Bioremediation of Contaminated Soils by *Echinacea purpurea* and Arbuscular Mycorrhizal Fungi

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Abstract

As a potential bioremediation system for contaminated soils, I evaluated the use of an arbuscular mycorrhizal (AM) fungus, *Glomus intraradices* on roots and shoots uptake of polycyclic aromatic hydrocarbons (PAHs), alkyl PAHs, and toxic metals in *Echinacea purpurea*, in using a controlled 20-week greenhouse study and a complimentary 2-year field study. *E. purpurea* seeds were either inoculated with the mycorrhizal fungus (AM) or not inoculated (non-AM) and grown in soil provided by the National Capital Commission (NCC) that have known contamination.

In the greenhouse study, AM inoculation increased the uptake of alkyl PAHs in the roots of *E. purpurea*. The AM inoculation showed no effect on root uptake of PAHs and toxic metals over the 20-week study period. However, when I calculated the uptake rates (k_1) for PAHs between both treatments, the AM treated roots ha 10-fold higher k_1 values than non-AM treated roots. The soil concentrations of PAHs were found to increase over time with AM inoculation, suggesting, that AM fungi are causing a solvent depletion through root uptake of minerals and carbon, which concentrates the more hydrophobic PAHs in soils. Alkyl PAHs and metals showed no change over time amongst any of the treatments.

Assessing the performance of AM fungi on the uptake of contaminants under field conditions, only PAHs showed increased bioaccumulation in the shoots of *E. purpurea* with AM inoculation. Alkyl PAHs and metals in plant material were unaffected by the AM inoculation, but increased significantly from year 1 to year 2. The uptake rates among treatments were similar, with non-AM roots having slightly greater uptake. Soil concentrations of PAHs and alkyl PAHs were unaffected over the course of the experiment. Our control soil, however, showed significant increases in concentration from year 1 to year 2 with alkyl PAHs.

These results quantified the influence of AM hyphae-mediated uptake of organic and inorganic contaminant transfer from soil to plants and the bioaccumulation kinetics for contaminants by *E. purpurea* that will be useful for environmental models and phytoremediation strategies.

Résumé

Cette étude visait à évaluer le potentiel de bioremédiation de sols contaminés par des hydrocarbures aromatiques polycycliques (HAP et HAP alkyles) et des métaux toxiques par la symbiose d'un champignon mycorhizien arbusculaire (MA), *Glomus intraradices*, et d'une plante, *Echinacea purpurea*. Nous avons réalisé une étude en serre de vingt semaines ainsi qu'une étude sur le terrain de deux années. La croissance des plantes, pré-inoculées ou pas, a été réalisée dans du sol contaminé d'un site, l'île Victoria, Ottawa, Commission de la capitale nationale.

Notre étude en serre a montré que l'inoculation MA a accru l'absorption des HAP alkyles dans les racines d'*E. purpurea*, mais sans effet sur les HAP et les métaux. Le calcul des taux d'absorption (k_1) des HAP a indiqué que l'inoculation MA a augmenté le taux d'absorption racinaire d'environ 10 fois plus par comparaison aux racines non inoculées. Les concentrations en HAP dans le sol ont augmenté avec l'inoculation MA dans le temps ; ce qui suggère que les champignons MA ont pu engendrer un effet de dissolution par l'entrée des minéraux et du carbone dans les racines, ce qui aurait concentré les HAP les plus hydrophobes.

Les résultats de terrain ont montré que l'inoculation MA n'a pas été concluante quant à l'absorption des contaminants à l'exception de la bioaccumulation des HAP dans les systèmes aériens d'*E. purpurea.* Les masses des HAP alkyles et des métaux chez les plantes n'ont pas été influencées par le traitement MA mais ont augmenté significativement de la 1ère à la 2ème année. Les taux d'absorption étaient similaires bien qu'un peu plus élevés chez les plantes MA. Les concentrations en HAP et HAP alkyles du sol n'ont pas changé mais les HAP alkyles ont augmenté significativement de la 1ère à la 2ème année.

L'ensemble des résultats a ciblé des pistes de recherche sur le transfert des contaminants du sol aux plantes via les hyphes mycorhiziens ainsi que sur la cinétique de bioaccumulation de contaminants utiles aux modèles environnementaux et aux stratégies phytoremédiatrices.

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Table of Contents

List of Figures

Figure 3.1	Field plot on Victoria Island, Ottawa, ON.
Figure 3.2	Mean (±SE) ΣAlkyl-PAH content (ng g^{-1}) in soil over 1 year of growth in the field on Victoria Island. Data was analyzed using a one-way ANOVA [F (5, 12)=6.6, p=0.004]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (p=0.19) and Levene's test (p=0.93) for homogeneity of variance. Control Soil, AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.
Figure 3.3	Mean (±SE) ΣPAH content (ng) over 1 year of growth in *Echinacea purpurea* on Victoria Island. Data was analyzed using a one-way ANOVA for roots [F (3, 8)=13.8, p=0.002] and shoots [F (3, 8)=6.1, p=0.02]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (SW) and Levene's test (LT) for homogeneity of variance in roots (SW p=0.02, LT p=0.21) and shoots (SW p=0.68, LT p=0.96). AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.
Figure 3.4	Log transformed uptake rates measured in roots of AM and non-AM plants during year 1 (Y1) of the greenhouse experiment. AM roots showed no significant relationship (slope= -0.000033, r^2=0.12, p=0.22) while non-AM roots showed a significant negative relationship (slope=-0.000025, r^2=0.39, p=0.018). Log KOW are given for the following PAHs: acenaphthene, fluorene, phenanthrene anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3- cd]pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene.
Figure 3.5	Mean (±SE) total metal content (mg•kg^{-1} dry weight) in soil samples from year 2 at Victoria Island field site. CS (Control Soil), AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.
Figure 3.6	Mean (±SE) metal content (mg dry weight) in root sample of *Echinacea purpurea* from year 2 at Victoria Island field site. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.
Figure 3.7	Mean (±SE) metal content (mg dry weight) in shoot sample of *Echinacea purpurea* from year 2 at Victoria Island field site. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

List of Abbreviations

Alkyl	Alkylated
ANOVA	Analysis of variance
AMF	Arbuscular Mycorrhizal Fungi
ASE	Accelerated Solvent Extraction
ES	Emission Spectroscopy
GC	Gas Chromatography
HMW	High Molecular Weight
ICP	Inductively Coupled Plasma
LMW	Low Molecular Weight
LSD	Least significant difference
MS	Mass Spectrometry
NCC	National Capital Commission
PAH	Polycyclic Aromatic Hydrocarbons
PVLG	Polyvinyl alcohol-lactic acid glycerol

Chapter 1

1.1 Introduction

The release of potentially toxic chemicals into the environment through industrial, agricultural or military activities has become a major concern for human and environmental health. As a result, there is increased interest in natural remediation technologies like bioremediation, recognized as one of the most cost effective, reliable and promising technologies for reclaiming contaminated soils (Gao and Zhu 2005; Juwarkar and Jambhulkar 2008; Suresh and Ravishankar 2004). Bioremediation involves the use of organisms for the restoration and reclamation of polluted sites either *in situ* or *ex situ*. Some examples of bioremediation technologies in use today are phytoremediation, bioventing, bioleaching, land farming, bioreactors, biofilters, composting, bioaugmentation, rhizofiltration and biostimulation (Frazar 2000).

In this chapter I will identify the unique characteristics of AM fungi that make them useful in bioremediation of organic and inorganic contaminants in soils. In particular, I will focus on polycyclic aromatic hydrocarbons (PAHs), alkyl PAHs, and heavy metal contaminants. Furthermore, I will review current knowledge of the AM fungal association with roots and its function in ecosystems.

1.2 Polycyclic Aromatic Hydrocarbons (PAHs)

Environmental contaminants that are of particular concern are organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Harms et al. 2011). PAHs are a composed of two to seven aromatic units, with benzene as a central unit (CCME 2010). PAHs can be separated into pyrogenic and petrogenic classes. Pyrogenic PAHs are derived from incomplete combustion, and petrogenic PAHs are derived from low temperature diagenetic processes (Manzetti 2013). Both chemical classes have natural and anthropogenic sources. The petrogenic PAHs can be found in oil, refineries and spills. The pyrogenic PAH sources include volcanic eruptions, vehicle emissions, smoking, wood burning and industrial combustion of fossil fuels (Lima et al. 2005). Many PAHs have been listed by IARC as probable or suspected human carcinogens, with others showing endocrine disrupting properties, or a link to birth defects and reproductive problems (ATSDR 1995). Currently, regulations on PAHs do not fall under the Stockholm Convention, which legally requires the signatory countries to eliminate the use, emissions and production of persistent organic pollutants (POPs). Instead, the United Nations Economic Commission for Europe Long-Range Transboundary Air Pollution (LRTAP) protocol, the United States Environmental Protection Act (US EPA), or Canadian Environmental Protection Act (CEPA) regulates PAH emissions in their respective countries. These pollutants have raised global concern due to their toxic effects, bioaccumulation, persistence, and their ability for long-range transport in the atmosphere (UNEP 2001).

1.3 Toxic metals

In general, toxic metals can by classified as having a relatively high density of 5g/cm^3 and atomic number higher than 20, low water solubility, high affinity for thiolic, aminic, phoric and carbocylic group of organic compounds and tendency to form complex compounds (Sinicropi et al. 2010; Sparks 2005). Generally, this describes metals and semimetals (metalloids) associated with toxicity or ecotoxicity (Duffus 2002). There has been an effort to replace the term “heavy metal” with chemically sound terminology, because it includes elements required by organisms at low concentrations, and the name implies that the pure metal and all its compounds have the same physiochemical, biological and toxicological properties (Adriano 2001; Duffus 2002; Sparks 2005). Currently thirteen trace metals and metalloids are listed as priority pollutants and are derived from both natural and anthropogenic sources. Metals occur naturally in the Earth's crust or atmosphere and cannot be degraded, but can be sequestered by organisms. These metals are released into the environment from parent rocks, metallic minerals, agriculture, fertilizers, mining, and energy production (Adriano 2001). The use of metals from early civilizations up until present has allowed metals to accumulate or be transported into terrestrial and aquatic environments around the world causing harm to animals, plants and humans (Sparks 2005).

1.4 Chemical Structure and Properties

Polycyclic aromatic hydrocarbons (PAHs) have both pyrogenic and petrogenic sources. They are composed of two or more fused aromatic rings containing carbon (C) and hydrogen (H) atoms that can be arranged in a linear, angular or clustered pattern (Gan et al. 2009). Petrogenic PAHs are fairly similar to pyrogenic PAHs in that they have the same ring structures composed of C and H atoms, however, petrogenic PAHs possess alkylated substitutions on the ring. Crude oils are found to contain a high percentage of petrogenic PAHs due to diagenesis and low temperatures favoring alkylated PAH homologs (Irwin 1997: Haritash and Kaushik 2009; Lima et al. 2005). Pyrogenic PAHs are the result of incomplete combustion, which favors high percentages of unsubstituted PAHs. Currently, only 16 PAHs compounds are currently listed as hazardous under the U.S. EPA with no current regulation of alkyl PAHs (Appendix E).

PAHs consist of over 100 individual compounds, all with varying chemical properties. The molecular weight and structure of PAHs affects their environmental behavior, fate and concentration, vapor pressure, boiling points and aqueous solubility. Other factors include their molecular weight and chemical structure. PAHs can be classified into two molecular weight classes, low molecular weight (LMW) and high molecular weight (HMW). LMW PAHs are defined as consisting of two to three fused rings, and when compared to HMW PAHs, they have higher water solubility, lower Log K_{OW}, higher vapor pressure, and less recalcitrance within the environment (Wick et al. 2011).

PAHs are a world-wide concern because many are known carcinogens and/or mutagens. Due to their high lipid solubility, they are readily absorbed by the GI tract of mammals (Cerniglia 1984). Many PAHs have highly reactive sites called K and bay regions, which often exhibit higher carcinogenicity due to their ability to form DNA adducts. Until recently, most information on PAH studies have been focused on the toxicity of the 16 priority PAHs with little information on the alkyl substituted PAHs (CCME 2010).

Metals can be a part of a variety of chemical structures but the two main factors that influence the toxicity of a particular metal are its chemical form and its binding capability. Metals can be found as elemental, organic or inorganic which can affect the absorption distribution, metabolism and excretion in cellular and intracellular targets (Sinicropi et al. 2010). Organic metals are those that contain carbon, such as MeHg. This makes them highly lipophilic. Metals are non-biodegradable, thus their toxicity must be handled by sequestering them to keep them from harming the natural environment. The accumulation of some metals in organisms can cause severe oxidative stress leading to cell death (Sinicropi et al. 2010).

1.5 Bioavailability

The bioavailability of PAHs and metals in the environment is dependent on their physico-chemical properties, soil characteristics and the organism exposed. Physico-chemical properties such as the molecular weight of PAHs can either decrease or increase their bioavailability. LMW PAHs, which have decreased hydrophobicity and increased vapor pressure, are generally more bioavailable than HMW PAHs, which have a lower vapor pressure and hydrophobicity. Consequently, the bioavailability of PAHs decreases logarithmically with increasing molecular mass (Johnsen et al. 2005). Bioavailabilities of metals are also influenced by other physico-chemical properties. For instance, if the metal enters the soil as free ions or complexed to inorganic or organic ligands it could be more or less bioavailable.

The environment in which contaminants are found has profound impacts on bioavailability. Over time, soil becomes weathered, which can make contaminants less bioavailable (Uyttebroek and Spoden 2007). PAHs and metals are susceptible to weathering stages, characterized by different profiles and availabilities over time. When PAHs are first introduced into the soil matrix, they are degraded or removed based on their physico-chemical properties. The remaining PAHs become less bioavailable by sorption onto organic matter, partitioning into the nonaqueous phase liquids (NAPLs) and sequestration in soil micropores. Metals such as As, Cd, Co, Mn, Ni, and Zn among others are also subject to weathering. (Alexander 2000). With increasing contact time, metals in soil would become more stable and less bioavailable (NRC 2003).

Uptake and remediation can depend on the organism exposed to or in contact with the contaminant. The release of stable solid phase contaminants by an organism is common in natural systems (NRC 2003). Some organisms degrade complex contaminants through the release of enzymes, while the stimulation of other microorganisms can transform contaminants such as metals through oxidation-reduction and methylation, which greatly affects metal mobility and toxicity (Pilon-Smits 2005; Sparks 2005). Plants possess inducible mechanisms to protect themselves from heavy metal poisoning. To prevent the metal ion from entering the cytosol of the cell it is complexed, which prevents the metal from inactivating structural proteins (Zenk 1996).

1.6 AM Fungi

The arbuscular mycorrhizal (AM) fungi comprise 214 species in four orders, 13 families and 19 genera, in the class Glomeromycetes of the phylum Glomeromycota (Muthukumar et al. 2009). Although these organisms are small in size and look fragile, mycorrhizae have been found in sedimentary deposits dating back to the early Devonian period which formed 410 million years ago (Remy et al. 1994). AM fungi in particular were important in contributing to plant nutrition in the nutrient depleted hostile environments of our early Earth (Willis et al. 2013). The reason fungi are particularly successful is the ability of their hyphae to penetrate soil pores and rock matrices as small as 2 µm (Allen 2007; Bornyasz et al. 2005). This effective foraging strategy of explorative growth in poor nutrient conditions allows the organism to maximize its survival (Ritz and Young 2004). The phylum is represented in all major terrestrial biomes (Treseder and Cross 2006) with almost all tropical plants and herbaceous plants being AM mycotrophic (Janos 1987). The fungal species are not considered host specific in their associations (Giovanetti and Hepper 1985) although some pairings show preference (Croll et al. 2008; Sinclair et al. 2013; Vandenkoorhuyse et al. 2002). The dominance of fungi in many environments is an indication of the enormous quantities of organic matter they process. Their contribution to the global cycling of carbon and nitrogen is not completely known, but evidence suggests they play a significant role (Govindarajulu et al. 2005; Jones et al. 2009; Read and Perez-Moreno 2003). Identification of AM fungi used to be based primarily on morphological characteristics but recently molecular methods have dominated. In particular, quantitative real-time PCR has enabled research into spatial, temporal and functional symbiotic activities of AM fungi (Robinson-Boyer et al. 2009; Konig et al. 2010).

The AM fungi, the most ubiquitous of all mycorrhizal fungal types, are known to enhance plant growth and their tolerance to organic contaminants and toxic metals (Audet and Charest 2006; Liu and Dalpé 2009; Volante et al. 2005). These symbionts are found in over 90% of vascular plants and more than 80% of all terrestrial plants (Wang and Qui 2006). The use of AM fungi in bioremediation is becoming increasingly popular. AM fungi have the ability to aerate, bind and stabilize soils, and to enhance plant nutrient uptake (Dalpé 2003). These mycorrhizal associations provide benefits to plants and extend soil capacities of rhizoremediation (Jeffries et al. 2003), in addition to their synergistic effects with other rhizospheric microorganisms (Liu and Dalpé 2009).

1.6.1 Development of Hyphal Networks

The life cycle of AM fungi begins as a spore. Although they are considered obligate biotrophs, they can germinate in the absence of host plants. However, in order to complete their lifecycle they require a living photoautotrophic partner to produce the next generation of spores (Parniske 2008). When a spore germinates, the hyphae begin exploring the soil matrix until they are presented with plant-derived signals. The hyphae of a spore may grow up to 20-30 mm, but if a host root is not found within 15-20 days it will cease growth and withdraw its metabolites (Willis et al. 2013). Individual AM fungi show no host specificity to different plants, and many AM fungal species can colonize the same root (Fitter 2005; Santos-Gonzalez 2007). Until recently, the hormones involved in the branching of AM fungi were unknown. In two landmark papers, strigolactones were found to induce the presymbiotic stage characterized by branching and the alteration of fungal physiology and mitochondrial activity (Akiyama et al. 2005; Besserer et al. 2006). It is theorized that hyphae are able to discern the concentration gradient and determine the proximity of the host root (Parniske 2005). When the hyphae get close to the root epidermis, they release mycorrhizal (Myc) factors, diffusible molecules, which are found to induce calcium oscillations in root epidermal cells (Kosuta et al. 2008) as well as transcriptional activation of symbiosis related genes (Kosuta et al. 2003). Hyphae then form hyphopodia, a special type of appressoria, on the root epidermis. Four to five hours after the formation of the hyphopodium on the root epidermis, a prepenetration apparatus (PPA) is produced by the plant cells, which predetermines the path of fungal growth through the plant cells. Subsequently, hyphae from the hyphopodium enter the PPA, and are guided through the roots cells towards the inner cortex. The tunnel is composed of endoplasmic reticulum membranes which allow the synthesis of a perifungal membrane. Once in the root cortex the hyphae leave the plant cell and branch laterally along the root axis. The branching hyphae stimulate the development of PPA structures in the inner cortex (Genre et al. 2008) and later enter the cells to form arbuscules.

The intraradical hyphal modifications in roots are nutrient exchange sites between the fungi and host plant. The fungi will exchange phosphate, ammonia/ammonium across an H+-ATPase pathway in a specialized membrane of the cortex cells (Kobae and Hata 2010). The host plant then provides the AM fungi with 10-30% of its net carbon fixation in the form of photosynthates (Allen et al. 2003). The nutrient absorbing hyphae extend outwards from the root and decrease in diameter from 20 to 2 μm (Friese and Allen 1991). Due to their small size, the hyphae are able to extract nutrients that are inaccessible to root hairs.

The hyphal networks have been shown to have a length ranging from up to 10^2 m to 10^4 m per gram of soil. The range in hypal length has been attributed to the type of land they are found in. For example, arable land has been found to have the smallest hyphal length and forests have the largest. However, species still differ in the degree of soil volume they occupy (Abbott and Robson 1985) and the distance they travel from host plant roots (Munkvold et al. 2004).

1.6.2 Transport

Once hyphal networks have formed in the soil matrix, the hyphae begin translocating compounds both passively and actively (Govindarajuli et al. 2005). The symbioses between the host plant and mycorrhizae requires effective fungal uptake of nutrients and transfer to the host plant. Allen et al. (2003) showed that mycorrhizae provided up to 80% phosphorus, 60% copper, 25% nitrogen and zinc, and 10% of the potassium absorbed by plants. The host plant provides carbon in the form of sugars through passive efflux in exchange for the macromolecules provided by the AM fungi. It is believed that the AM fungi take hexoses from the plant and convert them into lipids and glycogen before transport to the extra-radical mycelium (Bago et al. 2003). Through isotopic labelling conducted by Bago et al. (2002), it was discovered that the plants were translocating lipids at a speed of 11mm s^{-1}, or 1.3 mg $hour^{-1}$ $hyphae^{-1}$ to the extraradical mycelium. The process in which the plant and AM fungi provide each other with excess nutrients or lipids above the maintenance requirements proves to be beneficial to both. However, it has been shown that an increase in nutrients such as phosphorus in the soil from agricultural fertilizers can reduce the plant's lipid supply. In doing so, the mycorrhizal colonization and diversity decreases (Johnson and Pfleger 1992).

1.6.3 Degradation of Contaminants

Sites that are too polluted, too acidic, or too dry for organisms can make contaminants biochemically or physically inaccessible because the pollutant structures are too complex or toxic for specific degradation (Singh 2009). It has been shown that AM fungi are capable of tolerating these inhospitable conditions and hosting specific endomycotic bacteria (Bonfante and Anca 2009). Not only can AM fungi mobilize nutrients, they are able to transport hydrophobic organic chemicals inside their hyphae (Wick et al. 2010). This activity is not confined to just organics, as they are able to incorporate and/or transform metals in the host plant or immobilize contaminants in the mycorrhizosphere (Gohre and Paszkowski 2006). It has already been shown that fungi are able to transform organic contaminants metabolically or co-metabolically (Pinedo-Rilla et al. 2009). They can also transfer plant-derived organic substrates to non-symbiotic soil bacteria as well as allow the bacterial movement to pollutants within the soil matrix (Kohlmeier et al. 2005).

AM fungi contribute to the protection of the host plant from stress. Apart from their ability to improve the soil structure through particle aggregation and stabilization with the release of glycoproteins such as glomalins, they also provide plants with resistance against metals (Gaur and Adholeya 2004; Rillig and Mummey 2006). Several studies have shown that the AM fungi have mechanisms not completely understood, that allow for metal accumulation in roots (Giasson et al. 2005) but prevent shoot translocation. In other cases, AM fungi cause a greater translocation into the shoots (Davies et al. 2001; Trotta et al. 2006). Audet and Charest (2006) proposed a reason for the variation in translocation of metals. If the concentration was high in the soil there would be phytostabilization, and the roots would have a higher metal concentration. If the soil concentration was low, phytoextraction would be increased with metals translocating to the shoots. AM fungi help improve phytoremediation through the enhancement of phytostabilization, phytoextraction, phytodegradation and rhizodegradation.

Phytostabilization is the reduction in mobility of pollutants, i.e. reducing a contaminants' bioavailability can be achieved by decreasing wind and water erosion. Contaminant mobility can be influenced by plant roots through absorption or adsorption, precipitation in the rhizosphere, or hydraulic control with large amounts of water being transpired by plants (Pilon-Smits 2005; Salt et al. 1998). AM fungi exert their influence beyond the immediate vicinity of the plant roots in what is known as the mycorrhizosphere (Barea et al. 2005). The major influencers in the mycorrhizosphere are the extracellular immobilization of metals through adsorption to the fungal wall (Zhou 1999) and chelation by functional groups (González-Chávez et al. 2004). The cell walls of AM fungi are composed of polysaccharides and chitin. The cell wall has free amino acids with hydroxylic, carboxylic functional groups providing a negative charge to the structure. This negative charge allows for the control of metal ions and solutes into the cell (Ahalya et al. 2003) as well as the binding of ionic elements such as most toxic metals (Zhou 1999). Other mechanisms used by AM fungi include metal exclusion by permeability barrier, intracellular sequestration using metallothioneins, active transport away from the cell, enzymatic detoxification, and reduction in metal sensitivity (Meier et al. 2012). AM fungi also produce extracellular glycoproteins (Cornejo et al. 2008) such as glomalin. This glycoprotein is produced in the hyphae of AM fungi and may have a role in resistance to soil microorganisms (Purin and Rillig 2007). The molecular structure still has to be established, but it is agreed that it consists of monomeric structures linked through hydrophobic interactions (Nichols 2003), which bind contaminants through functional groups on its structure (González-Chávez et al. 2004; Vodnik et al. 2008). The binding capacity of glomalin is influenced by a variety of factors including soil type, pH, and redox potential (Chern et al. 2007; Nichols 2003). When AM fungi release glomalin in the soil it binds the toxic metals (González-Chávez et al. 2004), therefore fungal strains that produce high amounts of glomalin would be advantageous to bioremediation efforts.

In addition to phytostabilization, another component of remediation is phytoextraction, i.e. the use of plants to concentrate contaminants into the above-ground plant tissue for harvest. Consequently, the removal of plant biomass also removes the contaminants. The harvested plants can be used to produce energy through combustion or metals can be recaptured (phytomining). The largest constraint to phytoextraction is plants that do not produce large biomass or that don't have a high root-to-shoot transfer. A possible way to accelerate the root to shoot transfer and increase biomass is to inoculate host plants with AM fungi. In a study by Leung et al. (2006), arsenic uptake in *Pteris vittata* was highest in AM inoculated plants. However in several cases, if plant and AM fungi were not chosen carefully there was an accumulation of contaminants in the roots which could interfere with efficient phytoextraction (Gohre and Paszkowski 2006).

Phytodegradation is the breakdown of contaminants taken up by plants, achieved through metabolic processes within the plant or the breakdown of contaminants surrounding the plant roots. Ultra et al. (2006) found arsenic was converted to organic forms in the mycorrhizosphere by *Glomus* inoculated sunflower plants, suggesting an active reducing mechanism by the AM fungi.

Rhizodegradation is the stimulation of pollutant-degrading bacteria that live around the water film found on hyphae of AM fungi (Figure 3). The growing hyphae are able to penetrate air-filled pores in the soil matrix that are usually inaccessible to pollutant-degrading bacteria. Polycyclic aromatic hydrocarbons (PAHs) have been shown to be good substances from an energetic perspective, with low molecular mass PAHs becoming readily degraded by bacteria (Johnsen et al. 2005). However it has been shown that as molecular mass increases, so does PAH recalcitrance.

