UNIVERSITY OF BIRMINGHAM

PHYTOREMEDIATION POTENTIAL FOR CO-CONTAMINATED SOILS

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M.Sc, B.Sc

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY (Ph.D)

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August 2013

Abstract

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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Phytoremediation is a plant-based remediation process for treating contaminated soils. The overall aim of this thesis was to determine whether phytoremediation could be applied to co-contaminated soils. Copper (Cu) and pyrene, and Chromium (Cr) and Benzo[a]pyrene (B[*a*]P) were used as contaminants.

The first study involved the joint effect of Cu and pyrene or Cr and B[*a*]P on the early seedling growth of *Lolium perenne*. Results suggest that co-contamination showed several types of interactions for seedling growth with different combinations of the pollutants. The second study involved the role *Brassica juncea* and *Zea mays* during the remediation of Cu and/or pyrene, and Cr and/or B[*a*]P co-contaminated soils respectively. *Brassica juncea* and *Z. mays* showed contrasting results for metal and polycyclic aromatic hydrocarbon (PAH) remediation. The third study compared freshly spiked soils and aged soils. Ageing affected the plant biomass, metal phytoextraction and PAH dissipation in different ways when compared to fresh soils. Finally, the efficiency of ethylenediaminetetraacetic acid-EDTA and/or citric acid as chelators in co-contaminated soils was studied. The combined application of EDTA and citric acid was more effective in co-contaminated soils.

The overall findings from the four studies suggest that phytoremediation could be applied to co-contaminated soils.

Acknowledgements

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me. It is only possible to mention some of them here.

I would like to thank my lead supervisor, Dr. Lesley Batty for the patient guidance, encouragement and advice she has provided throughout my time as a student. I have been extremely lucky to have a supervisor who showed so much interest in my work and responded to my queries so promptly. I would also like to thank my second supervisor, Dr. Rebecca Bartlett and also Prof. Ian Fairchild who helped me in my supervisors absence.

I must express my gratitude to Chinie for her continuous support, encouragement and willingness to proof read countless of un-interesting studies. To my friend Emmanuel Nduka for squeezing out time to accompany me for site visits; to Sharifa and Stine who always found my work interesting even though they knew nothing about them and to Larai, Ada and Karima for just being in that little office.

Completing this work would have been all the more difficult were it not for the support provided by Richard Johnson, Christopher Stark, Eimear Orgill and Dr. Gilian Kingstonfor their invaluable assistance in everything technical.

Finally, to my dad, Sir Johnson, his wife Cecelia and my wonderful siblings for making life a lot easier.

Chigbo Chibuike

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Introduction

CHAPTER 1

Introduction

1.1 Background

Contaminated land could be described as surface environments which have been affected by both natural and anthropogenic sources (US EPA 2008). It is a global problem that has both environmental and health implications. Internationally, contaminated land has occurred more extensively since the 1800s but its risk management is a 1970s phenomenon (Rivett *et al*. 2002). As the population increases globally, the need for land is likely to rise for purposes such as housing, production of food and energy and other activities. An estimated one-third of a million sites have been identified as contaminated in England and Wales (Ashworth *et al.* 2005). Due to the excessive need for land, many countries are encouraging the use of brownfield land rather than exploiting undisturbed land. These brownfield lands could have been previously contaminated with substances that are hazardous or could be potentially harmful. In United States of America for example, land development for both residential and commercial use in 2003 increased by 48 percent from its level in 1982 while population still increased (US EPA 2008). Therefore the need to re-develop or re-use brownfield lands would mean the application of remediation technologies for its specific use. The need for sustainable development and land recycling has been increased by a number of governments since the Bruntland report was published in 1987. As an example, the UK government agreed that by 2008, previously developed lands should be used for building three out of five new homes (Batty and Anslow 2008) and about three out of four new homes by 2010. Also in developing countries, for example Nigeria where there are large cases of land contamination by its oilfield activities, there is a

need for improved treatment of the affected land (Chigbo 2009). Therefore, although the technological advances for land remediation have been improved, most of them are environmentally not sustainable as they mostly involve certain processes like 'dig and dump', which is the removal of soil to landfill (Batty and Anslow 2008). This method although effective just moves the contaminated soil (problem) elsewhere and due to the implementation of the European Union landfill directive (99/31/EC) that aims at reducing the negative impact of landfilling of waste to the environment, has become an unviable process (Jones and Hills 2002). Alternative methods, which include the application of other substances that could be potentially harmful to the environment or even thermal treatment, which destroys soil structure, are all unsustainable.

Sustainable approaches to land remediation, which include the use of plants and microbes to transform or uptake toxic substances, have been proposed. These techniques have not been widely applied due to time restraints (Cost and risk), the identification of species that are appropriate for remediation and the cost in the case of use of microbes (Chen *et al.* 2004). As much as these remediation technologies (both sustainable and unsustainable) have shown promise for individual pollutants (Gao and Zhu 2004), it has not been the case for sites that are contaminated by more than one single pollutant. This has been the challenge for soil remediation as many contaminated sites do not contain one single pollutant but instead a number of different substances (Lin *et al.* 2008, Zhang *et al.* 2011). Therefore combinations of traditional techniques are used to remediate these soils, which of late have included methods that use microbes. This combination of techniques uses energy in many cases and with the emphasis on sustainability, low energy and environmentally friendly technologies are required and phytoremediation could solve this problem.

1.1.1 Phytoremediation Potential for contaminated land

Phytoremediation is the use of green plants and their associated microbes to remove environmental pollutants or to render them harmless (Kambhampati and Vu 2013). It is a plant-based technology that enhances environmental clean-up (Pilon-Smiths 2005, Cook and Hesterberg 2013). It has the advantage of being less expensive than most traditional methods (the fact that it is carried out in-situ), environmentally sustainable since solar energy drives the process and aesthetically pleasing (Singer *et al*. 2007). However phytoremediation has limitations: for example, it is limited to sites where contamination is low and confined to rooting depth and the remediation process could affect the food chain when chemicals are degraded or taken up by plants (Pilon-Smiths 2005). Phytoremediation makes use of natural processes where the plants in combination with their microbial rhizosphere degrade and take up pollutants (organic and inorganic). It has shown to be effective in clean up of both organic and inorganic pollutants (Pilon-Smiths 2005). Most organic pollutants that are released into the environment are anthropogenic and are xenobiotic to organisms showing evidence of toxicity and carcinogenicity (Meagher 2000). The uptake or degradation of organics by plants is dependent on the pollutant properties such as their recalcitrant nature and solubility in water (Smith *et al*. 2006). Some organics such as Trichloroethylene (TCE) (Newman *et al.* 1997, Lewis *et al*. 2013), the herbicides atrazine (Burken and Schnoor 1997, Murphy and Coats 2011), petroleum hydrocarbons (Schnoor *et al.* 1995, Cook and Hesterberg 2013), polychlorinated biphenyls (Harms *et al*. 2003, Li *et al*. 2013) have been successfully removed using plants.

In comparison to organics, inorganics are non-degradable and can only be stabilized or taken up by plants during the phytoremediation process. The science and technology of phytoremediation of inorganics has progressed significantly over recent years because of

the ease of inorganic detection in various plants (Salt *et al*. 1998, French *et al*. 2006, Zhu *et al.* 2012). Plant micronutrients, trace elements, non-essential elements and radioactive elements can be remediated by this method in solid, liquid and gaseous forms (Pilon-Smiths 2005). This process relies on the ability of some plants to tolerate and accumulate high concentration of inorganics or in some cases, to be able to transform or stabilize the inorganics. In other words, the plants would be able to either tolerate or avoid uptake of the inorganic compounds.

With land contamination, where both organic and inorganic substances are present, there has been limited work carried out to understand the potential use of phytoremediation for clean up. When studying these sites, it is very important to consider the interactions of both organic and inorganic substances with respect to those that can affect the form and availability of pollutants. Certain concentration of metals has been shown to inhibit the biodegradation of organics by microorganisms (Sandrin and Maier 2003, Thavamani *et al.* 2013) and also interact with organic pollutants to affect metal bioavailability (Gao *et al*. 2006). Plant growth could also be affected by a combination of pollutants. For example, Lin *et al*. (2006) concluded in their research that combinations of PCP and Cu sometimes exerted a toxic antagonistic effect on the growth of *Raphanus sativus* and *Lolium perenne.* Also, Zhang *et al.* (2009) showed that the toxicity of Cd to *Z. mays* was not alleviated by pyrene. However, research in this area of combined effects has been limited and the methods of interaction are still unclear.

In as much as there are these negative effects, some literatures have reported positive effects of co-contaminants (organic and inorganic). Chen *et al.* (2004) showed that the reduction in the uptake of zinc in the shoot of rye grass was caused by the presence of 2,4-dichlorophenol while Lin *et al.* (2008) concluded that at 50, 100 and 500mg kg^{-1}, pyrene

showed the tendency to alleviate Cu inhibition to growth of *Zea mays* L. Therefore the question is, if organics reduce the uptake of metals in accumulators, could the potential of accumulators as phytoremediating plants be compromised?

