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Potential of Five Plant Species for Phytoremediation of Metal-PAH-Pesticide Contaminated Soil

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Abstract

Phytoremediation of contaminated soils has gained great attention as a low-cost and environmentally friendly remediation option. Given the desired advantages of phytoremediation, the present research evaluates the potential of established phytoremediation plants (alfalfa, oat, ryegrass, Indian mustard, sunflower, tall fescue and switch grass) to remediate mixed metal-PAH-pesticide contaminated soil in greenhouse pot experiments. Mixed contaminated soil was prepared by spiking soil with copper (Cu), lead (Pb), pyrene and DDT as model compounds. Prior to the pot experiments, a phytotoxicity test was conducted to determine preliminary toxicity effects of combined contaminants on plants. The results eliminated tall fescue and switch grass from further consideration. Alfalfa, oat, ryegrass, Indian mustard and sunflower were grown in triplicates for 72 days in pots containing clean soil and soil contaminated with mixed contaminants. The results showed that sunflower and Indian mustard were the most tolerant plants to the studied mixed contaminants. Furthermore, sunflower was able to simultaneously remove metals, PAH and pesticide. Oat was identified as unsuitable for phytoremediation of metal-PAH-pesticide contaminated soil due to its ability to increase exchangeable Cu compared to unplanted soils. Overall the work supports the use of phytoremediation as a potential remedial option for soils contaminated with mixed contaminants.

Key words: Phytoremediation, Copper (Cu), Lead (Pb), DDT, Pyrene, Mixed contaminated soil, Sunflower, Indian mustard, Oat, Alfalfa, Ryegrass

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Table of Contents

Abstract.....	i
Acknowledgements	ii
List of Tables	vii
List of Figures.....	viii
List of Appendices.....	xi
List of Abbreviations	xii
CHAPTER 1	1
1.0 Introduction	1
1.1 Environmental contamination.....	1
1.2 Soil remediation.....	2
1.3 Problem statement and justification.....	6
1.4 Objectives of the research	9
1.5 Thesis Format.....	10
CHAPTER 2	11
2.0 Literature Review	11
2.1 Fate of contaminants in soil	11
2.2 Phytoremediation of contaminated soils	14
2.3 Phytoremediation Techniques.....	15
2.4 Phytoremediation of organic compounds	16
2.5 Phytoremediation of inorganic contaminants	18

2.6 Phytoremediation of organic-inorganic mixed contaminated soils	20
2.7 Conclusion	25
CHAPTER 3	26
3.0 Phytoremediation of Metal-PAH-Pesticide Contaminated Soil	26
3.1 Materials and Methods.....	26
3.1.1 Contaminant selection	26
3.1.2 Plant selection.....	29
3.1.3 Physical and chemical properties of soil	29
3.1.4 Soil spiking procedure	29
3.1.5 Toxicity test	30
3.1.6 Plant growth and harvesting	31
3.1.7 Soil pore water extraction.....	32
3.1.8 Metal analysis in soil and plant	32
3.1.9 Pyrene and DDT analysis in soil and plants	33
3.1.10 Statistical analysis and quality control	35
3.2 Results.....	36
3.2.1 Soil properties.....	36
3.2.2 Preliminary toxicity test of mixed contaminated soil on plants: Effect of contamination on percentage germination	38
3.2.3 Preliminary toxicity test of mixed contaminated soil on plants: Effects of contamination on root and shoot length	39

3.2.4	Metal uptake by plants.....	41
3.2.5	Fate of metals in Soil pore water.....	44
3.2.6	Fate of metals in soil.....	45
3.2.7	Fate of Organic Contaminants in Soil.....	50
3.2.8	Organic contaminants uptake by plants.....	51
3.2.9	Plant growth response to mixed contamination.....	54
3.3	Discussion.....	60
3.3.1	Preliminary toxicity test.....	60
3.3.2	Metal uptake by plants.....	61
3.3.3	Soil metal fractions.....	63
3.3.4	Fate of organic contaminants.....	65
3.3.5	Plant growth performance.....	68
3.4	Conclusion.....	68
3.5	Limitation of study.....	69
CHAPTER 4	71
4.0	Conclusion and Recommendation.....	71
4.1	Significance of Study.....	71
4.2	Recommendations.....	72
References	74
APPENDICES	101

Curriculum Vitae116

List of Tables

Table 1-1: Some applied remediation techniques. The list includes examples of contaminants targeted by each technique and the effectiveness of remediation.	3
Table 3-1: Concentration limits for selected contaminants in industrial soil and the concentrations used as the experimental treatment.....	27
Table 3-2: Physical and chemical properties tested for the study soil.....	28
Table 3-3: Steps for sequential extraction of metals from soil.....	34
Table 3-4: Physical and chemical properties of the natural study soil (mean \pm SE, n=3).....	37
Table 3-5: pH _{CaCl2} (mean \pm SE, n=3) of planted and unplanted mixed contaminated soil. Different letters indicate a significant difference between planted and unplanted soil ($p \leq 0.05$).....	38
Table 3-6: Translocation Factor (mean \pm SE) of Cu and Pb in plants grown on contaminated soil. Different letters indicate a significant difference between TF of plants for Cu and Pb ($p \leq 0.05$).	43
Table 3-7: Metal concentration (mean \pm SE) in soil pore water after phytoremediation.....	44
Table A-1: HPLC setup for pyrene.....	104
Table B-1: HPLC setup for DDT	107
Table C-1: Metal concentration (mean \pm SE, n=3) of plants grown in clean soil.....	110
Table D-1: Two way analysis of variance for Cu fractions in soil. DF=Degree of freedom, SS=Sum of squares, MS=Mean square.....	112
Table D-2: Two way analysis of variance for Pb fractions in soil. DF=Degree of freedom, SS=Sum of squares, MS=Mean square.....	112
Table E-1: List of statistical analysis of all data sets.....	114

List of Figures

Figure 3-1: Germination percent (mean \pm SE, n=3) of plants in clean soil and contaminated soil. Different letters indicate a significant difference between clean and contaminated soil and between plants ($p \leq 0.05$).....	39
Figure 3-2: Root length (mean \pm SE, n=3) of plant species in clean soil and contaminated soil. Different letters indicate a significant difference between clean and contaminated soil and between plants ($p \leq 0.05$).....	40
Figure 3-3: Shoot length (mean \pm SE, n=3) of plant species in clean soil and contaminated soil. Different letters indicate a significant difference between clean and contaminated soil and between plants ($p \leq 0.05$).....	40
Figure 3-4: Metal concentration (mean \pm SE) in plant tissues after growth in contaminated soil (a) Cu (b) Pb. Different letters indicate a significant difference between Cu and Pb uptake by plants ($p \leq 0.05$).	43
Figure 3-5: Metal accumulation (mean \pm SE) in plants after growth in contaminated soil. Metal speciation in soil. Different letters indicate a significant difference between total Cu and Pb accumulation by plants ($p \leq 0.05$).	44
Figure 3-6: Metal concentration (mean \pm SE) in planted and unplanted soil. (a) Cu and (b) Pb. Different letters indicate a significant difference between Cu and Pb content in planted and unplanted soils ($p \leq 0.05$).	47
Figure 3-7: Comparison of effect of plant growth on Cu speciation in mixed contaminated soil.F1: exchangeable fraction, F2: carbonates-bound fraction, F3: Fe-Mn oxides-bound fraction, F4:	

organic fraction, F5: residual fraction	
Figure 3-8: Comparison of effect of plant growth on Pb speciation in mixed contaminated soil. F1: exchangeable fraction, F2: carbonates-bound fraction, F3: Fe-Mn oxides-bound fraction, F4: organic fraction, F5: residual fraction.....	48
Figure 3-9: Comparison of pyrene concentration in planted and unplanted soil before and after phytoremediation treatment. Different letters indicate a significant difference between pyrene degradation in planted and unplanted soil ($p \leq 0.05$).	50
Figure 3-10: Comparison of 4, 4'- and 2, 4'-DDT concentration in planted and unplanted soil before and after phytoremediation treatment. Different letters indicate a significant difference between DDT isomers and their degradation in planted and unplanted soil ($p \leq 0.05$).	51
Figure 3-11: Total DDT concentration (mean \pm SE) in plant tissues after growth in contaminated soil (a) Shoot (b) Root. Different letters indicate a significant difference between Cu and Pb uptake by plants ($p \leq 0.05$).	53
Figure 3-12: Germination percent (mean \pm SE) of plants in clean and contaminated soil. Different letters indicate a significant difference between DDT isomers and their degradation in planted and unplanted soil ($p \leq 0.05$).	55
Figure 3-13: Plant survival (mean \pm SE) as a ratio of final germination to initial germination of plants in contaminated soil. Different letters indicate a significant difference between DDT isomers and their degradation in planted and unplanted soil ($p \leq 0.05$).	56
Figure 3-14: Increase in plant height with time (mean \pm SE) in clean and contaminated soil. * indicate a significant difference between plant growth in clean and contaminated soil (Time $p \leq 0.05$).	58
Figure 3-15: Root and shoot biomass (mean \pm SE) of plants in clean (CS) and contaminated soil (TS). Different letters indicate a significant difference between clean and contaminated soil and between plants (Total biomass $p \leq 0.05$).	59

Figure A-1: Chromatograph for pyrene 100mg/l.....	104
Figure A-2: Calibration curve for pyrene.....	105
Figure B-1: Chromatograph for DDT 10mg/l	108
Figure B-2: Calibration curve for DDT.....	108
Figure F- 1: Yellowing and drying up of leaves observed in sunflower and Indian mustard plants grown in contaminated soils.....	116

List of Appendices

Appendix A: Plant metal concentrations in clean soil.....	103
Appendix B: HPLC details for pyrene.....	106
Appendix C: HPLC details for DDT.....	109
Appendix D: Analysis of variance for metal fractioning in soil.....	111
Appendix E: List of parametric and non-parametric statistical tests used for data analysis.....	113
Appendix F: Photographs of effects of contamination on plant growth.....	115

List of Abbreviations

ANOVA	Analysis of variance
BTEX	Benzene, toluene, ethylbenzene and xylene
DDD	Dichloro-diphenyl-dichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
DOC	Dissolved organic carbon
HCH	Hexachlorocyclohexane
HPLC	High performance liquid chromatography
OCP	Organochlorine pesticides
PAH	Polycyclic aromatic Hydrocarbon
PBDE	Polybrominated Diphenyl Ethers
PCB	Polychlorinated Biphenyls
PCDD/FS	Polychlorinated Dibenzop-Dioxins and Polychlorinated Dibenzofurans
PHC	Petroleum Hydrocarbons
PPCP	Pharmaceuticals and Personal Care Products
TF	Translocation Factor
TNT	2,4,6-trinitrotoluene

TPH

Total Petroleum Hydrocarbon

TPH

Total petroleum hydrocarbon

VOC

Volatile Organic Compound

CHAPTER 1

1.0 Introduction

1.1 Environmental contamination

Soil contamination is a major problem in Africa (Donkor, Bonzongo, Nartey, & Adotey, 2005; Jonker & Olivier, 2012), Asia (Moore, Dehghan, & Keshavarzi, 2014; Zhao, Ma, Zhu, Tang, & McGrath, 2015), Australia (Martley, Gulson, & Pfeifer, 2004; McGrath, Morrison, Sandiford, Ball, & Clarke, 2016), Europe (Douay et al. 2008; Lage, Wolterbeek, and Almeida 2016; Global Soil Forum 2013), North America (Eagles-Smith et al., 2016; McClintock, 2012) and South America (Mochungong & Zhu, 2015). Soil contamination has been attributed largely to rapid expansion of human activities in the form of agriculture, mining, industrialization, urbanization and globalization, to sustain the increasing world population. These activities have led to continuous release into the environment of xenobiotic chemicals whose concentrations and behaviors alter the natural state of the environment. Contamination of soil is of key interest because contaminants can be easily transferred to other natural resources (surface water, ground water and air) via leaching, run-off and evaporation, and to the food chain through uptake by plants, thereby compromising human health. Alongside reports of occurrence of these contaminants are findings of their adverse effects on the ecosystem, such as loss of aquatic life, loss of soil organisms, mutation during reproduction of organisms at various trophic levels of the ecosystem and cancer in humans (CCME 1999a).

In general, contaminants may be classified as being either organic or inorganic. Organic contaminants include pesticides, PHC (petroleum hydrocarbons), PCB (polychlorinated biphenyls), HCH (hexachlorocyclohexane), PBDE (polybrominated diphenyl ethers), and PPCP

(pharmaceuticals and personal care products). Inorganic contaminants include metals, metalloids, nanomaterials, radionuclides and nutrients. It is important to note that some of these contaminants occur naturally in soil and are only considered as contaminants when they occur in forms and concentrations that are detrimental to the ecosystem. The most problematic types of contaminants are the volatile organic contaminants (due to their easy transfer from soil or water to air, creating inhalation risks), hydrophobic organic compounds (due to their low solubility in water, which makes them immobile and persistent in soil) and metals (due to their inability to undergo microbial or chemical degradation) (Lee et al. 2002; Saichek and Reddy 2005).

Increasing occurrence of contaminants in the environment as well as their adverse effects led to establishment of soil pollution prevention measures such as banning the use of some contaminants and establishing legislation to prevent soil pollution (CCME 1999a; U.S EPA 2016). Over time, it was observed that even with adoption of pollution prevention measures, contaminants persisted in soil, due to their resistance to natural degradation processes, and have found their way into water courses and other constituents of the ecosystem. This created the need for research on possible remediation techniques.

1.2 Soil remediation

Established soil remediation techniques fall into four categories (Castelo-Grande, Augusto, Monteiro, Estevez, & Barbosa, 2010; Cheng-Kim, Bakar, Mahmood, & Abdullah, 2016). 1) Biological techniques, which include the use of microorganisms that can degrade the contaminant and establishing conditions that encourage increased microbial activities; e.g., bio-piles and land farming, bioventing, bio-stimulation, bio-augmentation, composting, natural attenuation and phytoremediation (the use of plants to take up contaminants from soil into their biomass).

Table 1-1: Some applied remediation techniques. The list includes examples of contaminants targeted by each technique and the effectiveness of remediation.

Remediation technique	Type of remediation	Contaminants	Treatment description	Outcome	Reference
Biological techniques	Biostimulation with hydrogen peroxide, oleophilic fertilizer and surfactant (Bioversal HC)	PHC(8.77mg/kg)	Field-scale	Complete degradation of linear alkanes and reduction of cyclic and branched compounds after 4 months	(Menendez-Vega et al., 2007)
	Bioaugmentation by multiple inoculation with indigenous bacteria	Fuels- diesel oil and aircraft fuel(6188mg/kg)	Field-scale	80-98% removal of TPH (total petroleum hydrocarbon) after 5months	(Łebkowska et al., 2011)
	Natural attenuation	Co-contaminated soil with heavy metals (Cu, Pb and Zn at 87, 100 and 110 mg kg ⁻¹) and petroleum hydrocarbons (3800 mg kg ⁻¹)	Pot experiments	37% reduction in TPH (total petroleum hydrocarbon) Zero reduction in metals	(Agnello, Bagard, Van Hullebusch, Esposito, & Huguenot, 2016)
Phytoremediation with alfalfa (<i>Medicago sativa</i> L.)	47% reduction TPH (total petroleum hydrocarbon) Zero extraction of metals but considerable phytostabilization				

Chemical techniques	Soil washing with 2M H ₃ PO ₄ , 2M NaOH and 0.1M Dithionite in 0.1M EDTA for 24hours	As (165.5mg/kg)	Laboratory study	90% reduction in As content of soil	
	Soil washing with fish extracts for 60mins per cycle	PCDDs (polychlorinated dibenzo-p-dioxins), PCDFs (Dibenzofurans) (22µg/kg)	Laboratory study - ultrasonification and mechanical double-blade stirring	94.12% removal of contaminants in moderately contaminated soils (5 washing cycles) and 94.51% removal of contaminants highly contaminated soils (10 washing cycles)	(Vu et al., 2017)
	Enhanced electrokinetic treatment with citric acid and ethylenediaminetetraacetic acid (EDTA)	Co, Zn, Cd, Cu, Cr, Pb and Hg(10406mg/kg)	Laboratory study	Migration of metals to soil section closer to the cathode	(Figueroa, Cameselle, Gouveia, & Hansen, 2016)
	Unenhanced electrokinetic treatment	Pyrene (261.3mg/kg in sandy soil and 259.8mg/kg in loam soil)	Laboratory study	57% and 20% removal of pyrene from sandy and loam soil	(Xu, Guo, Wu, Li, & Li, 2014)
Physical techniques	Thermal treatment by resistive heating	Trichloroethylene(273mg/kg)	Laboratory study-Tank reactors	99.8% reduction in TCE after 37 days	(Heron, Van Zutphen, Christensen, & Enfield, 1998)
	Thermal treatment by heated air	PAH(2308mg/kg) and VOC(4105mg/kg)	Field trial	71% reduction in PAH and 74% reduction in VOC	(CL:AIRE, 2006)

2) Chemical techniques that exploit the chemical properties of contaminants and soil to enhance degradation; e.g., soil vapor extraction, airsparging, dechlorination, soil washing/flushing, solidification/stabilization, electrokinetics (use of low voltage electric current to immobilize contaminants) and solvent extraction. 3) Physical techniques which mainly refer to soil replacement (complete removal of contaminated soil or mixing contaminated soil with non-contaminated soil) and thermal desorption (high temperatures in the presence of oxygen are used to breakdown contaminants). 4) Primary action techniques (use of passive and reactive barriers to prevent contaminant migration). These remediation techniques mentioned above have been applied in a number of laboratory and field studies, some with promising results and others with little success as demonstrated by recent studies (Table 1-1).

Limitations to field application of remediation techniques could include cost of technology, social factors, site accessibility, climatic and soil conditions, biological factors, depth and location of contaminants, types of contaminants, combination of contaminants, and regulations. To implement remediation techniques successfully, these limitations must be addressed. An important limitation which has hardly been addressed in remediation studies is the occurrence of a mixture of various classes of contaminants.

Typically, soil contamination does not only involve one class of contaminants (Arjoon, Olaniran, & Pillay, 2013; DCS Limited, 2002; Loper, Breen, Zimmerman, & Clunne, 2009; Treasury Board of Canada, 2016). In the USA, approximately 40% of hazardous sites in the National Priority List (NPL) of the U.S Environmental Protection Agency are contaminated with a mixture of organic and inorganic contaminants (Sandrin, Chech, and Maier 2000). Similarly, in Canada a majority of the 23,111 contaminated sites contain mixed contaminants (Treasury Board of Canada, 2016).

In these sites, metals are the most common inorganic contaminants while petroleum hydrocarbons, chlorinated solvents and pesticides are common organic contaminants (Sandrin and Hoffman 2007). These contaminant mixtures are very common in factories, gas stations, waterfront properties (port lands) formerly used for industrial and commercial activities (MOECC, 2016), agricultural lands impacted by industrial activities (Vácha et al., 2015) and recently in urban landfills (XL Group Insurance, 2014). Some organic and inorganic contaminant mixtures are PHCs-metals, BTEX-PHCs-metals, PAHs-BTEX-metals, PAHs-PHCs-BTEX-pesticides, PHC-Pesticides, PAHs-PHCs, PHCs-BTEX, PHCs-PAHs-metals, PHCs-PAHs-metals-PCBs-PCDD/FS, PAHs-PHCs-metals-BTEX, PAHs-metals, PHCs-Halogenated hydrocarbon-PAHs-metals-BTEX, PAHs-metals-pesticides (Riely, Zachara, & Wobber, 1992; Treasury Board of Canada, 2016). Certain contaminant mixtures are associated with certain locations or activities. For example, PAHs, metals and pesticides are commonly found at dump sites (Reddy and Chirakkara 2013) and in cattle market soil (Adeyi, Omidiran, & Osibanjo, 2014); PAH and metals are found in gas plant sites, sewage sludge dump sites, roadside soils and wood preservation sites; TPHs and metals in petrochemical units; DDT and metals (arsenic) in sheep and cattle dip sites; PAHs, PCBs and metals in electronic waste processing sites; nitro compounds and arsenic in military sites; PAHs, TPHs and metals in railway corridors; PAHs, PCBs and metals in river sediments; OCPS, PAH and metals in areas around coal-fired power plants (Thavamani et al., 2013); and PBDE, PCB, PAHS and metals in electronic waste sites (Ye et al., 2015).

1.3 Problem statement and justification

The occurrence of more than one class of contaminants in soil further complicates and limits field application of remediation processes due to the difference in physical and chemical characteristics of different classes of contaminants. Interactions among different groups of contaminants in soil

can be unpredictable and may result in synergistic or antagonistic effects during remediation. Hence there is a need to understand the interaction of contaminants in mixed contaminated soil as well as their response to remediation techniques.

One of the most common ways mixed contaminants in soil are remediated is by excavation and disposal in landfills. With the excessive cost of finding new landfill sites and increasing regulatory requirements on quality of soil to be disposed in landfills, it is becoming necessary to develop low cost, environmentally friendly and socially acceptable techniques of remediating sites with mixed contaminants.