1.7 *Echinacea sp.*

Echinacea sp. was first mentioned in the *Flora Virginica* in 1762 by Clayton, but was known and used by Native American Nations long before the European settlers colonized North America (Barrett 2003; Flannery 1999; Foster 1991). In modern times it has been recognized as an economically important species with an estimated $1.3 billion annual worldwide sales (Blumenthal 2003), and major production occurring in Europe, North America, South America and Australia (Galambosi 2004; Yu and Kaarlas 2004). According to McGregor taxonomy, *Echinacea* consists of 9 species and 2 varieties, with three species used medicinally: *E. purpurea* (L.) Moench, *E. angustifolia* (DC.) Hell and *E. pallida* (Nutt.)

Nutt. However, *Echinacea* was recently reclassified into four species and eight varieties using morphometric analysis (Binns et al. 2002a). All species are drought resilient, herbaceous perennial wildflowers that are grouped within the Asteraceae family (Mistríkova and Vaverkova 2007). Of the three medicinal species, the most studied and well known is *E. purpurea*, which is the focus of my lab and field research. Although most studies have focused on the medicinal benefits of *E. purpurea*, some research has shown *E. purpurea* has bioremediation potential. Liu et al. (2012) found the purple coneflower had one of the highest removal rates of total PAHs among 14 ornamental species.

Echinacea comes from the Greek word "echinos" which translates to urchin or hedgehog, which was likely given due to the flower, the most striking characteristic of *Echinacea sp.*. The flower forms a cone that is composed of many inner florets ending in spines. This is surrounded by a droopy outer ray, which ranges from white to pink, to a deep purple (Mistríkova and Vaverkova 2007). The stalks can reach heights of 1.8 meters with alternate ovate to ovate-lanceolate basal leaves, alternate cauline leaves with coarse hairs and solitary cones with purple bracts (Barrett 2003; Mistríkova and Vaverkova 2007). The roots of *E. purpurea* are different to *E. pallida* and *E. angustifolia*, in that they are fibrous and not tap roots. This allows them greater coverage in the soil in which they grow.

Echinacea, like many other plants are known to produce secondary metabolites in their various plant tissues as a response to herbivory and physiochemical stressors in their environment. These secondary compounds have been used to make natural health products from the roots, shoots and flower heads depending on the species of *Echinacea*. Each of these tissues is made up of various constituents that are thought to have immunological activity in humans (Barnes et al. 2005). Of the three medicinally used varieties, *E. purpurea* has been favoured due to its low production cost, increased biomass per hectare, and ease of cultivation (Barrett 2003; Matthias et al. 2008). *E. purpurea* is known to have over 216 different medicinally active compounds (Murch et al. 2006), consisting of alkamides, caffeic acid derivatives, polysaccharides and alkenes (Barnes et al. 2005). Combinations and concentrations of these compounds are known to differ based on species as well as growing and harvesting conditions (Binns et al. 2002a, b). The only constituents known to be bioavailable to humans are alkamides (Matthias et al. 2004, 2005). Araim et al. (2009) found that the concentrations of proteins and phenolics produced in the roots of *E. purpurea* were increased with inoculation of *Glomus intraradices.*

1.8 Victoria Island, Ottawa

Victoria Island is situated within the Ottawa River, and at the boundary of Ottawa, Ontario and Gatineau, Québec. The majority of Victoria Island is occupied by green space with grass-covered land and some shrubs and trees. The sites geological condition consists of bedrock ranging from depths of 0.2 to 5.3 meters and made up of fractured grey crystalline limestone with narrow black shale horizons. Above the bedrock lies heterogeneous fill materials and a discontinuous layer of heterogeneous clay and gravel till. Groundwater is found within locally saturated zones where thicker overburden intervals are present and within the fractured bedrock.

For nearly a century, from the late 1800’s until 1960 the site has served as an industrial area. Industrial activities have included foundries, pulp and paper mill, a scrap iron company, a calcium carbide factory, and housed military buildings and offices. In the early 1960’s, the National Capital Commission (NCC) acquired Victoria Island as part of the

LeBreton expropriation. Since the expropriation most industrial activities, with the exception of the hydroelectric power station on the western portion of the island, have been abandoned. The site is predominantly urban parkland and includes an Aboriginal Experience area. The southeastern portion of the island has served as a traditional meeting area/stopover beneath the Chaudière Falls for the First Nations community who used the waterway route for trade/travel.

1.9 Rationale, Objectives and Hypotheses

This research has focused on the use of AM fungi with echinacea plants in the remediation of soils contaminated with PAHs, alkyl PAHs and toxic metals from Victoria Island, Ottawa, ON. My first objective was to determine the combined effects of *Glomus intraradices* and *Echinacea purpurea* on the uptake of PAHs, alkyl PAHs and toxic metals in a greenhouse experiment. My second objective was to conduct the same experiment under field conditions at the contaminated site on Victoria Island, Ottawa.

The goal of this study was to determine whether AM fungi would increase the mass of contaminants in *Echinacea purpurea*, thus becoming a better bioremediation strategy than the use of plants alone.

My three hypotheses were:

1) The presence of AM fungi increases the uptake of PAHs, alkyl PAHs and toxic metals by *Echinacea purpurea* through increased absorption and adsorption via AM hyphae in a greenhouse environment.
2) The presence of AM fungi increases the uptake of PAHs, alkyl PAHs and toxic metals by *Echinacea purpurea* under field conditions on Victoria Island.

3) The presence of AM fungi decreases the concentration of PAHs, alkyl PAHs and toxic metals in soil planted with *Echinacea purpurea.*

Chapter 2

Greenhouse Study: Bioremediation of PAHs, alkyl PAHs and toxic metals from aged-industrial soil

2.1 Introduction

Soil contamination with organic and inorganic pollutants is increasing with industrialisation, intensive agriculture, and large-scale use of xenobiotic compounds (Harms et al. 2011; Rayu et al. 2012). Exposure to these contaminants poses a significant risk to human and ecological health (Singh 2009; Suresh and Ravishankar 2004) and the need for remediating harmful contaminants has become a major priority for most countries. To date, bioremediation has been recognized as one of the most cost-effective, reliable and promising technologies for the remediation of contaminated soils (Gao and Zhu 2005; Juwarkar and Jambhulkar 2006; Suresh and Ravishankar 2004). Initial estimates by Glass (1999) considered a market of $34-54 billion U.S. worldwide, however, nothing near this has materialized in the past decade (Conesa 2012).

Bioremediation exploits plants and microbes for the decontamination of polluted sites. Plants can facilitate enhanced degradation of organics and increased uptake of inorganics by creating favourable conditions for microbial degradation, and accessing contaminants through their root system (Gao et al. 2011; Suresh and Ravishankar 2004). Plant-microbe associations are gaining considerable attention with enhanced remediation, positive effects on plant establishment and survival of plants in contaminated soil (Jeffries et al. 2003; Joner et al. 2001; Meier et al. 2011). In particular, arbuscular mycorrhizal (AM) are of interest due to their formation of extraradical hyphae, which have access to fine soil pores that are unavailable to plant roots (Gao et al. 2010).

AM fungi are complex, ancient organisms that are ubiquitous in nature, found in over 90% of vascular plants and more than 80% of all terrestrial plants (Wang and Qui 2006). Their presence is deeply involved in all aspects of soil ecology and they have a huge impact, both spatially and temporally on all biomes. They play a fundamental role in nutrient acquisition, tolerance mechanisms, microbial communities, carbon cycles and host success in all environments (Willis et al. 2013). AM fungi have been shown to increase uptake of both organic and inorganic contaminants from soil. In previous studies by Cheng et al. (2008) and Debiane et al. (2009), alfalfa roots colonized by AM fungi had increased PAH accumulation. Further studies by Gao et al. (2010) have shown AM fungi increase the uptake of PAHs in the roots of ryegrass.

Polycyclic aromatic hydrocarbons (PAHs) and their alkyl homologs are organic pollutants that are of particular environmental concern (Harms et al. 2011). These contaminants are persistent and occur due to natural and anthropogenic activities. They are the focus of many bioremediation programs due to their acute toxicity, carcinogenicity, mutagenicity, teratogenicity, their effects on endocrine function, their persistence and bioavailability, and their prevalence in the environment (Cerniglia 1992; Freeman and Cattell 1990; IARC 1983; Sudip et al. 2002; U.S. Dept. of HHS 1995). PAHs consist of two to seven fused benzene rings that are arranged in various structural configurations. Alkyl PAHs usually have one to four saturated carbon atoms and can produce many different structural isomers and homologs for each hydrocarbon family. PAHs can be separated into pyrogenic and petrogenic classes. Pyrogenic PAHs are derived from incomplete combustion of organic molecules, and petrogenic PAHs are derived from oil (Freeman et al. 1990; Lim et al. 1999; Manzetti 2013). The type of PAH, petrogenic or pyrogenic, is also linked to the temperature at which these compounds are formed. Low temperatures form petrogenic PAHs characterized by alkyl-substitutions on the rings, whereas high temperatures form pyrogenic PAHs without alkyl substitutions on the rings (Lima et al. 2005). Their hydrophobic nature leads to increased accumulation and enrichment in soils which is cause for remediation of contaminated sites.

Toxic metals are another group of contaminants that are of concern. Currently, the U.S. EPA considers 13 metals and metalloids priority pollutants. These are derived from natural sources such as the Earth's crust and atmosphere or anthropogenic sources including mining, electronics, agriculture and industry (Adriano 2001; Sinicropi et al. 2009). Some metals are considered "essential" such as copper (Cu), zinc (Zn), iron (Fe), nickel (Ni), and manganese (Mn), and are used for the catalyzing of various enzymatic and redox reactions (Zenk 1996). In contrast, there are also "non-essential" metals such as chromium (Cr), lead (Pb), and arsenic (As), which are known as toxic. However, for both classifications, the concentrations determine toxicity (Sinicropi et al. 2010). At high concentrations "essential" metals can interfere with enzyme function and protein structure (Miransari 2011). Elemental contaminants are immutable and thus cannot be broken down into completely non-toxic forms. The only strategy is to accumulate the metals into plant tissues in a process known as hyperaccumulation. In order for plants to detoxify their environment, they either release root exudates to chelate metals, or produce intracellular phytochelatins and metallothioneins which possess a high affinity for metals that are then sequestered in their vacuoles (Hall

2002). Taking advantage of this process can create a viable option for remediating contaminated soils.

The aim of this study was to evaluate the use of AM and non-AM plants for the bioremediation of soils contaminated with PAHs, alkyl PAHs and toxic metals. Industrial contaminated soils from Victoria Island, Ottawa were provided by the National Capital Commission. The purple coneflower was selected based on previous experiments by Liu et al. (2009) who screened ornamental plants for high effectiveness in remediating PAH contaminated soil. They found the purple coneflower (*Echinacea purpurea*, L.) had one of the highest removal rates of total PAHs among 14 ornamental species. Further advantages of using *E. purpurea* were its positive response to a commercially available inoculum of *Glomus intraradices*, an AM fungi (Araim et al. 2009), its drought tolerance and its fibrous root system, which allows greater contact with soil (Mistíkova 2007). Using a factorial design greenhouse experiment, we predicted that AM inoculation would increase the uptake of PAHs, alkyl PAHs and toxic metals in *Echinacea purpurea* through enhanced bioaccumulation via AM hyphae.

2.2 Materials and Methods

2.2.1 Experimental Design

A 20-week greenhouse study was conducted (March to August 2013) using *Echinacea purpurea* (L.) Moench, inoculated with or without AM fungi, and grown in homogenized soil samples collected by NCC from ten test pits (1m deep) from Victoria Island, Ottawa, ON (45° 25’ 15” N, 75° 42’ 50” W). The soil characteristics were analyzed for its content of carbon, nitrogen, organic matter, pH, mineral content, percent native AM, metals, PAHs and alkyl PAHs.

2.2.2. Inoculation of Plants

Echinacea purpurea plants were grown from seeds (Ontario Seed Company, Waterloo, ON) for 20 weeks in soil from Victoria Island that was premixed from 10 test pits (each 1 meter deep) and sown with or without AM fungal propagules of *Glomus intraradices* Schenck and Smith, DAOM 181602 (MYKE PRO Greenhouse-G, Premier Tech, Rivière-du-Loup, QC). The fungal inoculum was integrated as a 3-cm thick substrate layer on top of 1 L of soil, and then covered with soil as determined by Audet and Charest (2012). Eight seeds were sown ~1cm deep in the soil mixture and thinned after 2 weeks to one plant per pot as determined by Araim et al. (2009). The seeds were previously surface sterilized by a 10% (v/v) solution of hydrogen peroxide for 10 min, rinsed with sterile distilled water and pre-germinated on moist filter paper overnight before sowing.

2.2.3. Pot Experiment

The factorial design of the study [i.e. 1 plant sp. x 2 treatments (non-AM and AM) x 2 harvests (week 10 and week 20) x 5 reps] provided a total of 20 plants, 1 per pot. Five replicates per harvest of un-seeded pots with soil from Victoria Island were also collected to compare contaminant concentrations over time in bare soil. The greenhouse conditions were maintained with a photoperiod of 16:8 L:D provided by natural light and high-pressure sodium lamps (PL Light Systems, Beamsville, ON, Canada), a day/night temperature regime of approximately 27˚C/23˚C and a relative humidity of 40%. The average light intensity

(408 µmol s^{-1} m^{-2}) was measured using a light meter with a quantum sensor (LiCor LI-250A and LI -190SA, Lincoln, NE).

2.2.4. Watering and Fertilization

Plants were watered with dH_2O on a daily basis as required, without water leaking through the pots. Fertilization began after the third week following germination with 20 mL of ½ ammonium nitrate type Long Ashton Nutrient Solution (LANS) (Hewitt and Smith 1975), once a week for two weeks (the 4th and 5th weeks). The plants were then fertilized with the full LANS (50 mL at the 6th week, and 100 mL until harvest). The nutrient solution contained 2.0 mM K_2SO_4, 4.0 mM $CaCl_2$ anhydride, 1.5 mM $MgSO_4 \cdot 7H_2O$, 1.5 mM $NaH_2PO_4 \cdot H_2O$, 5.0mM NH_4NO_3, 0.01 mM $MnSO_4 \cdot 4H_2O$, 1.0 µM $CuSO_4 \cdot 5H_2O$, 1.0 µM $ZnSO_4 \cdot 7H_2O$, 0.05 mM H_3BO_3, 0.09 mM NaCl, 0.5 µM $Na_2MoO_4 \cdot 2H_2O$ and 5.7 ppm EDTA-Fe.

2.2.5. Sample Preparation

Plants were harvested at 10 and 20 weeks of growth. Any part of the plant above the soil surface was considered shoot material, and anything below was included as the roots. In order to obtain all root material, the soil was carefully sieved and added to root samples. Roots were then washed with distilled water to remove any soil particles, and blotted dry and weighed. Every harvest, five pots were collected for each treatment; three pots per treatment were analyzed with the rest frozen if needed for further analysis.

2.2.6. Pre-experimental Soil Analyses

To determine the pre-experimental mineral content of the soil, 2 g of soil was dissolved in 10 mL of 1:1 HNO_3 and Milli-Q H_2O. Samples were heated on a graphite block (DigiPREP MS block digestion system, SCP Science, QC, Canada) for 30 minutes at 90 °C. Once cooled to room temperature 30% H_2O_2 was added and then heated at 90 °C for 3 hours. Samples were then diluted with ultra pure Milli-Q H_2O up to 50 mL mark. All samples were filtered using a 45μm *Digi*Filter (SCP Science, QC, Canada) to remove any particulate. Filtered samples were then diluted 10:1 using ultra pure Milli-Q H_2O. In the same manner, standard reference material (Buffalo River Sediment #8704) from the National Institute of Standards and Technology (NIST, USA) and blanks consisting of HNO_3, H_2O_2 and Milli-Q H_2O were also prepared to ensure quality and accuracy of the metal analysis. The digested extracts were then analyzed by Dr. N. De Silva at the University of Ottawa using ICP-ES using an Agilent ICP-MS 7700 series, which was run using a reactive gas for the removal of known interferences in a complex unknown mixture without loss of sensitivity.

Soil pH was analyzed directly from 10 g samples suspended in dH_2O with a soil:solution ratio of 1:2 (*m/v*) according to Hendershot et al. (2008).

2.2.7. Percent Organic Carbon and Nitrogen

Percent organic carbon and nitrogen were determined at the G.G. Hatch Stable Isotope Lab, University of Ottawa. The determination of percent organic carbon and nitrogen was done using CO_2 and N_2, produced by combustion on a VarioEL III Elemental Analyser followed by "trap and purge" separation and on-line analysis by continuous-flow with a DeltaPlus Advantage isotope ratio mass spectrometer coupled with a ConFlo II.

2.2.8. Native Soil AM Analyses of Victoria Island

The native AM fungal species in the soil samples taken from Victoria Island were identified by Dr. Y. Dalpé, Agriculture and Agri-Food Canada, Ottawa. Soil samples from 0.1m -0.4 m deep at each of the ten test pits were weighed out to 10 g. Each sample was sieved through 300, 150 and 38 µm sieves. Sieved soil was recovered, distributed into centrifugation tubes and mixed with 10 mL of water. Using a syringe, 50% sucrose solutions were added to the bottom of the centrifuge tubes. Samples were centrifuged at 2500 rpm for 4 minutes. After centrifuging, the supernatants were recovered and spores concentrated with a 30µm sieve. Spores were suspended into 10 mL of water, and poured into 9 cm diameter Petri dishes and then mounted on slides. Recovered spores were observed under a compound microscope (Eclipse-Nikon) at 60x for identification.

2.2.9. Assessment of AM Root Colonization

Root samples were cleaned, rinsed and stained with an aniline blue 0.02% dye solution (6.78 mM aniline blue, 500 mL glycerol, 450 mL H2Od, and 50 mL 1% HCl) according to Dalpé (1993). Fifty root segments, ~1–2 cm long, were mounted on slides (10/slide) and examined at 10x and 40x magnification using a compound microscope. AM colonization was estimated by determining the counts and relative density of fungal structures evidenced by the presence of hyphae, vesicles, arbuscules or spores that provide insight distribution and intensity of AM root colonization (Allen 2001). The formulas for relative density is defined as:

$$\frac{\#\ Structures_{AM}}{Root\ Length_{AM}} \qquad (1)$$

2.2.10. Analysis of Polycyclic Aromatic Hydrocarbons (PAHs), and Alkyl PAHs

All plant and soil samples were homogenized with elemental copper and Hydromatrix^{TM} diatomaceous earth (Varian Inc., Palo Alto, CA, USA). Samples were then spiked with ^{13}C labeled PAHs (Cambridge Isotope Laboratories Inc., Andover, MA, USA) and extracted using accelerated solvent extraction module (ASE-350, Dionex Corporation, Sunnyvale, CA, USA) at 140°C using 1:1 hexane: acetone mixture following methods US EPA Method 3640A. Extracts then underwent liquid-liquid extraction with hexane to remove organic compounds from the co-extracted water. Following liquid-liquid extraction, extracts were concentrated using a TurboVap (Biotage, Charlotte, NC, USA) under a gentle nitrogen stream. Clean up with US EPA Method 3630C was adapted for use on 6ml (1g) Superclean^{TM} LC-Si solid-phase extraction cartridges. Samples were further concentrated to approximately 1 mL, which was the final extract volume for all samples. Internal standard p-terphenyl-d14 was added to all final extracts.

Analysis of the final extract was done by injecting 1µL of sample into an HP 6890 gas chromatograph) coupled with a HP 5973 N (Agilent Technologies, Santa Clara, CA, USA) mass selective detector. Separation was completed on a DB5-MS 30 m x 0.250 µm x 0.25 µm column (Agilent Technologies) with H_2 as the carrier gas.

All compounds monitored for PAHs and alkyl PAHs are detailed in Appendix D, Table D.1, and all method limits of quantification were based on a signal to noise ratio of 3:1. All samples were blank corrected to remove background contamination, and replicate extractions were carried out on Standard Reference Material ® (SRM) 1941b – Organics in

Marine Sediment from the National Institute of Standards and Technology (Gaithersburg, MD, U.S.A.).

2.2.11. Total Metal Analyses

After the 10 and 20-week growth period, metals were determined using ICP-MS. Samples of root, shoot and soil were individually digested using a hot nitric acid solution following a modified version of US-EPA method 3050. This method allows the determination of total recoverable analytes in solid samples and will not allow complete dissolution of samples. Total metals were then analysed from the acid extracts following US-EPA method 200.8. Samples were dissolved in 10 mL of 1:1 HNO_3 and HCl and heated on a graphite block (DigiPREP MS block digestion system, SCP Science, QB, Canada) for 30 minutes at 90 °C. Once cooled to room temperature, 30% H_2O_2 was added and then heated at 90 °C for 3 hours. Samples were then diluted with ultra pure Milli-Q H_2O up to 50 mL mark. All samples were filtered to remove any particulate. Filtered samples were then diluted 10:1 using ultra pure Milli-Q H_2O. In the same manner, standard reference material (Buffalo River Sediment #8704) from the National Institute of Standards and Technology (NIST, USA) and blanks consisting of HNO_3 and HCl were also prepared to ensure quality and accuracy of the metal analysis. The digested extracts were then analyzed using an Agilent ICP-MS 7700 series, which was run using a reactive gas for the removal of known interferences in a complex unknown mixture without loss of sensitivity.

2.2.12. k_1 Uptake Rate of PAHs in Roots

The rate of uptake for PAH compounds in AM and non-AM roots were determined using the following formula that was modified from Gobas and Morrison (2000):

$$k_1 = \frac{(dCr/dt)}{Cs} \qquad (2)$$

Where k_1 is the rate constant for the uptake of the PAH compound in units day^{-1}. dCr is the initial change in concentration of PAH compound in the root, dt is the time interval (days) and Cs is the soil concentration. This formula assumes no significant depuration or loss of PAH by excretion of metabolism, so we calculate the uptake during the first 10 weeks of exposure when these losses are minimized due to low PAH concentrations in the roots.

2.2.13. Statistical Analyses

One and two-way ANOVA's were performed for native AM fungi, PAH concentrations, and metal concentrations. An independent t-test was used for AM colonization and simple linear regressions were used for K_1 uptake rates. Comparison among means for the ANOVA's was performed using Tukey's test. Shapiro-Wilk and Levene's tests were used to verify the normality of distribution and the homogeneity of variance. The data were log transformed as required to meet the assumptions of parametric analysis. All statistical analysis was done using R statistical software (version 3.1.2).

2.3 Results

2.3.1. pH, Isotope and Mineral Content of Soils

The pre-experimental soil that was homogenized from Victoria Island showed a slightly acidic pH of 6.77 ± 0.10, with a 4.01 ± 0.44 % carbon and a 0.15 ± 0.11 % nitrogen.

Mineral soil composition showed the most abundant elements were Ca, P, and Ti (42,400 ±603.74 mg kg^{-1}, 1,040±28.4 mg kg^{-1}, 631±282.19 mg kg^{-1}, respectively), and the least abundant were Fe (13.8 ± 0.23 mg kg^{-1}), Na (0.315 ± 0. mg kg^{-1}) and Sr (0.0899 ± 0.001 mg kg^{-1}), as summarized in Table A.1.

2.3.2. Soil AMF Analyses

The predominant native species of AM fungi identified in the soil sampled from Victoria Island were *Funneliformis geosporum* (41%), *Glomus constrictum* (39%) and *Funneliformis mosseae* (13%). The lowest were *Glomus rubiforme* (4%), *Scutellospora calospora* (2%) and *Acaulospora cavernata* (1%) (Figure A.1). A one-way ANOVA was conducted to compare the percent AM fungal spores between species in ten test pits. There was a significant effect among the species of AM fungal spores [F (5, 54)=31.8, P<0.001)]. Post hoc comparisons using the Tukey HSD test indicated that *Glomus rubiforme*, *Scutellospora calospora* and *Acaulospora cavernata* were not significantly different, whereas there was a significant difference in *Funneliformis geosporum* (group b) *and Glomus constrictum* (group c). *Funneliformis mosseae* shared similarities with both groups a (*Acaulospora cavernata, Glomus rubiforme, and Scutellospora calospora*), and c (*Glomus constrictum*).

2.3.3. AM Root Colonization

Overall, mycorrhizal colonization was found in both the inoculated and non-inoculated roots following weeks 10 and 20 (Figure 2.1). However, the inoculated plants consistently had higher colonization counts shown in the formation of hyphae, vesicles and spores. Week 10 shows AM plants have ~4 times greater relative density of hyphae and ~14 times more vesicles. Non-inoculated AM roots at week 10 had no spores relative to AM roots which had 0.01 $mm^{-1.}$ At week 20, the AM structures decreased in density, but were still higher than in non-AM roots. There were ~14 times more hyphae and ~5 times more vesicles density in AM roots when compared with non-AM roots. Spores had a higher density in non-AM roots (0.02 mm^{-1}) compared with AM roots. The colonization counts are summarized in Table B.1 for week 10 and week 20. Although there were large differences in AM structures, there was only significantly more spores in AM treated roots at week 10 ($F(1,4) = 10.3$, $p<0.05$) and hyphae in AM treated roots at week 20 ($F(1, 4) = 80.1$, $p<0.001$).

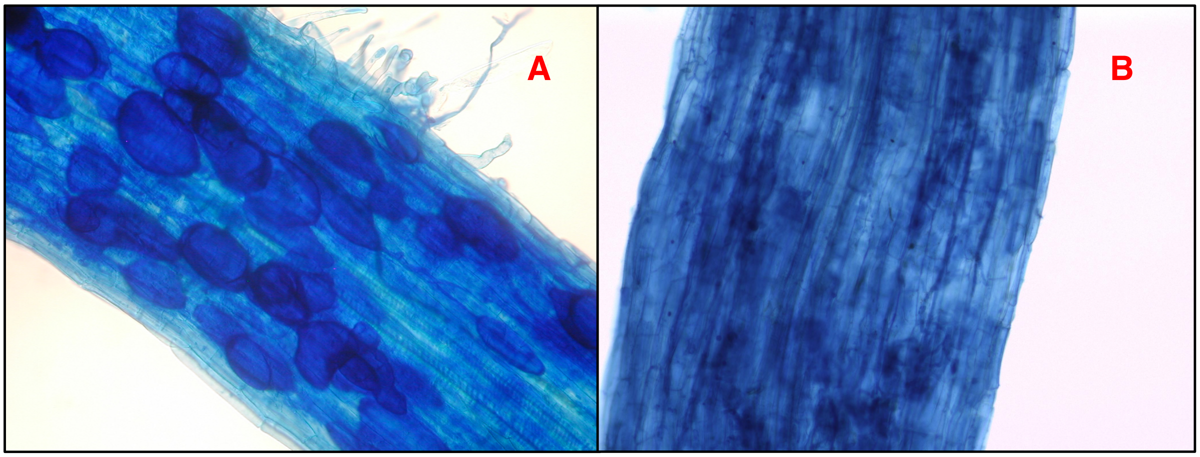

Figure 2.1: Representative microscope images of AM (A) and non-AM (B) *Echinacea purpurea* roots stained with aniline blue solution shown at 40X magnification.