It is widely acknowledged that differences in plant species richness could cause resource partitioning and therefore affect ecosystem processes (Hooper 1998). Total resource capture could be increased by plants in more diverse communities (Macdonald *et al*. 2012). There was an observed increased productivity associated with increasing species richness, and mixed cultures of ryegrass, white clover and celery significantly helped in the dissipation of PAH more than mono cultures (Meng *et al*. 2010). Jiang *et al.* (2010) also showed increased growth stimulation and increased metal uptake (Cd and Zn) for the individual plants during co-planting in metal contaminated soils. The rates of respiration for microorganisms were also found to be dependent upon the composition and functional group of plant communities (Johnson *et al.* 2008). Therefore, there could be an argument that if diverse plant communities are provided which would in turn increase microbial diversity, there could be an enhanced degradation of individual pollutants and multiple contaminants in a co- contaminated sites. Presently there is limited research carried out to investigate this. For example, *Echinochloa crusgalli, Helianthus annuus Abutilana vicennae* and *Aeschynomene indica* removed TNT irrespective of it being mixed or monoculture as shown in the experimental phytoremediation of 2,4,6-trinitrotoluene, Cd and Pb co-contaminated soil carried out by Lee *et al.* (2007) for 180 days. They also reported that mixed species of *Echinochloa crusgalli* extracted more of Cd and Pb although all other mixed species extracted Cd and Pb in low to negligible amounts probably due to competition between species. Therefore, there is a need for research on a thorough understanding of the complex interactions between plant species.

In order to enhance the availability of metals and PAHs, as well as the translocation of metals from root to shoot, enhancing chemicals have proven to be efficient. For example, different kinds of chelates like EDTA, ethylenediamine disuccinic acid (EDDS), nitrilotriacetic acid (NTA) have all been effective in enhancing metal uptake by plants (Huang *et al*. 1997, Chen *et al*. 2003). Similarly, surfactants like Tween 80 improved the dissipation rate of fluoranthene and phenanthrene in PAH contaminated sewage sludge (Zheng *et al*. 2007). With soils co-contaminated with metal and PAH, there has been limited research to understand the role of enhancing chemicals during phytoremediation. Therefore the question is, if enhancing chemicals are applied to co-contaminated soils, will contaminants availability be enhanced? And will there be a simultaneous uptake of metals and PAH dissipation?

As pollutants occur in aged soils in many brownfield sites (Olson *et al.* 2003), they may be highly soluble and unlikely to be immobilised. There could be questions as to why remediation would be necessary giving the non-bioavailability of the contaminants, but the interaction between pollutants which could be of particular concern in mixed contaminated sites could affect their bioavailability and subsequent uptake or removal by plants (Chen *et al.* 2004). This is a concern as many of the contaminants such as PAH and metals remain extremely persistant and toxic (Hamdi *et al*. 2012, Zhong *et al*. 2012)). Research in this area still remains unclear.

1.2 Statement of problem

Most of the methods used for contaminated soil remediation in emerging countries are adaptations of technologies originally developed in industrialized countries (Adam and Guzman-Osorio 2008). These methods were designed for areas with different kinds of

economies, culture and mostly different physical and biological environments. Implementation of these methods is extremely costly as it requires materials, machinery and skills importation. However, in many developing regions especially in the humid tropical and subtropical environment, physical, chemical and biological processes occur which can be applied to remediate contaminated soils. Both in developed and developing countries, large amount of lands are contaminated mostly by more than one single pollutant. These multiple pollutants could be of the same form e.g., land contamination with different kinds of metal or with different kinds of PAHs etc or could be of different forms e.g. land contamination with organic pollutants (e.g. PAH) mixed with inorganic pollutants (e.g. metals). Research in this area is very limited and there have been gaps in knowledge on the use of plants to remediate the latter land types. As of present, most studies on phytoremediation of organic and inorganic contaminants in soil are mostly treated as two distinct topics both in the manner in which the contaminants behave and the remediation prospects As phytoremediation has been mostly used on single contaminants or multiple contaminants of same type, this research work will try to address the problems posed by the mixture of organic and inorganic contaminants during phytoremediation of co-contaminated industrial soils.

1.3 Aims and objectives.

The overall aim of this research is to determine whether phytoremediation could be applied to co-contaminated sites.

Within this overall aim there are several objectives:

- To determine whether single and mixed contaminants affect seed germination rate and early seedling growth of selected plant species.

- To establish which plant species used in phytoremediation are tolerant of mixed contaminants
- To determine whether uptake of metals by accumulating species is reduced in the presence of organic pollutants.
- To determine whether soil amendments can facilitate phytoremediation in co-contaminant soils
- To determine the effect of soil ageing on phytoremediation of co-contaminated soils

An understanding of the complex interactions between organic and inorganic pollutants within a contaminated land will be gained from this research work.

1.4 Structure

The chapter 2 of this thesis consists of an introductory literature review that outlines the mainstream concept of contaminated soils and methodologies for remediation. It also briefly described plant mechanisms responsible for the phytoextraction of metals and dissipation of PAHs in contaminated soils. Chapter 3 contains the description of soil localities, classifications and characterization for sites and soils used for this study. The description of sample preparation, greenhouse studies and analytical methods common to studies described in subsequent chapters are also included.

The four main objectives of this thesis are individually addressed in chapters 4, 5, 6 and 7. Two model contaminant mixture (Cu+ pyrene and Cr + B[*a*]P) are used for each of the study and as such, each of the chapters are made up of two separate individual study. Chapter 4 evaluated the effect of Cu and pyrene, and Cr and B[*a*]P on the germination and early seedling growth of *L. perenne* using growth media; Chapter 5 investigated the role of *B. juncea* or *Z. mays* as model plants for the remediation of soils co-contaminated with Cu

and pyrene, and Cr and B[*a*]P respectively; Chapter 6 compared freshly spiked soils and aged soils of Cu and pyrene, and Cr and B[*a*]P contaminated soil using *B. juncea* and *Z. mays* respectively; and Chapter 7 assessed the role of EDTA and citric acid as chelates in soils contaminated with Cu and pyrene, and Cr and B[*a*]P using *M. sativa* and *Z. mays* as model plants respectively. The findings from chapters 4 to 7 were individually discussed at the end of each study while chapter 8 concluded the thesis with a general discussion and approaches for further study.

2

Literature review

CHAPTER 2

Literature review

2.1 Soils - Characteristics

Soils are formed by the physical, chemical and biological weathering of rocks to small particles (Chapin III *et al*. 2002). They are composed of three major phases: solid, liquid and gas. The phases are arranged in different ways to produce different soil types (Lozet and Mathieu 1991). The combination of the mineral component and the organic matter forms the solid phase. The exact rock from which a soil is derived is reflected by the chemical components of a particular soil. Silica is the dominant component of the soil and it is present in silt, sand and clays (Derry *et al*. 2005), although some soils consists of very high organic matter with no inorganic mineral matter (Feurstenau 2003). The organic matter in soil is made up of plants, animals, humus microorganisms and their metabolites. The spaces between the solid articles are filled with water, which is made up of weak solution of salts and forms the solvent system by which plants and microorganisms assimilate nutrients (Feurstenau 2003). The soil biological community ismade up of the microbiota (includes the algae, bacteria, fungi, protozoa etc), the mesobiota (includes the nematodes, small oligochaete worms, smaller insects and larvae etc) and the macrobiota (includes the root of plants, the larger insect, earthworms, other larger organisms etc).

2.2 Contaminated land

2.2.1 Overview

In the UK, land is classified as contaminated only when there is a risk of significant harm to humans, ecosystem, controlled waters, buildings, etc based on the existence of a pathway by which pollutants may impact on these receptors (Giusti 2013). Contaminated land is a global problem that constitutes significant threat to human and environmental health both in the present and future. As a result of increasing agricultural, civil and industrial practices, soils are severely degraded (Vamerali *et al*. 2010). Contaminated land has been an important topic in many areas of research, practice and policy within different countries, which has also been extended internationally (CLARINET 2002). The attached importance to contaminated land has been increasing over the years. Contaminated land is a term used to describe sites, including wider land areas with increased concentration of chemicals or other substances as a result of both anthropogenic and natural causes: the former being the major cause (CLARINET 2002). In many countries, government are actively encouraging non-exploitation of agricultural land, rather the use of sites that have been previously contaminated with potentially harmful substances (Urban Task Force 2005).However, before this land can be reused, it has to undergo some remedial work to make it fit for the purpose.In the UK, due to its long history of industrialization, many areas of land have become contaminated in different ways over a long period of time. For example arsenic can be found in high levels and in less bioaccessible forms in some areas of North Devon due to their long history of mining and smelting of arsenic and metalliferous ores (Palumbo-Roe and Klinck 2007). Contamination could be from point sources or non-point/diffuse sources. The point sources are from localized zones of high concentration of contaminant or a major release from a location that is defined whereas in the non point sources, the contaminants

are spread over a wide area, e.g. fallout from the atmosphere during smelting (Nathaniel and Bardos 2004). Soil contamination mostly affects the buffering, transforming and filtering abilities of the soil (EEA 2003).