Phytoremediation, a biological remediation technique, has received a lot of attention over the past few years mainly because of its low cost, which varies depending on the type of contaminant, depth and area of contamination. The energy requirements (dependency of the process on solar energy) and the costs of establishing and maintaining plants are lower compared to removal and disposal of contaminated soil or remediation by other remediation options (Marques, Rangel, & Castro, 2009; Wan, Lei, & Chen, 2016). For example, the cost of phytoremediating mercury-contaminated soils was estimated by Garbisu and Alkorta (2001) to be one-tenth to one-hundredth the cost of other traditional engineering methods such as landfilling, thermal treatments, and chemical extraction. In addition to low cost, the aesthetic appeal of plants compared to chemical plants and bulldozers has given phytoremediation wide acceptance by the public (Ali, Khan, & Sajad, 2013; Sharma & Reddy, 2004).

Phytoremediation of soils contaminated with a single contaminant has been successfully established for contaminants such as zinc, chromium, lead (Barbosa et al., 2015; Romeh, Khamis, & Metwally, 2016), aroclor (PCB)(Zeeb et al., 2006), crude oil (Couto, Pinto, Basto, &

Vasconcelos, 2012), azoxystrobin (Romeh, 2015), atrazine (Balsamo et al., 2015; Murphy & Coats, 2011), and DDT (Paul, Rutter, & Zeeb, 2015). Phytoremediation performance in soil co-contaminated with members of the same class of contaminants has also been evaluated; zinc and arsenic (An et al., 2005); zinc, copper, lead and manganese (Padmavathiamma & Li, 2009); cadmium, chromium, copper, nickel, lead and zinc (Chang, Ko, Tsai, Wang, & Chung, 2014); 16 PAHs prioritized by the US EPA (Sun et al., 2011); and 4 organophosphorous pesticides (Ji. Gao, Garrison, Hoehamer, Mazur, & Wolfe, 2000).

A few studies have gone a step further to examine the effectiveness of phytoremediation on co-contamination and the possibility of concurrent uptake of contaminants during phytoremediation of two classes of contaminants. These studies used one exemplary contaminant for each class of contaminant and one plant species and they reported plant tolerance in co-contaminated soil alongside reduced biomass due to co-contamination. In the case of metal and PAH co-contamination, significant reduction in PAH toxicity was observed while metal and PAH accumulation in plants were minimal (Zhang et al. 2009; Chigbo 2013). Similar observations were made in the case of metals and PCP and metals (Hemchi et al. 2013). In the case of metals and PHC, significant uptake of metals was observed alongside degradation of PHC (Ramamurthy and Memarian 2012).

Chirakkara and Reddy (2015) pushed further by considering 2 to 3 exemplary contaminants (metal and PAH) mixed in contaminated soil at concentrations like those in industrial areas and using 8 plant species. They observed low survival rates in all plants, significant uptake of metals and enhanced degradation of PAH by some plants.

No studies have investigated phytoremediation of a mixture of the most commonly found contaminants in soil, which according to the Ashraf, Maah, & Yusoff (2014), Rathoure (2016) and US EPA (2004), are metals, pesticides, and petroleum-based hydrocarbons. Furthermore, no studies have considered a mixture with more than one class of organic contaminant in soils.

Hence, this research is focused on identifying plant species with the potential of phytoremediating soil contaminated with a mixture of metals, PAH and a pesticide through laboratory experiment.

1.4 Objectives of the research

This research seeks to achieve the following objectives

- a. Review relevant literature on phytoremediation of contaminated soil to select plants that have been successful in remediating mixed contaminated soils.
- b. Determine the performance of the selected plant species for phytoremediation of metals - PAH - pesticides contaminated soils (Cu and Pb, pyrene, DDT, respectively as model contaminants) by measuring
 - Germination and growth rate of plant species in contaminated soil compared to uncontaminated soil.
 - Residual contaminant concentration in soil after phytoremediation.
 - Uptake and accumulation of contaminants by plants after phytoremediation.
 - Mobility of contaminants in soil after phytoremediation

1.5 Thesis Format

This thesis contains four chapters (including the current chapter). Chapter 2 is a detailed literature review in the area of contaminants fate and phytoremediation. Chapter 3 contains the experimental setup and methods adopted to achieve research objectives (section 1.4) and the outcomes of the study. Chapter four cover concluding thoughts on this study and recommendation for future studies.

CHAPTER 2

2.0 Literature Review

2.1 Fate of contaminants in soil

Organic contaminants are retained in soil either by adsorption to the surface of the natural material or dissolution into the molecular network of the matrix (Chiou, 2002). Dissipation of organic contaminants in a soil profile depends on their mobility and degradation, which in turn depend on properties of the organic contaminants, soil properties and weather conditions (Nicholls, 1986). The physical and chemical properties of organic contaminants contribute largely to sorption interaction, which is a significant factor responsible for movement of organic contaminants in soil. Mechanisms of sorption for organic compounds in soil can include one or a combination of hydrophobic interaction, water solubility, ligand exchange, ion exchange, charge transfer or hydrogen bonding (Nicholls, 1986). Hydrophobic interaction occurs with lipophilic organic contaminants, which are not water soluble but soluble in oil, fats, lipids, and non-polar solvents. Sorption of such organic contaminants increases with organic matter content and lipophilicity and is measured by the octanol-water partition coefficient (K_{ow}) and the sorption per unit weight of soil organic matter (K_{oc}). For soluble organic contaminants, the sorption mechanism is related to soil pore water solubility; they tend to partition into soil pore water at the limit of their water solubility value, the undissolved quantity remains in the soil and is degraded slowly (Nicholls, 1986). Ligand exchange only occurs when ligands formed between organic compounds and soil are as strong as the bond between water and soil. This sorption mechanism occurs for organic contaminants such as pesticides that contain atoms of nitrogen, oxygen or phosphorus which are potentially capable of forming coordinate bonds with ions of metals such as iron, aluminum, manganese and copper present in soil (Nicholls, 1986). Charge transfer is only a significant sorption mechanism in field soil for aromatic molecules that are highly activated towards

electrophilic substitution. Ion exchange occurs for cationic organic compounds, as these can be exchanged at cation exchange sites in soil (clay minerals and humic surfaces).

The fate and transport of inorganic contaminants is dependent on the chemical form and speciation of the contaminant. In soil, they are adsorbed by initial fast reactions (minutes, hours), followed by slow adsorption reactions (days, years) and are then redistributed into different chemical forms with varying bioavailability, mobility, and toxicity. This distribution is believed to be controlled by reactions in soils such as mineral precipitation and dissolution, ion exchange, adsorption and desorption, aqueous complexation, redox reactions, biological immobilization and mobilization, and plant uptake (Wuana and Okieimen 2011).

In mixed contaminated soils, there is an interaction between dissipation processes of organic contaminants and those of inorganic contaminants. These interactions may be additive, synergistic or antagonistic (Onyema, 2013; Wuana, Okieimen, & Vesuwe, 2014). For metal-metal mixtures, mobility, adsorption and accumulation of metals in soil is strongly influenced by competitive interactions. Metals with like atomic radii and valencies can easily be interchanged on the surfaces of soil particles and those with higher valency can displace those with lower valency. Similarly, in organic-organic contaminant mixtures competitive displacement is largely responsible for the partitioning of organic contaminants between liquid and solid phases of the soil. According to Xing et al. (1996), mixtures of organic contaminants in soils may reverse their adsorption in soil; a competing organic contaminant can displace an already adsorbed organic contaminant, which was formerly unavailable to the environment, into soil solution. In soil manifesting non-linear sorption behavior, this phenomenon makes prediction of organic contaminant transport and soil remediation efforts difficult (McGinley et al., 1993). Reactions between metals and organic compounds can result to chemical bonding between carbon atom of the organic

compound and metals leading to the formation of organometallic compounds and ligands which are ions, molecules or molecule fragments bound to a central atom usually metal atom (usually metal is sandwiched between the organic compounds)(Spessard & Miessler, 2010). The compounds formed from metal-organic compound interactions may be soluble in soil pore water leading to simultaneous increase in the mobility of metals and inhibition of biodegradation of organic contaminants. Such an effect was reported by Chen et al. (2004) who observed an increase in Zn and Cu mobility due to co-contamination with an organic contaminant, 2,4-dichlorophenol (DCP), and attributed this to an increase in DOC (dissolved organic carbon) due to the addition of DCP. The increased mobility of metals due to the presence of organic contaminants has also been attributed to the formation of metal organic and inorganic complexes that do not adsorb to surfaces of solid soil particles, competition with other contaminants for sorption sites and increased metal association with mobile colloidal-sized particles (McGinley et al., 1993). This is not always the case, Dubé, Galvez-Cloutier, and Winiarski (2002) found that an increase in mobility of Cd, Cu and Pb in the presence of LNAPL(Light Non-Aqueous Phase Liquid) in carbonaceous soil was due to changes in soil hydrodynamics induced by LNAPL rather than chemical interaction between metals and LNAPLs. Interference of biodegradation of organic contaminants by metals is largely due to metal toxicity to microorganisms responsible for biodegradation of organic contaminants (Thavamani et al., 2011).Alternatively, compounds formed from metal-organic compounds interaction may also be insoluble in water and hence adsorb to soil solid phase making metals unavailable for plant uptake and persistent in soil.

Overall, the interactions between organic and inorganic contaminants are unpredictable and dependent on physicochemical properties of contaminant and soil.

2.2 Phytoremediation of contaminated soils

Phytoremediation is a bioremediation system that uses plants for in-situ removal of contaminants from soils, sludge, sediments and ground water (Ramamurthy & Memarian, 2012). This is not a new concept, it has been applied to wetlands, reed beds and floating-plant systems for treatment of wastewater for many years (Cunningham et al., 1995). Over the last few decades, studies on phytoremediation for the removal of different contaminants from soil has shown promising prospects.

Some of its major advantages are that it is usually carried out in-situ, allowing for reduced risk of exposure to contaminated soil for humans and other parts of the environment (Marques et al., 2009), and contaminants are removed from soil without affecting soil properties (Ali et al., 2013; Zihms et al., 2013), allowing for reuse of soil. On the other hand, phytoremediation is not without limitations, this technology is largely dependent on plants and bioavailability of contaminants, thus properties such as contaminant concentration, pH, salinity and the presence of other toxins in soil must be within the limits of plant tolerance (Cunningham et al., 1995; Hellström, 2004). This limitation makes this technology mainly suitable for shallow contamination (within the rooting zone) at non-excessive concentrations (Ramamurthy & Memarian, 2012). Despite this, phytoremediation has been recommended for very large soil contamination sites which otherwise would involve high remediation cost with other technologies (Ali et al., 2013; Cunningham et al., 1995). In addition, phytoremediation is slower than physio-chemical remediation processes and is usually considered to be a long-term remediation strategy (Wong, 2004). One major risk posed by phytoremediation is the introduction of remediated contaminants into the food chain by consumption of plants used for phytoremediation.

2.3 Phytoremediation Techniques

The diverse ways in which plants interact with contaminants for eventual removal or degradation can be referred to as phytoremediation techniques. Phytoremediation can occur by phytoextraction, phytofiltration, phytostabilization, phytovolatilization, phytodegradation, rhizodegradation or phytodesalination (Hussain et al. 2009; Pilon-Smits 2005; Hellström 2004; Ali, Khan, and Sajad 2013; Ghosh and Singh 2005). These techniques are not mutually exclusive.

Phytoextraction has also been called phytoaccumulation, phytoabsorption and phytosequestration. It results in the uptake of contaminants from soil or water by plant roots, translocation to and accumulation in aboveground biomass.

Phytofiltration is the removal of contaminants from water or wastewater by plants. This may be rhizofiltration (using plant roots), blastofiltration (using plant seedlings) or caulofiltration (using excised plant shoots). This is the dominant mechanism of remediation in wetlands.

Phytostabilization (also known as phyto immobilization), as the name suggests, is the use of plants to stabilize contaminants in soil, thus reducing mobility and bioavailability in the environment. This prevents migration of contaminants to groundwater and the food chain. Phytostabilization can either prevent erosion, leaching, and runoff or convert contaminants to less bioavailable forms (Pilon-Smits, 2005). It is more a containment technique than a decontamination technique.

Phytovolatilization is the uptake of pollutants from soil by plants, followed by their conversion to a volatile form and subsequent release into the atmosphere. The limitation faced by this technique is that the contaminants are transferred from one environmental medium (soil) to another (air).

Phytodegradation is the breakdown of contaminants by plants with the help of plant enzymes (e.g. dehalogenase and oxygenase) and other molecules in root exudates. This technique is limited to organic contaminants as inorganic contaminants are not readily biodegradable.

Rhizodegradation (also called phytostimulation) is the breakdown of organic pollutants in soil by microorganisms in the rhizosphere of plants. In the rhizosphere, soil microbial activity is stimulated to about 10-100 times by secretion of plant root exudates containing carbohydrates, amino acids, and flavonoids (Ali et al., 2013). These exudates provide additional carbon and nitrogen sources for soil microorganism, thus facilitating microbial growth.

Phytodesalination refers to the use of halophytic plants for removal of salts from salt-affected soils to enable them to support normal plant growth

Phytodegradation and rhizodegradation are removal mechanisms specific to organic contaminants while phytoextraction is specific to inorganic contaminant (Ghosh and Singh 2005), although some organic contaminants such as DDT and PCB can also be phytoextracted. Phytovolatilization, rhizofiltration and phytostabilization apply to both organic and inorganic contaminants (Sharma & Reddy, 2004).

2.4 Phytoremediation of organic compounds

As summarized above, phytoremediation of organic contaminants occurs either by direct phytoremediation and/or by phytoremediation explanta. The former involves direct uptake and accumulation of xenobiotics from soil (phytoextraction) and the latter is based on secretion of root exudates and enzymes by plants (rhizodegradation and phytodegradation) (Chirakkara and Reddy 2015; Cunningham et al., 1995).

In direct phytoremediation, movement of organic contaminants into and within plants is primarily driven by diffusion in the liquid phase (Alkorta & Garbisu, 2001; Pilon-Smits, 2005). Direct uptake of organic

compounds by plant is limited by low bioavailability of organic contaminants and evapotranspiration rate in plants(Alkorta & Garbisu, 2001). Bioavailability is strongly related to the octanol-water partition coefficient (K_{ow}) and volatility (expressed by Henry's law constant, H_i) of the organic contaminant. Organic contaminants with moderate hydrophobicity ($\log K_{ow} = 0.5-3$) such as BTEX, chlorinated solvents, and short-chain PAH can be directly taken up by plants and stored in plant structures via lignification, or mineralized to water and carbon dioxide by plants(Schnoor, Light, McCutcheon, Wolfe, & Carrier, 1995). They are hydrophobic enough to move through lipid bi-layers of membranes and water soluble enough to travel through cell fluids(Pilon-Smits, 2005); although they are generally considered to be phloem immobile(unless modified by plants before uptake) and xylem mobile (Cunningham & Berti, 1993; Hellström, 2004). Compounds that are very soluble in water ($\log K_{ow} < 0.5$) are not sufficiently sorbed to roots or actively transported; accumulation of such organic contaminants by plants is inversely related to passive influx of the transportation and transpiration system in the soil(Cunningham & Berti, 1993; Schnoor et al., 1995). Hydrophobic compounds ($\log K_{ow} > 3$) are very strongly bound to plant roots and are not easily translocated within the plants(Schnoor et al., 1995). For such organic contaminants, plant root exudates and enzymes help to enhance their degradation and immobilization(Alkorta & Garbisu, 2001). Organic contaminants with $H_i > 10^{-4}$ will tend to move in soil air spaces and those with $H_i < 10^{-6}$ will tend to move in soil pore water. Organic contaminants between these two H_i values will move in both soil air and soil pore water.(Hellström, 2004).Evapotranspiration rates vary greatly across plant species and are reported to have significant effects on the uptake of organic contaminants (Burken & Schnoor, 1996). Plants with higher evapotranspiration rates will take up more water, thus take up more contaminants that move with the bulk flow of water.

In phytoremediation explanta, the root exudates secreted by plants support the growth of diverse microbial activities in the rhizosphere by serving as a carbon and nitrogen source for microorganisms. Carbon and

nitrogen sources in root exudates are from organic compounds, such as phenolics, organic acids, alcohols, and proteins and the chemical composition of root exudates and rates of exudation differ significantly among plant species(Alkorta & Garbisu, 2001). In addition to root exudates, plants also secrete enzymes that degrade organic compounds. Plant-derived enzymes that have been proven to be responsible for degradation of organic contaminants include laccases, halogenases, nitroreductases, nitrilases and peroxidases(Alkorta & Garbisu, 2001).

2.5 Phytoremediation of inorganic contaminants

Inorganic contaminants such as metals are either transformed to harmless forms, such as metal oxides or metal phosphates (phytostabilization) or accumulated in the plant tissue (phytoaccumulation)(Chirakkara & Reddy, 2015b). The transport process in plants utilized for uptake and distribution of soil nutrients are also used for uptake and translocation of metals because they are chemically similar to plant nutrients, in fact some metals are essential plant nutrients. The uptake of inorganics in plants is facilitated through chelating agents produced by plant roots and are capable of inducing pH and oxidation-reduction potential (E_h) changes in soils surrounding the rhizosphere, resulting in solubilisation of soil bound inorganic contaminants (Tangahu et al., 2011). After uptake by roots, transport within plants is achieved through an active transport process involving generation of electrochemical potential gradients, co- and anti-membrane transporter proteins and transport channels (Tangahu et al., 2011; Thakur et al., 2016).

Just like organic contaminants, bioavailability of inorganic contaminant is of key importance to plant uptake from soil. The majority of metals occur naturally in soil and at varying bio-availabilities. According to Prasad (2003), metals can be categorized as readily bioavailable(Cd, Ni, Zn, As, Se, Cu), moderately bioavailable (Co, Mn, Fe) and least bioavailable(Pb, Cr, U). In general, inorganic contaminants occur as cations or anions and are considered hydrophilic. Bioavailability of cations is controlled by soil's cation

exchange capacity (CEC), which is a measure of the availability of binding sites for ions, thus cations will be less bioavailable in soils with higher CEC. But at lower pH the bioavailability of cations increases due to replacement of cations on soil CEC sites by H^+ (Hellström, 2004). In general, bioavailability of metals has been known to increase with decrease in soil pH. Another factor that controls the bioavailability of inorganic contaminants is the oxidation-reduction potential of soils. Depending on the oxidation-reduction (E_h) state of soil, heavy metals can occur in a variable oxidation state which may or may not be readily taken up by plants. For example, inorganic As and Cr forms available in soil for plant uptake are arsenite AsO_3^{-3} /As(III), arsenate AsO_4^{-3} / As(V), Cr(III) and Cr(VI). As (V) and Cr (III) are considered relatively immobile because they are more stable and strongly retained in soil while Cr(VI) and As(III) are unstable and easily mobilized irrespective of pH (Kabata-Pendias, 2000; Rinklebe, Knox, & Paller, 2017). In a reducing soil environment (more negative E_h), As(III) and Cr(III) are prevalent, whereas in an oxidizing soil environment (more positive E_h), As(V) and Cr(VI) are prevalent (Delaune & Reddy, 2005). Thus, under reducing soil conditions bioavailability of As is expected to increase because of the dominance of As (III), whereas Cr bioavailability is reduced due to dominance of Cr (III). In reality, controlled bioavailability of metals in soils by interaction between CEC, pH and E_h is expected as opposed to influence by a single factor

Metal availability can be modified by root exudates and microbial soil activities. For example exudation of siderophores will increase iron solubility and exudation of low molecular weight organic acids (such as citrate and malate) will increase the solubility of aluminum, cadmium, copper, nickel and zinc (Li, Ye, & Wong, 2010; Nascimento, Amarasiriwardena, & Xing, 2006; Sessitsch et al., 2013). The activities of microorganisms in the rhizosphere can also increase solubility of metals by impacting soil pH, increase the transfer of soluble metals from the rhizosphere to the plant or increase the root surface area and hair production (Alford et al., 2010; Ali et al., 2013).

Plants can be categorized as either metal excluders, indicators or accumulators (Ghosh and Singh 2005; Ali, Khan, and Sajad 2013). Metal accumulators absorb metals from soil and concentrate them in their roots, shoots and/or leaves, possibly at levels exceeding soil concentration. Metal excluders prevent metals from entering their aerial parts or maintaining low and constant concentrations of soluble/exchangeable metal fraction in soil, they typically accumulate metals in their roots. Metal indicators accumulate metals in their tissues at levels that reflect soil concentration.

