class and genera levels. For fungi, the metal contaminated and reference sites shared 58.00% of the families identified; 6.00% of the fungal families were specific to the contaminated sites, and 10.00% to the reference sites. The same proportions were observed at class and genera levels. These suggest that some bacteria and fungi groups need some metals to grow while other cannot survive in the presence of metals. The study

also suggests that organic matter and CEC are key factors involved in changes in microbial communities.

2.7 Acknowledgments

We would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC), Vale and Sudbury Nickel Operations (Glencore Limited) for their financial support. Thanks to FAME Lab Microbial ID, Inc. Newark and MR DNA, Shallowater, Texas, USA for assistance with PLFA and pyrosequencing analysis, respectively.

CHAPTER 3: MICROBIAL RESPONSE TO SOIL LIMING OF DAMAGED ECOSYSTEMS IN THE GREATER SUDBURY REGION REVEALED BY PYROSEQUENCING AND PHOSPHOLIPID FATTY ACID ANALYSES

3.1 Abstract

The main objective of the present study was to assess the effects of dolomitic limestone applications on soil microbial communities' dynamics and bacterial and fungal biomass, relative abundance, and diversity in metal reclaimed regions. The study was conducted in reclaimed mining sites and metal uncontaminated areas. The limestone applications were performed over 35 years ago. Total microbial biomass was determined by phospholipid fatty acids. Bacterial and fungal relative abundance and diversity were assessed using 454 pyrosequencing. There was a significant increase of total microbial biomass in limed sites (342.15 ng/g) compared to unlimed areas (149.89 ng/g). Chao1 estimates followed the same trend. But the total number of OTUs (Operational Taxonomic Units) in limed (463 OTUs) and unlimed (473 OTUs) soil samples for bacteria were similar. For fungi, OTUs were 96 and 81 for limed and unlimed soil samples, respectively. Likewise, Simpson and Shannon diversity indices revealed no significant differences between limed and unlimed sites. Bacterial and fungal group's specific to either limed or unlimed sites were identified. Five major bacterial phyla including *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Firmicutes,* and *Proteobacteria* were found. The latter was the most prevalent phylum in all the samples with a relative abundance of 50.00%. *Bradyrhizobiaceae* family with 12 genera including the nitrogen fixing *Bradirhizobium* genus was more abundant in limed sites compared to unlimed areas. For fungi, *Ascomycota* was the most predominant phylum in unlimed soils (46.00%) while *Basidiomycota* phylum represented 85.74% of all fungi in the limed areas. Detailed analysis of the data revealed that although soil liming increases significantly the amount of microbial biomass, the level of species diversity remains statistically unchanged even though the microbial compositions of the damaged and restored sites are

different. Soil liming still has significant beneficial effects on soil microbial abundance and composition > 35 years after dolomitic limestone applications.

Keywords: Microbial biomass; Bacterial and fungal relative abundance and diversity; Phospholipid fatty acid analysis; Pyrosequencing; Soil liming; Metal stress.

3.2 Introduction

Mining activities in the Greater Sudbury Region (GSR) in Northern Ontario (Canada) that started over a century ago have resulted in serious damages to surrounding ecosystems. Logging and ore smelting led to a large scale of SO_2 (sulphur dioxide) emissions and metal contamination (copper, Cu; iron, Fe; nickel, Ni, and zinc, Zn) [15,34,38,40,212]. These have resulted in damaged ecosystems with reduced plant growth and population diversities within the region [15,38,40]. Remediation projects were initiated to restore the affected lands and to decrease industrial emissions. Building of a super chimney in 1972 reduced metal particulates and SO_2 emissions by 50% and 85%, respectively [15]. To decrease acidity, 10 tons of limestone per hectare was applied in 1978. This liming was followed by land fertilization, grass and legume seeding [15,212]. In addition, since 1979, 12 million trees have been planted to complete the reclamation process [15,38,40].

Several studies investigating the effects of metal contamination on soil biology reported mixed results. Some found no changes in microbial biomass while others reported a decrease in microbial relative abundance associated with high levels of soil metal accumulation [33–35,213]. Extensive research has been conducted on the effects of liming on agricultural soils within the context of integrated soil management [15,39,171,214–216]. Overall, these studies showed that liming resulted in the mineralization of the soil solution due to its saturation with calcium (Ca) and in some cases with magnesium (Mg).

The addition of liming lowered soil acidity, reduced soil erosion and metal mobility, and resulted in an increase in organic matter [38–40]. Recent analysis revealed that soil liming in the GSR increased plant species richness and abundance and the overall plant ecosystem health

[25,26,47]. This has led to improvement of soil fertility in limed sites compared to unlimed areas [25,26,47]. The relationship between soil pH and its effect on the activity and composition of microbial populations has been discussed in various reports [34,35,39,61,93,213,214,217]. Low pH inhibits the growth of soil bacteria in favor of more resistant fungi [31,93]. Liming of acidic soils creates improved environmental conditions for the development of acid-intolerant microbes, resulting in increased microbial biomass and soil respiration [15,216,218]. Also, soil type and management affect microbial and chemical responses once lime is applied [31]. Enhanced soil respiration as well as nitrogen mineralization have also been documented when lime is added to weakly acidic soils [214,219]. Pawlett *et al*. [215] reported that liming significantly altered PLFA profile (reduced bacterial fatty acids and did not affect fungal PLFA signature) and increased respiration rates. Other studies reported that application of fertilizers and lime resulted in the activation of biochemical processes; an increase in the relative abundance of prokaryotes, *Bacilli* and *Actinomycetes*; and a decrease in micromycetes [39,50]. In contrast, there are reports on the inhibitory effect of lime on some groups of prokaryotes such as actinomycetes that facilitate the mineralization of soil organic matter (SOM) [39,213].

Most of these studies focused on bacterial relative abundance in agricultural soil. We have a poor understanding of how liming affects microbial relative abundance, diversity and dynamic within a context of land reclamation of severely damaged ecosystems. Moreover, studies on the variation of fungi communities in response to soil liming are lacking. We hypothesize that liming increases bacterial and fungal biomass, relative abundance, diversity, and community composition.

The present report discusses for the first time the effect of soil liming on belowground diversity using PLFA and pyrosequencing analyses in the GSR. The main objectives of this study were to assess the long term effects of liming on 1) soil microbial biomass and composition (bacteria, fungi, and actinomycetes) and 2) bacterial and fungal population dynamic and diversity in reclaimed ecosystems.

3.3 Materials and Methods

3.3.1 Site Characterization and Sampling

The study was conducted in reclaimed mining sites in Sudbury, Northern Ontario, Canada (46°30′ N, 80°00′ W). The topography of the region is characterized by mosaic of rock outcrops, glacial till deposits, numerous lakes, and narrow valleys resulting from the Wisconsin glaciations [15]. The dominant soil type in the GSR area are podzols, which are well drained soils on sandy till with an organic top layer, a grey middle, and a reddish-brown lower layer [163]. Liming of this region was completed >35 years ago through the Sudbury Regional Land Reclamation Program (Regreening Program) using dolomitic limestone [15,37].

Soil sampling was performed at four selected locations each with a reclaimed site (limed site) and its adjacent un-reclaimed or unlimed area. These targeted sites include Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway (Fig. 10). The GPS coordinates of these sites are given in table S1. At each of the four limed and four unlimed sites, 20 soil samples were collected in 2014 from the organic layer (0-5 cm in depth). Plant materials, stones, and residues were removed. Soil samples from each site were pooled and stored for a short period (in incubator at 30°C for soil chemical analyses; freezer for PLFA analysis for no more than 10 days before analysis). Microbial DNA was extracted from fresh samples within hours after soil sampling.

All the samplings took place on Laurentian University research fields and Crown lands. No specific permission was required to access the lands and collect samples.

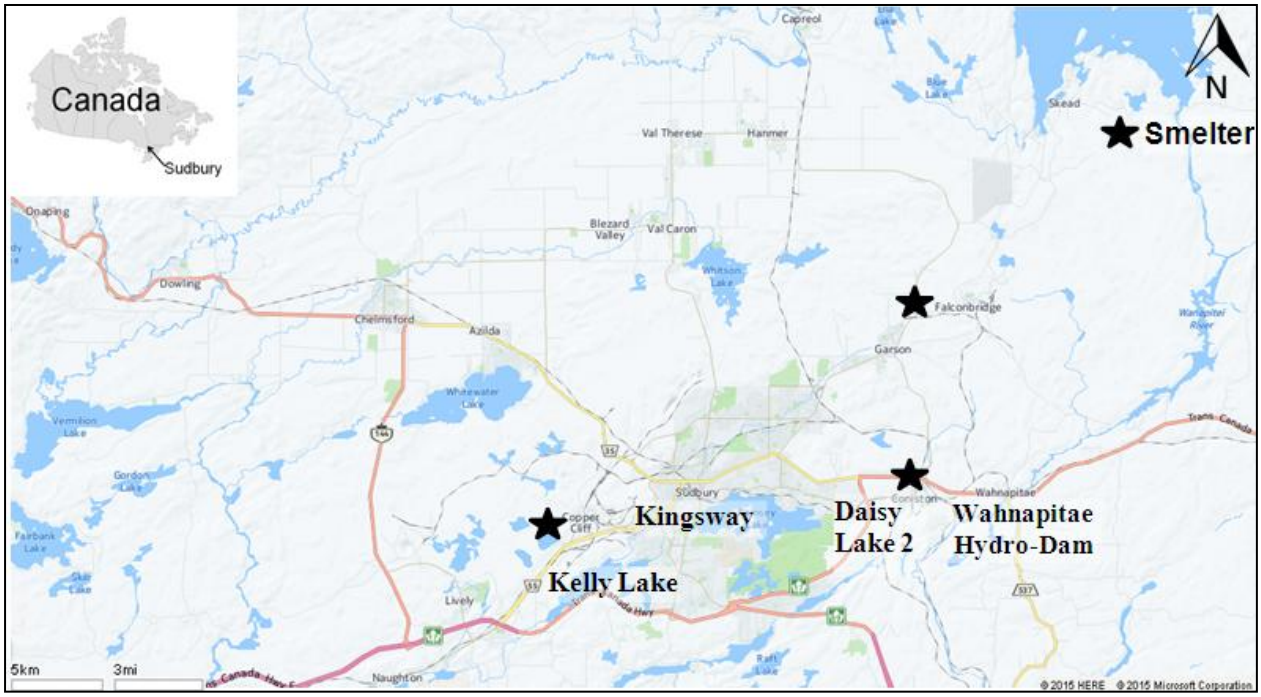


Figure 10: Geographical locations of the sampling area from the Greater Sudbury Region (GSR) in Northern Ontario.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

3.3.2 Soil Chemistry Analysis

Soil subsamples were sieved using a 2 mm mesh and air dried. Cation exchange capacity (CEC) was measured by using ammonium acetate extraction method at pH 7 [167]. CEC is an intrinsic property of soil defining the concentration of negatively charged sites on soil colloids that can adsorb exchangeable cations. The exchangeable cations that included aluminum, Al^{3+}; calcium, Ca^{2+}; iron, Fe^{3+}; potassium, K^{+}; magnesium, Mg^{2+}; manganese, Mn^{2+}; and sodium, Na^{+} were quantified by inductively coupled plasma mass spectrometry (ICP-MS) [25]. Soil pH was measured in de-ionized water and in neutral salt solution (0.01 M $CaCl_2$) as described previously by Nkongolo *et al*. [25].

3.3.3 Phospholipid Fatty Acid (PLFA) Analysis

Soil samples (from four limed and four unlimed sites) were analyzed following the protocol described by Buyer and Sasser [86]. Mole percentage of each PLFA was used to determine bacterial and fungal biomass in soil. Total PLFA extracted from soil was used as an index of living microbial biomass [86]. The selected PLFAs for bacterial biomass include i15:0, a15:0, i16:0, 16:1ω9, 16:1ω7c, cy17:0, i17:0, a17:0, 18:1ω7 and cy19:0, while PLFA 18:2ω6 and 18:1ω9 were used for fungi. PLFAs such as 10Me, 20:3ω6/20:4ω6 and 12:0/16:1ω7/18:2ω9/18:2ω12/18:3ω9/18:3ω12/18:3ω15/polyunsaturated fatty acids were used for the identification of actinomycetes, protozoa, and eukaryotes, respectively.

3.3.4 Microbial DNA Extraction and Purification

Microbial DNA was extracted from approximately 10 g of fresh soil (four limed and four unlimed) using the PowerMax® soil DNA isolation kit for soil from MO BIO (cat # 12988-10) with the bead-beating protocol supplied by the manufacturer. The concentration of the DNA was

determined using the fluorochrome Hoechst 33258 (busdensimide) fluorescent DNA quantification kit from Bio-Rad (cat # 170-2480). Fluorescence intensity was measured using a BMG Labtech FLUOstar Optima microplate multi-detection reader in fluorescence detection mode. The quality was determined by running the samples on a 1% agarose gel. DNA samples were stored at -20°C until further analysis.

3.3.5 PCR Amplification and 454 Pyrosequencing

Amplicon sequencing was performed at the MR DNA Molecular Research DNA laboratory (Shallowater, Texas, USA). Amplicon based analysis of the soil bacterial and fungal microbiota was assessed by high throughput sequencing of 16S rRNA gene and internal transcribed spacer (ITS) region. Tag-encoded FLX-titanium 16S rRNA gene amplicon pyrosequencing (bTEFAP) was performed using 16S universal Eubacterial primers 530F (5' GTG CCA GCM GCN GCG G) and 1100R (5' GGG TTN CGN TCG TTR) for amplifying the 600 bp region of 16S rRNA genes [108]. Fungal tag-encoded FLX amplicon pyrosequence (fTEFAP) was determined using ITS specific primers ITS1F (5' TCC GTA GGT GAA CCT GCG G) and ITS4R (5' TCC TCC GCT TAT TGA TAT GC) to amplify 600 bp fragment of the fungal ITS region [103]. The sequencing library was generated using a one-step PCR of 30 cycles using HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 min followed by 28 cycles of 94°C for 30 sec, 53°C for 40 sec and 72°C for 1 minute, and a final elongation step at 72°C for 5 min [108]. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads. Tag-encoded FLX amplicon pyrosequencing analysis was performed using a Roche 454 FLX instrument with titanium reagents following the manufacturer's guidelines with 3,000 sequence depth.

3.3.6 Data Processing

The sequencing data were processed using a proprietary analysis pipeline (MR DNA). Sequences were depleted of barcodes and primers. Short sequences (< 200 bp), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp were removed. Sequences were then denoised and chimeras were removed. OTUs (Operational Taxonomic Units) were defined following removal of singleton sequences, clustering at 3% divergence (97% similarity). OTUs were taxonomically classified using BLASTN (Basic Local Alignment Search Tool for Nucleotides) against a curated GreenGenes database (http://greengenes.lbl.gov; version 2011) and compiled into each taxonomic level.

3.3.7 Statistical Analyses

Data was tested for normality using Shapiro-Wilk test and analyzed using SPSS statistics version 20 for windows. Association between soil pH and exchangeable cations were determined based on Pearson *r* correlation coefficients ($p \leq 0.05$). t-test was used to determine significant differences between limed and unlimed sites for CEC, microbial biomass (total PLFA), and composition (bacterial, actinomycetes and fungal PLFAs).

Chao1, Shannon index, Simpson index, and species richness and evenness for bacterial and fungal species diversity were analyzed using QIIME (version 1.8.0) [99,108,112,220]. Differences in frequency distribution for bacterial and fungal groups between limed and unlimed soils samples based on Kolmogorov-Smirnov test (non-parametric test) were determined using SPSS.

Pair wise comparisons (beta diversity) among the sites were performed using similarity indices (based on the presence/absence data), including Jaccard and Sorensen indices.

Dissimilarity indices (based on abundance or relative abundance) including Bray-Curtis and Whittaker indices were calculated. Beta diversity was also estimated by computing weighed UniFrac (Qualitative) distances among sites. Weighed UniFrac variants are widely used in microbial ecology and they account for abundance of observed organisms. QIIME (version 1.8.0) was used to compute the within community diversity (alpha diversity), between community diversity (beta diversity), and UniFrac distances [111,199,220].

3.4 Results

3.4.1 Soil Chemistry

The pH in limed soils was significantly higher (pH 6.3) even >35 years after dolomitic limestone applications (Table 6). A similar trend was observed for cation exchange capacity (CEC). Correlations between soil pH and CEC were positive ($p \leq 0.05$) for limed soil samples and negative (-0.64; $p \leq 0.05$) for samples from unlimed areas (Fig. 11). As expected, the highest values of exchangeable Ca^{2+} and Mg^{2+} were recorded in limed soils (Table 6). Whereas, low values were obtained for exchangeable K^{+} and Na^{+} in both types of soils (Table 6). Total sum of exchangeable cation (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}) values were 13.89 cmol/kg and 1.55 cmol/kg for limed and unlimed soils, respectively (Table 6).

3.4.2 Phospholipid Fatty Acid Analysis

PFLA revealed a significant high level ($p \leq 0.05$) of total microbial biomass in limed sites compared to samples from unlimed areas (Table 7). The relative abundance levels of gram negative and gram positive bacteria, arbuscular mycorrhizal (AM), and other fungi followed the same trend (Table 7). There were positive correlations ($p \leq 0.05$) between gram negative bacterial biomass and pH ($r = 0.71$) as well as between AM fungi and pH ($r = 0.59$). But negative correlations ($p \leq 0.05$) were observed between gram positive bacterial biomass and pH ($r = -0.49$) and between fungal biomass and pH ($r = -0.22$).

The ratio of gram positive to gram negative bacterial biomass was lower in the limed soil samples compared to unlimed (Table 8). The opposite trend was observed for 18w to 19cyclo ratio (Table 8). The ratio between fungal and bacterial biomass was very low for the two soil types (Table 8).

Table 6: Mean values of basic cations (Ca^{2+}, Mg^{2+}, K^{+} and Na^{+}), sum of cations, CEC and pH.

Characteristics	Limed sites	Unlimed sites
Ca^{2+} (cmol/kg)	10.52a ± 3.82	0.99b ± 0.48
Mg^{2+} (cmol/kg)	3.13a ± 1.36	0.26b ± 0.14
K^{+} (cmol/kg)	0.20a ± 0.04	0.25a ± 0.13
Na^{+} (cmol/kg)	0.04a ±0.01	0.05a ± 0.03
Sum of Cations (cmol/kg)	13.89	1.55
CEC (cmol/kg)	16.30a ± 5.50	1.80b ± 0.90
pH (H_20)	6.30a ± 0.40	4.70b ± 0.20
pH (0.01M $CaCl_2$)	5.80a ± 0.40	4.30b ± 0.30

Results are expressed as mean values ± standard error.

Means in rows with a common letter are not significantly different based on t-test ($p \geq 0.05$).

CEC: cation exchange capacity.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

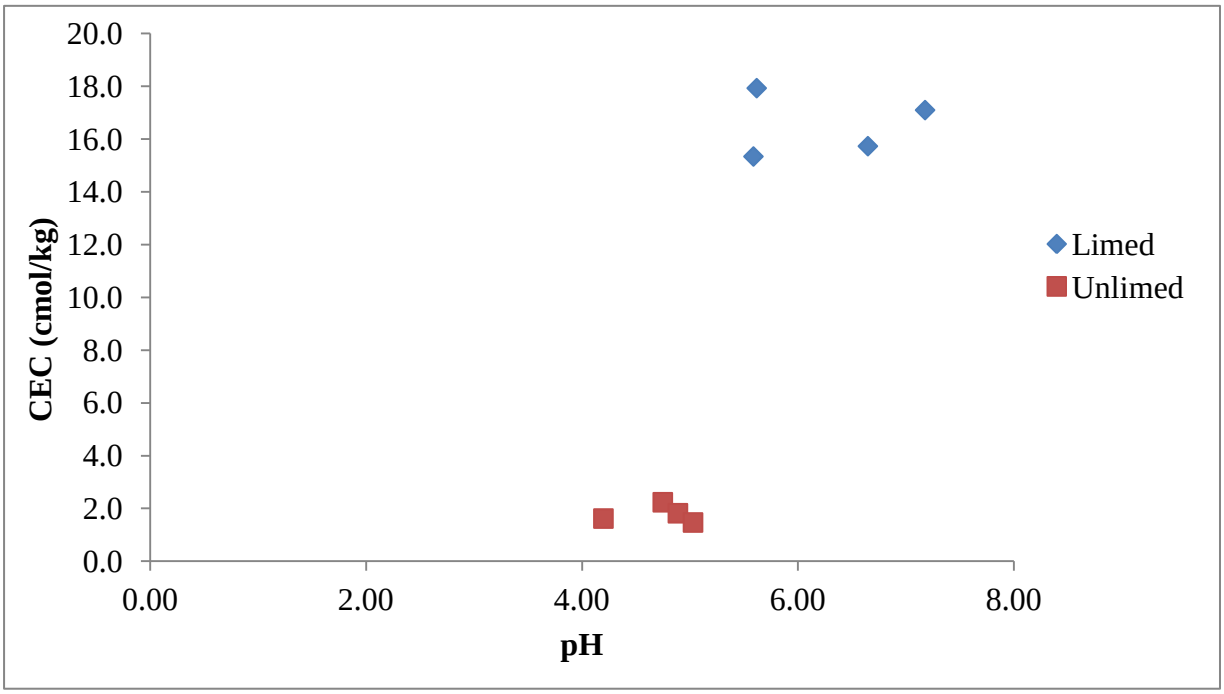


Figure 11: Patterns of pH and Cations Exchange Capacity (CEC) in samples from limed and unlimed sites in the Greater Sudbury Region (GSR) in Northern Ontario.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

Table 7: Microorganisms identified using phospholipid fatty acid (PLFA) analysis in soil samples from the Greater Sudbury Region (GSR). Data in ng/g.

