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Strucutural, Chemical and Bacteriological Characterization of the Rhizosphere of Zea Mays Grown in Metal-Contaminated Soil

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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ABSTRACT

Plants grown in metal-contaminated soils may take up and transfer metals to higher trophic levels of the food web. This project developed a new embedding technique to investigate the interaction among metals, bacteria and corn (*Zea mays*) roots in soils from Blackfrairs Community Garden and a home in Hamilton, using ICP-AES, Biolog Ecoplates^{TM}, scanning electron microscopy (SEM) and synchrotron analyses (SRA). The bacterial numbers were lower than those reported in uncontaminated soils. The concentrations of bioavailable metals varied among soils and increased with time. The bacterial community in bulk and rhizosphere soils used all of the carbon sources on Ecoplates^{TM}; however, there were no differences in the bacterial activities representing bulk soils and the functional diversity in both treatments. The SEM and SRA detected grains containing high concentrations of metals. Copper, iron and manganese were accumulated in both rhizospheres. In plants from Blackfrairs, zinc was distributed throughout the root tissue.

Keywords: Metal solubility, Metal bioavailability, Rhizosphere, Plant root, Bacterial community, Corn, *Zea mays*, Toxicity, Scanning electron microscopy (SEM), Synchrotron radiation analyses (SRA)

I dedicate my thesis to my mom, dad and sister

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TABLE OF CONTENTS

Certificate of Examination **ii**

Abstract **iii**

Dedication **iv**

Acknowledgements **v**

Table of Contents **vi**

List of Tables **viii**

List of Figures **ix**

List of Abbreviations **xi**

1 Chapter 1. Introduction

1.1 Trace Metals in the Soil Environment 1

1.1.1 Metal Solubility 2

1.1.2 Factors Affecting Metal Availability 2

1.1.3 Assessment of Trace Metal Forms in Soil 4

1.2 Plant-Microbe and Metal Interaction 4

1.3 Plant Nutrients and Toxicity 6

1.3.1 Metal Uptake by Plants 7

1.3.2 Potential Risk of Trace Metals 8

1.4 Mechanisms of Tolerance 14

1.4.1 Extracellular Tolerance Mechanisms 14

1.4.2 Intracellular Tolerance Mechanisms 16

1.5 Rationale 17

1.6 Project Objective and Hypothesis 18

2 **Chapter 2. Materials and Experimental Procedures**

2.1 Laboratory Model 20

2.2 Soil Sampling and Initial Analyses 20

2.3 Germination and Growth Conditions 25

2.4 Chemical Analyses of the Soils 25

2.5 Soil Microbial Biodiversity 27
2.6 Investigating the Intact Rhizosphere 31
2.7 Data Analysis 35

3 Chapter 3. Results and Discussion

3.1 Initial Metal and Microbial Content of the Soils 37
3.2 Growth and Chemical Analyses of the Boulk and Rhizosphere Soils 39
3.3 Carbon Source Utilization 44
3.4 New Embedding Technique 48
3.5 SEM and SRA Analyses 52

4 Chapter 4. General Discussion, Conclusions and Future Prospects

4.1 General Discussion and Conclusions 64
4.2 Directions for Future Research 68

References 70
Curriculum Vitae xii

LIST OF TABLES

1. Chapter 1 Introduction

1.1 Agricultural soil quality guidelines for the protection of environmental and human health and common range of some metals in soils 9

2. Chapter 2 Materials and Experimental Procedures

2.1 Laboratory model to study trace metals in bulk and rhizosphere soils 21

2.2 Sole carbon sources in Biolog EcoPlates™ 28

3. Chapter 3 Results and Discussion

3.1 pH values of bulk and rhizosphere soils over 30 days of the experiment 44

LIST OF FIGURES

1. Chapter 2 Materials and Experimental Procedures

2.1 Location of the study area 22

2.2 Bulk and rhizosphere soils grown in growth chamber and Biolog Ecoplate™ 26

2.3 Impregnation procedure and embedded bulk and rhizoshere soils 33

2. Chapter 3 Results and Discussion

3.1 Concentrations of trace metals in control soils 38

3.2 Estimated bioavailable concentrations of metals in soils among days of the treatment 43

3.3 Mean substrate utilization patterns for soils among days of the treatment 45

3.4 Absorbance values of carbon substrates on Ecoplates for soils among days of the treatment 47

3.5 Shannon Diversity Index (H') and evenness (E) values for soils among days of the treatment 49

3.6 Cross section of a soil core containing a corn (*Zea mays*) root and rhizosphere 50

3.7 Metal concentrations and microorganisms in the wash-water of the new embedding procedure 51

3.8 SEM micrographs highliting an example of distingtive metal grains found in soils 54

3.9 SEM micrographs of embedded polished cross section of bulk soils on day 0 55

3.10 EM micrographs of embedded polished cross section of bulk and rhizosphere soils on day 15 from Black friars 56

3.11 SEM micrographs of embedded polished cross section of bulk and rhizosphere soils on day 15 from Hamilton 57

3.12 SEM micrographs of embedded polished cross section of 58

rhizosphere soils on day 30
3.13 Typical x-ray fluorescence maps for trace metals in soils from Black friars 60
3.14 Typical x-ray fluorescence maps for metals in soils from Hamilton 61
3.15 Typical x-ray fluorescence maps for trace metals in melt grain from the rhizosphere of Hamilton soil 63

LIST OF ABBREVIATIONS

AWCD	Average well colour development
CCME	Canadian Council of Ministers of the Environment
CFU	Colony forming unit
CLPP	Community level physiological profiling
E	Evenness
EDAX	Energy dispersive x-ray spectrometer
H’	Shannon diversity index
ICP-AES	Inductively coupled plasma-atomic emission spectroscopy
kPa	Kilopascals
kV	Kilo-volt
R	Substrate richness
SE	Standard error
SEM	Scanning electron microscopy
SEM-BSE	Scanning electron microscopy-backscattered electron
SEM-SE	Scanning electron microscopy-secondary electron
Spp	Species
SRA	Synchrotron radiation analysis
WDAX	Wave length dispersive x-ray spectrometer
XANES	X-ray absorption near edge structure
XRF	X-ray fluorescence spectroscopy

CHAPTER 1

1. Introduction

1.1 Trace Metals in the Soil Environment

Trace metals are typically present naturally in the soil environment at fairly low concentrations, but can occur at significant concentrations (up to 1000 mg kg^{-1} or more; He et al., 2005; Wuana and Okieimen, 2011) resulting in polluted soil (Wang and Jia, 2010). Arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn) are the most commonly enriched metals in soils and the greatest cause for concern worldwide (He et al., 2005; Basar, 2009; Wang and Jia, 2010; Wuana and Okieimen, 2011). Because metals cannot be degraded by biological or chemical reactions, they can remain within soil for a long time and reach excessive concentrations (Nies, 1999; Cheng, 2003; Wunan and Okieimen, 2011). High concentrations of metals may negatively affect soil function (*e.g.*, aeration and nutrient cycling), the quality of agricultural products and human health (Athar and Ahmad, 2008; Meier et al., 2011).

The level and distribution of these elements in soils are increasing with large variations in concentration based on site conditions *e.g.*, physical and chemical soil characteristics (Dube et al., 2000; He et al., 2005; Wang and Jia, 2010) and due to natural phenomena (*e.g.*, volcanoes), geochemical processes (*e.g.*, rock erosion), agricultural applications (*e.g.*, fertilizers), energy fuel production, waste sludge, ore refining, mining, electroplating in addition to a wide range of other industrial activities (Lasat, 2000; Yan-de et al., 2007; Meier et al., 2011).

1.1.1 *Metal Solubility*

Metals are heterogeneously distributed across the different soil horizons, where they exist in a variety of chemical forms with different valence states and solubilities. They can also exist as free ions or in molecular complexes in the soil solution. Metals in soils range from exchangeable ions, which can be adsorbed to organic or inorganic molecules, to mineral fractions, which are essentially constituents of the solid phases of the bulk soil *e.g.*, oxides, carbonates, sulphates and nitrates (Giller et al., 1998; Lasat, 2000; Dube et al., 2000; Violante et al., 2010; Gadd, 2010). Based on the chemical forms in which metals are found, metals may vary in solubility, mobility, bioavailability and toxicity to all domains of living organisms (Cataldo and Wildung, 1978; Giller et al., 1998; Lin et al., 2004; Violante et al., 2010). For example, metals associated with solid phases, which make up, on average, 45% of the bulk soil (Dube et al., 2000) are less mobile and unavailable to plants and soil microorganisms (Lasat, 2000; Lasat, 2002; Reichman, 2002), often because their exchange with the soil solution (solubility) is limited.

An interesting property of metals in soil is that they interact with the soil through fast and slow reactions, including mineral precipitation, ion exchange, aqueous complexation, oxidation and reduction (reviewed in: Cataldo and Wildung, 1978; Giller et al., 1998; Wunan and Okieimen, 2011). After such reactions, the soil ultimately comes to equilibrium with the dominant chemical conditions in the soil solution. However, as soil is dynamic, metal mobility can be equally active.

1.1.2 Factors Affecting Metal Availability

Previous studies have shown that the total concentration of trace metals in soil alone can not function as a predictor of the toxic effect on plant activities and organism functions

(Basar, 2009; Laing et al., 2009). Metal solubility, mobility, distribution and transportation through soil to soil microorganisms and to plant tissues are governed by several soil and environmental parameters. These factors include pH, cation exchange capacity, content of organic matter, biota, plant species and rhizosphere processes, *e.g.*, root exudates and microbial activity (Cataldo and Wildung, 1978; Reichman, 2002; Cheng, 2003; Nwuche and Ugoji, 2008; Basar, 2009; Meire et al., 2011). However, pH and organic matter are two of the most important factors that may affect metal bioavailability in the soils.

1.1.2.1 pH

In soil solution, the H^+ ions are strongly adsorbed to negatively charged surfaces of the soil particles (*e.g.*, clay minerals). The presence of high concentrations of H^+ in the soil solution (low pH), dissolves metal hydroxides that tend to form at high pH values (Alloway, 1995). In this sense, as the availability of metal ions increases, the risk of trace metal ions uptake, *e.g.*, phytotoxicity, increases.

1.1.2.2 Organic Matter

Soil organisms and plant roots release a wide range of organic compounds that may contribute to increased or decreased soil nutrient availability by altering soil chemistry and soil biological processes. The presence of high concentrations of dissolved organic matter in soil can enhance the mobility and reduce the toxic effects of some metals in solution (Alloway, 1995; Gregory, 2006). However, many other soil properties (*e.g.*, aeration, type and water content) also govern the degree of mobilization (Gregory, 2006).

1.1.3 Assessment of Trace Metal Forms in Soil

Only bioavailable metals present in the soil solution are taken up by plants, where they can be potentially concentrated in the food chain (Cataldo and Wildung, 1978; Lasat, 2000; Wunan and Okeimen, 2011). As a result, bioavailability is a key consideration in the investigation of metal uptake by plants that can be assessed by the solubility of these elements in soil solution (Cataldo and Wildung, 1978; Basar, 2009), as they may create potential ecological risk to organisms within the contaminated environment and may even be hazardous to human health (Athar and Ahmad, 2001; Murray et al., 2011).

The forms of metals in soil can be determined by using sequential chemical extraction methods. A wide range of extractants can be used to define soluble and bioavailable metal fractions. Different solvents (extractants) that vary in their strength are used, depending on the nature of the metal under investigation. For example, solvents that contain oxygen (O) are better for extracting hard metals (species with high charge states) while solvents that contain phosphorus (P) or sulphur (S) are better solvents for extracting soft metals (species with low charge states) (Basare, 2009; Dube et al., 2000; and see sections 2.2.1 and 2.4.2).

1.2 Plant-Microbe-Metal Interactions

Many researchers are interested in understanding the dynamic system that exists below ground, in the absence or presence of plants that affects metal bioavailability, uptake and accumulation. Consequently, many researchers have concentrated on metals in the rhizosphere, which is known to have different properties as compared to the bulk soil

(Morel et al., 1986; Mench and Martin, 1991; April and Keller, 1999; Clemens et al., 2002; Naftel et al., 2006).

When plant roots grow into the soil, they create a rhizosphere. This concept was first introduced by Hiltner in 1904 as the portion of the soil attached to the root surface where microbial growth and activity are stimulated (Linderman, 1988; Morgan et al., 2005). However, this term has been modified to also take into account the bio-physico-chemical properties of the soil that adheres to the root (ectorhizosphere, which extends up to 2 mm away from the root surface) and the root tissues colonized by micro-organisms (the rhizoplane, which includes the root surface with the epidermis plus mucilaginous polysaccharide layers, and the endorhizosphere, which includes the endodermis plus the cortical layers) (Bertin et al., 2003; Morgan et al., 2005). In this thesis, the rhizosphere is defined as the volume of the soil influence by plant roots.

Vital physiological processes in the rhizosphere include microbial activities, which are enhanced by an extensive range of root exudates, as well as the uptake of mineral nutrients. The release of plant metabolites promotes the growth of microorganisms within the rhizosphere, where they, in turn, alter the geochemical environment through the release of their metabolic products. Using plate counts, the estimate of bacterial populations in a single gram dry weight of rhizosphere soil is 10^9 (Foster, 1986), compared to 10^8 in the bulk soil (Curl and Truelove, 1986). These microorganisms can also increase the solubilisation of essential nutrients and some symbiotic organisms can promote metal tolerance (Xu et al., 2008). This interaction among plant roots and microorganisms occurring in the rhizosphere system shifts in response to changes in soil properties (Kamnev and Lelie, 2000) such as biological (*e.g.*,

increased microbial populations and activities) and physiochemical (*e.g.*, decreased pH, enhanced nutrient availability and exudation of organic molecules) properties, hence drastically affecting the diameter of the rhizosphere, metal solubility and bioavailability as compared to the bulk soil (Hinsinger, 1998). The effects of changing environmental conditions on the activity of the microbial communities can be investigated using a wide range of phenotypic methods, *e.g.*, Biolog® plate technology. The idea of this technique, called community-level physiological profiling (CLPP), is based on spectrophotometric measurements of the utilization (oxidation) of different types of carbon sources by the microorganisms (Garland, 1997; and see section 2.5).

1.3 Plant Nutrients and Toxicity

Only 17 out of 90 common elements in soil ecosystems are required for the growth of plants (Schützendübel and Polle, 2002; Morgan et al., 2005). These elements are divided into two categories: (i) macronutrients (calcium, Ca; carbon, C; hydrogen, H; potassium, K; magnesium, Mg; nitrogen, N; O; P and S), which are needed in large quantities (between 1-4% dry weight of the plant) and (ii) micronutrients including trace metal ions (Cu; iron, Fe; manganese, Mn; Ni; Zn; boron, B; chlorine, Cl; and molybdenum, Mo), which are needed in small quantities (Bruins et al., 2000; He et al., 2005; Solymosi and Bertrand, 2011). However, the required amounts of metal ions vary depending on plant type, species, as well as the type of element. In addition, several biochemical reactions that occur at the cellular level require an adequate supply of trace metals for proper function. Such reactions include photosynthesis, as well as protein and nucleic acid metabolism (Bruins et al., 2000; Reichman, 2002; Nwuche and Ugoji,

2008; Solymosi and Bertrand, 2011). In addition to involving some essential metals such as Cu, Fe and Ni (these metals are known also as transition metals) in redox processes, plants can use elements to aid in maintaining the ionic balance of cells (Burins et al., 2000; Clemens, 2001; Nwuche and Ugoji, 2008). Other metal ions, such as As (either arsenite, AsO_3^{3-}, As^{3+} or arsenate, AsO_4^{3-}, As^{5+}), Cd, cobalt (Co), Cr, Hg and Pb, are non-essential or have no known physiological role (Schützendübel and Polle, 2002; Memon and Schroder, 2009).

1.3.1 Metal Uptake by Plants

The uptake of metals varies based on plant species as well as the nature of the element. Generally speaking, organisms are able to take up a small portion of metals that are present in the soil solution (Murray et al., 2009). There are three principle processes that control metal movement and uptake by organisms. In soil, for example, the mineral nutrients move via mass flow and simple diffusion towards the root surface. Only a small portion of the dissolved ions enter the root by crossing the cell membranes, which are highly selective (facilitated diffusion). Uptake of metal ions is likely controlled by secondary transporters such as transport proteins (also called carrier proteins) and ion channels (also called channel proteins), within the membranes. Specific ions combine with carrier proteins and are transported to the other side of the membrane. Channel proteins allow specific ions to pass across the membrane based on the size of hydrated ion and its charge. When metal ions enter the cell, a high concentration of ions can be found. In this case, active transport pumps protons (H^+) to maintain a balance between the concentration of solutes in and outside the cell (Hopkins and Hüner, 2008). Once

metal ions are transported to the xylem, they may then move to aboveground parts, where metals are stored either as hydrated ions or chelate complexes (Clemens et al., 2002).

1.3.2 Potential Risk of Trace Metals

In terms of toxicity, the concentrations of bioavailable trace metals in soil solution are crucial components that threaten the growth of plants and organisms (Reichman, 2002; Wang and Jia, 2010). Excessive concentrations of free metal ions in the soil, regardless of their necessity, can trigger various symptoms of toxicity (Giller et al., 1998). For example, metals aid in the production of hydroxyl radicals, which have the capacity to alter various biological molecules like proteins and lipids (Hall, 2002; Clemens, 2006; Solymosi and Bertrand, 2011). Also, trace metals pose the risk of displacing essential metals from their native binding sites or through complex compounds, by binding to sulphydryl, O and N atoms (Nies, 1999; Bruins et al., 2000; Hall, 2002; Schützendübel and Polle, 2002). Such alterations result in detrimental effects to the permeability of cellular membranes, inactivate enzymes, damage DNA and accelerate cell death (Briat and Lebrun, 1999; Bruins et al., 2000; Solymosi and Bertrand, 2011; Li et al., 2011). According to the Canadian Council of Ministers of the Environment (CCME) and He et al. (2005), the acceptable concentrations of different metals in soil differ among different type of soils and countries. Table 1 compares the agricultural soil quality guidelines (modified from CCME, 1997; CCME, 1999) and common range for some trace metals (modified from Lindsay, 1979).