2.3.4. Polycyclic Aromatic Hydrocarbons (PAHs) and Alkylated PAHs

Root and shoot PAH and alkyl-PAH concentrations (ng g^{-1}) were multiplied by mass (g) of respective tissue weights to find the content of PAHs and alkyl PAHs in each. Soil was measured as concentration (ng g^{-1}) dry weight. The treatments were taken in triplicate. All compounds analyzed are listed in Appendix D. One-way ANOVAs were run for hydrocarbon content (ng) vs. treatment for roots and shoots, and hydrocarbon concentration (ng g^{-1}) vs. treatment for soil from week 10 to week 20.

Soil ΣPAH concentrations were significantly different for each treatment (p=0.002) over the course of the greenhouse experiment as seen in Figure 2.2. Control soil had the lowest mean concentration at week 10 (6830 ± 134 ng g^{-1}), while AM soil had the highest mean concentration at week 20 (14,000 ± 1020 ng g^{-1}). Soil showed no significant differences between the three treatments in ΣAlkyl PAH concentrations over the course of the greenhouse experiment (Figure B.1).

ΣPAH content that accumulated in roots was significantly different between AM and non-AM roots (p=0.006) over the 20 week greenhouse experiment. Examination of the mean content of ΣPAHs in AM roots shows AM roots accumulated ~23 times more at week 10 and ~4 times more at week 20 compared to non-AM roots (Figure B.2). *E. purpurea* shoots did not show significant accumulation (p=0.16) of ΣPAHs over the 20 week experiment between AM and non-AM treatments.

Root ΣAlkyl PAHs content was significantly different for each treatment (p=0.002) over the course of the greenhouse experiment. AM roots had the highest accumulation of ΣAlkyl PAHs at week 20 as seen in Figure 2.3. ΣAlkyl PAHs had accumulated ~4.5 times more in AM roots (107, 000 ± 14,400 ng) to non-AM roots (24, 800 ± 9490 ng) at 20 weeks.

ΣAlkyl PAH content showed no significant difference between treatments in the shoots of *E. purpurea* (p=0.31) over the 20-week experiment (Figure 2.3).

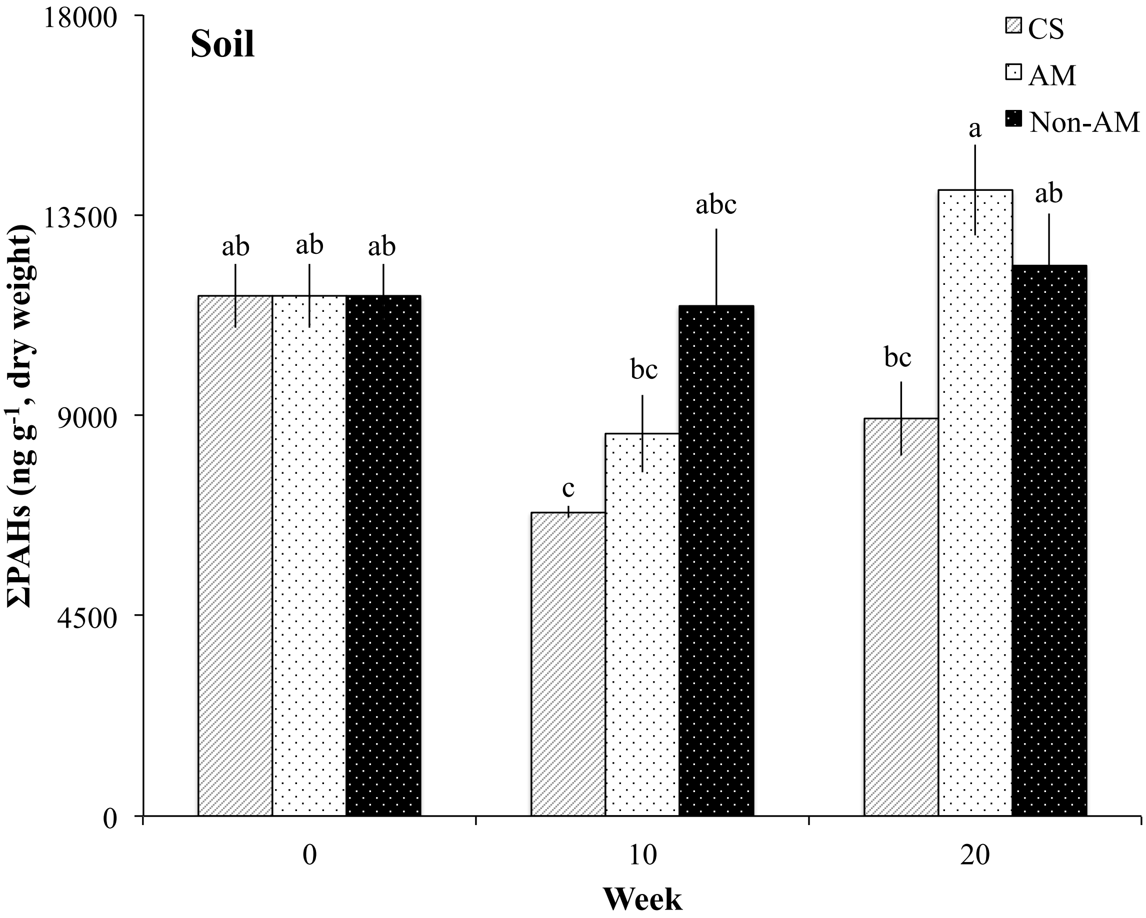

Figure 2.2: Mean (±SE) total PAH content (ng g^{-1}) over 20 weeks of growth in soil in the greenhouse. Data was analyzed using a one-way ANOVA [F(8, 18)=5.3, p=0.002]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (p=0.24) and Levene's test for homogeneity of variance (p=0.89). CS: Control Soil, AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

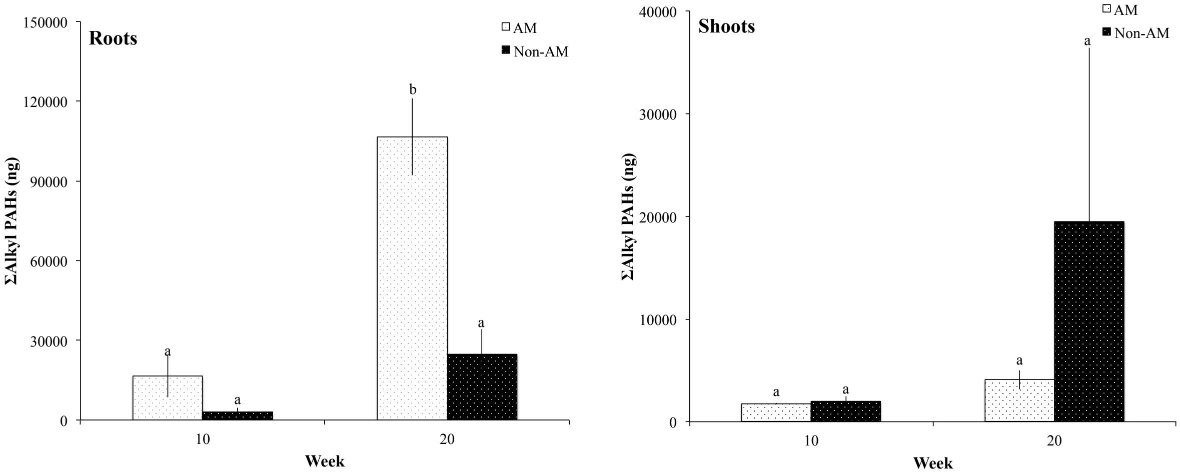

Figure 2.3: Mean (±SE) ΣAlkyl PAHs (ng) in roots and shoots over 20 weeks of growth in *Echinacea purpurea* in the greenhouse. Data was analyzed using a one-way ANOVA for roots [F(3, 8)=23.9, p=0.0002] and shoots [F(3, 8)=1.4, p=0.31]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (SW) and Levene's test (LT) for homogeneity of variance in roots (SW p=0.008, LT p=0.36) and shoots (SW p=0.000003, LT p=0.44). AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

2.3.5. k_1 Uptake Constant

The k_1 rates for AM roots show a significant negative relationship (slope= -0.17, r^2=-0.47, $p<0.05$) with log K_{OW}, indicating that accumulation rates are slower for more hydrophobic compound when AM are present (Figure 2.4). Non-AM roots had ~10 x lower $k_1$values compared to AM roots, and were not correlated with log K_{OW}. Fluorene showed the greatest uptake rate at 0.02 day^{-1} in AM roots at week 10 with a log K_{OW} of 4.02. Benzo[k]fluoranthene had the highest uptake rate for non-AM roots at week 10 at 0.001 day^{-1} at a log K_{OW} of 6.11.The lowest uptake at week 10 was found to be benzo[g,h,i]perylene with a rate of 0.003 day^{-1}for AM roots and 0.0004 day^{-1}at a log K_{OW} of 6.7.

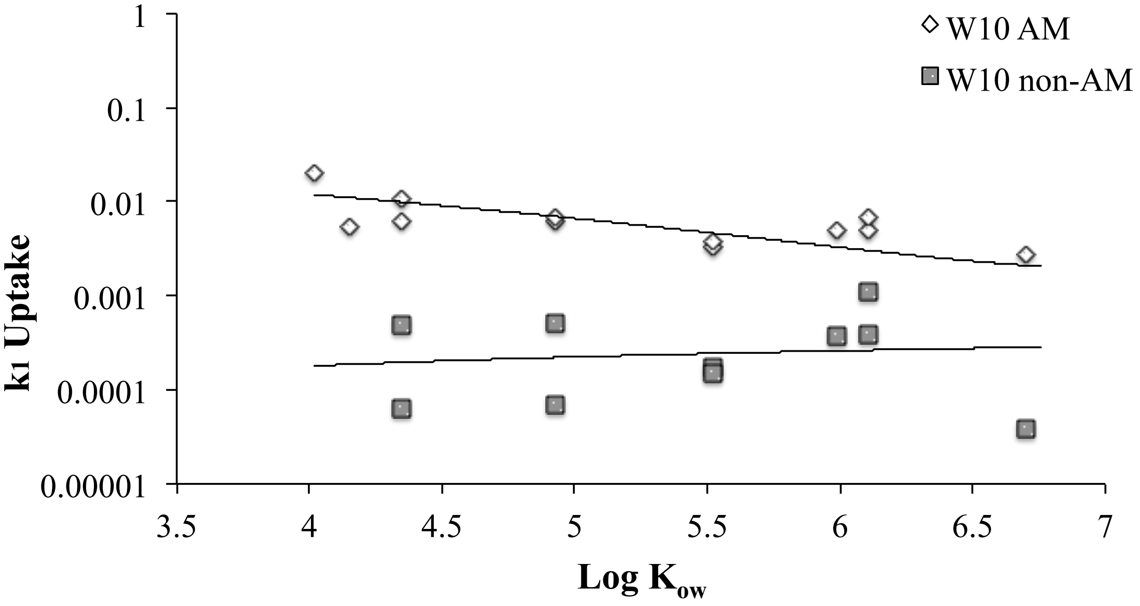

Figure 2.4: Uptake rates on a logarithmic scale measured in roots of AM and non-AM plants during week 10 (W10) of the greenhouse experiment plotted against the Log K_{OW}'s of selected PAHs. AM roots show a significant negative relationship (slope= -0.17, r^2=-0.47, p=0.014) while non-AM roots showed no significance (slope=0.0095, r^2=0.00026, p=0.97). Log K_{OW} are given for the following PAHs: acenaphthene, fluorene, phenanthrene anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3- cd]pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene.

2.3.6. Metals

Root and shoot metal concentrations (mg kg^{-1}) were multiplied by mass (kg) of respective tissue weights to find the metal content (mg). Soil was measured as concentration (mg kg^{-1}) dry weight. The treatments were taken in triplicate. All compounds analyzed are listed in Appendix B. Two-way ANOVAs were run for metal content (mg) vs. treatment for roots and shoots, and metal concentration (mg kg^{-1}) vs. treatment for soil from week 10 to week 20 (Table B.4).

Overall, the concentration of Sb in soil was significantly reduced ($p<0.01$) between week 10 and week 20, whereas the other soil metal concentrations showed no significant change over the 20-week period or between treatments. Week 20 soil metal concentrations are shown in Figure 2.5.

The content of Al, As, Cd, Co, Cr, Ni, Se, Th, and V in *E. purpurea* roots significantly increased between week 10 and week 20 ($p<0.05$), but there was no significant difference between treatments (Figure 2.6).

Cd and Mo showed significant reduction ($p<0.05$) in the shoots of *E. purpurea* between week 10 and week 20 with no significance between treatments (Figure 2.7).

The interaction between week and treatment was significant for Cu ($p<0.01$), Cr, Mn, Ni, and Sb ($p<0.05$) in the shoots of *E. purpurea*. Examination of the mean content of each metal showed AM treatments increased from week 10 to week 20, and non-AM treatments decreased from week 10 to week 20. Ni was the only metal that showed a decrease in metal content for both AM and non-AM treatments from week 10 to week 20.

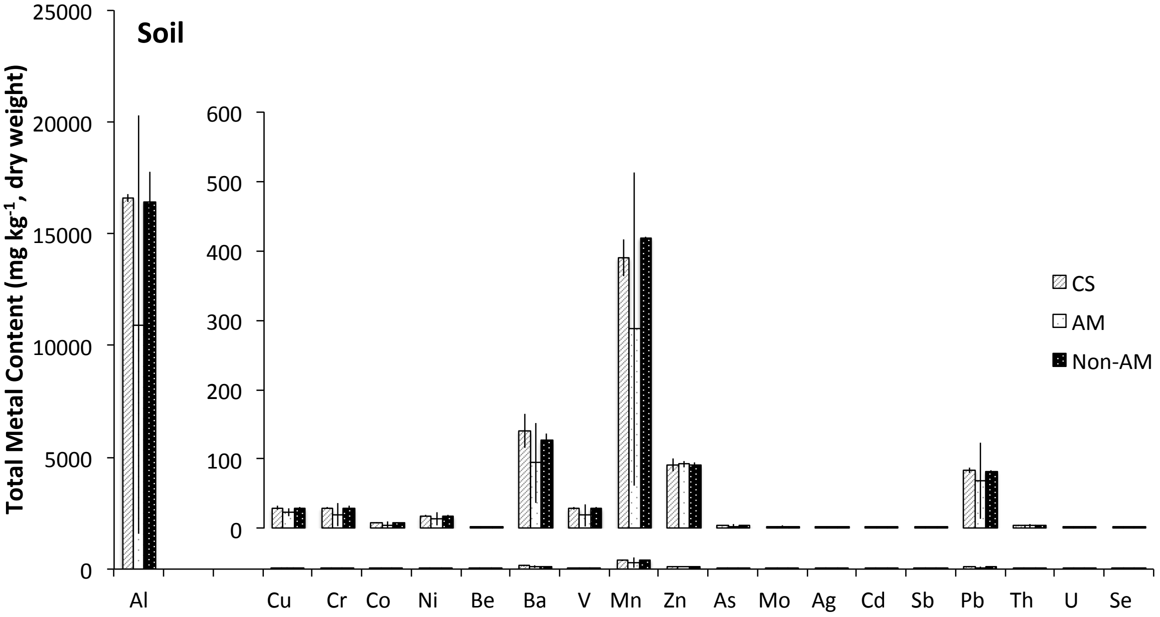

Figure 2.5: Mean (±SE) total metal content (mg kg^{-1} dry weight) in soil samples from week 20 for the greenhouse experiment. CS (Control Soil), AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

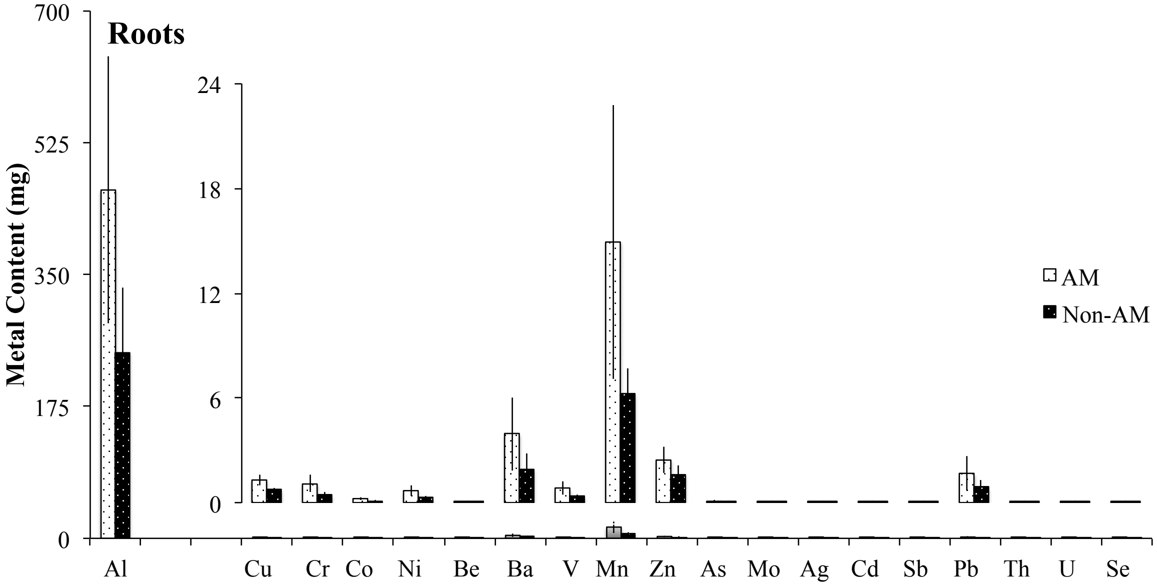

Figure 2.6: Mean (±SE) metal content (mg dry weight) in root samples from week 20 from the greenhouse experiment. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

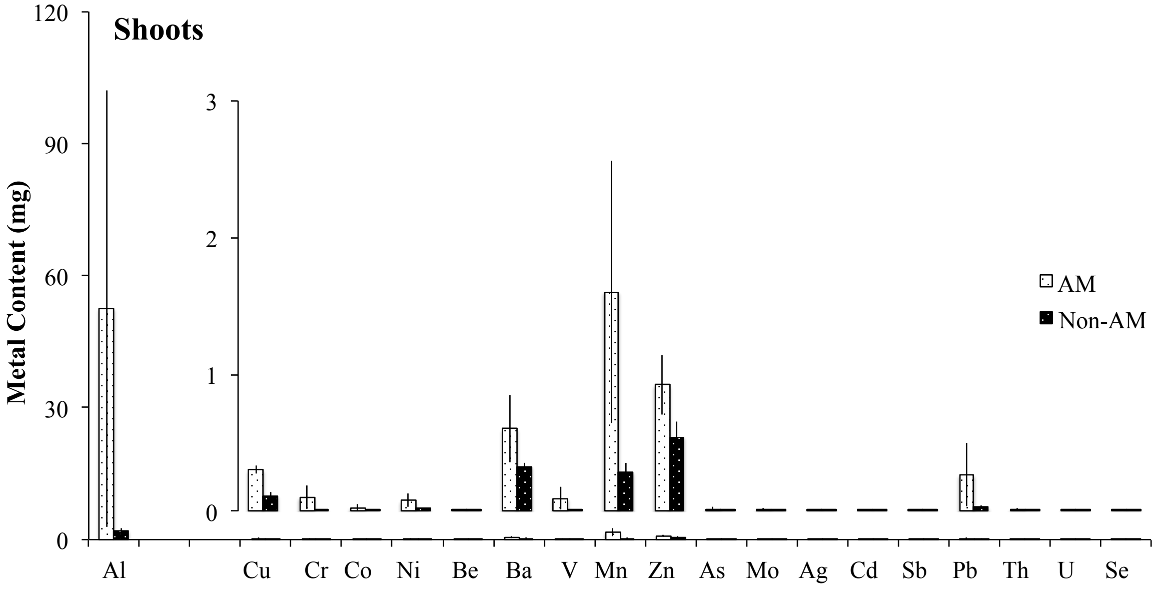

Figure 2.7: Mean (±SE) metal content (mg dry weight) in shoot samples from week 20 from the greenhouse experiment. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

2.4 Discussion

This study investigated whether AM fungal innoculation would enhance the uptake of PAHs, alkyl PAHs and toxic metals in *Echinacea purpurea* from contaminated soil. Our first findings show *E. purpurea* inoculated with *G. intraradices* increased the uptake of PAHs and alkyl PAHs compared to non inoculated *E. purpurea*. Twenty weeks after the inoculation and growth of *E. purpurea,* roots accumulated PAHs and alkyl PAHs four times higher compared to non-AM roots. However, alkyls PAHs were the only contaminant that showed significantly higher uptake after 20 weeks in inoculated roots. There was no difference among treatments in shoot concentrations, even though measurable amounts were found. We also showed inoculation of *E. purpurea* with AM fungi did not appear to increase the uptake of metals. However, the number of weeks was an important factor for the uptake of metals. This is supported by our observation that the mean metal content increased in the roots from week 10 to week 20 regardless of the treatment.

Increasing content of PAHs in *E. purpurea* due to inoculation with *G. intraradices* can be explained through several mechanisms that have been found in the literature. The first mechanism is increased absorption with AM inoculated *E. purpurea*. AM fungi act as an extension of the roots and increase the surface area of the root system, making it more efficient for absorption of contaminants through simple diffusion. This is the same system used to exchange nutrients and carbohydrates between AM fungi and the plant (Finlay 2008). The increased root surface area can enhance uptake of contaminants with increasing soil concentrations of PAHs or alkyl PAHs. In other studies, it has been shown that plant concentrations of PAHs generally increase with increasing soil PAH concentrations (Gao and Ling 2006; Sung et al. 2001). This holds true for our experiment when looking at PAHs but not for alkyl PAHs. PAH concentrations in the soil were higher than alkyl PAHs in the soil at the start of the experiment. After 10 weeks, the concentrations of PAHs in plants would be expected to follow the same pattern based on simple diffusion, but instead the highest accumulation rates in roots were observed for alkyl PAHs, followed by parent PAHs. This trend continued into week 20, which means that something other than simple diffusion must be occurring. Measuring the uptake rate between both treatments, AM inoculated roots had higher uptake rates for all parent PAHs.

Another mechanism to explain the higher PAHs and alkyl PAHs in roots with AM treatments could be the release of exudates from the roots or AM fungi. Exudates could be enhancing the bioavailability of alkyl and parent PAHs, which supports current findings (Banks et al. 1999; Binet et al. 2000; Thomas et al. 2012). The root exudates could cause a transformation of the alkyl and parent PAHs metabolically or co-metabolically (Pinedo-Rilla et al. 2009), using exo-enzymes to make them water soluble (Johnsen et al. 2005). Although the roots of *E. purpurea* had high concentrations of parent and alkyl PAHs, the shoots showed very low concentrations with the exception of alkyl PAHs. Heitkamp and Cerniglia (1987) and Garrett et al. (1998) found that alkyl-substituted PAHs degrade slower than parent compounds. This could explain why such high concentrations were found in the plant tissues. Our study was not designed to determine metabolic transformation of the PAHs into other compounds.

Our study not only measured whether AM fungi would enhance the uptake of PAHs, alkyl PAHs and toxic metals in *E. purpurea,* but investigated whether contaminants concentrations in the soil were decreasing over time. By week 20 soil planted with *E. purpurea* and inoculated with AM fungi had an increase in PAH concentrations that exceeded pre-experimental concentrations (Figure 2.2). PAH concentrations in the soil declined at week 10 for all three treatments with significant difference shown for control soil. At week 20, AM treated soil had the highest concentration of PAHs followed by non-AM soil, and control soil respectively. Originally, we thought increasing soil PAH concentrations could be attributed to carbon reduction in the soil, thus leading to the phenomenon of "solvent depletion" described by MacDonald et al. (2002). If the soil carbon pool or mineral content were decreasing, contaminant concentrations would gradually increase due to the diminishing supply of organic carbon. This was not found to be the case because organic matter content in the soils remained relatively stable (3.30-3.75%) over the course of the experiment. These results suggest that plants may be releasing contaminants back into the soil matrix, which could have major implications for the viability of bioremediation strategies.

Our studies on soil metal uptake by *E. purpurea* showed that metal levels were relatively unaffected by the presence or absence of the AM fungus, but were significantly affected by duration of the exposure in soil. More than 60% of the toxic metals responded significantly to week as a factor and less than 40% of the compounds showed no significance with any of the factors tested. The 'enhanced uptake' (Audet and Charest 2006; Davies et al. 2001) hypothesis, which predicts that AM inoculated plants have a greater metal uptake when compared to non-AM inoculated plants is not supported in our study. This could be explained by non-AM treatments having some inoculation by native AM fungi, but not to the same degree as AM treated roots. Another possibility would be in line with other studies showing metals sequestered in the AM fungal tissues are prevented from transferring into the roots (Audet and Charest 2012; Joner et al. 2000). The reduction in heavy metal uptake prevents binding to sulfhydryl groups in proteins. It can also cause the formation of free radicals and reactive oxygen species (ROS), resulting in inhibition of activity or plant toxicity (Dietz et al. 1999; Hall 2002). The AM protection of plants is complimented by intrinsic strategies such as efflux pumping of metals that have entered the cytosol, use of metallothioneins for metal binding, and chelation of metals by organic acids and amino acids (Hall 2002). In further studies it would be beneficial to look at which exudates AM fungi are releasing into the contaminated soil, to determine what is driving the increased number of metal species being taken up. Some of the exudates that increase in *E. purpurea* when inoculated with *G. intraradices* have been highlighted in a few studies looking into secondary metabolite production for plant defense. These compounds are exploited for human use in many ailments, but in plants they are used as defense, attraction of pollinators, UV protection, structural support, nutrition storage, hormonal regulation and signalling agents in plant-microbe relationships (Herms and Mattson 1992). Araim et al. (2009) using *E. purpurea* and *G. intraradices*, found that phenolic concentrations, particularly cynarin, cichoric, caftaric and chlorogenic acids do significantly increase. This was also supported by several other studies (Fester et al. 1999; Maier et al. 1999; Toussaint et al. 2007), which concluded that AM colonization induces the synthesis of secondary compounds (phenolics, cyclohexane derivatives, blumenin, rosmarinic and caffeic acids). Another process that could be having an effect would be the release of extracellular glycoproteins (Cornejo et al. 2008), such as glomalin from AM fungi. Glomalin, whose biochemical nature is yet to be revealed, is believed to stabilize aggregates in the soil, but more importantly has been shown to sequester toxic elements through functional groups in its structure (González-Chávez et al. 2004; Purin and Rillig 2007). It has been shown to have a high binding capacity for metals such as Cu, Cd and Pb (Chern et al. 2007; González-Chávez et al. 2004; Vodnik et al. 2007).