2.6 Phytoremediation of organic-inorganic mixed contaminated soils

Like many other remediation methods, a large number of phytoremediation studies is focused on one class of contaminants despite the abundance of evidence of mixed contamination in soils. The complexity of inorganic-organic contaminant interactions supports the need to investigate effectiveness of phytoremediation for mixed contaminated soils as well as interactions among organic contaminants, inorganic contaminants and plants. These interactions control mobility, uptake, bioavailability and degradation of contaminants. Outcomes of these interactions are different from phytoremediation of a single class of contaminants. There is a paucity of studies on phytoremediation of mixed contaminated soil. Available studies have shown highly variable outcomes relating to plant growth, contaminant transport within plants, contaminant accumulations by plants and degradation of contaminants. Some studies have discovered that plant response during phytoremediation of mixed contaminated soils will differ from plant to plant. This was demonstrated by Batty and Anslow (2008) who examined the effect of PAH(pyrene, 1000 mg/kg) on phytoremediation of a metal (Zn, 8000mg/kg) contaminated soil using *Brassia juncea* (Indian mustard) and *Festuca arundinacea* (tall fescue) after 12 weeks of plant growth. The two plant species responded differently under the same soil conditions. Growth (growth rate and wet biomass) of tall fescue was unaffected by the addition of pyrene while the growth of Indian mustard was significantly reduced by approximately 50%. The plants also accumulated Zn differently in mixed Zn-pyrene

contaminated soil, compared to single Zn contamination. Zn concentration in Indian mustard (concentrated mostly in shoot) was increased by ~79% in mixed contaminated soil but that of tall fescue (concentrated mostly in root) remained the same compared to control. At the end of the study Zn removal efficiency of tall fescue was improved by pyrene but residual Zn in co-contaminated soil was not different between pots planted with either plants. Even though Zn removal efficiency was the same in both plants, the tolerance of tall fescue and its ability to concentrate Zn in its roots makes it a better candidate than Indian mustard, as the risk of contaminant transfer to the food chain is reduced. Similar synergistic effects on plant growth were observed by Zhang et al. (2009) and Sun et al. (2011). Zhang et al. (2009) studied the remediation of soil co-contaminated with pyrene (10, 50, or 100 mg/kg) and cadmium (2, or 4.5 mg/kg) using *Zea mays* L. (maize). After 8.6 weeks, the growth of maize was reduced by 0-8.90% in co-contamination with increasing pyrene concentration as well as in single Cd contaminated soil. Although plant growth was reduced by co-contamination, concurrent dissipation of pyrene and removal of Cd was achieved by maize. But the degradation of pyrene was greatly limited by Cd concentration, as demonstrated by an increase in residual pyrene with increased Cd concentration until similar values to unplanted soil was observed in combined Cd (4.5 mg/kg) and pyrene (100 mg/kg). A similar trend was observed for Cd concentration and accumulation in maize, which was reduced with increased pyrene concentration. A similar concurrent removal and growth pattern was observed by Sun et al. (2011) in a comparative study of phytoremediation of single contamination of B[a]P (benzo[a]pyrene) 2,5,10,50 mg/kg and that of B[a]P 5mg/kg co-contaminated with Cd 20,50mg/kg, Cu 100,500mg/kg or Pb 1000mg/kg,3000mg/kg using *Tagetes patula* (marigold). At the end of the 13-week growth period, translocation of organic and inorganic contaminants by marigold was observed in B[a] P and Cd mixed contaminated soil only, Pb and Cu were concentrated largely in roots. These studies show that synergistic effects were not only observed for plant growth but also for contaminant removal.

Although mixed contamination can lead to synergistic effects, antagonistic effects have also been observed. Jeelani et al. (2017) exposed *Acorus calamus* (sweet flag) to Cd (0, 10, 20mg/kg) and two PAH (phenanthrene and pyrene 0, 50+25,100+50mg PAH/kg) for 8.6 weeks. They showed that plant biomass production and plant height increased by 0 – 140% and 0 - 42.86 % ($p < 0.05$ and $p < 0.01$, respectively) with co-contamination compared to uncontaminated soil, and a single 20mg/kg Cd contaminated soil (in which plant growth was similar to that in clean soil). Highest Cd accumulation was observed in soil co-contaminated with low Cd-PAH soil contamination (10mg Cd/kg-50+25mg PAH/kg) and high Cd-PAH soil contamination (20mg Cd/kg-100+50mg PAH/kg). Cd translocation was generally poor irrespective of the treatment and Cd was largely concentrated in roots. These results imply that antagonistic effects such as improved plant growth and phytoremediation efficiency of contaminants depend on concentrations of contaminants in mixed contaminated soil. Irrespective of improvements in tolerance and Cd accumulation of sweet flag in mixed contaminated soil, this plant unfortunately was unable to improve the degradation of PAH in mixed contaminated soil. Chen et al. (2013) observed similar interaction in phytoremediation of hexachlorocyclohexane (HCH) -Cd contaminated soil using *Allium sativum L.* (garlic). They explained that the antagonistic effects of mixed contamination are partly due to formation of metal-organic complexes between metals and organic contaminants.

Depending on the physiological function of metals, uptake and translocation of metals in mixed contaminated soil might be improved. Chigbo, Batty, and Bartlett (2013), studied phytoremediation of Cu (0, 50 and 100 mg/kg) and pyrene (0, 250 and 500 mg/kg) using indian mustard and found phyto-toxic effects on the plant (in terms of reduced biomass) after 9.2 weeks of growth, the study showed improvements in metal translocation within Indian mustard. At low Cu concentration (50mg/kg), increasing pyrene concentration led to a 36% ($p < 0.05$) increase in Cu-concentration in the plant compared to single Cu-contamination of 50mg/kg. At high Cu (100mg/kg) and incremental addition of pyrene, a 19%-70%

increase in Cu concentration in the shoot was also observed compared to single Cu-contamination of 100mg/kg. In other words, with incremental concentration of pyrene and Cu in soil, the ability of Indian mustard to transport Cu from root to shoot seemed to improve. This demonstrates that biological functions of the metal in question (in this case Cu is a micronutrient) might contribute to improved translocation (as opposed to findings from Batty and Anslow (2008)) during phytoremediation of mixed contaminated soils. Irrespective of increased metal transport within plant in co-contaminated soil, overall accumulation of metal reduced drastically by 90% and 94% at low co-contamination (Cu 50mg/kg and pyrene 250mg/kg) and 86.5% and 83.5% at high co-contamination (Cu 100mg/kg and pyrene 500mg/kg) due to reduction in plant biomass. Furthermore, this study showed that degradation of pyrene was better in planted soil compared to unplanted soil. In planted soil, pyrene degradation was significantly reduced by incremental co-contamination with Cu compared to planted single pyrene contaminated soil. Residual pyrene in soil increased from 37.05mg/kg (at single 500mg/kg of pyrene) to 98.48mg/kg when 50mg/kg Cu was added and to 111.9mg/kg (greater than value in unplanted soil) when 100mg/kg Cu was added. The results of this study indicate that the presence of metal can inhibit biodegradation of organic contaminants and, most importantly, concurrent removal of contaminants is possible by phytoremediation. Lin et al. (2006) obtained similar outcomes in their study of effects of inorganic contaminant (represented by Cu 0,150,300mg/kg) on degradation/dissipation of pentachlorophenol (PCP) (0, 50,100mg/kg) in the presence of *Lolium perenne* (rye grass) and *Raphanus sativa* (Radish) after growth of 12weeks. Growth of plants and dissipation of PCP increased with incremental addition of Cu but was limited to low Cu(100mg/kg), which is about the same range of Cu as that used by Chigbo et al.(2013), and increased concentration of Cu reduced percent PCP removal.

A much more undesirable outcome of co-contamination is the increased mobility of contaminants in mixed contaminated soil. This was observed by Chen et al. (2004), who examined the effect of 2,4-

dichlorophenol(DCP), 100 mg/kg (organic contaminants), on uptake of Zn, 2978 mg/kg, and Cu, 1086 mg/kg, (inorganic contaminant) during 4 weeks of phytoremediation with *Lolium preenne* (rye grass). They found that the presence of 2, 4-dichlorophenol (DCP) increased the mobility of Zn and Cu by (as indicated by an increase in soluble and exchangeable soil metal fractions) in planted soil alongside reduced Zn accumulation and no significant effect on Cu accumulation in plant tissue when compared to unplanted soil. Increased mobility of metal was attributed to reduced uptake of metal by plants and an increase in dissolved organic carbon (DOC) due to the presence of DCP and a further increase in DOC due to the growth of rye grass in the co-contaminated soil. In addition, they found that the presence of DCP did not affect the growth (in terms of biomass production) of ryegrass.

Outcomes of phytoremediation of mixed contaminated soils may vary due to age-related changes in the physicochemical properties of soil. Chigbo and Batty (2013) demonstrated this by comparing performance of Indian mustard in freshly spiked and aged Cu-pyrene contaminated soil after 8.6 weeks. Biomass of Indian mustard decreased (>50%) in freshly spiked soil compared to aged soil. Probably because the bioavailability of contaminants decreases with time. As expected, the accumulation of Cu in shoot was reduced by 60-88% in aged soil. But there was no significant effect of planting on degradation of pyrene in aged soil.

Others have tried to screen plants based on their tolerance and contaminant removal. Chirakkara and Reddy (2015), conducted a study to select plants suitable for concurrent uptake of phenanthrene(100mg/kg), naphthalene(50mg/kg), Pb(500mg/kg), Cd (50mg/kg) and Cr (200mg/kg) by examining the phytoremediation efficiency of sunflower, indian mustard, field mustard, marigold, oat, rye grass, tall fescue, alfalfa, green onion, white clover, black nightshade and green gram (growth duration of 9 weeks). The concentrations they used were similar to those found in U.S superfund sites that have mixed contamination in soil. Sunflower, oat plant, rye grass, tall fescue and green gram were the only plants that

survived in the experimental conditions, although percentage survival and plant biomass were significantly reduced compared to that in clean soil. Removal efficiency of metals was in the order of Cr>Pb>Cd with uptake of Cr by all surviving plant species. Pb reduction was achieved only by sunflower (29%) and Cd reduction achieved by sunflower (18%) and Green gram (7%). Also, significant reductions in phenanthrene and naphthalene were observed for all surviving plant species. Similarly, Huang et al. (2011) screened 23 genotypes of *Ricinus communis* (castor) for remediation of Cd-DDT contaminated soil after 8.6 weeks and found concurrent accumulation of Cd and DDT by some genotypes, even higher concentrations than previously reported for any other plant. Lee et al. (2007) examined phytoremediation of Cd-Pb-2,4,6-trinitrotoluene(TNT) contaminated soil using *Echinochloa crusgalli* (barnyard), *Abutilon avicennae* (Indian mallow), *Aeschynomene indica* (Indian joint vetch) and *Helianthus annuus* (sunflower) for 26.7 weeks. All plants simultaneously removed Cd and TNT completely but Pb was not removed due to low exchangeable and soluble Pb in soil.

2.7 Conclusion

Based on the studies reviewed above, it may be concluded that typical rules that apply to single contaminated soils may not hold true for mixed contaminated soil and it is difficult to predict the outcomes of phytoremediation of mixed contaminated soil because of the many variables that affect the process. These variables include individual plant tolerance to contamination, type and concentration of contaminants in the mixture, and physiochemical properties of soil. Identifying plants with the potential to phytoremediate specific mixtures of contaminants in soils is a foundational step to providing insights in the area of phytoremediation of mixed contaminated soils.

CHAPTER 3

3.0 Phytoremediation of Metal-PAH-Pesticide Contaminated Soil

3.1 Materials and Methods

3.1.1 Contaminant selection

Two metals were selected to represent inorganic contaminants and two compounds were selected to represent organic contaminants. The Canadian Federal Contaminated Site inventory lists several active sites with soils contaminated by metals, pesticides and PAH. Pb and Cu were selected to represent two classes of metals, non-essential and essential metals, based on their roles in biological systems and they are the most commonly found metals at contaminated sites(He et al., 2015). Pyrene and DDT were selected to represent two classes of organic contaminants. DDT was selected because of its environmental significance as a pesticide that is persistent in the environment long after its production and use has been banned. Pyrene was selected because it is typically the most abundant PAH(World Health Organization, 2003). All contaminants selected for this study are on the US EPA's priority pollutants list.

The concentrations of the contaminants were selected such that they were within the range of concentrations used in studies reviewed in Chapter 2. They were above maximum concentrations prescribed for soils in industrial areas by the Ontario Environmental Protection Act (MOECC, 2011) and protection of ecological receptors in the environment and human health in industrial areas(CCME 1999b,1999c, 1999d,2010), since cases of mixed contamination are associated with areas with history of industrial activities (Table 3-1).

Table 3-1: Concentration limits for selected contaminants in industrial soil and the concentrations used as the experimental treatment.

Contaminants	Concentration (mg/kg)	Concentration (mg/kg)	Concentrations (mg/kg)	Concentrations used (mg/kg)
Lead	600 (CCME 1999b)	120 (MOECC, 2011)	500- 3000 (Chirakkara & Reddy, 2015b; Sun et al., 2011)	650
Copper	500 (CCME 1999c)	230 (MOECC, 2011)	50- 1086 (Chen et al., 2004; Chigbo et al., 2013)	550
DDT	12 (CCME 1999d)	1.4 (MOECC, 2011)	0.61- 30 (Mo et al., 2008; Wang, 2008)	20
Pyrene	100 (CCME 2010)	96 (MOECC, 2011)	10-1000 (Batty & Anslow, 2008; Zhang et al., 2009)	200

Table 3-2: Physical and chemical properties tested for the study soil

Property	Method	Analyzing laboratory
Grain size distribution	ASTM C136/C136M (2014) for sieve analysis and ASTM D7928 (2017) for hydrometer test. Soil classification was done using soil texture triangle.	Western Geotechnical Lab
Specific gravity	Water Pycnometer according to ASTM D854 (2014b).	Western Geotechnical Lab
Hydraulic conductivity	ASTM D5856 (2015)	Western Geotechnical Lab
Moisture content	ASTM D2216 (2010)	Western Geotechnical Lab
Organic matter content	Loss on ignition at 360°C	A&L Laboratories Canada
pH	Electrometric measurement of 1:1 soil: water extract	A&L Laboratories Canada
Nitrate content	0.01M K ₂ SO ₄ extract, cadmium reduction to NO ₂ , colorimetric measurement	A&L Laboratories Canada
P, K, Mg, Ca, S, Zn, Mn, Fe, Cu, B, Al, Na	Mehlich 3 extraction (plant available micro- and macro nutrients in soil) and ICP-OES	A&L Laboratories Canada
Nitrogen content	Combustion and thermal conductivity	A&L Laboratories Canada
Available potassium and phosphorous	Ammonium citrate buffer extraction and ICP-OES	A&L Laboratories Canada
Metal content	EPA Methods 6010,6020,7196A and 7471A	Caduceon Environmental Laboratories

3.1.2 Plant selection

Based on a review of the literature on phytoremediation (Chapter 2) the following plants were selected because of their tolerance and contaminant removal abilities (Chirakkara and Reddy 2015; Lunney et al. 2004; Paul et al. 2015): *Panicum virgatum* (switch grass), *Lolium perenne* (rye grass), *Avena sativa* (oat), *Medicago sativa* (alfalfa), *Brassica juncea* (Indian mustard), *Helianthus annuus* (sunflower) and *Festuca arundinacea* (tall fescue). Seeds of switch grass, oat and sunflower were purchased from Hawthorn Farm organic seed, ON Canada; alfalfa, ryegrass and tall fescue from ProRich Seeds ON, Canada; and Indian mustard from Eagleridge Seeds BC, Canada.

3.1.3 Physical and chemical properties of soil

Four physical and seven chemical properties of soil were determined according to methods listed in Table 3-2. Most of the chemical properties of the soil were determined by A&L Laboratories Canada and Caduceon Environmental Laboratories Canada. Physical properties were conducted in the Geotechnical Engineering Laboratory at Western University. All tests were performed in triplicates.

3.1.4 Soil spiking procedure

Soil was collected from pits operated by AAROC Aggregates, London, Ontario, and air dried for 7 days after which the soil was pulverized, passed through a 2-mm sieve, mixed and divided into portions of 1000 g each. Subsamples (250 g each) of these soil portions were first contaminated with the acetone-soluble DDT and pyrene prior to adding the water-soluble metals (Pb and Cu) and generating the mixed contaminant test soil.

For the acetone-soluble compounds, 25 mg of two forms of DDT (68.51% 4, 4'- DDT and 31.49% 2, 4'- DDT) and 210 mg pyrene were dissolved in 100 mL of acetone and added to 250 g of soil. The soil was allowed to dry in a fume hood for 4 days and turned daily to ensure complete evaporation of acetone.

Appropriate amounts of $\text{Pb}(\text{NO}_2)_3$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (depending on what the initial concentration of lead and copper was in a given batch) were dissolved in 75g of distilled water and added to the organic-contaminated soil to achieve a moisture content of 30%. The remaining portion (750 g) of clean soil was mixed with 225 g of water to achieve a similar moisture content. The clean soil (750 g) and contaminated soil (250 g) were then mixed together for 3 hours using a soil mixer to achieve a final concentration of 20 mg/kg, 200 mg/kg, 650 mg/kg and 550 mg/kg of DDT, pyrene, Pb and Cu, respectively. Contaminated soils were stored in moisture tight containers for 1 month before planting of seeds in order to achieve equilibrium between the solid phase and liquid phase of the soil. Although adsorption, fractioning and speciation of contaminants in soil phases involves a combination of fast and slow reaction which may take as little as a few hours or as much as a few years, the time constraint of this study permits 1 month to allow for stabilization of these reactions.

Cu salt was purchased from Caledon Laboratories Canada, all other spiking compounds were purchased from Sigma-Aldrich Canada.

3.1.5 Toxicity test

Seed germination or root and stem elongation tests are the simplest type of toxicity test, typically used to determine preliminary effects of toxicity of contaminants on plants and can give a fair idea of plant tolerance to a specific level of contamination. The procedure for seed germination and the root and stem elongation test was adopted from ASTM (2009) and Greene et al. (1996).

Spiked (contaminated) soil and clean soil (control) were placed in a Petri dish. Prior to planting, seeds were aerated in water until the first sign of germination to ensure uniform germination among seeds. Each Petri dish was sown with 10 seeds each for every plant species. The Petri dishes were then covered and sealed with Para film and placed in a growth chamber. The chamber was set to 22°C and 16:8 hours of light: dark, with a relative humidity of 60%. It is difficult to provide optimum growth conditions (photoperiod, day-

night temperature and relative humidity) for each plant species. Most plant species have been found to grow actively between a minimum of 12-hour photoperiod, an average relative humidity of 50%. (Blankendal et al., 1972), and an average daily temperatures of 5-35°C, with the general assumption being that to achieve optimal growth that temperatures at night should be less than day temperatures by 3-10°C (Poorter et al., 2012) but positive effects of lower night temperature has been found to be negligible or detrimental to plant growth (Rajan & Blackman , 1975), Thus, the selected growth chamber conditions for this study is satisfactory for plant growth.

Each plant species had 3 replicates for spiked soil as well as for clean soil. The Petri dishes were monitored for 7 days and the number of germinated seeds recorded. After 7 days the root and shoot lengths of plants were measured and the final germination percentage calculated.

3.1.6 Plant growth and harvesting

Based on the results of the germination test, alfalfa, ryegrass, sunflower, oat and Indian mustard were selected for the phytoremediation studies. Pots (8 cm diameter) were filled with clean soil (control) or contaminated soil, as described in Section 3.4. For each plant species, 3 pots of clean soil and 3 pots of spiked soil were prepared. Seeds (10 for alfalfa, ryegrass, oat and Indian mustard and 7 seeds of sunflower, to avoid overcrowding of plants in pots over the duration of this study) were placed at a depth of approximately 1 cm below the soil surface.

Plants were grown in a growth chamber (same conditions as in Section 3.5). The height of plants was measured every 7 days and the number of germinated seeds and surviving plants recorded. The plants were watered once every 2 days to maintain a moisture content of 40% across pots. Exactly 3.3 g of slow releasing fertilizer (N: P: K =12:4:8) was added two weeks after planting to all pots. All plants were grown until constant height was observed in some plants (harvesting only plants that showed constant heights at

72 days will make comparison of performance difficult as there is no known method of correcting for variation in plant growth duration for phytoremediation studies)

At the end of 72 days, the plants were harvested, shoots were separated from the roots and roots were washed with distilled water to remove soil particles. Plant tissue and soil were oven-dried at a temperature $\leq 40^{\circ}\text{C}$ until constant weight was achieved. Plant root and shoot weight were measured and reported as root and shoot biomass.

3.1.7 Soil pore water extraction

Pore water was extracted from soil using a pneumatic pore water squeezer. The squeezer cylinder was washed with distilled water and dried, and approximately 140 g of wet soil was loaded into the clean cylinder and a hydraulic press was set to a maximum pressure of 125 MPa. Filter paper was placed at the base of the cylinder to prevent soil particles from being collected along with the pore water. After 24 hours, pore water was collected and stored at 4°C prior to testing. The cylinder was washed thoroughly with soap and rinsed a few times with distilled water and acetone between samples to avoid cross contamination.

3.1.8 Metal analysis in soil and plant

Pb and Cu were extracted from the soil matrix by microwave-assisted acid digestion using Method 3051A by U.S. EPA (2007a) and Tighe et al. (2004). The oven-dried soil was pulverised and 0.5g of soil weighed into the microwave express vessel. Ten millilitres (10 mL) of concentrated nitric acid (Sigma Aldrich Canada Omni Trace) was added to the vessel and then transferred into the microwave with temperature set to ramp to 175°C over 6.5 minutes and held for another 15 minutes. The vessels were allowed to cool at room temperature. The samples were filtered and diluted to 50 mL.