Visible symptoms of toxicity vary among different organisms, individuals of a certain species, organs, metals and even doses (Reichman, 2002; Mohsenzdeh et al., 2011). The effects of trace metals, whether acting singly or in combination, on the

Table 1.1: Agricultural soil quality guidelines for the protection of environmental and human health (modified from CCME 1997; CCME 1999) and common ranges of some metals in soils (modified from Lindsay, 1979).

Metal	**Metal Abbreviations**	**Maximum Allowable Concentration in Agricultural Soil (mg kg^{-1} dry weight)**	**Common Range for Soils (mg kg^{-1})**
Arsenic	As	12	1-50
Cadmium	Cd	1.4	0.01- 0.70
Copper	Cu	63	2-100
Iron	Fe	-	7,000-550,000
Lead	Pb	70	2-200
Manganese	Mn	-	20-3,000
Zinc	Zn	200	1-300

growth of plants and microorganisms have long been of great interest to many researchers (Ellis et al., 2001; Athar and Ahmad, 2002; Nwuche and Ugoji, 2008; Wang and Jia, 2010). Symptoms of toxicity in plants are seen either at the whole plant level in reduced growth and productivity (Athar and Ahmad, 2002; Celmens, 2006; Wang and Jia, 2010) or at the organ level in root, leaf and fruit.

Generally, plants exposed to high metal doses reduce their root growth and exhibit leaf roll, chlorosis and necrosis (Sanita di Toppi and Gabbrielli, 1999; Reichman, 2002; Celmens, 2006; Mohsenzdeh et al., 2011). This is a consequence of the inhibition of mitotic activity in the meristematic root tip (Briat and Lebranne, 1999), water imbalance, impaired nutrition and reduced protein content within tissues (Sanita di Toppi and Gabbrielli, 1999; Ahmad and Athar, 2001; Clemens, 2006).

Soil microorganisms can also be adversely affected by elevated concentrations of metals (Gadd and Griffiths, 1978; Gadd, 2005; Gadd, 2010; Li et al., 2011), which can be a problem because different species of bacteria, actinomycetes, fungi, and algae are responsible, together or individually, for assimilation of organic residues, nitrogen fixation, nutrient transformation and soil aeration (Nwuche and Ugoji, 2008; Iram et al., 2009; Gadd, 2010). From a microbiological perspective, high concentrations of metals contribute to altered microbial communities and reduced microbial biomass, populations and activities, *e.g.*, soil respiration (Ellis et al., 2001; Ye et al., 2005; Nwuche and Ugoji, 2008; Li et al., 2011), each of which could negatively impact plant survival.

1.3.2.1 Arsenic (As)

Arsenic (As) is a gray or yellow metalloid belonging to period 4 and group 15 (VA) of the periodic table with atomic number 33, atomic mass 74.9 and density 5.72 g cm^{-3}

(http://www.webelements.com). It is naturally found in four oxidation states as arsine (As^{3-}), arsenic metal (As^{0}), As^{+3}, and As^{5+}. Arsenic metals are present in inorganic and organic forms as a mineral combined with other metals such as Cu, Pb, and Zn (Wunana and Okieimen, 2011). The more mobile and most often encountered forms of As in soils are (1) inorganic As^{3+}, which can adsorb with metal sulfates under high pH (ranging from 7- 10) and oxidizing conditions, and (2) As^{5+}, which can adsorb with (Fe) hydrous oxides or volatilized to arsine (AsH_3) under low pH (ranging from 4- 7) and reducing conditions (CCEM, 1997; Garg and Singla, 2011; Wunana and Okieimen, 2011). Frankenberger and Tabatabai (1981) reported that exposure of the soil to As^{3+} caused decline in enzyme (urease) activity. In plants, As^{5+} is known to inhibit the growth, reduce yield and may also lead eventually to plants' death (Garg and Singla, 2011).

1.3.2.2 Cadmium (Cd)

Cadmium is a soft and silvery gray metalloid, belonging to period 5 and group 12 (IIB) of the periodic table with atomic number , atomic mass and density of 44, 112.4 and 8.65 g cm^{-3}, respectively (http://www.webelements.com). In soil nature, Cd is present as a divalent cation (Cd^{+2}) (CCME, 1999; Wunana and Okieimen, 2011). Also cadmium can form complex ions (Alloway, 1995). Soil pH has a great effect on govern the availability of Cd in the soil. The solubility and uptake of Cd are increased at low pH (Wunana and Okieimen, 2011).

1.3.2.3 Copper (Cu)

Copper is a bronze metalloid that belongs to period 4 and group 11 (IB) of the periodic table. The atomic number, atomic mass and density of Cu are 29, 63.5 and 8.96 g cm^{-3}, respectively (http://www.webelements.com). In soil, because of the limited mobility of Cu as it strongly bound to mineral or co-precipitated by silicate and non- silicate clays , Cu presents as $Cu(H_2O)_6^{2+}$ ions in the soil solution. At low pH, Cu^{2+} is the most soluble and dominant ion in soil solution. However, various organic complexes and forms of Cu *e.g.*, Cu^{2+}, Cu^{+}, $CuSO_4^0$, $CuCl^0$ may exist in soil solution over a range in pH (Alloway, 1995).

1.3.2.4 Iron (Fe)

Iron is a bronze metalloid that belongs to same period of the periodic table with As and Cu, but a different group (8 [VIIIB]). It has an atomic number of 26, atomic mass of 55.84 and density of 7.87 g cm^{-3} (http://www.webelements.com). Iron in soil makes up 0.5 to 5% of the total Fe. In soil, most of the Fe is found in silicate mineral, oxides and hydroxide forms, which are not available to plants and organisms (Brimecombe et al., 2001). Ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron are also forms of Fe in soil solution which are mainly governed by the pH of the soil and organic matter. The solubility of Fe in soil solution decreases as the pH increases (pH ranging from 7.4-8.5). Organic compounds (*e.g.*, root and microorganisms exudates) also can reduce Fe^{3+} to Fe^{2+}, which is the more soluble form in soil solution (Schulte, 2004).

1.3.2.5 Lead (Pb)

Lead is a bluish-white lustrous metal in period 6, group 14 (XIV) with the following characteristic: atomic number 82, atomic mass 207.2 and density 11.4 g cm^{-3} (http://www.webelements.com). Lead is present as a mineral collective with other elements such as sulphur (lead sulfide, PbS; lead sulfate $PbSO_4^{2-}$) or oxygen (lead carbonates, $PbCO_3$). In soils, the most encountered soluble Pb forms are $PbSO_4^{2-}$, Pb^{2+}, which are the most stable forms of Pb, whereas the most insoluble forms are lead acetate compounds including basic salts such as lead phosphates $Pb_3(PO_4)_2$ and $PbCO_3$ (form at $pH \geq 6$), and strong oxidants such as lead oxides (PbO_2) and lead hydroxides ($Pb(OH)_2$. Because of the very low solubility of lead in soil, plants may take up only small quantities of it when it is present in excessive concentrations (Wuana and Okieimen, 2011).

1.3.2.6 Manganese (Mn)

Manganese is a hard silvery metal belonging to period 4, group 7 with atomic number 25, atomic mass 54.94, and density 1.51 g cm^{-3} (http://www.webelements.com). In soils, it commonly occurs as the oxide mineral forms with different redox states (ranging from +2 to +7) and solubility. Also, it is associated with other metals such as Fe. However, in plants, Mn is mostly taken up as Mn^{2+} by the roots. The uptake of Mn^{2+} increases in nutrient-rich (containing organic exudates from roots and microorganisms) and acidic soils (Alloway, 1995).

1.3.2.7 Zinc (Zn)

Zinc is a bluish pale grey metal located in period 4, group 12. It has an atomic number of 30, an atomic mass of 65.38 and a density of 7.14 g cm^{-3} (http://www.webelements.com). Zinc in nature exists either in insoluble compounds including oxides, carbonates, phosphates, and silicates, and soluble compounds including sulphates and chlorides (Alloway, 1995). However, Zn becomes highly available to microorganisms and plants at acidic pH (especially below pH 4) (CCME, 1999; Alloway, 1995).

1.4 Mechanisms of Metal Tolerance

Extensive reviews have summarized the responses of plants and microorganisms in soil to excess metal concentrations (Gadd and Griffiths, 1978; Giller et al., 1998; Briat and Lebrun, 1999; Sanita di Toppi and Gabbrielli, 1999; Clemens, 2000; Lasat, 2000; Hall, 2002; Lasat, 2002; Gadd, 2005; Clemens, 2006; Gadd, 2010; Gomathy and Sabarinathan, 2010). Plants and microorganisms have evolved several mechanisms capable of either altering the bioavailability of metals for uptake into their cells or maintaining the metal within subcellular compartments, keeping cytosolic metals below toxic levels. These are classified as extracellular and intracellular mechanisms.

1.4.1 Extracellular Tolerance Mechanisms

In plants, the principle mechanisms of tolerance have first been linked with extracellular defense systems, including mycorrhizae, root exudates and adsorption to the cell wall. Mycorrhizal fungi, which form symbiotic associations with plant roots, play important roles in stabilizing soils, enhancing nutrient uptake, particularly phosphorus (P), altering metal bioavailability and reducing root to shoot translocation (Linderman, 1988;

Schützendübel and Polle, 2002; Cicatelli et al., 2012). Schützendübel and Polle (2002) suggested that the tolerance to Cd stress of *Pinus* roots that were mycorrhizal with *Paxillus* could result from the elevated concentrations of secondary metabolites (*e.g.*, glutathione, ferulic acid and phenolics) that were reported in the mycorrhizal roots. However, the mechanism (s) behind the augmented tolerance of mycorrhizal plants to trace metals toxicity has not yet been explained (reviewed in Hall, 2002; Schützendübel and Polle, 2002; Cicatelli et al., 2012).

Plant roots release substances into the rhizosphere that can chelate and detoxify metal ions (Lasat, 2000; Hall, 2002). Such substances include low molecular weight amino acids, organic acids, phenolics and sugars, and high molecular weight mucilage and proteins (Lasat, 2000; Bais et al., 2006). Corn roots (*Zea mays*), for instance, exude an extensive range of substances, such as organic acids (*e.g.*, uronic, citric, acetic and malic acids) including amino acids (*e.g.*, aspartic acid), sugars (*e.g.*, glucose and polysaccharides) and proteins (Mench et al., 1987; Mench and Martin, 1991; Carvalhais et al, 2011).

Low molecular weight organic acids are important components of root exudates as they play a significant role in metal availability within the rhizosphere. Under a broad range of soil conditions, metals ions, which have a positive charge, are capable of strongly binding to organic acids, which have a negative charge. As a result, metal concentrations in the soil solution may increase in the presence of dissolved organic acids (Han et al., 2006) as in the case with acetic and malic acids influencing the elevated uptake of metals by maize (*Zea mays L. cv. TY2*) roots. Microorganisms, such as some yeast (Gadd and Griffiths, 1978) and bacteria (*e.g.*, *Azotobacter* spp.; Gomathy and

Sabarinathan, 2010) have also been shown to exude metal-binding chelators (e.g., siderophores) that are involved in metal homeostasis and detoxification. Siderophores can bind to the metal and increase its solubility (*e.g.*, Fe) under deficiency conditions and can form stable complexes with other metals, such as Al, Cd, Cu, Pb and Zn (Rajkumae et al., 2010).

The plant cell envelope is composed of the plasma membrane and cell wall. It occurs at the interface between a plant and its extracellular environment, and can bind metals and prevent or reduce metal uptake (Briat and Lebrun, 1999; Lasat, 2000; Hall, 2002). The positively charged metals in the external environment can adsorb to the negative charge on the outer surface of cell walls. For instance, Sanita di Toppi and Gabbrielli (1999) showed that Cd ions are mainly bound by hystidyl groups and pectin sites of the cell wall in the roots and leaves of bush beans. Moreover, the plasma membrane acts to moderate metal ions entering into the cells (cytosol) through either repair or efflux mechanisms (Hall, 2002).

1.4.2 Intracellular Tolerance Mechanisms

As soon as metals pass into the plant cytosol, several metal-binding proteins with varying complexity (*phytochelatins* and *metallothioneins*) may be synthesized in order to limit the free circulation of metal ions inside the cytosol (Briat and Lebrun, 1999; Sanita di Toppi and Gabbrielli, 1999; Clemens et al., 2002), and sequester metals in the vacuole, which impedes their translocation to other parts of the organism (Gadd and Griffiths, 1978; Briat and Lebrun, 1999; Hall, 2002). In this sense, tolerance to metal stress may be

attributable to several mechanisms rather than a single one (Hall, 2002; Memon and Schroder, 2009).

1.5 Rationale

The distribution of metals at the soil/root interface and into plant tissues has long been of great interest to environmental, plant and soil scientists who have used several techniques to answer questions as to where the metal is located in the plant and what forms of metal (speciation) exist in the rhizosphere, and whether the dynamic root/microbial system plays a role in metal behavior, specially, the solubility, mobility and bioavailability.

Recent research has attempted to further evaluate the uptake of metals by plants and examine the distributions and accumulation of these metals in the rhizosphere and plant tissues using scanning electron microscopy (SEM) (Martin et al., 2004), and synchrotron radiation analysis (SRA) (Lin et al., 2010; Naftel et al., 2006; Martin et al., 2006; Naftel et al., 2001). In these methods, high energy particles, *e.g.*, electrons (JEOL, 2009) or x-rays (Templeton and Knowles, 2009), interact with the metals, and produce specific signals that can be used to obtain images (SEM) or maps (SRA) of the distributions of elements. Also, SRA-XANES (x-ray absorption near edge structure) can provide information about the oxidation state and speciation of metals (Naftel et al., 2006). However, the results of these studies have been limited due to difficulties with separating the rhizosphere from the soil (McLaughlin et al., 2011) during sample preparation. In several studies (Tippkotter et al., 1986; Martin et al., 2004; Naftel et al., 2006), the roots were taken out of the soil, shaken to remove the loose soil, and then washed gently with water. These authors defined the rhizosphere as the soil particles that

remained firmly attached to the surface of the roots after such treatment. Not only would this procedure result in a loss of root-associated microbes, only a fraction of the original rhizosphere would remain with the sample since the rhizosphere is believed to extend up to 2 mm from the root surface (Bertin et al., 2003).

Preparation of roots or soil for SEM or SRA typically involves chemical fixation, dehydration using different techniques (*e.g.*, acetone, ethanol, critical point drying or freeze drying), and embedding in a solid resin and sectioning (Campbell and Rovira, 1973; Tippkotter et al., 1986; Camuti and McGuire, 1999; Naftel et al., 2001; Martin et al., 2004; Naftel et al., 2006). Even if the rhizosphere remained attached to roots pulled from the soil, the major problem with these techniques is the dehydration step, which can (1) cause plant cells, microorganisms, and the soil to shrink and distort as water is removed (Tippkotter et al., 1986) and (2) potentially leach soluble metals and wash microbes from the soil and rhizosphere.

1.6 Project Objectives and Hypotheses

The overall goal of this research project was to develop a method to investigate the interface between soil, microorganisms and plant roots in the rhizosphere. In this thesis, I studied corn (*Zea mays*) grown in two metal-contaminated garden soils. The need for studying these soils is magnified by their current use as vegetable gardens; the soils have been used for growing vegetables for at least 5 years and the plants look very healthy (Macfie, personal communication). Corn was chosen because of its rapid and abundant root growth, and its well-documented rhizosphere (Mench et al., 1987; Mench and Martin, 1991; Carvalhais et al, 2011). Therefore, the key objective of this research

project was to examine the "intact" rhizosphere. This was achieved by answering the following research questions:

I. What effect does the presence of corn (*Zea mays*) roots have on soil pH?

II. To what extent are the bacterial activities and functional diversity in metal contaminated soils affected by the presence of corn roots?

III. What are the distributions of trace metals in the bulk soil versus in the rhizosphere of corn?

My hypotheses include the following:

I. The pH of the rhizosphere will be lower than the pH of the bulk soil.

II. The activity and functional diversity of the bacterial community will be inversely proportional to the solubility of metals in soil.

III. Trace metals will be uniformly distributed in the bulk soil and lower concentrations will be found around the corn root.

CHAPTER 2

2. Materials and Experimental Procedures

2.1 Laboratory Model

Plant species and environmental factors can play a significant role in the formation of the rhizosphere. In this study, the factors including plant species, amount of soil, pot size, plant age, irrigation, temperature, humidity, and light were controlled in the system. Each treatment was performed in four replicates (n= 4) under controlled environmental conditions (See Table 2.1). The two garden soils that were used in this study are described in Section 2.2, and the experimental treatments in Section 2.3. In a preliminary experiment, I monitored the pH every 2 days and noticed a decrease in the pH after two weeks. After 33 days, corn leaves turned completely yellow. Therefore, selected chemical (Section 2.4) and bacteriological (Section 2.5) variables were measured on days 0, 15 and 30 of the experiment (n= 3). The final component involved developing a new technique for preserving intact soil cores (n= 1) (Section 2.6). Data analyses are described in section 2.7.