Of further interest is soil pH, since it influences the bioavailability of nutrients and metals. If metals are more bioavailable, they could cause metal toxicity within organisms, which could reduce degradation or uptake of PAHs (Wick et al. 2011). Audet and Charest (2012) reported that AM inoculated plants showed a pH buffering while non-AM soil became more acidic. Although it is known that hyphae can induce the alkalinisation of proximal soil due to organic chelators, it seems there was no real change in pH of the soil we tested in both AM and non-AM treatments.

2.5 Conclusion

The presence of AM fungi significantly accelerated absorption of PAHs into echinacea roots. AM fungi showed a preference in accumulating both PAHs and alkyl PAHs, with the highest concentrations in the roots. AM roots had a greater uptake rate (k_1) of PAHs when compared to non-AM roots. Although we did not observe significant declines in the concentration of PAHs and alkyl PAHs from soils with echinacea or AM fungal inoculation, it may be attributable to the decline in soil mass (during root and plant expansion), which was difficult to precisely quantify. The mass of metals accumulated in plant tissue showed no significance with AM fungi. Instead, metal uptake was shown to increase significantly from week 10 to week 20. This greenhouse study shows promise for additional field-testing of bioremediation strategies. With a greater understanding of the complex symbioses between plants and AM fungi, there can be better exploitation of fungal remediation of hazardous contaminants globally.

Chapter 3
Field Experiment: Bioremediation of PAHS, Alkyl PAHS And Toxic Metals

3.1 Introduction

Environmental remediation has come to the forefront as industrialization and large-scale use of xenobiotic compounds are creating a negative impact on environmental and human health. Engineering based methods such as incineration, solvent extractions, and chemical treatments are not only expensive but they strip the soil of important nutrients and microorganisms (Pilon-Smits 2005; Rayu et al. 2012). Bioremediation, which uses organisms for the restoration and reclamation of polluted sites, either *in situ* or *ex situ,* offers an environmentally friendly and economically feasible option. Organic and inorganic contaminants that are of particular concern are PAHs and toxic metals. Interest in PAHs dates back at least 200 years, when Percival Potts found chimney sweeps exhibiting increased occurrence of scrotal cancer in London (Pott 1775). It was not until 1933 when the link between PAHs and cancer was strengthened with the isolation of benzo[a]pyrene; a known carcinogen. Toxic metals have also been gaining attention due to their association with contamination from agriculture and industry, and their potential toxicity and ecotoxicity (Duffus 2002).

Organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), and their alkyl homologs are environmental contaminants of concern (Harms et al. 2011). PAHs and alkyl PAHs are persistent environmental contaminants, which occur due to natural and anthropogenic activities. They consist of two to seven fused benzene rings in various structural configurations. Alkyl PAHs are structurally similar with the exception of alkyl side chains. Pyrogenic PAHs are derived from incomplete combustion of organic molecules, and petrogenic PAHs are derived from oil (Freeman et al. 1990; Lim et al. 1999; Manzetti 2013). Temperature plays an important role in PAH formation. Low temperatures usually favor the formation of petrogenic PAHs, characterized by alkyl-substituted PAHs, whereas high temperatures favor pyrogenic PAHs (Lima et al. 2005).

Another group of contaminants that are of concern are toxic metals. Toxic metals can be classified as having a relatively high density of $5g/cm^3$ and atomic number higher than 20, low water solubility, high affinity for thiolic, aminic, phoric and carbocylic group of organic compounds and tendency to form complex compounds (Sinicropi et al. 2010; Sparks 2005). The use of metals from early civilizations up until present has allowed metals to accumulate or be transported into terrestrial and aquatic environments around the world causing harm to animals, plants and humans (Sparks 2005). Currently, thirteen trace metals and metalloids are listed as priority pollutants and are derived from both natural and anthropogenic sources. These metals are released into the environment from parent rocks, metallic minerals, agriculture, fertilizers, mining, and energy production (Adriano 2001).

Arbuscular mycorrhizal (AM) fungi, the most ubiquitous of all mycorrhizal fungal types, are known to enhance plant growth and their tolerance to organic contaminants (Liu and Dalpé 2009; Volante et al. 2005). Studies have shown the potential of AM fungi in phytoremediation programmes with the degradation of numerous PAHs, including phenanthrene, anthracene, chrysene, dibenz(a,h)anthracene, pyrene, and benzopyrene (Cheema et al. 2010; Joner et al. 2001; Liu et al. 2004 as cited in Gao et. al. 2010; Xiao et al. 2009). AM fungi have the ability to aerate, bind and stabilize soils, and to enhance plant nutrient uptake (Dalpé 2003). These mycorrhizal associations provide benefits to plants and extend soil capacities of rhizoremediation (Jeffries et al. 2003), in addition to their synergistic effects with other rhizospheric microorganisms on PAH degradation (Liu and Dalpé 2009).

The aim of this study was to evaluate the use of AM fungi with *Echinacea purpurea* (L.) Moench in a field experiment for the bioremediation soil contaminated with PAHs, alkyl PAHs and toxic metals on Victoria Island, Ottawa. For the experiment we chose the purple coneflower, based on the work of Liu et al. (2009) as well as our findings in chapter 2. In a study by Liu et al. (2009), it was determined that purple coneflower (*Echinacea purpurea*, L.) had one of the highest removal rates of total PAHs among 14 ornamental species of plants. Further advantages of using *E. purpurea* was its positive response to a commercially available inoculum of *Glomus intraradices* an AM fungi (Araim et al. 2009), its drought tolerance and its fibrous root system which allows greater contact with soil (Mistíkova 2007). Using a factorial design we predicted that under field conditions AM inoculation would increase the uptake of PAHs, alkyl PAHs and toxic metals in *Echinacea purpurea* through enhanced bioaccumulation via AM hyphae.

3.2 Materials and Methods

3.2.1 Experimental Design

A 1-year factorial block field study was conducted (August 2013 to August 2014) using *Echinacea purpurea* (L.) Moench, inoculated with or without AMF, and grown in tilled soil on Victoria Island, Ottawa (45° 25’ 15” N, 75° 42’ 50” W). The plot was of 6 m x 4 m and contained 5 blocks. Each block was 1.3 m x 1.2 m and comprised of three rows; control soil, non-AM treatment and AM treatment each 0.3 m wide with spacing of 0.2 m between rows (Appendix F). Compost was applied as a 0.1 m thick layer and covered with an equally thick layer of natural cedar mulch.

Figure 3.1: Field plot on Victoria Island, Ottawa, ON.

3.2.2. Inoculation of Plants

Echinacea purpurea plants were grown from seeds (Ontario Seed Company, Waterloo, ON) in autoclaved PRO-MIX all purpose garden soil (Premier Tech, Rivière-du-Loup, QC) for 6-weeks and sown with or without AM fungal propagules of *Glomus intraradices* Schenck and Smith, DAOM 181602 (MYKE PRO Greenhouse-G, Premier Tech, Rivière-du-Loup, QC) before being transplanted into the field site on Victoria Island. The fungal inoculum was integrated as a 3-cm thick substrate layer on top of 0.5 L of soil, and then covered with more soil as determined by Audet and Charest (2012). Eight seeds were sown ~1cm deep in the soil mixture and thinned after 2 weeks to one plant per pot as determined by Araim et al. (2009). The seeds were previously surface sterilized by a 10% (v/v) solution of hydrogen peroxide for 10 min, rinsed with sterile distilled water and pre-germinated on moist filter paper overnight before sowing. The greenhouse conditions were maintained with a photoperiod of 16:8 L:D provided by natural light and high-pressure sodium lamps (PL Light Systems, Beamsville, ON, Canada), a day/night temperature regime of approximately 27˚C/23˚C and a relative humidity of 40%. The average light intensity (408 μmol s^{-1} m^{-2}) was measured using a light meter with a quantum sensor (LiCor LI-250A and LI -190SA, Lincoln, NE).

3.2.3. Field Experiment

The plants were grown for 6 weeks in the greenhouse prior to being transplanted in the field. This was chosen over direct seeding to ensure survival from seed predators. In the field plants were removed from there pots and loose soil removed from roots. The factorial design of the study [i.e. 1 plant sp. x 2 treatments (non-AM and AM) x 2 harvests (year 1 and year

2) x 5 reps] provided a total of 20 plants. Five replicates per harvest of un-seeded soil from Victoria Island were also collected to compare contaminant concentrations over time in bare soil. A representative diagram is shown in Appendix F

3.2.4. Watering and Fertilization

For the first 6-weeks of growth in the greenhouse, plants were watered with dH_2O on a daily basis as required, without water leaking through the pots. Fertilization began after the third week following germination with 20 mL of ½ ammonium nitrate type Long Ashton Nutrient Solution (LANS) (Hewitt and Smith 1975), once a week for two weeks (the 4th and 5th weeks). The plants were then fertilized with the full LANS (50 mL at the 6th week). The nutrient solution contained 2.0 mM K_2SO_4, 4.0 mM $CaCl_2$ anhydride, 1.5 mM $MgSO_4{\cdot}7H_2O$, 1.5 mM $NaH_2PO_4{\cdot}H_2O$, 5.0mM NH_4NO_3, 0.01 mM $MnSO_4{\cdot}4H_2O$, 1.0 µM $CuSO_4{\cdot}5H_2O$, 1.0 µM $ZnSO_4{\cdot}7H_2O$, 0.05 mM H_3BO_3, 0.09 mM NaCl, 0.5 µM $Na_2MoO_4{\cdot}2H_2O$ and 5.7 ppm EDTA-Fe. In the field, plants were watered with dH_2O twice a week unless there was sufficient rainfall.

3.2.5. Sample Preparation

After 10 weeks of growth in the field, the plants were harvested and considered year 1 of growth. Approximately 1 year later (considered year 2) the second harvest occurred. Any part of the plant above the soil surface was considered shoot material, and anything below was included as the roots. In order to obtain all root material, the soil was carefully sieved and added to root samples. Roots were then washed with distilled water to remove any soil particles, and blotted dry and weighed. Every harvest, five plants/soil were collected for each treatment; three plants/soil per treatment were analyzed with the rest frozen if needed for further analysis. In year 2, collection of soil, roots and shoots was done with some variation. Due to the large plant size, soil and root cores were collected in triplicate from each plant, and shoot samples were collected randomly from each plant to ensure consistency.

3.2.6. Soil pH

Soil pH was analyzed directly from 10 g samples suspended in dH_2O with a soil:solution ratio of 1:2 *(m/v)* according to Hendershot et al. (2008).

3.2.7. Percent Organic Matter

Percent organic matter was determined using sequential loss on ignition (LOI) method outlined in Heiri et al. (2000). The determination of percent organic matter was done by oxidizing organic matter at 550 °C to CO_2 and ash using a muffle furnace (Barnstead|Thermoline, 30400 Furnace,). The weight loss was determined by weighing the samples on an analytical balance (Mettler Toledo, AG104) before and after heating. Percent organic matter was detmined using LOI_{550} (1) and percent carbonate content was determined usingLOI_{950} (2):

$$\left(\frac{DW105-DW550}{DW105}\right)\times 100 \qquad (1)$$

$$\left(\frac{DW550-DW950}{DW105}\right)\times 100 \qquad (2)$$

3.2.8. Assessment of AM Root Colonization

Root samples were cleaned, rinsed and stained with an aniline blue 0.02% dye solution (6.78 mM aniline blue, 500 mL glycerol, 450 mL H2Od, and 50 mL 1% HCl) according to Dalpé (1993). Fifty root segments, ~1–2 cm long, were mounted on slides (10/slide) and examined at x 10 and x 40 magnification using a compound microscope. Non-AM roots were also observed to ensure their non-mycorrhizal status. AM colonization was estimated by determining the relative density of fungal structures evidenced by the presence of hyphae, vesicles, arbuscules or spores that provide insight distribution and intensity of AM root colonization (Allen 2001). The formula's for relative density is defined as:

$$\frac{\#\, Structures_{AM}}{Root\ Length_{AM}} \qquad (3)$$

3.2.9. Analysis of Hydrocarbons (Polycyclic Aromatic Hydrocarbons, PAHs, and Alkyl PAHs)

All plant and soil samples were homogenized with elemental copper and Hydromatrix^{TM} diatomaceous earth (Varian Inc., Palo Alto, CA, USA). Samples were then spiked with ^{13}C labeled PAHs (Cambridge Isotope Laboratories Inc., Andover, MA, USA) and extracted using accelerated solvent extraction module (ASE-350, Dionex Corporation, Sunnyvale, CA, USA) at 140°C using 1:1 hexane: acetone mixture following methods US EPA Method 3640A. Extracts then underwent liquid-liquid extraction with hexane to remove organic compounds from the co-extracted water. Following liquid-liquid extraction, extracts were concentrated using a TurboVap (Biotage, Charlotte, NC, USA) under a gentle nitrogen stream. Clean up with US EPA Method 3630C was adapted for use on 6ml (1g) Superclean^{TM} LC-Si solid-phase extraction cartridges. Samples were further concentrated to approximately 1 mL, which was the final extract volume for all samples. Internal standard p-terphenyl-d14 was added to all final extracts.

Analysis of the final extract was done by injecting 1µL of sample into an HP 6890 gas chromatograph) coupled with a HP 5973 N (Agilent Technologies, Santa Clara, CA, USA) mass selective detector. Separation was completed on a DB5-MS 30 m x 0.250 µm x 0.25 µm column (Agilent Technologies) with H_2 as the carrier gas.

All compounds monitored for PAHs and alkyl PAHs are detailed in Appendix D, Table D.1, and all method limits of quantification were based on a signal to noise ratio of 3:1. All samples were blank corrected to remove background contamination, and replicate extractions were carried out on Standard Reference Material ® (SRM) 1941b – Organics in Marine Sediment from the National Institute of Standards and Technology (Gaithersburg, MD, U.S.A.).

3.2.10. Total Metal Analyses

After year 1 and year 2 growth period, metals were determined using ICP-MS. Samples of root, shoot and soil were individually digested using a hot nitric acid solution following a modified version of US-EPA method 3050. This method allows the determination of total recoverable analytes in solid samples and will not allow complete dissolution of samples. Total metals were then analysed from the acid extracts following US-EPA method 200.8. Samples were dissolved in 10 mL of 1:1 HNO_3 and HCl and heated on a graphite block (DigiPREP MS block digestion system, SCP Science, QB, Canada) for 30 minutes at 90 °C. Once cooled to room temperature, 30% H_2O_2 was added and then heated at 90 °C for 3 hours. Samples were then diluted with ultra pure Milli-Q H_2O up to 50 mL mark. All samples were filtered to remove any particulate. Filtered samples were then diluted 10:1 using ultra pure Milli-Q H_2O. In the same manner, standard reference material (Buffalo River Sediment #8704) from the National Institute of Standards and Technology (NIST, USA) and blanks consisting of HNO_3 and HCl were also prepared to ensure quality and accuracy of the metal analysis. The digested extracts were then analyzed using an Agilent ICP-MS 7700 series, which was run using a reactive gas for the removal of known interferences in a complex unknown mixture without loss of sensitivity.

3.2.11. k_1 Uptake Rate of PAHs in Roots

The uptake rate constant of PAH compounds in AM and non-AM roots of was determined using the following formula modified from Gobas and Morrison (2000):

$$k1 = \frac{(dCr/dt)}{Cs} \qquad (2)$$

Where k_1 is the rate constant for the uptake of the PAH compound in units day^{-1}. dCr is the initial change in concentration of PAH compound in the root, dt is the number of days and Cs is the soil concentration. This formula assumes no significant depuration or loss of PAH by excretion or metabolism, so we calculate the uptake during the first 10 weeks of exposure when these losses are minimized due to low PAH concentrations in the roots.

3.2.12. Statistical Analyses

One and two-way ANOVA's were performed for native AM fungi, PAH concentrations, and metal concentrations. An independent t-test was used for AM colonization and correlations were used for K1 uptake rates. Comparison among means for the ANOVA's was performed using Tukey's test. Shapiro-Wilk and Levene's tests were used to verify the normality of distribution and the homogeneity of variance. The data were log transformed to meet the assumptions of parametric analysis. All statistical analyses were done using R statistical software (version 3.1.2).

3.3 Results

3.3.1. Soil pH

In general, soil pH was maintained roughly constant for all three treatments over the course of the field experiment as seen in Table C.1. Control soil and AM soil had a slight increase from year 1 to year 2. Control soil went from 7.45 to 7.68, an increase of 0.23, and AM soil went from 7.47 to 7.50 a slight increase of 0.03. Non-AM treated soil was the only soil to show decrease in pH becoming slightly more neutral with a drop from 7.46 to 7.38 by the second year.

3.3.2. Organic Matter

The organic content from year 1 to year 2 was quite constant in the control soil (4.40 to 4.04%) but decreased in the AM treatment (8.56 to 6.76%). However, in the non-AM treatment, there was a dramatic increase in soil organic matter (4.91 to 9.13%), an increase of 4.22%, almost doubling organic matter content in the soil as seen in Table C.2.

3.3.3. AM Root Colonization

At the end of the field experiment, mycorrhizal colonization of roots was confirmed for both AM and non-AM treatments. Root colonization (Table C.3) of the AM treatment was consistently higher for hyphae, vesicles and spores when compared to non-AM treated roots.

There were ~3.5 times more vesicles, ~2.5 times more spores and ~1.3 times more hyphae in the AM treated roots. However, after an independent t-test was run on mean counts between AM and non-AM, there were no significant differences between AM and non-AM counts in the roots.

3.3.4. Polycyclic Aromatic Hydrocarbons (PAHs) and Alkylated PAHs

Root and shoot ΣPAH and ΣAlkyl PAH concentrations (ng g^{-1}) were multiplied by mass (g) of respective tissue weights according to previous findings by Muntean et al. (1990, 1991) to find the mass of PAHs in each. Soil was measured as concentration (ng g^{-1}) dry weight. Each treatment was analyzed in triplicate, and all compounds analyzed are listed in Table D.1. One-way ANOVAs with a Tukey post hoc were run for hydrocarbon mass vs. treatment for roots and shoots, and hydrocarbon concentration vs. treatment for soil from year 1 to year 2.

Soil ΣPAH concentrations were not significantly different between treatments ($p=0.51$) over the course of the field experiment as seen in Figure C.1. However, soil ΣAlkyl PAH concentrations did show significant increase ($p=0.004$) from year 1 to year 2 in all three treatments. Control soil had the highest mean concentration at year 2 (2010 ± 308 ng g^{-1}), and was the only treatment that was shown to be significantly different to all other Σalkyl PAH concentrations (Figure 3.2).

ΣPAHs content significantly increased in roots and shoots ($p=0.002$ and $p=0.02$ respectively) from year 1 to year 2 in the roots, and between treatments in the shoots. Roots showed no significant differences between treatments (Figure 3.3), while shoots showed no difference in year except for AM treatment at year 2 which had ~2 times more ΣPAHs than the other non-AM shoots.

ΣAlkyl PAHs content in roots and shoots significantly increased ($p=0.013$ and $p=0.000002$ respectively) from year 1 to year 2 (Figure C.2). Shoots preferentially accumulated higher quantities of ΣAlkyl PAHs compared to roots. At year 2, AM shoots had ~7 times higher masses while non-AM had ~6 times greater mass of ΣAlkyl PAHs in their shoots. The highest content of ΣAlkyl PAHs was in non-AM roots 21,700 ± 7650 ng and non-AM shoots 119,000 ± 21,700 ng at year 2.

All treatments were compared between year 1 and year 2 looking at the uptake of individual PAHs and alkyl-PAH compounds. PAH concentrations from year 1 to year 2 saw a slight increase in both parent and alkyl PAHs before correcting for organic content in the soil. The largest percent increase from year 1 to year 2 in AM soil (Figure C.4) was C1-Benzofluoranthene/Benzopyrene with a 338% increase. The largest increase in non-AM soil (Figure C.5) was C4-Naphthalene with an increase of 463%. The largest reduction from year 1 to year 2 in AM soil was Naphthalene (44%) and in non-AM it was C2-Naphthalene (99%). The total hydrocarbons increased from year 1 to year 2 in both AM (61%) and non-AM soil (24%) (Figure C.1). The same trend was clear when the soil was normalized for organic matter. AM and control soil saw an increase in TPAHs in year 2 (111% and 26% respectively), but non-AM soil saw a decrease of 35%. Organic matter decreased for the control soil (0.36%) and AM soil (1.8%) from year 1 to year 2. Non-AM soil did the opposite and increased its organic matter 4.22% from year 1 to year 2, almost doubling its original concentration of organic matter (4.91 to 9.13%).

Overall, alkyl PAHs had higher concentrations in the roots at year 2, 28.0 ± 5.11 ng g^{-1} C3-Flourene and 52.5 ± 44.3 ng g^{-1} C4-Dibenzothiophenes for AM (Figure C.6) and non-AM respectively (Figure 2.7). At year 1 the highest concentrations were lower with C2-Phenanthrene/Anthracene being the highest concentrations for both AM (18.7 ± 4.55 ng g^{-1}) and non-AM (23.5± 8.12 ng g^{-1}). The highest concentrations reached with individual PAH compounds in year 2 were 12.7 ± 2.54 ng $g^{-1}$for AM and 15.1 ± 4.10 ng g^{-1} for non-AM, both with the same alkyl-PAH, Benzo[b]fluoranthene. Year 1 saw the highest concentrations at 17.6 ± 17.6 ng g^{-1}Naphthalene for AM and 2.16 ± 0.35 ng g^{-1}Benzo[b]fluoranthenefor non-AM roots.

Shoot concentrations were 52.2 ± 9.55 ng g^{-1} C2-Naphthalene for AM (Figure C.8) and 39.3 ± 8.43 ng $g^{-1}$for non-AM (Figure C.9) in year 1 for alkyl-PAH C2-Naphthalene. In year 2, the highest concentrations were 37.3 ± 9.99 ng g^{-1} C2-Naphthalene for AM, and 51.3 ± 30.2 ng g^{-1} C4-Dibenzothiophenes for non-AM. Parent compounds in years 1 and 2 showed Phenanthrene had the highest concentrations for AM (14.4 ± 4.73 ng $g^{-1}$and 30.2 ± 4.17 ng g^{-1}, respectively) and non-AM (14.0 ± 3.03 ng g^{-1} and 15.0 ± 4.65 ng g^{-1}, respectively).