Sites	Total microbial biomass	AM Fungi	Other Fungi	Gram Negative	Gram Positive	Other Eukaryote	Anaerobe	Actinomycetes
Limed sites	342.15a	16.10a	37.18a	164.90a	78.10a	8.49a	3.83a	33.56a
	±35.61	±4.63	±6.10	±21.87	±9.38	±0.54	±0.68	±4.17
Unlimed sites	148.98b	5.67b	13.45b	58.92b	42.57b	4.35b	2.39a	21.63a
	±61.47	±2.11	±5.34	±28.82	±16.82	±1.54	±0.84	±6.65

Results are expressed as mean values ± standard error.

Means in columns with a common letter are not significantly different based on t-test ($p \geq 0.05$).

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

Table 8: Phospholipid fatty acid (PLFA) ratios of main microbial groups in soil samples from the Greater Sudbury Region (GSR)

Sites	Fungi/ Bacteria	Predator/ Prey	Gram +/ Gram -	Saturated/ Unsaturated	Mono/ Poly	16w/ 17 cyclo	18w/ 19 cyclo
Limed sites	0.23a ±0.05	0.04a ±0.01	0.47a ±0.02	0.87a ±0.07	4.06a ±0.98	2.20a ±0.30	1.97a ±0.47
Unlimed sites	0.20a ±0.03	0.05a ±0.00	0.72b ±0.17	1.46b ±0.10	3.19b ±0.36	2.29a ±0.08	0.63b ±0.11

Results are expressed as mean values ± standard error.

Means in columns with a common letter are not significantly different based on t-test ($p \geq 0.05$).

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

Palmitic acid (16:0) was the most common fatty acid averaging 12.98% for all soil samples. Other detected fatty acids included a15:0 (3.00%), i15:0 (6.73%), 16:1ω7c (7.21%), 16:1ω3c (3.30%), 10Me16:0 (4.53%), c17:0ω7c (3.25%), 18:0ω7c (11.26%), 18:1ω9c (8.40%), 18:2ω6c (7.49%), and c19:0ω7c (7.88%). These fatty acids were present in all the sites and made up about 78% and 73% of total fatty acid content in the limed and unlimed soil samples, respectively. Overall, monounsaturated fatty acids were more prevalent in the targeted sites followed by saturated, branched, and cyclo chain fatty acids.

3.4.3 Pyrosequencing Analyses

Overall, 63,814 and 42,979 sequences were generated for bacteria and fungi, respectively. The number was reduced to 61,630 and 41,588 for bacterial 16S rRNA and fungal ITS after trimming, removing chimeras, and omitting sequences shorter than 200 bp. Relative abundances of bacterial and fungal groups were estimated at the phylum, family, class and genus levels. All the bacterial sequences were classified while fungal sequences were unclassified. In fact, for the *Ascomycota* phylum, 48% of the sequences were not classified and for the *Basidiomycota*, this portion was 0.02%. Relative abundances of bacterial and fungal species are illustrated in Tables S3 and S4. The reads generated in this project have been deposited in the NCBI Short Read Archive database (Accession number: SRP071853).

3.4.3.1 Bacterial Community Composition and Diversity Analysis

Five major bacterial phyla were identified in the soil samples analyzed. They include *Actinobacteria*, *Acidobacteria*, *Chloroflexi*, *Firmicutes*, and *Proteobacteria*. They accounted for 96.01% of the phyla present in the soil samples. *Firmicutes* and *Chloroflexi* were the least

abundant in all soil samples with a relative average abundance of 3.23% and 4.62%, respectively. They were mostly found in unlimed soils (Table 9). The most abundant phylum was *Proteobacteria* with a prevalence of 52.48%, followed by *Acidobacteria* (28.30%), and *Actinobacteria* (7.34%). *Acidobacteria* were more prevalent in unlimed soils compared to limed soils ($p \leq 0.05$) (Table 9). The most preponderant families in this phylum included *Bradyrhizobiaceae, Rhizobiaceae, Rhodobacteraceae, Rhodobiaceae, Rhodocyclaceae,* and *Rhodospirillaceae*. *Bradyrhizobiaceae* family with 12 genera including the nitrogen fixing *Bradirhizobium* genus was more abundant in limed sites compared to unlimed areas. In total, 31 bacterial classes, 80 families, and 133 genera were identified. Venn diagrams show different bacterial groups and their distribution (Figs. 12-14).

No differences in the frequency distribution for bacterial taxonomies (family: $p=0.74$; class: $p=0.29$ and genera: $p=0.80$) were observed between limed and unlimed soil samples based on Kolmogorov-Smirnov test (non-parametric test). At the genus level, *Solirubrobacter* and *Nocardioides* belonging to the *Actinobacterial* phylum were more abundant in limed sites compared to unlimed soil samples. Interestingly, *Nitrospira* class was found only in limed site whereas *Anaerolineaea, Chloroflexi, Jungermanniopsida, lentisphaeria, Oscillatoriophycideae* classes were found only in unlimed areas. Likewise, there were 25% of genera specific to limed sites and 16% to unlimed (Fig. 14).

In total, 149 bacterial groups were identified in all the targeted sites (Table S10) of which 121 were found in limed sites and 108 were present in unlimed soil samples (Table S10). Further analysis revealed that 20 bacterial groups were present only in limed sites and 21 in unlimed soils (Table S8). *Afipia broomeae, Afipia felis, Arenimonas* spp., *Bradyrhizobium* spp.,

Candidates alysiosphaera spp., *Defluviicoccus* spp., *Ktedonobacter* spp., *Labrys* spp., *Pedomicrobium* spp., *Pilimelia* spp. and *Pseudolabrys* spp. were more abundant ($p \leq 0.05$) in limed sites compared to unlimed areas (Table S10). On the other hand, *Acidobacterium* spp., *Aquicell* spp., *Caldanaerobacter thermoanaerobacter tengcongensis*, *Candidates koribacter* spp., *Conexibacter* spp., *Filomicrobium* spp., *Gemmatimonas* spp., *Geobacillus* spp., *Mycobacterium riyadhense*, *Planctomyces* spp., *Sciscionella* spp., *Skermanella* spp., *Sorangium* spp., *Thermosporothrix* spp., *Thioalkalispira* spp. and *TM7* were prevalent in unlimed areas compared to soils from limed sites (Table S10).

The average estimated minimum number of species (OTUs), Chao1 index, Shannon's index, Simpson index, and species richness and evenness for the two soil types (limed and unlimed) are described in Table 10. Final dataset yielded 619 OTUs of which 463 were observed in limed and 473 in unlimed sites (Table 10). Chao1 values were higher in limed compared to unlimed soil samples (Table 10). However, there was no significant difference for Simpson index, Shannon index, and species richness and evenness between microbial populations from limed and unlimed sites ($p \geq 0.05$) (Table 10). In the present study, Jaccard and Sorenson's similarity indices were low (Table 11). Whereas, high values were recorded for Bray-Curtis and Whittaker dissimilarity indices (Table 11). The results from similarity and dissimilatiry indices suggest that microbial communities are closely related to each other in the two sites (limed and unlimed) (Table 11). Further, we compared the sites with one another using clustering based on the weighed UniFrac (incorporates relative abundance) distances (Table S11).

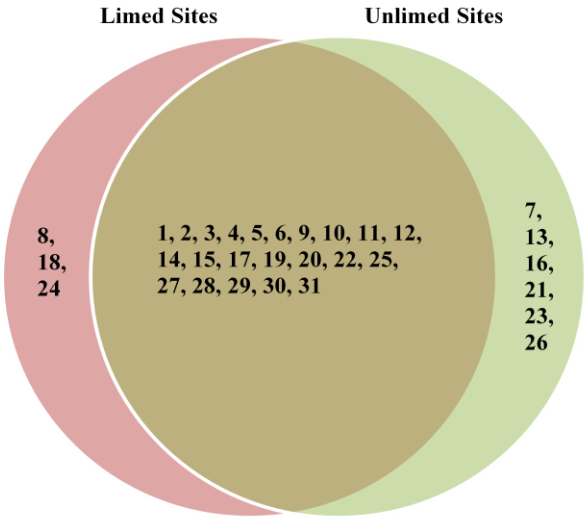
Table 9: The main phylogenetic groups of bacteria and fungi, and their relative abundance in soil samples from the Greater Sudbury Region (GSR).

Phylum	Limed sites	Unlimed sites
Bacteria		
Actinobacteria	10.90a ± 3.81	3.77b ± 1.11
Acidobacteria	18.53a ± 6.17	38.15b ± 3.34
Chloroflexi	1.17a ± 0.67	8.06a ± 5.12
Firmicutes	1.88a ± 1.39	4.59a ± 2.24
Proteobacteria	64.74a ± 3.88	40.22b ± 5.45
Fungi		
Ascomycota	13.79a ± 3.92	46.00b ± 14.52
Basidiomycota	85.74a ± 4.07	49.20b ± 12.03
Zygomycota	0.46a ± 0.20	4.80b ± 1.92

Results are expressed as mean percentages ± standard error

Means in rows with a common letter are not significantly different based on t-test ($p \geq 0.05$).

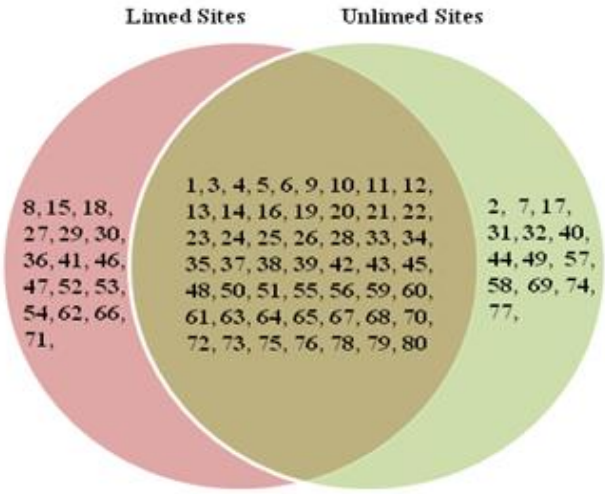
Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.



1. *Acidimicrobiia*
2. *Acidobacteria* (unclassified)
3. *Acidobacteria* (class)
4. *Actinobacteria* (subclass)
5. *Actinobacteria* (class)
6. *Alphaproteobacteria*
7. *Anaerolineae*
8. *Armatimonadia*
9. *Bacilli*
10. *Betaproteobacteria*
11. *Bryopsida*
12. *Caldilineae*
13. *Chloroflexi*
14. *Class* unspecified
15. *Clostridia*
16. *Cytophagia*
17. *Deltaproteobacteria*
18. *Flavobacteria*
19. *Gammaproteobacteria*
20. *Gemmatimonadetes*
21. *Jungermanniopsida*
22. *Ktedonobacteria*
23. *Lentisphaeria*
24. *Nitrospira*
25. *Opitutae*
26. *Oscillatoriophycideae*
27. *Planctomycetacia*
28. *Spartobacteria*
29. *Sphingobacteria*
30. *Thermoleophilia*
31. *TM7*classes

Figure 12: Venn diagram showing distribution of bacterial classes identified in limed and unlimed soil samples from the GSR.

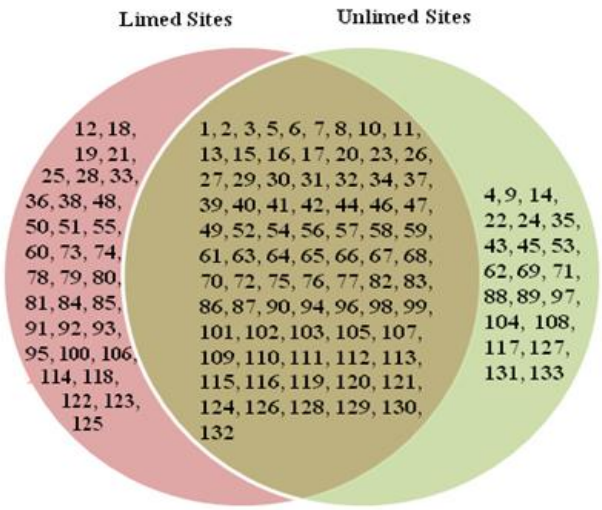
Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.



1. *Acetobacteraceae*
2. *Acidimicrobiaceae*
3. *Acidimicrobiales* families
4. *Acidobacteriaceae*
5. *Acidothermaceae*
6. *Alcaligenaceae*
7. *Anaerolineaceae*
8. *Armatimonadaceae*
9. *Bacillaceae*
10. *Beijerinckiaceae*
11. *Bradyrhizobiaceae*
12. *Burkholderiaceae*
13. *Caldilineaceae*
14. *Candidatus alysiosphaera*
15. *Candidatus captivus*
16. *Candidatus chloracidobacterium*
17. *Candidatus chlorothrix*
18. *Candidatus microthrix*
19. *Candidatus solibacter*
20. *Caulobacteraceae*
21. *Chitinophagaceae*
22. *Chromatiaceae*
23. *Climaciaceae*
24. *Colwelliaceae*
25. *Comamonadaceae*
26. *Conexibacteraceae*
27. *Corynebacteriales* families
28. *Coxiellaceae*
29. *Cryomorphaceae*
30. *Cryptosporangiaceae*
31. *Cytophagaceae*
32. *Dermatophilaceae*
33. *Ectothiorhodospiraceae*
34. *Enterobacteriaceae*
35. *Frankiaceae*
36. *Gallionellaceae*
37. *Gemmatimonadaceae*
38. *Hyphomicrobiaceae*
39. *Hyphomonadaceae*
40. *Iamiaceae*
41. *Kineosporiaceae*
42. *Koribacteraceae*
43. *Ktedonobacteraceae*
44. *Lepidoziaceae*
45. *Methylocystaceae*
46. *Methylophilaceae*
47. *Micrococcaceae*
48. *Micromonosporaceae*
49. *Mitochondria*
50. *Mycobacteriaceae*
51. *Nannocystineae*
52. *Nitrospinaceae*
53. *Nitrospiraceae*
54. *Nocardioidaceae*
55. *Opitutaceae*
56. *Oxalobacteraceae*
57. *Parvularculaceae*
58. *Phormidiaceae*
59. *Planctomycetaceae*
60. *Pseudonocardiaceae*
61. *Rhizobiaceae*
62. *Rhodobacteraceae*
63. *Rhodobiaceae*
64. *Rhodocyclaceae*
65. *Rhodospirillaceae*
66. *Sneathiellaceae*
67. *Solirubrobacteriaceae*
68. *Sorangiineae* families
79. *Sphingobacteriaceae*
70. *Sphingomonadaceae*
71. *Streptomycetaceae*
72. *Thermoanaerobacteraceae*
73. *Thermodesulfobiaceae*
74. *Thermogemmatisporaceae*
75. *Thermosporotrichaceae*
76. *TM7* families
77. *Victivallaceae*
78. *Xanthobacteraceae*
79. *Xanthomonadaceae*
80. *Xiphinematobacteraceae*

Figure 13: Venn diagram showing distribution of bacterial families identified in limed and unlimed soil samples from the GSR.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.



1. *Achromobacter*
2. *Acidicaldus*
3. *Acidimicrobiales*
4. *Acidimicrobium*
5. *Acidiphilium*
6. *Acidisphaera*
7. *Acidobacterium*
8. *Acidocella*
9. *Acidomonas*
10. *Acidothermus*
11. *Acidovorax*
12. *Actinoplanes*
13. *Afipia*
14. *Anaerolinea*
15. *Anderseniella*
16. *Aquicella*
17. *Arenimonas*
18. *Armatimonas*
19. *Arthrobacter*
20. *Azospira*
21. *Azovibrio*
22. *Bazzania*
23. *Blastochloris*
24. *Blastomonas*
25. *Bosea*
26. *Bradyrhizobium*
27. *Burkholderia*
28. *Byssovorax*
29. *Caldanaerobacter*
30. *Caldilinea*
46. *Conexibacter*
47. *Coprothermobacter*
48. *Corynebacteriales*
49. *Crossiella*
50. *Cryptosporangium*
51. *Cupriavidus*
52. *Defluviicoccus*
53. *Dermatophilus*
54. *Dongia*
55. *Dyella*
56. *Edaphobacter*
57. *Escherichia shigella*
58. *Ferruginibacter*
59. *Filomicrobium*
60. *Fluviicola*
61. *Frankia*
62. *Geitlerinema*
63. *Gemmata*
64. *Gemmatimonas*
65. *Geobacillus*
66. *Granulicella*
67. *Haliangium*
68. *Hirschia*
69. *Hymenobacter*
70. *Hyphomicrobium*
71. *Iamia*
72. *Inquilinus*
73. *Kineosporia*
74. *Kribbella*
75. *Ktedonobacter*
90. *Nitrosococcus*
91. *Nitrospira*
92. *Nocardioides*
93. *Novosphingobium*
94. *Opitutus*
95. *Parasegetibacter*
96. *Parvularcula*
97. *Pedomicrobium*
98. *Phenylobacterium*
99. *Pilimelia*
100. *Pirellula*
101. *Planctomyces*
102. *Pleomorphomonas*
103. *Polaromonas*
104. *Pseudolabrys*
105. *Rhizobium*
106. *Rhodobacter*
107. *Rhodobium*
108. *Rhodopirellula*
109. *Rhodoplanes*
110. *Rhodovastum*
111. *Rhodovibrio*
112. *Roseomonas*
113. *Sciscionella*
114. *Sideroxydans*
115. *Simplicispira*
116. *Singulisphaera*
117. *Skermanella*
118. *Sneathiella*
119. *Solirubrobacter*

31. *Candidatus koribacter*
32. *Candidatus alysiosphaera*
33. *Candidatus captivus*
34. *Candidatus chloracidobacterium*
35. *Candidatus chlorothrix*
36. *Candidatus entotheonella*
37. *Candidatus koribacter*
38. *Candidatus microthrix*
39. *Candidatus solibacter*
40. *Candidatus xiphinematobacter*
41. *Caulobacter*
42. *Climaciaceae*
43. *Collimonas*
44. *Colwellia*
45. *Comamonas*
76. *Labrys*
77. *Leptothrix*
78. *Luteibacter*
79. *Magnetospirillum*
80. *Marmoricola*
81. *Massilia*
82. *Methylocystis*
83. *Methylosinus*
84. *Methylotenera*
85. *Methyloversatilis*
86. *Metyhlovirgula*
87. *Mitochondria*
88. *Mucilaginibacter*
89. *Mycobacterium*
120. *Sorangiineae*
121. *Sorangium*
122. *Sphingomonas*
123. *Streptomyces*
124. *Telmatospirillum*
125. *Tepidamorphus*
126. *Thermogemmatispora*
127. *Thermomonas*
128. *Thermosporothrix*
129. *Thioalkalispira*
130. *Tistlia*
131. *TM7*
132. *Victivallis*
133. *Zavarzinella*

Figure 14: Venn diagram showing distribution of bacterial genera/species identified in limed and unlimed soil samples from the GSR.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

Table 10: Microbial diversity index values for bacterial and fungal communities from the Greater Sudbury Region (GSR).

	Chao 1	# of OTUs	Simpson Index	Shannon Index (H')	Species Evenness	Species Richness
Bacteria species						
Limed sites	257a ± 24.93	463	0.86a ± 0.08	5.68a ± 0.22	0.52a ± 0.02	121
Unlimed sites	153b ± 39.38	473	0.78a ± 0.04	4.29a ± 0.87	0.51a ± 0.01	108
Fungi species						
Limed sites	37a ± 6.54	96	0.81a ± 0.12	2.59a ± 0.30	0.51a ± 0.01	59
Unlimed sites	27b ± 9.07	81	0.78a ± 0.03	2.32a ± 0.51	0.54a ± 0.03	51

Results are expressed as mean values ± standard error

Means in column with a common letter are not significantly different within bacteria and fungi species based on t-test ($p \geq 0.05$).

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

Table 11: Pair wise microbial diversity analysis for bacteria and fungi communities in the Greater Sudbury Region (GSR).

Sites	Jaccard's Index (β_j)	Sorenson's Index (β_{sor})	Bray-Curtis Index (β_b)	Whittaker Index (β_w)
Bacteria species				
Limed and Unlimed sites	0.26	0.41	0.53	0.59
Fungi species				
Limed and Unlimed sites	0.27	0.42	0.81	0.58

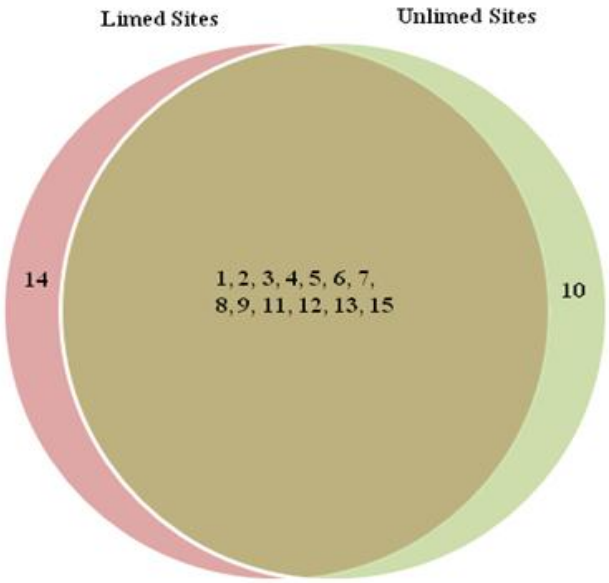
Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

3.4.3.2 Fungal Community Composition and Diversity Analysis

Pyrosequencing analysis identified three phyla of fungi that included *Ascomycota, Basidiomycota*, and *Zygomycota* (Table 9). *Dibaeis* (lichen-forming fungi), *baeomyces* (lichenized fungi), *Dermateaceae* sp., (plant pathogens and decay plant materials), *Fusarium oxysporum* (plant parasites), *Phialocephala fortini* (mycorrhizal fungi), and *Pezizales* sp. (saprophytes) were the most dominant groups of *Ascomycota*. The majority of sequences in the *Basidiomycota* phylum matched ectomycorrhizal (EM fungi) and wood rotting fungi.