2.2 Soil Sampling and Initial Analyses

Two metal-contaminated garden soils (Figure 2.1) were selected based on soil type, organic matter content and metal concentrations. One soil was collected from the Blackfriars Community Garden in London, Ontario (42. 99 N 81.26 W). Organic matter made up 12.2 ± 1.3% of the soil and the texture of this soil has been determined as loamy

Table 2.1: Laboratory model. The experiment involved five experimental treatments for each of two different soils; soil on day 0 (n= 8), bulk soil collected on each of days 15 (n= 8) and 30 (n= 8), and rhizosphere soil collected on each of days 15 (n= 8) and 30 (n= 8). Three of the syringes from each bulk soil (n= 3) and rhizosphere soil (n= 3) treatments were used for measurement of pH, metal bioavailability and bacterial community (*e.g.*, activities and functional diversity); only one syringe from each treatment (n=1) was fixed and embedded for SEM and SRA analyses. Soils on day 0 (n= 6) were studied for metal content and bioavailability, and for bacterial community.

Sampling Sites	**Sampling Times (Days)**	**Treatments**	**Analyses for Each Treatment**
Blackfriars	0 15 30	Bulk soil (soil only) Rhizosphere soil (soil in which a seedling grew)	1- pH, ICP-AES, bacterial community analysis (n= 3) 2- Embedding into LR white resin (SEM and SRA) (n= 1)
Hamilton	0 15 30	Bulk soil (soil only) Rhizosphere soil (soil in which a seedling grew)	1- pH, ICP-AES, bacterial community analysis (n= 3) 2- Embedding into LR white resin (SEM and SRA) (n= 1)

Figure 2.1: Soils were collected from Blackfriars community garden in London and a private vegetable garden in Hamilton. Locations of London and Hamilton are shown in the inset (modified from https://www.presentationmall.com and http://www.weldingschoolsofontario.com).

sand (Murray et al., 2011). The other soil sample was collected from a private garden in Hamilton, Ontario (approximately 43.25 N 79.88 W). The Hamilton soil possessed 7.4 ± 0.4% organic matter and the texture of this soil has been classified as sandy loam (Pinchin et al., unpublished data).

The two garden soils were sampled from both sites in September, 2011. Samples were taken from a depth of 5 to 15 cm after removing the surface vegetation. The fresh, moist soils were sieved separately through a 4 mm mesh sieve, homogenized using a hand trowel, then stored in a covered plastic box at 4°C. Prior to use, the pH of the soils was determined to be 8.02 ± 0.01 and 7.11± 0.01 for Blackfriars and Hamilton soils respectively, using a 1:1 soil: deionized water ratio.

2.2.1 Metal content

The total metal content of the soils was determined using the United States Environmental Protection Agency (US EPA) test method SW-846 (US EPA 2005). The metals were extracted by digesting 1 g of dried ground soil at time zero in 1 mL of pure nitric acid (HNO_3; Omni-Trace®, EM Science) overnight. Digesting in HNO_3 does not extract the fraction of elements that are strongly bound in the matrix of silicates (Carmen et al., 1932). After overnight acid digestion, samples were heated to 100°C (until the vapors were clear). Once the digested samples cooled down, they were filtered using a 9 cm filter paper (VWR International, Grade 413 filter paper). Then, the solutions were diluted to a volume of 50 mL with deionized water and were analysed for As, Cd, Cu, Fe, Pb, Mn and Zn using Inductively Coupled Plasma-Atomic Emission Spectroscopy at the Biotron at the University of Western Ontario (ICP-AES; Perkin Elmer Optima 3300 Dual

View ICP-AES). The detection limits were: As 0.1 μg mL^{-1}, Cd <0.001 μg mL^{-1}, Cu 0.01 μg mL^{-1}, Fe 0.01 μg mL^{-1}, Mn 0.02 μg mL^{-1}, Pb <0.001 μg mL^{-1} and Zn 0.02 μg mL^{-1}. Controls of distilled water added to HNO_3 (blank), and Montana Soil (Standard Reference Material 2711, National Institute of Standards and Technology, Gaithersburg, USA) were treated the same. The blank value of HNO_3 (brought up to 50 mL using distilled water) was negligible for all elements under consideration, which indicated the high purity of the reagent used. Duplicate samples were within 0 to 0.19% of each other, indicating good reproducibility of the technique.

2.2.2 Bacteria in the Soils

The initial bacterial communities in the garden soils were studied within three days of soil collection. The bacterial community in each soil was analyzed using the spread plate method on Nutrient Agar (Nutrient Agar; Difco Laboratories, Detroit, USA) for the growth of bacteria. For soil dilutions and plate counts, 1 g of each of the fresh soils was placed into 10 mL of 0.85% (w/v) sodium chloride (NaCl) in 16 x 150 mm test tubes possessing push caps, vortexed for 1 min and left to settle. Sequential aliquots (1 mL) were transferred through a ten-fold dilution series (up to 10^{-7}). Then, a 0.1 mL aliquot of each dilution was spread onto duplicate Nutrient Agar plates (Petri dishes; Greiner Bio-One, Monroe, USA) using a sterilized plastic spreader. The plates were incubated in an inverted position at room temperature for 48 hours. After incubation, standard plate count methods (using a colony counter (New Brunswick Scientific, USA)) were carried out to count the total number of bacteria (as number of colony forming units "CFUs" per

gram of soil) and diversity ($D= - \Sigma pi \ln pi$) where pi is the proportion of each colony type on the plate, using colony morphology to distinguish "types".

2.3 Germination and Growth Conditions

Corn (*Zea mays*) seeds (Pioneer 34 R06, ≥ 10 years old, stored at 4ºC) were germinated in the dark on moist filter paper placed in a Petri dish for 36 hours. Then, the seeds were transferred to 60 mL syringes (Becton Dickinson, Franklin Lakes, USA) containing ~90 g of soil. The syringes (bulk and rhizosphere soils) were wrapped with foil to provide a dark environment for the roots and rhizosphere. Syringes were placed in a controlled environment chamber set at 60% humidity, 119 ± 20 µmol m^{-2} s^{-1} photon light intensity and 16/8 hours day/night cycle at 21ºC (Figure 2.2 A). The samples were watered daily with 5 mL of tap water (pH= 7.93 ± 0.3). Samples were harvested on days 15 and 30, and the following chemical (Section 2.4) and microbial (Section 2.5) analyses were performed.

2.4 Chemical Analyses of the Soils

2.4.1 pH

The pH of 1 g from both the bulk and rhizosphere soil samples at each time point suspended in 1 ml deionized water was measured using a glass electrode (Denver Instrument Company, USA).

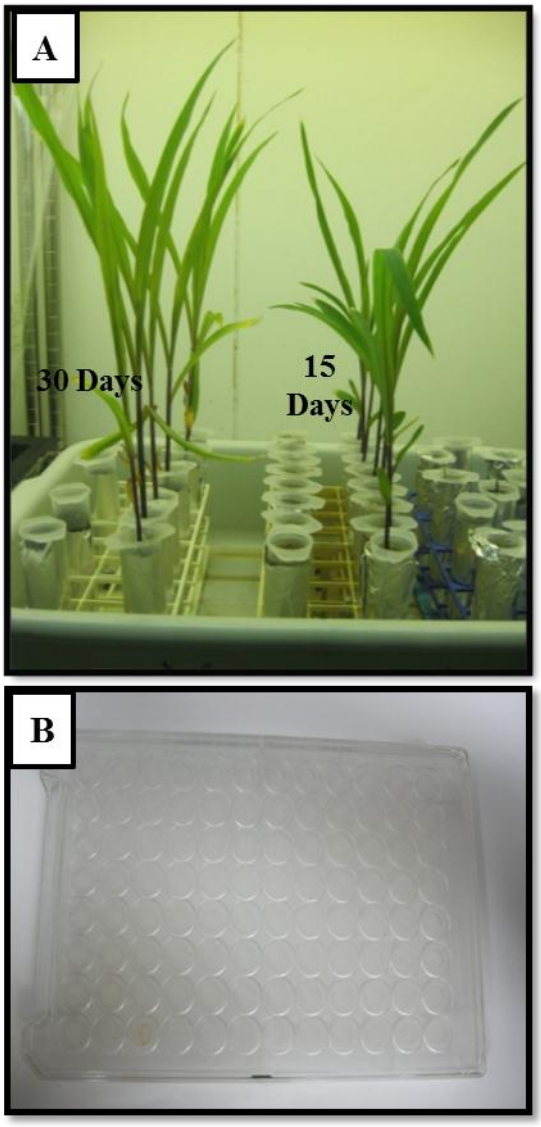


Figure 2.2: (A) Soil and plant samples grown in a growth chamber; (B) Biolog Ecoplate™.

2.4.2 Bioavailable Metal

For each treatment, 10 g of dried, ground soil was placed in 50 mL Falcon tubes with the addition of 20 mL of 0.01 M calcium chloride ($CaCl_2$) (pH= 6.99± 0.03). The tubes were then capped tightly and shaken at 250 rpm for 20 min. After extraction, the supernatants were filtered into clean test tubes through a 9 cm filter paper (VWR International, Grade 413 filter paper), and re-filtered under a gentle vacuum using a 0.45 µm membrane filter (Gelman Sciences, Ann Arbor, Michigan, USA) The extracts were then acidified with 0.2 mL of ultrapure nitric acid and diluted to a volume of 10 mL with deionized water. Quality control was assured through the analysis of duplicate samples (0- 0.19%) and blanks. The bioavailable fractions of As, Cd, Cu, Fe, Pb, Mn and Zn in the prepared solutions were quantified using ICP-AES as described in section 2.2.1.

2.5 Soil bacterial Biodiversity

The Biolog EcoPlate^{TM} system (Biolog Inc., Hayward, California, USA) was employed to characterize the physiological diversity of the bacterial community in the bulk and rhizosphere soil samples (Figure 2.2 B). In this technique, 96 well microtiter plates, each possessing 3 sets of 31 carbon sources (Table 2.1) which are considered to be root exudates (Shengnan et al., 2011), and a non-carbon (water) reference well, were inoculated with soil bacteria. Each well also contained tetrazolium dye. The community level physiological profiling (CLPP) was based on a colour change within each well, which was caused by reducing the colourless of the tetrazolium dye and oxidation of a carbon source as the bacteria utilized the carbon source within that well (Garland, 1999 and Preston-Mafham et al., 2002). The metabolic fingerprint of the microbial

Table 2.1: Sole carbon sources in the Biolog EcoPlate™ (modified from Garland and Mills, 1991 and Preston-Mafham et al., 2002).

Carbon Sources and Chemical Formula			
Amino Acids and Amines		**Carboxylic Acids**	
Glycyl-L- Glutamic Acid	$C_7H_{12}N_2O_5$	**α**- Keto Butyric Acid	$C_4H_6O_3$
L- Arginine	$C_6H_{14}N_4O_2$	D- Galactonic Acid γ-	$C_6H_{10}O_6$
L- Asparagine	$C_4H_8N_2O_3$	Lactone	
L- Phenylalanine	$C_9H_{11}NO_2$	D- Galacturonic Acid	$C_6H_{10}O_7$
L- Serine	$C_3H_7NO_3$	D- Glucosaminic Acid	$C_6H_{13}NO_6$
L- Threonine	$C_4H_9NO_3$	D- Malic Acid	$C_4H_6O_5$
Phenylethylamine	$C_8H_{11}N$	2- Hydroxy Benzoic Acid	$C_7H_6O_3$
Putrescine	$C_4H_{12}N_2$	4- Hydroxy Benzoic Acid	$C_7H_6O_3$
		γ- Hydroxy Butyric Acid	$C_4H_8O_3$
Carbohydrates		Itaconic Acid	$C_5H_6O_4$
α- D- Lactose	$C_{12}H_{22}O_{11}$		
β- Methyl-D- Glucoside	$C_7H_{14}O_6$	**Miscellaneous**	
D- Cellobiose	$C_{12}H_{22}O_{11}$	D,L- α- Glycerol Phosphate	$C_3H_9O_6P$
D- Mannitol	$C_6H_{14}O_6$	Glucose-1- Phosphate	$C_6H_{13}O_9P$
D- Xylose	$C_5H_{10}O_5$	Pyruvic Acid Methyl Ester	$C_4H_6O_3$
i- Erythritol	$C_4H_{10}O_4$		
N- Acetyl-D- Glucosamine	$C_8H_{15}NO_6$	**Polymeric Compounds**	
		α- Cyclodextrin	$C_{36}H_{60}O_{30}$
		Glycogen	$(C_6H_{10}O_5)_n$
		Tween 40	
		Tween 80	

communities can thus be measured spectrophotometrically at a wavelength (λ) of 590 nm (Preston-Mafham et al., 2002) because the intensity of the colour change is proportional to the utilization of the carbon source. The optical density of each well, also known as the absorbance value, was determined using a microtitre plate reader.

Cell suspensions were obtained from each sample by adding 1 g of soil (bulk or/and rhizosphere) to 1 mL of sterile saline solution (0.85% (w/v) NaCl) and vortexing for 2 min to help separate microorganisms from soil or/and root surfaces. The samples were then left for a few minutes to allow the large particles to settle out. Aliquots of 400 µL of supernatant were diluted in 19.6 mL of saline. Then, 150 µL of the cell suspension was inoculated into each well of the Biolog EcoPlate^{TM} using a pipette. To ensure that the aliquots were representative of the bacterial community, duplicate subsamples from each suspension were analysed. All plates were placed in polyethylene bags to reduce desiccation, and then incubated in the dark for seven days at room temperature. All solutions, transfer equipment, and glassware were sterilized with an autoclave at 101.3 kilopascals (kPa) and 121°C for 20 minutes prior to use. All work during plate preparation was done in a laminar flow hood (1300 Series A2, Thermo Fisher Scientific, USA) to minimize the risk of contamination.

The median absorbance value of each set of duplicate wells was calculated to obtain a single value for each replicate. Prior to calculating the mean value for each experimental treatment, the absorbance values from the control wells were subtracted from the absorbance values for the corresponding wells containing the 31 carbon sources (Eq 1).

$$\text{Absorbance value} = W - C \quad (1)$$

where W is the absorbance value of one well and C is the mean value of the control well.

Absorbance values in wells that were negative or below 0.06 were set to zero following Shengnan et al. (2011). Average absorbance values were then calculated for each carbon source.

The functional diversity of the soil bacteria from each treatment was expressed as substrate richness (R), which is the number of oxidized carbon substrates among the 31 carbon sources. Bacterial activity (*e.g.*, the extent to which the carbon sources were utilized) was assessed by calculating the average well colour development (AWCD) and was determined by dividing the sum of total absorbance values for the 31 carbon sources over the 31 carbon sources on the Ecoplate™ using the following formula (Shengnan et al., 2011):

$$\text{AWCD} = \sum(W - C)/R \quad (2)$$

Where $\sum(W - C)$ is the sum of 31 absorbance values and R is substrate richness.

The extent to which a bacterial community utilized each individual carbon source relative to its utilization of all carbon sources is the proportional colour development, which was calculated using the following formula (Garland 1999):

$$pi = (W - C) / \sum(W - C) \quad (3)$$

where (W- C) is the absorbance value in each well over the sum of total absorbance values.

The sum of all of the individual well pi values from each replicate equaled one. The functional diversity of each bacterial community was quantified using the mean

Shannon Diversity Index (H') and Evenness (E). The Shannon Diversity Index takes into account both the richness (reveals the number of different substrates used by the community) and evenness (varies from close to 0 to 1, the latter representing a perfectly even community; measures the homogeneity of bacterial community activities using particular carbon sources). These are common measures to evaluate the CLPP of environmental samples (Garland, 1997). Indices were calculated as follows:

$$H' = -\Sigma\, p_i \ln p_i \qquad 4$$

where pi is the proportional colour development of the substrate i over total colour development for all substrates.

$$E = H'/\ln C \qquad 5$$

where H' is the Shannon Diversity Index and C is the number of the carbon source on the Eco-plate (C= 31).

2.6 Investigating the Intact Rhizosphere

A new scientific approach for sample preparation that minimizes 'degradation' of the rhizosphere was required prior to investigating the chemistry of metals in the rhizosphere using various microscopic techniques.

2.6.1 Embedding Procedure

I modified the method used by Tippkötter et al. (1986) for fixing, dehydrating and embedding samples. In their method, the root with its intact soil was fixed for 2-3 h in 0.1 M Sörensen phosphate-buffered saline with 2.5% (v/v) glutaraldehyde, then dehydrated rapidly in a graded series of acetone (50%, 70% then double changes of 100

%), then triplicate changes of pure acetone at low pressure (-20 kPa) and the soil was impregnated with Crystic 17449 resin (Tippkötter et al., 1986). I modified this method to reduce the potential disruption of the rhizosphere by (i) eliminating phosphate because it will precipitate with divalent and trivalent cations, (ii) using a low concentration of glutaraldehyde, (iii) reducing the dehydration steps using a less severe chemical, ethanol, and finally (iv) doing all the impregnation steps in the 60 mL syringes in which the plants were grown. A burette was used to drop all the liquids through the column (Figure 2.2).

In this project, soil and rhizosphere cores were fixed with 20 mL of 0.1% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA. 19440), and incubated at room temperature overnight. In a preliminary experiment, different concentrations of glutraldehyde (0.1 -3.5%) were used to identify the optimal concentration for preserving the biological material (corn roots); since there was no difference among all concentrations that were used in term of preserving the root cells structure, the lowest concentration was selected for use. Samples were dehydrated with 30 mL of 100% ethanol (Ethanol; Green Field Ethanol Inc, Brampton, ON, CA) three times for approximately two hours each. Following dehydration, 30 mL of a 1:1 mixture of LR White resin (Electron Microscopy Sciences, Hatfield, PA. 19440) and 100% ethanol was added drop wise onto the top of the soil column with 2 hr incubation (twice) before adding 30 mL of pure resin drop wise onto the column. The samples were cured at 60ºC for 24 hours. Then samples were mounted on microscope slides as polished cross sections approximately 30 µm thick (Figure 2.3).