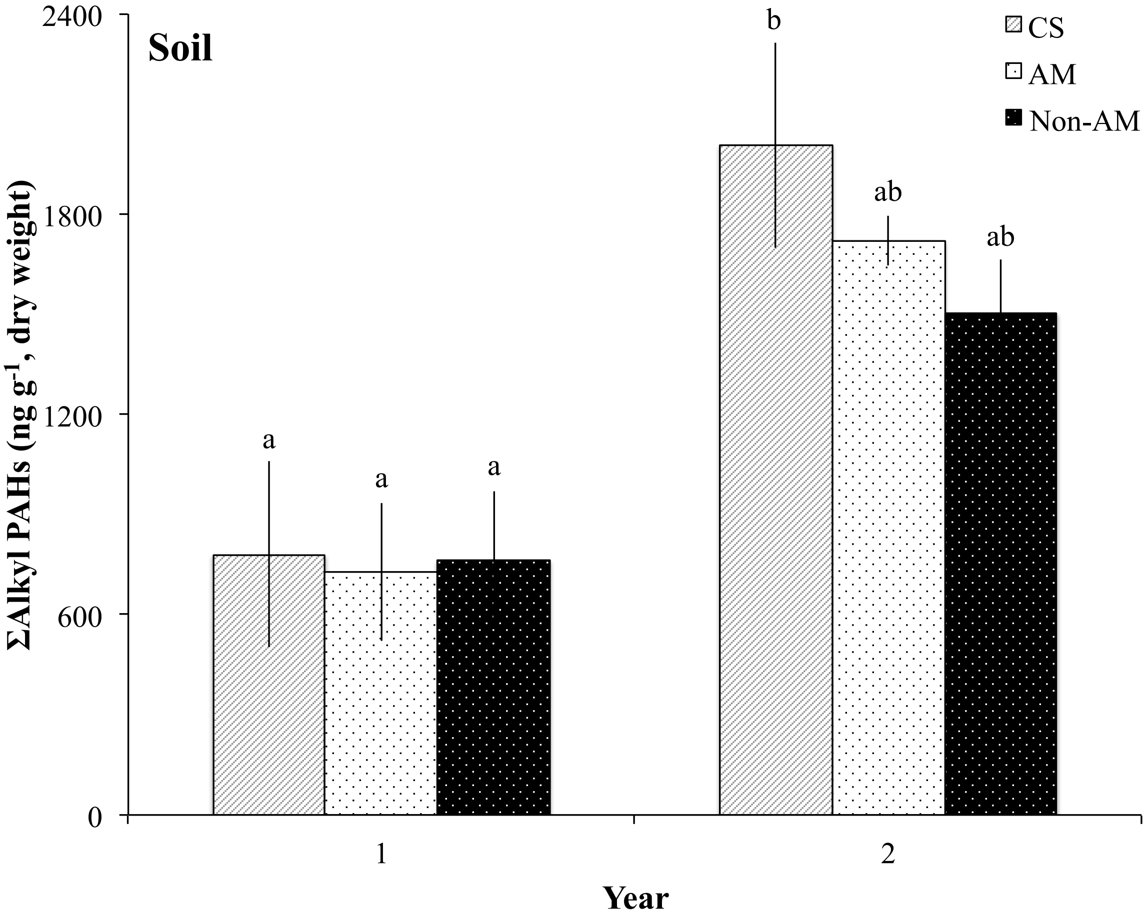

Figure 3.2: Mean (±SE) ΣAlkyl PAH content (ng g^{-1}) in soil over 1 year of growth in the field on Victoria Island. Data was analyzed using a one-way ANOVA [F (5, 12)=6.6, p=0.004]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (p=0.19) and Levene's test (p=0.93) for homogeneity of variance. Control Soil, AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

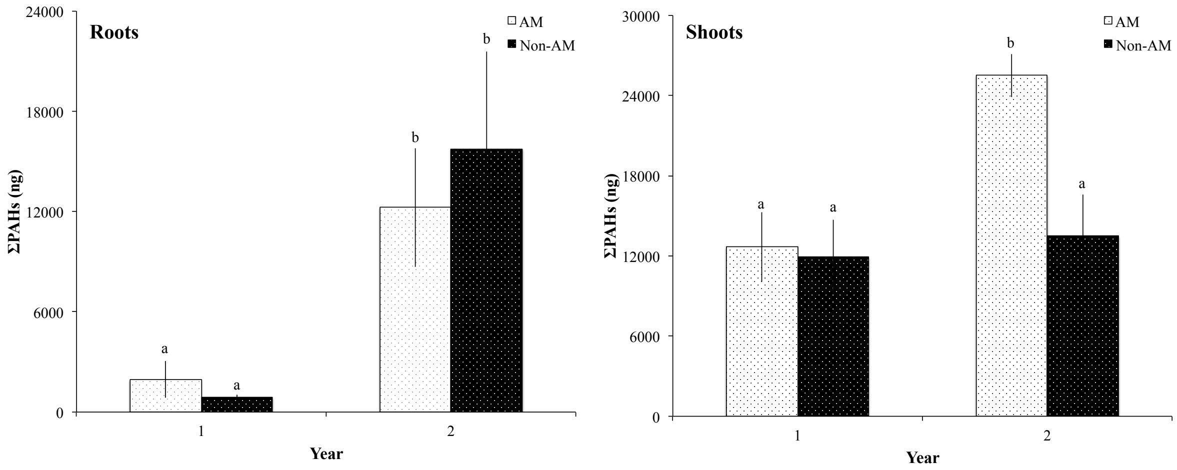

Figure 3.3: Mean (±SE) ΣPAHs content (ng) over 1 year of growth in *Echinacea purpurea* on Victoria Island. Data was analyzed using a one-way ANOVA for roots [F (3, 8)=13.8, p=0.002] and shoots [F (3, 8)=6.1, p=0.02]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (SW) and Levene's test (LT) for homogeneity of variance in roots (SW p=0.02, LT p=0.21) and shoots (SW p=0.68, LT p=0.96). AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

3.3.5. k1 Uptake Constant

AM roots had lower k_1 rates and were not correlated with log K_{OW}. The k_1 rates for non-AM roots showed a significant negative relationship (r^2=0.39, p=0.02) with log K_{OW} (Figure 3.4). This indicates that accumulation rates are slower when hydrophobic compounds are present. The highest uptake rate for AM roots was acenaphthene (0.0008 day^{-1}) and the lowest was fluorene (0.000001 day^{-1}) at log K_{OW} of 4.15 and 4.02 respectively. Acenaphthene had the highest uptake rate for non-AM roots at 0.0008 day^{-1} at a log K_{OW} of 4.15.The lowest uptake for non-AM roots was found to be benzo[g,h,i] perylene with a rate of 0.00001 day^{-1} at log K_{OW} of 6.7.

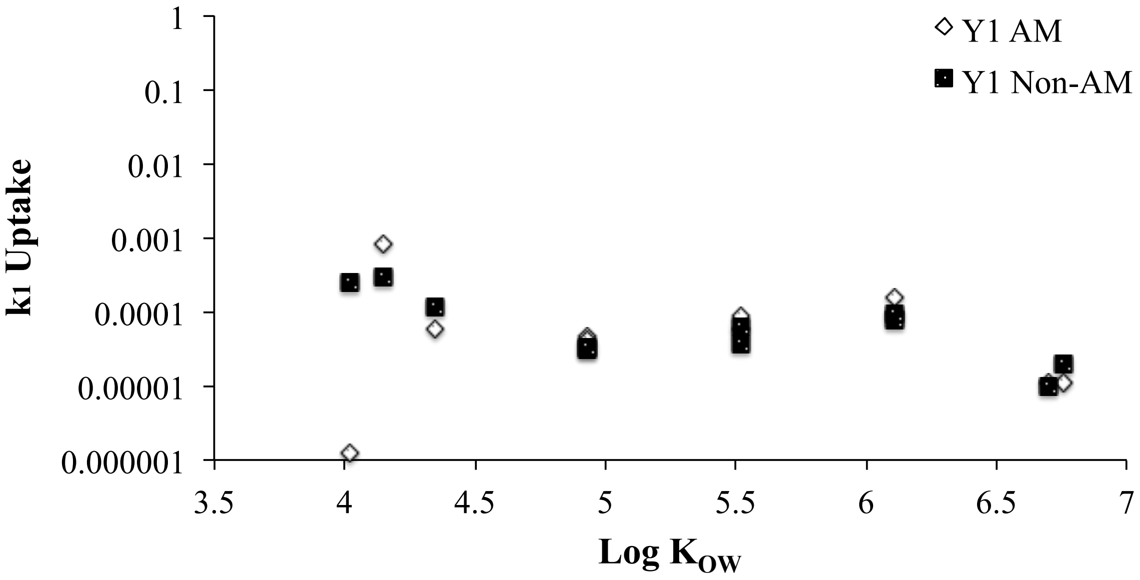

Figure 3.4: Uptake rates on a logarithmic scale measured in roots of AM and non-AM plants during year 1 (Y1) of the greenhouse experiment. AM roots showed no significant relationship (slope= -0.000033, r^2=0.12, p=0.22) while non-AM roots showed a significant negative relationship (slope=-0.000025, r^2=0.39, p=0.018). Log KOW are given for the following PAHs: acenaphthene, fluorene, phenanthrene anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3- cd]pyrene, dibenz[a,h]anthracene, and benzo[g,h,i]perylene.

3.3.6. Total Metals

Root and shoot metal concentrations (mg kg^{-1}) were multiplied by mass (kg) of respective tissue weights to find the metal content (mg). Since we did not have total weights of *E. purpurea,* the total fresh weights were determined from Muntean et al. (1990 and 1991). Soil was measured as concentration (mg kg^{-1}) dry weight. The treatments were taken in triplicate. All compounds analyzed are listed in Table C.4 in Appendix C. Two-way ANOVAs were run for metal content (mg) vs. treatment for roots and shoots, and metal concentration (mg kg^{-1}) vs. treatment for soil from year 1 to year 2 (Table C.4).

Overall, the concentration of toxic metals in the soil significantly decreased between year 1 and year 2 for Zn ($p<0.001$), Al ($p<0.01$), Co and Cr ($p<0.05$), whereas Ag, Ba, Be, Mn, and V significantly increased ($p<0.05$). All the other soil metal concentrations showed no significant change over the course of the field experiment or between treatments (Figure 3.5).

Ag, Al, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, U, V ($p<0.001$) and Be, Mo, Se, Th ($p<0.01$) and Zn ($p<0.05$) content significantly increased from year 1 to year 2 in the roots. Every heavy metal increased significantly from year 1 to year 2 in the roots or *E. purpurea*, but none of the metals showed significant differences between treatments (Figure 3.6).

Total As, Ba, Cr, Cd, Cu, Mn, Mo, Sb, Se, V, and Zn in shoots of *E. purpurea* increased significantly ($p<0.05$) from year 1 to year 2. However, none of the metals showed significant differences in metal uptake between treatments in the shoots (Figure 3.7).

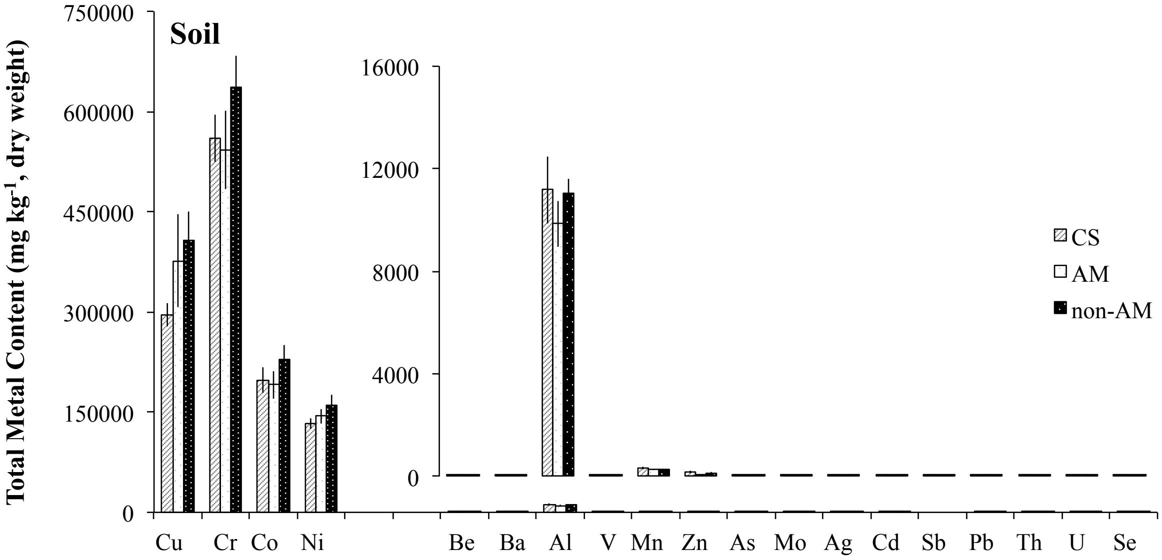

Figure 3.5: Mean (±SE) total metal content (mg kg^{-1} dry weight) in soil samples from year 2 at Victoria Island field site. CS (Control Soil), AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

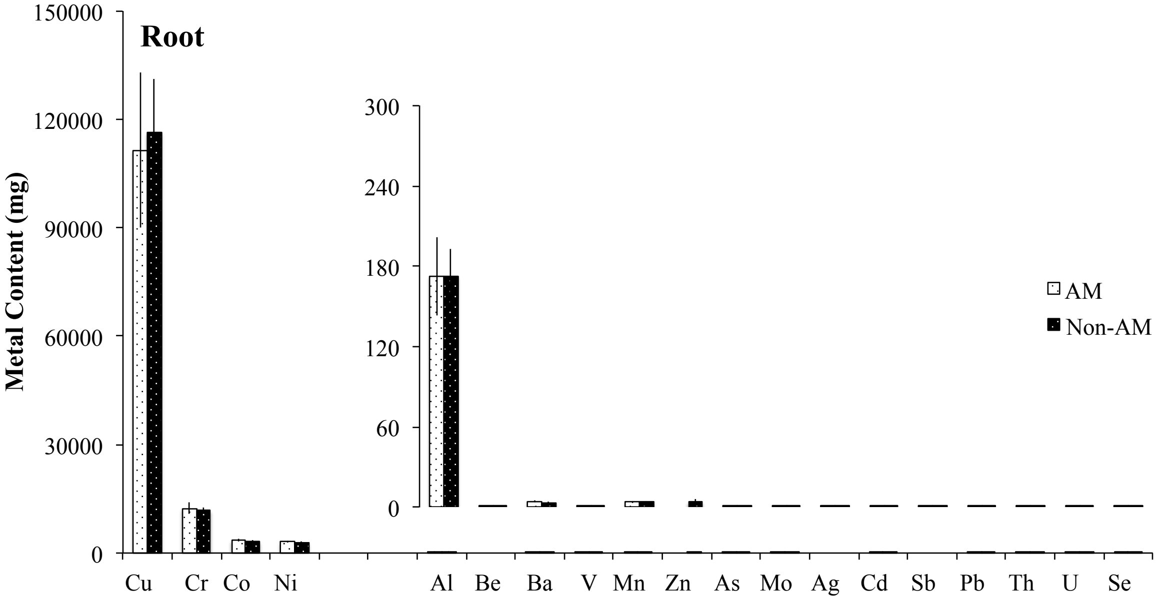

Figure 3.6: Mean (±SE) metal content (mg dry weight) in root sample of *Echinacea purpurea* from year 2 at Victoria Island field site. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

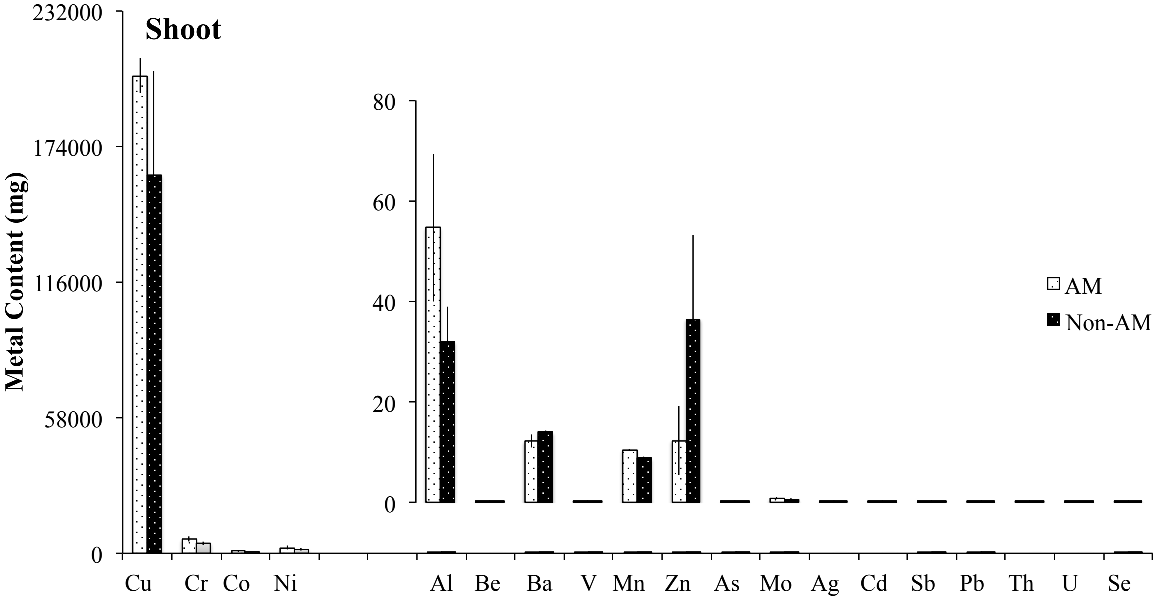

Figure 3.7: Mean (±SE) metal content (mg dry weight) in shoot sample of *Echinacea purpurea* from year 2 at Victoria Island field site. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

3.4 Discussion

Initially, the results from the greenhouse study indicated that PAH and alkyl-PAH uptake by *Echinacea purpurea* was significantly increased if treated with AM fungi. However, our field experiment showed no significant increase in uptake of PAHs or alkyl PAHs with AM inoculation in roots and shoots (Figure C.1 and Figure 3.2). Soil and plant material both showed increases in PAH concentrations and content from year 1 to year 2 in all treatments. In the studies of Gao and Ling (2006) and Sung et al. (2001), it was shown that plant concentrations of PAHs increase with the increasing soil PAH concentrations. This holds true for parent compounds but not for alkylated groups. In all three treatments, PAH concentrations were ~4 times more concentrated than the alkyl PAHs in the soil, yet concentrations of alkyl PAHs were the highest in plant roots and shoots. The increased concentrations of alkyl PAHs could be explained by a number of factors, including slow decomposition rates of alkyl PAHs (Heitkamp and Cerniglia 1987), exudates or enzymes increasing bioavailability metabolically or co-metabolically (Banks et al. 1999; Binet et al. 2000; Pinedo-Rilla et al. 2009; Thomas et al. 2012), or the plants inability to metabolize alkyl PAHs once concentrated in plant tissues.

When determining the concentration of individual hydrocarbons in the soil, it was found that the concentrations increased in all three treatments over time. To determine the PAH concentrations more accurately, these were normalized to organic matter concentrations in the soil. Between the three treatments, AM and control soil had a decrease in organic matter content while non-AM soil saw almost a doubling in organic matter. When the hydrocarbon concentrations were normalized to organic matter, the same trends remained for control soil and AM soil but non-AM soil saw a reduction in PAH and alkyl PAH concentrations. Cheng et al. (2012) demonstrated that AM fungi have the capacity to diminish carbon pools in soil rather than sequester them over a short term under elevated CO_2. The decreasing pool of carbon in the soil matrix would increase the concentration of PAHs, then reflecting a process known as "solvent depletion" (MacDonald et al. 2002). With less carbon available in the soil matrix, the concentrations of PAHs and alkyl PAHs are amplified. The AM fungi could be causing solvent depletion either directly, by breaking down soil C stores through root exudates or organic acids, or through stimulation of bacterial heterotrophs in the soil matrix. AM fungi are known to behave physiologically different under metal stress (Miransari 2011), so the same could be true for hydrocarbon stress.

The log K_{OW} impacted the uptake of individual PAHs. Log K_{OW} for individual PAH compounds was found to be inversely related to uptake in both AM and non-AM roots at year 1. As the log K_{OW} increases so does the molecular weight and hydrophobicity. However, contrary to the greenhouse experiment we did not see an increased uptake rate in AM treated roots. Both treatments showed similar uptake rates, with non-AM having slightly higher k1. This could be explained by native AM fungi colonizing the roots of non-AM roots. Studies have shown that microorganisms that exist in contaminated sites are more efficient at uptake and degradation of contaminants in the soil (Joner and Leyval 2001; Leung et al. 2006).

Our next major finding in the field experiment was that treatment had no significance in uptake of metals, but instead toxic metal uptake was more affected by year as a factor. AM fungi enhanced the number of metals taken up when compared to the non-AM treatments. The 'enhanced uptake' hypothesis, which predicts AM inoculated plants will have a greater metal uptake when compared to non-AM inoculated plants showed no significance in this study when looking at individual toxic metals. As discussed in chapter 2, the reduction in soil corresponded to increased uptake in AM treated plant roots and shoots, with mycorrhizae allowing greater access to the metals while increasing the transfer of metals into the plant.

3.5 Conclusion

This field study demonstrated the uptake of PAHs, alkyl PAHs and toxic metals from contaminated soil by AM fungi and *Echinacea purpurea*. Although AM treated *Echinacea purpurea* saw no significant difference in PAH or metal uptake in the roots and shoots, there was still measurable uptake of both. We believe part of this can be explained through carbon depletion in soil leading to higher soil concentrations, and colonization of non-AM plants in the field by native AM fungi which are more suited to the contaminated environment. This study provides new views on mixed contaminated soils containing all 16 Priority PAHs, as well as the lesser-known alkyl PAHs and 19 different metals. Understanding how complex mixtures of contaminants are taken up in a field setting provides valuable information into for the perfecting of bioremediation strategies in the future.

4.0 General Conclusion and Perspectives on Future Research

This current research set out to investigate if AM fungi could enhance the uptake of PAHs, alkyl PAHs and toxic metals from contaminated soil. This study was unique in being one of the few that focuses on a complex mixture of contaminants, rather than individual compounds, in the soil matrix. In the greenhouse experiment, I determined AM treated roots increased the uptake of alkyl PAHs in *E. purpurea.* However, AM fungi had no effect on the accumulation of PAHs and metals. Despite no significant accumulation of PAHs in inoculated *E. purpurea*, the uptake rates for AM roots were 10 x greater than non-AM roots. Thus the AM fungi's fine hyphae were increasing the absorptive capacity of the roots. Although measurable quantities of contaminants were accumulating in *E. purpurea*, the toxic metals were the only contaminant decreasing in concentration within the soil. Alkyl PAHs remained unchanged from week 10 to week 20, but PAHs increased to higher concentrations than the original concentration. These results would support the phenomenon of solvent depletion. The AM fungi are possibly taking up minerals and organic carbon faster than PAHs, thus creating higher concentration of PAHs in the soil.

Moreover, we conducted the same study under field conditions at Victoria Island, Ottawa. In this two-year study, we quantified the accumulation of PAHs, alkyl PAHs and toxic metals in *E. purpurea*, and again the concentrations of contaminants in the soil. AM treatments increased the uptake of PAHs in the shoots of *E. purpurea*. Yet, AM inoculation had no significant accumulation of alkyl PAHs or metals over the two years. Further, the most important factor was time, with alkyl PAHs and toxic metals accumulating significantly more from year 1 to year 2. Contrary to the greenhouse study, AM roots did not have greater uptake rates of PAHs when compared to non-AM roots. To explain the lower uptake rates, we found non-AM roots of *E. purpurea* were being colonized to a lesser extent by native AM fungi. Previous studies have shown native microbes are better suited to the contaminated conditions and can increase plant accumulation of contaminants. Meanwhile, the soil concentrations of PAHs and alkyl PAHs remained the same, with toxic metals decreasing. Again, this could be due to solvent depletion. If the minerals and organic carbon are being taken up faster than the contaminants, the contaminant concentrations will appear higher. Another possibility is we were unable to control contaminant inputs to the field site. There could atmospheric and water runoff deposition of contaminants to the field site making it difficult to determine remediation benefits.

The key to understanding bioremediation is the complex and intriguing interactions that occur in the rhizosphere. This interface between plant roots and soil is considered one of the most dynamic interfaces that affect biogeochemical cycling, plant growth, and tolerance to biotic and abiotic stress. The release of nutrients, exudates, border cells, and mucilage from the roots of plants attracts and supports bacteria, viruses, fungi, oomycetes and archaea (Philippot et al. 2013). Physico-chemical characteristics of soil can play an important role in in plant physiology and root exudation patterns which in turn can influence the community structure of rhizosphere microorganisms, thus there is a strong need to understand the mechanisms underlying the selection of specific populations of microorganisms living in the soil. With the use of next generation sequencing, we can discover microbial taxa in the rhizosphere and determine the important communities that aid in the degradation and uptake of environmental contaminants. Selecting for the appropriate bacterial communities with AM fungi could enhance bioremediation further and improve the overall process.

The need for more studies investigating the various mechanisms at play between plants and microorganisms in mixed contaminated soil are important to elucidate better remediation strategies.

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Appendices -List of Tables

Appendices-List of Figures

Figure B.10 Mean (±SE) total metal content (mg kg^{-1} dry weight) in soil samples from week 10 for the greenhouse experiment. CS (Control Soil), AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

Figure B.11 Mean (±SE) metal content in root samples from Week 10 from the greenhouse experiment in mg dry weight. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

Figure B.12 Mean (±SE) metal content (mg dry weight) in shoot samples from week 10 from the greenhouse experiment. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

Figure C.1 Mean (±SE) ΣPAH content (ng g^{-1}) in soil over 1 year of growth in the field on Victoria Island,.Data was analyzed using a one-way ANOVA [F (5, 12)=0.9, p=0.51]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (p=0.33) and Levene's test (p=0.99) for homogeneity of variance. Control Soil, AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

Figure C.2 Mean (±SE) ΣAlkyl PAH content (ng) over 1 year of growth in *Echinacea purpurea* on Victoria Island. Data was analyzed using a one-way ANOVA for roots [F (3, 8)=6.7, p=0.013] and shoots [F (3, 8)=98.4, p=0.000002]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (SW) and Levene's test (LT) for homogeneity of variance in roots (SW p=0.05, LT p=0.46) and shoots (SW p=0.012, LT p=0.22). AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

Figure C.3 Mean (±SE) hydrocarbon content (ng g) profile for control soil from year 1 (Y1) to year 2 (Y2) on Victoria Island field site. n=3.

Figure C.4 Mean (±SE) hydrocarbon content (ng g) profile for AM soil from year 1 (Y1) to year 2 (Y2) on Victoria Island field site. n=3.

Figure C.5 Mean (±SE) hydrocarbon content (ng g) profile for non-AM soil from year 1 (Y1) to year 2 (Y2) on Victoria Island field site. n=3.

Figure C.6 Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM roots from year 1 (Y1) to year 2 (Y2) on Victoria Island field site. n=3.

Figure C.7 Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM roots from year 1 (Y1) to year 2 (Y2) on Victoria Island field site. n=3.

Appendix A: Pre-Experimental Soil Characteristics

Table A.1: Pre-experimental soil composition of freeze-dried and homogenized soil from Victoria Island, Ottawa.

Parameter	**Value**
pH*	6.77 (0.10)
C (%)**	4.01 (0.44)
N (%)**	0.15 (0.11)
Ca (mg kg^{-1})	42400 (603.74)
Fe (mg kg^{-1})	13.8 (0.23)
Na (mg kg^{-1})	0.315 (0.01)
P (mg kg^{-1})	1040 (28.04)
Sr (mg kg^{-1})	0.0899 (0.001)
Ti (mg kg^{-1})	631 (282.19)

Note: Means (±SE). n=5 , *n=3, **n=5

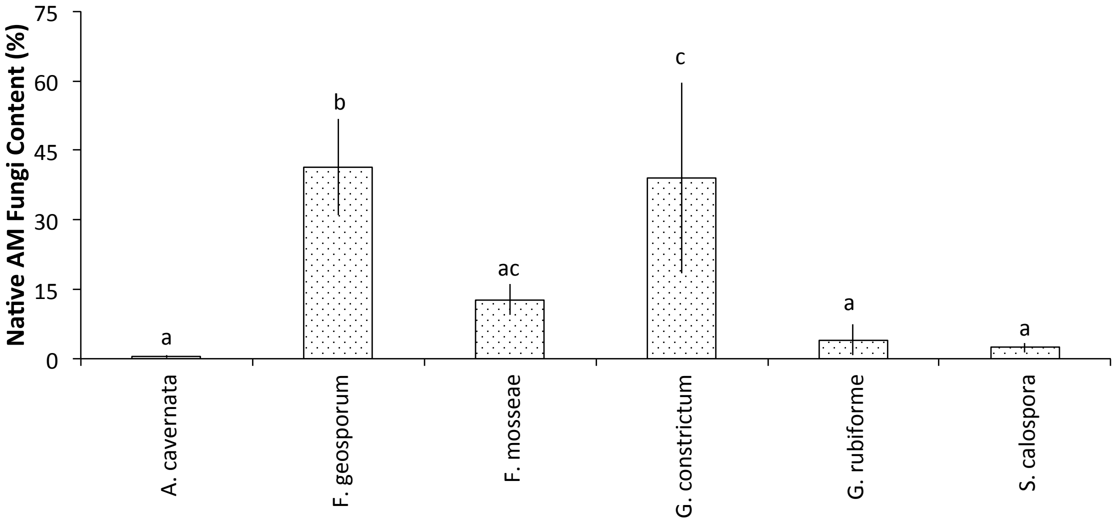

Figure A.1: Mean (±SE) percent native AM fungi averaged from 10 test pits from Victoria Island, Ottawa, using soil from 0.1-0.4m layer in each of the ten test pits. (n=10). Data was analyzed using a one-way ANOVA [F (5, 54)=31.8, p<0.001]. Different letters indicate significant differences between the species according to Tukey's post-hoc test.