Metal content of plant tissues was determined using a U.S EPA acid digestion method modified by Ahkter & Macfie (2012). The dried plant tissues were hand chopped into fine pieces and 0.1g was placed in a 15ml test tube and covered using glass marbles to prevent evaporation and allow pressure to be released during

heating. All the test tubes were placed in a rack and 1 mL pure nitric acid (OmniTrace®, EM Science, USA) was added to each test tube to digest the organic matter. The samples were left overnight at room temperature. The following day, the test tube rack was placed in a shallow tray filled with sand and heated to 90-100°C on a hot plate until the vapors became transparent. Samples were allowed to cool to room temperature before being filtered into 50 ml sterile disposable centrifuge tubes and diluted to 25 mL using reverse osmosis water.

Metal fractions in soil (exchangeable fraction, carbonates-bound fraction (or acid-extractable fraction), Fe-Mn oxide bound fraction (or reducible fraction), organic-bound fraction (or oxidizable fraction) and residual fraction) were determined using the sequential extraction procedure outlined in Table 3-3 using 1g of soil. The procedure for extracting the various fractions of metals was originally developed by Tessier et al. (1979) but the modification by Reddy et al. (2017) was adopted for this project. The extractant solution was recovered for each fraction by centrifugation (5000 rev/min for 20 minutes) and the supernatant carefully withdrawn with a pipette. The residue was then rinsed with milli Q water, centrifuged, and the resulting supernatant discarded. Shaking was done with an orbital shaker (Thermo Scientific MaxQ 2000) at 300 rpm.

All samples were stored at -4°C until analyzed by ICP- OES (Inductively Coupled Plasma-Optical Emission Spectroscopy).

3.1.9 Pyrene and DDT analysis in soil and plants

Microwave Assisted Extraction (MAE) base method for the extraction of organic compounds from soil is described in Method 3546 by U.S. EPA (2007b). A modification of this method by Wang et al. (2007) for simultaneous extraction of PAH and organochlorine pesticides was adopted for extracting pyrene, DDT and its metabolites .

5 g of oven dried soil was weighed into the microwave vessel and 25ml mixture of acetone and n-hexane (1:1) was added to the vessel. Vessel was sealed and put into the microwave. The extraction was performed at a microwave power of 100% (1200W), with temperature at 110⁰C and programmed to ramp to 110⁰C for 10 minutes and held at 110⁰C for another 10 minutes. Vessels were allowed to cool at room temperature for a minimum of 5 minutes, contents centrifuged at 4000 rpm for 20 minutes to separate soil particles from extract solution.

The supernatant was collected in a clean centrifuge tube and taken to an evaporator and evaporated to dryness. The residue left behind after evaporation was dissolved in 1900 µL of acetonitrile (Sigma Aldrich Canada HPLC grade) and 100 µL of dichloromethane (Caldeon Laboratories Canada) for DDT and 2000 µL of acetonitrile for pyrene. Pre-concentrated extract was stored in HPLC vials at 4 °C prior to testing (for a maximum of 4 days). Samples were analysed using an Agilent 2000 series HPLC with UV-diode-array detector (DAD) and Eclipse C18 reverse phase column (25 cm × 4.6 mm, 5 µm) made by Agilent.

Table 3-3: Steps for sequential extraction of metals from soil

Fraction	Procedure
Exchangeable fraction	8 mL of 1M sodium acetate solution (pH 8.2) was added to soil sample and mixed continuously
Carbonates-bound fraction	Residue from above plus 8 ml of 1M sodium acetate (pH=5, adjusted with acetic acid) and mixed continuously for 5 hours.
Fe-Mn oxides-bound	Residue from above plus 20mL of 0.04 M hydroxylamine hydrochloride (NH ₂ OH.HCl) in 25 % (v/v) of acetic acid and heated to 96 °C with occasional stirring for 6 hours.

Organic-bound	Residue from above plus 3 mL of 0.02 M nitric acid and 5 mL of 30% hydrogen peroxide (pH=2, adjusted with nitric acid) and mixed continuously for 3 hours and allowed to cool, 5mL of 3.2 M ammonium acetate in 20% nitric acid is added and diluted to 20 mL with distilled water and mixed continuously for 30 minutes.
Residual fraction	EPA 3050B

The Method 8310 by U.S EPA (1986) was used; it gives the fundamental procedure and conditions for the use of HPLC in the detection of organic compounds. HPLC was calibrated using a stock solution of 300 mg/L prepared by dissolving pyrene in acetonitrile and a stock solution of 80 mg/L prepared by dissolving DDT (68.51% 4, 4'- DDT and 31.49% 2, 4'-DDT) in acetonitrile and was diluted accordingly with acetonitrile.

Sample HPLC chromatographs and calibration curves for pyrene and DDT are shown in Appendix A and B, respectively. All retention times were below relative standard deviation of 5% (relative standard deviation is the ratio of standard deviation and mean expressed as a percentage).

Plant samples were sent to Agriculture and Food Laboratory University of Guelph for total DDT analysis using gas chromatography.

3.1.10 Statistical analysis and quality control

All parametric and non-parametric statistical tests were performed using Sigma plot 11.0 with α (significance level) at 0.05. Significant difference is shown using uppercase letters or asterisk (*), where bars and numbers with same letter indicate no significant difference, numbers and bars with

different letter or * indicate significant difference). Blanks and samples spiked with known concentrations were analysed alongside all samples during acid digestion, sequential extraction and extraction of organic compound. Recoveries from spiked samples were between 80- 120% and blanks did not indicate any signs of cross-contamination.

3.2 Results

3.2.1 Soil properties

The physical and chemical properties of the study soil are presented in Table 3-4. The soil is predominantly sandy with low organic matter content and circumneutral pH. The soil can be classified as loamy sand. Also, the contaminants of interest are below the specified concentrations in natural soils in Ontario(MOECC 2011). Typically the most productive agricultural soils are those with high clay content because they have a higher water holding capacity.(Hillard & Reedyk, 2014).Unfortunately, this type of soil is not the characteristic soil of the London, Ontario area where this study was conducted.

The pH of soil before contamination, after contamination and after phytoremediation is given in Table 3-5. Soil pH was maintained within the neutral range after phytoremediation.

Table 3-4: Physical and chemical properties of the natural study soil (mean \pm SE, n=3).

Properties	Values
Physical properties	
%clay	4.22% \pm 0.84
%silt	10.26% \pm 1.90
%Sand	85.52% \pm 1.07
Specific gravity	2.92 \pm 0.05
Hydraulic conductivity (cm/s)	7.59 x 10 ⁻⁴ \pm 1.79 x 10 ⁻⁶
Chemical Properties	
CEC(meq/100g)	23.9 \pm 2.40
pH _{water}	7.6 \pm 0.12
Organic matter (%)	2.2 \pm 0.23
Nutrient content	
P (mg/kg)	20 \pm 2.60
K(mg/kg)	70 \pm 10.97

NO ₃ -N(mg/kg)	15 \pm 1.73
S(mg/kg)	144 \pm 23.21
Mg (mg/kg)	350 \pm 30.88
Ca(mg/kg)	4130 \pm 529.66
Fe(mg/kg)	77 \pm 15.86
Bo(mg/kg)	0.9 \pm 0.20
Mn(mg/kg)	41 \pm 5.92
Zn(mg/kg)	3.8 \pm 0.66
Cu(mg/kg)	1.7 \pm 0.52
Mo(mg/kg)	<1
Contaminants of interest	
Copper(mg/kg)	14 \pm 4.44 (92)
Lead (mg/kg)	28 \pm 9.37(120)
Total DDT	<D.L (1.5)
Pyrene	<D.L (1)

() values in bracket are background concentrations for soils in Ontario

Table 3-5: pHcacl₂ (mean \pm SE, n=3) of planted and unplanted mixed contaminated soil. Different letters indicate a significant difference between planted and unplanted soil ($p \leq 0.05$).

Soil	Pre-contamination	Post-contamination	Post-Remediation
Unplanted	7.4 \pm 0.00 ^C	7.3 \pm 0.00 ^D	7.4 \pm 0.06 ^C
alfalfa	7.6 \pm 0.00 ^A	7.6 \pm 0.00 ^A	7.5 \pm 0.03 ^{BC}
Oat	7.3 \pm 0.00 ^D	7.3 \pm 0.00 ^D	7.4 \pm 0.03 ^C
Ryegrass	7.5 \pm 0.00 ^B	7.4 \pm 0.00 ^C	7.2 \pm 0.03 ^E
Indian mustard	7.5 \pm 0.00 ^B	7.4 \pm 0.00 ^C	7.3 \pm 0.00 ^D
Sunflower	7.4 \pm 0.00 ^C	7.3 \pm 0.00 ^D	7.3 \pm 0.06 ^D

3.2.2 Preliminary toxicity test of mixed contaminated soil on plants: Effect of contamination on percentage germination

Seed germination for this study was defined as having a 1 mm radical emergence. Figure 3-1 shows percent germination for various plants. At the end of 7 days, no significant ($p \leq 0.05$) effect of soil treatment was observed. Irrespective of the lack of a significant effect of contamination on seed germination, differential plant response in both clean and contaminated soil was observed. Specifically, switch grass did not

germinate in both soils and tall fescue had the lowest percentage germination (27.5% in clean soil and 25% in contaminated soil) compared to the other viable plant species in both clean and contaminated soil.

3.2.3 Preliminary toxicity test of mixed contaminated soil on plants: Effects of contamination on root and shoot length

As with percent germination, the responses of plant roots (Figure 3-2) and shoots (Figure 3-3) varied from species to species. A significant effect ($p \leq 0.05$) of contamination on plant root length was observed for all plants except for sunflower. The percentage reduction in root length was as follows: 63% for tall fescue, 72% for rye grass, 45% for Indian mustard and 32% for oat; while a percentage increase of 41% was observed for alfalfa. The effect of contamination on shoot length was significant ($p \leq 0.05$) only in oat with an increase of 135% compared to shoot length in clean soil. Slight non-significant effects were observed for ryegrass, alfalfa, Indian mustard and tall fescue, each of which showed an increase of shoot length, while sunflower shoot length decreased in contaminant soils.

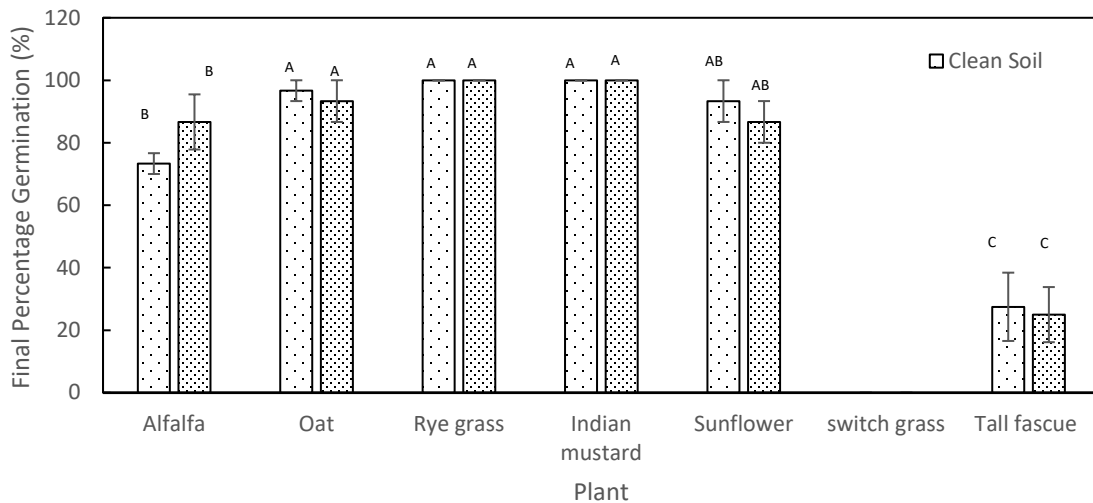


Figure 3-1: Germination percent (mean \pm SE, n=3) of plants in clean soil and contaminated soil. Different letters indicate a significant difference between clean and contaminated soil and between plants ($p \leq 0.05$).

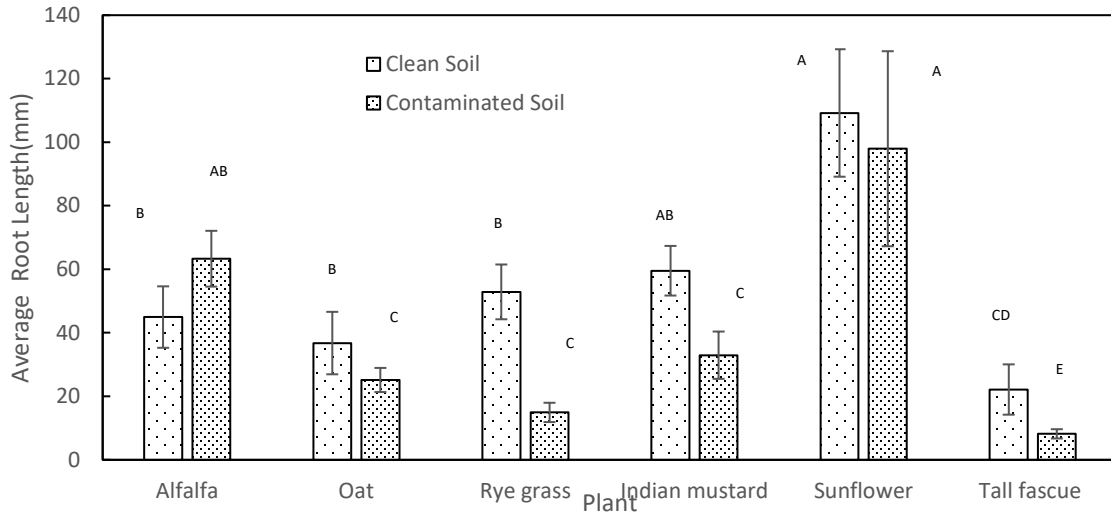


Figure 3-2: Root length (mean \pm SE, n=3) of plant species in clean soil and contaminated soil. Different letters indicate a significant difference between clean and contaminated soil and between plants ($p \leq 0.05$).

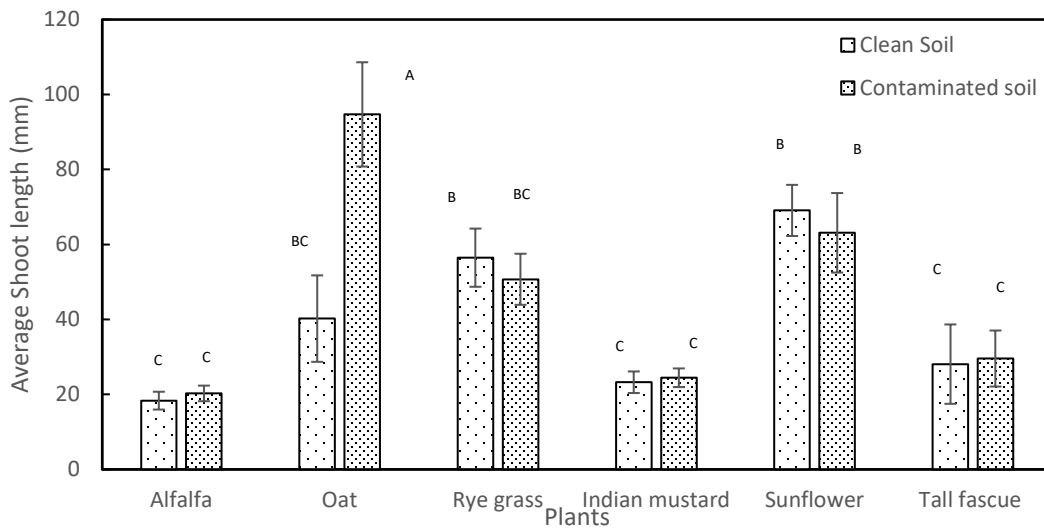


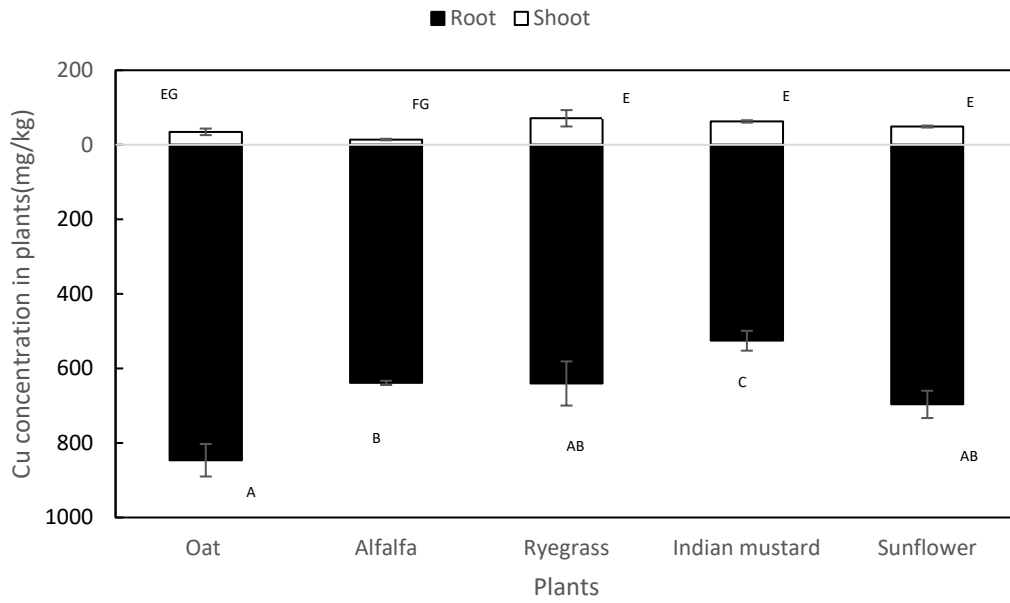
Figure 3-3: Shoot length (mean \pm SE, n=3) of plant species in clean soil and contaminated soil. Different letters indicate a significant difference between clean and contaminated soil and between plants ($p \leq 0.05$).

3.2.4 Metal uptake by plants

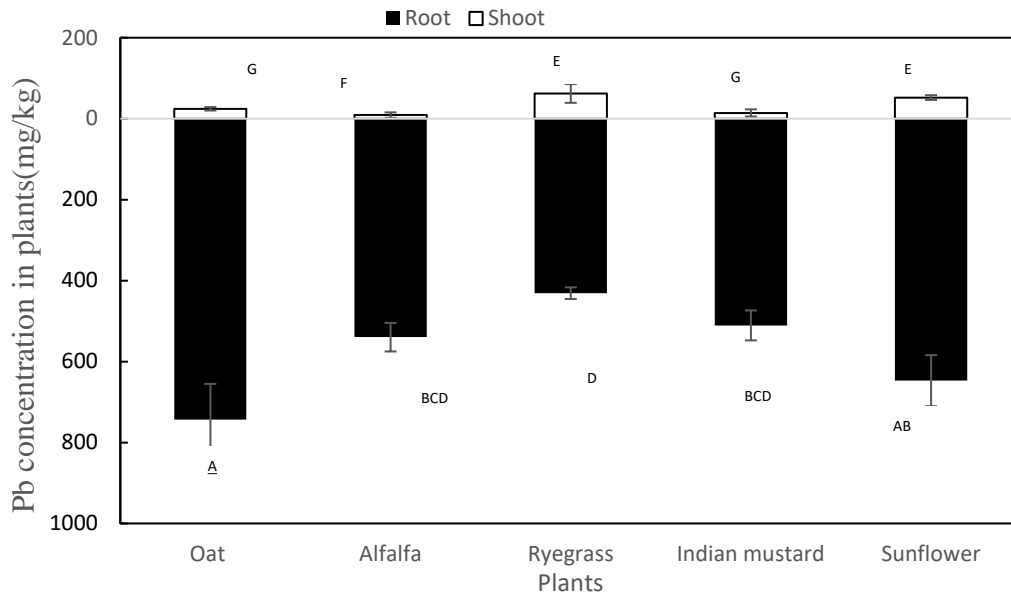
The concentration of metals in plants grown in contaminated soils were significantly higher than those grown in clean soils (Table A-1). The Cu and Pb concentrations in plant tissues are shown in Figure 3-4 (a) and (b), respectively. For all plants, 89-94% of Cu and 87-97% of Pb taken up from soil were concentrated in plant roots. No statistically significant difference was observed between Pb and Cu concentrations in plant tissues, except for ryegrass whose Cu concentration was 65% higher than its Pb concentration. Even though a statistically significant difference between Pb and Cu concentrations in plant tissues was absent for the remaining plants, they tended to accumulate more Cu than Pb; oat, alfalfa, Indian mustard and sunflower accumulated 15, 19, 12 and 7% more Cu than Pb, respectively. The translocation factor (TF is an indication of contaminant movement from roots to shoot or leaves and it is calculated as a ratio of metal concentration in stem or leaves and metal concentration in roots) of metals were generally low (less than 15%) in all plants (Table 3-6). The TF of Cu was higher than TF of Pb for alfalfa, oat, Indian mustard and sunflower, but the opposite was observed for ryegrass. Variation in metal TF was significant ($p \leq 0.05$) only for Indian mustard whose TF for Cu was 4.3 times higher than that of Pb.

$$TF = \frac{\text{Concentration of contaminant in shoot}}{\text{concentration of contaminant in root}}$$

A much clearer indicator of performance of plants for the purposes of phytoremediation is total metal accumulation (Figure 3-5), which is a function of metal concentration in plant tissue and plant biomass (dry weight of harvested plants). Like plant metal concentration, plants accumulated similar amounts of Cu and Pb (no statistical difference between amount of accumulated Cu and Pb was observed). However, amounts of Pb and Cu accumulated was significantly different across plants except for Indian mustard and sunflower, which accumulated similar amounts of both metals.