Overall, 15 fungal classes, 42 families, and 59 genera were identified. Venn diagrams show different fungal groups and their distribution (Figs. 15-17). No differences in the frequency distribution for fungal groups were observed between limed and unlimed soil samples based on Kolmogorov-Smirnov test (non-parametric test) (family, $p=0.84$; class, $p=0.77$; and genera, $p=0.71$). In total, 70 fungal species belonging to 59 genera were identified in limed and unlimed sites. They are described in Table S11. In addition, 15 site-specific species were observed of which 10 were present only in limed and 5 in unlimed soil samples (Table S9).

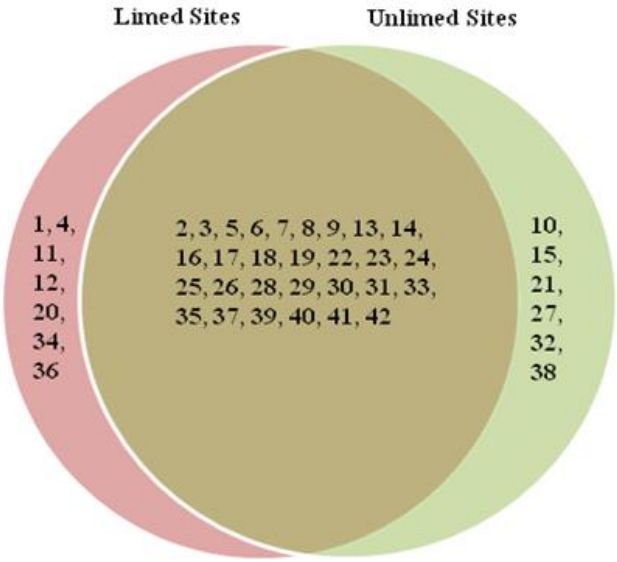
Several fungal species were more abundant in limed soils compared to unlimed sites. They include *Amanita muscaria, Cryptococcus podzolicus, Penicillium montanense*, *Pezizomycotina* sp., *Russula gracilis* and *Thelephoraceae* sp. (Table S12). On the other hand, *Ascomycot*a sp., *Calicium salicinum, Dermateaceae* sp., *Dothideomycetes* sp., *Helotiaceae* sp., *Helotiales* sp., *Herpotrichiellaceae* sp., *Laccaria proxima, Leotia viscose*, *Mortierellales* sp., *Oidiodendron maius, Russula* sp., *Russula sphagnophila* and *Sebacinaceae* sp. were prevalent in unlimed areas compared to limed sites ($p \leq 0.05$) (Table S12). In addition, several fungal genera known for their roles as human, plant or animal pathogens were found. They include *Dothideomycetes,*



1. *Agaricomycetes*
2. *Ascomycota* classes
3. *Basidiomycota* classes
4. *Dothideomycetes*
5. *Eurotiomycetes*
6. *Fungi* classes
7. *Lecanoromycetes*
8. *Leotiomycetes*
9. *Mortierellomycotina* classes
10. *Mucoromycotina* classes
11. *Pezizomycetes*
12. *Pezizomycotina* classes
13. *Sordariomycetes*
14. *Taphrinomycotina* classes
15. *Tremellomycetes*

Figure 15: Venn diagram showing distribution of fungal classes identified in limed and unlimed soil samples from the GSR.

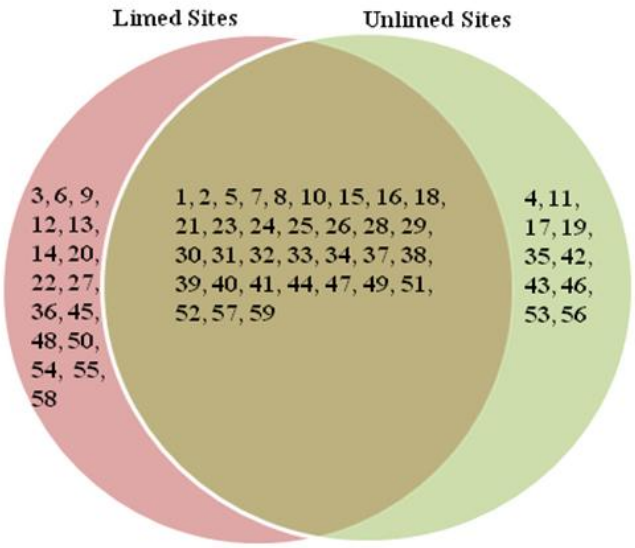
Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.



1. *Agaricales* families
2. *Agaricomycotina* families
3. *Amanitaceae*
4. *Archaeorhizomycetaceae*
5. *Ascomycota* families
6. *Atheliaceae*
7. *Basidiomycota* families
8. *Boletaceae*
9. *Caliciaceae*
10. *Cladoniaceae*
11. *Clavulinaceae*
12. *Cortinariaceae*
13. *Dermateaceae*
14. *Dothideomycetes* families
15. *Elaphomycetaceae*
16. *Fungi* families
17. *Helotiaceae*
18. *Helotiales* families
19. *Herpotrichiellaceae*
20. *Hypocreales* families
21. *Icmadophilaceae*
22. *Leotiaceae*
23. *Leotiomycetes* families
24. *Magnaporthales* families
25. *Mortierellaceae*
26. *Mortierellales* families
27. *Mucoromycotina* families
28. *Myxotrichaceae*
29. *Pezizomycotina* families
30. *Pyronemataceae*
31. *Russulaceae*
32. *Sclerodermataceae*
33. *Sebacinaceae*
34. *Sordariales* families
35. *Sordariomycetes* families
36. *Suillaceae*
37. *Thelephoraceae*
38. *Tremellaceae*
39. *Tremellales* families
40. *Trichocomaceae*
41. *Tricholomataceae*
42. *Venturiales* families

Figure 16: Venn diagram showing distribution of fungal families identified in limed and unlimed soil samples from the GSR.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.



1. *Agaricomycotina*
2. *Amanita*
3. *Archaeorhizomyces*
4. *Ascomycete*
5. *Ascomycota*
6. *Atheliaceae*
7. *Basidiomycota*
8. *Calicium*
9. *Cenococcum*
10. *Chaetomella*
11. *Cladonia*
12. *Clavulinaceae*
13. *Cortinariaceae*
14. *Cortinarius*
15. *Cryptococcus*
16. *Dermateaceae*
17. *Dibaeis*
18. *Dothideomycetes*
19. *Elaphomyces*
20. *Ericoid*
21. *Fungi*
22. *Fusarium*
23. *Gyoerffyella*
24. *Helotiaceae*
25. *Helotiales*
26. *Herpotrichiellaceae*
27. *Inocybe*
28. *Laccaria*
29. *Lactarius*
30. *Leotia*
31. *Leotiomycetes*
32. *Magnaporthales*
33. *Mortierella*
34. *Mortierellales*
35. *Mucoromycotina*
36. *Myrothecium*
37. *Myxotrichaceae*
38. *Oidiodendron*
39. *Penicillium*
40. *Pezizomycotina*
41. *Phialocephala*
42. *Piloderma*
43. *Pyronemataceae*
44. *Russula*
45. *Russulaceae*
46. *Scleroderma*
47. *Sebacinaceae*
48. *Sordariales*
49. *Sordariomycetes*
50. *Suillus*
51. *Thelephoraceae*
52. *Tomentella*
53. *Tremella*
54. *Tricholoma*
55. *Tricholomataceae*
56. *Tylospora*
57. *Venturiales*
58. *Wilcoxina*
59. *Xerocomus*

Figure 17: Venn diagram showing distribution of fungal genera identified in limed and unlimed soil samples from the GSR.

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway.

Fusarium, Herpotrichiellaceae, Leotiomycetes, Magnaporthales, Mortierella, Pezizales, Scleroderma and Sordariomycetes. Overall, 27% of genera were specific to limed sites and 17% to unlimed (Fig. 17).

A total of 119 OTUs were identified of which 96 were found in limed and 81 in unlimed sites (Table 10). Estimated Chao1 values revealed significant differences between limed and unlimed sites ($p \leq 0.05$) (Table 10). But no significant differences between limed and unlimed sites were observed based on Simpson index, Shannon index, and species evenness data ($p \geq 0.05$) (Table 10). The study showed no change in species richness among limed and unlimed soil samples (Table 10). Further analysis indicated a weak negative correlation between all the fungal diversity parameters and pH. Pair wise comparisons among soil samples revealed low similarity indices or high dissimilarity indices (Table 11). Distance matrix values were high among samples (Table S13).

3.5 Discussion

The pH in limed soils was significantly higher than in unlimed areas reflecting the addition of dolomitic limestone to soils >35 years ago. These results are consistent with data reported in other studies [25,34,35,47]. Likewise, strong positive correlations between soil pH and CEC reflect the data documented in other soil analysis under different conditions [12,26,172]. PLFA and pyrosequencing data demonstrate that microbial biomass and relative abundance were significantly higher in limed sites compared to the adjacent unlimed areas. Although several bacterial and fungal groups were present in all the sites, 25% of bacterial genera were specific to limed sites and 16% to unlimed areas. Similarly, the proportions of fungal genera specific to limed and unlimed sites were 27% and 17%, respectively. Chao1 values were higher for limed sites compared to unlimed areas for both bacteria and fungi. But the number of OTUs, and the levels of Simpson and Shannon diversity indices, and species evenness were not affected by liming.

3.5.1 Phospholipid Fatty Acid Analysis

PLFA analysis was used to determine soil microbial responses to liming. The results revealed an increase in total microbial biomass, AM fungi, other fungi, other eukaryotes, actinomycetes, gram positive, and gram negative bacteria in limed sites compared to unlimed soil samples. The differences in microbial biomass between limed and unlimed soils can be attributed to soil pH [31,38,40,91]. Soils treated with different types of limes have also been found to change PLFA composition [39,91].

The relative proportion of branched PLFAs (17:0 and 18:0) and iso- and anteiso branched PLFAs (i15:0, i16:0, i16:1, and i17:0) were higher in limed soils compared to samples from

unlimed areas suggesting that liming stimulated gram positive bacterial populations. A similar trend was observed for gram negative bacteria which were more abundant in limed soils compared to unlimed soil samples. PLFAs that are used as indicators of gram negative bacteria include 16:1ω5, 16:1ω9, 17:1ω9, cy17:0, 18:1ω7 and cy19:0 [91,92]. Overall, a predominance of gram negative over gram positive bacteria was observed in the two types of soils. This is usually common under stress conditions [40,91,92].

It has been demonstrated that acidic soil conditions enhance the development and activity of fungi relative to those of bacteria [13,31,40,91,93]. In the present study, 16:0 and 18:1ω9 PLFA considered as reliable indicators of fungal biomass were present in higher concentrations in unlimed soils compared to limed sites. But the concentration of PLFA 18:2ω6 another indicator of fungal biomass was lower in unlimed areas compared to limed sites. PLFA 18:1ω9 and 18:2ω6 are not exclusive to fungi since they are present in many eukaryotic organisms, including plants [40,90]. Therefore, some of these PLFAs identified in fungi especially in limed sites, might be from plant materials. In general, 16:1ω5 and 18:1ω7 are good indictors of AM fungi [94,95]. Both PLFAs were slightly more abundant in limed sites compared to unlimed samples. Overall, fungal biomass was significantly higher in limed compared to unlimed soil samples. In addition to the biomass and community structure determinations, PLFA analysis has been used to get an insight into the physiological status of the microbes. The increase in trans/cis, saturated/unsaturated, and cy17:0/16:1ω7c ratios in the unlimed sites are consistent with stressed environmental conditions [92]. Low ratio of trans to cis-monoenoic unsaturated fatty acids, fungi to bacteria, and a high proportion of cyclopropyl fatty and gram negative PLFAs in all soil samples confirm that the region is still under environmental stress.

3.5.3 Pyrosequencing Analysis

Pyrosequencing has been used to analyze bacteria population dynamics [99,111,112]. In the present study, analysis of two types of soil samples showed a higher relative abundance of bacterial species and genera compared to fungi. In total, 149 bacterial groups were identified belonging to 133 genera. For fungi, 70 fungal groups corresponding to 59 genera were identified in the targeted sites.

Bacterial communities are directly influenced by soil pH as most bacterial taxa exhibit narrow growth tolerances [26,33,93,221]. Rousk *et al*. [93] and Ferandez-Calvino and Baath [221] reported that deviations of 1.5 pH units can reduce bacterial community's activity by 50%. The shifts in the relative abundances of specific taxonomic groups across pH gradient are similar to the pH responses observed in other studies [93,221]. For instance, the relative abundance of *Acidobacteria* has been shown to increase when soil pH decreases [93,216,222]. Rousk *et al*. [93] and Lauber *et al*. [111] reported a strong positive correlation between relative abundance of *Bacteriodetes* and pH. But the results of the present study showed an opposite trend. The increase of *Bacteriodetes* in unlimed sites could be related to site characteristics such as carbon availability or soil moisture. The high relative abundance of *Proteobacteria* in limed sites is in agreement with Rousk *et al*. [93] and Lauber *et al*. [111] who reported an increase of these bacteria associated with a higher availability of carbon and high pH. *Proteobacteria* are members of photosynthetic and nitrogen fixing bacteria. The bacterial communities of those functional species play an important role in carbon, nitrogen cycles and in maintaining integrity of the ecosystem [111]. In fact, the targeted limed sites showed a high level of forest complexity and diversity compared to unlimed areas [47].

Bacterial community composition was also examined at the genus level. *Acidobacterium*, *Afipia*, *Aquicella*, *Bradyrhizobium*, *Geobacillus*, *Granulicella*, *Nitrosococcus*, *Rhodoplanes*, *Skermanella*, *Solirubrobacter*, *Thermosporothrix*, and *Thioalkalispira* were the most predominant genera in all the sites. The composition and distribution of these 12 common bacterial genera varied between limed and unlimed soil samples. Photosynthetic bacteria, such as *Rhodobacter* were found only in limed soils. These bacteria are known to play an irreplaceable role in carbon cycling and material transformation, and their diversities affect the process of nitrogen cycle [99]. *Rhizobium* present in all soil samples is a major contributor to the global nitrogen cycle as it forms a symbiotic nitrogen fixation with many plants. It also improves soils fertility, promotes circulation of soil materials and increases soil microbial activity [99]. The relative abundance of *Burkholderia* observed in the unlimed soil is consistent with the recent classification of this genus as an acid tolerant group [223]. *Bradyrhizobia* are often found in acid soils and they were identified in both limed and unlimed soil samples. In addition, several acid tolerant bacterial species were identified in both soil types.

Bacterial groups that grow in stressed environment and are involved in nitrogen fixation, carbon cycling and iron oxidation, were found in targeted sites at various levels of relative abundance. They are usually present in sludge and waste water treatments. *Nitrospira* bacteria that were observed only in limed sites in the present study are part of a nitrification process which is important in the biogeochemical nitrogen cycle. Overall, a higher percentage of gram negative bacterial species compared to gram positive were identified in all the sites. This is in agreement with PLFA analysis data.

3.5.3.1 Fungal Populations

While many reports have limited their pyrosequencing analysis only on bacterial communities this study assessed fungal populations in all the sites as well. Recent studies using pyrosequencing technique have revealed that relative abundance of fungi is not affected by pH and that fungal diversity is only weakly associated to pH [93,218,224]. We confirmed that the composition of fungal community is weakly related to soil pH. This demonstrates that fungi exhibit wider pH ranges for optimal growth. The optimum level of pH varies from 5 to 9 [13,34,35,218]. Overall, pyrosequencing data are consistent with PLFA results revealing a much lower fungal biomass compared to bacteria in limed and unlimed sites.

The relative abundance of *Basidiomycota* was higher in limed soils compared to samples from unlimed sites. An opposite trend was observed for *Ascomycota*. The main *Ascomycota* detected in this study were classified as lichen forming fungi (*Calicium salicinum*, *Cladonia coniocraea*, *Dibaeis baeomyces*), little/wood decomposers (*Dermateaceae* sp., *Sordariomycetes* sp., *Tremella diploschistina*), plant parasites (*Dothideomycetes* sp., *Fusarium oxysporum*), human pathogens (*Herpotrichiellaceae* sp.), endophytes (*Gyoerffyella* sp., *Phialocephala fortinii*) and saprotrophs (*Venturiales* sp.). *Basidiomycetes* included EM fungi and decay organisms of plant residues. EM fungi colonize plant roots and help plants to access nutrients such as phosphorous from soil [112,225]. Several decomposers involved in the decomposition of hard woody organic matters and in the conversion of organic matters into fungal biomass, carbon dioxide (CO_2) and organic acids were also present in the targeted sites. Compared to *Ascomycota* and *Basidiomycota,* a low relative abundance of *Zygomycota* was observed in all the soil samples analyzed. *Mortierella sp.* and *Mortierellales* sp. from the phylum *Zygomycota* were more

abundant in unlimed compared to limed samples. These species mineralize readily dissolved organic substrates rather than breaking down soil litter polymers [226]. Members of the *Taphrinomycotina* were found only in limed sites. In general they range from simple and yeast-like to filamentous. Their role in the environment is not well documented.

3.5.3.2 Bacterial and Fungal Diversity

Estimates of diversity based on DNA sequencing are more accurate than culture techniques. In this study, relative abundance and diversity were estimated by the number of OTUs, Chao1, Shannon index and Simpson index. OTU is a widely used construct of clustered sequence data where each OTU represents a different microbial (bacterial or fungal) population in the community. These approaches are providing estimates of diversity at > 10,000 species (OTUs) of bacteria per gram of soil [81]. Dimitriu and Grayston [216] looked at bacterial diversity across reclaimed and natural boreal forest in British Columbia and reported 577 OTUs for bacteria. This is consistent with the results of the present study on the damaged and reclaimed sites in the GSR. Chao1 estimates species richness from the rarefaction of observed sequences present in a community. This index is based on the number of rare classes (i.e. OTUs or species) in a sample [199]. Chao1 estimates for bacterial species richness in soils were higher compared to fungi. Importantly, Chao1 estimates were higher in limed sites compared to unlimed areas for both bacteria and fungi. But this study revealed that liming has no effects on overall bacterial and fungal diversity based on Shannon and Simpson indices. The level of diversity in fungal communities was lower than bacterial diversity based on Shannon diversity index.

For beta measures, Jaccard and Sorenson indices are based on the presence/absence data [220,227]. Low values for Jaccard and Sorenson indices, for both bacteria and fungi indicate

high dissimilarity (high beta diversity) between limed and unlimed areas. High values for Bray-Curtis and Whittaker dissimilarity indices indicate that there is a high number of species that are different between the two types of sites (high beta diversity). Overall, both similarity and dissimilarity indices reveal significant difference between limed and unlimed sites.

In addition, weighed UniFrac distances which take into account differences in relative abundance of taxa between limed and unlimed sites showed that for bacterial communities, limed sites are closely related to each other. Whereas for fungal communities, high distance values indicated that the sites are different from each other.

In the present study, both alpha and beta diversity indices were used. Our data show low levels of diversity within each group of sites (limed and unlimed) and low similarity among the two types of sites. This indicates that although liming increases significantly the amount of microbial biomass, the level of species diversity in limed compared to unlimed areas remain unchanged (and low) even though the microbial compositions are not similar. Ecological and soil properties such as vegetation type, soil texture, soil type, moisture and nutrient concentrations could play a significant role in microbial community composition and relative abundance.

3.5.4 Pyrosequencing and PFLA Comparison

In general, PLFA-based methods are rapid, inexpensive, sensitive and reproducible. There are a number of scientific publications based on PFLA analysis that have increased our understanding of the soil ecosystem. Unlike pyrosequencing, PLFA has limitations such as overlap in the composition of microorganisms and the specificity of PLFAs signature. For the present study, PFLA data revealed significant difference in microbial biomass among the limed and unlimed sites. But these differences were not always detected by pyrosequencing. In this

study, the combination of PLFA and pyrosequencing analyses provided a more complete assessment of microbial relative abundance, structure, and diversity.

3.6 Conclusions

In the present study, soil microorganisms were studied to assess the effect of liming on bacterial and fungal communities. This study is novel as it describes the effect of liming on microbial relative abundance and diversity >35 years after dolomitic limestone applications. The combination of both PFLA and pyrosequencing validate our findings. Soil analysis revealed an increase in soil pH (6.3) and CEC (16.30 cmol/kg) in limed sites compared to unlimed areas. Total microbial biomass, bacterial and fungal relative abundance were higher in limed sites compared to unlimed samples. Chao 1 estimates followed the same trend. But the total number of OTUs in limed and unlimed soil samples for bacteria and fungi were similar. Likewise, Simpson and Shannon diversity indices revealed no significant differences between limed and unlimed sites. Bacterial and fungal group's specific to limed and unlimed sites were identified. Overall, the results of the present study show that soil liming increases the amount of microbial biomass but has limited effects on bacterial and fungal diversities > 35 years after dolomitic applications. Further studies will target in detail the effects of organic matter content and metal contamination on microbial relative abundance and diversity.