Appendix B: Greenhouse Study

Table B.1: AM root colonization count and density measurements

		Count			Density (mm^{-1})		
Week	**Treatment**	**Hyphae**	**Vesicles**	**Spores**	**Hyphae**	**Vesicles**	**Spores**
10	AM	227.0 (35.3)	1128.7 (1002.1)	4.3 (1.2)	0.45(0.07)	2.26(2.00)	0.01(0.00)
	Non-AM	92.7 (50.3)	80.0 (73.6)	0.3 (0.3)	0.1 (0.10)	0.16(0.15)	0.00(0.00)
20	AM	71.3 (6.4)	623.67 (273.22)	6.7 (2.0)	0.14(0.01)	1.25(0.55)	0.01(0.00)
	Non-AM	6.7 (3.3)	130.7 (54.7)	11.0 (9.0)	0.01(0.01)	0.26(0.11)	0.02(0.02)
t-values and levels of significance							
10	Treatment	2.2ns	1.0ns	3.2ns			
20	Treatment	8.9**	1.8ns	-.5ns			

Note: Each treatment is 150, 1cm root segments (10 per slide) observed under a compound microscope. Means (n=3) and (SE) are shown for each treatment.
ns: not significant
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Table B.2: Soil pH over measured at week 0, 10 and 20 homogenized soils from the greenhouse experiment.

Week	Treatment	pH
0	CS	6.77 (0.18)
	AM	6.77* (0.18)
	Non-AM	6.77* (0.18)
10	CS	6.99 (0.04)
	AM	7.01 (0.03)
	Non-AM	7.03 (0.04)
20	CS	7.34 (0.03)
	AM	7.25 (0.04)
	Non-AM	7.25 (0.07)

Note: Means (n=3) and (SE) are shown for each treatment.
*Week 0 AM and non-AM soils have the same pH as control soil since the same homogenized soil was used for all treatments.

Table B.3: Percent organic matter in greenhouse study soil by LOI

Week	Treatment	Percent Organic Matter
10	**CS**	3.55 (0.07)
	Non-AM	3.75 (0.21)
	AM	3.64 (0.04)
20	**CS**	3.30 (0.25)
	Non-AM	3.43 (0.06)
	AM	3.50 (0.10)

Means (n=3) and (SE) are shown for each treatment.

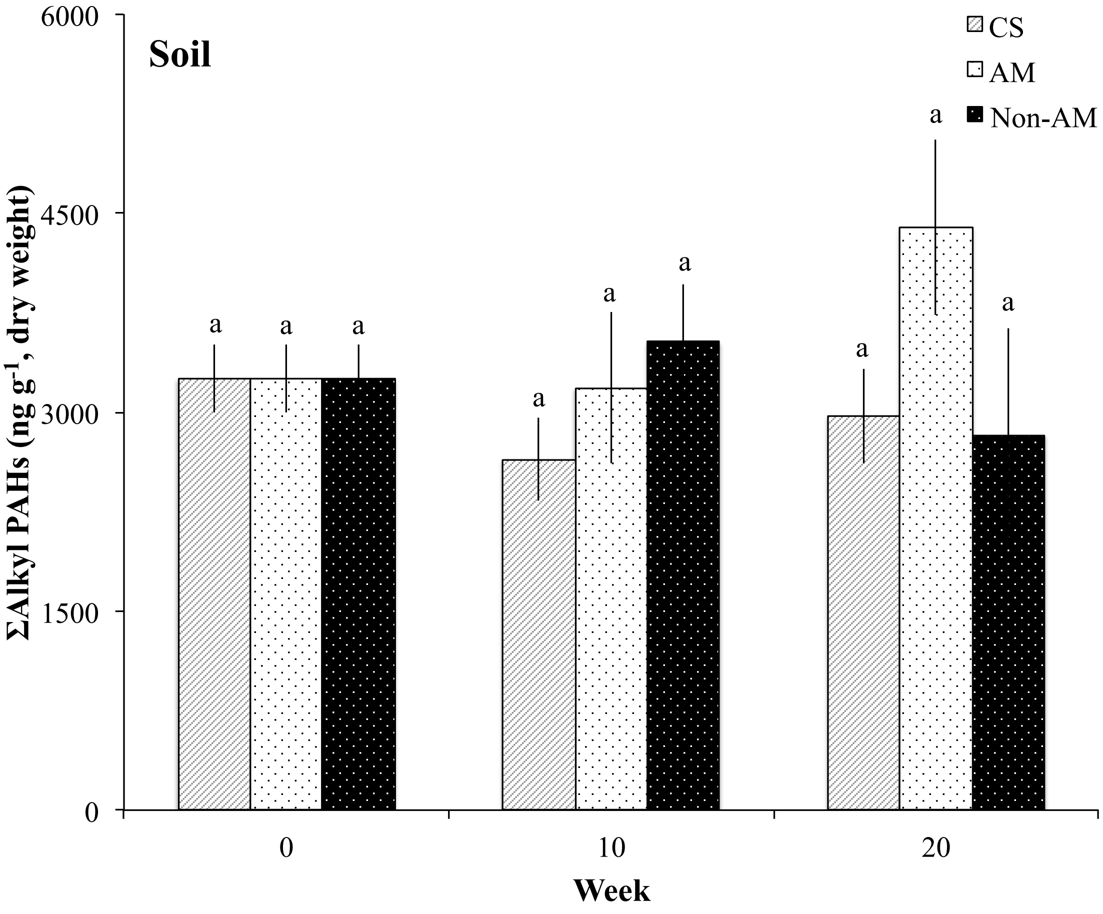

Figure B.1: Mean (±SE) ΣAlkyl PAH content (ng g^{-1}) over 20 weeks of growth in soil in the greenhouse. Data was analyzed using a one-way ANOVA [F (8, 18)=1.1, p=0.39]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (p=0.65) and Levene's test for homogeneity of variance (p=0.82). CS: Control Soil, AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

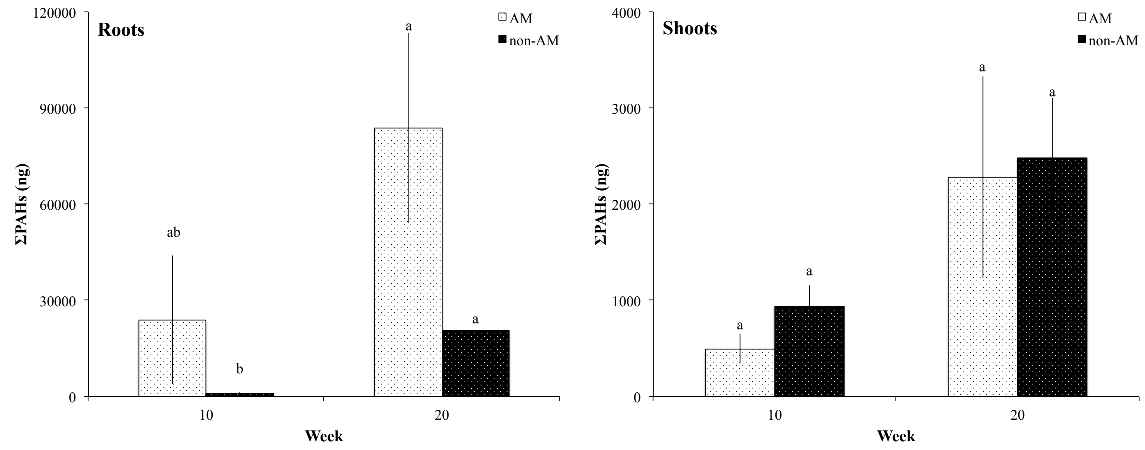

Figure B.2: Mean (±SE) ΣPAH content (ng) over 20 weeks of growth in *Echinacea purpurea* in the greenhouse. Data was analyzed using a one-way ANOVA for roots [F(3, 8)=9.0, p=0.006] and shoots [F(3, 8)=2.2, p=0.16]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (SW) and Levene's test (LT) for homogeneity of variance in roots (SW p=0.039, LT p=0.36) and shoots (SW p=0.65, LT p=0.42). AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

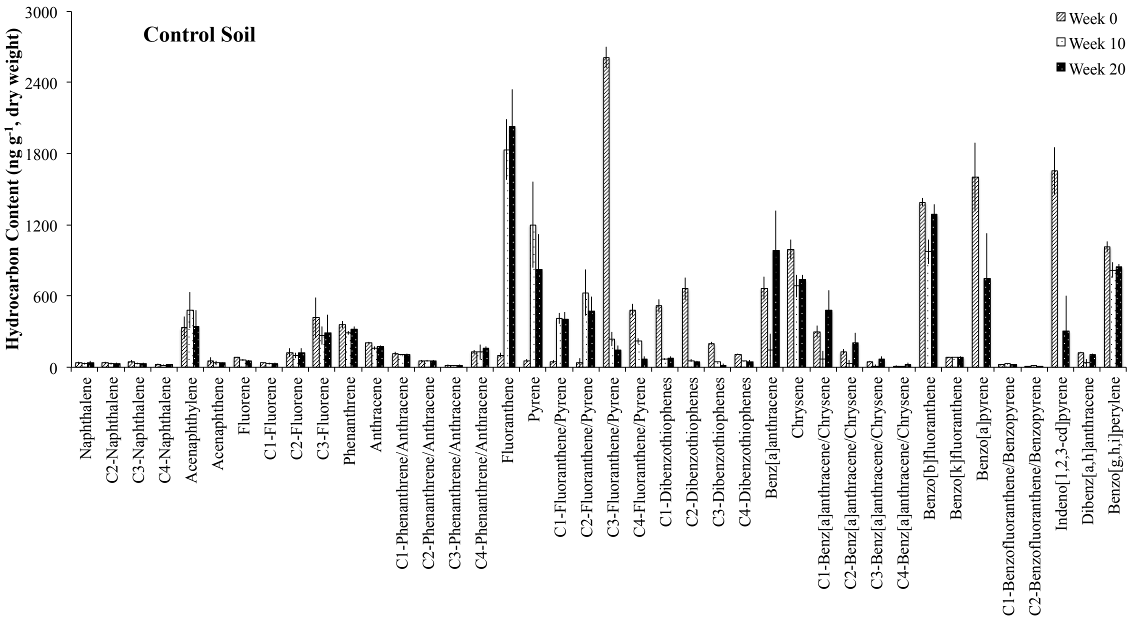

Figure B.3: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for Control Soil (CS) from week 0 to week 20 in the greenhouse study. n=3.

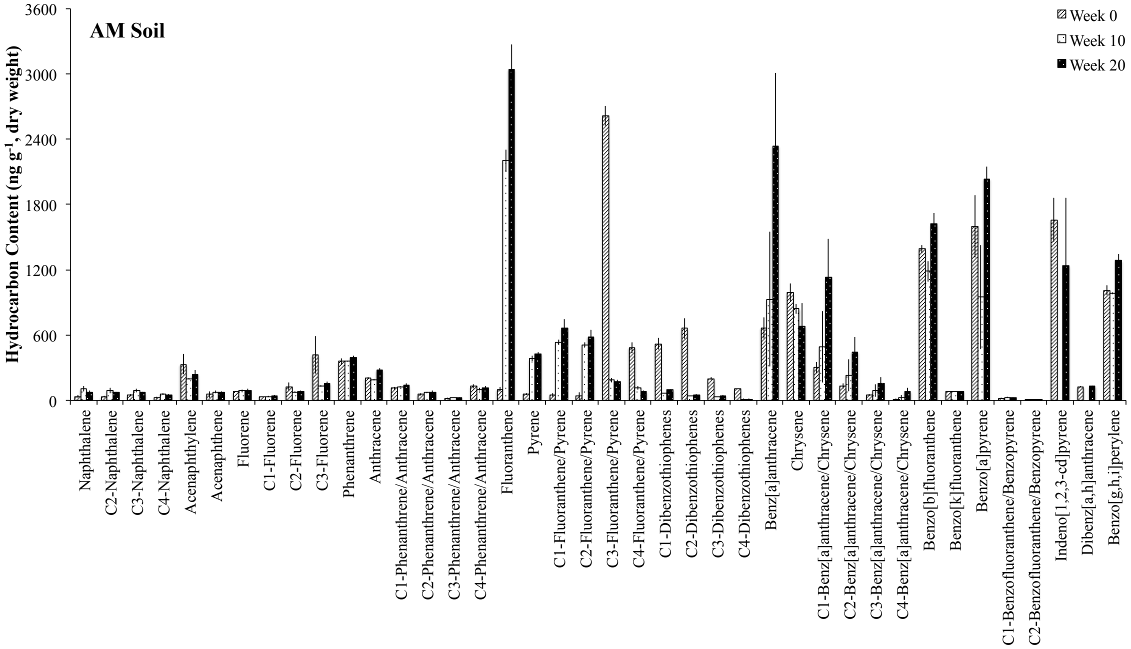

Figure B.4: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM soil from week 0 to week 20 in the greenhouse study. n=3.

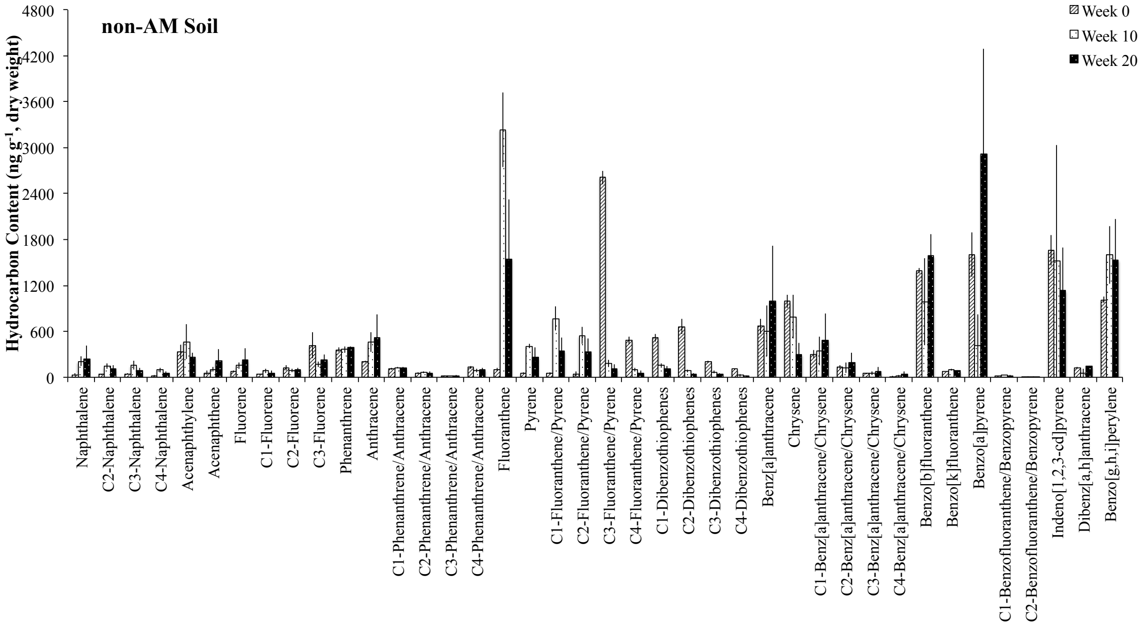

Figure B.5: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM soil from week 0 to week 20 in the greenhouse study. n=3.

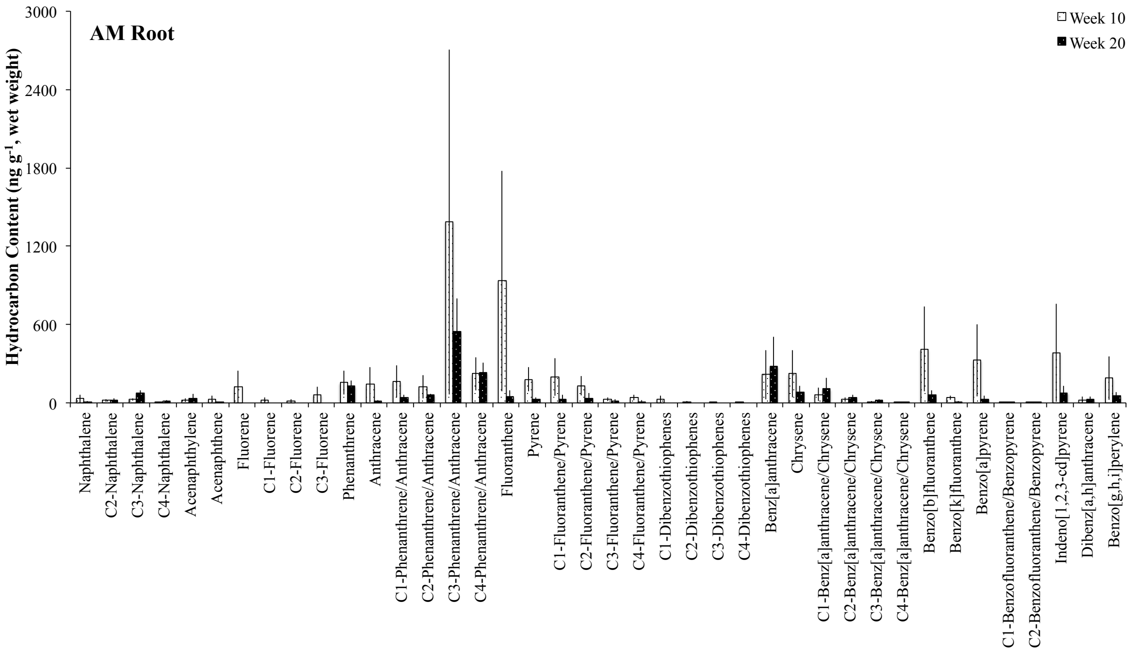

Figure B.6: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM root from week 10 to week 20 in the greenhouse study. n=3.

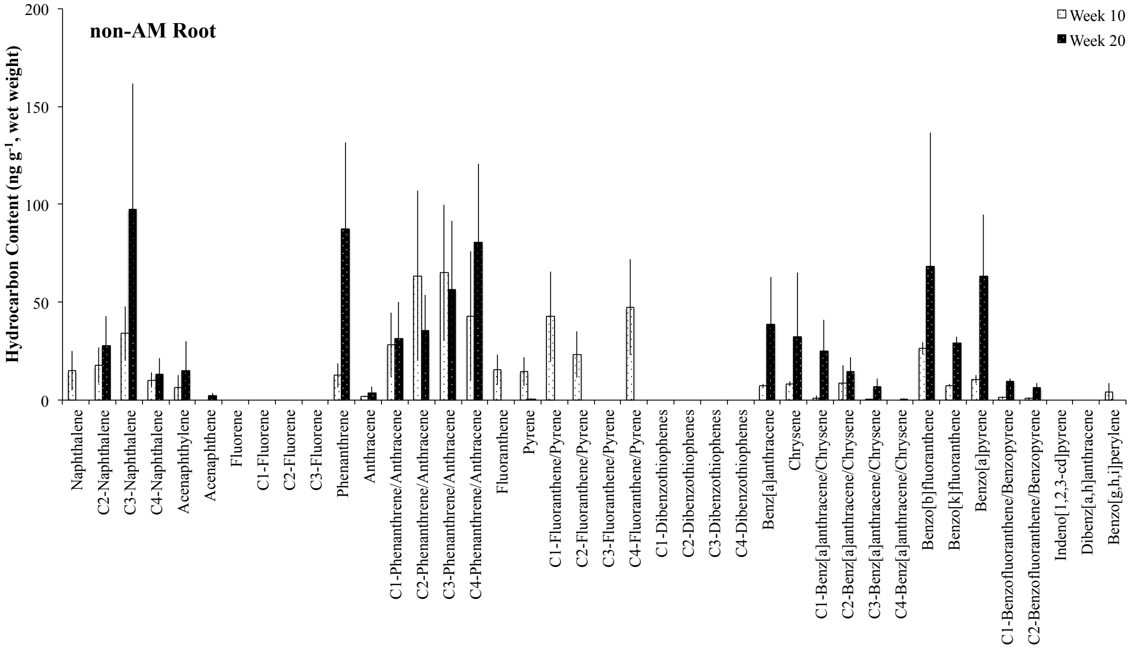

Figure B.7: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM root from week 10 to week 20 in the greenhouse study. n=3.

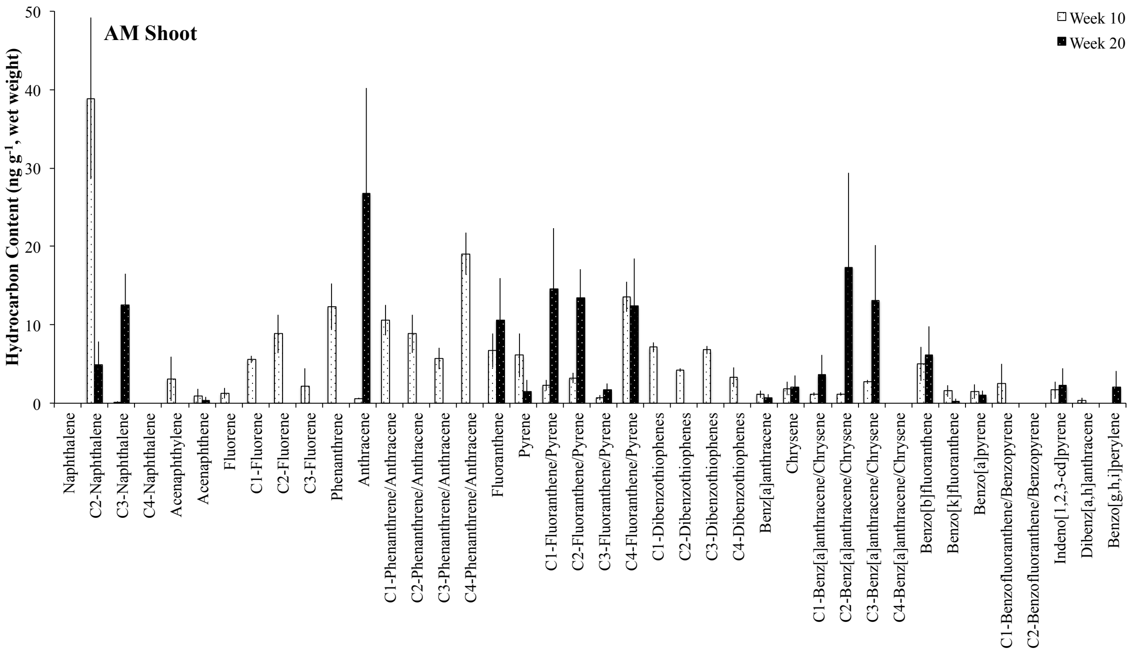

Figure B.8: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM shoot from week 10 to week 20 in the greenhouse study. n=3.

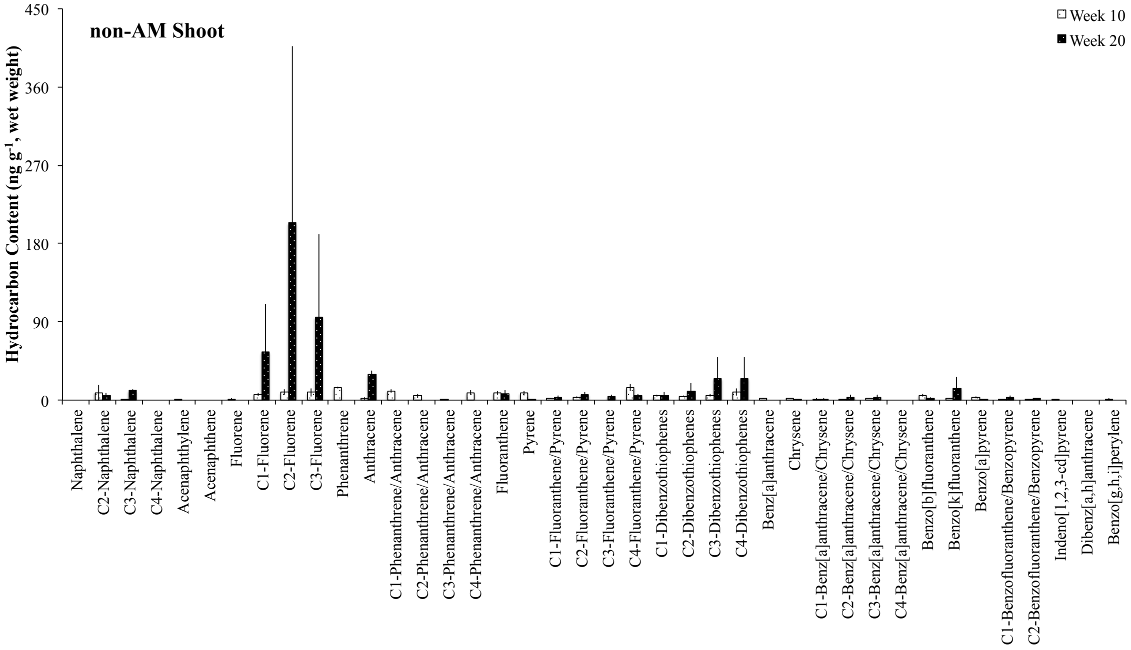

Figure B.9: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM shoot from week 10 to week 20 in the greenhouse study. n=3.