2.2.2 Impacts of contaminated land

Certain environmental impacts are associated with land contamination. These include,

1. Health impact on humans
2. Impact on the ecosystem
3. Impact on groundwater and surface water quality
4. Impact on archaeology and building conditions

These impacts lead to economic and social impacts, not only the cost of remediation, but the wider effects it has on the value of land and the well being of local inhabitants. In the past, some of these impacts received more attention than the others. Therefore, tools and/or technologies required for the assessment of these impacts and the choice of solution are not always perfect or mostly not available to address the emerging problems (CLARINET 2002).

2.2.3 Policy approaches

Approaches to policy on contaminated land are often viewed in two perspectives.

1. Protection perspective- This relates to the effects/impacts of land contamination on human health and also impacts on quality of the environment.

2. Planning perspective- This relates to the management of the impacts of contaminated land by the way land is used, .e.g. regeneration of old industrial areas(Ferguson 1999).

The major aim of policy development is to address the above-mentioned perspectives simultaneously, but there is no consistency in the way that these perspectives are used by different countries to influence their legal requirements (Ferguson 1999). Whereas some countries use environmental legislation as the main means of avoiding contaminated land impacts, others use planning legislation. However, a more holistic approach to urban development management is linked to economic issues (Vegter *et al.* 2003). This includes changes to land valuation and the use of market to drive environmental improvements. However, this holistic approach should ensure sustainable development. For example, the need to consider the timing of any intervention as well as the future environmental, economic, social and cultural consequences of any particular solution (Vegter *et al.* 2003).

2.2.4 Brownfields

There is no fixed definition for brownfield. In Europe, it is used in different contexts and the meaning varies whereas in some countries, brownfield (complexity and context) is not recognised. Brownfield sites could be defined as sites that have been affected by its former use or the use of surrounding land, are neglected, have real or apparent contamination issues, and require intervention to restore it to beneficial use (CLARINET 2002). The most widely used definition of brownfields is provided by US EPA as 'abandoned, idled, or under-used industrial and commercial facilities where expansion or redevelopment is complicated by real or perceived environmental contamination' (De Sousa 2003). They are known as localized contaminated soils (French *et al.* 2006), since previous use of land

mainly for industrial purposes may have caused contamination problems. As brownfield sites are used in reference to sites of known or perceived/suspected contamination probably because of former use, the problem could be extensive especially in terms of remediation and redevelopment. Previous research has shown that greening of brownfields improves the social well-being of residents in associated areas in different ways (De Sousa 2003). Therefore in as much as the major focus environmentally is to remediate these sites, using plants (phytoremediation) to achieve this could also improve residents well being by reducing stress for example (Kaplan 1993).

2.3 Co-contaminated sites

Co-contaminated sites could be described as sites that are simultaneously contaminated with pollutants of different nature (Almeida *et al.* 2009). For example, sites that are contaminated with trace metals are frequently contaminated with other chemicals of different nature such as petroleum hydrocarbon, pesticides, surfactants, etc. It is difficult to quantify the extent to which land is contaminated. Over two million sites have been termed as contaminated in Europe (European Environment Agency 2005) and about a sixth of that in the UK (Ashworth *et al*. 2005). About 30,000 to 40,000 sites covering an area of about 55,000-80,000 ha have been identified as being affected by contamination in England and Wales according to the recent research carried out by the Environment Agency, Department of Environment Food and Rural Affairs (DEFRA) and the Welsh assembly, excluding Scotland and northern Ireland as similar exercise are yet to be carried out in these areas (Land contamination,United Kingdom: statistics and related) and from calculations, about 750 sites covering an area of 30 ha is thought to be newly contaminated from 2001 to present day (Ashworth *et al.* 2005). As with the case of brownfield sites, records of use and potential contamination may be very limited most especially in cases where there is a long

history and therefore it could be difficult to ascertain the exact mix of pollutants associated with the contaminated land if thorough testing is not accomplished. This is in part due to the multiple use of land that was not recorded. Co-contaminated sites are in abundance and those contaminated with organic and inorganic compounds are most difficult to remediate. According to the review by Sandrin and Maier (2003), about 40% of the waste sites in US are co-contaminated with organic and inorganic compounds. This forms part of the 37% of contaminated sites in US reported to contain both organic and inorganic pollutants (Springael *et al*.1993). Depending on how land has been and/or is presently used, contamination would be of different combinations. For example, petrol stations could contain PAHs, benzene, toluene, ethyl benzene, xylene and solvents (Herwijnen and Hutchings 2005). In land that served as a timber industry in its former use, about 2/3 of the sites contain a mixture of organics and inorganics due to timber treatments used (Ensley 2000). In cases where there is an existence of multiple contaminants in soil, the type of compound varies extensively as well as their concentrations and the background soil type.

2.3.1 Assessment of contaminated sites

Most brownfield sites in the UK do not contain high amount of contaminants and may not even be classified as contaminated land according to statutory definition (French *et al.* 2006). This makes remediation a low priority. The decision on the need for remediation is based upon the source-pathway-receptor model which assesses the likely exposure of adults and children in contact with the contaminated site. DEFRA and Environment Agency have developed a model known as Contaminated Land Exposure Model (CLEA) and it is in use in UK for this assessment. It predicts the likely exposure of a child or adult in use of a land based on soil contaminant toxicity and exposure estimates and generates maximum safe limits for humans (DEFRA 2006). This approach focuses solely on human health and risk

to the environment is not covered. The direct measurement of bioavailable fractions are not included as well as potential interactions between contaminants which can affect their form, thereby altering the exposure and associated risks. For example work carried out by Chen *et al.* (2004) showed remarkable changes in the bioavailability of metals in the presence of organics (2,4- dichlorophenol). When pollutants are superficial and less bioavailable, it makes for the unsuitability of many of the existing remediation technologies (Chen 2009), likely due to high cost and the difficulty in removing the low levels of contaminants that may not be bioavailable (Chen 2009). Also in brownfield sites, most contaminants occur in aged soil and could be more recalcitrant and more difficult to remediate than in newly contaminated soils because contaminants are highly insoluble and are unlikely to be mobilized (Olsen *et al*. 2003). Therefore it could be asked that if contaminants are not bioavailable, what is the rationale for its remediation? The reason is that the evaluation of risk was based on total values and not bioavailability or the interaction between contaminants. Secondly, the environmental conditions change with time, which could impact upon the form in which contaminants occur, therefore affecting the way they behave within the environment. Therefore it is important that remediation of these low-level contaminated soils be dealt with appropriately.

2.3.2 Remediation of contaminated land

There are various ways a contaminated land can be remediated. According to Stegmann *et al.* (2001), they include, mechanical, thermal or biological processes such as-

1. Restricting the use of the contaminated land and leaving the contaminants as they are.
2. Encapsulation of the contaminated land (complete or partial).

3. Landfilling: carried out after excavation of the contaminated soil.
4. In-situ or ex-situ treatment of contaminated soil.

The processes listed above could be classified into two categories: isolation/containment and decontamination (Lambert *et al*.1997). The first three methods refer to the former as they do not remove contaminants from the soil but rather restricts the use of the contaminated soil. Based on the four processes listed above, different remediation methods have been developed in the last three decades due to the risk of contaminants to groundwater and air (Stegmann *et al*. 2001). These methods are discussed below which are otherwise known as traditional remediation technique-

The physical method of remediation uses impermeable physical barriers to isolate and contain the contaminants, preventing/reducing their movement/permeability to less than 0.0000001 m s^{-1} as required by US EPA (Mulligan *et al*. 2001). Soil washing is a well practised ex-situ physical technique in the U.S and Europe. It removes organic, inorganic or radioactive pollutants accumulated in the fine fraction of the soil matter by dissolving/suspending them in wash solution. Following the Landfill Regulation (2002) and the increased cost of disposing contaminated land this method may become more desirable in the UK.

Chemical extraction is a technique that uses chemicals to extract contaminants from the soil. Solvent extraction uses organic solvents while acid extraction uses different types of acids for extracting different contaminants. For example, using a leaching solvent to remediate petroleum contaminants via partitioning (Friend 1996, Schifano and Thurston 2007) and using citric acid, ethylenediaminedisuccinic acid (EDDS) and

methylglycinediacetic acid to efficiently extract Cu, Pb and Zn from soil (Arwidsson *et al.* 2010, Wuana *et al.* 2010).

Reductive/oxidative remediation detoxifies metal contaminants (Evanko and Dzombak 1997) using hypochlorite, H_2O_2 and chlorine gas in the oxidation process or Na_2SO_3 salts, sulphur dioxide and ferrous sulphate in the reduction process. When carried out in-situ, the chemical agents for both the oxidation/reduction process should be selected carefully to prevent further soil contamination (Mulligan *et al.* 2001).