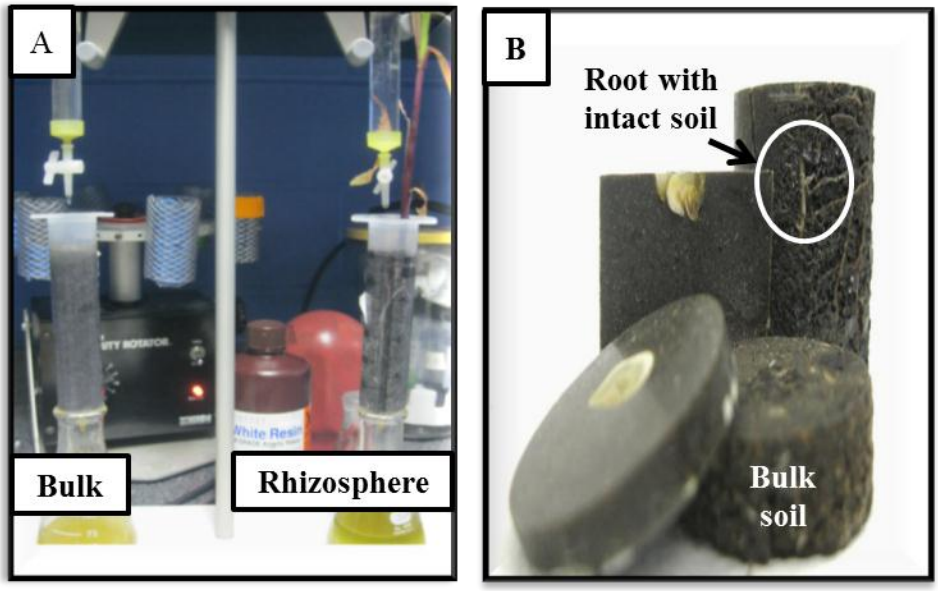


Figure 2.3: (A) Impregnation steps; (B) Bulk and rhizosphere soils in plastic.

One of the duplicate soil columns was leached with distilled water under the same flow conditions as in the impregnation steps and examined for the presence of metals by ICP-AES and microorganisms by filtering the leachate through a 0.45 μm membrane filter to determine whether any water soluble metals were present in the soil extract and if the microbes (*e.g.*, fungal hyphae) were washed out.

2.6.2 Imaging and Analysis Techniques

Polished, embedded soil samples were examined using a light microscope combined with a digital camera (Nikon Eclipse LV100POL, Japan) to enhance the contrast of the soil and the root cells for photography. Methods used to characterize the chemistry of bulk and rhizosphere soils included (i) scanning electron microscopy (SEM) combined with an energy dispersive x-ray spectrometer (EDAX) and a wave length dispersive x-ray spectrometer (WDAX), and (ii) x-ray fluorescence spectroscopy (XRF).

2.6.2.1 Scanning Electron Microscopy (SEM)

Roots and metals were targeted using secondary electron (SEM-SE) and Backscattered Electron (SEM-BSE) imaging, respectively. Elemental composition and distribution were determined quantitatively using an Oxford Instruments' INCAx-sight EDAX and WDAX operating at a 15 kV accelerating voltage. EDAX point analyses of bright BSE phases, <1mm away from the roots were taken at a low (200-500 X) magnification. When a phase contained As, Cd, Cu, Fe, Pb, Mn and Zn, a zoomed-in BSE image was taken, and EDAX point analyses were taken at the higher magnification. A WDAX map was taken of a random area in the control soil samples and an area around a root in the

rhizosphere samples. Each WDAX map was done based on EDAX at a very low magnification (180X). Standards were used to calibrate the peak location for accurate mapping. Thin sections were sputter coated with a thin (5 nm) conductive coating of osmium using an osmium plasma coater (Filgen; SPI) prior to SEM analysis in order to reduce charging effects.

2.6.2.2 X-ray Fluorescence Spectroscopy (XRF)

Based on the data obtained from the SEM-EDAX analysis, thin sections were selected for synchrotron radiation analysis (SRA). X-ray fluorescence spectroscopy (XRF) was used to determine the localization of each metal in the control soil (on day 0) and the rhizosphere soil (on day 30) from the Blackfriars and Hamilton soils. The analyses were conducted at beamline 20-ID-B at the Advanced Photon Source, Argonne National Laboratory Illinois, USA. The Si (111) monochromator was calibrated to 10000 or 8000 eV for XRF mapping. The thin sections were horizontally and vertically mapped using 4 micron steps with a counting time of 0.5 seconds per step to obtain detailed maps of metal (Cu, Fe, Mn, and Zn) distributions.

2.7 Data Analysis

2.7.1 Soils and Microbial Analyses

A *t*- test was performed for the evaluation of the means of the initial bacterial counts within the two soils. Three-way factorial analyses of variance (ANOVAs), as three factors in the experiment were time (0, 15 and 30 days), sample site (Blackfriars and Hamilton) and treatment (soil only, rhizosphere soil), with a further Tukey test were

performed for the evaluation of the means between (i) the two treatments within time, (ii) each treatment separately among all time points, and (iii) sample sites within time, using SigmaPlot version 12 software. Normality and homogeneity of variance were assessed prior to data testing to satisfy the assumptions of normality and variance homogeneity. Significance among means of the three replicates was calculated on the basis of homogeneous groups at the 95 percent level when P values were less than or equal to 0.05. Graphical work was made using SigmaPlot for Windows version 12 software packages. Data (from sections 2.2, 2.4 and 2.5) are means and standard error (SE) of the three replicates.

2.7.2 XAS Data Analysis

Elemental composition and spectra for the corresponding areas of interest were determined using EDAX software INCA Oxford Instruments' operating at a 15 kV accelerating voltage. XRF data was processed using 2D ScanPlot (LabView, version 7.1; National Instruments, 2004).

CHAPTER 3

3. Results and Discussion

3.1 Initial Metal and Microbial Content of the Soils

Acid digestion followed by ICP-AES indicated that both soil sites contained some metal concentrations above the Canadian Soil Quality Guidelines (Figure 3.1). In both soils, concentrations of Cd, Pb and Zn were over the maximum acceptable limits, while Cu was below the maximum acceptable limits for agricultural soil as established by the Canadian Council of Ministers of the Environment (CCME) (1999). Arsenic was below the detection limits, while Fe was outside of the instrument's calibration range of 50 μg/mL (even when it was diluted three times). In general, Hamilton soil contained higher concentrations of all trace metals than Blackfriars soil.

Bacteria are some of the smallest and most abundant microbes in the soil. Based on plate count estimates, there can be more than 100 million (10^8) viable bacterial cells in a single gram (dry weight) of healthy soil (Curl and Truelove, 1986). Changes in population numbers, activities and diversity (*e.g.*, species and function) of soil organisms can be used as bioindicators of a change in the environment. The Shannon Diversity Index of the bacterial community (based on the morphology of the colonies) did not differ between the Blackfriars soil (0.43 ± 0.08) and the Hamilton soil (0.66 ± 0.02), $P > 0.05$. However, it is apparent from the present experiment that, overall, the bacterial counts were slightly higher within the Blackfriars soil (8.5×10^5 CFU/g) as compared to the Hamilton soil (3.9×10^5 CFU/g).

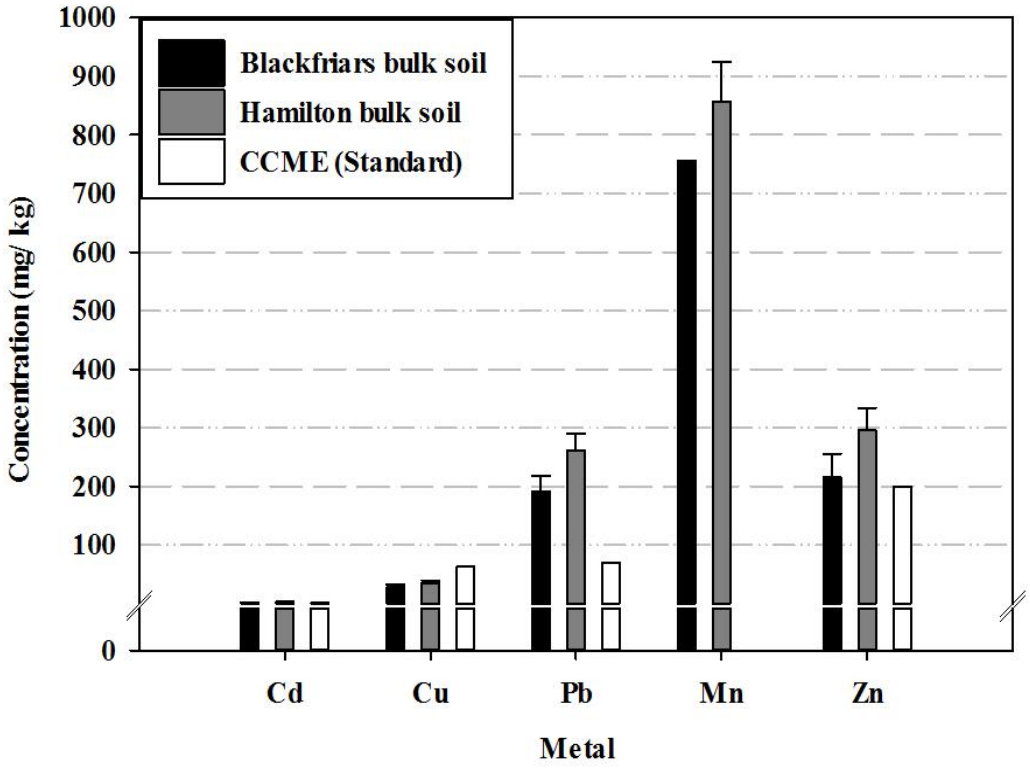


Figure 3.1: Concentrations of Cd, Cu, Pb, Mn and Zn in Blackfriars and Hamilton soils on day 0 (n= 6). The maximum acceptable values for agricultural soil, as established by the Canadian Council of Ministers of the Environment (CCME 1999) are shown for comparison; the CCME has not set a maximum acceptable limit for Mn. Error bars are SE of three replicates.

The bacterial population in two metal contaminated soils were three orders of magnitude below those expected in a healthy soil (based on values from Curl and Truelove, 1986) and this is probably due to (1) the method used to isolate bacteria from the soils (2) metals may have a harmful effect on the natural bacterial population (Kamnev and Lelie, 2000), or (3) fewer nutrients were available for the organisms. Other studies have reported similar results for different soil microorganisms including: Al-Gaidi (2010) who found that soil contaminated with different concentrations of Cd (1.5, 3 and 6 mg kg^{-1}) substantially deceased the total bacterial counts (plated out on Nutrient agar medium) relative to soil contaminated with different concentrations of Pb (40, 80 and 160 mg kg^{-1}); Bisessar (1982) who found decreased numbers of microorganisms (bacteria plated out on 2% malt extract agar medium, actinomyces plated out on water agar medium and fungi plated out on malt extract with bacteriostatic rose bengal agar medium) in soil contaminated with 57 mg kg-1 As, 5 mg kg-1 Cd, 73 mg kg-1 Cu and 703 mg kg-1 Pb and Hiroki (1992) who found that the number of bacteria and actinomycetes (bacteria and actinomycetes plated out on an albumin agar medium and the fungi plated out on a Martin's rose bengal agar medium) strongly decreased in soil contaminated with low concentration of trace metals ranging from 1.1- 2.7, 310- 751 and 234-571 mg kg^{-1} soil for Cd, Cu and Zn, respectively.

3.2 Growth and Chemical Analyses of the Soil and Rhizosphere

Leaves of corn plants started to turn yellow at 21 days (See Figure 2.2 B in Chapter 2), which may indicates that metals were very mobile or the soil provided inadequate nutrition.

The initial pH of the soils measured at time 0 days were alkaline in Blackfriars (8.02± 0.01) and circum-neutral (7.11± 0.01) in Hamilton soil. In both bulk and rhizosphere Hamilton soil, the pH increased after 15 days, while the rhizosphere of Blackfriars soil was unchanged (Table 3.1). After 30 days of treatment, the pH of all bulk and rhizosphere soils returned to initial values (Table 3.1). When comparing the means of treatments (bulk and rhizosphere) among the three time points within both sites (Blackfriars and Hamilton), the pH of Hamilton soil was significantly higher than the pH of Blackfriars ($p < 0.001$), and a significant interaction between treatment within each soil site and times was seen ($P < 0.001$). This may imply that watering the samples with tap water of pH 7.93± 0.3 may have kept the pH high despite the plant's exudates due to the dissolution of carbonates in these soils (*e.g.*, calcium carbonate, $CaCO_3$). Also, the decrease in the pH after 30 days in the presence of corn (*Zea mays*) roots could be attributed to the root exudates.

This research clearly demonstrates and confirms previous reports that changes in pH can occur within bulk (control) soil as well as in the rhizosphere. These changes likely arise due to exudates from soil microorganisms or/and plant roots. Li and Wong (2010) suggested that the decrease in pH resulting from the inoculation of bacteria in bulk soil could be attributed to the secretion of soluble low molecular weight organic compounds and protons through bacterial metabolic activities. The explanation for a decrease of pH in the rhizosphere is that this is due to interactions between substrate supply (substrates released by the corn roots) and soil microbial dynamics within the rhizosphere. This is in agreement with Greger and Landberg (2008) who reported that the pH of bulk soil was always higher than the pH of the rhizosphere of 24 different

Table 3.1: pH values over 30 days of the experiment. Small and capital letters refer to the significant difference ($P < 0.05$) between treatments (Bulk and Rhizosphere) within Blackfriars and Hamilton soils respectively. Error terms are SE of three replicates.

Time/days	**Bulk and Rhizosphere Soils pH**			
	Black friars		**Hamilton**	
	Bulk	**Rhizosphere**	**Bulk**	**Rhizosphere**
0	8.02 ± 0.01 a		7.11 ± 0.01 A	
15	8.15 ± 0.02 b	8.02 ± 0.09 a	7.57 ± 0.02 B	7.48 ± 0.04 B
30	8.02 ± 0.09 a	7.99 ± 0.03 a	7.4 ± 0.01 B	7.16 ± 0.05 A

cultivars of *Triticum aestivum*, *Triticum durum*, and *Triticum spelta*. Cervantes et al. (2011) revealed similar results indicating a lower pH in the rhizospheres of *Sarcocornia fruticosa* and *Phragmites australis* than the pH of the bulk soils during 6 months of their experiment.

Estimates of concentrations of some bioavailable metals (*e.g.*, Cd, Cu, Fe, Mn, Pb and Zn) varied among soil sites and increased with time in both bulk and rhizosphere soils (Figure 3.2). In addition, higher values of bioavailable Cu and Fe were found in the rhizosphere of Blackfriars soil as compared to the corresponding bulk soils, whereas bioavailable concentrations of metals except Pb in Hamilton soil were unaffected by the presence of the plant (Figure 3.2). Low concentrations of bioavailable Pb in Hamilton soils were expected due to Pb's very low bioavailability (Jung, 2008) and the high pH of that soil. Concentrations of bioavailable As were below the detection limit (0.03 μg/mL) in all cases.

Three way ANOVA showed that Hamilton soil had higher concentrations of bioavailable Cd, Pb and Zn compared to Blackfriars soil among sampling times ($P<0.001$). In contrast, Blackfriars had higher concentrations of bioavailable Fe ($P= 0.015$) and Mn ($P= 0.001$). However, no significant difference was seen in the case of Cu. All of the values for bioavailable concentration were very low, suggesting that the plants were unlikely to accumulate high concentrations of any metal if metal solubility was solely based on abiotic processes. Given the neutral (Hamilton) to slightly alkaline (Blackfriars) pH of the soils, low solubility of the metals was expected.

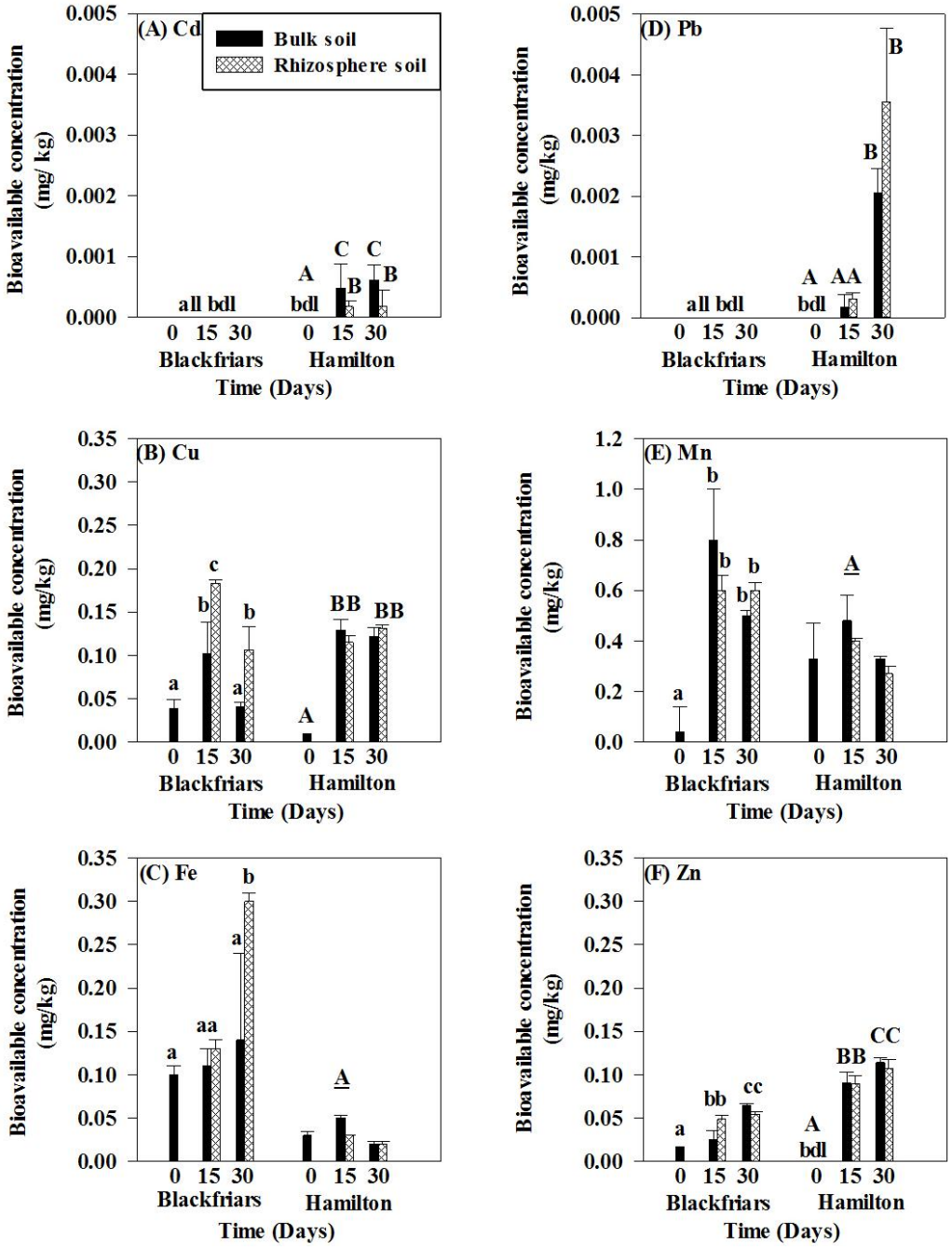


Figure 3.2: Estimated bioavailable concentrations of metals in Blackfriars and Hamilton control and rhizosphere soils at 0, 15 and 30 days. bdl denotes that the metal concentration was below the detection limit for ICP-AES. Small and capital letters refer to the significant difference ($P < 0.05$) between treatments (Bulk and rhizosphere) within Blackfriars and Hamilton soils respectively. Error terms are SE of three replicates.