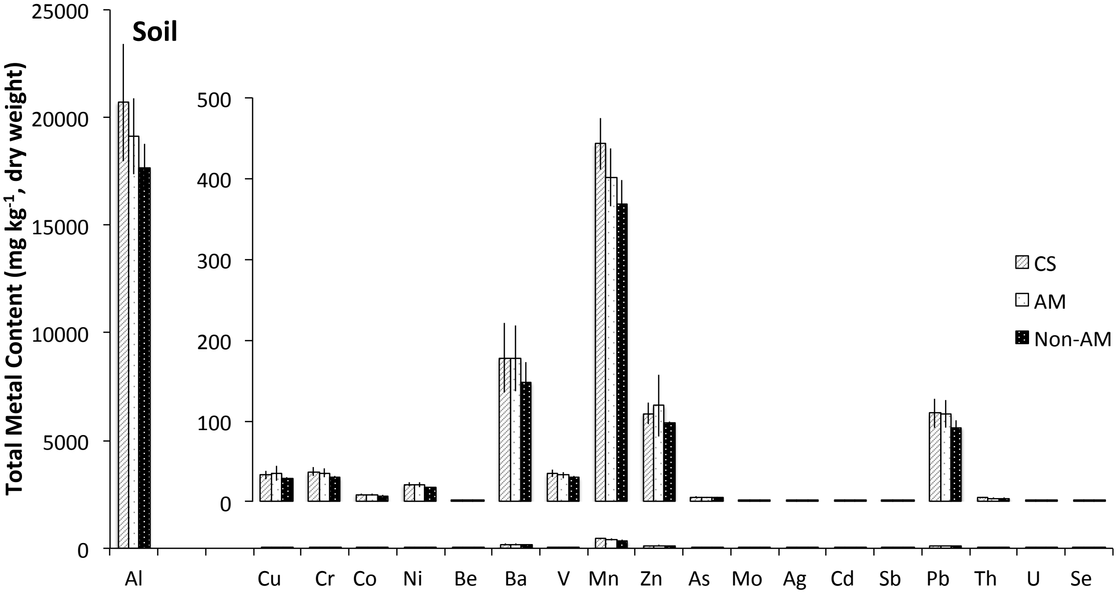

Figure B.10: Mean (±SE) metal content (mg kg^{-1} dry weight) in soil samples from week 10 for the greenhouse experiment. CS (Control Soil), AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

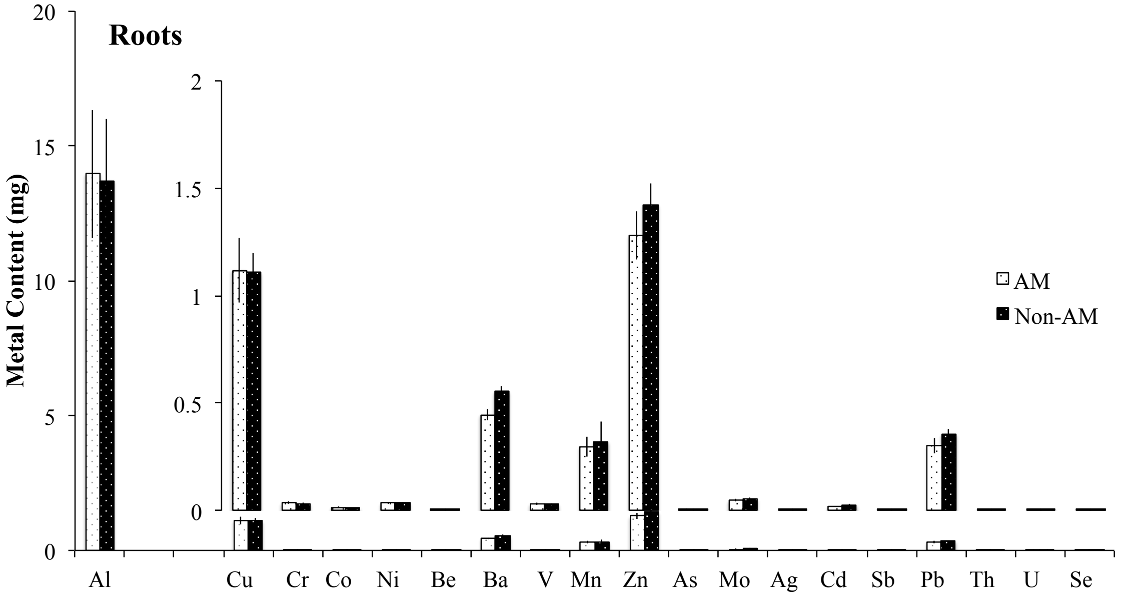

Figure B.11: Mean (±SE) metal content (mg dry weight) in root samples from Week 10 from the greenhouse experiment. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

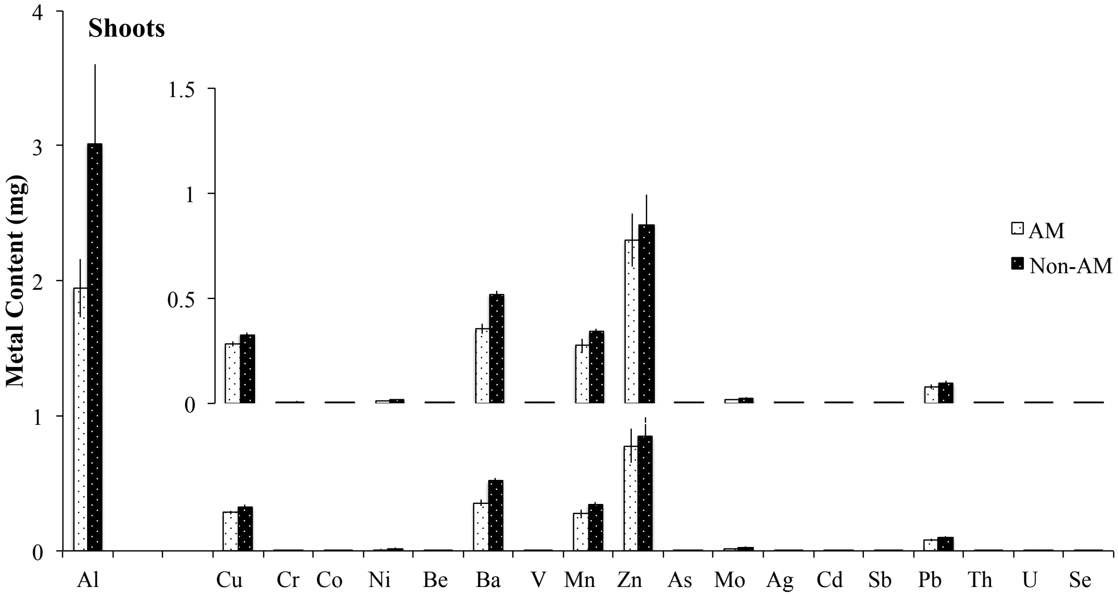

Figure B.12: Mean (±SE) metal content (mg dry weight) in shoot samples from week 10 from the greenhouse experiment. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

Table B.4: Two way ANOVA F-values and levels of significance for greenhouse metal concentrations (soil) and content (root and shoot).

F-Values and Significance				
		Soil	**Root**	**Shoot**
Ag	Week	0.4	2.1	0.2
	Treatment	0.1	0.1	0.2
	Week:Treatment	0.8	0.0	0.5
Al	Week	0.1	36.1***	0.3
	Treatment	0.0	0.2	1.4
	Week:Treatment	1.0	0.6	2.6
As	Week	0.2	14.2**	0.8
	Treatment	0.1	1.2	1.8
	Week:Treatment	0.9	1.3	2.7
Ba	Week	0.4	4.5	2.9
	Treatment	0.2	0.8	4.1
	Week:Treatment	1.2	1.2	4.9
Be	Week	0.0	30.2	2.0
	Treatment	0.0	0.1	1.2
	Week:Treatment	0.9	0.4	2.2
Cd	Week	2.1	9.0*	7.3*
	Treatment	0.3	1.4	1.5
	Week:Treatment	1.2	1.6	1.8
Co	Week	0.0	25.6***	1.1
	Treatment	0.0	0.1	1.8
	Week:Treatment	1.0	0.5	2.9
Cr	Week	0.1	29.0***	0.4
	Treatment	0.0	0.2	3.8
	Week:Treatment	1.1	0.9	6.7*
Cu	Week	0.5	3.0	33.7***
	Treatment	0.5	1.1	9.5*
	Week:Treatment	2.0	2.8	18.9**
Mn	Week	0.1	27.3	0.3
	Treatment	0.1	0.2	3.6
	Week:Treatment	1.0	0.4	7.0*

Mo	Week	0.6	1.8	9.5*
	Treatment	0.2	7.7*	2.1
	Week:Treatment	2.1	12.8**	2.0
Ni	Week	0.1	23.7**	0.0
	Treatment	0.1	0.7	4.0
	Week:Treatment	1.1	1.5	5.3*
Pb	Week	0.2	1.5	2.6
	Treatment	0.1	0.3	0.9
	Week:Treatment	0.8	0.4	1.1
Sb	Week	20.6**	1.9	4.9
	Treatment	1.4	1.1	10.8*
	Week:Treatment	0.8	1.7	8.6*
Se	Week	0.3	8.4*	3.5
	Treatment	0.0	0.2	3.0
	Week:Treatment	0.9	0.3	4.4
Th	Week	0.1	21.1**	0.3
	Treatment	0.0	0.0	0.1
	Week:Treatment	0.6	0.1	0.6
U	Week	0.1	3.6	0.8
	Treatment	0.0	0.2	1.3
	Week:Treatment	1.0	0.5	2.5
V	Week	0.1	26.5***	0.4
	Treatment	0.0	0.2	2.4
	Week:Treatment	1.1	0.5	4.1
Zn	Week	1.8	0.0	0.9
	Treatment	0.9	0.6	0.6
	Week:Treatment	0.4	0.9	0.9

* $p<0.05$

** $p<0.01$

*** $p<0.001$

Appendix C: Field Plot

Table C.1: Soil pH measured at year 1 and year 2 collected at Victoria Island, Ottawa, ON.

Year	Treatment	pH
1	**CS**	7.45(0.06)
	AM	7.47(0.08)
	Non-AM	7.46(0.05)
2	**CS**	7.68 (0.04)
	AM	7.50 (0.02)
	Non-AM	7.38 (0.01)

Note: Means (n=3) and (SE) are shown for each treatment.

Table C.2: Percent organic matter based on LOI

Year	Material	Percent Organic Matter
1	**CS**	4.40 (0.61)
	Non-AM	4.91 (0.73)
	AM	8.56 (0.95)
2	**CS**	4.04 (0.67)
	Non-AM	9.13 (2.19)
	AM	6.76 (0.74)

Note: Means (n=3) and (SE) are shown for each treatment.

Table C.3: AM root colonization counts after 1 year from field plot

	Count			Density (mm-1)		
Treatment	**Hyphae**	**Vesicles**	**Spores**	**Hyphae**	**Vesicles**	**Spores**
AM	34.7 (8.2)	286.7 (178.8)	2.7 (0.7)	0.07 (0.02)	0.57 (0.36)	0.005 (0.00)
Non-AM	26.0 (3.0)	84.3(57.7)	1.0 (0.6)	0.05 (0.01)	0.17 (0.12)	0.002 (0.00)
t-values and significance						
Treatment	1.0ns	1.1ns	1.9ns			

Note: Each treatment is 150, 1cm root segments (10 per slide) observed under a compound microscope. Means (n=3) and (SE) are shown for each treatment.
ns: not significant
* $p<0.05$
** $p<0.01$
*** $p<0.001$

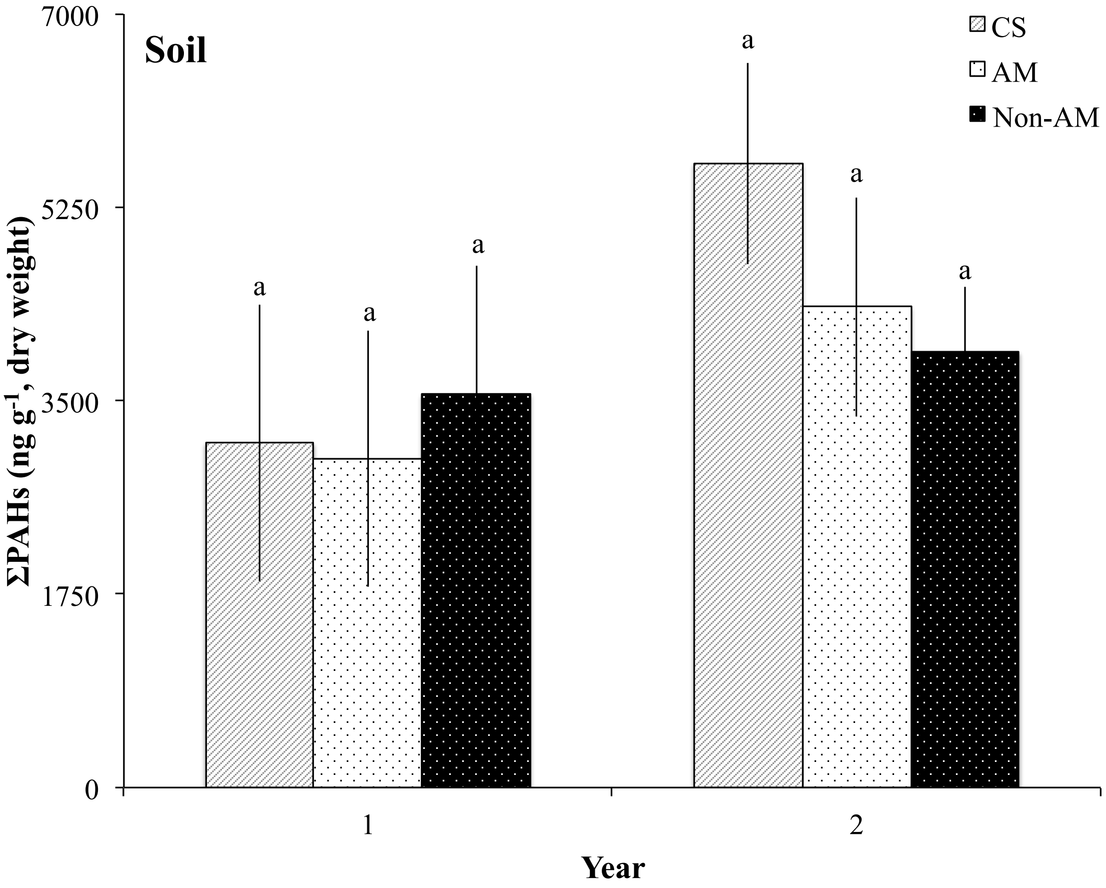

Figure C.1: Mean (±SE) ΣPAH content (ng g^{-1}) in soil over 1 year of growth in the field on Victoria Island. Data was analyzed using a one-way ANOVA [F (5, 12)=0.9, p=0.51]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (p=0.33) and Levene's test (p=0.99) for homogeneity of variance. Control Soil, AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

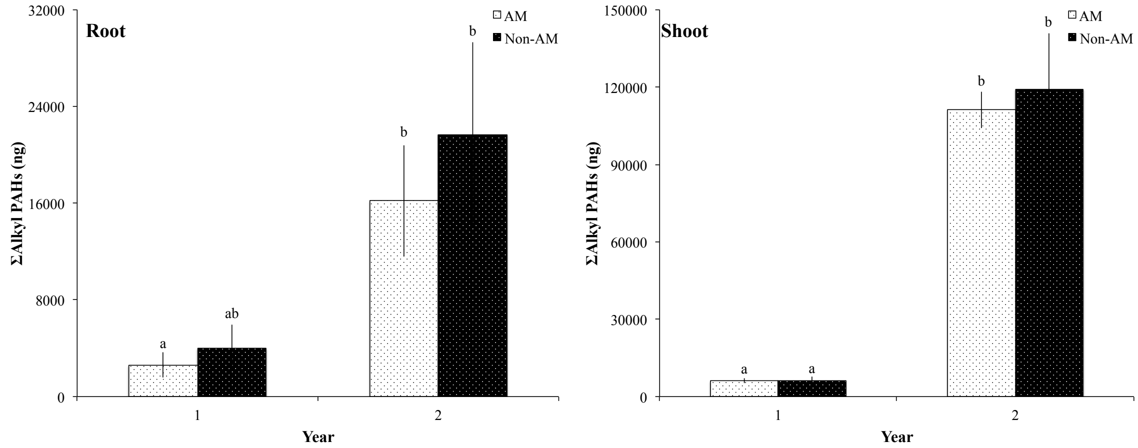

Figure C.2: Mean (±SE) ΣAlkyl PAH content (ng) over 1 year of growth in *Echinacea purpurea* on Victoria Island. Data was analyzed using a one-way ANOVA for roots [F (3, 8)=6.7, p=0.013] and shoots [F (3, 8)=98.4, p=0.000002]. Different letters indicate significant differences according to Tukey's post-hoc test. Normality was tested using Shapiro-Wilk (SW) and Levene's test (LT) for homogeneity of variance in roots (SW p=0.05, LT p=0.46) and shoots (SW p=0.012, LT p=0.22). AM: *Echinacea purpurea* inoculated with *Glomus intraradices*, non-AM: *E. purpurea* only. n=3.

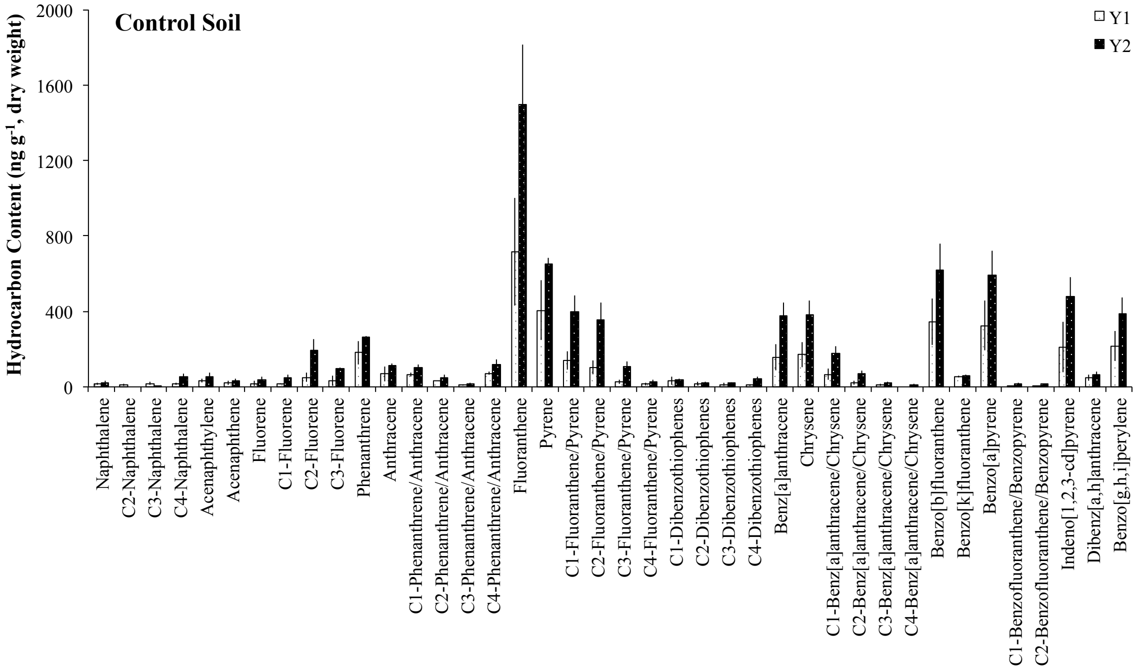

Figure C.3: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for control soil from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

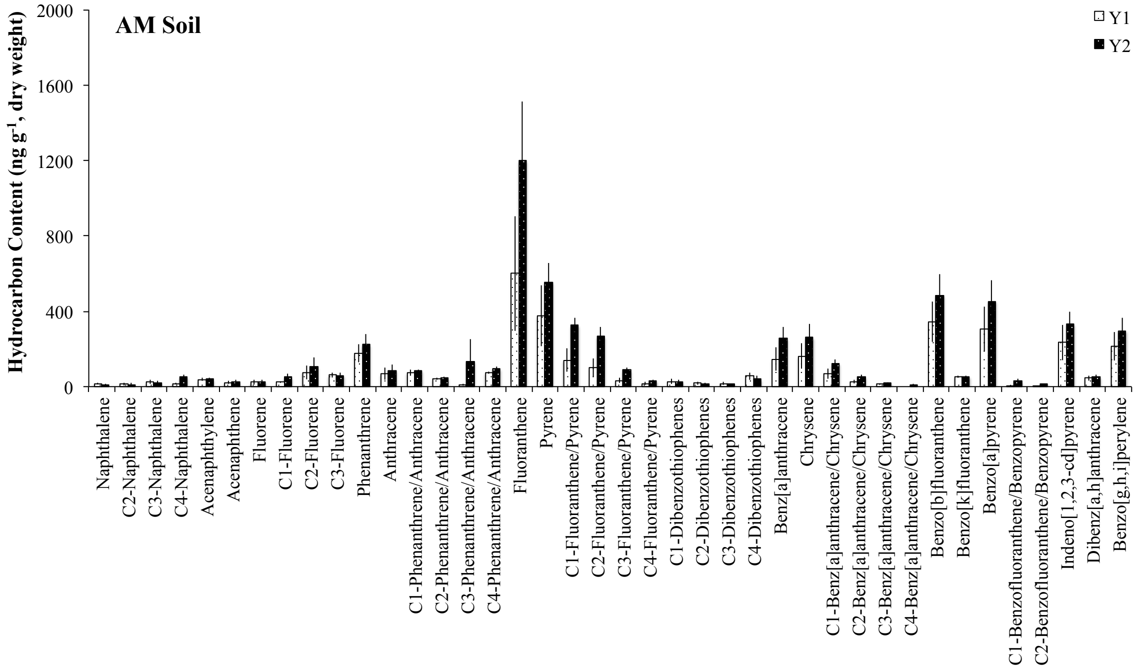

Figure C.4: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM soil from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

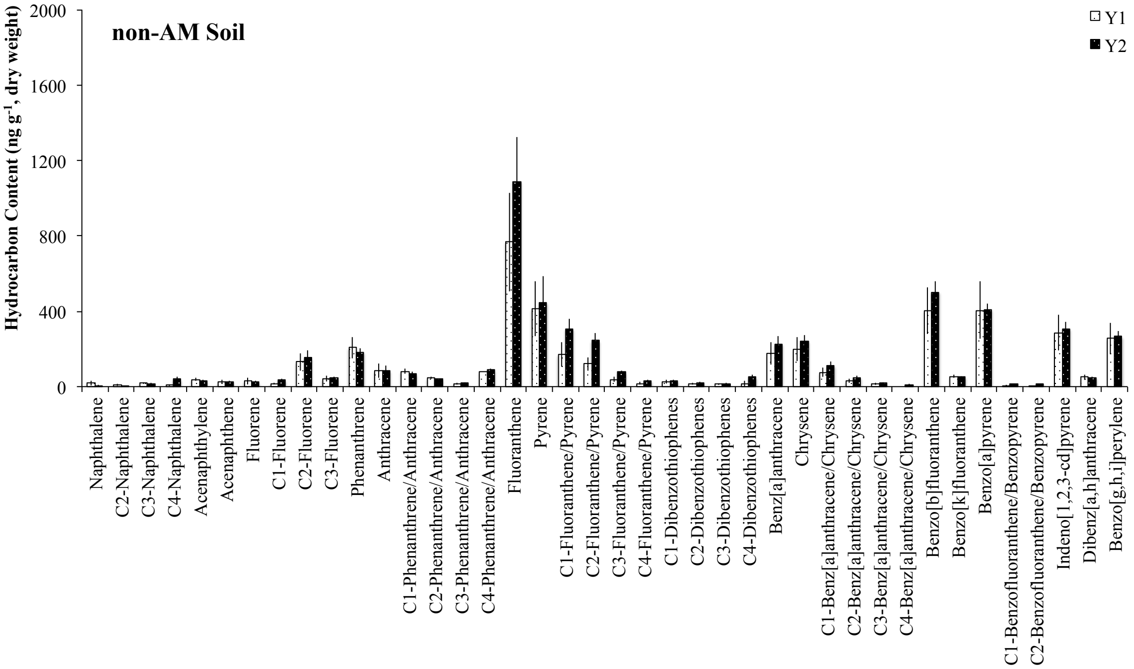

Figure C.5: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM soil from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

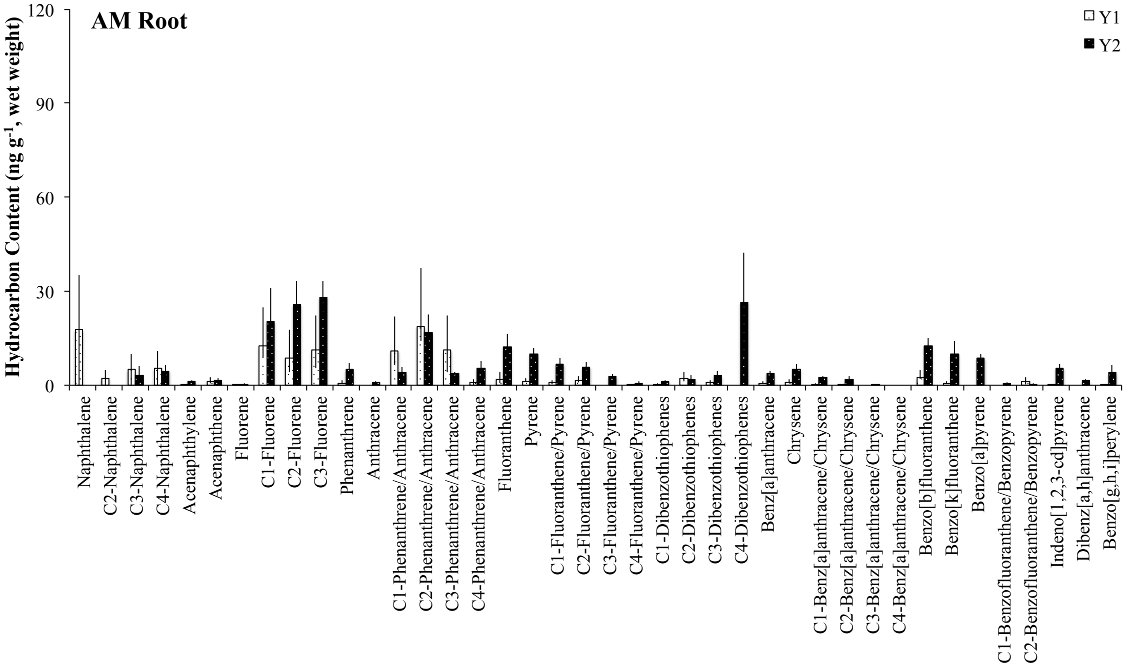

Figure C.6: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM roots from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