The thermal decontamination technique involves heating the contaminated soil between 150 ^{o}C and 500 ^{o}C to induce the transfer of the pollutants to a gas stream physically separating these pollutants from the soil (thermal desorption) or uses higher temperatures between 600 ^{o}C and 900 ^{o}C to induce the chemical modification of the contaminants (thermal destruction) (Merino and Bucala 2005). According to Risoul *et al.* (2002), Larsen *et al.* (1994) and Gilot *et al.* (1997), the properties of the contaminants, soil characteristics and the operating conditions are key parameters for thermal decontamination of organic and inorganic pollutants.

2.4 Bioremediation

Bioremediation is a process that allows the remediation of harmful/toxic chemicals by natural processes (US EPA 2001). It exploits the metabolic diversity and adaptation of microbes for degrading and transforming various organic and inorganic contaminants (Cunningham and Philip 2000). For practical application of bioremediation to be considered, there should be a demonstration that the removal of contaminants is the primary effect of biodegradation and that the degradation rate is greater than the natural rate of decontamination (Bento *et al*. 2005). Since microbes existing in soils/groundwater feed on

certain chemicals, the complete digestion of these chemicals by microbes convert them into water and gases such as CO_2 (US EPA 2001). Commonly used organisms for this purpose are bacteria, fungi or protozoa either naturally occurring or genetically modified (Mathew 2005). Organisms have been widely studied and shown to destroy organic chemicals, whereas they can either remove or convert metals to a stable form. For bioremediation to be successful, it is important to ensure that the correct environmental conditions are in place to maximise the growth and activity of the microbes. These conditions include nutrient content, soil structure and texture, temperature and oxygen content as well as the correct assemblage of the microorganisms (Baptista *et al.* 2005). If these conditions are not met, the microbes could grow too slowly, die or even create more harmful chemicals (US EPA 2001). Different kinds of bioremediation methods have been developed to reduce the time required for degradation and reduce cost by increasing the degradative activity of native microbial populations (Perfumo *et al.* 2007). These approaches include the following, which can be in-situ or ex-situ:

2.4.1 Biostimulation

This involves the addition of oxygen or mineral nutrients to stimulate the numbers and activities of natural populations, usually bacteria and fungi so that they can break down pollutants into harmless products (Perfumo *et al.* 2007). In most environments, the presence of nitrogen and phosphorous is limited, even when total concentrations are high, it may be in a mineral form that is biologically unavailable (Hazen 2010). Therefore, biostimulation accerlerates the decontamination rate as the addition of one ore more rate limiting nutrients improves the microbes degrading potential (Nikolopoulou and Kalagerakis 2009). Nitrogen and phosphorous has been widely used in biostimulating processes to support growth of microorganisms. For example, Sakar *et al*. (2005) showed

that the addition of nitrogen and phosphorous as inorganic fertilizers and the addidtion of biosolids enhanced the biodegradation of petroleum hydrocarbon by up to 96%. However in some cases, addition of nutrients can negatively affect the microorganisms and biodegradation is suppressed. For example work carried out by Johnson and Scow (1999) showed that phenanthrene mineralization rates were depressed or remained the same with the addition of nitrogen and phosphorus to phenanthrene-contaminated soil. This could be as a result unbalanced or inappropriate level of nutrients, adsorption of the pollutant to the medium (soil) that prevents the availability of the pollutants for destruction or inactivity of the indigenous microbes caused by high concentration of pollutants.

2.4.2 Bioaugumentation

The success of bioremediation usually requires the application of strategies that are specific to the particular environmental conditions of the contaminated sites. Bioaugumentation which includes the addition of pre-adapted consortium, introduction of genetically engineered bacteria or the addition of biodegradation relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms plays a major role during biodegradation (EL Fantroussi and Agathos 2005). From an application perspective, using microbial consortioum instead of a pure culture for bioaugumentation is advantageous (Nyer *et al.* 2002). Two factors limit the use of added microbial pure cultures for contaminated land treatment. Firstly, the non-indigenous cultures are unable to compete properly with the indigenous population to develop useful population levels and secondly, most soils that have been exposed to biodegradable contaminants for a long period have indigenous microorganisms that can effectively degrade the contaminant if treatment is properly managed (Vidali 2001). Although this method of remediation is simple, there have been many records of failures. For example, Bouchez *et al.* (2000) showed that there was

no improvement on nitrogen removal when a nitrifying batch reactor was inoculated two times with aerobic denitrifying bacteria even after addition of acetate as a nutrient. Nevertheless, some work has shown promise for the strategy of combining both bioaugumentation and biostimulation to enhance bioremediation (El Fantroussi and Agathos 2005). Alisi *et al*. (2009) successfully obtained complete degradation of diesel oil and phenanthrene to an overall 75% reduction of the total hydrocarbon in 42 days. Indigenous and exogenous microbes could benefit from the addition of energy sources or electron-acceptors. For example, Silva *et al.* (2004) with their development of a combined bioaugumentation and biostimulation process for treatment of site highly contaminated with atrazine, showed that bioaugumentation with *Pseudomonas* sp. strain ADPand citrate or succinate biostimulation increased attrazine mineralisation.

2.4.3 Composting

This method is based on ancient method of turning household waste into usable organic amendments. It uses the biological system of microbes in the compost to breakdown or transform contaminants in soil/water (USEPA 1997). This method of remediation has received little attention although it has been used for treatment of contaminated soils for many years (Atagana 2008). Most of the work has been carried out on low levels of contamination although compost has shown potential for remediation of heavily contaminated sites (Garcia Gomez *et al*. 2003). Many contaminants like PAH (Cajthaml *et al*. 2002), heavy metal (Barker and Bryson 2002) and pesticides (Frazer 2000) have been remediated by this method. For example Cajthanel *et al.* (2002) showed that after 42 days of composting, 35 to 68% of both 3 to 4 rings and other higher molecular mass PAH were removed. Metallic contaminants are not degradable, therefore during composting, they are converted into organic combinations that are less bioavailable than the mineral combination

of the metals (Barker and Bryson 2002). For example, Paré *et al.* (1999) showed that during composting of biosolids and municipal wastes, there was a decline in soluble components of metals like Zinc (Zn), Cr and Cu and an increase in residual, organically bound forms. Compost remediation works in the same way as the biological process of soil remediation. As there is increased temperature in compost than in soil, there is increased solubility of contaminants in compost which increase the destruction of contaminants helped with an increase and diversity of microbial population (Barker and Bryson 2002). Microbes play an important role from the beginning to the end of composting. Their increase and diversity are controlled by changes in levels of moisture, temperature and nutrients, these ensure that the contaminants are exposed to a wider range microbe-environment conditions (Ling and Isa 2006). Although composting has been effective (Carcama and Powers 2002, Ahiamadu and Suripno 2009), its vulnerability to high concentration (> 2500 ppm) of heavy metal (microbial growth could be inhibited), its requirement for impermeable liners and its requirement for large area of land for treatment has limited its use (Kalogerakis 2005).

2.4.4 Land Farming

This is an ex-situ remediation method that involves the application of contaminated soil to the land surface and with periodic mixing with agricultural equipment contaminant biodegradation is achieved (Irvine and Frost 2003). Contaminants such as total petroleum hydrocarbon (TPH) can also be volatilized using this process (Paudyn *et al*. 2008). Tilling of the soil periodically disrupts the aggregate, which accelerates nutrient and contaminant distribution throughout the soil while providing oxygen to the soil (Irvine and Frost 2003). As microbes in soil have diverse catabolic activities, adding compounds containing microbes to the contaminated site leads to pollutant degradation (Mmom and Deekor 2010). Cover crops could be planted during land farming remediation. This would enhance

rhizosphere degradation (Frazer 2000). Detailed assessment of rhizospheric degradation will be discussed in the phytoremediation section. Land farming is slow because the conditions that affect degradation such as temperature and rainfall are uncontrolled (EPA 1994), but it is a low cost technology. The rate of application is calculated and the size of land is based on the application rate. This is done to avoid concentrations that would be detrimental to the soil (Frazer 2000). Most land farming remediation requires the addition of nutrients to accelerate degradation by indigenous microbes. For example, with the addition of fertilizer, biodegradation was enhanced and enough hydrocarbons were degraded when land farming remediation was applied on hydrocarbon-contaminated soil in the Canadian Arctic (Paudyn *et al*. 2008). Although land farming is an effective remediation technique, it has its shortfalls. These include its remediation potential for inorganics since they are non-biodegradable and the presence of heavy metal concentration of 2500 mg kg^{-1} or more may inhibit microbial growth. Also, volatile constituents may evaporate during aeration and could cause air pollution and it requires large land area for treatment. Similarly, dust generated could cause air pollution and it is difficult to achieve more than 95% contaminant reduction (EPA 1994).