3.7 Acknowledgements

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CHAPTER 4: CHANGES IN ENZYMATIC ACTIVITIES IN METAL CONTAMINATED AND RECLAIMED LANDS IN THE GREATER SUDBURY REGION

4.1 Abstract

Metal and sulphur dioxide (SO_2) contaminations in Northern Ontario (Canada), especially in the Greater Sudbury Region (GSR) caused by mining activities have resulted in severe environmental degradation. A long term restoration program has led to significant landscape changes and healthy ecosystems. The objective of this study was to assess variation in enzymatic activities and soil respiration in metal contaminated and reclaimed ecosystems. Soil respiration analysis revealed that respiration rates were higher in limed soils (65 ppm) compared to unlimed soils (35 ppm). The respiration rate in metal contaminated sites (55 ppm) was significantly lower compared to reference sites (90 ppm). β-glucosidase (BG), cellobiohydrolase (CBH), β-N-acetylglucosaminidase (NAGase), aryl sulfatase (AS), acid phosphatase (AP), alkaline phosphatase (AlP), glycine aminopeptidase (GAP), and leucine aminopeptidase (LAP) activites were significantly higher in limed compared to unlimed sites. Metal contamination significantly reduced the activities of these enzymes with the exception of LAP. An opposite trend was observed for peroxidase (PER) enzyme activity that was higher in unlimed and metal contaminated sites compared to limed and reference sites. Further analysis revealed that soil respiration and enzyme activities are linked to soil organic matter (SOM) and pH.

Keywords: Soil respiration; Soil enzyme activity; pH; Soil organic matter; Soil quality; Liming; Metal contamination.

4.2 Introduction

Mining activities for over a century have led to a decrease in soil organic matter (SOM) content and hence to a decline in soil quality in Greater Sudbury Region (GSR) [15,25,26,38,40]. This was a direct effect of metalliferous ores (since 1800) that released enormous amounts of sulphur dioxide (SO_2) and various metals into the atmosphere resulting in severe contamination and acidification of soils and water in the GSR [15,25,26,38,40]. In the last 40 years legislated controls and industrial technology development reduced SO_2 emissions by 90% which resulted in improved air quality and natural recovery of damaged ecosystems [25,38,40]. In addition, a regreening program that consisted in soil liming and planting of over 12 million trees in the GSR led to the increase of SOM and microbial biomass [15,35,38,40,47]

Chemical analysis of soils from the GSR indicates that soil pH and cation exchange capacity (CEC) are still lower (acidic) in unlimed soils compared to limed areas [25,26,34,35]. Also, low pH values were documented in metal contaminated soils compared to reference sites [25,40]. In addition, plant population diversity showed a similar trend as unlimed and metal contaminated sites had lower tree species richness compared to limed and reference sites, respectively [25,26,40]. Moreover, phospholipid fatty acid analysis (PLFA) revealed that total microbial biomass, fungal and bacterial abundance were significantly lower in unlimed and metal contaminated sites compared to limed and reference sites, respectively [26,35,40]. These studies did contribute to our understanding of the dynamics of soil chemistry and ecosystems in the GSR. But biochemical indicators are more informative as they provide information on microbial activities and functions [213,228–231]. Soil respiration and enzyme activities involved

in organic matter turnover, nutrient cycling and plant nutrition have been used to study soil fertility and health [213,228–231].

Enzyme activities in soils have been useful tools to understanding the biochemistry of decomposition and nutrient cycling (carbon: C, nitrogen: N, phosphorous: P and sulfur: S) [114,213,230,231]. In addition, enzyme activities have been associated with microbial ecology, biogeochemical process, and soil processes and health [114,150,213,228,231]. The most widely assayed enzymes are those involved in the degradation of cellulose and lignin (abundant components of plant litter), as well as enzymes that hydrolyze proteins, chitin, and peptidoglycan (reservoirs of organic N) [50,228,230]. In addition, phosphatases which play a role in mineralizing P from nucleic acids, phospholipids, and other ester phosphates have been analyzed under different agricultural conditions [150,213,230,232].

The main objective of this study was to assess the effect of liming and metal contamination on soil enzymatic activities and respiration.

4.3 Experimental Procedures

4.3.1 Study Sites

Study sites were located at Laurentian University research areas and Crown (public) lands that are not within a park or conservation reserve in the GSR and sites away from GSR (Table S1). Reclaimed mining sites in the GSR where dolomitic limestone were applied >40 years ago were selected for this study. Soil sampling was performed at four distant locations in reclaimed sites (limed) and their respective adjacent un-reclaimed areas (unlimed). These sites included Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake, and Kingsway (Table S1) (Fig. 10).

To determine the effects of metals on enzymatic activities, three sites close to smelters with high levels of metal contaminations were selected. They include Laurentian, Kelly Lake and Kingsway (Table S1) (Fig. 1). Three metal uncontaminated sites located >50 km from smelters (Onaping Falls, Capreol and Killarney) were used as controls (Table S1) (Fig. 1). The GPS coordinates for all the sites are given in table S1.

For all the sites, weather conditions such as temperature and rain falls, were presumed similar based on literature (Brown, 2006; Historical Weather, 2015; Time and Date, 1995). Data on metal concentration, pH, organic matter contents, cation exchange capacity (CEC), and microbial abundance for these sites have been previously reported [25,38,40].

4.3.2 Soil Sampling

At each site of approximately 5 km^2 in size, three soil samples (each consisting of 15 sub-samples) were collected from organic layer (0-5 cm in depth). Plant materials, stones and residues were removed and sieved using a 2 mm mesh. Soil samples were divided into two equal parts for further analysis.

4.3.3 Soil Respiration

For soil respiration, samples were completely dried, labeled and stored prior to analysis. Soil respiration analysis was performed as described by Narendrula and Nkongolo [40]. Each soil sample was analyzed in triplicates.

4.3.4 Enzyme Activities

Soil samples were stored at 4°C and enzyme activity was analyzed within six days. The potential activities of nine enzymes, involved in catalyzing the cleavage of a range of organic matter compounds and their importance in nutrient cycling in soils were investigated (Table 12). All enzymes were assayed at their optimal pH values. The activities of β-glucosidase (BG), cellobiohydrolase (CBH), β-N-acetylglucosaminidase (NAGase), aryl sulfatase (AS), acid phosphatase (AP), alkaline phosphatase (AlP), glycine aminopeptidase (GAP), leucine aminopeptidase (LAP), and peroxidase (PER) were analyzed in details.

Assays were conducted using *p*-nitrophenol (*p*NP) linked substrates and measured in a 96-well plate reader (Fluostar optima). Glycine aminopeptidase and leucine aminopeptidase activity were assayed using *p*-nitroanilide whereas peroxidase was assessed using DOPA (L-3,4-dihydroxyphenylalanine) (Table 12). Substrate concentrations of 5 mM were used for all enzymes, except cellobiohydrolase (CBH) and β-N-acetylglucosaminidase (NAGase) due to their solubility (2 mM) and cost. Original protocols for all assays are available on the Environment RCN webpage (http://enzymes.nrel.colostate.edu).

For enzyme activity, 4 g of soil was mixed with 40 mls of 50 mM sodium acetate buffer (pH 5.0) and vortexed on high speed for 3 minutes. Aliquots (200 µl) of slurry were transferred to polypropylene tubes to which 200 µl of substrates were added. For the peroxidase activity,

Table 12: Enzyme assays measured, their function and the substrate used.

Enzymes assayed	Function	Substrate
β-glucosidase (BG)	Cellulose degradation, carbon cycling	*p*NP β-D-glucopyranoside
Cellobiohydrolase (CBH)	Cellulose degradation and other beta-1,4 glucans, carbon cycling	*p*NP-β-D-cellobioside
β-N-acetylglucosaminidase aka chitinase (NAGase)	Chitin degradation, carbon/nitrogen cycling	*p*NP-N-acetyl-β-D-glucosaminide
Arylsulfatase (AS)	Produces plant available sulfates, sulfur cycling	*p*NP sulfate
Acid phosphatase (AP)	Produces plant available phosphates, phosphorus cycling	*p*NP phosphate (buffer pH 5.0)
Alakaline phosphatase (AlP)	Releases ester bound phosphates, phosphorous cycling	*p*NP phosphate (buffer pH 9.0)
Glycine aminopeptidase (GAP)	Degrades protein into amino acids, nitrogen cycling	Glycine-*p*-nitroanilide
Leucine aminopeptidase (LAP)	Degrades leucine and other hydrophobic amino acids from protein, nitrogen cycling	L-Leucine-*p*-nitroanilide
Peroxidase (PER)	Lignin and tannin (polyphenols) degradation, carbon cycling	L-3,4-dihydroxyphenylalanine (DOPA)

*p*NP: 4-nitrophenyl

10 µl of 0.3% H_2O_2 (hydrogen peroxide) was added. The tubes were then capped and placed on a rotary shaker for 2 hrs at 25 °C. Following incubation, tubes were centrifuged at 3200 × g for 4 min and aliquots (100 µl) of supernatant were taken from each tube and transferred into microplate. For *p*NP substrates, 5 µl of 1.0 M NaOH (sodium hydroxide) was added to the wells to stop the reaction. Microplates were read at 405 nm for *p*NP and *p*-nitroanilide and at 450 nm for peroxidase. Substrate and sample controls were used and all assays were done in triplicates, and repeated twice. The absorbance of the assay was corrected by subtracting the combined absorption results for the sample and substrate controls. Enzyme activity was expressed as nmol h^{-1} g $soil^{-1}$.

4.3.5 Statistical Analysis

Respiration and enzyme activity data were tested for normality using the Shapiro–Wilk test ($p \leq 0.05$). Both data showed normal distribution, hence statistical analyses were conducted using SPSS version 20 for windows (IBM, NY, USA). Independent sample t-tests were used to determine significant difference for soil respiration and enzyme activity between limed and unlimed sites and between metal contaminated and reference sites.

4.4 Results

4.4.1 Soil Respiration

Results of soil respiration are illustrated in figures 18 and 19. Soil respiration rates (CO_2-C) were significantly higher ($p \leq 0.05$) in limed sites compared to unlimed sites. In fact, respiration rates were 67.36 ppm (parts per million) in limed sites and 37.07 ppm in unlimed areas (Fig. 18). Similarly, higher ($p \leq 0.05$) respiration rates were observed in reference sites (93.54 ppm) compared to metal contaminated sites (55.55 ppm) (Fig. 19).

4.4.2 Soil Enzyme Activities

Enzyme activity data are illustrated in figures 18 to 23. Overall, soil samples from limed areas exhibited significantly higher ($p \leq 0.05$) enzymatic activity for β-glucosidase (BG), cellobiohydrolase (CBH), β-N-acetylglucosaminidase (NAGase), aryl sulfatase (AS), acid phosphatase (AP), alakaline phosphatase (AlP), glycine aminopeptidase (GAP), and leucine aminopeptidase (LAP) compared to unlimed samples (Figs. 20, 21). In contrast, there was a decrease of peroxidase (PER) activity in limed sites (19.14 nmol h^{-1} g $soil^{-1}$) compared to in unlimed areas (47.69 nmol h^{-1} g $soil^{-1}$) (Fig. 22).

Likewise, enzyme activity was significantly higher ($p \leq 0.05$) for BG, CBH, NAGase, AS, AP, AlP and GAP in reference sites compared to metal contaminated sites (Figs. 23, 24 and 25). LAP activities in metal contaminated (3.57 nmol h^{-1} g $soil^{-1}$) and reference sites (4.92 nmol h^{-1} g $soil^{-1}$) were not significantly different (Fig. 25). But, PER activity was higher ($p \leq 0.05$) in metal contaminated sites (49.03 nmol h^{-1} g $soil^{-1}$) compared to reference sites (17.28 nmol h^{-1} g $soil^{-1}$) (Fig. 25).

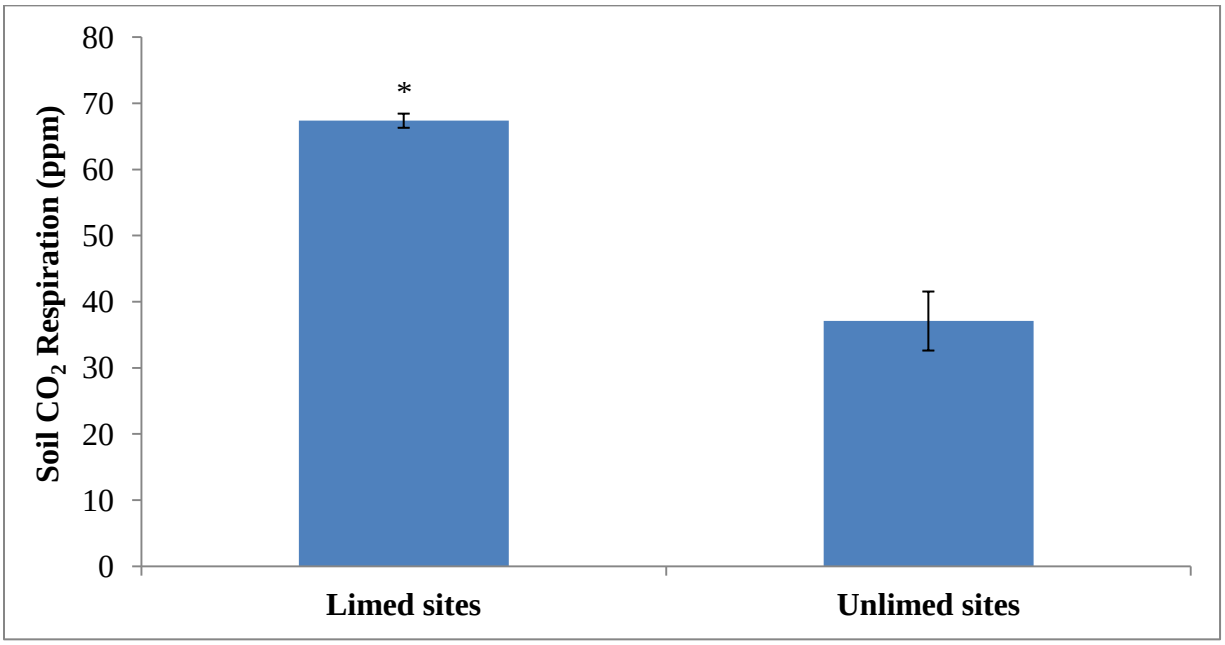

Figure 18: Mean soil respiration rates in the organic layer (0-5cm) from various limed and unlimed sites from GSR.

Means (±SE) are given (n=16).

* represents significant differences between limed and unlimed sites based on t-test ($p \leq 0.05$)

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake and Kingsway

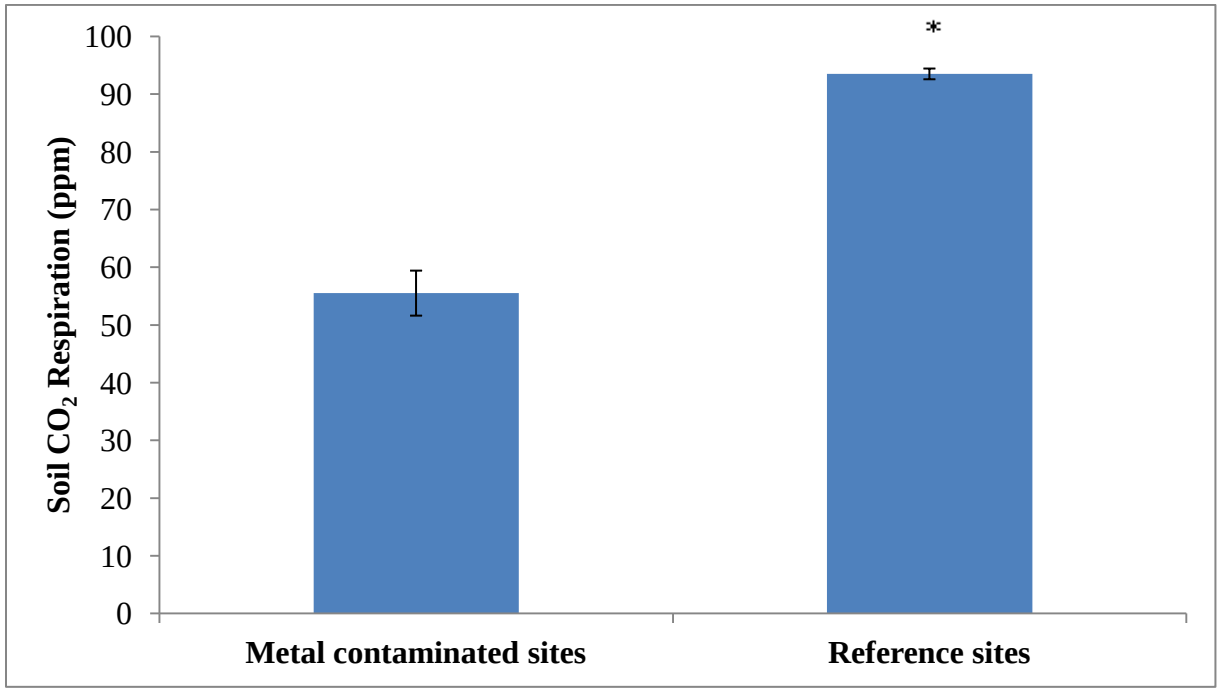

Figure 19: Mean soil respiration rates in the organic layer (0-5cm) from various metal contaminated and reference sites from GSR.

Means (±SE) are given (n=12).

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney

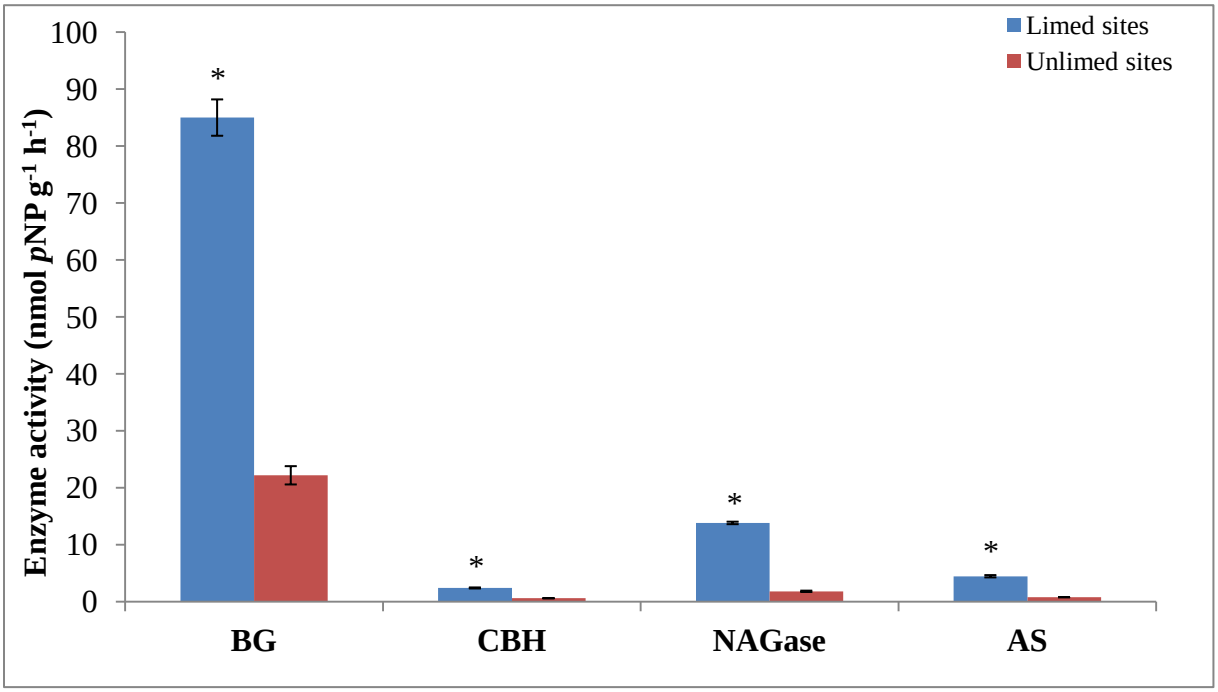

Figure 20: The activities of selected hydrolytic enzymes from limed and unlimed soil samples from the GSR (n = 72) using *p*-nitrophenol (*p*NP) linked substrates. BG = β-glucosidase; CBH = Cellobiohydrolase; NAG = β-N-acetylglucosaminidase; AS = Aryl sulfatase.

* represents significant differences between limed and unlimed sites based on t-test ($p \leq 0.05$)
Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake and Kingsway

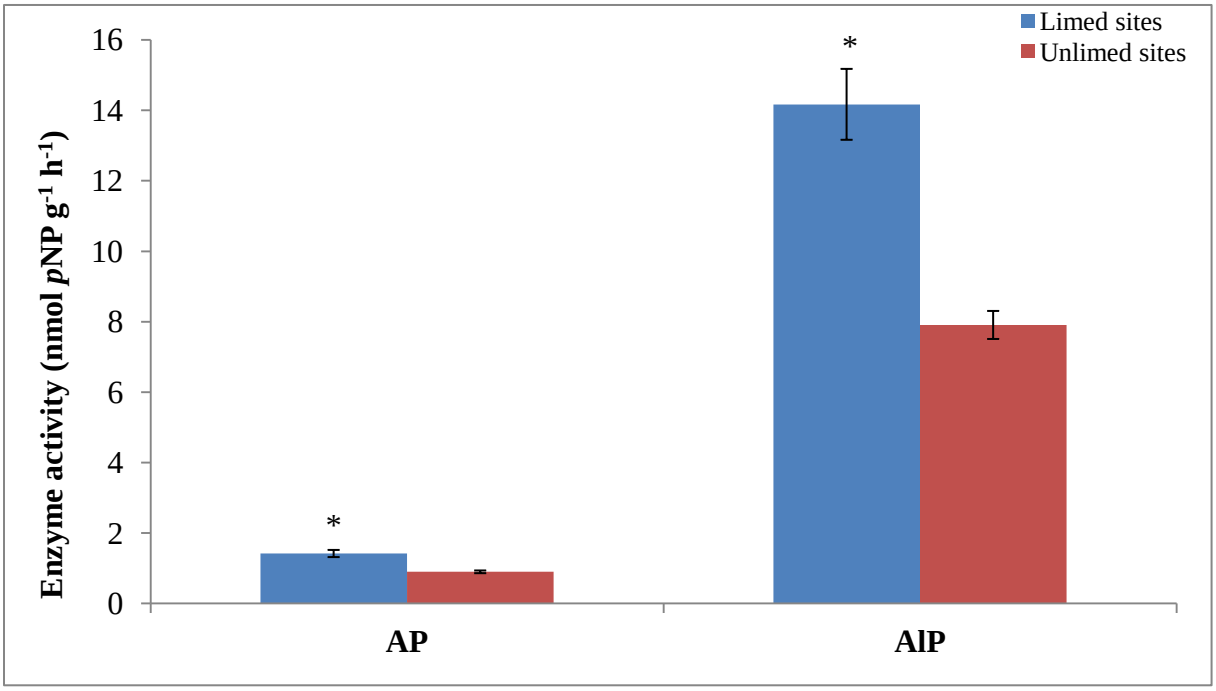

Figure 21: The activities of selected hydrolytic enzymes from limed and unlimed soil samples from the GSR (n = 72) using *p*-nitrophenol (*p*NP) linked substrates.