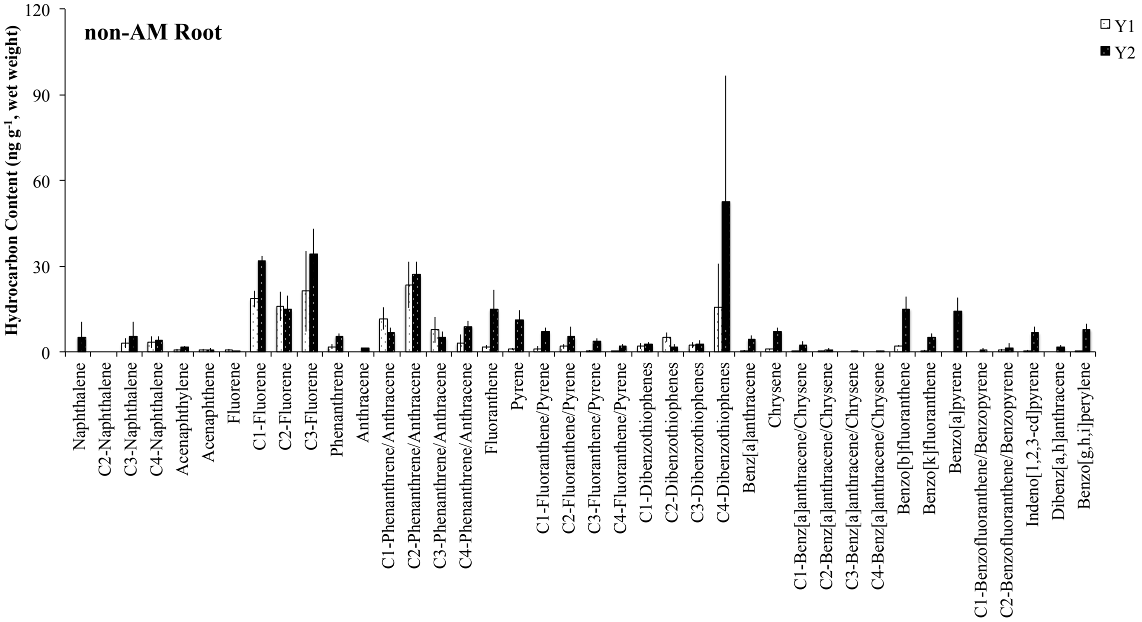

Figure C.7: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM roots from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

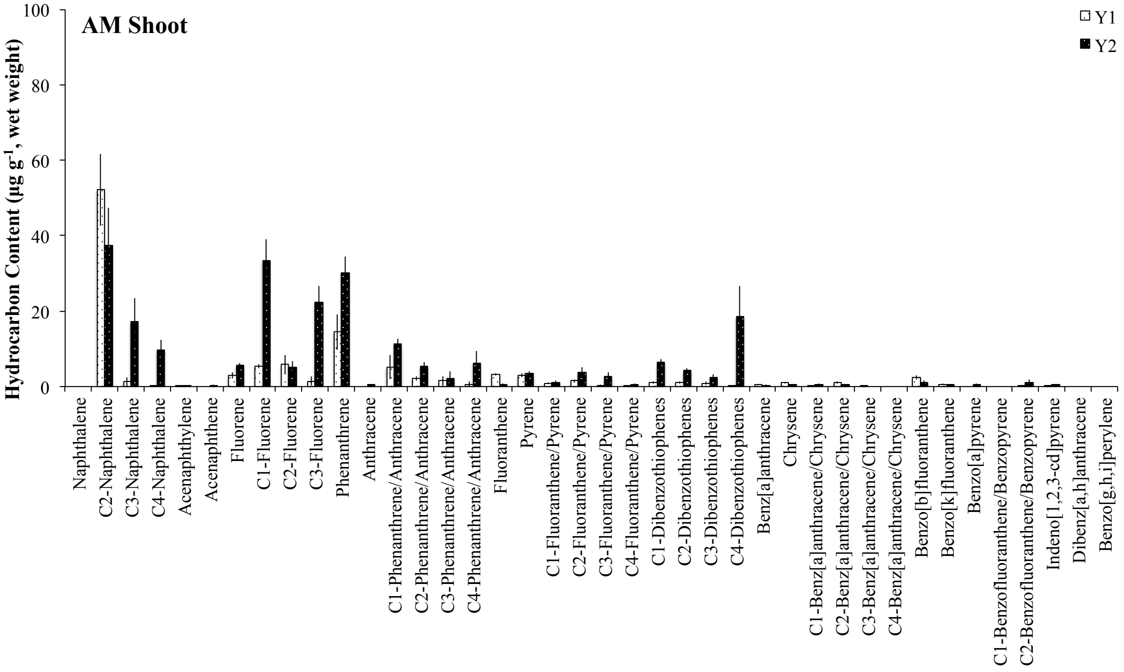

Figure C.8: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for AM shoots from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

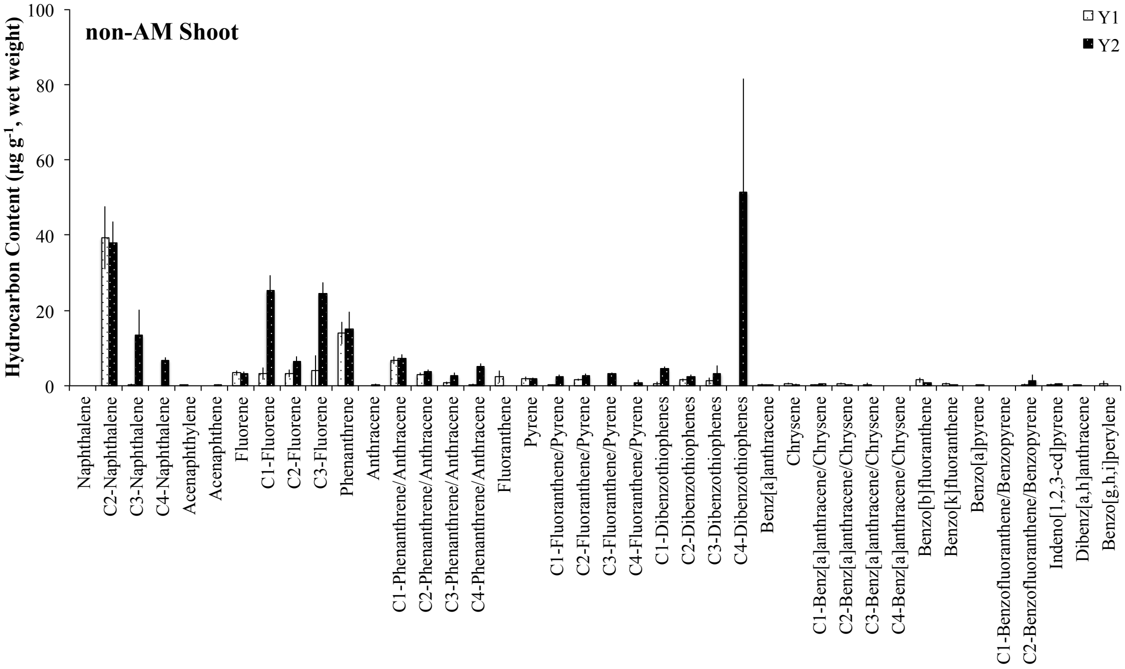

Figure C.9: Mean (±SE) hydrocarbon content (ng g^{-1}) profile for non-AM shoots from year 1 (Y1) to year 2 (Y2) on Victoria Island, ON field site. n=3.

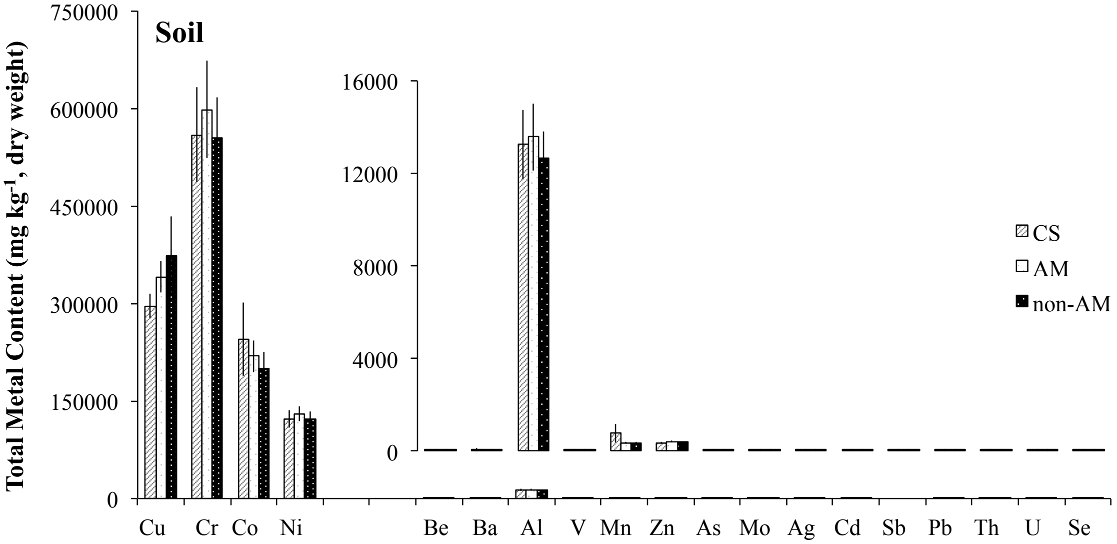

Figure C.10: Mean (±SE) total metal content (mg kg^{-1} dry weight) in soil samples from year 1 at Victoria Island field site. CS (Control Soil), AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

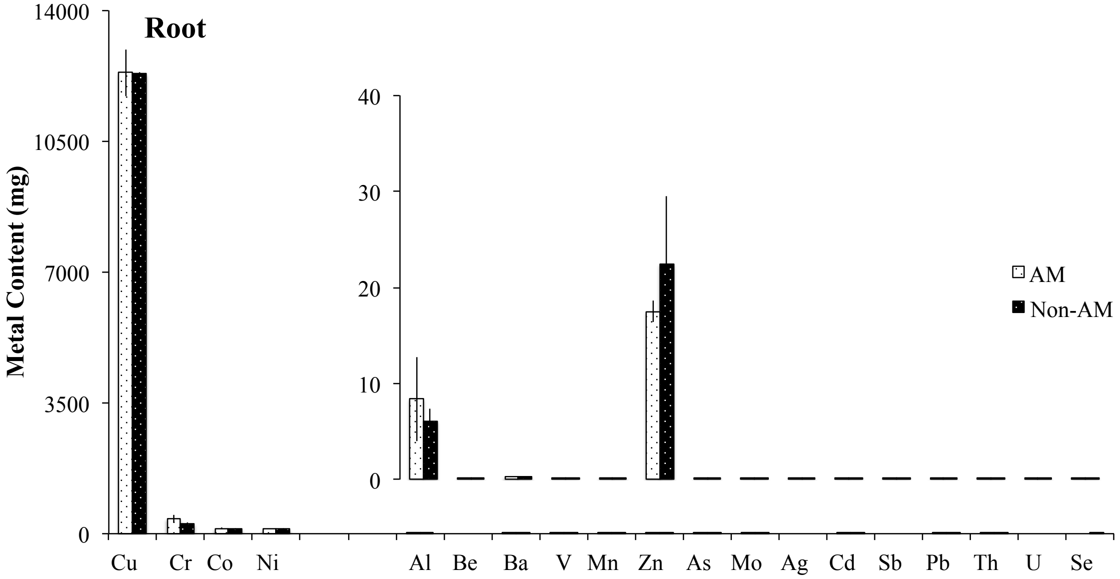

Figure C.11: Mean (±SE) metal content (mg dry weight) in root sample of *Echinacea purpurea* from year 1 at Victoria Island field site. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

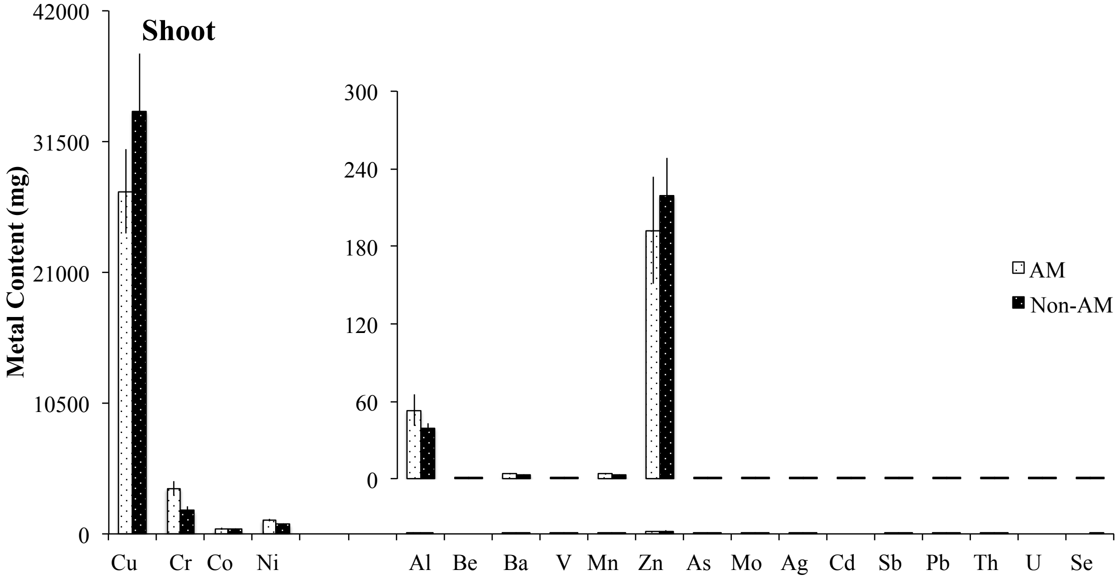

Figure C.12: Mean (±SE) metal content (mg dry weight) in shoot sample of *Echinacea purpurea* from year 1 at Victoria Island field site. AM (inoculated with *Glomus intraradices*), non-AM (not inoculated), n=3. Inset is an enlargement of the concentrations.

Table C.4: Two way ANOVA F-values and levels of significance for field plot metal concentrations (soil) and content (root and shoot).

F-Values and Significance				
		Soil	**Root**	**Shoot**
Ag	Week	7.2*	26.3***	1.1
	Treatment	0.2	0.9	0.7
	Week:Treatment	0.2	0.5	0.3
Al	Week	20.6**	78.6***	0.6
	Treatment	0.3	0.1	0.0
	Week:Treatment	0.7	0.1	0.3
As	Week	0.0	92.9***	9.0*
	Treatment	0.2	4.8	0.1
	Week:Treatment	0.4	3.1	0.1
Ba	Week	5.8*	255.2***	95.1***
	Treatment	0.3	0.0	4.4
	Week:Treatment	0.9	0.4	4.0
Be	Week	6.8*	21.9**	2.5
	Treatment	0.2	0.2	0.3
	Week:Treatment	0.7	0.1	0.3
Cd	Week	4.5	67.1***	10.9*
	Treatment	0.5	1.7	0.1
	Week:Treatment	0.6	3.6	0.7
Co	Week	7.3*	420.8***	2.3
	Treatment	0.8	0.5	0.2
	Week:Treatment	1.5	0.2	0.9
Cr	Week	6.2*	248.4***	12.7**
	Treatment	0.1	1.6	3.7
	Week:Treatment	1.3	0.9	1.0
Cu	Week	0.0	163.9***	37.0***
	Treatment	1.1	0.0	1.7
	Week:Treatment	0.5	0.1	2.2
Mn	Week	9.4*	195.3***	169.6***
	Treatment	1.9	0.4	3.2
	Week:Treatment	1.2	0.1	0.4

Mo	Week	1.1	20.2**	36.4***
	Treatment	0.5	2.8	0.0
	Week:Treatment	0.4	2.5	0.2
Ni	Week	2.3	447.5***	5.2
	Treatment	0.0	0.0	0.4
	Week:Treatment	1.6	0.1	0.0
Pb	Week	3.6	91.3***	0.8
	Treatment	0.1	1.5	0.0
	Week:Treatment	0.7	0.8	0.0
Sb	Week	4.0	33.7***	176.6***
	Treatment	0.0	1.0	3.1
	Week:Treatment	0.4	0.9	3.8
Se	Week	4.0	19.8**	26.1***
	Treatment	0.1	0.5	0.2
	Week:Treatment	0.8	0.3	0.2
Th	Week	0.6	17.1**	0.0
	Treatment	0.0	0.3	0.0
	Week:Treatment	0.0	0.2	0.1
U	Week	2.8	98.0***	3.9
	Treatment	0.0	1.3	0.2
	Week:Treatment	0.2	1.2	0.2
V	Week	10.0*	502.9***	12.5**
	Treatment	0.7	0.7	5.3
	Week:Treatment	1.3	0.4	0.1
Zn	Week	27.1***	8.6*	6.1*
	Treatment	0.0	0.0	0.5
	Week:Treatment	0.4	0.1	1.4

* $p<0.05$
** $p<0.01$
*** $p<0.001$

Appendix D: List of all PAH and Alkyl PAHs Analyzed

Table D.1: List of all compounds analyzed using GC-MS.

Type	PAH Compound
Low Molecular Weight (LMW)	Naphthalene
	Acenaphthylene
	Acenaphthene
	Fluorene
	Phenanthrene
	Anthracene
	Fluoranthene
High Molecular Weight (HMW)	Pyrene
	Benz[a]anthracene
	Chrysene
	Benzo[b]fluoranthene
	Benzo[k]fluoranthene
	Benzo[a]pyrene
	Indeno[1,2,3-cd]pyrene
	Dibenz[a,h]anthracene
	Benzo[g,h,i]perylene
Alkyl PAHs	C2-Naphthalene
	C3-Naphthalene
	C4-Naphthalene
	C1-Fluorene
	C2-Fluorene
	C3-Fluorene
	C1-Phenanthrene/Anthracene
	C2-Phenanthrene/Anthracene
	C3-Phenanthrene/Anthracene
	C4-Phenanthrene/Anthracene
	C1-Dibenzothiophenes
	C2-Dibenzothiophenes
	C3-Dibenzothiophenes
	C4-Dibenzothiophenes
	C1-Fluoranthene/Pyrene
	C2-Fluoranthene/Pyrene
	C3-Fluoranthene/Pyrene
	C4-Fluoranthene/Pyrene
	C1-Benz[a]anthracene/Chrysene
	C2-Benz[a]anthracene/Chrysene
	C3-Benz[a]anthracene/Chrysene
	C4-Benz[a]anthracene/Chrysene
	C1-Benzofluoranthene/Benzopyrene
	C2-Benzofluoranthene/Benzopyrene

Appendix E: Structure of 16 U.S. EPA Priority Pollutant PAH Compounds

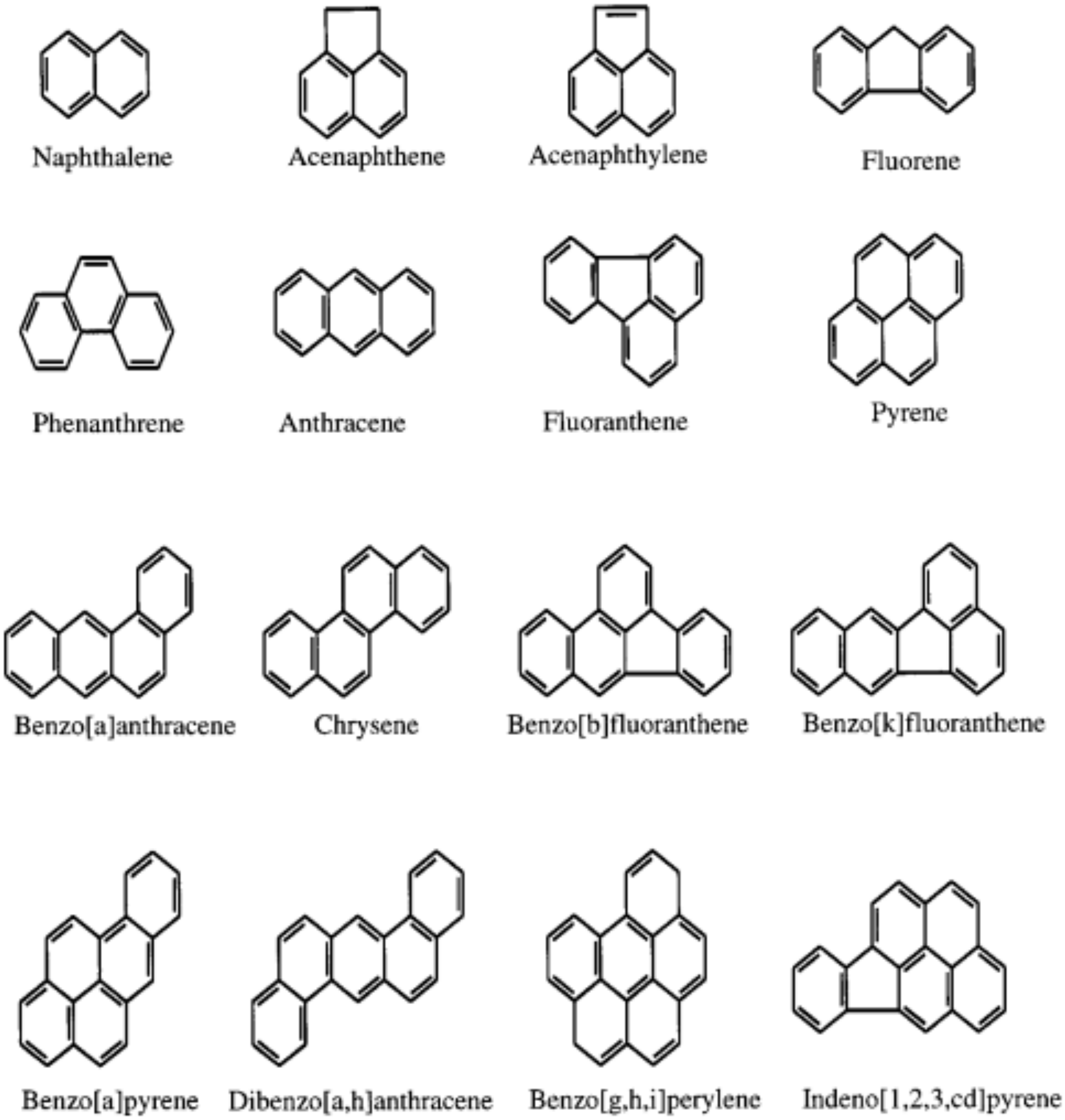
Naphthalene
Acenaphthene
Acenaphthylene
Fluorene
Phenanthrene
Anthracene
Fluoranthene
Pyrene
Benzo[a]anthracene
Chrysene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Benzo[a]pyrene
Dibenzo[a,h]anthracene
Benzo[g,h,i]perylene
Indeno[1,2,3,cd]pyrene

Figure E.1: Structure of 16 U.S. EPA Priority Pollutant PAH compounds.

Appendix F: Factorial Block Design

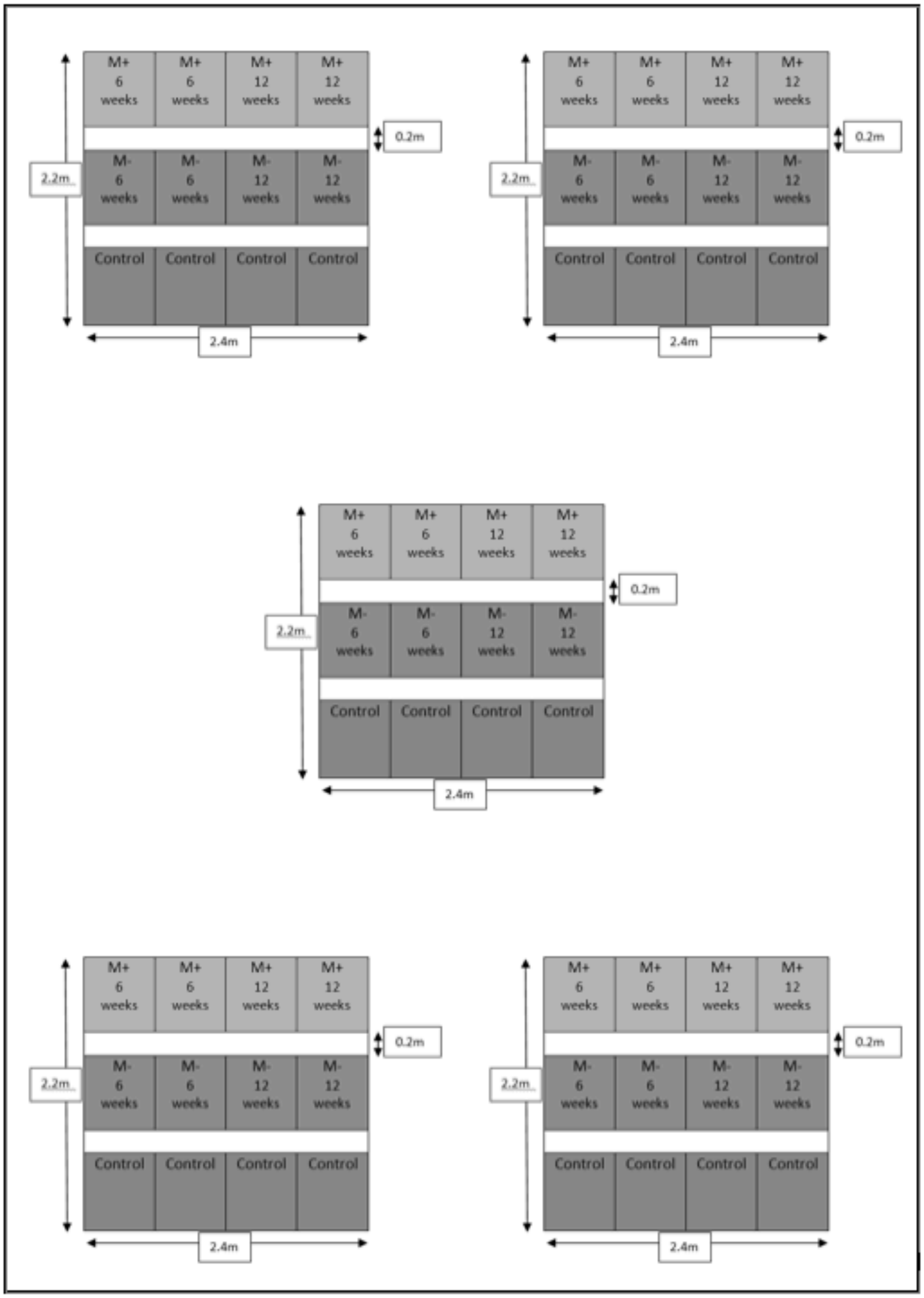

Figure F.1: Factorial block design used for the field plot.