2.5 Phytoremediation

2.5.1 Definition

Phytoremediation is a broad term that incorporates all the different processes that plants use to remove, transform or stabilize pollutants in soil, water or atmosphere. It is a plant based remediation technology that is applied to both inorganic and organic contaminants in soil, water and sediments globally (Nwoko 2010). Natural processes by which plants and their associated microbes degrade and/or sequester inorganic and organic pollutants are

incorporated in this technology which makes it a cheaper and environmentallysustainable option to mechanical and chemical methods of removing contaminants from soil (Nwoko 2010). It also generates fewer secondary wastes and less environmental impact than would be obtained using other traditional methods (Mohanty *et al.* 2010). Results of research for phytoremediation potential show that it is applicable to a broad range of contaminants including metals (Jadia and Fulekar 2009), radionuclides (Kaushik *et al*. 2008), organic compounds e.g, chlorinated solvents, BTEXbenzene, toluene, ethylbenzene and xylene (Weishaar *et al*. 2009), polychlorinated biphenyl (Chen *et al.* 2010), PAHs (Denys *et al.* 2006) and pesticides (Chang *et al.* 2005).

The term phytoremediation was not used until the 1980s although the use of plants to remediate radionuclide-contaminated soils was explored in the 1950s (Gerhardt *et al*. 2009). According to Green and Hoffangle (2004), numerous laboratory and greenhouse studies are carried out to determine plant toxicities and contaminant uptake abilities. In order for phytoremediation to achieve global acceptance as a remedial method, field scale applications need to be carried out and documented. There have been extensive studies on application of constructed wetlands and vegetative covers in the field to demonstrate their phytoremediation capabilities as well as field scale studies of the use of plants for ground water and soil remediation (Olsen *et al*. 2003).

2.5.2 Processes of phytoremediation

The success of phytoremediation depends on the plants' ability to assimilate and/or accumulate organic and inorganic contaminants in their cellular structures and to carry out deep oxidative degradation of organic xenobiotics (Kvesitadze *et al.* 2006). These are based

on some natural processes that are carried out by plants and according to Ralinda and Miller (1996), they include:

1. The uptake of metals and some moderately soluble organic compounds like BTEX from soil and water.

2. Accumulation or assimilation of organic contaminants through lignification, metabolization, volatilization and mineralization with CO_2 and water as end products.

3. Break down of complex organic molecules into simpler ones by enzymes.

4. Increasing the carbon and oxygen content of the soils around the roots through the release of exudates and decay of root tissues. This helps in promoting microbial activity.

5. Capture of groundwater (contaminated or otherwise) and using it for plant processes.

2.5.3 Phytoremediation Technologies

Depending upon the processes by which plants remove or reduce the toxic effects of contaminants in soil, phytoremediation technology can be classified as follows:

a) **Phytostabilization**

Berti and Cunningham (2007) described phytostabilization as the use of plants to stabilize contaminants in soil. This could be either by preventing erosion, leaching or run off, or by transforming contaminants to less bioavailable forms through rhizospheric precipitation and preventing their migration into the food chain or ground water (Pillon-Smits 2005). The microbiology and the chemistry of the root zone helps the course of phytostabilization as soil pH may be changed by plant root exudates which leads to the alteration of the soil

environment or contaminant chemistry (EPA 2000). When this happens, there can be change in metal solubility or mobility (Salt *et al*. 1995) as well as dissociation of organic compounds if organic contaminants are present. Decrease in soluble arsenic (As) and cadmium (Cd) in soil as well as increase of soil pH with high concentration of the metals (As and Cd) present in root suggested that *Lupinus albus* can phytostabilize contaminants in soil in a field trial carried out by Vazquez and co workers in 2006. Phytostabilization has promising advantages as a soil remediation technology. Soil removal is unnecessary, leading to lower cost and less disruption to the environment (Pierzynski *et al.* 2002). With revegetation, the ecosystem is enhanced and there is no need for disposal of hazardous materials (EPA 2000). However, there are limitations to this technology. The contaminants remain in place and there could be a need for extensive monitoring (if the specific contaminant inhibits the reversal of the stabilization process), fertilization or amendment of vegetation since contaminants with an elevated toxic effect can affect plant growth drastically (Pierzynski *et al.* 2002). Plants used for this remediation technique should be tolerant to metals in metal contaminated sites (King *et al.* 2008) and/or the particular growing conditions of the site, have a shallow root system that enables stabilisation of the soil as well as uptake of soil water and should not be able to take up contaminants into plant parts above ground. Certain plants have been used to achieve phytostabilization. For example, poplars have a remediation potential of about 5 to 10 ft depth of a soil (EPA 2000, Pierzynski *et al.* 2002), *Lupinus albus* for Cd and As (Vazquez *et al.* 2006), *Festuca* cultivars for various heavy metals (Krzyzak *et al*. 2006), *Agrostis tenuis* for Zn (Panfili *et al*. 2005) and research have shown that native plants in Montana like *Poa ampla*, *Elymus trachycaulus* and *Vicia americana* can be used for phytostabilization of mine tailings (Neuman *et al*. 2005). An important aspect of phytostabilization is the identification of the

factors limiting the growth of plant. For example, poor soil properties (physical and chemical), phytotoxicity of metals, very high or low pH, salinity and inadequate plant nutrients can all affect plant growth. For successful vegetation establishment, these limiting factors need to be addressed (Pierzynski *et al.* 2002). However, studies have shown that amendments can address the issue of physical and chemical properties of soil and phytotoxicity. For example, Alvarenga *et al.* (2009) showed that sewage sludge, municipal waste and garden waste imomobilized Cu, Zn and Pb and decreased their mobile fractions in a highly acidic metal contaminated soil. Also, research carried out by Zanuzzi and Cano in 2010 showed changes in plant colonization, reduction in Pb and soil characteristics when organic and lime amendments was used for phytostabilization of a Pb polluted site. Inorganic fertilizer alone did not improve plant growth on a metal mine contaminated site when compared to animal wastes (Hetrick *et al.* 1994) although it improved growth of *Thysanlaena maxima* when grown on a lead-contaminated site (Rotkittkhun *et al.* 2006).

b) **Phytoextraction**:

Phytoextraction is the use of plants to remove inorganic (mostly metals) contaminants (Lasat 2002) and/or organic contaminants (Utmazian and Wenzel 2006) from contaminated soils, water, sludges or sediments. In practice, the contaminants are taken up by the plant roots, and moved to the above ground parts (EPA 2000). For this technique to be successful, there should be soil-to-metal-to-plant interactions, which could be dependent on the extent of the soil contamination, bioavailability of contaminants, and plant accumulation capacity for contaminants in above ground parts (Lasat 2002). Plants have a natural tendency of taking metals (e.g. Ag, Cu, Co, Fe, Ni, Zn, Hg, Mo, Pb), non-metals (e.g. B, radionuclides such as ^{90}Sr, ^{137}Cs, ^{239}Pu, ^{238}U, ^{234}U), metalloids (e.g. As and Se) and organics (e.g. TNT and PCBs) (EPA 2000, Alkorta and Garbisu 2001, Lasat 2002, and

Ficko *et al.* 2010). The roots of plants play an important role in phytoextraction. As phytoextraction is limited to the zone influenced by the roots of plants, the depth and size of the root determines the depth of phytoextraction (Keller *et al.* 2003). During a phytoextraction process, there should be restricted access to plants as well as proper disposal of harvested plant materials; this is because phytoextraction allows for accumulation of toxic levels of contaminants in the above ground parts of the plant (EPA 2000). There are two basic strategies employed for efficient phytoextraction (Salt *et al.* 1998). It can be continuous/natural using hyperaccumulators or accumulators or induced using chelates to increase bioavailability in soil (Utmazian and Wenzel 2006). However, some authors have reported that when EDTA was used as chelate, metal complexes remained in the soil water for several weeks, hence environmental issues could be a problem (Lombi *et al.* 2001, Wenzel *et al*. 2003). Hyperaccumulators/accumulators and chelates will be discussed later. For an efficient phytoextraction process, soil conditions must be appropriate for plants to grow and allow for contaminant migration with no metal leaching. Therefore to achieve this, soil pH could be adjusted to allow for contaminant bioavailability and subsequent uptake by plants (EPA 2000). Extracted contaminants in harvested plant biomass in a phytoextraction process could be a resource. For example, harvested plant biomass containing selenium could be used for animal feed (Banuelos and Mayland 2000). However, there are some limitations such as, the slow growth of hyperaccumulators, phytotoxic effect of contaminants on plants, and the need for plants to be harvested and disposed of (Prasad and Freitas 2003).

c) **Phytovolatilization:**

Phytovolatilization entails the uptake and transportation of contaminants by plants with subsequent release of the contaminant in normal or modified form to the atmosphere