* represents significant differences between limed and unlimed sites based on t-test ($p \leq 0.05$)

Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake and Kingsway

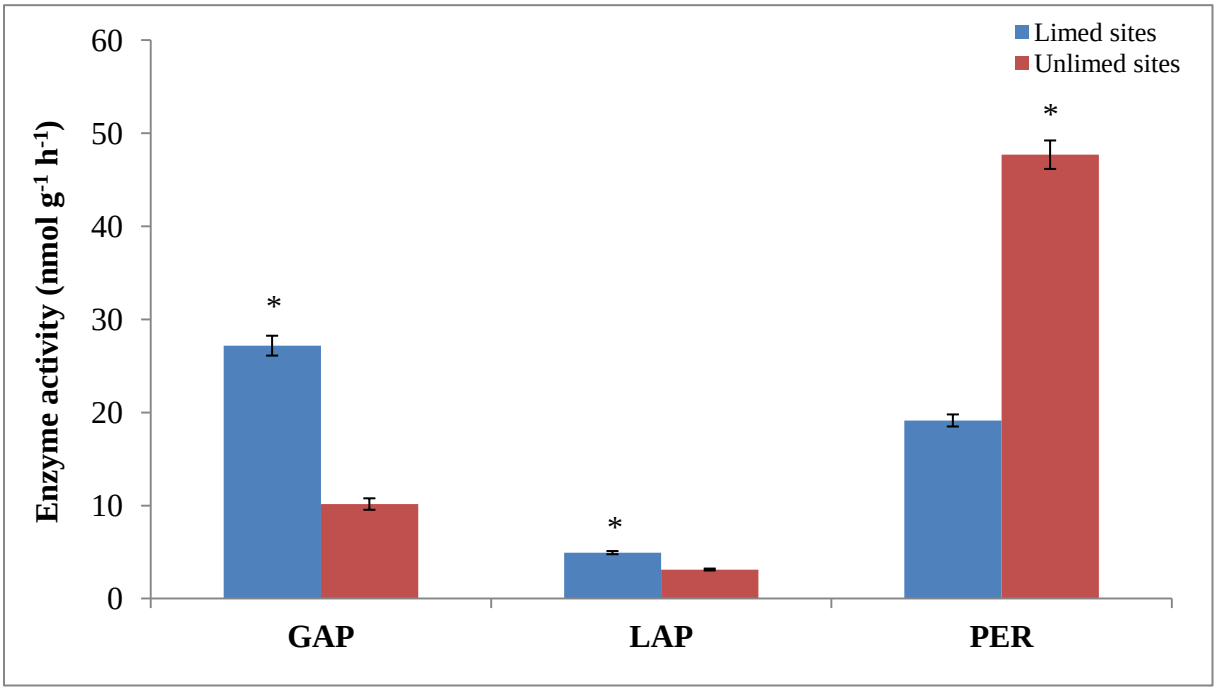

Figure 22: The activities of selected hydrolytic and oxidoreductase enzymes from limed and unlimed soil samples from the GSR (n = 72) using *p*-nitroanilide linked substrate for glycine and leucine aminopeptidase (GAP and LAP), and L-3, 4-dihydroxyphenylalanine (DOPA) linked substrates for peroxidase (PER) activity.

* represents significant differences between limed and unlimed sites based on t-test ($p \leq 0.05$)
Limed and Unlimed sites: Daisy Lake 2, Wahnapitae Hydro-Dam, Kelly Lake and Kingsway

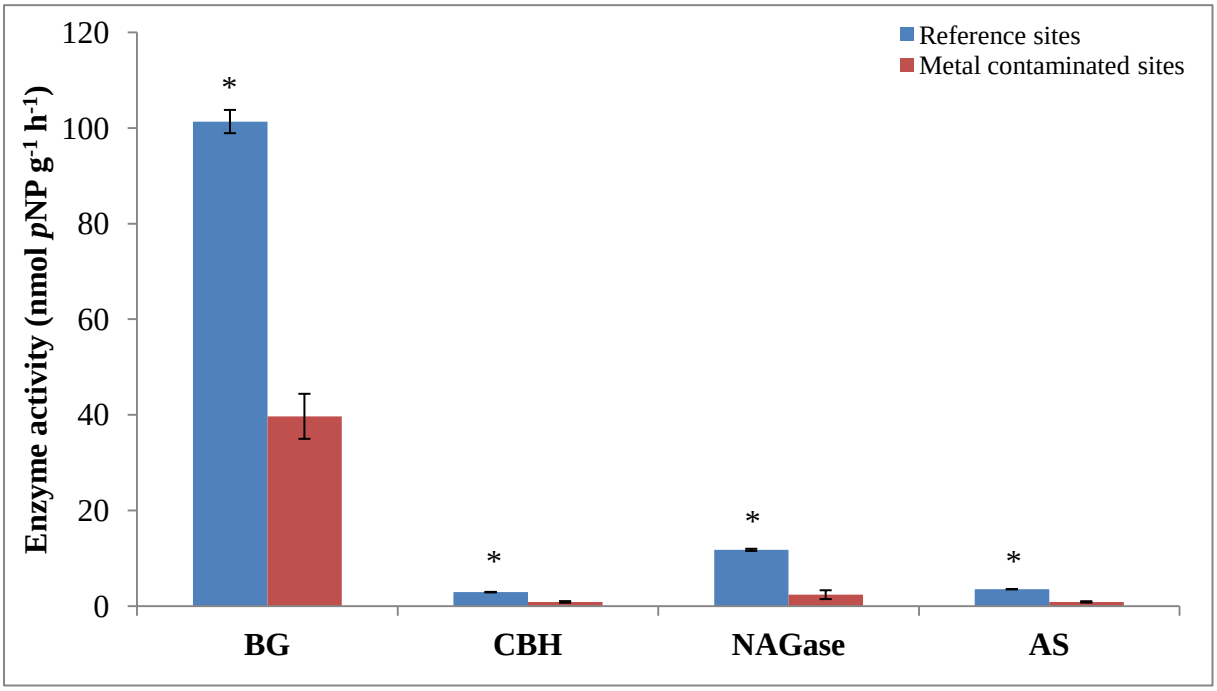

Figure 23: The activities of selected hydrolytic enzymes in metal contaminated and reference soil samples from the GSR (n = 54) using *p*-nitrophenol (*p*NP) linked substrates. BG = β-glucosidase; CBH = Cellobiohydrolase; NAGase = β-N-acetylglucosaminidase; AS = Aryl sulfatase.

* represents significant differences between metal contaminated and referece sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney

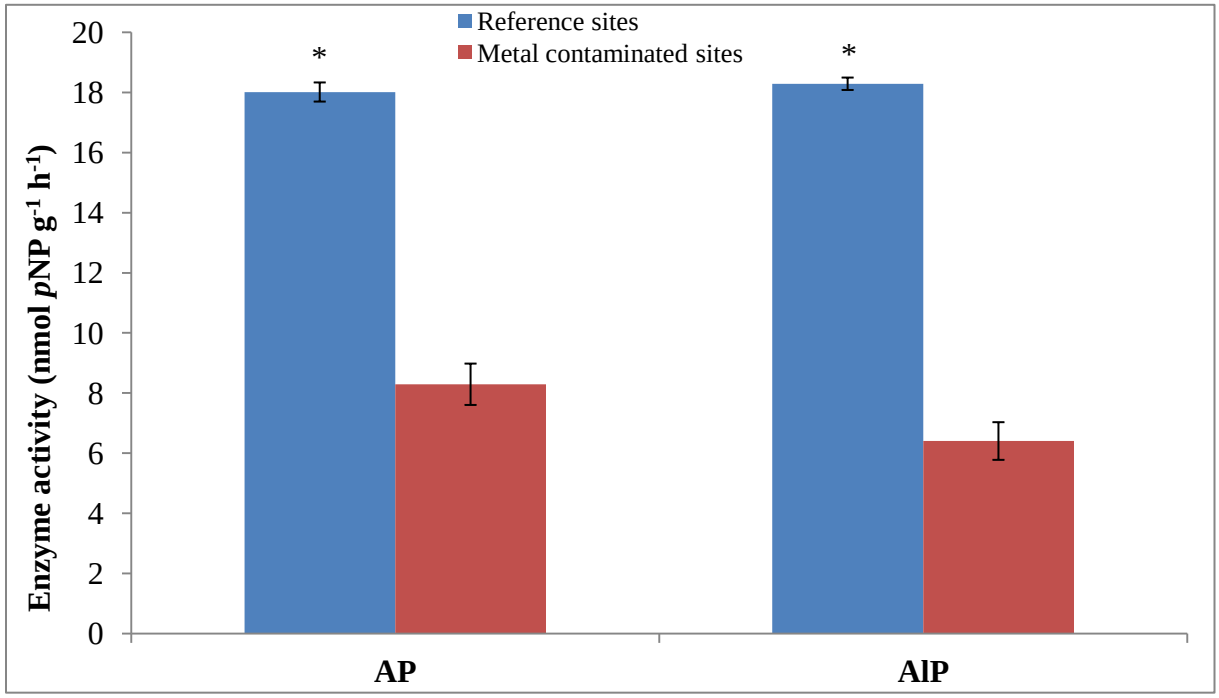

Figure 24: The activities of selected hydrolytic enzymes in metal contaminated and reference soil samples from the GSR (n = 54) using *p*-nitrophenol (*p*NP) linked substrates.

* represents significant differences between metal contaminated and referece sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney

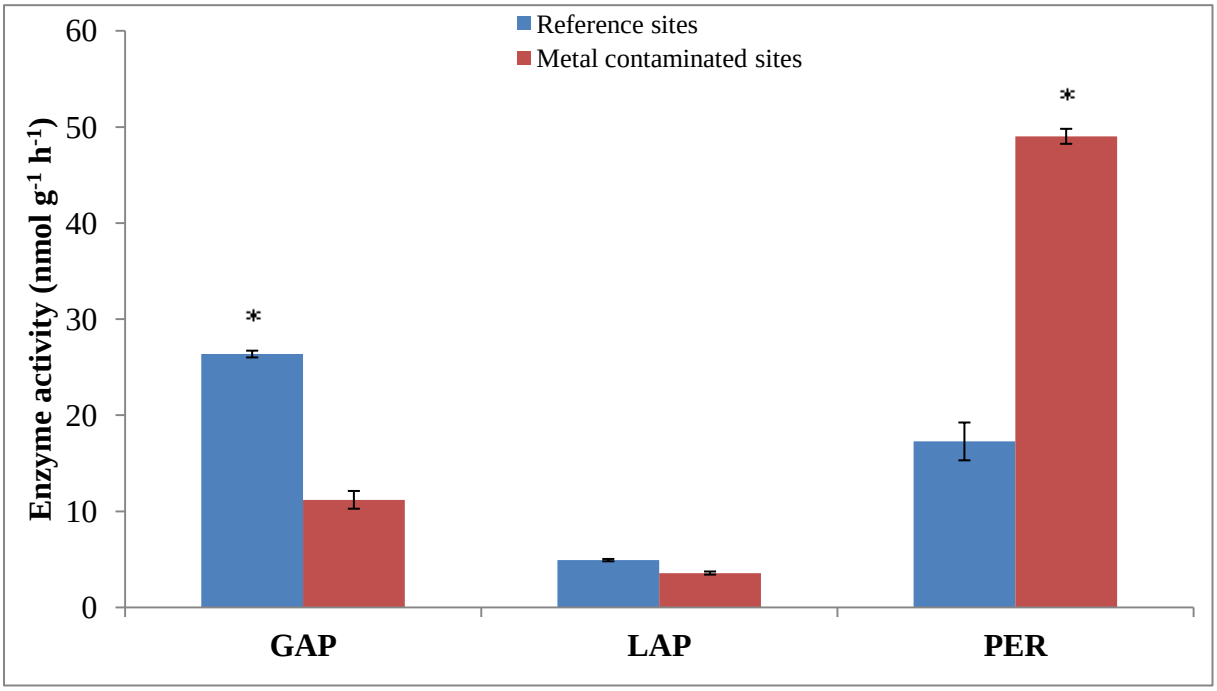

Figure 25: The activities of selected hydrolytic and oxidoreductase enzymes in metal contaminated and reference soil samples from the GSR (n = 54) using *p*-nitroanilide linked substrate for glycine and leucine aminopeptidase (GAP and LAP), and L-3, 4-dihydroxyphenylalanine (DOPA) linked substrates for peroxidase (PER) activity.

* represents significant differences between metal contaminated and referece sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney

4.5 Discussion

4.5.1 Soil Respiration

Soil respiration regulates atmospheric CO_2 concentration and climate dynamics, mainly produced by soil microorganisms and plant roots. It is strongly affected by various biological (microorganisms, vegetation), environmental (pH, temperature, moisture) and man-made factors [34,40,126,234]. It is an important phenomenon in soil ecosystems as it is the only pathway of soil carbon pool and is an important source of atmospheric CO_2. Many studies have highlighted that soil respiration is an indicator of soil health, fertility, and microbial activity [115,234].

4.5.1.1 Effects of Liming

Results show that soil liming increases soil respiration even >40 years after dolomitic limestone application. This is consistent with other studies [60]. CO_2 released from the soil after liming is a result of a chemical (dolomite lime hydrolysis) and biological processes (increased microbial activity) [214,235]. Liming improved chemical conditions in soil profile (pH, CEC and exchangeable cations) which in turn favored microbial activities (abundance) [26,34]. Changes in soil chemical conditions, such as reduction in acidity, increase of pH, Ca^{2+} and Mg^{2+} content have been observed in limed sites [236]. These factors led to an increase in respiration rate, soil CO_2 emission, and the development of acid-intolerant microorganisms. Recent evaluation of the same sites showed that microbial populations, bacterial, and fungal biomasses were higher in limed sites compared to the unlimed [236]. These results were consistent with previous reports in other soil conditions [26,34,35,214,235,237].

4.5.1.2 Effects of Metal Contamination

Respiration rates were significantly lower in metal contaminated sites compared to reference sites. Our previous studies showed high concentrations of As, Cu, Ni, and Zn in metal contaminated sites compared to reference sites [25,40,45–47]. In addition, pH values were consistent with the acidity levels documented for soils on the Canadian Shield. The data also confirm that the pH is low in metal contaminated soils compared to reference soil [25,40,47]. At low pH levels (< 4.5) many metals remain in soluble form and can be lost in the leachate soil system. Although some heavy metals are required for life's physiological processes, their excessive accumulation can be detrimental to plants and affect soil microbial composition and diversity [13,35,40]. Studies have shown that long term metal contamination of soils even at low dose might have harmful effects on soil microbial activities, especially microbial respiration [60,161].

Soil microbial compositions are often used as indicators of soil quality and contamination. Reduced microbial activity and abundance in microbial community structure have been reported in metal contaminated soils [60,90,238]. In our recent study, phospholipid fatty acids analysis (PLFA) and 16S profile of metal contaminated sites revealed a decline in microbial biomass without a significant decrease of microbial diversity and composition [40,239]. Other studies have reported lower community diversity in soils with high metal concentrations [60,161,238].

4.5.2 Soil Enzyme Activity

Enzymes in soil play an important role in maintaining soil health and its environment. Enzyme activities in addition to various chemical and physical components contribute to maintaining soil health. Soil pH and metal concentrations play a role in soil weathering and plant community composition which affects the distribution of enzyme activity through changes in soil

organic matter, nutrient availability, and microbial community composition [114]. Ellert *et al*. [240] reported that healthy soils are essential for the integrity of the ecosystem to remain intact and to recover from disturbances. As soil supports all terrestrial life forms, a thorough understanding of soil enzyme activities is an important factor in monitoring soil health.

4.5.2.1 Activity of β-glucosidase

β-glucosidase (BG) is a well-studied enzyme due to its universal distribution and well defined wide variety of substrates [230]. It catalyzes the hydrolysis of glycosidic linkages, thereby degrading oligosaccharides and glycoconjugates [114,130,230,241]. This enzyme plays an important role in the degradation of organic C compounds in soil and their products (sugars) are important energy sources for soil microorganisms [114,230,242]. In the present study, the activity of BG was higher in limed sites compared to unlimed sites. Similar studies in other conditions have reported higher activity of BG in limed soils as the activity is positively correlated with pH [117,230]. In metal contaminated soils, BG activity was lower compared to reference sites. Lee *et al*. [243] reported lower activity of this enzyme in metal contaminated soils where the pH and organic matter were lower. Shukla and Verma [117] showed that increased CO_2 led to a high microbial biomass and BG.

Studies have shown that the most important sources of BG are microscopic fungi [117, 244]. In fact, β-glucosidase are produced from filamentous fungi (*Aspergillus niger*, *A. oryzae*, *Penicillium brasilianum*, *P. decumbens*, *Phanerochaete chrysosporium*, *Paecilomyces* sp., etc), yeasts (majority from *Candida* sp.), and few bacteria [244]. In coniferous forests, BG activities are reduced with a decrease of microbial biomass suggesting a strong correlation between BG activities and microbial biomass [117].

4.5.2.2 Activity of Cellobiohydrolase

Cellobiohydrolase (CBH) is a cellulase that degrades cellulose by hydrolysis of 1,4-β-D-glycosidic bonds [114,230,241]. Many studies have reported a variety of specialized microorganisms that have evolved to produce enzymes that carry out the hydrolysis of cellulose. *Trichoderma reesei* is a widely studied fungus that is known to produce CBH [244]. Likewise, *Basidiomycetes* produce rich arrays of cellulose degrading enzymes [117]. This is consistent with our previous analysis that revealed that *Basidiomycetes* are higher in limed and reference sites compared to unlimed and metal contaminated sites, respectively [236,239].

Saiya-cork *et al*. [245] reported an increase of CBH activity in soils with high organic N content. Previous analysis of these sites showed a higher organic N in limed and reference soils compared to unlimed and metal contaminated sites [236,239]. Similarly, Guo *et al*. [246] observed a higher CBH activity in fertilized soils. Other studies reported that a lower CBH activity was positively related to lower microbial biomass [117].

4.5.2.3 Activity of β-N-acetylglucosaminidase

β-N-acetylglucosaminidase (NAGase) degrades chitin by hydrolyzing β-1,4-glycosidic bonds of chitooligosaccharides into N-acetylglucosamine [114,241]. Chitin is found primarily in fungal walls and also in exoskeletons of some arthropods. It contains approximately 6% nitrogen and is relatively abundant in soils, making it an important source of organic nitrogen in terrestrial ecosystem [247]. NAGase activity was higher in limed and reference sites compared to unlimed and metal contaminated sites.

In N-limited systems, microbes degrade labile forms of N-containing organic matter. They produce enzymes responsible for degrading more forms of N, such as β-1,4-N-acetyl-glucosamine [248]. Guo *et al*. [246] reported an increase in polyphenol activity after inorganic N fertilization. Chemical analysis of soils in the GSR indicate that organic N is higher in limed and reference sites compared to controls which is in agreement with an increase in NAGase activity observed.

4.5.2.4 Activity of Arylsulfatase

Arylsulfatse (AS) enzyme catalyzes the hydrolysis of organic sulfate esters (organic S form) [230,249]. Sulfur occurs in organic and inorganic forms in soils, with >95% of total S being organic in nature. In soil, 40-70% of total S in surface soils is in the form of ester sulfates, which act as substrates for arylsulfatase [249]. AS enzyme has been identified in plants, animals, microorganisms, and soils. Ekenler and Tabatabai [250] reported that AS activity is positively correlated with soil pH following liming treatment.

AS activity is highly correlated with levels of organic C, total S and microbial biomass [249,250]. Like with many other enzymes studied, the high level of AS activity observed is associated with a higher organic C, organic S, microbial abundance and biomass in limed and reference soils [26,35,40,236,239].

4.5.2.5 Activity of Acid and Alkaline Phosphatase

Phosphatases are enzymes that catalyze the hydrolysis of phosphate ester bonds leading to the release of phosphate (P), which is taken up by plants or microorganisms [114,232,250,251]. Like many hydrolases, activity of phosphatases depends on several factors such as plant cover,

soil properties (pH, organic matter, metal concentration), soil organisms and microbial community [114,150,230,251].