(a)



(b)

Figure 3-4: Metal concentration (mean \pm SE) in plant tissues after growth in contaminated soil (a) Cu (b) Pb. Different letters indicate a significant difference between Cu and Pb uptake by plants ($p \leq 0.05$).

Table 3-6: Translocation Factor (mean \pm SE) of Cu and Pb in plants grown on contaminated soil. Different letters indicate a significant difference between TF of plants for Cu and Pb ($p \leq 0.05$).

Plants	Translocation factor(TF) %	
	Cu	Pb
Alfalfa	2.19 ± 0.30^E	1.66 ± 0.33^E
Oat	4.07 ± 0.94^D	3.30 ± 0.11^D
Ryegrass	9.61 ± 2.98^C	14.23 ± 2.92^C
Indian mustard	11.92 ± 0.03^A	4.07 ± 0.22^B
Sunflower	7.07 ± 0.58^A	4.07 ± 0.20^{AB}

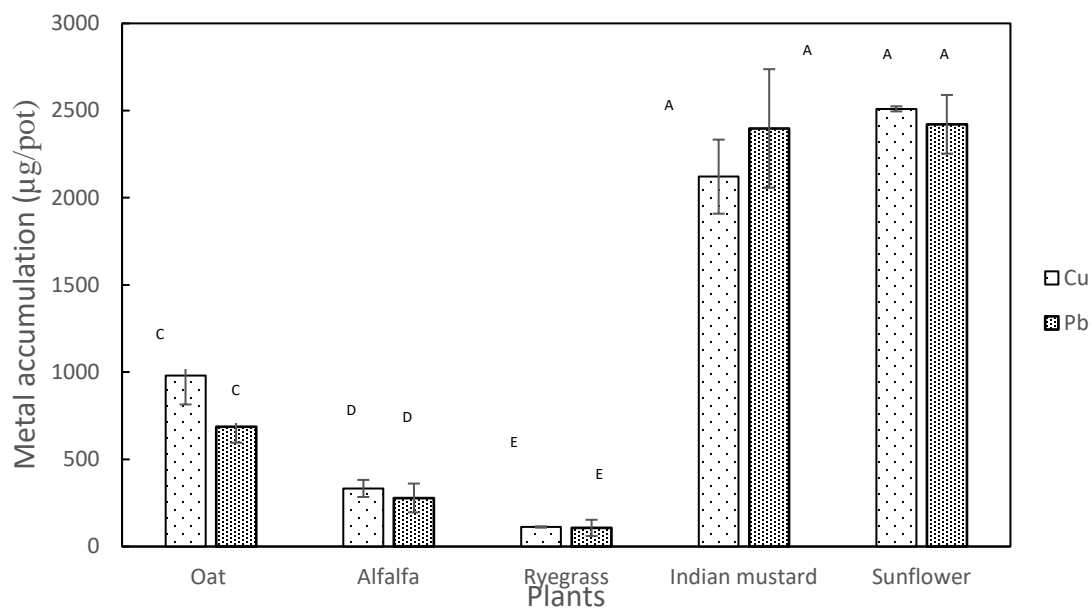


Figure 3-5: Metal accumulation (mean \pm SE) in plants after growth in contaminated soil. Metal speciation in soil. Different letters indicate a significant difference between total Cu and Pb accumulation by plants ($p \leq 0.05$).

3.2.5 Fate of metals in Soil pore water

Metal concentration in soil pore water is given in Table 3-7. All planted soil significantly ($P \leq 0.05$) mobilized more metals into soil pore water compared to unplanted soil except for soil planted with alfalfa. From the results of metal concentration in pore water, plants mobilized 1.3 -8.5 times more Cu than Pb with the concentration of Cu in pore water observed to be consistent with the Cu concentration in plant tissues whereas Pb concentration in plant tissues did not reflect its concentration in pore water.

Table 3-7: Metal concentration (mean \pm SE) in soil pore water after phytoremediation

Soil	Average metal concentration (mg/l)	
	Cu	Pb
Unplanted	0.079 \pm 0.017 ^B	0.008 \pm 0.006 ^D
Alfalfa	0.043 \pm 0.003 ^B	0.051 \pm 0.005 ^{BD}
Oat	0.292 \pm 0.035 ^A	0.04 \pm 0.019 ^D
Ryegrass	0.254 \pm 0.034 ^A	0.03 \pm 0.010 ^D
Indian mustard	0.204 \pm 0.061 ^A	0.152 \pm 0.018 ^C
Sunflower	0.283 \pm 0.083 ^A	0.178 \pm 0.033 ^{AC}

3.2.6 Fate of metals in soil

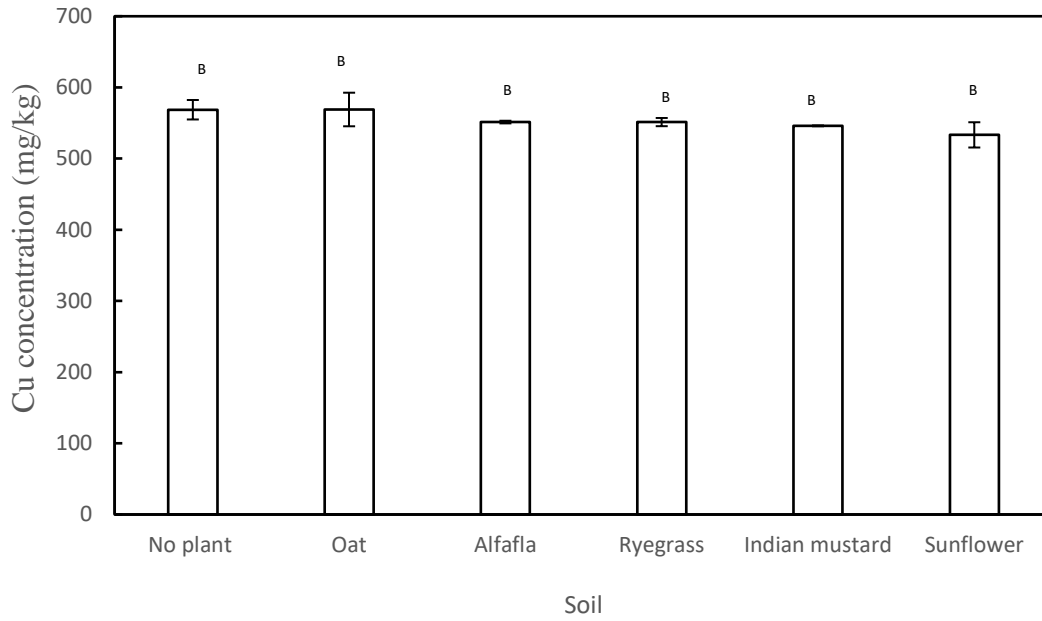
After 72 days of plant growth, no significant ($p < 0.05$) reduction in total metal content of contaminated soil was observed (Figure 3-6). But plant growth affected metal speciation in soils in diverse ways.

Figure 3-7 shows the effect of plant growth on Cu speciation in soil sown with the five plant species compared to unplanted soil. For soil planted with alfalfa, there was no statistical difference ($p \leq 0.05$) in Cu speciation when compared to unplanted soil. The growth of Indian mustard and sunflower in contaminated soil significantly reduced exchangeable metal by 17% and 39%, respectively. In soil planted with ryegrass, 100% of exchangeable Cu was redistributed to the organic fraction, causing a 35% increase in organic bound fraction of Cu. Oat increased exchangeable Cu by 70% by redistributing the carbonate bound Cu fraction to exchangeable Cu. Residual and Fe-Mn oxide-bound fraction of Cu in all planted soils was similar to that of unplanted soil.

The exchangeable Pb fraction was significantly reduced by 37%, 14%, 16%, 24%, and 31% in soils planted with oat, alfalfa, ryegrass, indian mustard and sunflower, respectively, when compared to unplanted soil (Figure 3-8). Carbonates, Fe-Mn oxides, organic and residual fractions in planted soil were the same as in unplanted soil.

Regardless of plant growth, Cu was associated mainly with the Fe-Mn oxide-bound fractions (33-40%) followed by residual (26-29%), carbonate bound (18-21%), organic-bound (11-20%) and exchangeable (0-0.9%) fractions, while most of the Pb was associated with the carbonates-bound fraction (38-45%) followed by Fe-Mn oxides-bound (29-35%), residual (14-23%), organic (4-8%) and exchangeable fraction (0.4-0.8%). The percent of total soil Cu in the organic-bound fraction was 86-120% higher ($p \leq 0.05$) than Pb for the same fraction.

(a)



(b)

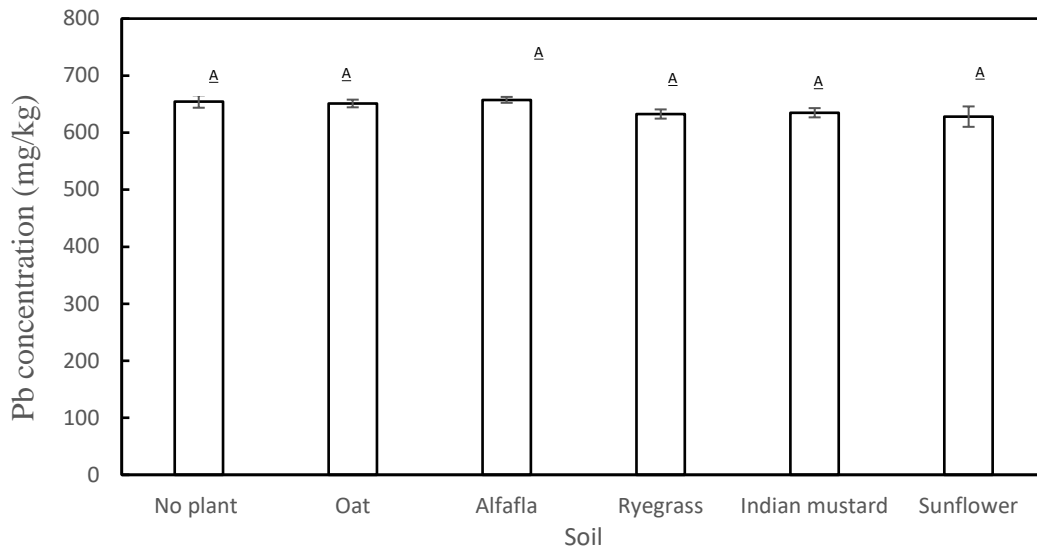


Figure 3-6: Metal concentration (mean \pm SE) in planted and unplanted soil. (a) Cu and (b) Pb. Different letters indicate a significant difference between Cu and Pb content in planted and unplanted soils ($p \leq 0.05$).

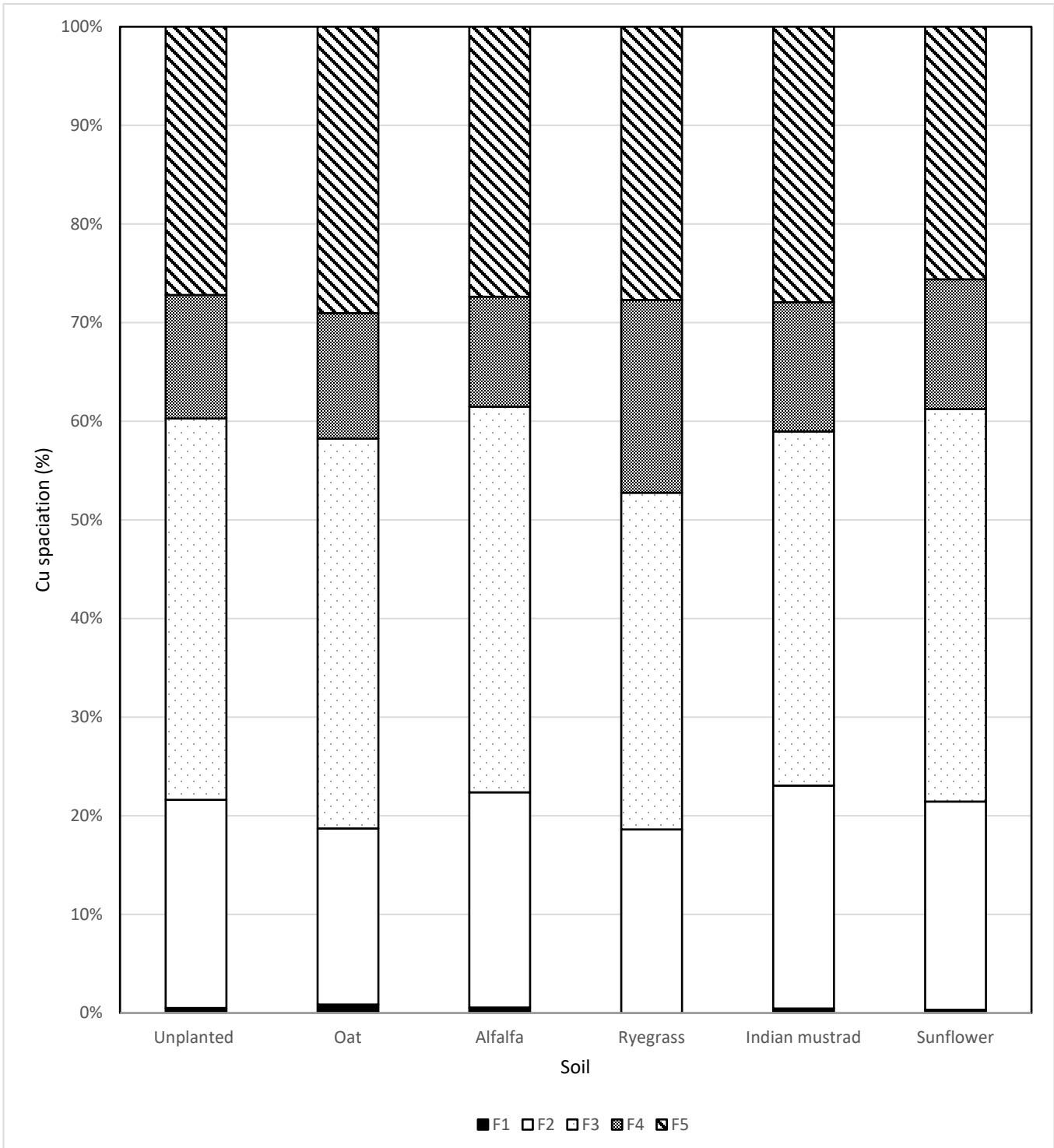


Figure 3-7: Comparison of effect of plant growth on Cu speciation in mixed contaminated soil. F1: exchangeable fraction, F2: carbonates-bound fraction, F3: Fe-Mn oxides-bound fraction, F4: organic fraction, F5: residual fraction

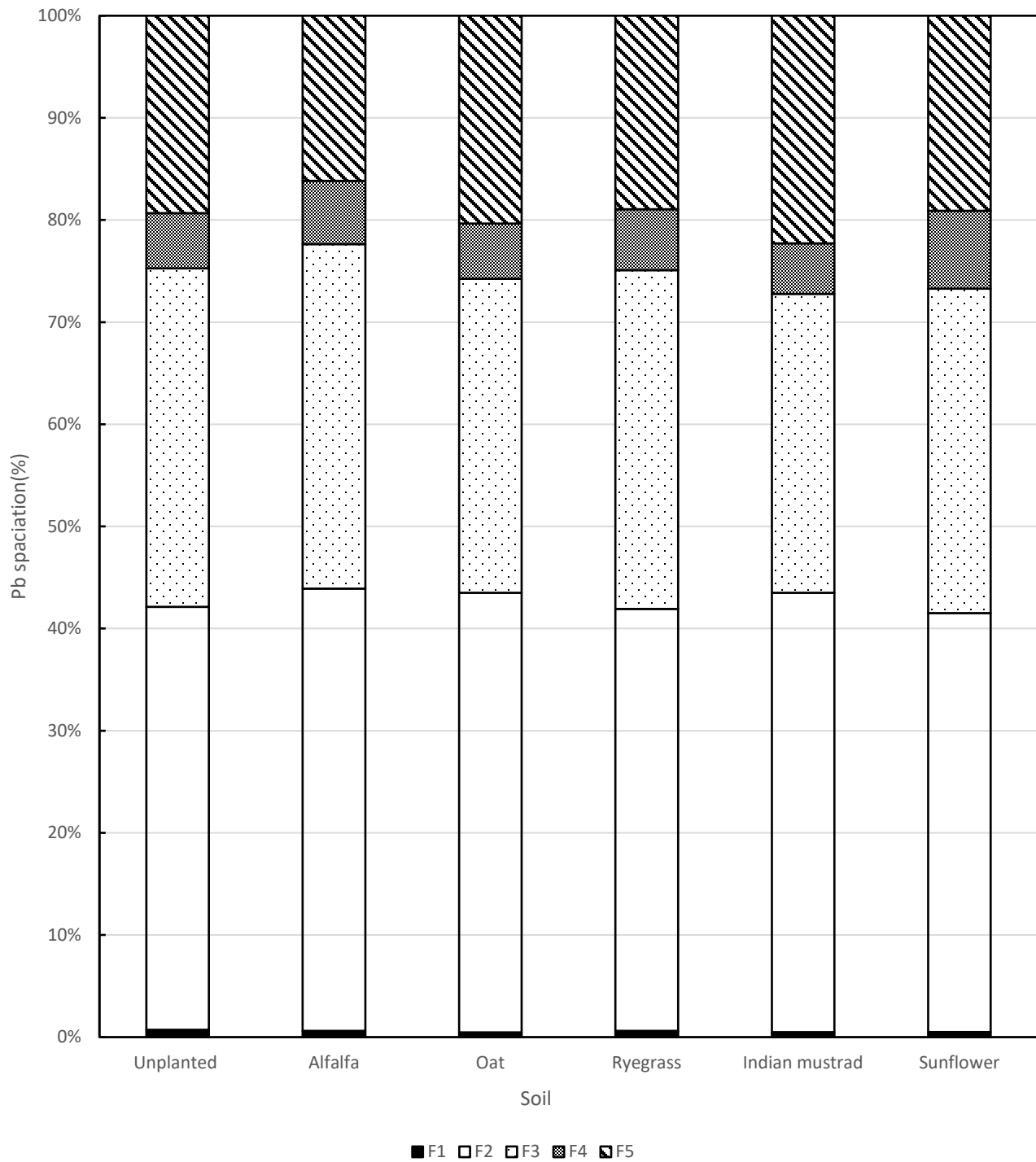


Figure 3-8: Comparison of effect of plant growth on Pb speciation in mixed contaminated soil. F1: exchangeable fraction, F2: carbonates-bound fraction, F3: Fe-Mn oxides-bound fraction, F4: organic fraction, F5: residual fraction.

3.2.7 Fate of Organic Contaminants in Soil

Residual organic contaminant in soil after phytoremediation is shown in Figure 3-9 for pyrene and Figure 3-10 for DDT. Significant reduction (~65%) in soil pyrene levels was achieved without the aid of plant. Additional pyrene was removed in the presence of alfalfa (17%) and sunflower (25%). Oat and Indian mustard seemed to slow down natural degradation of pyrene while ryegrass did not interfere with pyrene degradation.

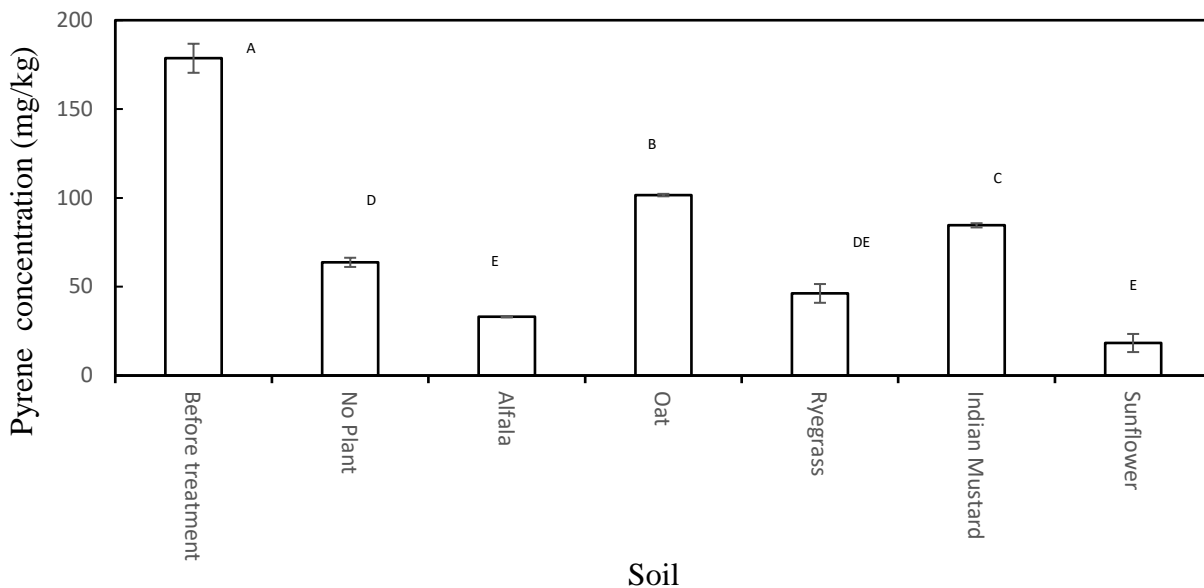


Figure 3-9: Comparison of pyrene concentration in planted and unplanted soil before and after phytoremediation treatment. Different letters indicate a significant difference between pyrene degradation in planted and unplanted soil ($p \leq 0.05$).