The estimation of bioavailable metals in soil is needed in order to estimate the potential for metal uptake by plant roots and avoid the risk of toxicity. The highly mobile elements (*e.g.*, Cu, Fe, Mn and Zn) increased in the soil solutions of each treatment (both bulk and rhizosphere soils) with increases in time, presumably due to the slight decrease in pH. In general, metal bioavailability uptake by plants is increased by a decrease in pH (Reichman, 2002; Yen-de et al., 2007; Pietri and Brookes, 2008; Murray et al., 2011; Cervantes et al., 2011), which may facilitate the release of metal cations from the soil matrix.

3.3 Carbon Source Utilization

Functional diversity of the bacteria, which includes substrate richness (R), a measure of the number of different carbon sources utilized, and extent of substrate metabolism were compared between bulk and rhizosphere soils (Figure 3.3). Consumption of some of the substrates on the Ecoplate started in the first 2 days of incubation, and consumption of other substrates increased over the 7 day incubation (data not shown). Substrate richness was at or near the maximum value of 30 in bulk and rhizosphere soils across all sampling times, except for the rhizosphere of Blackfriars soil, which was reduced by nearly half at the end of the 30 day experiment (Figure 3.3A).

Qualitatively, soil bacterial community activity, as judged by the colour in the Ecoplate wells, continued to increase over the 7 day incubation for both soil sites and types (personal observation). This result could be consistent with previous studies conducted by Ellis et al. (2001) who found that each of AWCD, maximum absorbance

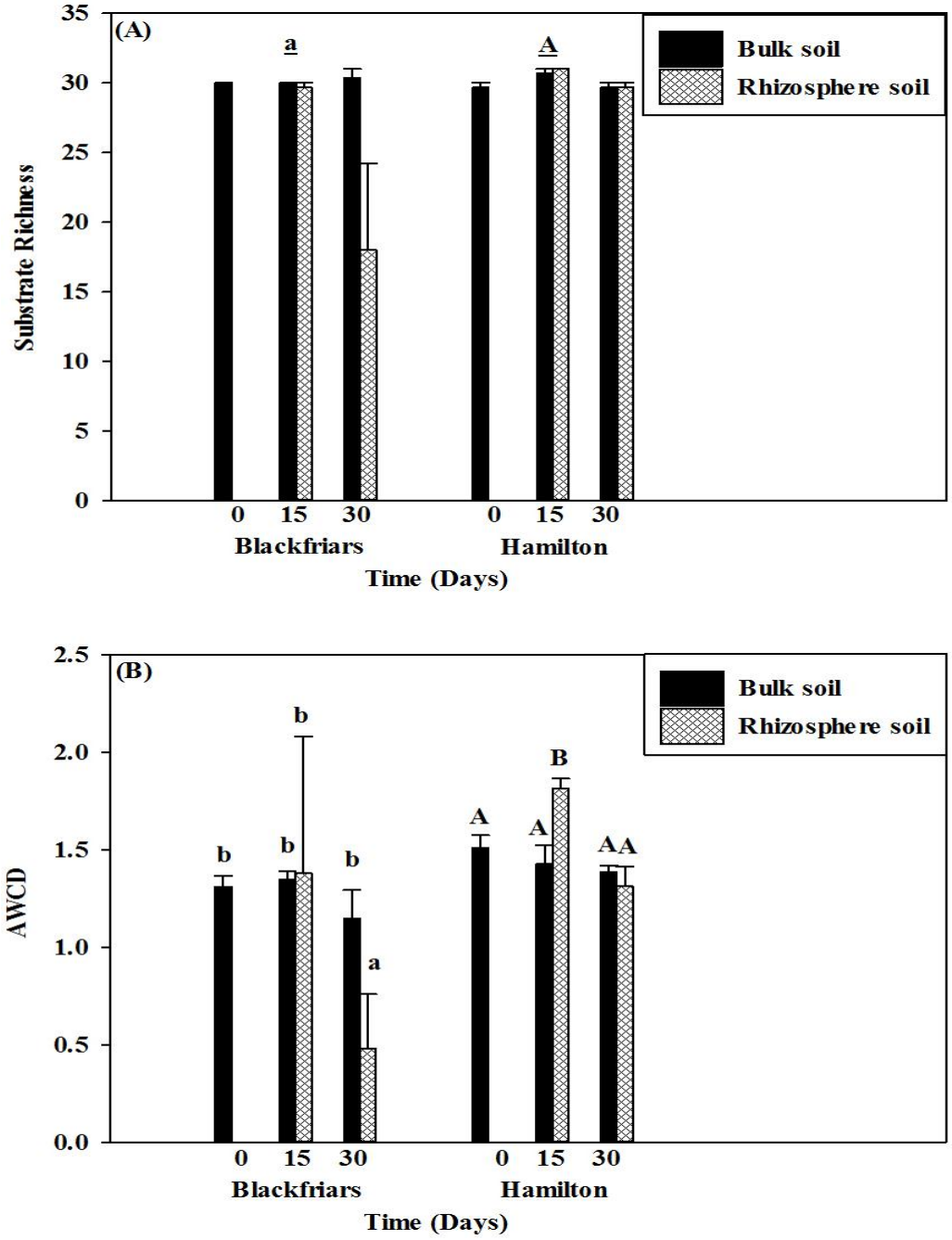


Figure 3.3: Mean substrate utilization patterns for Blackfriars and Hamilton soils among days of the treatment. (A) Number of carbon sources utilized by each community (richness); (B) Average Well Colour Development (AWCD). Small and capital letters refer to the significant difference ($P < 0.05$) between treatments within Blackfriars and Hamilton soils, respectively. Error terms are SE of three replicates.

values as well as number of substrates on the Biolog GN plate, which contains 95 carbon sources suitable for identification of gram negative bacteria (Stefanowicz, 2006), increased over 6 days of incubation; and Shengnan et al. (2011) who did a number of measurements of AWCD on Ecoplates over 10 days of incubation and found that bacterial community activity in soil continued to increase during the whole incubation. No growth of fungal mycelium was observed in wells of the plates (personal observation).

In this study, AWCD was measured on the 7^{th} day of incubation and there was no difference in AWCD representing bulk soils from the different sampling times, with equal values for Blackfriars and Hamilton soils (Figure 3.3B). However, a decrease in AWCD for the bacterial community inhabiting the rhizosphere of Blackfriars soil and a spike in AWCD for the bacterial community inhabiting the rhizosphere of Hamilton soil were seen at day 15 (Figure 3.3B). This result indicates that corn (*Zea mays*) had an effect on the activity of the bacterial community in both soils, even though the soils had different concentrations of toxic metals.

Substrate utilization patterns can be more reliable indicators of the presence of a particular physiological group of bacteria within a soil sample. Further investigation into substrate utilization patterns for five types of carbon sources reveals that soil bacterial communities from both soil types preferred similar carbon sources (Figure 3.4A-E), with higher values being for carbohydrates and carboxylic acids (Figure 3.4A and B). Each of the amino acids/amines (except L- Arginine and L- Asparagine), carbohydrates, carboxylic acids (except D- Galactonic Acid γ-Lactone and D- Galacturonic Acid), miscellaneous (except glucose-1-phosphate) and polymeric compounds were well utilized

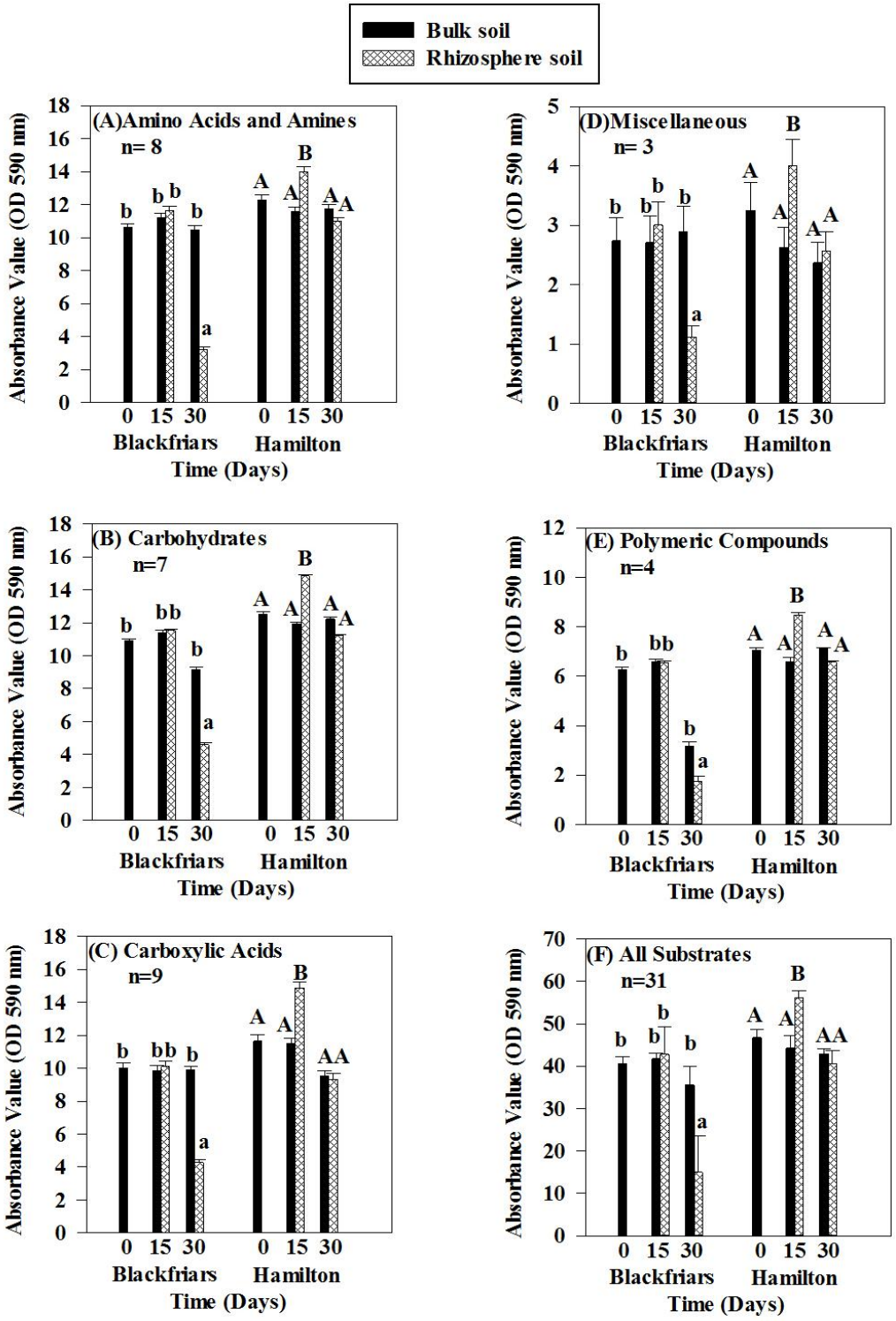


Figure 3.4: Absorbance values of carbon substrates on Ecoplates for Blackfriars and Hamilton soils among days of the treatment. Small and capital letters refer to the significant difference ($P < 0.05$) between treatments (Bulk and rhizosphere) within Blackfriars and Hamilton soils, respectively. Error terms are SE of three replicates.

(See Table 2.2 in chapter 2 for the list of all carbon sources) by the bacterial communities sampled from both soil sites and treatments, with significantly higher values obtained from the Hamilton rhizosphere ($P= 0.001$) (Figure 3.4F).

On the basis of the patterns of utilization carbon substrates by the bacteria from each treatment at each time point, the Shannon Diversity Index (H'), which incorporates measurements of the types of carbon sources and how well they are utilized, and Evenness (E), which corresponds to the extent to which the community uses all types of carbon equally well and can be considered as the functional diversity of the community, were calculated from the Ecoplate data (Figure 3.5). Three way ANOVA indicated that H' and E did not change with time in any of the treatments except in the Blackfriars rhizosphere soil, in which they were reduced by 0.7 and 0.21 units on day 30 for H' and E, respectively. This implies that biodiversity was not affected by the growth of corn. However, Hamilton soil had a significantly higher H' than Blackfriars ($P< 0.001$) based on colony morphology, which could indicate that similar functional potential (*e.g.*, ability to use different carbon sources) between the two sites may be provided by different bacterial communities (See bacterial diversity in soil characterization in section 3.1).

3.4 New Embedding Technique

The microscopy images showed that the impregnation procedure successfully preserved the root and rhizosphere for subsequent SEM and SRA analysis (Figure 3.6).

High concentrations of some metals, especially Cu and Mn, and lower values for Pb and Zn, were leached from the column during embedding (Figure 3.7A); As and Fe were below the detection limits for ICP-AES. Additionally, the microorganisms

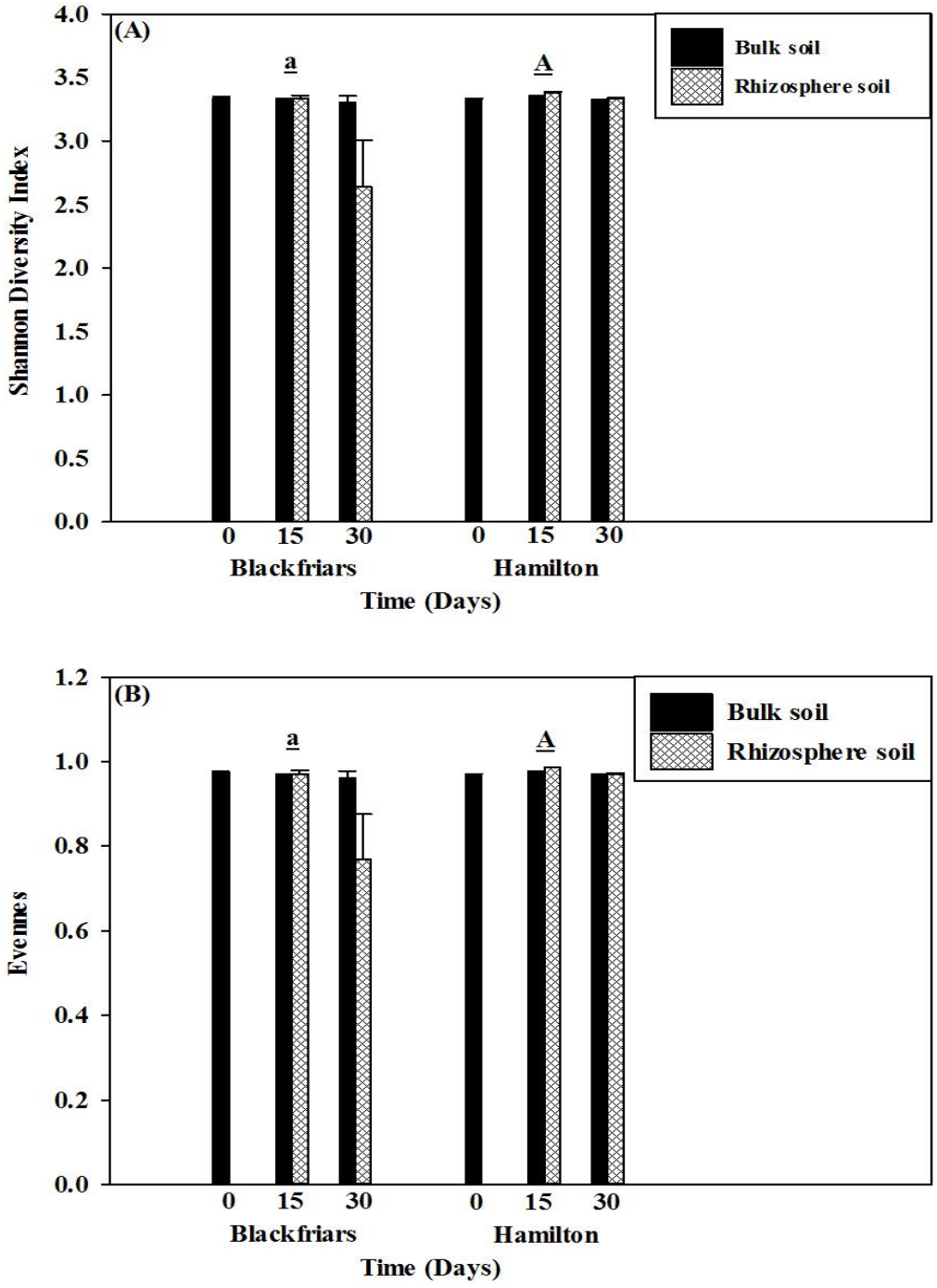


Figure 3.5: Functional diversity of the bacterial communities as measured by (A) Shannon Diversity Index (H') and (B) evenness (E) values for Blackfriars and Hamilton soils among days of treatment. Small and capital letters refer to the significant difference ($P < 0.05$) between treatments within Blackfriars and Hamilton soils, respectively. Error terms are SE of three replicates.