Activities of soil phosphomonoesterases that include acid and alkaline phosphomonoesterases (known as acid and alkaline phosphatase) have been among the most studied [114,230,241,251]. These enzymes hydrolyze monoester bonds including mononucleotides and sugar phosphates. When soil microorganisms are P limited, they produce acid or alkaline phosphatase that release inorganic phosphate from organic matter [114,230,251]. Nannipieri *et al*. [251] reported that acid phosphatase activity prevails in acidic soils, whereas alkaline phosphatase activity is high in alkaline soils. Other studies have reported increased phosphatase activity with high microbial activity [117,251]. Our results revealed higher AP and AlP activity in limed and reference soils where pH [25,26,34,40] and microbial biomass [25,26,34,40] were higher compared to unlimed and metal contaminated soils. Arbuscular mycorrhizal (AM) fungi stimulate the release of acid phosphatases [252]. Recent reports on Sudbury ecosystems revealed a higher level of AM in limed and reference soils compared to unlimed and metal contaminated soils [236,239]. It is established that the main source of alkaline phosphatase activity are bacteria whereas acid phosphatase activity is from bacteria, fungi and plants [251, 252].

4.5.2.6 Activity of Aminopeptidase

Aminopeptidases are widely distributed in bacteria, fungi, plants and animals [253,254]. These enzymes are involved in degradation of intracellular or extracellular peptides to amino acids for new protein syntheses and play a role in nitrogen cycling [114,253,254]. Leucine aminopeptidase (LAP) hydrolyzes leucine and other hydrophobic amino acids from N terminus

of polypeptides and the activity of LAP is broadly used as an indicator of peptidase potential [114]. Similarly, glycine aminopeptidase (GAP) enzyme specifically hydrolyzes Gly-X (amino acids, peptide or arylamide) bonds [254]. Both LAP and GAP have broad substrate specificity [254]. Ito *et al*. [254] reported that GAP has been found in various fungal and bacterial species such as *Actinomucor elegans*, *Aspergillus oryzae*, *Aspergillus sojae*, *Escherichia coli* and *Penicillium citrinum*.

LAP and GAP activities were higher in limed soils compared to unlimed soils. In metal contaminated sites, GAP activity was lower compared to reference sites whereas, LAP showed no difference between the two sites. Aminopeptidase activities showed a stronger relationship with soil pH compared to SOM [114]. Ramirez *et al*. [248] reported that N additions alter the composition and diversity of soil microbial communities and impact microbial C dynamics which in turn could affect enzyme activity. In relation to N distribution, studies have reported lower aminopeptidase activity with N amendment but other studies found no response [114].

4.5.2.7 Activity of Peroxidase

The degradation of polyphenols (lignin, tannin and their degradation products) is an oxidative process [114,255,256]. Two classes of enzymes, phenol oxidase (POX, eg: laccases) and peroxidases (PER, eg: lignin peroxidase, Mn peroxidase) are well characterized, [114,257]. PER describes activity of an enzyme that uses H_2O_2 or secondary oxidants to degrade aromatic compounds [114,257]. They are ubiquitous and abundant oxidative enzyme in all forms of life [257].

White rot (*Basidiomycetes*) and soft rot (*Ascomycota*) fungi are best known for producing extracellular PER [256,257]. Bacterial species belonging to *actinomycetes*, α- and γ-*proteobacteria* are also producers of extracellular PER [256]. These bacterial and fungal species produce oxidative enzymes and are considered as efficient degraders of lignin and humus [114,256,257]. These organisms are most abundant in areas where plants have high lignin concentrations [114,257]. The activities of PER within these ecosystems are shown to increase with secondary successions [114]. Studies have also shown that at global scale, edaphic conditions and near optimal pH (acidic range: 4.0 – 5.0) promote PER activities, even though *Basidiomycetes* are relatively uncommon [114,257]. In addition, decline in PER activity has been observed in N amended soils [114,248,257]. The present study showed similar results, where higher PER activity were documented in unlimed and metal contaminated soils which had low pH and lower organic N concentrations compared to limed and reference sites.

4.6 Conclusions

Liming increases soil respiration, β-glucosidase, cellobiohydrolase, β-N-acetylglucosaminidase, aryl sulfatase, acid phosphatase, alkaline phosphatase, glycine aminopeptidase, and leucine aminopeptidase activities. However it decreased peroxidase activity. An opposite trend was observed with metal contamination that decreases soil respiration and enzymatic activities with the exception of peroxidase.

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CHAPTER 5: GENERAL CONLUSIONS

The present study aimed at analyzing soil biogeochemical properties within the GSR in metal contaminated and reclaimed sites. Chemical analysis revealed that concentrations of total metals were within the OMEE (Ontario Ministry of Environment and Energy) guidelines. Results also confirmed that only a fraction of total metals were in the bioavailable form. The pH was low in metal contaminated soils compared to reference sites. Liming increased significantly soil pH even > 35 years after dolomitic limestone application. At low pH, many metals remain in soluble form thereby providing a good potential for transport in the leachate soil system. In acidic soils, several elements (Ca, Mg, and P) become deficient whereas Al and Mn are abundant and sometimes reach toxic levels. Low pH and metal deficiency/abundance can be detrimental to plants and affect soil microbial composition and diversity. A strong positive correlation was observed between soil pH and CEC for all the sites.

Low CEC and SOM were observed in metal contaminated sites. PLFA and pyrosequencing analysis revealed significantly lower levels of microbial biomass and relative abundance, in metal contaminated sites compared to reference sites. Overall, 62% of bacterial families identified were common to metal contaminated and the reference sites. But 11.50% were specific to metal contaminated sites and 10.00% to the reference sites. This trend was also observed at class and genus levels. For fungi, metal contaminated and reference sites shared 58% of the families identified; while 6% of the families were specific to metal contaminated sites, and 10% to reference sites. The same proportions were observed at class and genus levels.

Microbial biomass and relative abundance were significantly higher in limed sites compared to the adjacent unlimed areas. Although several bacterial and fungal groups were present in all the sites, 25% of bacterial genera were specific to limed sites and 16% to unlimed

areas. Similarly, the proportions of fungal genera specific to limed and unlimed sites were 27% and 17%, respectively. For both bacteria and fungi, Chao1 values were higher for limed sites compared to unlimed areas. But the number of OTUs, and the levels of Simpson and Shannon diversity indices, and species evenness were not affected by liming.

Soil respiration and enzyme activities data for the targeted sites in the GSR provided a frame of reference for comparing ecosystems and allowing scientistis to relate soil microbial community function to global patterns of microbial biomass composition, pH, nutrients and organic matter content. Detailed analyses showed that liming and metal contamination resulted in changes in soil respiration and enzyme activities. Liming increased soil respiration and activities of β-glucosidase, cellobiohydrolase, β-N-acetylglucosaminidase, aryl sulfatase, acid phosphatase, alakaline phosphatase, glycine aminopeptidase, and leucine aminopeptidase. But it decreased significantly peroxidase activities. An opposite trend was observed for soils from metal contaminated sites where a decrease in soil respiration and enzymatic activities were observed with the exception of peroxidase activties.

Overall, results of the present study demonstrate that long-term exposure to metals reduce microbial biomass and relative abundance but had no effect on microbial diversity. Similarly, soil liming increased the amount of microbial biomass but had limited effect on bacterial and fungal diversities. Soil respiration and enzyme activities showed different ranges of variation and distribution in relation to ecosystem variables. We found that organic matter, pH, and CEC are key factors involved in changes in microbial communities.

Future studies will involve PLFA and pyrosequencing analyses of microbial communities from reclaimed and unreclaimed tailings developed by Cu and Ni mining companies in the GSR.

Such studies will determine if treatments of tailings that are highly contaminated with metals can result in sustainable ecosystems.

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APPENDICES

Table S1: Study locations and their coordinates.

Sites	GPS Coordinates
Daisy Lake 2	46’27’50’N/80’53’1’W
Wahnapitae Hydro-Dam	46’28’31’N/80’49’14’W
Kelly Lake	46’26’42’N/81’3’18’W
Kingsway	46’29’54’N/80’58’14’W
Laurentian	46’28’5’N/80’58’35’W
Onaping Falls	46’35’32’N/81’23’3’W
Capreol	46’45’28’N/80’55’21’W
Killarney	46’13’12’N/80’47’43’W

Table S2: **Site-specific bacterial groups and their relative abundance identified in metal contaminated and reference soil samples from the GSR.**

	Metal contaminated sites	Reference sites
1	*Acidimicrobium* spp. (3.67)	*Armatimonas* spp. (13.00)
2	*Acidovorax* spp. (1.67)	*Azovibrio* spp. (2.00)
3	*Afipia* spp. (2.67)	*Blastochloris viridis* (3.67)
4	*Anaerolinea* spp. (3.00)	*Bosea* spp. (1.00)
5	*Anderseniella* spp. (1.33)	*Caldilinea* spp. (1.00)
6	*Bazzania trilobata* (0.33)	*Candidatus entotheonella spp. (21.00)*
7	*Blastomonas* spp. (3.66)	Candidates xiphinematobacter spp. (10.67)
8	*Burkholderia caryophylli* (0.67)	*Chelatococcus* spp. (6.67)
9	*Comamonas* spp. (1.33)	Corynebacteriales spp. (4.33)
10	*Geitlerinema* spp. (1.67)	*Cupriavidus pinatubonensis* (0.33)
11	*Ktedonobacter* spp. (2.67)	*Herbiconiux* spp. (1.00)
12	*Mycobacterium insubricum* (0.33)	*Hgci clade* spp. (1.67)
13	*Mycobacterium* spp. (2.67)	*Hymenobacter* spp. (3.67)
14	*Parvularcula* spp. (2.00)	*Kineosporia* spp. (0.33)
15	*Pleomorphomonas oryzae* (0.33)	*Limnobacter* spp. (1.33)
16	*Polaromonas* spp. (7.33)	*Massilia* spp. (6.33)
17	*Rhodopirellula* spp. (6.67)	*Massilia timonae* (0.33)
18	*Thermomonas* spp. (7.00)	*Meganema* spp. (1.33)
19		*Microvirga bosea thiooxidans* (3.00)
20		*Mycobacterium celatum* (9.33)
21		*Nitrosovibrio tenuis* (8.00)
22		*Novosphingobium* spp. (0.33)
23		*Pedomicrobium* spp. (1.00)
24		*Pseudonocardia* spp. (1.33)
25		*Rhodomicrobium* spp. (2.00)
26		*Rhodovastum* spp. (2.67)
27		*Roseomonas ruber* (11.67)
28		*Sandarakinorhabdus* spp. (2.67)
29		*Schlesneria* spp. (4.00)
30		*Singulisphaera* spp. (2.67)
31		*Spam (candidate division)* (1.67)
32		*Sphingomonas* spp. (1.67)
33		*Tepidamorphus* spp. (0.33)
34		*Thalassospira* spp. (6.00)
35		*TM6* (3.67)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.

Table S3: All the bacterial groups and their relative abundance identified in metal contaminated and reference soil samples from the GSR.

	Bacterial species	Metal contaminated sites	Reference sites
1	*Achromobacter* spp.*	34.00 ± 2.18	21.33 ± 4.70
2	*Acidicaldus* spp.*	77.33 ± 11.73	32.33 ± 10.04
3	*Acidimicrobiales* spp.*	1.33 ± 1.33	29.67 ± 3.03
4	*Acidimicrobium* spp.	3.67 ± 3.67	0.00 ± 0.00
5	*Acidiphilium* spp.	1.67 ± 1.00	2.67 ± 1.19
6	*Acidisphaera* spp.*	15.67 ± 2.96	4.33 ± 2.33
7	*Acidobacterium mine drainage*	0.33 ± 0.33	1.67 ± 1.67
8	*Acidobacterium* spp.*	701.33 ± 47.06	387.67 ± 29.73
9	*Acidocella* spp.*	27.00 ± 12.28	10.33 ± 1.20
10	*Acidothermus* spp.	30.67 ± 5.76	24.67 ± 8.54
11	*Acidovorax* spp.	1.67 ± 1.67	0.00 ± 0.00
12	*Afipia broomeae*	445.33 ± 16.94	430.00 ± 6.17
13	*Afipia felis**	5.33 ± 2.40	14.00 ± 0.94
14	*Afipia* spp.	2.67 ± 1.76	0.00 ± 0.00
15	*Anaerolinea* spp.	3.00 ± 2.22	0.00 ± 0.00
16	*Anderseniella* spp.	1.33 ± 1.33	0.00 ± 0.00
17	*Aquicella* spp.	158.33 ± 7.70	146.33 ± 8.60
18	*Arenimonas* spp.*	0.33 ± 0.33	14.67 ± 0.31
19	*Armatimonas* spp.*	0.00 ± 0.00	13.00 ± 3.26
20	*Azospira* spp.*	35.00 ± 4.98	11.33 ± 0.88
21	*Azovibrio* spp.	0.00 ± 0.00	2.00 ± 2.00
22	*Bazzania trilobata*	0.33 ± 0.33	0.00 ± 0.00
23	*Blastochloris* spp.*	4.33 ± 0.96	9.67 ± 0.89
24	*Blastochloris viridis**	0.00 ± 0.00	3.67 ± 0.73
25	*Blastomonas* spp.	3.67 ± 3.67	0.00 ± 0.00
26	*Bosea* spp.	0.00 ± 0.00	1.00 ± 1.00
27	*Bradyrhizobium* spp.*	49.33 ± 8.50	75.67 ± 8.41
28	*Burkholderia caryophylli*	0.67 ± 0.33	0.00 ± 0.00
29	*Burkholderia* spp.	11.33 ± 2.03	8.00 ± 2.65
30	*Caldanaerobacter thermoanaerobacter tengcongensis**	26.67 ± 11.86	5.67 ± 3.67
31	*Caldilinea* spp.	0.00 ± 0.00	1.00 ± 1.50
32	*Candidatus alysiosphae*ra spp.	6.00 ± 3.46	5.67 ± 2.85
33	*Candidatus chloracidobacterium* spp.*	26.00 ± 3.65	43.33 ± 3.33
34	*Candidatus entotheonella* spp.*	0.00 ± 0.00	21.00 ± 3.75
35	*Candidatus koribacter* spp.*	117.00 ± 6.09	195.67 ± 3.38
36	*Candidatus koribacter versatilis*	2.67 ± 1.76	2.33 ± 1.20
37	*Candidatus solibacter* spp.*	294.00 ± 5.69	490.33 ± 4.00

38	*Candidatus xiphinematobacter* spp.	0.00 ± 0.00	10.67 ± 8.74
39	*Caulobacter* spp.*	30.00 ± 2.58	13.33 ± 3.10
40	*Caulobacter vibrioides*	13.67 ± 8.82	6.67 ± 1.45
41	*Chelatococcus* spp.	0.00 ± 0.00	6.67 ± 6.67
42	*Climaciaceae climacium dendroides*	9.33 ± 4.33	5.33 ± 2.67
43	*Collimonas fungivorans*	3.00 ± 3.00	0.33 ± 0.33
44	*Colwellia* spp.	49.67 ± 20.54	28.67 ± 7.84
45	*Comamonas* spp.	1.33 ± 1.33	0.00 ± 0.00
46	*Conexibacter* spp.*	63.67 ± 8.06	32.67 ± 3.02
47	*Coprothermobacter* spp.*	5.00 ± 1.52	9.67 ± 1.60
48	*Corynebacteriales* spp.	0.00 ± 0.00	4.33 ± 2.96
49	*Crossiella* spp.	6.00 ± 5.03	3.33 ± 0.67
50	*Cupriavidus pinatubonensis*	0.00 ± 0.00	0.33 ± 0.33
51	*Defluviicoccus* spp.	18.00 ± 17.50	4.33 ± 3.84
52	*Dongia* spp.	16.67 ± 1.45	13.00 ± 3.34
53	*Dyella* spp.	0.33 ± 0.33	1.00 ± 0.58
54	*Edaphobacter modestum**	28.67 ± 3.94	32.00 ± 2.00
55	*Edaphobacter* spp.	12.00 ± 7.21	13.67 ± 5.24
56	*Escherichia shigella* spp.	0.67 ± 0.33	2.00 ± 1.15
57	*Ferruginibacter* spp.*	27.67 ± 3.18	13.67 ± 2.68
58	*Filomicrobium* spp.*	88.33 ± 8.35	3.33 ± 0.88
59	*Frankia* spp.	5.00 ± 1.00	3.00 ± 1.15
60	*Geitlerinema* spp.	1.67 ± 1.67	0.00 ± 0.00
61	*Gemmata* spp.	6.67 ± 4.18	5.67 ± 2.73
62	*Gemmatimonas* spp.	0.67 ± 0.33	8.67 ± 3.36
63	*Geobacillus* spp.	27.67 ± 24.69	45.67 ± 29.20
64	*Granulicella* spp.	212.33 ± 84.84	286.00 ± 42.34
65	*Haliangium* spp.	0.67 ± 0.67	7.00 ± 4.04
66	*Herbiconiux* spp.	0.00 ± 0.00	1.00 ± 1.00
67	*Hgci clade* spp.	0.00 ± 0.00	1.67 ± 1.67
68	*Hirschia* spp.	29.33 ± 3.53	22.00 ± 5.86
69	*Hymenobacter* spp.	0.00 ± 0.00	3.67 ± 3.67
70	*Hyphomicrobium* spp.	3.67 ± 3.67	2.33 ± 1.33
71	*Iamia* spp.	1.67 ± 1.20	1.67 ± 1.67
72	*Inquilinus* spp.*	1.67 ± 0.88	7.33 ± 3.48
73	*Kineosporia* spp.	0.00 ± 0.00	0.33 ± 0.33
74	*Ktedonobacter* spp.	2.67 ± 2.67	0.00 ± 0.00
75	*Labrys* spp.*	18.33 ± 9.39	0.67 ± 0.33
76	*Leptothrix* spp.	4.33 ± 1.20	4.67 ± 3.71
77	*Limnobacter* spp.	0.00 ± 0.00	1.33 ± 1.33
78	*Marmoricola* spp.	0.33 ± 0.33	3.00 ± 2.52
79	*Massilia* spp.	0.00 ± 0.00	6.33 ± 5.84

80	*Massilia timonae*	0.00 ± 0.00	0.33 ± 0.33
81	*Meganema* spp.	0.00 ± 0.00	1.33 ± 0.88
82	*Methylocystis* spp.*	19.00 ± 4.93	31.67 ± 5.33
83	*Methylosinus sporium*	3.33 ± 3.33	1.67 ± 1.67
84	*Methylosinus trichosporium**	8.67 ± 1.67	0.33 ± 0.33
85	*Metyhlovirgula* spp.*	15.67 ± 11.79	27.33 ± 24.84
86	*Microvirga bosea thiooxidans*	0.00 ± 0.00	3.00 ± 3.00
87	*Mycobacterium celatum**	0.00 ± 0.00	9.33 ± 3.36
88	*Mycobacterium insubricum*	0.33 ± 0.33	0.00 ± 0.00
89	*Mycobacterium riyadhense**	39.00 ± 3.21	13.67 ± 10.68
90	*Mycobacterium* spp.	2.67 ± 1.76	0.00 ± 0.00
91	*Nitrosococcus* spp.	115.00 ± 40.99	93.00 ± 28.54
92	*Nitrosovibrio tenuis*	0.00 ± 0.00	8.00 ± 8.00
93	*Novosphingobium* spp.	0.00 ± 0.00	0.33 ± 0.33
94	*Opitutus* spp.	9.33 ± 8.84	7.33 ± 3.18
95	*Parvularcula* spp.	2.00 ± 1.53	0.00 ± 0.00
96	*Pedomicrobium* spp.	0.00 ± 0.00	1.00 ± 0.58
97	*Phenylobacterium* spp.*	3.67 ± 2.73	27.33 ± 11.46
98	*Pilimelia* spp.*	0.33 ± 0.33	23.67 ± 7.88
99	*Planctomyces* spp.	11.00 ± 3.00	1.00 ± 1.00
100	*Pleomorphomonas oryzae*	0.33 ± 0.33	0.00 ± 0.00
101	*Polaromonas* spp.	7.33 ± 7.33	0.00 ± 0.00
102	*Pseudolabrys* spp.	4.33 ± 0.33	8.00 ± 3.79
103	*Pseudonocardia* spp.	0.00 ± 0.00	1.33 ± 1.33
104	*Rhizobium* spp.	1.33 ± 0.88	3.00 ± 2.52
105	*Rhodomicrobium* spp.	0.00 ± 0.00	2.00 ± 2.00
106	*Rhodopirellula* spp.	6.67 ± 3.76	0.00 ± 0.00
107	*Rhodoplanes* spp.*	173.00 ± 7.51	239.00 ± 7.64
108	*Rhodovastum atsumiense*	2.00 ± 1.00	1.33 ± 0.88
109	*Rhodovastum* spp.	0.00 ± 0.00	2.67 ± 2.67
110	*Rhodovibrio* spp.	14.67 ± 7.86	2.67 ± 0.88
111	*Roseomonas ruber*	0.00 ± 0.00	11.67 ±11.67
112	*Sandarakinorhabdus* spp.	0.00 ± 0.00	2.67 ± 2.67
113	*Schlesneria* spp.	0.00 ± 0.00	4.00 ± 4.00
114	*Sciscionella* spp.	9.00 ± 6.66	0.33 ± 0.33
115	*Sideroxydans* spp.	1.00 ± 1.00	0.33 ± 0.33
116	*Simplicispira* spp.	4.67 ± 4.67	4.67 ± 4.67
117	*Singulisphaera* spp.	0.00 ± 0.00	2.67 ± 2.67
118	*Skermanella* spp.	86.00 ± 64.04	20.00 ± 5.13
119	*Solirubrobacter* spp.	18.33 ± 3.28	34.00 ± 15.13
120	*Sorangiineae* spp.*	14.67 ± 4.84	6.33 ± 2.60
121	*Spam (candidate division)*	0.00 ± 0.00	1.67 ± 1.67
122	*Sphingomona*s spp.	0.00 ± 0.00	1.67 ± 1.20
123	*Telmatospirillum* spp.	0.67 ± 0.33	2.67 ± 2.67

124	*Tepidamorphus* spp.	0.00 ± 0.00	0.33 ± 0.33
125	*Thalassospira* spp.	0.00 ± 0.00	6.00 ± 6.00
126	*Thermomonas* spp.	7.00 ± 1.00	0.00 ± 0.00
127	*Thermosporothrix* spp.*	120.00 ± 36.61	1.67 ± 0.88
128	*Thioalkalispira* spp.*	69.33 ± 14.20	22.00 ± 8.96
129	*TM6*	0.00 ± 0.00	3.67 ± 3.67
130	*TM7* *	36.33 ± 8.65	9.67 ± 4.06
131	*Zavarzinella* spp.	0.67 ± 0.67	2.00 ± 2.00

Results are expressed as mean values ± standard error

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney

Table S4: The weighed UniFrac distance matrix between sites for bacterial community.