Degradation of DDT to its metabolites DDD (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethane) produced from biotic degradation and DDE (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene) produced from abiotic degradation was not observed. There was also no significant ($p < 0.05$) reduction in 4, 4- and 2, 4- DDT of unplanted soil compared to the initial DDT value. Plant growth did not generate any significant reduction

of 4, 4'-DDT in soil but 2, 4' DDT was reduced ($p < 0.05$) in soils planted with alfalfa, Indian mustard and sunflower by 40%, 38% and 30%, respectively.

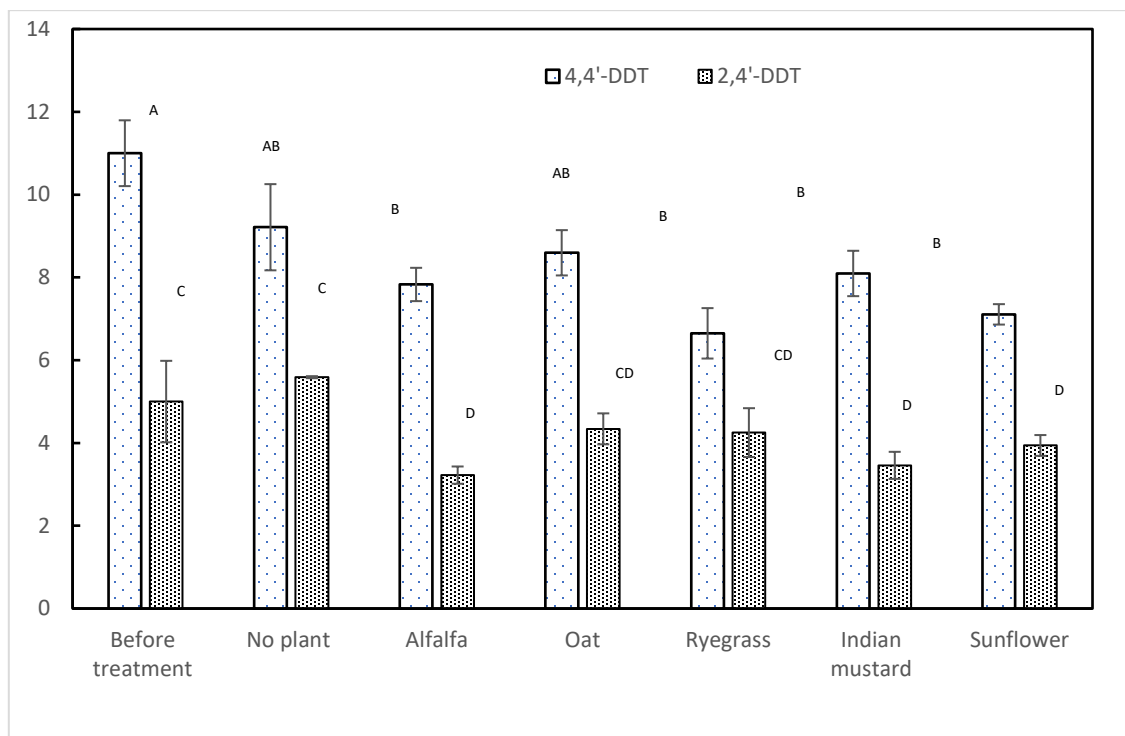


Figure 3-10: Comparison of 4, 4'- and 2, 4'-DDT concentration in planted and unplanted soil before and after phytoremediation treatment. Different letters indicate a significant difference between DDT isomers and their degradation in planted and unplanted soil ($p \leq 0.05$).

3.2.8 Organic contaminants uptake by plants

Plant uptake of organic contaminants is well established (Paul et al., 2015; White, 2000; Zeeb et al., 2006). For the most part, plant uptake of PAH from contaminated soil accounts for a small portion (usually less than 0.1%) of total PAH decrease during phytoremediation (Hechmi, Aissa, Abdennaceur, & Jedidi, 2013; Lin, Shen, Zhao, & Li, 2008). Hence, determination of pyrene content of plants was considered unnecessary in this study. For DDT, studies on phytoremediation of DDT in contaminated soil have shown that plants

can accumulate a considerable amount of DDT and its metabolites ranging from 0 -77% of total DDT lost from soil (Huang et al., 2011; Lunney et al., 2004; Mo et al., 2008; Paul et al., 2015; Wang, 2008).

The concentration of DDT in plant tissue is shown in Figure 3-11. Although DDD and DDE were absent in planted soils after phytoremediation, both isomers of DDD and DDE were observed in all plant tissue. DDD and DDE accounted for 56 – 73% and 1-7% of total DDT concentration (sum of DDT, DDD and DDE) in plant respectively. Sunflower had the highest concentration of DDT (4.81mg/kg) and DDD (8.96mg/kg) while oat had the highest DDE concentration (0.49 mg/kg). DDT distribution in plant tissues were similar to the pattern observed for Cu and Pb distribution in plant tissue in that DDT was concentrated mostly in plant root. DDT, DDD and DDE concentration in plant shoot ranged from 0 mg/kg in Indian mustard to 0.57 mg/kg in ryegrass, 0.14 mg/kg in Indian mustard to 0.32 mg/kg in sunflower and 0 mg/kg in all plants respectively. Root concentration values ranged from 1.93 mg/kg of DDT in Indian mustard to 4.5 mg/kg of DDT in sunflower, 5 mg/kg of DDD in alfalfa to 8.6mg/kg in sunflower and 0.12mg/kg of DDE in ryegrass to 0.49 mg/kg in oat. DDE concentration across plants was not significantly ($p < 0.05$) different, but significant differences in DDD and DDT concentration was observed.

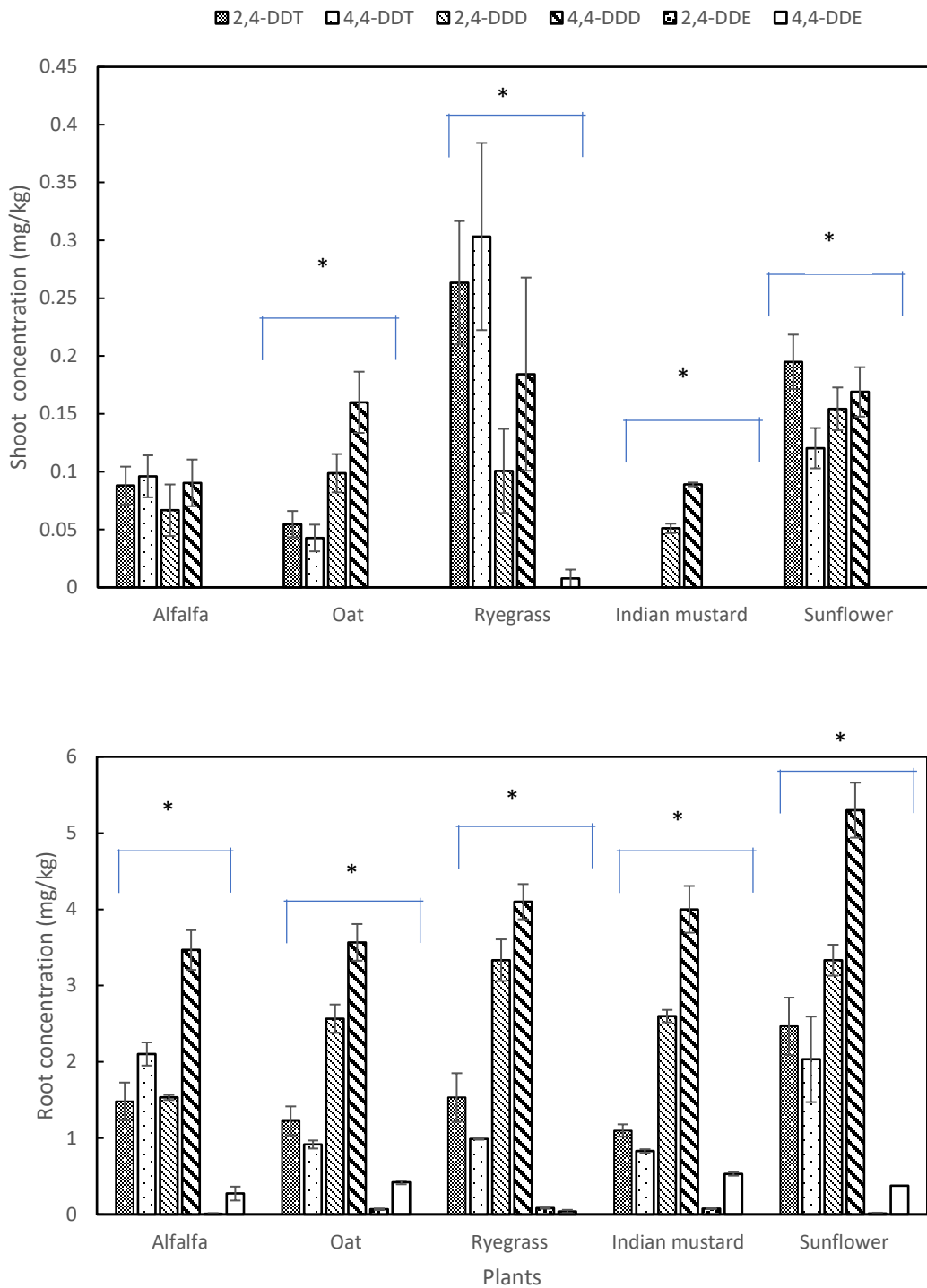


Figure 3-12: Total DDT concentration (mean \pm SE) in plant tissues after growth in contaminated soil (a) Shoot (b) Root. * indicate a significant difference between DDT, DDD and DDE concentration within plants ($p \leq 0.05$).

3.2.9 Plant growth response to mixed contamination

Percent germination in contaminated soil compared to clean soil is shown in Figure 3-12 and is seen to be similar to that obtained during the toxicity test (Figure 3-1) where no significant ($p < 0.05$) effect of contamination on plant percent germination was observed. By the end of 72 days growth, percent survival of all plants in clean soil and that of oat, ryegrass and sunflower in contaminated soil remained the same but that of alfalfa and indian mustard in contaminated soil was significantly reduced (Figure 3-13) by 32% and 33% of their percent germination value at the beginning in contaminated soil. All plants developed extensive root systems that filled the entire soil volume, except for ryegrass whose roots were sparsely distributed in the soil.

A significant reduction ($p < 0.05$) in growth rate in response to soil contamination was also observed for all plants except for sunflower (Figure 3-14). For Indian mustard, reduction of plant growth in contaminated soil was first observed on day 14 but by day 28 the plant went back to the same growth rate as in clean soil. Final height of plants in contaminated soil were significantly ($p < 0.05$) lower than the plant height in clean soil by 36% for alfalfa, 22% for oat, 28% for rye grass and 5% for sunflower.

In addition to growth rate, biomasses produced in contaminated soil were significantly ($p < 0.05$) lower than those produced in clean soil for all plants except Indian mustard (Figure 3-15). Contamination induced reductions in biomass of 92, 34, 69 and 61% for ryegrass, sunflower, alfalfa and oat, respectively. No effect on Indian mustard biomass was observed.

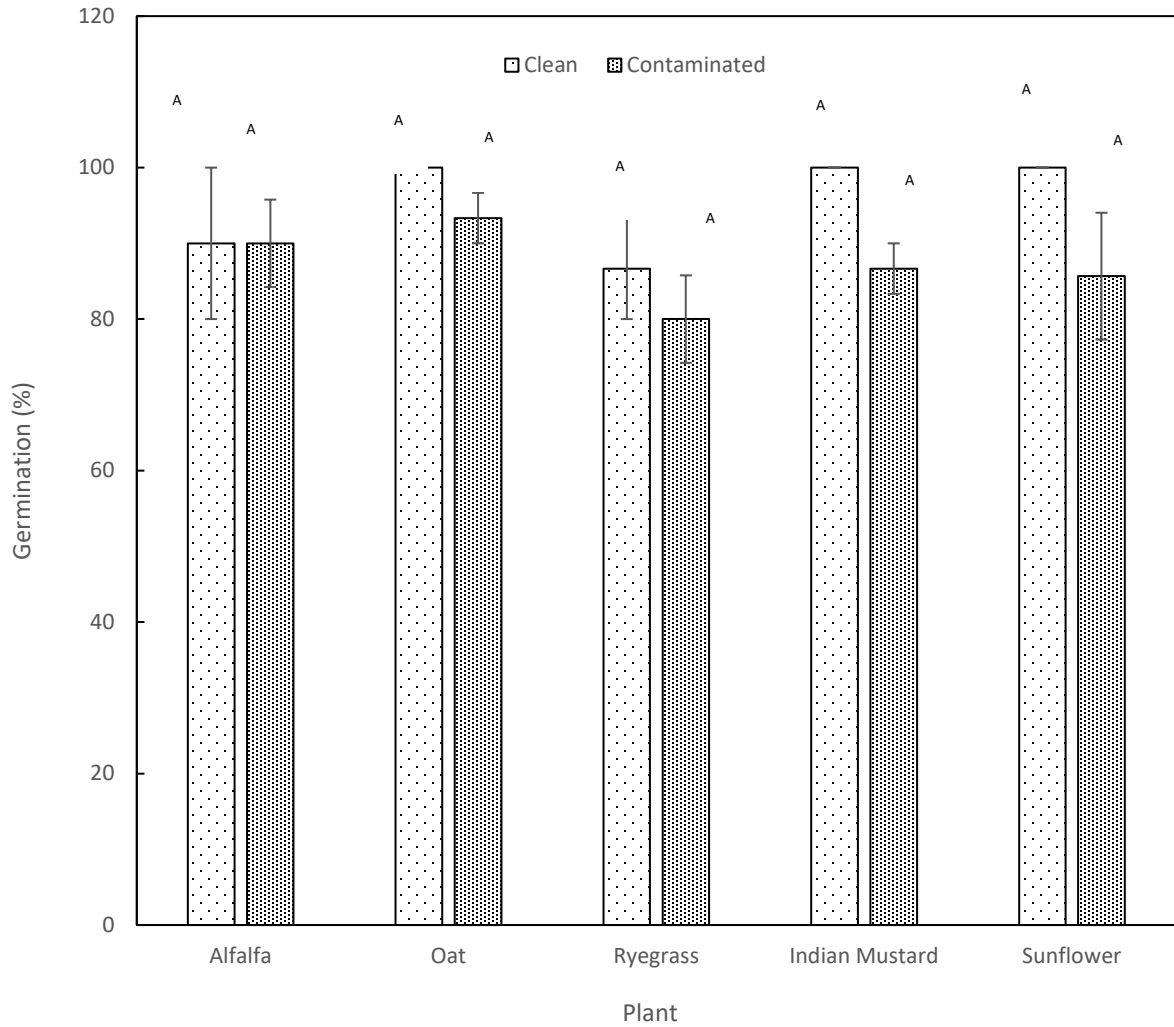


Figure 3-12: Germination percent (mean \pm SE) of plants in clean and contaminated soil. Different letters indicate a significant difference between DDT isomers and their degradation in planted and unplanted soil ($p \leq 0.05$).

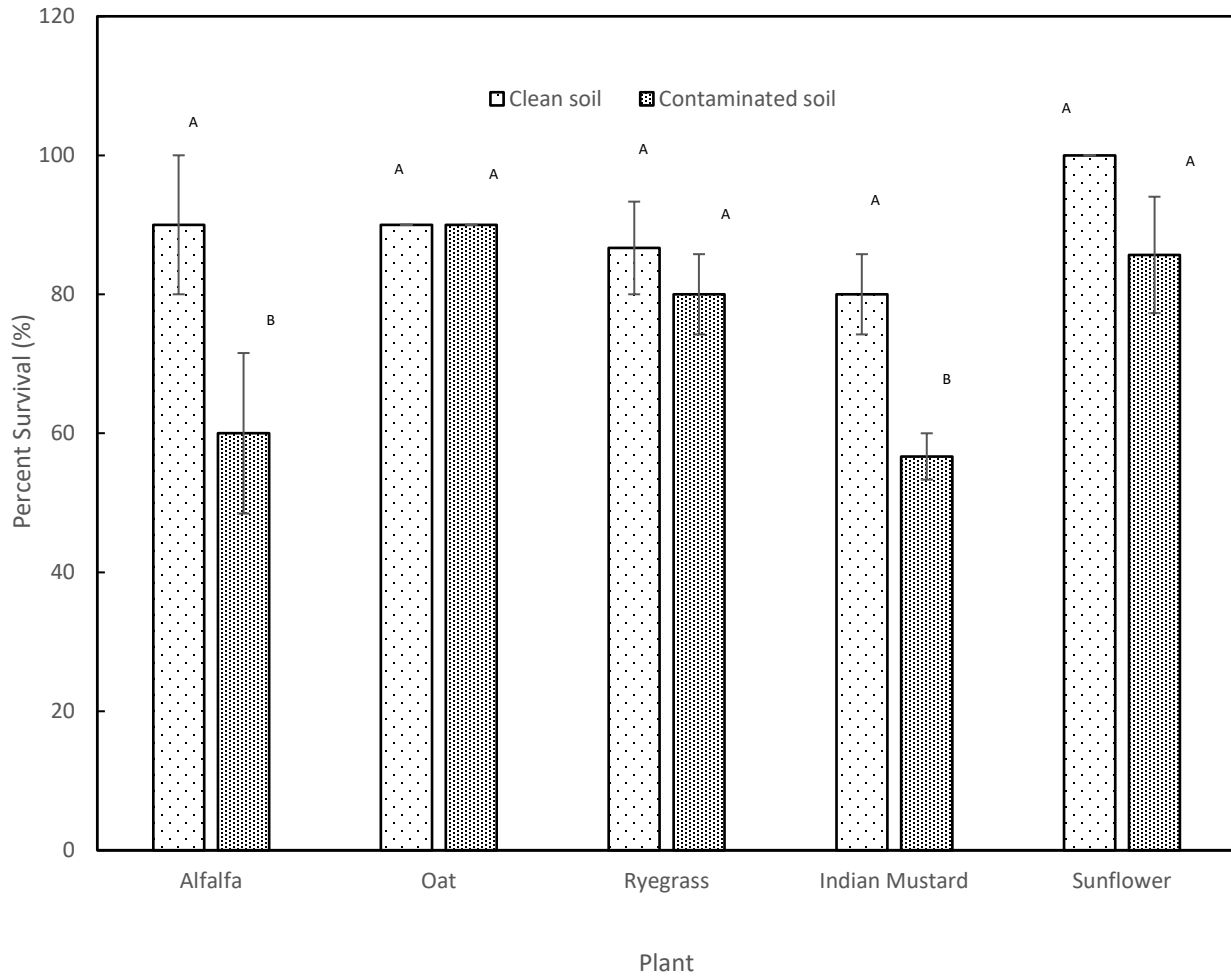


Figure 3-13: Plant survival (mean \pm SE) as a ratio of final germination to initial germination of plants in contaminated soil. Different letters indicate a significant difference between DDT isomers and their degradation in planted and unplanted soil ($p \leq 0.05$).