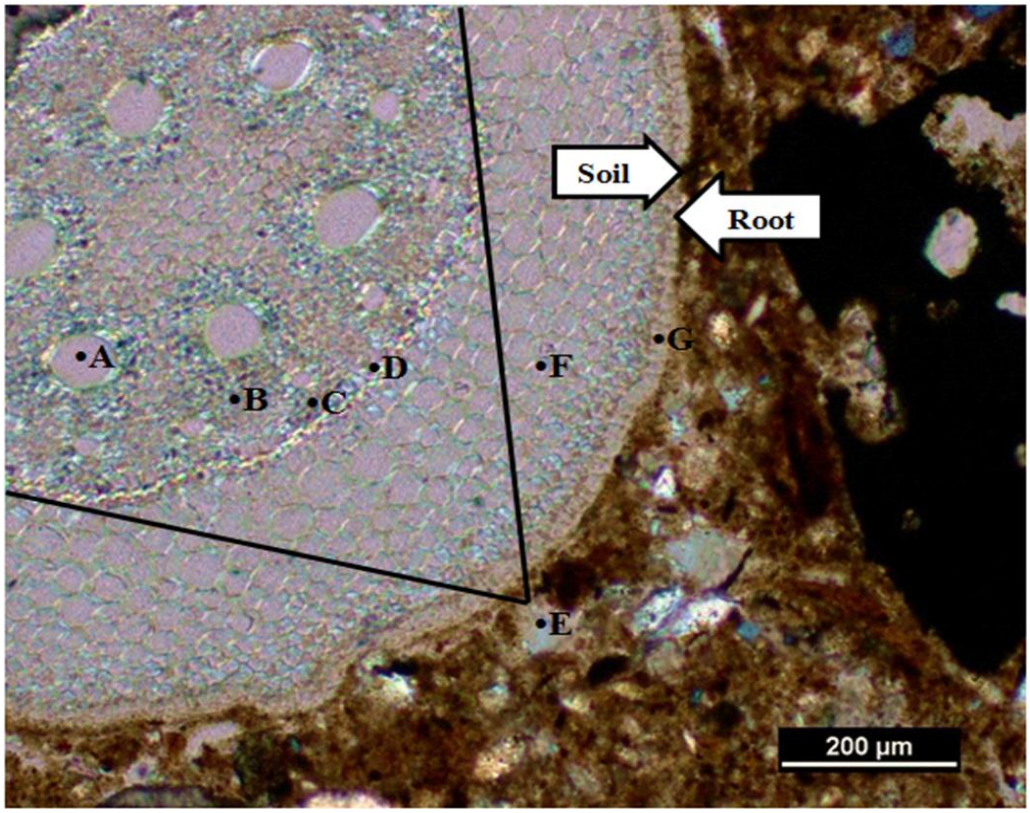
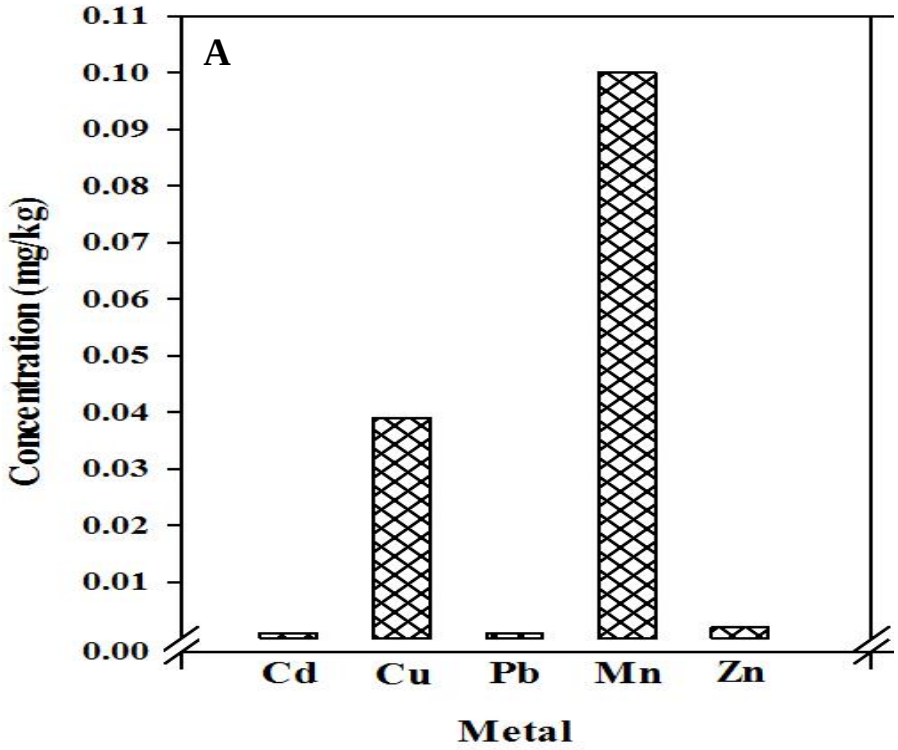


Figure 3.6: Cross section of a soil core containing a corn (*Zea mays*) root and rhizosphere (A xylem, B phloem, C pericycle, D endodermis, E stele, F cortex and G epidermis); observed with a light microscope.



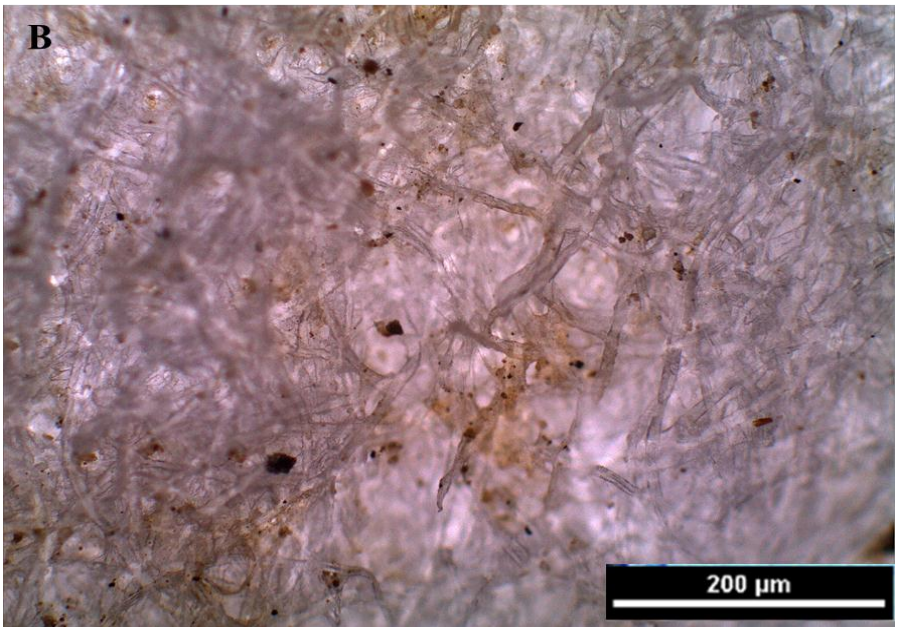


Figure 3.7: (A) ICP-AES for the wash-water of the new embedding procedure (bulk soil at time zero) (n= 2) and (B) a filter paper, observed with a light microscope.

(*e.g.*, fungal hyphae) could not be observed on the surface of a filter paper through which embedding leachate liquids were passed (Figure 3.7B) under the light microscope or in the thin section under either the light microscope or SEM, which is likely due to thickness of polished thin sections (approx. 30 µm thick).

The application of the SEM to study the rhizosphere has been limited due to difficulties in preventing distortion during sample preparation (the sample is required to be completely dry since the sample chamber is at low vacuum). Numerous dehydration techniques (*e.g.*, graded series of acetone or ethanol, freeze-drying, critical point drying) and impregnation substances such as Epoxy (April and Keller, 1990), polyester resin (Camuti and McGuire, 1999), Crystic 17449 resin, Vestopal 150 resin (Tippkötter et al., 1986), have failed to preserve the root-soil continuum for SEM and SRA analyses, *e.g.*, preserving the rhizosphere, as the root is pulled out of the soil. Tippkötter et al. (1986) recommended dehydration using a graded series of acetone:water mixtures (70, 90 and double changes of 100% acetone) because rapid dehydration with 100% acetone caused the sample to collapse and shrink. In this research, a rapid dehydration with 100% ethanol followed by impregnation in LR white resin gave superior results compared with previously published methods by preserving the rhizosphere (root-soil continuum), as the roots were embedded within the soil.

3.5 SEM and SRA Analyses

3.5.1 Scanning Electron Microscopy (SEM) Analysis

BSE images of bulk (control) and rhizosphere samples at 0, 15 and 30 days confirmed the presence of particles with high atomic mass, and some grains that look structurally

distinctive (Figure 3.8). In this thesis, these particles are referred to as melt grains; melt grains are natural, fine grains in the soil that form under specific conditions (*e.g.*, high temperature, microbial activities) and during this process they bind with other elements. These particles varied in size and shape, as some had smooth edges and some were rough and jagged, but all were located close to the roots (in the rhizosphere soils). The micrographs also show several gaps of different size dispersed within the soil (bulk and rhizosphere) matrices (Figures 3.8 to 3.12). EDAX identified aluminum (Al), Ca, Cu, Fe, K, Pb, Mg, Mn, O, silica (Si), titanium (Ti) and Zn fluorescence peaks (Figures 3.8 to 3.12). These elements are expected as part of the natural composition of the aluminosilicate minerals in soil (Reyes-Gutiérrez et al., 2007), and some of them were observed in ICP-AES (*e.g.*, Cu, Fe, Mn, Pb and Zn) (See Figure 3.1). Also, there was a peak overlap between Pb L-alpha and osmium (Os) L-beta of the osmium coat, which made the observation of other peaks of metals difficult (Figure 3.12). WDAX mapping of a single root section failed to detect these metals (other than the particles detected in the EDAX point analyses).

Most previous studies on metals in the rhizosphere focused their investigation on how metals change within the rhizosphere, providing analysis of materials that occurred up to 2 mm away from the root. However, this work was limited to only a few soil grains that were attached to the roots following all of the preparation steps, and thus previous techniques could not provide information about changes in metal speciation from the bulk soil towards the roots. In this research, embedding the plant roots right into the soil where they grew allowed us to investigate the distribution and movement of metals toward the plant roots.

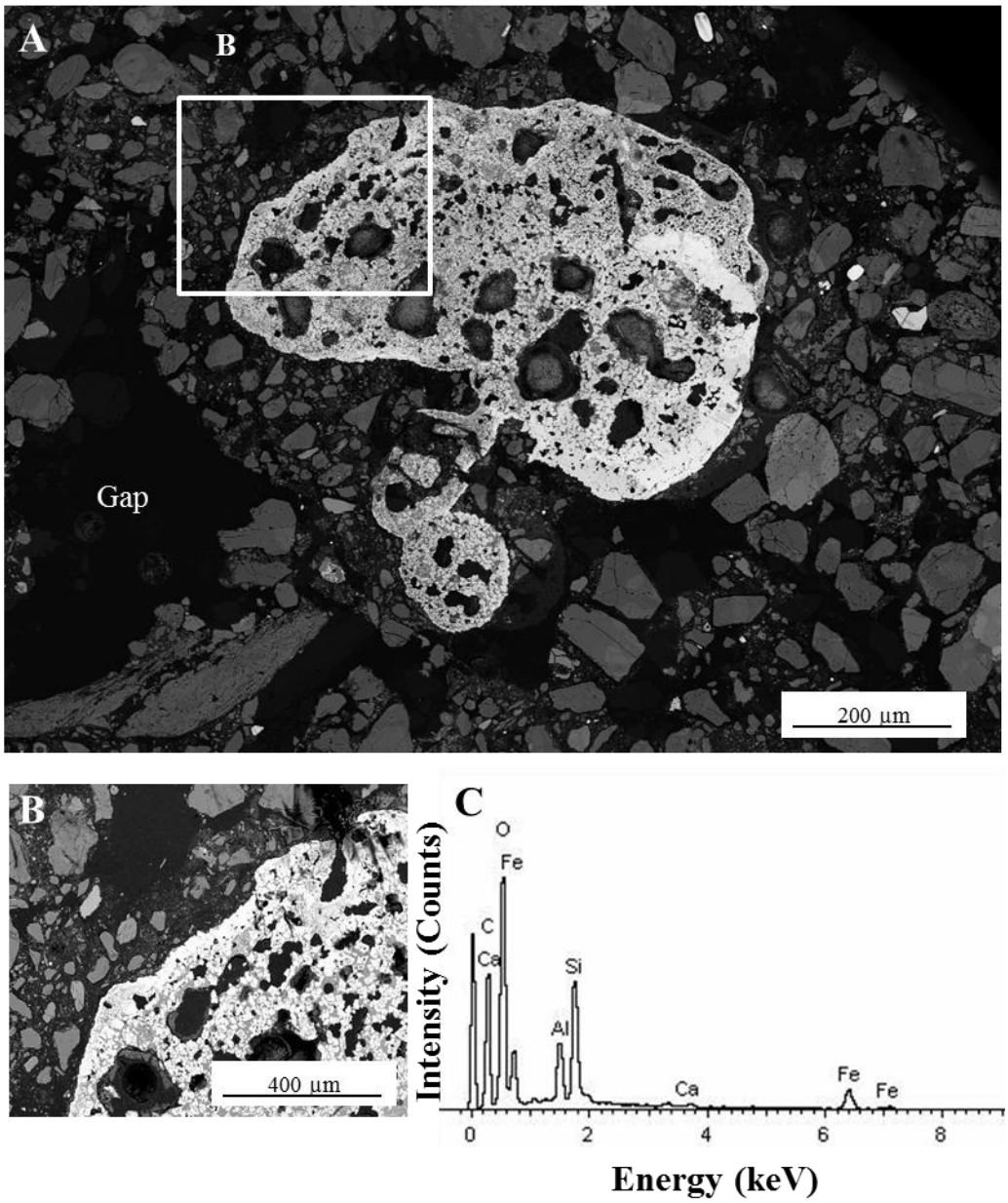


Figure 3.8: SEM micrographs highlighting an example of distinctive metal grains found in soils; (A) Metallic grain from the rhizosphere (on day 30) of Hamilton sites imaged under BSE-SEM, and (B) a magnified view of the edge of the grain, with (C) corresponding spectrum highlighting some particles possessing a high atomic mass element (s).

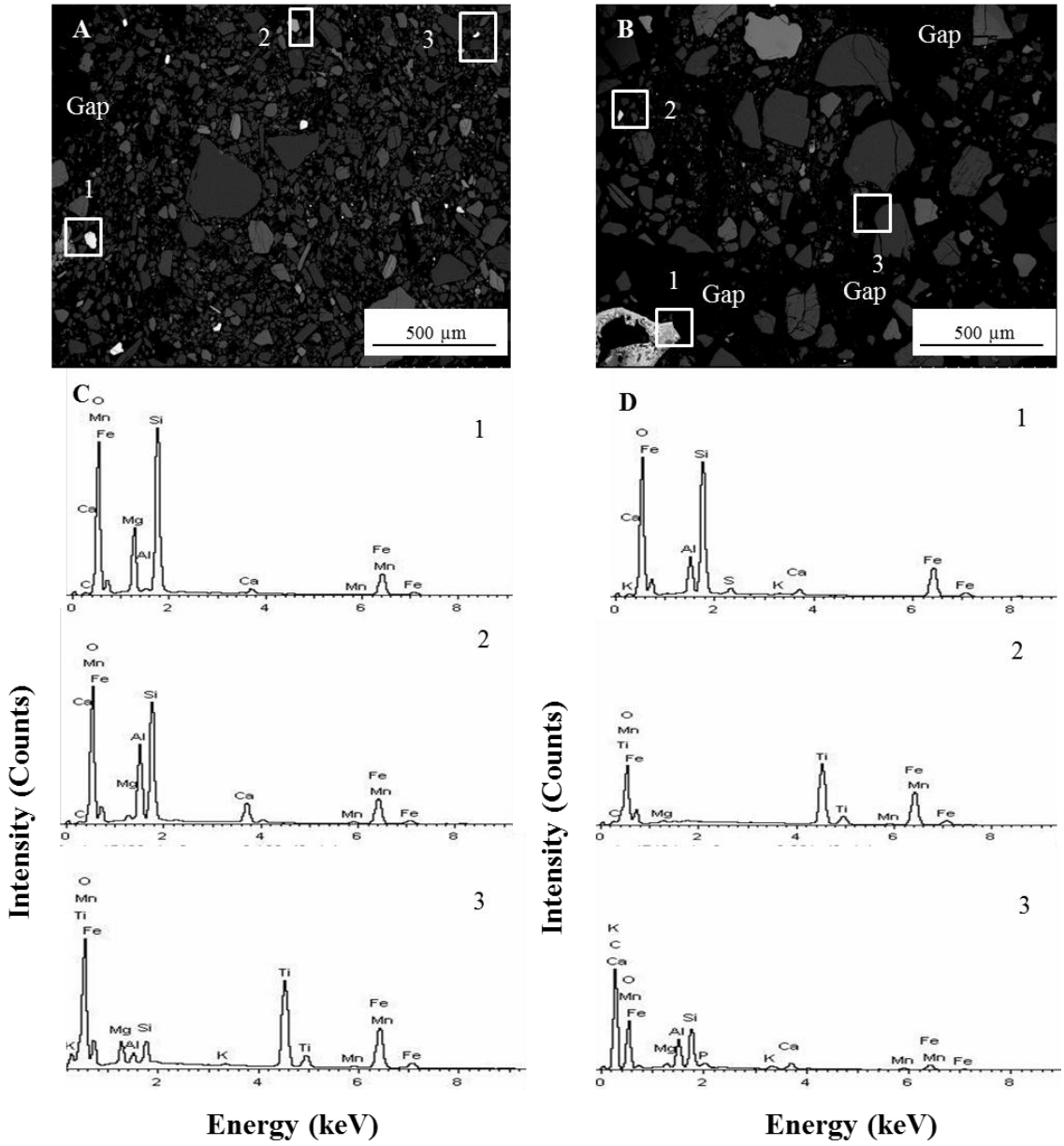


Figure 3.9: Embedded polished cross section of bulk metal contaminated garden soils on day 0 from (A) Blackfriars and (B) Hamilton sites viewed under SEM-BSE with corresponding spectra (C and D) highlighting some particles possessing a high atomic mass element (s).