Sites	Laurentian	Kelly Lake	Kingsway	Onaping Falls	Capreol	Killarney
Laurentian	0.00	0.22	0.20	0.15	0.14	0.13
Kelly Lake		0.00	0.21	0.22	0.19	0.24
Kingsway			0.00	0.15	0.12	0.13
Onaping Falls				0.00	0.17	0.14
Capreol					0.00	0.15
Killarney						0.00

Table S5: Site-specific fungal groups and their relative abundance identified in metal contaminated and reference soil samples from the GSR.

	Metal contaminated sites	Reference sites
1	*Amanita muscaria* (5.33)	*Amanita* sp.(4.33)
2	*Ascomycete* sp. (18.50)	*Archaeosporales* sp. (158.67)
3	*Dothideomycetes* sp. (39.00)	*Atheliaceae* sp. (0.33)
4	*Leotia viscosaa* (48.33)	*Clavulina cinerea* (17.00)
5	*Magnaporthales* sp. (8.33)	*Clavulinaceae* sp. (34.33)
6	*Piloderma lanatum* (12.33)	*Cortinariaceae* sp. (0.33)
7	*Russula vesca* (306.00)	*Cortinarius camphorates* (1.00)
8	*Tremella diploschistina* (5.00)	*Elaphomyces decipiens* (64.00)
9	*Xerocomus badius* (12.33)	*Ericoid mycorrhizal* sp. (0.33)
10		*Geoglossum barlae* (91.33)
11		*Gyoerffyella* sp. (3.33)
12		*Hygrocybe miniata* (55.00)
13		*Inocybe lacera* (45.00)
14		*Inocybe lanatodisca* (34.00)
15		*Lactarius picinus* (8.00)
16		*Pezizales* sp. (182.33)
17		*Piloderma fallax* (223.33)
18		*Piloderma olivaceum* (155.33
19		*Russula gracilis* (36.33)
20		*Russulaceae* sp. (0.33)
21		*Saccharomycetes* sp. (79.00)
22		*Sordariales* sp. (10.67)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and Reference sites: Onaping Falls, Capreol and Killarney.

Table S6: All the fungal species and their relative abundance identified in metal contaminated and reference soil samples from the GSR.

	Fungi species	Metal contaminated sites	Reference sites
1	*Agaricomycotina* sp.*	173.00 ± 62.00	787.67 ± 46.29
2	*Amanita muscaria*	5.33 ± 5.33	0.00 ± 0.00
3	*Amanita* sp.	0.00 ± 0.00	4.33 ± 4.33
4	*Archaeosporales* sp.	0.00 ± 0.00	158.67 ± 158.67
5	*Ascomycete* sp.	1.67 ± 1.67	0.00 ± 0.00
6	*Ascomycota* sp.	43.00 ± 19.92	31.00 ± 13.23
7	*Atheliaceae* sp.	0.00 ± 0.00	0.33 ± 0.33
8	*Basidiomycota* sp.*	176.00 ± 17.57	133.67 ± 13.67
9	*Cenococcum geophilum*	287.67 ± 287.67	85.00 ± 85.00
10	*Chaetomella oblonga*	6.33 ± 4.88	0.67 ± 0.67
11	*Clavulina cinere*a	0.00 ± 0.00	17.00 ± 10.12
12	*Clavulinaceae* sp.	0.00 ± 0.00	34.33 ± 34.33
13	*Cortinariaceae* sp.	0.00 ± 0.00	0.33 ± 0.33
14	*Cortinarius camphoratus*	0.00 ± 0.00	1.00 ± 1.00
15	*Cryptococcus podzolicus**	49.00 ± 1.00	22.67 ± 4.26
16	*Dermateaceae* sp.*	75.67 ± 3.86	11.67 ± 9.74
17	*Dothideomycetes* sp.	39.00 ± 26.66	0.00 ± 0.00
18	*Elaphomyces decipiens*	0.00 ± 0.00	64.00 ± 64.00
19	*Elaphomyces muricatus*	2.67 ± 1.45	10.67 ± 10.67
20	*Ericoid mycorrhizal* sp.	0.00 ± 0.00	0.33 ± 0.33
21	*Fungi fungal* sp.	24.67 ± 8.67	38.00 ± 20.78
22	*Fungi mycorrhizal fungal* sp.	14.33 ± 13.84	17.00 ± 17.00
23	*Geoglossum barlae*	0.00 ± 0.00	91.33 ± 91.33
24	*Gyoerffyella* sp.	0.00 ± 0.00	3.33 ± 2.40
25	*Helotiaceae* sp.*	122.00 ± 10.64	0.33 ± 0.33
26	*Helotiales* sp.	48.33 ± 42.47	5.33 ± 3.18
27	*Herpotrichiellaceae* sp.	45.00 ± 17.62	5.00 ± 5.00
28	*Hygrocybe miniata*	0.00 ± 0.00	55.00 ± 55.00
29	*Inocybe lacera*	0.00 ± 0.00	45.00 ± 45.00
30	*Inocybe lanatodisca**	0.00 ± 0.00	34.00 ± 9.63
31	*Laccaria proxima**	259.67 ± 21.05	33.00 ± 6.56
32	*Laccaria* sp.*	23.67 ± 19.80	5.33 ± 3.53
33	*Lactarius camphoratus*	5.67 ± 5.67	92.00 ± 92.00
34	*Lactarius fuscus*	3.00 ± 3.00	21.00 ± 17.62
35	*Lactarius picinus*	0.00 ± 0.00	8.00 ± 8.00
36	*Leotia viscosa*	48.33 ± 48.33	0.00 ± 0.00

37	*Leotiomycetes* sp.	31.33 ± 29.36	7.00 ± 4.00
38	*Magnaporthales* sp.	8.33 ± 6.01	0.00 ± 0.00
39	*Mortierella* sp.	17.00 ± 17.00	3.67 ± 3.18
40	*Mortierellales* sp.*	80.00 ± 7.72	14.33 ± 7.17
41	*Mucoromycotina* sp.	38.33 ± 38.33	0.33 ± 0.33
42	*Myxotrichaceae* sp.	40.00 ± 24.25	25.67 ± 19.80
43	*Oidiodendron maius*	33.33 ± 19.88	17.00 ± 6.08
44	*Penicillium montanense*	56.00 ± 30.89	23.00 ± 18.68
45	*Pezizales* sp.*	0.00 ± 0.00	182.33 ± 78.84
46	*Pezizomycotina* sp.	31.67 ± 11.39	32.33 ± 19.70
47	*Phialocephala fortinii*	14.00 ± 14.00	2.00 ± 1.53
48	*Piloderma fallax*	0.00 ± 0.00	223.33 ±223.33
49	*Piloderma lanatum*	12.33 ± 12.33	0.00 ± 0.00
50	*Piloderma olivaceum*	0.00 ± 0.00	155.33 ± 155.33
51	*Pyronemataceae* sp.	33.67 ± 32.67	209.00 ± 199.58
52	*Russula aeruginea**	2.00 ± 0.58	5.33 ± 1.96
53	*Russula gracilis*	0.00 ± 0.00	36.33 ± 36.33
54	*Russula* sp.	833.33 ± 529.88	28.67 ± 27.18
55	*Russula sphagnophila*	336.00 ± 328.03	0.33 ± 0.33
56	*Russula vesca*	306.00 ± 158.55	0.00 ± 0.00
57	*Russulaceae* sp.	0.00 ± 0.00	0.33 ± 0.33
58	*Saccharomycetes* sp.	0.00 ± 0.00	79.00 ± 79.00
59	*Scleroderma citrinum*	71.67 ± 69.68	4.67 ± 4.67
60	*Sebacinaceae* sp.	60.00 ± 60.00	26.33 ± 18.91
61	*Sordariales* sp.	0.00 ± 0.00	10.67 ± 10.67
62	*Sordariomycetes* sp.*	0.33 ± 0.33	147.33 ± 41.84
63	*Thelephoraceae* sp.	0.33 ± 0.33	0.00 ± 0.00
64	*Tomentella* sp.*	10.67 ± 10.67	36.67 ± 16.62
65	*Tremella diploschistina*	5.00 ± 5.00	0.00 ± 0.00
66	*Tylospora asterophora*	19.00 ± 18.01	321.00 ± 321.00
67	*Venturiales* sp.	12.33 ± 7.54	39.67 ± 22.00
68	*Xerocomus badius*	12.33 ± 12.33	0.00 ± 0.00

Results are expressed as mean values ± standard error

* represents significant differences between metal contaminated and reference sites based on t-test ($p \leq 0.05$)

Metal contaminated sites: Laurentian, Kelly Lake and Kingsway and, Reference sites: Onaping Falls, Capreol and Killarney

Table S7: The weighed UniFrac distance matrix between sites for fungal community.

Sites	Laurentian	Kelly Lake	Kingsway	Onaping Falls	Capreol	Killarney
Laurentian	0.00	0.96	0.92	0.97	0.86	0.97
Kelly Lake		0.00	0.93	0.95	0.95	0.98
Kingsway			0.00	0.96	0.88	0.96
Onaping Falls				0.00	0.94	0.97
Capreol					0.00	0.96
Killarney						0.00

Table S8: Bacterial species and its relative abundance identified unique to sites from the GSR.

	Limed sites	Unlimed
1	*Actinoplanes ferrugineus* (3.25)	*Acidomonas baliensis* (1.00)
2	*Arthrobacter* spp. (4.00)	*Acidomonas methanolica* (1.50)
3	*Bradyrhizobium elkanii* (2.75)	*Afipia* spp. (2.00)
4	*Byssovorax* spp. (1.00)	*Anaerolinea* spp. (7.75)
5	*Candidates captivus* spp. (1.00)	*Blastomonas* spp. (6.25)
6	*Candidates microthrix* spp. (1.75)	*Caldanaerobacter thermoanaerobacter* sp. (12.00)
7	*Cryptosporangium japonicum* (0.25)	*Candidates chlorothrix* spp. (7.00)
8	*Kribbella* spp. (1.25)	*Comamonas* spp. (1.00)
9	*Luteibacter* spp. (1.00)	*Dermatophilus* spp. (2.75)
10	*Methylosinus* spp. (0.75)	*Geitlerinema* spp. (1.25)
11	*Methylotenera* spp. (6.75)	*Mitochondria marchantia polymorpha* (2.75)
12	*Methyloversatilis* spp. (3.25)	*Mucilaginibacter* spp. (3.25)
13	*Nitrospira freshwater sediment* (0.75)	*Mycobacterium insubricum* (0.25)
14	*Nocardioides* spp. (4.25)	*Mycobacterium* spp. (1.75)
15	*Parasegetibacter* spp. (1.00)	*Parvularcula* spp.(1.25)
16	*Pirellula* spp. (3.00)	*Polaromonas* spp. (5.50)
17	*Rhodobacter* spp. (8.00)	*Rhodobium orientis* (1.25)
18	*Rhodoplanes elegans* (3.75)	*Tistlia* spp. (1.25)
19	*Sphingomonas caulobacter leidyia* (1.5)	*Thermogemmatispora foliorum* (4.75)
20	*Streptomyces lincolnensis* (0.25)	*Thermogemmatispora onikobensis* (9.00)
21		*Victivallis* spp. (1.00)

Limed and Unlimed sites: Daisy Lake 2 (site 1), Wahnapitae Hydro-Dam (site 2), Kelly Lake (site 3), and Kingsway (site 4).

Table S9: Fungi species and its relative abundance identified unique to sites from the GSR.

	Limed sites	Unlimed sites
1	*Archaeorhizomyces finlayi* (16.75)	*Ascomycete* sp. (18.50)
2	*Cortinarius flos paludis* (636.00)	*Cladonia coniocraea* (9.50)
3	*Fusarium oxysporum* (7.75)	*Dibaeis baeomyces* (798.50)
4	*Inocybe abject* (78.00)	*Piloderma lanatum* (9.25)
5	*Inocybe fuscidula* (351.75)	*Tremella diploschistina* (3.75)
6	*Myrothecium cinctum* (18.50)	
7	*Suillus brevipes* (206.50)	
8	*Tricholomataceae* sp. (36.75)	
9	*Tricholoma ustale* (30.50)	
10	*Wilcoxina mikolae* (53.75)	

Limed and Unlimed sites: Daisy Lake 2 (site 1), Wahnapitae Hydro-Dam (site 2), Kelly Lake (site 3), and Kingsway (site 4).

Table S10: 149 bacterial species and their relative abundance identified from soil samples from the GSR.

Bacterial species		Limed sites	Unlimed sites
1.	*Achromobacter* spp.	18.75a (± 5.15)	12.75a (± 4.32)
2.	*Acidicaldus* spp.	30.00a (± 4.69)	38.75a (± 4.87)
3.	*Acidimicrobiales* spp.	13.75a (± 6.68)	0.50b (± 0.58)
4.	*Acidimicrobium* spp.	0.00a (± 0.00)	2.75a (± 3.18)
5.	*Acidiphilium* spp.	8.75a (± 8.99)	4.50a (± 3.42)
6.	*Acidisphaera* spp.	6.50a (± 2.45)	11.50b (± 4.73)
7.	*Acidobacterium* spp.	159.50a (± 10.74)	683.75b (± 11.78)
8.	*Acidocella* spp.	15.25a (± 5.70)	5.00b (± 2.94)
9.	*Acidomonas baliensis*	0.00a (± 0.00)	1.00a (± 1.15)
10.	*Acidomonas methanolica*	0.00a (± 0.00)	1.50a (± 1.73)
11.	*Acidothermus* spp.	4.75a (± 4.09)	8.50a (± 2.89)
12.	*Acidovorax* spp.	0.25a (± 0.29)	3.25a (± 2.28)
13.	*Actinoplanes ferrugineus*	3.25a (± 2.18)	0.00a (± 0.00)
14.	*Afipia broomeae*	423.25a (± 9.00)	314.75b (± 13.03)
15.	*Afipia felis*	12.75a (± 3.89)	4.75b (± 2.37)
16.	*Afipia* spp.	0.00a (± 0.00)	2.00a (± 1.63)
17.	*Anaerolinea* spp.	0.00a (± 0.00)	7.75a (± 6.26)
18.	*Anderseniella* spp.	2.00a (± 1.63)	3.50a (± 2.73)
19.	*Aquicella* spp.	49.75a (± 7.68)	300.75b (± 18.48)
20.	*Arenimonas* spp.	23.25a (± 4.88)	1.25b (± 1.09)
21.	*Armatimonas* spp.	0.25a (±0.29)	0.00a (± 0.00)
22.	*Arthrobacter* spp.	4.00a (± 4.62)	0.00a (± 0.00)
23.	*Azospira* spp.	20.75a (± 5.46)	38.00b (± 4.51)
24.	*Azovibrio* spp.	0.25a (± 0.29)	0.00a (± 0.00)
25.	*Blastochloris* spp.	1.00a (± 1.15)	1.00a (± 0.82)
26.	*Blastochloris viridis*	3.00a (± 3.46)	0.00a (± 0.00)
27.	*Blastomonas* spp.	0.00a (± 0.00)	6.25b (± 2.23)
28.	*Bosea* spp.	2.25a (± 2.23)	0.00a (± 0.00)
29.	*Bradyrhizobium elkanii*	2.75a (± 3.18)	0.00a (± 0.00)
30.	*Bradyrhizobium* spp.	69.75a (± 6.52)	29.50b (± 5.81)
31.	*Burkholderia caryophylli*	1.00a (± 0.47)	0.25a (± 0.29)
32.	*Burkholderia* spp.	2.25a (± 1.91)	11.00b (± 2.23)
33.	*Byssovorax* spp.	1.00a (± 1.15)	0.00a (± 0.00)
34.	*Caldanaerobacter thermoanaerobacter* sp.	0.00a (± 0.00)	12.00a (± 13.86)
35.	*Caldanaerobacter thermoanaerobacter tengcongensis*	5.50a (± 1.93)	20.00b (± 4.36)

36.	*Caldilinea* spp.	5.25 (± 6.06)	5.50 (± 6.35)
37.	*Candidatus koribacter versatilis*	0.25a (± 0.29)	2.00a (± 1.63)
38.	*Candidates alysiosphaera* spp.	20.50a (± 5.72)	5.50b (± 2.77)
39.	*Candidates captivus* spp.	1.00a (± 1.15)	0.00a (± 0.00)
40.	*Candidates chloracidobacterium* spp.	27.75a (± 7.93)	20.25a (± 4.44)
41.	*Candidates chlorothrix* spp.	0.00a (± 0.00)	7.00a (± 8.08)
42.	*Candidates entotheonella* spp.	70.75a (± 9.01)	0.00b (± 0.00)
43.	*Candidates koribacter* spp.	23.25a (± 9.68)	262.75b (± 16.79)
44.	*Candidates microthrix* spp.	1.75a (± 1.66)	0.00a (± 0.00)
45.	*Candidates solibacter* spp.	137.75a (± 12.37)	286.5b (± 14.54)
46.	*Candidates xiphinematobacter* spp.	5.00a (± 1.59)	0.75b (± 0.87)
47.	*Caulobacter* spp.	8.00a (± 3.74)	24.75b (± 4.77)
48.	*Caulobacter vibrioides*	4.25a (± 2.18)	10.25a (± 8.94)
49.	*Collimonas fungivorans*	0.00a (± 0.00)	2.25a (± 2.60)
50.	*Colwellia* spp.	7.50a (± 3.32)	40.00b (± 4.34)
51.	*Comamonas* spp.	0.00a (± 0.00)	1.00a (± 1.15)
52.	*Conexibacter* spp.	21.25a (± 7.61)	51.00b (± 8.39)
53.	*Coprothermobacter* spp.	0.75a (± 0.55)	3.25a (± 1.60)
54.	*Corynebacteriales* spp.	3.00a (± 3.46)	0.00a (± 0.00)
55.	*Crossiella* spp.	5.50a (± 3.84)	7.00a (± 4.76)
56.	*Cryptosporangium japonicum*	0.25a (± 0.29)	0.00a (± 0.00)
57.	*Cupriavidus pinatubonensis*	3.25a (± 3.75)	0.00a (± 0.00)
58.	*Defluviicoccus* spp.	25.50a (±10.34)	14.25a (± 3.95)
59.	*Dermatophilus* spp.	0.00a (± 0.00)	2.75a (± 3.18)
60.	*Dongia* spp.	5.00a (± 2.40)	12.75a (± 10.81)
61.	*Dyella* spp.	6.00a (± 6.93)	0.00a (± 0.00)
62.	*Edaphobacter modestum*	7.25a (± 2.80)	11.75a (± 3.95)
63.	*Edaphobacter* spp.	1.00a (± 1.15)	10.50b (± 2.74)
64.	*Escherichia shigella* spp.	1.75a (± 1.19)	5.50a (± 5.97)
65.	*Ferruginibacter* spp.	8.00a (± 1.25)	18.00b (± 2.38)
66.	*Filomicrobium* spp.	2.50a (± 2.89)	65.75b (± 7.39)
67.	*Fluviicola* spp.	0.75a (± 0.87)	0.00a (± 0.00)
68.	*Frankia* spp.	3.50a (± 1.45)	2.75a (± 1.44)
69.	*Geitlerinema* spp.	0.00a (± 0.00)	1.25a (± 1.44)
70.	*Gemmata* spp.	11.75a (± 3.24)	11.50a (± 2.37)
71.	*Gemmatimonas* spp.	8.00a (± 1.74)	18.75b (± 2.40)
72.	*Geobacillus* spp.	41.25a (± 3.81)	167.75b (± 13.03)
73.	*Granulicella* spp.	74.50a (± 10.48)	186.75b (± 16.68)
74.	*Haliangium* spp.	2.75a (± 2.47)	0.50a (± 0.58)
75.	*Hirschia* spp.	19.75a (± 3.67)	21.50a (± 2.81)