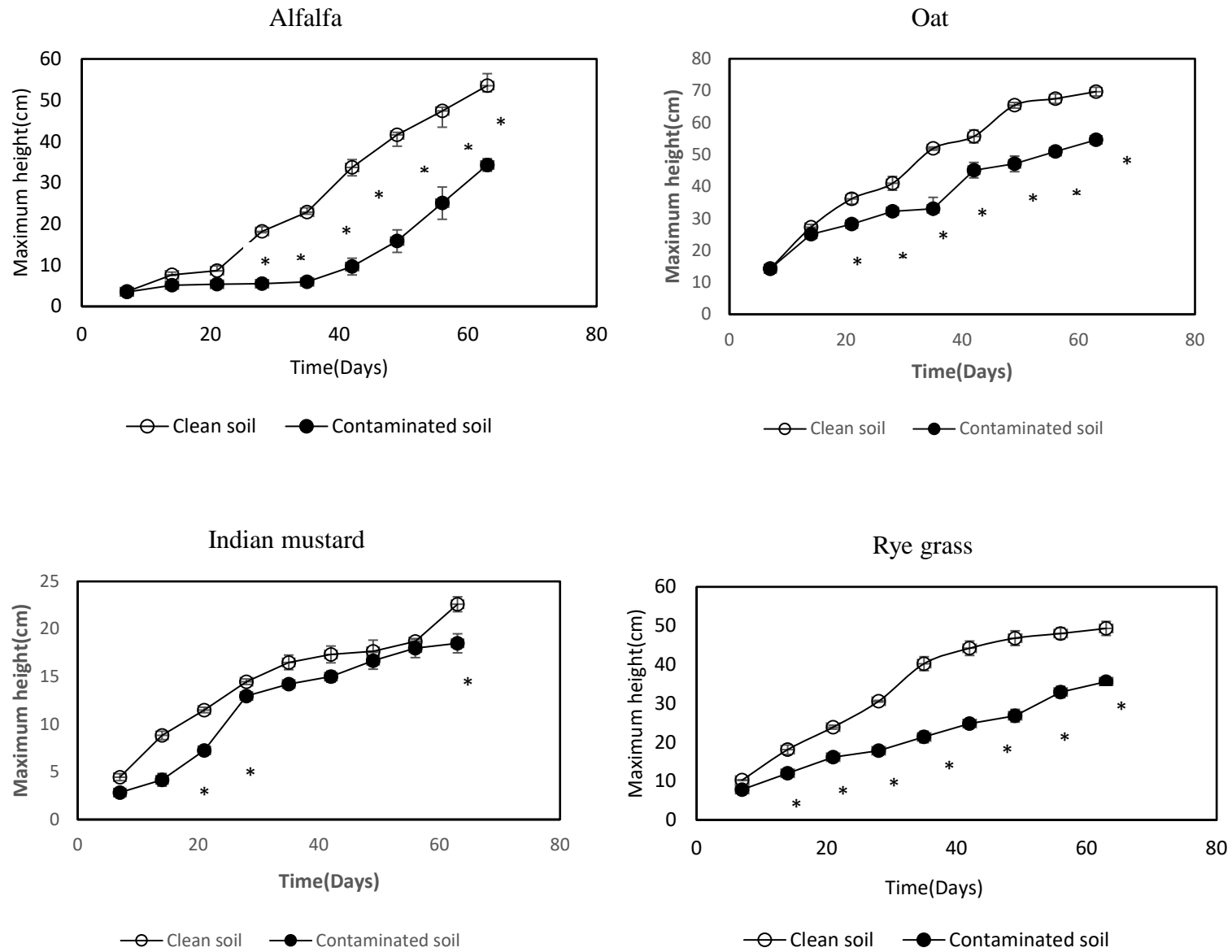


Figure 3-14: Increase in plant height with time (mean \pm SE) in clean and contaminated soil. * indicate a significant difference between plant growth in clean and contaminated soil (Time $p \leq 0.05$).

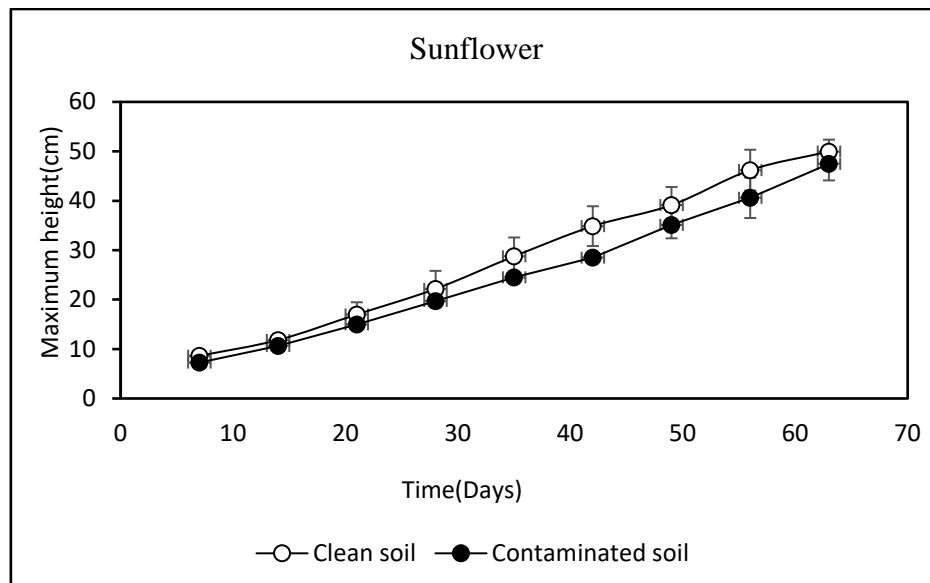


Figure 3-15 (Continued): Increase in plant height with time (mean \pm SE) in clean and contaminated soil. * indicate a significant difference between plant growth in clean and contaminated soil (Time $p \leq 0.05$).

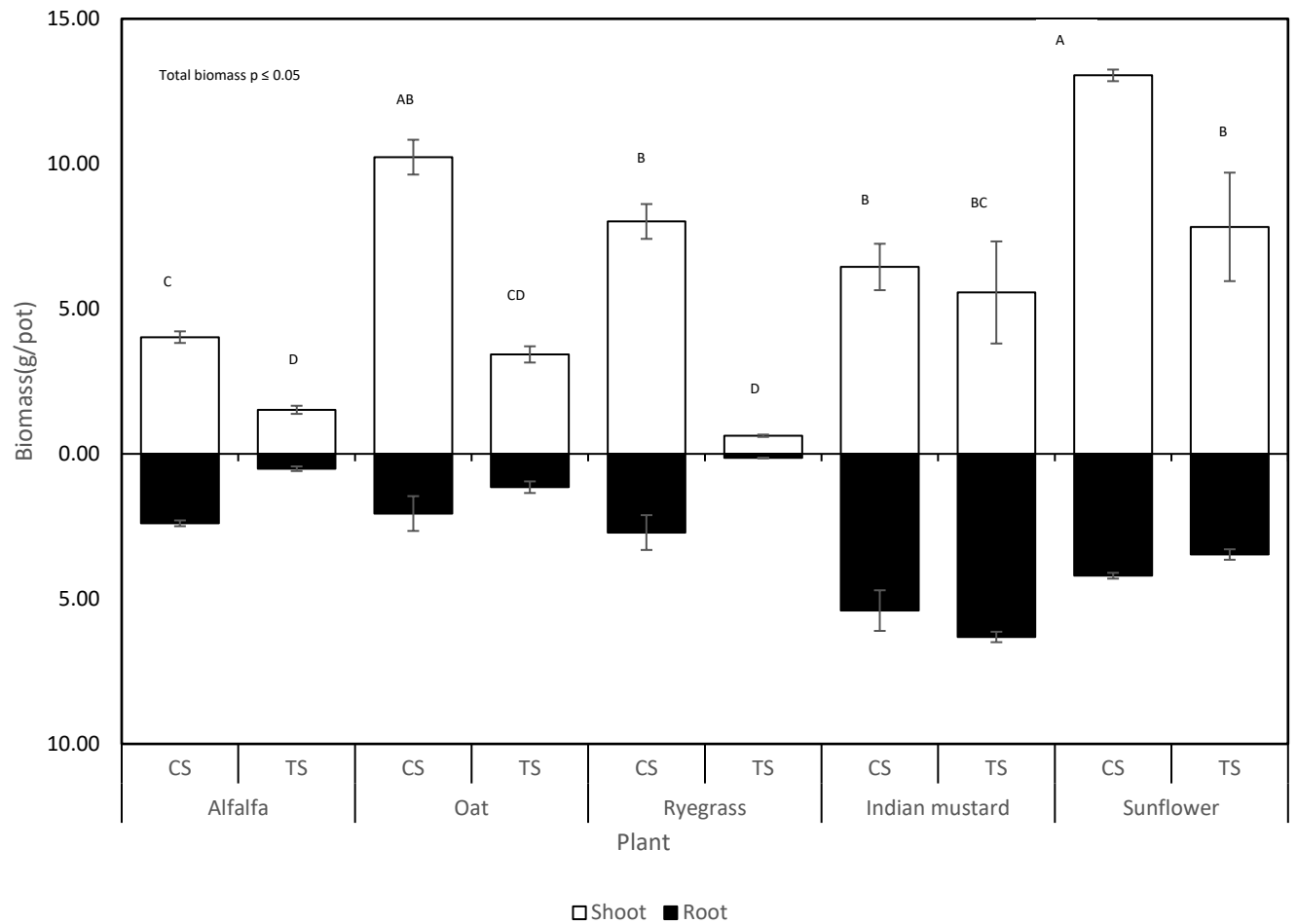


Figure 3-16: Root and shoot biomass (mean \pm SE) of plants in clean (CS) and contaminated soil (TS). Different letters indicate a significant difference between clean and contaminated soil and between plants (Total biomass $p \leq 0.05$).

3.3 Discussion

3.3.1 Preliminary toxicity test

Seed germination can typically represent the first step to effective phytoremediation because plant performance at the early stages sets the pace for root and shoot development as well as to determine the extent to which the soil environment may negatively or positively impact plant growth. The lack of significant effect of the contaminants on seed germination can be understood to mean that the seeds of these plants are resistant to penetrative phytotoxic stress of the contaminant combination and at the tested concentrations. Even though, at early growth stage, the nutritional needs of embryonic plants are not provided from the soil environment but internally from seed stored materials (Kapustka 1997), prevention of interference by contaminants with nutritional materials stored in plant seed is preferred. The ability of plants to prevent penetration of contaminants into the seed is attributed to the nature of the selective permeability of the seed coat (Klokk, 1984; Wierzbicka & Obidzińska, 1998). Plant seed coats act as a barrier between a plant embryo and the toxic environment, protecting the embryos from contamination until the embryonic roots start to develop (Kapustka 1997). The differential germination response of plants in contaminated soil is accounted for by the fact that the seed coat composition, as well as the permeability of seed coats, varies from plant to plant.

In addition to plant tolerance in a contaminated environment, a plant's above-ground mass and root structure are crucial for effective phytoremediation. Extensive roots and high above ground biomass are desirable qualities for phytoremediation. Longer roots increase the rhizosphere area thereby enhancing the ability to support soil microorganisms, improve contaminant uptake and reach contaminants at a deeper soil horizon (Harvey et al., 2002; Masarovičová & Král'ová, 2012). Larger shoot mass provides a larger area for transpiration, which improves metal transport from

root to shoot (Gleba et al., 1999). In the present toxicity test, root lengths of oat, rye grass, tall fescue and Indian mustard were adversely affected by contamination with tall fescue having the shortest root length in contaminated soil. Shoot lengths of most of the plants were larger for plants grown in contaminated soil, except for ryegrass; however, the shoot length of tall fescue in contaminated soil was one of the lowest. Over all, the sensitivity of plants to mixed contamination was in the order of roots>shoots> percentage germination.

By considering the importance of the plant roots and shoots as well as the relative tolerance in the contaminated soil alongside the results, switch grass, which didn't grow in either soil (probably because the batch of seeds were bad) , and tall fescue, which had the lowest germination percent and shortest roots and shoots, were eliminated from further consideration for phytoremediation.

3.3.2 Metal uptake by plants

The first step in phytoextraction of metals from soil is mobilization of metals from contaminated soil solid phase to the bulk pore water after application of additives(Wang et al., 2007) such as surfactants and chelating agents. Plant root exudates are well known chelating agents which explains the observed mobilization of metals in planted soils compared to unplanted soil. A similar observation was made by(Lombi, Zhao, Dunham, & McGrath (2000) using two species of *Thlaspi caerulescens* (alpine pennygrass), *J. Presl* and *C. Presl*, to phytoextract Zn and Cd from metal contaminated soil.

In the present study, the mobilization of more Cu compared to Pb in soil pore water maybe due to Cu being more soluble in water than Pb. This further supports the seemingly preferential uptake and transport of Cu over Pb, indicated by higher Cu concentration in plants (Figure 3-4). In addition, Cu being an essential element affords it plant specific membrane transporters in the root

cells that help bring in Cu compared to Pb which is not essential and does not have specific membrane transporters in plants (Mendoza-Cózatl, Jobe, Hauser, & Schroeder, 2011). This implies that for Pb to be taken up by plants, it has to slip through membrane proteins that are large enough to handle the 2+ charge on Pb^{2+} making Cu uptake by plants more likely than Pb. The order of Cu mobilization in soil was observed to be consistent with Cu plant concentration, surprisingly Pb uptake by plants was not related to Pb mobilization in pore water. This may point to the possibility of Pb ions competing with other ions in soil solution for plant uptake, since Pb will be travelling through non-specific transporters, Pb ions may compete with other ions in soil solution for access to these uptake channels, implying that the availability of Pb in soil solution may not translate into its uptake by plants.

The translocation factors (< 16%) for metals indicate that plants did not transfer metals from root to shoot. This sort of response is a tolerance mechanism in plants to reduce metal toxicity (Baker, 1981). Plant roots often act as a barrier to the uptake and transport of metals by binding contaminants outside the root surface which results in localization of metals in the root sometimes at metal concentrations higher than that of growth medium (Dalvi & Bhalerao, 2013; Inouhe, Hunag, Chaudhary, & Gupta, 2012; Kabata-Pendias, 2000).

Low total metal accumulation observed in this study can be attributed to the low concentration of bioavailable metals in the soil. Potentially most bioavailable forms of metal in soil are in the exchangeable fraction because they are weakly absorbed to soil and are easily converted to soluble forms (Narwal & Singh, 1998; Olaniran, Balgobind, & Pillay, 2013; Tessier et al., 1979). These soluble forms are readily taken up by plants from soil solution in the form of free ions or complexed forms (Kabata-Pendias, 2004). In the present study, the exchangeable fraction of metal in the

unplanted soil was 2.3mg/kg out of 550mg/kg of added Cu and 4.0mg/kg out of 650mg/kg of added lead. Both fractions are less than 1% of the total metal content leading to low amount of metals in soil solution and a considerable amount of metal in fractions that are not readily available for plant uptake. A similar observation was made by Chirakkara and Reddy (2015) after phytoremediation of a mixed contaminated soil (Pb, Cd, Cr, phenanthrene and anthracene) in which the lowest metal removal by plants corresponded to the metals with the lowest percent of exchangeable fraction. In their study, exchangeable forms of Pb, Cd and Cr were 4 mg/kg out of 500mg/kg added (<1%), 2.8mg/kg out of 50mg/kg (5.6%) and 30mg/kg out of 200mg/kg (15 %), respectively and the highest metal removal by plants was observed for Cr. In the face of low plant accumulation and preferential uptake of Cu over Pb, the highest amount of metals in the current study were accumulated by sunflower (2.5mg Cu and 2.4mg Pb) and Indian mustard (2.1 Cu and 1.7mg Pb).

3.3.3 Soil metal fractions

Metal speciation refers to the various chemical forms in which metals can exist in the environment. Tessier et al.(1979), identified exchangeable, carbonate, Fe-Mn oxide, organic and residual fractions as the metal species in soil that are likely to be affected by various environmental conditions. The exchangeable fraction of metals is generally considered to be mobile and bioavailable to plants for uptake and adsorption, whereas the carbonate, Fe-Mn oxide, organic and residual fractions are considered immobile and not readily available to plants (Shuman, 1985; U.S EPA, 2009). A principle controlling factor of metal speciation and bioavailability in soil is pH (Brown, Pickford, & Davison, 1984; Rieuwerts, Thornton, Farago, & Ashmore, 1998; T. Sandrin & Hoffman, 2007; U.S EPA, 2009).

In the present study, the low (<1%) exchangeable fraction of Cu and Pb can be related to the pH of soil. The pH of planted and unplanted soils ranged from 7.3 to 7.6 (Table 3-5). In general, the mobility of metals tends to increase at acidic pH and reduce at basic pH. This has been established to be true for cationic metals such Cu, Pb, Zn, Ni, etc. which at $\text{pH} > 7$ are adsorbed strongly to soil (McLean & Bledsoe, 1992) and are thus less likely to be mobilized. More specifically, Spurgeon et al. (2006), observed reductions in the extractable fractions of metals As, Cd, Cu, Hg, Pb and Zn at soil pH 7-8 compared to the extractable concentrations at soil pH 4 -6. Thus at the pH observed in this study, Cu and Pb are expected to be associated more with immobile fractions than mobile fraction.

Irrespective of soil pH, plants are capable of changing metal speciation in soil (Chirakkara & Reddy, 2015b; Padmavathi & Li, 2009). In the present study oat was able to redistribute a relatively immobile fraction of Cu to a mobile fraction and ryegrass redistributed a mobile fraction of Cu to an immobile fraction. An increase of metals in the mobile fraction can be undesirable because of increased risk of contaminant transfer to other parts of the environment. The ability of oat to increase exchangeable Cu can be attributed to the root exudates of oat. According to Adamczyk-Szabela et al. (2015), Chirakkara & Reddy (2015a) and Kabata-Pendias (2000), organic substances produced by plant roots and released to the soil as exudates can shift the equilibrium between different metal fractions and form soluble complexes with metals. This may explain why oat was able to increase exchangeable Cu in soil.

The reduction in exchangeable Pb by all plants and exchangeable Cu by Indian mustard and sunflower can be attributed to plant uptake. The difference in outcomes of Cu and Pb speciation in soil after growth of plants can be attributed to differences in the plants' response to contaminant

toxicity, differential binding mechanisms of Cu and Pb in soil, and the subsequent reactions with soil components. For example, Cu has a greater affinity to organic matter than Pb and hence forms stable complexes with organic matter unlike Pb (Kabata-Pendias, 2000; Q. Li et al., 2007). This is consistent with the present study, in which organic-bound Cu accounted for an average of 14% of total Cu in soil and organic-bound Pb accounted for an average of 6% of total Pb in soil.

3.3.4 Fate of organic contaminants

Organic contaminants can be removed from soil in one or more of the following ways: 1) Plant uptake, 2) Degradation by enzymes from plant roots or microorganisms in the rhizosphere, 3) Volatilization and 4) Incorporation into soil organic material (Lin, et al , 2006; Zhang et al, 2009).

The reduction in the amount of residual pyrene in unplanted soil compared to initial concentration at the end of the experiment implies degradation by soil micro-organisms and/or volatilization. Further reductions in pyrene concentration were observed in soils planted with alfalfa, ryegrass and sunflower, indicating plant-promoted biodegradation of pyrene. Plants are able to improve the degradation of organic contaminants by enzymes secreted by roots, which improves microbial activities in the rhizosphere. The same reason may explain higher pyrene concentrations in soils planted with oat and indian mustard, except that the root enzymes secreted by these plants may have reduced soil microorganism degradation activities by changing the metabolic capacity of micro-organisms (Phillips, Greer, Farrell, & Germida, 2012). An alternative explanation for increased pyrene content in some planted soils is that the movement of pyrene by mass flow or diffusion in the bulk flow of water towards the rhizosphere caused an increase in pyrene accumulation in the soils surrounding roots, which is expected to dissipate with time (Liste & Alexander, 2000).

The DDT concentrations measured in unplanted soil compared with the initial value indicates that loss of DDT via volatilization was negligible. In planted soils, the absence of main DDT metabolites (DDD and DDE) in soil was observed and can be considered desirable as DDE and DDD have similar toxic effects in the environment as DDT. Their absence can be attributed to toxicity to soil microorganisms by DDT itself or co-contamination with metals and pyrene. Toxicity of metals and DDT to soil microorganisms is well established. More specifically, metals have been reported to prevent degradation of DDT to DDE and DDD. Studies have shown that metals such as As can also inhibit breakdown of DDT to DDE and DDD, and Cu can prevent degradation to DDD (Gaw, Palmer, Kim, & Wilkins, 2003; Van Zwieten, Ayres, & Morris, 2003). Both studies observed that increase in metal and DDT concentration in soil was accompanied by reduction in microbial activities. Co-contamination may have resulted in pyrene, as opposed to DDT, being the preferred carbon source for soil microorganisms.

The outcome of not obtaining DDT metabolites is the persistence of DDT in the soil as no significant reduction in 4,4'-DDT in planted soil compared to unplanted soil and the initial value was observed. Soils planted with alfalfa, Indian mustard and sunflower on the other hand showed significant reductions in 2,4'-DDT, implying that these plants have a mechanism for assisting preferential degradation of 2,4'-DDT. Similar observations were made in bioremediation studies by Zhu et al., 2012 and Fang et al. 2010. In the former, *Sedum alfredi* accumulated 11.5 times more 2,4-DDT than 4,4-DDT. Similarly in the latter study, 78% removal of 2,4-DDT by pumpkin was observed compared to 13% recorded for 4,4-DDT. However, the mechanisms and processes in plants responsible for this preferential uptake and degradation is yet to be elucidated.

Comparing the amounts of DDT lost in soil to the amounts observed in plant tissue it is not clear what the dominant process of DDT removal was because of the observed transformation of DDT to DDD and DDE by plants and inability of the study set up to account for further breakdown of DDT to water and carbon(IV) oxide or other undetectable transformation products. Regardless, it is clear that the loss of DDT from soil can be attributed to more than one process, a possible combination of plant uptake (phytoextraction) , plant enzyme assisted degradation (rhizodegradation) and phytodegradation/phytotransformation. The processes involved in transformation of DDT in plants is not well known but some studies have reported similar transformation in plant tissue. Gao, Garrison, Hoehamer, Mazur, & Wolfe (2000) observed DDT transformation by axenically cultivated aquatic plants parrot feather (*Mariophyllum aquaticum*), duckweed (*Spirodela oligorrhiza*), and elodea (*Elodea canadensis*) to majorly DDD. This suggests that the transformation of DDT involves an enzymes mediated reaction(s) in plant cells. This is supported by results from an enzyme study by Chu, Wong, & Zhang (2006) showing the degradation of DDT in enzyme extract solution from the root, leaf and stem of common reed (*Phragmites australis*) and rice (*Oryza sativa* L.) to DDD and DDE with DDD being the main metabolite. The presence of DDD as the main metabolites in the present study as well as the previously mentioned studies further supports that the transformation is mediated by a biotic process as DDD is the major by product of biological breakdown of DDT (Chu et al., 2006).