(Vanek *et al*. 2010). It is mainly applied to groundwater but can be applied to soils, sediments and sludges (EPA 2000). It occurs as the plants take up water and contaminants (organic and inorganic) and the transport route could either be through open stomata in leaves with subsequent evaporation or by direct volatilization into the atmosphere from stems. For example, methyl tert-buthyl ether (MTBE) has the tendency to escape through the leaves, stem and even the barks of trees to the atmosphere (Hong *et al.* 2001, Ma *et al*. 2004). Phytovolatilization mostly works with both organic and inorganic contaminants such as BTEX, trichloroethylene (TCE), vinyl chloride, selenium (Se), mercury (Hg) and arsenic (As) (EPA 2000, Kamath *et al.* 2004). The common characteristic of these organic contaminants (BTEX, TCE and vinyl chloride) is that they have a Henry's constant greater than 10 atm m^3 water m^{-3} air. Most organic contaminants with lower values have low volatility (Kamath *et al.* 2004). With the release of these contaminants into the atmosphere, those with double bonds such as trichloroethylene (TCE) and perchloroethylene (PCE) could be quickly oxidised in the atmosphere by OH radicals (Kamath *et al.* 2004). However, in some situations such as poor atmospheric conditions, the contaminants can pose a risk of precipitation. For example, mertyl ert-butyl ether (MTBE) can pose a risk to shallow ground water due to precipitation because it stays long in the atmosphere (Morgan *et al*. 2005). Studies have shown that hybrid poplars have the ability to take up and transform organic contaminants such as TCE to several metabolic products such as trichloroethanol, trichloroacetic acid and dichloroacetic acid and therefore demonstrating its potential for in-situ phytoremediation (Newman *et al.* 1997,Orchard *et al.* 2000, Muller *et al*. 2011). Also indian mustard (*Brassica juncea L*.) and canola (*Brassica napus*) have been used to phytovolatilize inorganics such as Se where the Se is converted to dimethyl selenide that is less toxic to the environment and released to the atmosphere (De Souza *et al.* 1999,

EPA 2000, De Souza *et al*. 2002). However, because the transfer of contaminants is involved in phytovolatilization, its effect on the ecosystem should be addressed before carrying out the process and for transpiration to occur significantly, the soil must be able to transmit sufficient water to the plant. Phytovolatilization has its advantages as more toxic contaminants are biotransformed to non-toxic forms and when they are released to the atmosphere, they could be subject to a more effective degradation. However, there could be a passage of toxic metabolites in late products of plants like fruits (EPA 2000).

d) **Phytodegradation:**

Phytodegradation entails the breakdown of contaminants accumulated by plants through metabolic activities within the plants (EPA 2000) or the breakdown of contaminants directly by compounds produced by plants such as enzymes from roots (Greipsson 2011). It is used for the treatment of soil, sediments, groundwater, sludges and surface water (EPA 2000). In this process, contaminants are taken up from the soil or medium for remediation process by plant roots and metabolised into less toxic/non-toxic compounds in plant tissues by compounds produced by the plants (Salt *et al.* 1998, Singh and Jain 2003). The metabolic processes involved in phytodegradation are partly the same as that of human detoxification or elimination metabolic processes known as the 'green liver' model (Burken 2003, Singh and Jain 2003). When contaminants are moderately hydrophobic, their uptake is efficient whereas that cannot be said for hydrophilic or extremely hydrophobic contaminants that are not easily translocated within the plant because they are strongly bound to the roots (Singh and Jain 2003). Very soluble compounds are not sorbed onto roots and neither are they translocated within plants while lipophilic compounds can only be bound or partitioned to root surfaces but with no further translocation within the plants (Pevetz 2001). Plants can metabolize various types of organic compounds. These include

TCE (Newman *et al.* 1997), TNT (Medina *et al.* 2000, Zhu *et al*. 2012)), atrazine (Burken and Schnoor 1997, Wang *et al.* 2010), hexachlorobenzene (fungicide), dichloro-diphenyl-trichloroethane and polychlorinated biphenyls in plant cultures (EPA 2000). However, since phytodegradation requires the transformation of toxic contaminants to non/less toxic forms, the case of phytodegradation of TCE has been a concern as vinyl chloride which is highly toxic can be formed (Pevetz 2001), but work carried out by Newman *et al.* (1997) reported low levels of TCE metabolites in plants with no release of vinyl chloride. Organic compounds are mostly subject to phytodegradation although in addition inorganics can also be through uptake by plants and metabolism (EPA 2000, Pevetz 2001). The log K octanol-water ($\log K_{OW}$) of organic compounds determines their possibilities for phytodegradation because uptake by plants is dependent on the hydrophobicity, solubility and polarity of the organic compound. However, phytodegradation outside the plant does not depend on $\log K_{OW}$ and plant uptake. Plant-formed enzymes such as dehalogenase, nitroreductase, peroxidase, laccase and nitrilase have been discovered in soils and plant sediments (Pevetz 2001). For example, Schnoor *et al.* (1995) showed that the concentration of dissolved TNT in flooded soil decreased by over 92% in the presence of parrot feather (*Myriophyllum aquaticum*)that produces the nitroreductase enzyme. The advantages of this remediation method are rewarding. Contaminant degradation by enzymes from plants can take place without the need for microorganisms. Therefore when an environment lacks suitable microorganisms probably due to high contaminant level, phytodegradation could serve a useful remediation process and secondly, plants can grow in soil with high contaminant concentration that are not conducive for microorganisms (EPA 2000). However, the presence of metabolites within a plant might be a hard task to determine. Therefore confirmation of contaminant destruction could be difficult, although Newman *et al*. (1997)

determined the presence of metabolites of TCE like trichloroethanol, trichloroacetic acid and dichloroacetic acid in the degradation of TCE in their research using poplars and coupled with the transpiration of measurable amount of TCE, the possibility that degradation was produced by rhizosphere microorganisms were eliminated.

2.5.4 Phytoremediation of organic contaminants

Organic contaminants are those that contain carbon. They can be released into the environment via a range of industrial activities such as timber treatments (Mills *et al.* 2006, Robinson and Anderson 2007), oil exploration (Rogge *et al.* 1997), coal processing (Chmielewski *et al.* 2001) and gas works (Cofield *et al.* 2008). They vary widely in types which include the polyaromatic hydrocarbons (PAHs), trichloroethylene (TCE), petroleum hydrocarbons, 2,4,6 trinitrotoulene, benzene, toluene, ethylbenzene and xylene (BTEX), polychlorinated phenol, methyl-tert-butyl ether (MTBE), gasoline etc. PAHs are mostly common because they are widespread due to human activities and are by-products of major industrial processes such as pyrolysis reaction leading to charcoal formation and incomplete combustion of coal and gas (Ledesma *et al.* 2000, Barbosa *et al*. 2006).

Organic compounds exist in different structural form and chemical composition, and in order for phytoremediation to take place, the compounds needs to be mineralised into non-toxic compounds such as CO_2, NO_3^-, Cl^- and NH_4^+ (Meagher 2000), and also be in forms that are available to plants or microbes. Organic compounds have a wide range of chemical composition and structure and this affects both the potential for, and the mechanism of remediation and inorder for a successful phytoremediation, the compounds need to be in forms that are available to the plants and/or microbes (Parrish *et al.* 2005). Typical examples are PAHs, which are less soluble in water due to their non-polar nature (Nazzal

2007). Their solubility decreases with increase in molecular weight (Werner 2003) as they become increasingly hydrophobic and may become sorbed to the soil (Neuhauser *et al*. 2006). The more strongly sorbed they are, the less bioavailable and biodegradable they become (Neuhauser *et al*. 2006).

When plants absorb organic contaminants to their roots, the fate of the organic compound varies (Cunningham *et al*. 1996) and depending on the organic contaminant in question, the partitioning between the roots and the above ground tissues will vary (Alkorta and Garbisu 2001). They can be extracted, degraded, volatilized or stabilized (US EPA 2000, Greipson 2011) depending on the organic compounds' chemical nature, the external temperature, type of plant and the stage of growth of the plant (Kvesitadze *et al.* 2009). According to Gao and Zhu (2003), organic contaminant uptake in plant roots is complicated and occurs through active and/or passive transport and in the passive process, the pollutants accompany the transpiration water through the plant. Specific transporters such as carrier proteins are involved with active transport (Nardi *et al.* 2002). However, because organic compounds are man-made except for hydrocarbons which are naturally formed compounds but with increased accumulation in the environment through anthropogenic sources (Widdel and Rabus 2001), there are no transporters for uptake in plants; rather transport takes place by diffusion and are dependent on the hydrophilic or hydrophobic nature of the contaminants. The hemicelluloses in the cell wall and and the lipid bilayer of plant membrane have been shown to bind hydrophobic organic pollutants effectively (Cherian and Oliveira 2005). Hydrophobicity is determined by the octanol-water partition coefficient (Log K_{OW}) and a range of 0.5 to 3.0 is termed moderately hydrophobic (Alkorta and Garbisu 2001). This range is sufficient for organic contaminants to move through the lipid bilayer of membranes and taken up by plants (Pilons-Smith 2005). However, with a log

K_{OW} of less than 0.5, passage through the membranes and subsequent uptake becomes impossible. There are, however, disparities in organic contaminant uptake and translocation among plant species in addition to the factors that affects their bioavailability (Alkorta and Garbisu 2001). Cofield *et al*. (2008) found that although PAH concentration in soils decreased in the presence of tall fescue and switch grass, the nonlabile PAHs were unaffected,

The age of the compound plays an important role during phytoremediation of organic contaminants. According to Smith *et al.* (2006), the process of ageing of PAHs makes extraction by plants more difficult and thus compromising phytoremediation. This could be advantageous to living organisms in contact with the soil, as aged PAHs will be less accessible. The process of ageing starts with the binding or sorbing of PAHs to the humin, fulvic and humic acid components of the soil (Li and Liu 2005). This process (soil-PAH contact time) is very important to the fate and transportation of PAHs in soil (Hwang and Cutright 2002) by causing slow desorption of organic contaminants leading to low microbial degradation. If organic compounds age in the soil, there could be a decline in their lability and bioavailability with less effect on the total concentration. For example, Cofield *et al.* (2008) found that with the presence of *Festuca arundinacea* and *Panicum virgatum*, the non-labile PAHs were unaffected whereas the total PAHs in the soil reduced. In recent years, non-aged PAH spiked soils have been used for phytoremediation studies (Olsen *et al.* 2007). However some studies have shown that the age of the contaminants in the soil limits their degradation (Allard *et al.* 2000, Rezek *et al.* 2008). Therefore, spiking of soils with fresh PAH could likely give results that do not conform to the real environment sparking controversies in phytoremediation research. In as much as these controversies exist, phytoremediation of organic contaminants have been successful.