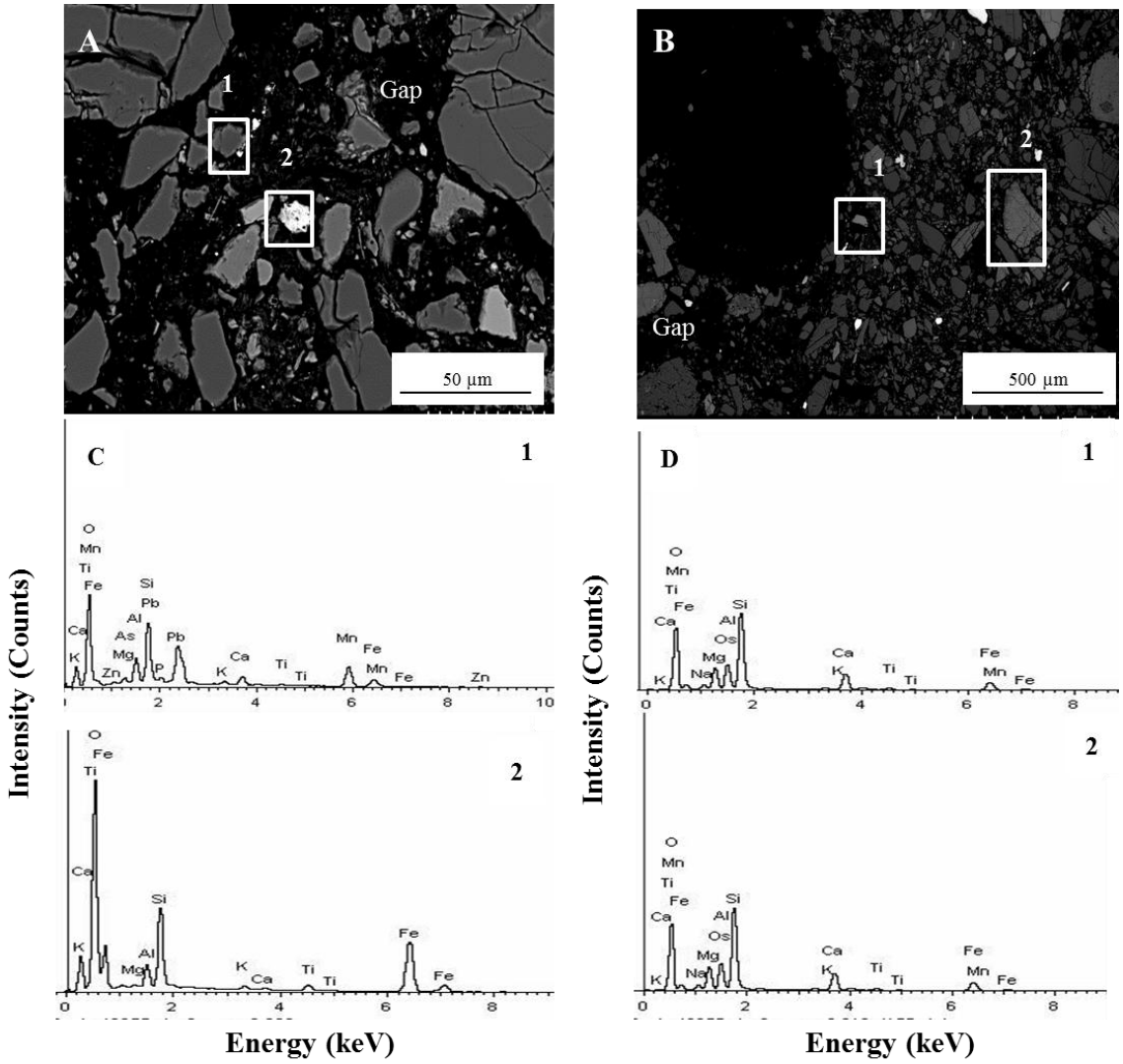


Figure 3.10: Embedded polished coross section of (A) bulk and (B) rhizosphere soils on day 15 from Blackfriars site viewed under BSE-SEM with corresponding spectra (C and D) highlighting some particles possessing a high atomic mass element (s).

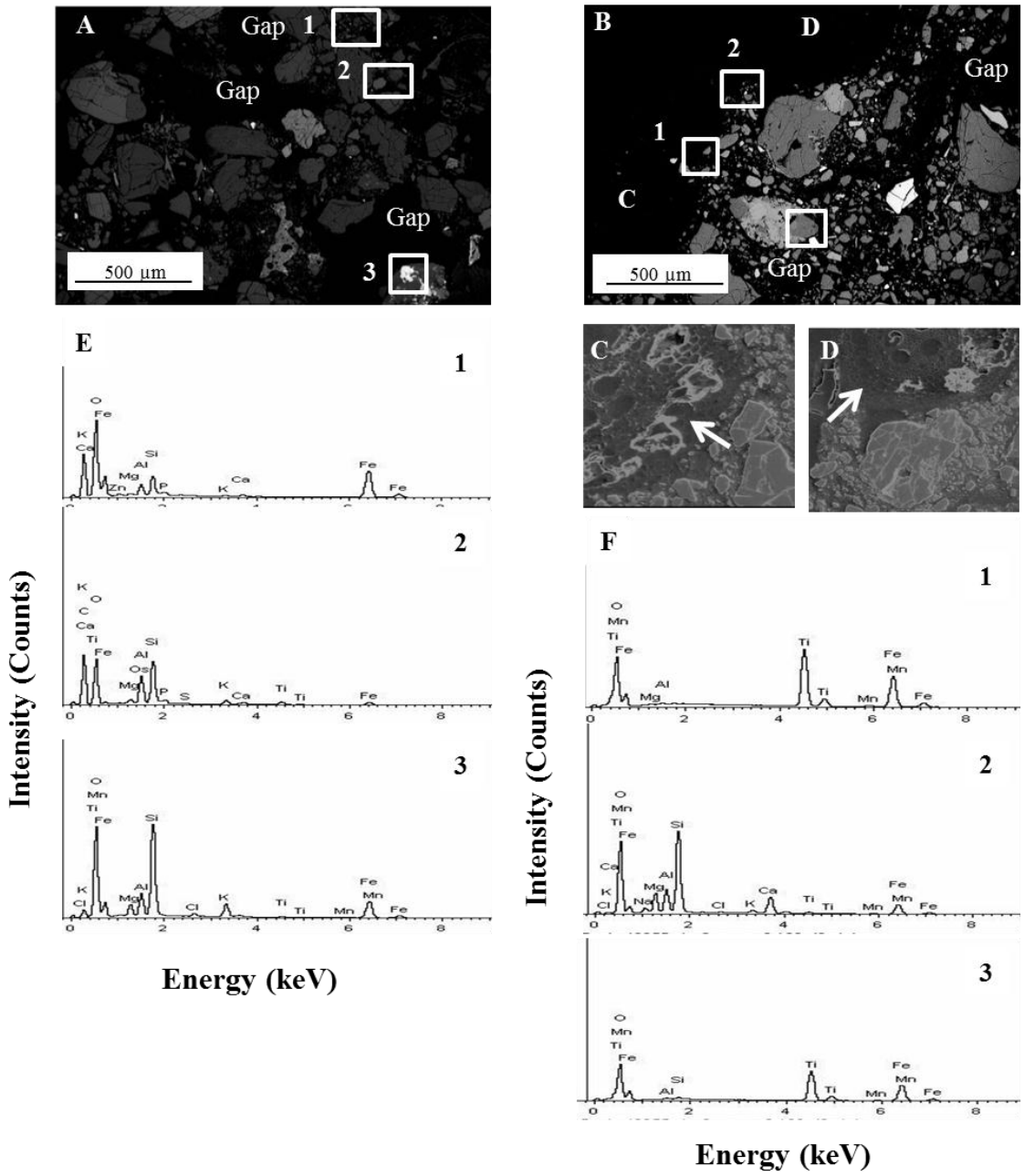


Figure 3.11: Embedded polished coross section of (A) bulk and (B) rhizosphere soils on day 15 from Hamilton site viewed under SEM-BSE (arrows on C and D are SEM-SE images for the roots) with corresponding spectra (E and F) highlighting some particles possessing a high atomic mass element(s).

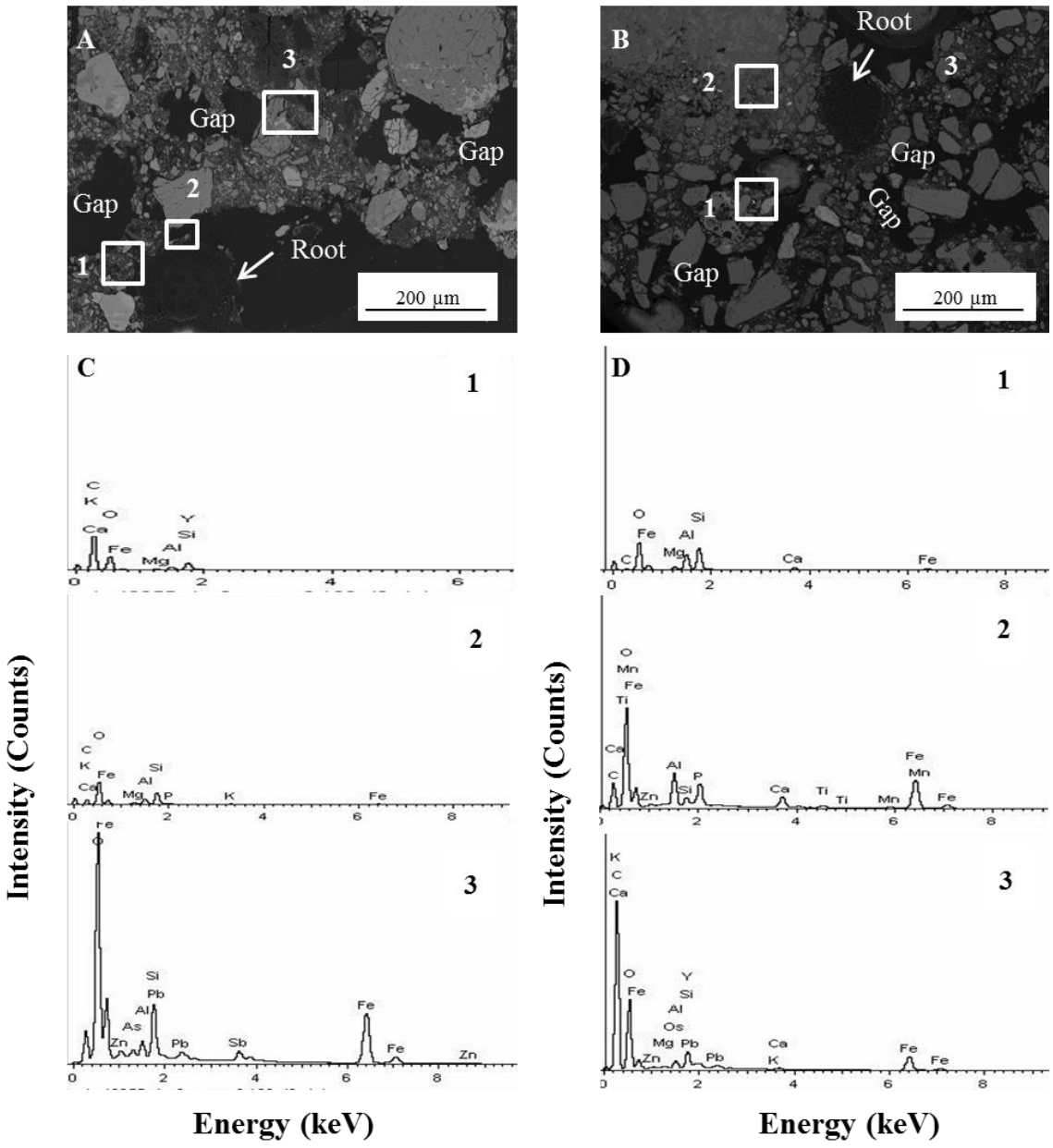


Figure 3.12: Embedded polished coross section of corn rhizosphere soils on day 30 from (A) Blackfriars and (B) Hamilton sites viewed under SEM-BSE with corresponding spectra (C and D are SEM-SE for the roots) highlighting some particles possessing a high atomic mass element (s).

3.5.2 Synchrotron Radiation Analysis (SRA)

Synchrotron x-ray fluorescence (XRF) has an ideal spatial resolution, the µm range, for studying metal distribution and speciation at the soil/ root interface and into the root cells. Synchrotron x-ray fluorescence detected the presence of more metals than did ICP-AES (*e.g.*, As, Ba and Cr) and more than were found using SEM (*e.g.*, Ba, Cr).

The x-ray fluorescence maps obtained for Cu, Fe, Mn and Zn (metals of interest) from the bulk and rhizosphere soils are presented in Figures 3.13- 3.14. Relative concentrations are presented with lighter colours denoting higher metal concentrations. The bulk soils contained all four of these metals, with higher concentrations occurring at a few localized places (arrows on Figure 3.13A and 3.14A). These likely represent at least three to four regions ("hot spots") that contain high concentrations of metals. Each of the four metals was distributed throughout the rhizosphere soils and on the root surface as well. Some of the Cu, Fe, Mn and Zn were detected in both the soil (rhizospheres) and root tissue, but with variances in the distribution (Figures 3.13B and 3.14B). Iron with Cu and Mn, and Zn were most concentrated on the root surface. However, in Blackfriars, Zn was also found with high concentration into the epidermis and endodermis of the root tissue, which is considered to be a concern as it can be translocated to aboveground tissues. A similar distribution for Cu, Mn, Fe and Zn was reported by Naftel et al. (2006) for aspen (*Populus tremuloides* Michx) roots collected from forested sites and analyzed by SRA. The XRF distribution maps for the metals above confirmed that Cu, Fe and Mn were mostly accumulated in the rhizosphere of aspen (at soil root interface) while Zn was mostly distributed throughout the root tissue. In this study of *Z. mays* (See Figures3.13B-

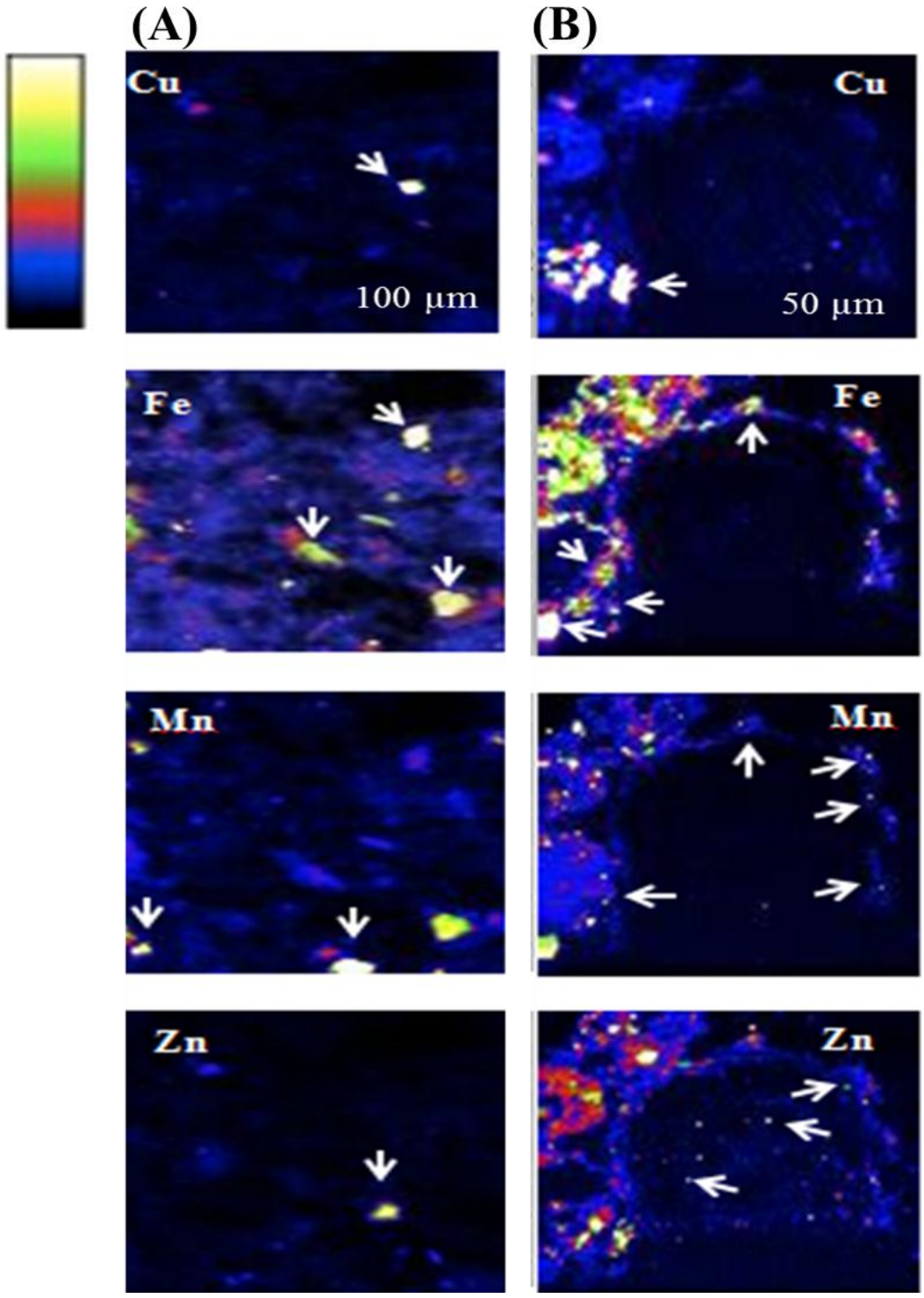


Figure 3.13: Typical x-ray fluorescence maps for Cu, Fe, Mn and Zn in (A) bulk (control on day 0) and (B) rhizosphere soil (on day 30) from Blackfriars community garden. Relative concentrations are presented, with lighter colours denoting higher metal concentrations.