Overall, it is difficult to compare plants performance in terms of accumulation of DDT in the current study to other studies that used similar plants to phytoextract DDT from DDT contaminated soils (Lunney et al., 2004; Mitton, Miglioranza, Gonzalez, Shimabukuro, & Monserrat, 2014). In addition to co-contamination of DDT with Cu, Pb and pyrene, the initial soil concentration of total DDT is 5-40 folds higher than those used in these studies.

3.3.5 Plant growth performance

Results of plants percent germination in potted seeds were similar to those obtained in petri dishes (toxicity test). Irrespective of the lack of any effect on germination by the contaminants, at the end of a 72 day growth period, reduced growth rate, biomass and survival in contaminated soil was observed in some plants as well as signs of phyto-toxicity such as yellowing and/or drying up of the leaves(Appendix F). Effects such as these can be attributed to the lack of soil nutrients or the presence of contaminants in soil. The possibility of nutrient deficiency was ruled out by the addition of slow-releasing fertilizer during plant establishment making the presence of mixed contaminants in soil most likely responsible for observed effects on plant growth.

Adverse effects of mixed contaminated soil on plants can be attributed to the presence of metals, organic contaminants or an interaction between both classes of contaminant. Metals such as Cu, Ni, Co, Zn and Pb are known to impact negatively on plant growth by reducing translocation and causing deficiency of essential nutrients within plants (Siedlecka, 1995).Also, organic contaminants such PAH and DDT have also been reported to adversely affect plant growth (Mitton et al., 2014; Smith, Flowers, Duncan, & Alder, 2006). Overall, growth performance and behavior varied significantly from species to species of plant.

3.4 Conclusion

At the end of phytoremediation, the biomass of Indian mustard was least affected by mixed contaminated soil while sunflower generated the highest biomass in mixed contaminated soil. Also compared to other plants used in this study, the growth rate of both plants was least affected by soil contamination. This implies the high tolerance of these plants in the presence of studied

contaminant mixture. Rye grass was most affected by mixed contamination and produced the least biomass.

In terms of contaminant removal and uptake, Indian mustard and sunflower also accumulated the highest amount of Cu and Pb in its tissues. Although Indian mustard slowed down the degradation of pyrene, it did improve the removal of 2, 4-DDT from soil and sunflower improved the removal of both pyrene and DDT from soil. Irrespective of ryegrass and alfalfa accumulating the least amounts of metals, ryegrass was able to redistribute exchangeable fraction of Cu to organic-bound fraction and alfalfa improved the removal of pyrene and 2, 4'-DDT from soil. All plants accumulated DDE and DDD in addition to DDT, even though metabolites of DDT were absent in soil.

All plants achieved reduction of exchangeable Pb thereby reducing the potential of increased mobility of Pb. But, exchangeable Cu was significantly increased by oat and completely redistributed to organic fraction in soils planted with ryegrass. The observed increase in exchangeable copper in soils planted with oat indicates a potential increase in mobility of Cu and the possibility of further contamination of groundwater during phytoremediation.

Overall considering plant growth in soil and contaminant removal from soil, sunflower demonstrated the greatest potential as a phytoremediation candidate in metal-PAH-pesticide contaminated soils.

3.5 Limitation of study

One of the limitations of this study is the duration of plant growth. The growth duration in this study is 72 days because ryegrass and oat attained constant height at 72days. The performance of the plants in this study is specific to maturity level attained by individual plants in 72days. The

outcomes may be different if plants were grown for a longer period of time or if plants maturity differs from those observed after 72 days. For example, maturity date of Indian mustard is 120-150 days, Hence growth and phytoremediation performance may have been different if Indian mustard was allowed to grow to maturity.

The sorption and desorption behavior of contaminants will vary depending of the physical and chemical characteristics of soil. Sorption and desorption characteristics of soils and contaminants can be affected by age related changes in soil. Freshly spiked soil aged for 1 month was used in the current study, the resulting removal efficiencies observed may be different if applied to metal-PAH-pesticide contaminated soils collected from sites that have been contaminated for a number of years. Furthermore site specific characteristics of contaminated soils such as photoperiod, contaminant concentrations, soil water holding capacity, day-night temperature, soil texture and relative humidity.

Finally, in this study Cu, Pb, pyrene and DDT were used as model contaminants for metals, PAH and pesticide. These model compounds and concentrations used in the current study cannot be used to generalize the behavior of all other metals, PAHs and pesticide in mixed contaminated soils. These compounds were chosen because of their environmental significant, frequency of occurrence and use in remediation study.

CHAPTER 4

4.0 Conclusion and Recommendation

4.1 Significance of Study

Relative tolerance (in terms of germination and survival) of plants examined in this study to the mixture of contaminants in soil highlights their potential as phytoremediation candidates for mixed contaminated soils. However, co-contamination did significantly affect plant growth in terms of reducing plant biomass and growth rate.

Sunflower stands out in this study because of its ability to improve the degradation of pyrene and 2, 4' DDT in soil alongside metal accumulation. This demonstrates the potential of sunflower for simultaneous remediation of metals, PAH and organochlorine pesticides in mixed contaminated soil.

The ability of ryegrass to redistribute exchangeable Cu to the relatively immobile organic fraction, reduce exchangeable Pb and improve phyto-degradation of pyrene (even though it was unable to achieve degradation of DDT), does qualify it as an excellent candidate for phyto-stabilization of metals in metal-pyrene-DDT contaminated soil. Soil amendments are recommended to improve growth and biomass production.

Oat accumulated more metals than ryegrass, but the accompanying undesirable outcome of increased Cu mobility (increase in exchangeable Cu) and failure to improve the degradation of pyrene and DDT makes oat unsuitable for phytoremediation of soils co-contaminated with metals, pyrene and DDT.

Alfalfa can be considered an excellent candidate for phyto-degradation subsequent to metal removal in combined remediation systems given its low potential for metal accumulation in the given mixed contaminated soil and its ability to enhance the removal of pyrene and 2, 4'-DDT.

Indian mustard has a similar potential as sunflower except that it slowed down removal of pyrene in planted soil and thus may require augmentation with pyrene degrading microorganisms or require further remediation technology for pyrene removal after phytoremediation with Indian mustard.

4.2 Recommendations

The current work focused solely on identifying plants with the potential to remediate metal-PAH-pesticide contaminated soils. Sunflower and Indian mustard were identified as the most tolerant of all the plants studied. In addition, sunflower was able to facilitate simultaneous removal of Pb and Cu, pyrene and DDT used as model compounds for metal, PAH and pesticide. A point of concern is the undesirable increase in exchangeable Cu observed in soils planted with oat. However, the characterization of plant enzymes and transport proteins involved in contaminant uptake, transport, degradation and metal speciation can provide a clearer understanding of the various adverse and favorable outcomes in phytoremediation. The examination of the physiology of these plants under the stress of mixed contamination is required to further elucidate the reasons for differential plant growth, given that metals, PAH and pesticides are common contaminant groups found in mixed contaminated soils as well as the interaction between these classes of contaminants.

Contaminant interactions in spiked soils is not always representative of field conditions, thus field application or green house studies with soil collected from contaminated sites is recommended to

further verify potential application of phytoremediation in mixed contaminated soils both in a short and long term.

In general, exchangeable/soluble metal fraction in soil is referred to as the most bioavailable forms of metal in soil, but some studies have identified plants like *Andropogon scoparius* (little blue stem) as being able to take up other forms of metals (in this case Fe-Mn oxides-bound fraction) from soil (Reddy et al., 2017). Therefore, analyzing metal speciation in plant tissue in addition to metal speciation in soils can provide a better understanding of how plants affect metal speciation in soil.

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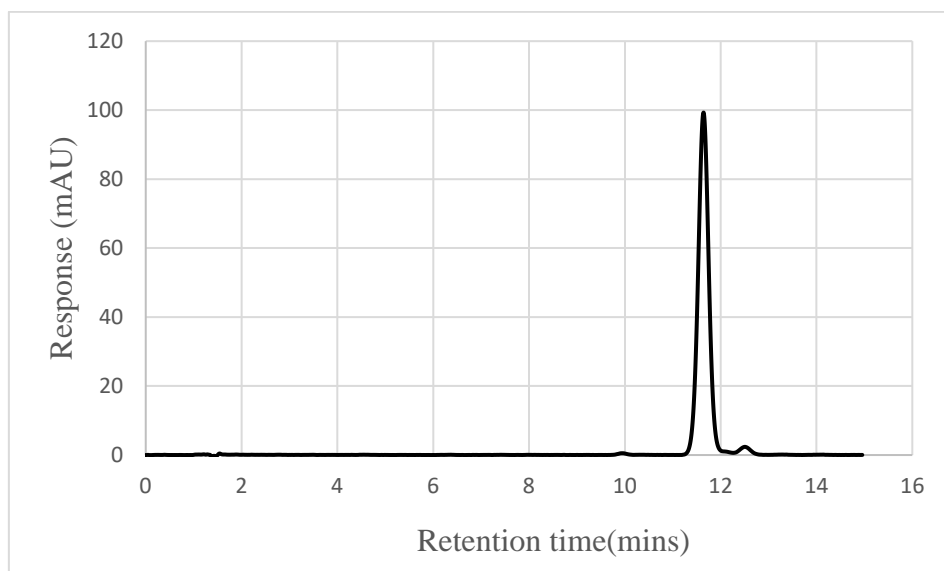
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APPENDICES

Appendix A: HPLC details for pyrene

Table A-1: HPLC setup for pyrene

Ref	Titato and Lancas 2006
Mobile phase	Acetonitrile: water
Elution programme	Isocratic elution 70% acetonitrile: 30% water
Flow rate	0.8ml/min
Column temperature	~30°C.
Detection wavelength	254nm
Retention time	11.62 ± 0.01 (n=5,±SD)

**Figure A-1: Chromatogram for pyrene 100mg/l**

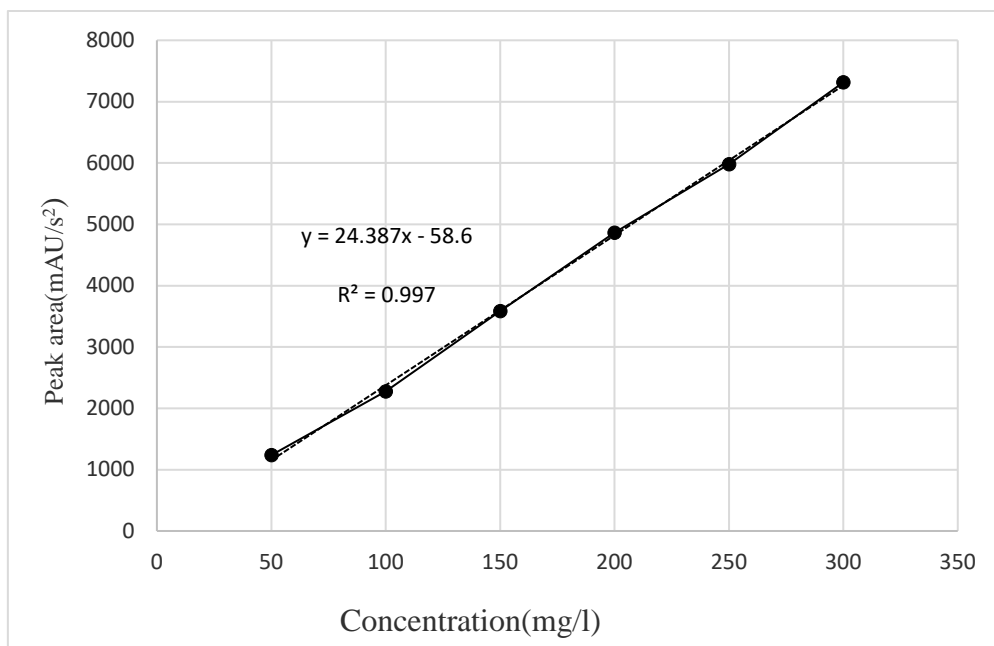


Figure A-2: Calibration curve for pyrene

Appendix B: HPLC details for DDT

Table B1: HPLC setup for DDT

Ref	(Wang, 2008)
Mobile phase	Acetonitrile: water
Elution programme	<p>Gradient elution</p> <p>At t=0 mins, 70% acetonitrile: 30% water</p> <p>At t=11 mins, 85% acetonitrile: 15% water</p> <p>At t=21 mins, 100% acetonitrile</p> <p>At t=31mins, 70% acetonitrile: 30% water</p> <p>AT t=41mins, 70% acetonitrile: 30% water</p>
Flow rate	1ml/min
Column temperature	~30°C.
Detection wavelength	235nm
Retention time	<p>10.83 ± 0.009 mins for 4,4'-DDT</p> <p>11.81 ± 0.01 mins for 2,4' DDT</p> <p>(n=4, ±SD)</p>

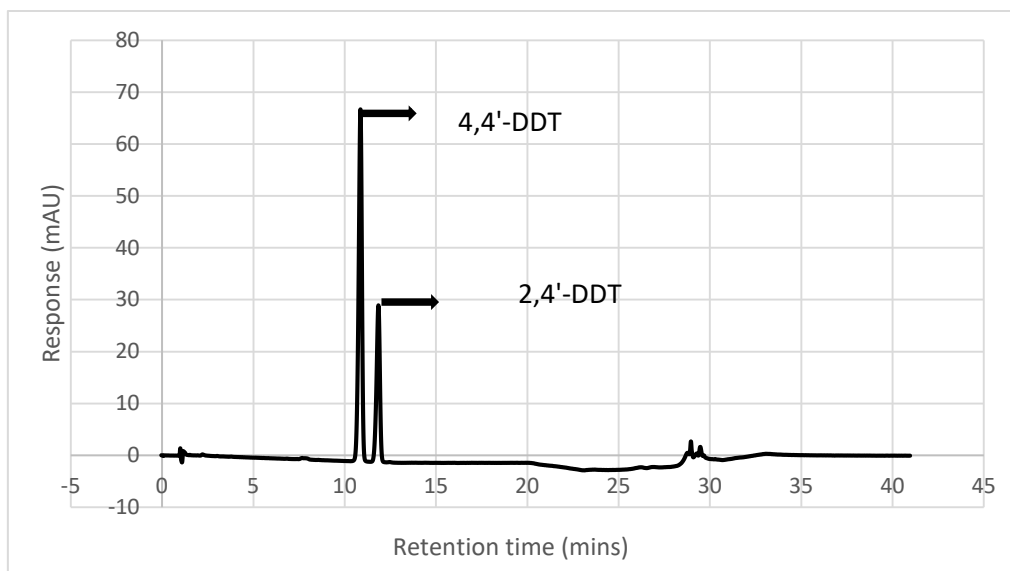


Figure B-1: Chromatogram for DDT 10mg/l

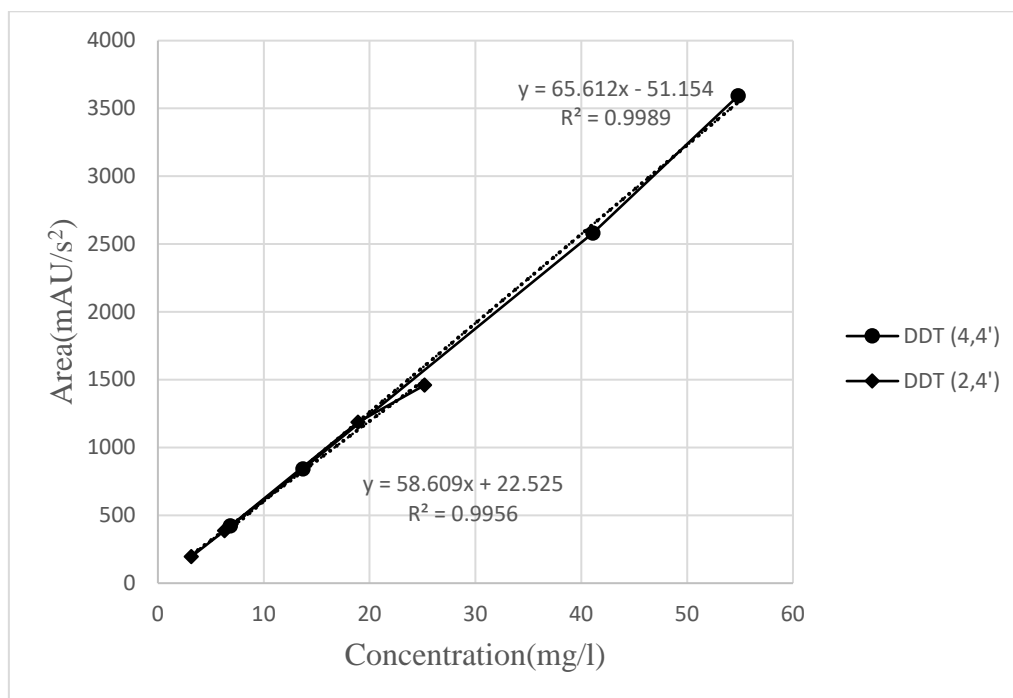


Figure B-2: Calibration curve for DDT

Appendix C: Metal concentrations of plant tissues in clean soil

Table C-1: Metal concentration (mean \pm SE, n=3) of plants grown in clean soil

Plant	Shoot Concentration (mg/kg)		Root Concentration (mg/kg)	
	Cu	Pb	Cu	Pb
Oat	5.89 \pm 0.36	0.17 \pm 0.17	43.77 \pm 2.90	22.49 \pm 1.31
Alfalfa	8.91 \pm 1.05	0.0 \pm 0.0	28.77 \pm 6.03	14.18 \pm 2.19
Ryegrass	15.90 \pm 4.0	1.72 \pm 0.35	31.15 \pm 7.73	17.01 \pm 1.41
Indian mustard	7.29 \pm 1.77	1.16 \pm 0.83	27.66 \pm 1.31	16.52 \pm 1.63
Sunflower	11.86 \pm 3.51	2.01 \pm 0.76	30.74 \pm 0.78	13.05 \pm 0.02

Appendix D: Analysis of variance for metal fractioning in soil

Table D-1: Two way analysis of variance for Cu fractions in soil. DF=Degree of freedom, SS= Sum of squares, MS=Mean square.

Source of variation	DF	SS	MS	F	P
Plant	5	0.522	0.096	12.648	<0.001
Metal fractions	4	50.94	15.947	1796.584	<0.001
Plant x Metal fraction	20	1.099	0.0297	12.924	<0.001
Residual	60	0.347	0.00891		
Total	89	68.497			

Table D-1: Two-way analysis of variance for Pb fractions in soil. DF=Degree of freedom, SS= Sum of squares, MS=Mean square.

Source of variation	DF	SS	MS	F	P
Plant	5	0.322	0.0644	8.647	<0.001
Soil metal fractions	4	46.718	11.680	1569.329	<0.001
Plant x Soil metal fractions	20	1.299	0.0649	8.724	<0.001
Residual	60	0.447	0.00744		
Total	89	48.785	0.548		

Appendix E: List of parametric and non-parametric statistical tests used for data analysis

Table E-1: List of statistical analysis of all data sets

Data	Text reference	Variance analysis	Pair wise comparisim
pH _{cacl2}	Table 3-5	Two way repeated ANOVA	Holm-Sidak
Preliminary toxicity test: Percent germination Root length Shoot length	Figure 3-1 Figure 3-2 Figure 3-3	Two way repeated ANOVA	Holm-Sidak
Metal concentration in plants	Figure 3-4	Two way ANOVA	Turkey (root) Holm-Sidak (shoot)
Total metal accumulation in plants	Figure 3-5	Two way ANOVA	Holm-Sidak
Pore water metal concentration	Table 3-7	Two way ANOVA	Holm-Sidak
Metal concentration in soil	Figure 3-6	Two way ANOVA	Holm-Sidak
Cu speciation in soil	Figure 3-7	Two way ANOVA	Turkey
Pb speciation in soil	Figure 3-8	Two way ANOVA	Turkey
Pyrene concentration in soil	Figure 3-9	Two way ANOVA	Holm-Sidak
DDT concentration in soil	Figure 3-10	Two way ANOVA	Holm-Sidak
Percent germination of plants in clean and contaminated soil	Figure 3-11	Mann-Whitney rank sum test and Kruskal-Wallis one way ANOVA on ranks	NA
Percent survival of plants in contaminated soil	Figure 3-12	Two way ANOVA	Holm-Sidak
Plant growth rate in clean and contaminated soils	Figure 3-13	Two way repeated ANOVA	Turkey
Plant biomass in clean and contaminated soil	Figure 3-14	Two way ANOVA	Holm-Sidak

Appendix F: Photographs of effects of contamination on plant growth



Figure F- 18: Yellowing and drying up of leaves observed in sunflower and Indian mustard plants grown in contaminated soils

Curriculum Vitae

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