76.	*Hymenobacter* spp.	0.00a (± 0.00)	10.75a (± 12.41)
77.	*Hyphomicrobium* spp.	1.50a (± 1.11)	2.75a (± 3.18)
78.	*Iamia* spp.	0.00a (± 0.00)	5.75b (± 1.98)
79.	*Inquilinus* spp.	2.00a (± 0.82)	3.50a (± 3.32)
80.	*Kineosporia* spp.	6.00a (± 2.79)	0.00b (± 0.00)
81.	*Kribbella* spp.	1.25a (± 1.44)	0.00a (± 0.00)
82.	*Ktedonobacter* spp.	12.00a (± 3.47)	3.25b (± 2.28)
83.	*Labrys* spp.	28.00a (± 4.65)	14.75b (± 3.91)
84.	*Leptothrix* spp.	1.00a (± 1.15)	2.75a (± 1.85)
85.	*Luteibacter* spp.	1.00a (± 1.15)	0.00a (± 0.00)
86.	*Magnetospirillum* sp.	1.75a (± 2.02)	0.00a (± 0.00)
87.	*Marmoricola* spp.	16.25a (± 15.16)	0.00a (± 0.00)
88.	*Massilia* spp.	2.00a (± 1.25)	0.00a (± 0.00)
89.	*Massilia timonae*	2.75a (± 3.18)	0.00a (± 0.00)
90.	*Methylocystis* spp.	8.50a (± 2.11)	7.75a (± 1.38)
91.	*Methylosinus sporium*	0.50a (± 0.58)	0.00a (± 0.00)
92.	*Methylosinus* spp.	0.75a (± 0.87)	0.00a (± 0.00)
93.	*Methylosinus trichosporium*	0.00a (± 0.00)	6.25b (± 2.84)
94.	*Methylotenera* spp.	6.75a (± 7.79)	0.00a (± 0.00)
95.	*Methyloversatilis* spp.	3.25a (± 1.44)	0.00b (± 0.00)
96.	*Metyhlovirgula* spp.	2.00a (± 1.41)	2.00a (± 1.94)
97.	*Mitochondria marchantia polymorpha*	0.00a (± 0.00)	2.75a (± 3.18)
98.	*Mucilaginibacter* spp.	0.00a (± 0.00)	3.25a (± 3.75)
99.	*Mycobacterium celatum*	1.00a (± 0.82)	0.00a (± 0.00)
100.	*Mycobacterium insubricum*	0.00a (± 0.00)	0.25a (± 0.29)
101.	*Mycobacterium riyadhense*	2.25a (± 2.60)	19.75b (± 6.88)
102.	*Mycobacterium* spp.	0.00a (± 0.00)	1.75a (± 1.36)
103.	*Nitrosococcus* spp.	56.00a (± 7.68)	81.50b (± 4.20)
104.	*Nitrospira freshwater sediment*	0.75a (± 0.87)	0.00a (± 0.00)
105.	*Nocardioides* spp.	4.25a (± 4.91)	0.00a (± 0.00)
106.	*Novosphingobium* spp.	2.50a (± 2.19)	0.00a (± 0.00)
107.	*Opitutus* spp.	2.25a (± 2.60)	0.25a (± 0.29)
108.	*Parasegetibacter* spp.	1.00a (± 1.15)	0.00a (± 0.00)
109.	*Parvularcula* spp.	0.00a (± 0.00)	1.25a (± 1.44)
110.	*Pedomicrobium* spp.	16.00a (± 2.88)	4.50b (± 3.12)
111.	*Phenylobacterium* spp.	17.50a (± 5.67)	3.00b (± 2.45)
112.	*Pilimelia* spp.	58.00a (± 3.32)	1.50b (± 1.37)
113.	*Pirellula* spp.	3.00a (± 0.05)	0.00b (± 0.00)
114.	*Planctomyces* spp.	0.25a (± 0.29)	10.00b (± 2.60)
115.	*Pleomorphomonas oryzae*	0.25a (± 0.29)	0.25a (± 0.29)

116.	*Polaromonas* spp.	0.00a (± 0.00)	5.50a (± 6.35)
117.	*Pseudolabrys* spp.	43.25a (± 7.49)	3.50b (± 0.75)
118.	*Rhizobium* spp.	2.75a (± 3.18)	3.00a (± 1.25)
119.	*Rhodobacter* spp.	8.00a (± 1.58)	0.00b (± 0.00)
120.	*Rhodobium orientis*	0.00a (± 0.00)	1.25a (± 1.44)
121.	*Rhodopirellula* spp.	1.00a (± 1.15)	6.00b (± 1.16)
122.	*Rhodoplanes elegans*	3.75a (± 4.33)	0.00a (± 0.00)
123.	*Rhodoplanes* spp.	115.75a (± 5.19)	120.5a (± 7.93)
124.	*Rhodovastum atsumiense*	0.50a (± 0.58)	1.00a (± 0.82)
125.	*Rhodovibrio* spp.	1.00a (± 0.82)	8.50a (± 8.35)
126.	*Roseomonas ruber*	0.75a (± 0.55)	1.50a (± 1.76)
127.	*Sciscionella* spp.	1.00a (± 1.15)	44.25b (± 4.25)
128.	*Sideroxydans* spp.	0.25a (± 0.29)	0.00a (± 0.00)
129.	*Simplicispira* spp.	0.50a (± 0.33)	3.50a (± 4.04)
130.	*Singulisphaera nostocoida limicola*	0.00a (± 0.00)	4.50a (± 5.20)
131.	*Skermanella* spp.	19.75a (± 10.84)	105.75b (± 25.88)
132.	*Sneathiella* spp.	2.25a (± 0.66)	0.00b (± 0.00)
133.	*Solirubrobacter* spp.	23.75a (± 3.02)	15.00b (± 5.94)
134.	*Sorangiineae* spp.	2.75a (± 3.18)	1.00a (± 1.15)
135.	*Sorangium* spp.	2.00a (± 1.05)	21.00b (± 6.82)
136.	*Sphingomonas caulobacter leidyia*	1.50a (± 1.73)	0.00a (± 0.00)
137.	*Sphingomonas* spp.	0.50a (± 0.58)	0.00a (± 0.00)
138.	*Streptomyces lincolnensis*	0.25a (± 0.29)	0.00a (± 0.00)
139.	*Telmatospirillum* spp.	0.25a (± 0.29)	2.50a (± 2.52)
140.	*Tepidamorphus* spp.	1.25a (± 1.44)	0.00a (± 0.00)
141.	*Thermogemmatispora foliorum*	0.00a (± 0.00)	4.75a (± 5.48)
142.	*Thermogemmatispora onikobensis*	0.00a (± 0.00)	9.00a (± 10.39)
143.	*Thermomonas* spp.	0.25a (± 0.29)	7.75b (± 2.84)
144.	*Thermosporothrix* spp.	12.75a (± 4.84)	343.75b (± 21.30)
145.	*Thioalkalispira* spp.	8.50a (± 5.11)	134.25b (± 10.00)
146.	*Tistlia* spp.	0.00a (± 0.00)	1.25a (± 1.44)
147.	*TM7*	7.75a (± 3.07)	24.50b (± 4.12)
148.	*Victivallis* spp.	0.00a (± 0.00)	1.00b (± 0.00)
149.	*Zavarzinella* spp.	0.00a (± 0.00)	2.50a (± 2.19)

Results are expressed as mean values ± standard error

Means in rows with a common letter are not significantly different based on t-test ($p \geq 0.05$).

Limed and Unlimed sites: Daisy Lake 2 (site 1), Wahnapitae Hydro-Dam (site 2), Kelly Lake (site 3), and Kingsway (site 4).

Table S11: The weighed UniFrac distance matrix between sites for bacterial community.

	Daisy Lake 2 Limed	Daisy Lake 2 Unlimed	Wahnapitae Hydro-Dam Limed	Wahnapitae Hydro-Dam Unlimed	Kelly Lake Limed	Kelly Lake Unlimed	Kingsway Limed	Kingsway Unlimed
Daisy Lake 2 Limed	0.00	0.31	0.24	0.30	0.16	0.23	0.21	0.20
Daisy Lake 2 Unlimed		0.00	0.35	0.47	0.37	0.30	0.36	0.41
Wahnapitae Hydro-Dam Limed			0.00	0.40	0.24	0.25	0.18	0.32
Wahnapitae Hydro-Dam Unlimed				0.00	0.30	0.36	0.34	0.19
Kelly Lake Limed					0.00	0.21	0.17	0.21
Kelly Lake Unlimed						0.00	0.21	0.29
Kingsway Limed							0.00	0.26
Kingsway Unlimed								0.00

Table S12: A total of 70 fungal species and their relative abundance identified from soil samples from the GSR.

Fungi species	Limed sites	Unlimed sites
1. *Agaricomycotina sp.*	109.25a (± 39.71)	306.50b (±18.61)
2. *Amanita muscaria*	70.75a (± 11.70)	4.00b (± 4.62)
3. *Amanita sp.*	1.00a (± 0.82)	0.00a (± 0.00)
4. *Archaeorhizomyces finlayi*	16.75a (± 19.34)	0.00a (± 0.00)
5. *Ascomycete sp.*	0.00a (± 0.00)	18.50b (± 7.57)
6. *Ascomycota sp.*	20.00a (± 19.74)	199.00b (± 19.28)
7. *Atheliaceae sp.*	0.50a (± 0.58)	0.00a (± 0.00)
8. *Basidiomycota sp.*	1.50a (± 1.37)	127.75b (± 14.51)
9. *Calicium salicinum*	2.25a (± 2.60)	21.00b (± 4.25)
10. *Cenococcum geophilum*	42.75a (± 4.84)	0.00b (± 0.00)
11. *Chaetomella oblonga*	2.75a (± 3.18)	1.00a (± 1.15)
12. *Cladonia coniocraea*	0.00a (± 0.00)	9.50a (± 10.97)
13. *Clavulinaceae sp.*	42.75a (± 49.36)	0.00a (± 0.00)
14. *Cortinariaceae sp.*	10.00a (± 11.55)	0.00a (± 0.00)
15. *Cortinarius flos paludis*	636.00a (± 34.39)	0.00b (± 0.00)
16. *Cryptococcus podzolicus*	87.50a (± 8.34)	25.75b (± 15.86)
17. *Dermateaceae sp.*	11.75a (± 7.52)	52.25b (± 4.18)
18. *Dibaeis baeomyces*	0.00a (± 0.00)	798.50b (± 22.03)
19. *Dothideomycetes sp.*	5.00a (± 2.54)	33.75a (± 24.68)
20. *Elaphomyces muricatus*	0.00a (± 0.00)	0.75a (± 0.55)
21. *Ericoid mycorrhizal sp.*	41.00a (± 4.49)	0.00b (± 0.00)
22. *Fungi fungal sp.*	5.75a (± 3.84)	16.25b (± 4.16)
23. *Fungi mycorrhizal fungal sp.*	2.25a (± 1.91)	10.50a (± 12.12)
24. *Fusarium oxysporum*	7.75a (± 8.20)	0.00a (± 0.00)
25. *Gyoerffyella sp.*	1.75a (± 1.66)	0.75a (± 0.55)
26. *Helotiaceae sp.*	65.00a (± 4.53)	96.75b (± 9.61)
27. *Helotiales sp.*	31.50a (± 5.36)	53.25b (± 7.63)
28. *Herpotrichiellaceae sp.*	3.50a (± 2.03)	23.75b (± 12.44)
29. *Inocybe abjecta*	78.00a (± 13.53)	0.00b (± 0.00)
30. *Inocybe fuscidula*	351.75a (± 40.17)	0.00b (± 0.00)
31. *Inocybe lacera*	0.25a (± 0.29)	0.00a (± 0.00)
32. *Laccaria proxima*	46.50a (± 16.01)	197.75b (± 17.12)
33. *Laccaria sp.*	12.50a (± 12.94)	15.75a (± 18.19)
34. *Lactarius camphoratus*	0.00a (± 0.00)	4.25a (± 4.91)
35. *Lactarius fuscus*	12.75a (± 12.52)	2.75a (± 2.47)
36. *Leotia viscosa*	1.00a (± 1.15)	36.25b (± 4.86)

37. *Leotiomycetes sp.*	1.00a (± 1.15)	23.50b (± 5.62)
38. *Magnaporthales sp.*	1.25a (± 1.44)	1.25a (± 1.44)
39. *Mortierella sp.*	2.50a (± 2.89)	14.00a (± 14.31)
40. *Mortierellales sp.*	8.00a (± 6.99)	59.25b (± 6.25)
41. *Mucoromycotina sp.*	0.00a (± 0.00)	28.75a (± 33.20)
42. *Myrothecium cinctum*	18.50a (± 21.36)	0.00a (± 0.00)
43. *Myxotrichaceae sp.*	22.00a (± 16.46)	21.00a (± 24.25)
44. *Oidiodendron maius*	9.00a (± 4.03)	26.50b (± 11.27)
45. *Penicillium montanense*	41.25a (± 4.63)	16.00b (± 5.08)
46. *Pezizomycotina sp.*	140.25a (± 10.70)	67.50b (± 15.66)
47. *Phialocephala fortinii*	9.25a (± 5.24)	15.50a (± 11.21)
48. *Piloderma lanatum*	0.00a (± 0.00)	9.25a (± 10.68)
49. *Pyronemataceae sp.*	0.00a (± 0.00)	24.75a (± 28.58)
50. *Russula aeruginea*	12.00a (± 9.80)	31.75a (± 35.13)
51. *Russula gracilis*	123.50a (± 20.70)	0.25b (± 0.29)
52. *Russula sp.*	170.75a (± 18.92)	454.25b (± 24.52)
53. *Russula sphagnophila*	44.50a (± 4.28)	86.75b (± 9.11)
54. *Russula ventricosipes*	0.25a (± 0.29)	5.00a (± 5.77)
55. *Russula vesca*	41.00a (± 47.34)	24.50a (± 28.29)
56. *Russulaceae sp.*	359.25a (± 414.83)	0.00a (± 0.00)
57. *Scleroderma citrinum*	0.00a (± 0.00)	80.50a (± 58.61)
58. *Sebacinaceae sp.*	14.25a (± 16.45)	159.50b (± 22.06)
59. *Sordariales sp.*	1.00a (± 0.82)	0.00a (± 0.00)
60. *Sordariomycetes sp.*	0.25a (± 0.29)	0.25a (± 0.29)
61. *Suillus brevipes*	206.50a (± 20.04)	0.00b (± 0.00)
62. *Thelephoraceae sp.*	1555.00a (± 277.48)	0.25b (± 0.29)
63. *Tomentella sp.*	1.50a (± 0.58)	8.00a (± 9.24)
64. *Tremella diploschistina*	0.00a (± 0.00)	3.75a (± 4.33)
65. *Tricholoma ustale*	30.50a (± 34.45)	0.00a (± 0.00)
66. *Tricholomataceae sp.*	36.75a (± 42.44)	0.00a (± 0.00)
67. *Tylospora asterophora*	0.00a (± 0.00)	0.50a (± 0.58)
68. *Venturiales sp.*	3.00a (± 2.05)	2.75a (± 3.18)
69. *Wilcoxina mikolae*	53.75a (± 37.51)	0.00a (± 0.00)
70. *Xerocomus badius*	0.25a (± 0.29)	9.25a (± 10.68)

Results are expressed as mean values ± standard error

Means in rows with a common letter are not significantly different based on t-test ($p \geq 0.05$).

Limed and Unlimed sites: Daisy Lake 2 (site 1), Wahnapitae Hydro-Dam (site 2), Kelly Lake (site 3), and Kingsway (site 4).

Table S13: The weighed UniFrac distance matrix between sites for fungal community.

	Daisy Lake 2 Limed	**Daisy Lake 2 Unlimed**	**Wahnapitae Hydro-Dam Limed**	**Wahnapitae Hydro-Dam Unlimed**	**Kelly Lake Limed**	**Kelly Lake Unlimed**	**Kingsway Limed**	**Kingsway Unlimed**
Daisy Lake 2 Limed	0.00	0.95	0.99	0.97	0.89	0.94	0.97	0.95
Daisy Lake 2 Unlimed		0.00	1.00	0.98	0.97	0.91	0.99	0.96
Wahnapitae Hydro-Dam Limed			0.00	1.00	0.99	1.00	0.88	1.00
Wahnapitae Hydro-Dam Unlimed				0.00	0.94	0.98	0.98	0.97
Kelly Lake Limed					0.00	0.90	0.95	0.91
Kelly Lake Unlimed						0.00	0.97	0.93
Kingsway Limed							0.00	0.75
Kingsway Unlimed								0.00

PUBLICATIONS

Manuscripts Published (Refereed):

1. **R. Narendrula-Kotha** and K.K. Nkongolo. 2017. Changes in enzymatic activities in metal contaminated and reclaimed lands in Northern Ontario (Canada). Ecotoxicology and Environmental Safety. Vol 140, pg: 241-248.

2. **R. Narendrula-Kotha** and K.K. Nkongolo. 2017. Bacterial and fungal community structure and diversity in a mining region under long-term metal exposure revealed by metagenomics sequencing. Ecological Genetics and Genomics. Vol 2, pg: 13-24.

3. **R. Narendrula-Kotha** and K.K. Nkongolo. 2017. Microbial response to soil liming of damaged ecosystems revealed by metagenomics and phospholipid fatty acid analyses. PLoS One. Vol 12, pg: 1–22.

4. K.K. Nkongolo, P. Michael, G. Theriault, **R. Narendrula**, P. Castilloux, K.N. Kalubi, P. Beckett and G. Spiers. 2016. Assessing biological impacts of land reclamation in a mining region in Canada: Effects of dolomitic lime applications on forest ecosystems and microbial phospholipid fatty acid signatures. Water, Air and Soil Pollution. Vol 227, Issue 4, pg: 1-13.

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7. A. Tran, K.K. Nkongolo, M. Mehes-Smith, **R. Narendrula**, G. Spiers and P. Beckett. 2014. Heavy metal analysis in Red oak *(Quercus rubra*) populations from a mining region in Northern Ontario (Canada): Effect of soil liming and analysis of genetic variation. American Journal of Environmental Science. Vol 10, Issue 4, pg: 363-373.

8. G. Mamba-Mbayi, K.K. Nkongolo, **R. Narendrula**, P. Tshilenge Djim and A. Kalonji-Mbuyi. 2014. Molecular relatedness and morpho-agronomic characteristics of congolese accessions of cassava (*Manihot esculenta Crantz*) for breeding purpose. British Biotechnology Journal. Vol 4, Issue, 5, pg: 551-565.

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12. K.K. Nkongolo, G. Spiers, P. Beckett, **R. Narendrula**, G. Theriault, A. Tran and K.N. Kalubi. 2012. Long term effects of liming on soil chemistry in stable and eroded upland areas in a mining region. Journal of Water, Air, and Soil Pollution. Vol 224, Issue 7, pg: 1-14.

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1. K.K. Nkongolo, P. Michael, G. Theriault, **R. Narendrula**, K. Kalubi and N.S. Kim. Molecular genetic monitoring of reclaimed metal-contaminated lands in mining regions. Paper presentation at International Conference of the Genetics Society of Korea held in Seoul, South Korea, December 2015.

2. **R. Narendrula**. Pyrosequencing analysis of bacterial and fungal diversity in reclaimed lands in a mining region. Paper presentation at ASA, CSSA and SSSA international annual meetings held in Minneapolis, Minnesota, USA, November 2015.

3. **R. Narendrula,** K.K. Nkongolo, G. Theriault, P. Beckett and G. Spiers. Assessing above and below ground diversity in a mining reclaimed region contaminated with metals. Paper presentation at the 6th Mining and Environmental international conference held in Sudbury, Ontario, Canada, June 2015.

4. K.K. Nkongolo, P. Michael, **R. Narendrula**. Analysis of soil microbial diversity and activities in mining reclaimed lands in Northern Ontario: Association with plant population structure. Paper presentation at ASA, CSSA and SSSA international annual meetings held in Long Beach, California, USA, November 2014.

5. M. Mehes-Smith, K.K. Nkongolo, P. Michael**, R. Narendrula**. Comparative analysis of metal distribution in Poplar (*Populus tremuloides*) and red maple (*Acer rubrum*) populations from reclaimed mining sites: analysis of gene expression. Paper presentation at ASA, CSSA and SSSA international annual meetings held in Long Beach, California, USA, November 2014.

6. **R. Narendrula** and K.K. Nkongolo. Cytological stability and molecular variation in *Picea mariana* x *Picea rubens* hybrid populations. Paper presentation at the 9th Canadian Plant Biotechnology conference held in Montreal, Quebec, Canada, May 2014.

7. K. Kalubi, M. Mehes-Smith**, R. Narendrula**, K.K. Nkongolo. Molecular analysis of red maple (*Acer rubrum*) populations from areas contaminated with metals in Northern Ontario, Canada. Paper presentation at ASA, CSSA and SSSA international annual meetings held in Tampa, Florida, USA, November 2013.

8. **R. Narendrula**. Ecological and genetic assessment of reclaimed sites in a metal contaminated region (Northern Ontario, Canada). Paper presentation at ASA, CSSA and SSSA international annual meetings held in Tampa, Florida, USA, November 2013.

9. M. Mehes-Smith, K.K. Nkongolo, **R. Narendrula**. Genetic and physiological responses of *Deschampsia Cespitosa* to soil metal contamination. Paper presentation at ASA, CSSA and SSSA international annual meetings held in Tampa, Florida, USA, November 2013.

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13. **R. Narendrula**, K.K. Nkongolo, P. Beckett. Comparative soil metal analyses in Sudbury (Canada) and Lubumbashi (DR-Congo): two contrasting mining cities with a similar legacy. Paper presentation at Science for a Changing North II, a Sudbury Restoration Workshop held in Sudbury, Ontario, Canada, February 2011.

14. **R. Narendrula**, M. Mehes-Smith, K.K. Nkongolo, C. Roussell, P. Beckett. Genetic analysis of natural and planted populations of *Picea glauca* and *Pinus strobus* growing from Northern Ontario. Paper presentation at Science for a Changing North II, a Sudbury Restoration Workshop held in Sudbury, Ontario, Canada, February 2011.

15. **R. Narendrula**. Effects of heavy metals on plants growing around the Greater Sudbury region. Paper presentation at Laurentian University open house held in Sudbury, Ontario, Canada, 2010.

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19. **R. Narendrula** and K.K. Nkongolo. Genetic and cytological analyses of wheat × triticale lines: Effects of gamma radiation. Oral and paper presentation at Laurentian University undergraduate seminar held in Sudbury, Ontario, Canada, April 2008.