Various kinds of plants including the grasses and legumes have successfully remediated organic contaminants. For example, the major mechanism of PAH dissipation in vegetated soil is associated with the microbial activity in the rhizosphere, therefore remediation varies across plant species and type of environment (Lee *et al.* 2008). The grass has been successful mainly because of the short growth season with large fibrous root system that results in increased rhizospheric soil and the legumes have the ability to germinate when nutrient availability is poor and are able to fix atmospheric nitrogen (Lee *et al.* 2008, Smith *et al.* 2006). Dzantor *et al.* (2000) showed that legumes like alfalfa (*Medicago sativa*), crown vetch (*Coronilla varia*), bush clover (*Lespedeza cuneata*) and flatpea (*Lathyrus sylvestris*) helped in trinitrotoluene (TNT) and pyrene dissipation in contaminated soils ranging from 51% for flatpea, 64-70% for bush clover and 80% for alfalfa and crown vetch. Also Lee *et al.* (2008) showed that the legumes (*Astragalus membranaceous* and *Aeschynomene indica*) withstood phenanthrene and pyrene contamination better than grasses (*Panicum bisulcatum* Thunb and *Eschinochloa crus-galli*). However the results are of limited value as comparisons were made with no indication of starting concentration. A variety of plant trials for remediation of organics have been completed and the most common include willows, grasses and herbs (Trapp and Karlson 2001).

Depending on the organic contaminant in question, phytoremediation can be achieved through uptake by plants. According to Gao and Zhu (2004), there were significant differences in phenanthrene and pyrene accumulation in shoot and root of *Glycine max, Phaseolus vulgaris, Capsicum annum, Solanum melongena, Brassica parachinensis, Lolium multiflorum, Amaranthus tricolor, Raphanus sativus, Ipomoea aquatica, Brassica chinenis, Brassica oleracea* and *Spinacea oleracia*. The probable uptake route could be through uptake of volatilized portion of contaminants from the soil as well as root to shoot

translocation. There is also evidence of removal of volatile organic compounds through volatilization for example the presence of trees in naphthalene contaminated site helped in direct volatilization of naphthalene to the atmosphere (Marr *et al.* 2006). But the key to the success of phytoremediation of organic contaminants is not the plant alone, but the interaction between the plants and the consortium of microorganisms in the rhizosphere also known as phytodegradation as discussed earlier. These microbes degrade the organic contaminants, which is enhanced with the presence of plants. For example, PAHs degraded faster in planted soils when compared to unplanted soils (White *et al*. 2006). This was as a result of increased microbial numbers, which results in increased activity (Lu *et al.* 2010). Also for TNT, vegetated plots had more microbial colonies forming in each gram of soil relative to unvegetated plots (Travis *et al.* 2008). When compared to the bulk soil, there are more PAH-degrading microorganisms in the rhizosphere (Parrish *et al*. 2005). For example, in the rhizosphere of Bermuda grass (*Cynodon dactylon),* there was a 400% increase in the number of pyrene degraders when compared to the bulk soil (Krutz *et al.* 2005). This increase could be associated with the release of phenolics and salicylates by plants (Chen and Aitken 1999) as flavones such as morusin, morusinol, and kuwanon c which are phenolic compounds have showed support for PCB degrading bacteria in some plant species (Leigh *et al.* 2002). In phytoremediation trials involving aliphatic and aromatic hydrocarbons, there have been clear correlations between the number of microbes in the rhizosphere and the dissipation of the hydrocarbon. For example, Günter *et al.* (1996) showed that the microbial plate count values increased with increased removal of artificially applied aliphatic hydrocarbon from the rhizosphere when planted with *Lolium perenne*, and similarly Fan *et al.* (2008) showed that residual pyrene concentration in soil planted with *Medicago sativa* was lower in the rhizosphere with increased microbial

numbers in this region. However in contrast work carried out by Kaimi *et al*. (2006), soil dehydrogenase activity was preferred to number of aerobic bacteria as the reason for total petroleum hydrocarbon (TPH) dissipation as there was no correlation between TPH and the number of aerobic bacteria. In this case, the plot was planted with *Medicago sativa*. There are suggestions that the microbial population differs according to plant species. Kirk *et al*. (2005) observed that after the seventh week of study, the microbial population was higher in plots planted with *Medicago sativa* than with *Lolium perenne* and that the combination of *Medicago sativa* and *Lolium perenne* showed the greatest microbial number differentiation from the bulk soil.

The degradation of organic contaminants is highly problematic. With few microorganisms with the ability to use high molecular weight PAHs as their sole source of carbon, it is expected that remediation with microbes independently will be likely inefficient (Huang *et al.* 2004). Even in cases when bacteria from PAH contaminated sites were used, or when nutrients were supplemented, they have been highly ineffective (Cunningham *et al.* 1996). The result of the work carried out by Huang *et al.* (2004) showed that bioremediation alone was ineffective for the removal of benzo[*a*]pyrene and dibenzo[*ah*] pyrene until the establishment of *Festuca arundinacea.* This shows that with the right plant, the rate of degradation of organic contaminants will be improved during bioremediation.

Table 2.0 shows some example of successful phytoremediation of organic contaminants

Table 2.0: Selected examples of phytoremediation trials for organic contaminants. All concentrations are maximum values except otherwise stated.

Pollutant	Soil concentration (mg kg^{-1})	Plant species	Growth condition	Amme-ndment /Fertilizer	Measure of success	Reference
Phenanth-rene, Pyrene	Phe-332.06 (av), Pyrene- 321.42 (av)	*Sorghum vulgare L.*	Greenhouse	None	Phenanthrene and Pyrene dissipated	Xin *et al.* 2009
PAH	1251.7	*Vicia faba, Zea mays, Triticum aesitivum*	Field	None	PAH dissipated	Diab 2008
Benzo[*a*] pyrene	100	*Medicago sativa L*	Glasshouse	None	B[*a*] P removal	Shiliang *et al.* 2004
Alkylated PAHs	9175	*Lolium arundinaceum, Lolium multiflorum, Cynodon dactylon.*	Field	Fertilized	Greater degradation for anthracenes and phenanthracenes	White *et al.* 2006
Pyrene	100	*Zea mays*	Greenhouse/ Spiked soil	NPK	Pyrene removal	Zhang *et al.* 2009a
TNT	80	*Vetiveria zizanioides*	Glasshouse/ Spiked	Urea	Removal of TNT helped by Urea.	Das *et al.* 2010
PAH	Unknown	*Festuca arundinacea, Lolium multiflorum*	Glasshouse	Compost	PAH removal	Parrish *et al.* 2005
TPH	6400	*Lolium perenne*	Glasshouse	None	Loss of TPH	Hou *et al.* 2010

Chrysene	500	*Lolium perenne, Trifolium repens L*	Glasshouse/ Spiked soil	None	Degradation of Chrysene	Johnson *et al.* 2004
Phenanthrene, Pyrene	Phenanthrene-87.56	*Panicum bisulcatum, Echinogalus crusgalli, Astragalus membranace-us, Aeschynomene indica*	Greenhouse/ Spiked soil	N	Greater pyrene removal	Lee *et al.* 2008
Pyrene	500	*Zea mays*	Greenhouse/ Spiked soil	N	Pyrene removal	Lin *et al.* 2008
Hydrocar-bons	11400 (av)	*Pinus sylvestris, Populus deltoids, Trifolium repens*	Field	Fertilized	65% hydrocarb-on removal	Palmroth *et al.* 2006
PAH (creosite)	3000	*Festuca arundinacea*	Greenhouse/ Spiked	N	Successful degradation of larger PAHs	Huang *et al.* 2004
PAH	753	*Poaceae, Verbenaceae, Polygonaceae Lamiceae, Germiniaceae, Fabaceae, Astraceae, Aslepiadaceae.*	Greenhouse/ aged soil	N	30% of PAH was degraded in the presence of ryegrass	Olsen *et al.* 2007

2.5.5 Phytoremediation of inorganics

Inorganic contaminants are mineral-based and unlike organics, they cannot be mineralised or degraded. Therefore their remediation must be either physical removal, conversion into biologically inert form or stabilization (Cunningham *et al.* 1996). However, as physical removal cannot be fully accomplished, conversion into biological inert form and stabilization should be a priority. Some plants have the ability of accumulating, transferring or stabilizing inorganic compounds. For the latter, the plant species only need to be tolerant to the inorganic compounds and avoid uptake, while for the former, hyperaccumulator plants have shown to accumulate high concentrations of inorganic compounds thereby removing the contaminants from the soil (Ghosh and Singh 2005).