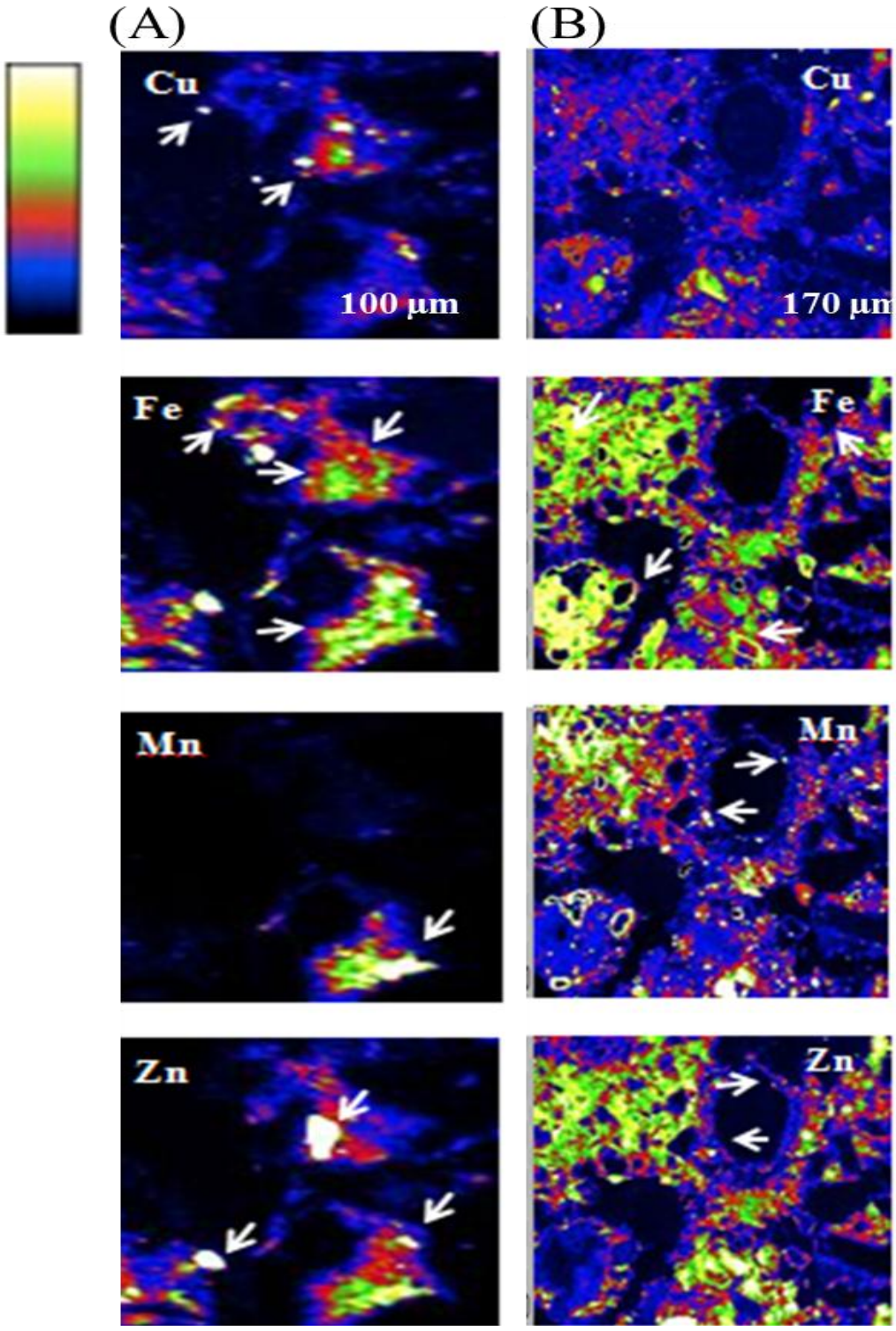


Figure 3.14: Typical x-ray fluorescence maps for Cu, Fe, Mn and Zn in (A) bulk (control on day 0) and (B) rhizosphere soil (on day 30) from Hamilton garden. Relative concentrations are presented, with lighter colours denoting higher metal concentrations.

3.14B), the higher concentrations of Cu were seen in the epidermis while higher concentrations of Zn were noticed in both the epidermis and endodermis.

Only one of the 'melt' grains that was found in the Hamilton rhizosphere soil (at 30 day) was selected (generally, this soil contains higher concentrations of all the metals than the Blackfriars soil) for identifying the mineral-rich soil material. The metal grain from which XRF maps for Cu, Fe, Mn and Zn were scanned is shown in Figure 3.15. This particle contains high concentration of Cu, Fe, Mn and Fe, with the highest concentrations being for Fe. This result is in agreement with the results obtained from ICP-AES (See section3.1) and EDAX (See Figure 3.8) analyses. This suggests that these particles could be the source of those metals present in the soils.

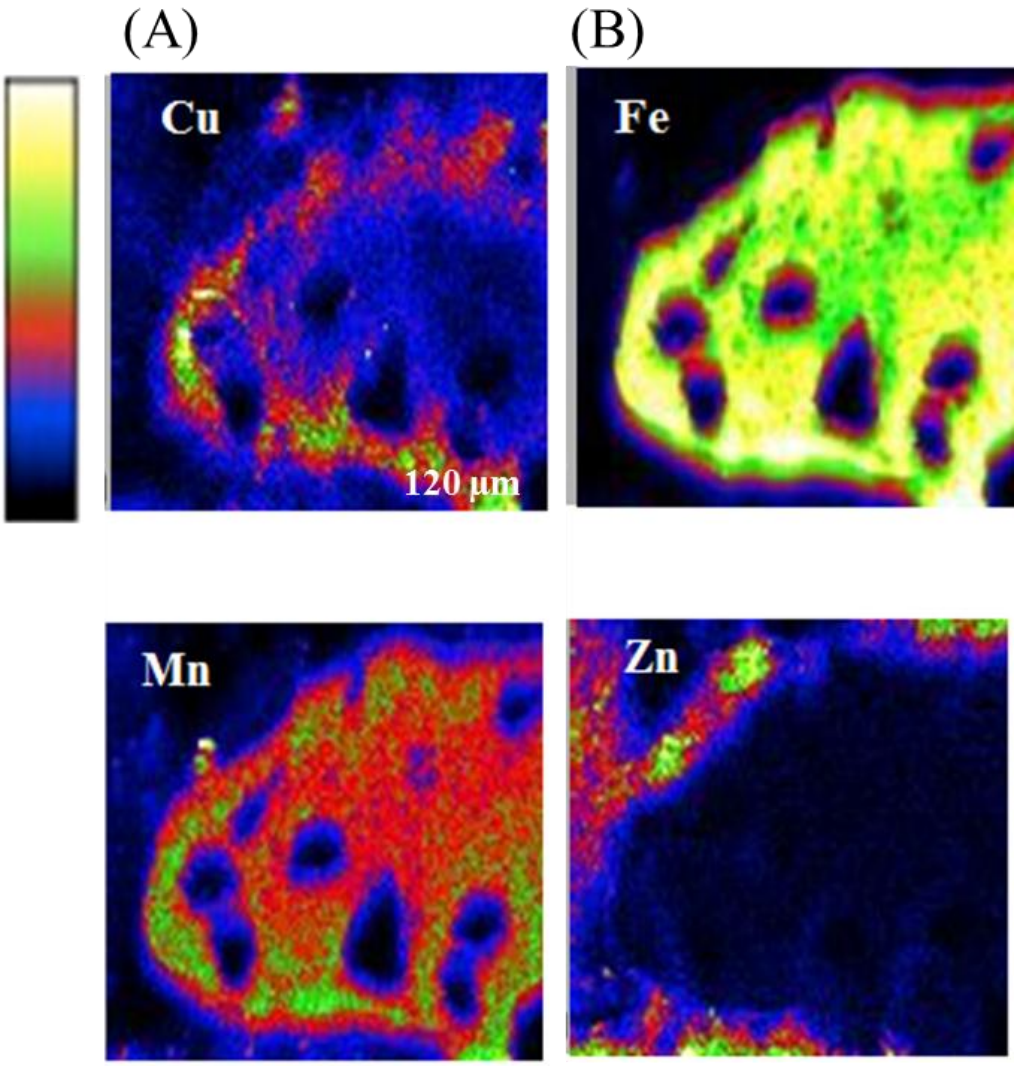


Figure 3.15: Typical x-ray fluorescence maps for Cu, Fe, Mn and Zn in melt grain from the rhizosphere (on day 30) of Hamilton soil (particle B in Figure 3.8). Relative concentrations are presented, with lighter colours denoting higher metal concentrations.

CHAPTER 4

4. General Discussion, Conclusions and Future Prospects

4.1 General Discussion and Conclusions

As human activities contribute to increased concentrations of trace metals in the landscape and garden soils, it has become increasingly more important to investigate the microbe-mineral interactions within the rhizosphere, where almost all tolerance mechanisms, which were described in section 1.4, occur.

Results from chemical and bacterial analyses described in sections 3.1- 3.3 demonstrated that the bacterial numbers in the two soils were lower than those reported in healthy agricultural soils. Plant-associated bacteria possessing a metal-sequestration system would be helpful for the plants as they can reduce metal phytotoxicity and enhance translocation of essential nutrients to the aboveground plant parts. Specifically, rhizosphere bacteria that produce organic acids and/ or siderophores can increase the availability of nutrient metals to plants as these compounds may significantly lower soil pH. However, there are many environmental factors (*e.g.*, temperature, moisture, pH, nutrient, and continued exposure to pollution) that may change the bacterial populations and communities. In this study, I would expect that the bacterial populations and communities would not be able to survive and flourish in the syringes (*e.g.*, bulk and rhizosphere) as compared to in a field, so the bacterial counts I obtained are likely to be lower than those in the gardens from which the soils were collected. However, I would expect the bacterial populations in the gardens to be fewer and smaller than those in a typical agricultural field because only those bacteria that are tolerant to toxic

concentrations of trace metals would survive in the Blackfriars and Hamilton garden soils.

Although the bioavailable metal concentrations in both soils, as estimated via $CaCl_2$ extraction and ICP-AES, varied between the two soil sites and treatments, higher values were found in the rhizosphere soils. This variability in metal bioavailability may have been due to the observed changes in pH. The amount and chemical form of organic compounds released by plants roots and organisms strongly affect the pH in the rhizosphere. One of the most important pH effects is the solubilization of nutrients, toxic elements and changes in microbial activity. However, in this study, the concentration of some metals (*e.g.*, Cd and Pb) in Blackfriars was below the detection limits for ICP and this could be explained by the low solubility of these metals due to high pH (See table 3.1 in chapter 3) of the soils, which may have been kept high due to added tap water of pH= 7.93± 0.3. Soils that have neutral pH are suitable for plants as a very high or very low pH can cause disease and be toxic to the plants. In home gardens, where fertilizers are frequently used, the soil pH may be low. Therefore, a test for soil pH is needed regularly to check whether the pH is decreasing due to fertilizer application. If this is the case, gardeners can use a lime (a fertilizer that has a high pH and contains a high amounts of Ca or Mg) to increase soil pH and reduce metal solubility.

City garden soils are laced with industrial pollutants (*e.g.*, mixture of trace metals), and people growing edible plants in such soils may not be aware of this contamination because the plants look healthy, but the plants may be tolerant of high concentrations of metals and grow slowly. Since many factors (*e.g.*, pH and organic matter) play a significant role in metal bioavailability to plants, these soils should be

tested to ensure that metals levels are acceptable for growing food. Also, plants growing in contaminated soil should be tested to ensure that they are metal-free and safe to be consumed.

This study also examined the activity and functional diversity of the bacterial community in two different metal-contaminated garden soils and the influence of corn (*Zea mays*) roots on the bacteria. The results indicated that corn roots had no effect on the bacterial community's ability to consume the carbon substrates on the Ecoplate™ or on its functional diversity (as measured by AWCD and H') except for the rhizosphere of Blackfriars treated for 30 days. It has been reported in many studies that metals become more available to organisms under acidic conditions, which may adversely affect the activity of organisms. The high concentration of bioavailable metals (*e.g.*, Fe) estimated in the rhizosphere of Blackfriars treated for 30 days may have contributed to the reduction in bacterial ability to consume all carbon sources on the Ecoplate™ and their activities as well. However, this assay does not provide information about what species exist in the soil and therefore it would be possible that the species diversity of the bacterial communities in the syringes were decreased as some species were less tolerant than others as the metal solubility increased. Lack of observed differences in AWCD among treatments could be explained if the remaining, metal-tolerant species were able to consume a diversity of carbon sources. I would expect, in garden soils, where different plants grow in a larger soil volume, the microbial community and activity shall be higher than I observed for one corn seedling grown in ~90 g of soil. Generally in this study, the bacterial functional diversity did not change during the experiment and this may indicate that the bacteria in these soils were already tolerant of the metals.

Even though the activity of the bacterial community was affected by the presence of the plant roots (as the colour in the wells on the Ecoplate^{TM} changed), there might be a need to test if metals could cause this change in the well colour by interacting with the dye on the Ecoplate^{TM}, for example. Nevertheless, my data did not support the first hypothesis of this research as it was expected that the bacterial community as well as its functional diversity would be adversely affected by increased metal solubility in the rhizosphere soils.

Metals are mobilized from the rhizosphere (as a result of soil microbial and plant root activities) and transported across the plasma membranes within the plant before being stored or integrated. Therefore, their concentration, location and chemical form or "speciation" (*e.g.*, bioavailable metals) are important because they provide information about the pathway (movement) and destination of a particular metal from the soil towards the plant tissue. In this thesis, I developed an embedding technique to prevent rhizosphere distortion during specimen preparation for SEM and SRA studies (Section 3.4) and have demonstrated the widespread distribution of metals in both of the soils (Blackfrairs and Hamilton) prior to growth of corn (*Zea mays*). Using SEM-EDAX, I was able to identify localized 'point-sources' of toxic metals that are undoubtedly contributing low levels of toxic metals (Section 3.5). By developing this new embedding procedure, I further was able to investigate the metal-distribution in soils and within plant roots using XRF maps and found that some trace metals such as Cu, Fe and Mn were generally distributed throughout the rhizosphere and most likely accumulated on the root surface, while in Blackfriars rhizosphere soil, Zn was found throughout the root tissue (Section 3.5). Taken together, these data did support the second hypothesis of this

research and suggest reasonable reaction flow-paths from these point-sources of metal towards the roots.

The combined results from chemical, bacterial, SEM and SRA experiments indicate that the new embedding technique may allow us to improve our understanding the roles of soil microorganisms and plant root exudates in affecting metal bioavailability and metal uptake by plants. This new embedding technique developed in this study could be applied to study plants grown in any agricultural soils under natural environment by sampling soil cores (instead of pulling the roots out of the soil) to prevent sample disruption following the same methods (See section 2.6.1 in chapter 2). Also, the results from this research may have a potential application in ecosystem health, food security and phytoremediation research.

4.2 Directions for Future Research

Data from this study emphasize the need for understanding metal movement from the bulk soil towards the plant roots and its distribution throughout the plant tissue. In addition, it will be important to determine the different forms of metals that exist in the bulk soil, rhizosphere and plant root, and if these forms of metals vary with distance from plant roots. This can be achieved by incorporating the use of XANES spectra to determine the oxidation state of each metal and metal complex from the bulk soil towards the plant roots.

Another possible avenue for future research is to use different types of Biolog® plates to investigate the activities of other microorganisms (*e.g.*, FF Biolog® plate to study fungi). Microorganisms that are exposed to toxic concentrations of trace metals in

soil environment suffer from increased stress. Mycorrhizal fungi are the most important group of soil organisms that assist plants with the absorption of sufficient minerals (single hyphae can span in length from a few cells to many yards) and they are more tolerant to metals than bacteria (Hartikainen et al., 2012). Therefore, I expect that mycorrhizal fungi would have a greater impact on metal bioavailability than bacteria.

It would be helpful for phytoremediation (*e.g.*, expedite the process) if we could determine exactly which species (or genera) of bacteria have the greatest influence on metal bioavailability, and to investigate the bacterial communities associated with different plant species.

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