According to Baker (1981) hyperaccumulators are plants (when growing in their natural habitat) with the ability to accumulate high concentration of metals without toxic effects when compared to other species or genotype and also the ratio of shoot to root or leaf to root concentration of metals accumulated is greater than one. Presently there are about 400 plants that hyperaccumulate metals. *Brassicaceae, Asteraceae, Caryophyliaceae, Cyperaceae, Cunouniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae and Euphorbiaceae* dominates the 45 families (Prasad and Freitas 2003). Most of the hyperaccumulators accumulate nickel while, manganese, cadmium, zinc and cobalt are accumulated by some. *Thlaspii caerulescens*, which is a hyperaccumulator of zinc and cadmium, has been identified as one of the most studied hyperaccumulators (Wang *et al.* 2006).

With the exception of mercury, the uptake of metals into plant occurs from aqueous phase. Therefore to control the uptake of metals, speciation of metals within the soil is very

important. In non-accumulating plants there are evidences of enhanced metal uptake even when some essential metals are not available. One way this happens is for the plants to cause rhizospheric changes such as the release of phytosiderophores or increase acidification to increase the mobility of some metals (Marschner 2002). However in hyperaccumulators there are limited processes for enhanced uptake. For example, increased acidification of the rhizosphere does not enhance metal uptake (Jing *et al.* 2007), but release of exudates have shown some promises in few studies (McGrath *et al.* 2001). Once metals are taken up into plants, they are stored within the tissues, which can be harvested. Some of these metals inside the plant are very insoluble and so do not freely move in the vascular system, and therefore carbonates, sulphates or phosphates are formed (Ghosh and Singh 2005). However, selenium and mercury can be transformed within plants and volatilized if released to the atmosphere (Meagher 2000), which is dependent on root uptake absorption as in the case of organics (Moreno *et al.* 2008). Some groups of plants are also able to survive and reproduce in highly metal contaminated soil without hyperaccumulating the metals. These are known as pseudometallophytes and they achieve this by developing tolerance through rhizospheric precipitation of metals. For example Dahmani-Muller *et al*. (2000) showed that metal (Pb, Zn, Cu and Cd) concentration in *Agrostis tenuis* was higher in roots than in leaves which suggested the immobilization of metals in roots. Amendment of soil increases the availability of metals for uptake during phytoremediation process. For example, *Pterocarpus indicus* and *Jatropha curcas. L* removed higher amount of chromium with addition of compost (Mangkoedihardjo *et al.* 2008) while ethylenediaminetetraacectic acid (EDTA) enhanced the uptake of Pb by *Vetiveria zizanioides* (Gupta *et al.* 2008). Other compounds such as citrate, oxalate, tartrate, malate, acetate and some synthetic chelates have been used as chelators of metals (Prasad

and Freitas 2003). However their effect on the microbial communities of the soil has not been fully assessed. The microbial communities within the rhizosphere can also play an important role during phytoremediation of inorganics. For example, the presence of rhizospheric bacteria increased the concentration of Zn in *Thlaspi caerulescens* (Whiting *et al*. 2001) and Ni in *Alyssum murale* (Abou-Shanab 2003). The increase in metal concentration in the respective plants is evidence of the role plant growth promoting rhizobacteria (PGPR) plays. They can affect heavy metal mobility and availability to plant by releasing chelating agents, acidification and redox changes or they can improve plant growth and nutrition though nitrogen fixing and transformation of nutrient elements (Jing *et al.* 2007). However, under high soil contaminant level, the growth of plant growth promoting bacteria can be inhibited. For example, Nie *et al*. (2002) showed a 30% germination of seeds of canola irrespective of the presence or absence of plant growth promoting bacteria.

Many glasshouse and laboratory studies on phytoremediation of inorganics have been successfully carried out as shown in table 2.1. However, full- scale application has been limited compared with organics. Some species that have been used include *Elsholtzia splendens* (Jiang *et al*. 2004) for Cu, *Salix sp, Populus sp and Alnus sp* (French *et al.* 2006) for phytoextraction of zinc and stabilization of nickel, and *Brassica juncea* and *Brassica carinata* for phytoextraction of Pb, Zn, Cu and Cd, although *Brassica juncea* showed better phytoextraction potential (Del Rio *et al.* 2000).

Table 2.1. Selected examples of successful phytoremediation trial for inorganic contaminants. All concentrations are maximum values except stated otherwise.

Pollutant	Soil concentration (mg kg^{-1})	Plant species	Growth condition	Ammendment /Fertilizer	Measure of success	Reference
Cu	unknown	*Brassica juncea* *Zea mays*	Glasshouse	EDTANa	Removal of Cu	Inoue *et al.* 2003
Cu	1200	*Elsholtzia splendens*	Glasshouse, Field	Urea, KH_2PO_4	Removal of Cu	Jiang *et al.* 2004
Cu, Pb, Mn, Zn	Cu- 640 Pb- 2400 Mn- 27000 Zn- 7800	*Brassica juncea*	Greenhouse	None	Removal of metals	Bennett *et al.* 2003
Zn, Cd	Zn- 25200 Cd- 170	*Thlaspi caerulescens*	Greenhouse	Compost	Removal of metals	Escarre *et al.* 2000
Cd, Zn, Pb	Cd- 20 Zn- 500 Pb- 1000	*Dianthus chinensis, Vetiveria zizanioides*	Greenhouse	EDTA	Removal of Metals greater with EDTA	Lai and Chen 2004
Cr	10	T*rigonella foenumgraecum. L, Spinacia oleracea, Brassica campestris*	Glasshouse	None	Removal of Cr	Dheri *et al.* 2007

Cr	90	*Pterocarpus indicus* *Jatropha curcas L.*	Glasshouse	Compost	Removal of chromium	Mangkoedihardjo *et al.* 2008
Zn, Ni	Ni- 109 Zn- 1300	*Salix sp* *Populus sp* *Alnus sp*	Field	N	Removal of Zn and stabilization of Ni	French *et al.* 2006
Cd	1.6 (av)	*Averrhoa carambola*	Field	N	Removal of metal	Li *et al.* 2009
Cd, Zn	Zn- 600 Cd- 8	*Pennisetum americanum, Pennisetum atratum*	Greenhouse, spiked soil	Basic fertilizer	Removal of metal	Zhang *et al.* 2010
Pb	20	*Vetiveria zizanioides*	Greenhouse	EDTA	Removal of metal	Gupta *et al.* 2008
As, Co, Cu, Pb, Zn	As- 886 (av) Co- 100 (av) Cu-1735 (av) Pb- 473 (av) Zn- 2404 (av)	*Populus alba* *Populus Nigra* *Populus tremula* *Salix alba*	Glasshouse, Field	NPK	Species successfully stabilized metals	Vameralli *et al.* 2009

2.5.6 Phytoremediation of mixed contaminants

Phytoremediation of mixed contaminated soils (mix of organic and inorganic) is poorly understood but very important as most sites are exposed to mixed contaminants (Zhang *et al.* 2011). The approaches for the remediation of these sites are different. It is very important to understand the interaction between both contaminants, which could affect their availability, and form.

When pollutants are mixed or combined, phytoremediation could be influenced as contaminants may interact with themselves as well as with plants and the rhizosphere (Almeida *et al.* 2005). Previous research has shown that an increase in metal bioavailability can occur when they interact with organic compounds (Chen *et al.* 2004, Gao *et al.* 2006). In addition severe inhibition of biodegradation of organic pollutants by toxic metals such as cadmium (Maslin and Maier 2000), as well as stimulation of microbial activity has been shown. A comprehensive review on the impacts of metals on biodegradation of organic pollutants was provided by Sandrin and Maier (2003). The review however highlighted a wide range of concentration of metals that could cause inhibitory effects. The non-specificity of concentrations was attributed to limited information on metal speciation as well as variety in experimental protocol.

Degradation of organic pollutants during phytoremediation depends extensively on the presence of suitable microbes as well as their activities. Therefore if metals cause any negative effect on microbes, limiting their activity, the success of phytoremediation could be severely compromised. Research carried out by Chaudri *et al.* (1993) and Dobbler *et al.* (2000) showed

that heavy metals decreased the number of specific populations of microbes and microbial diversity respectively. For example, inhibition of the activity of hydrocarbon degrading organisms was observed in the presence of heavy metals in work carried out by Al-Saleh and Obiekwe (2005). Also phenanthrene showed toxicity effects on *Allysum lesbiacum*, a hyper-accumulator of nickel in phenanthrene- nickel co-contaminated soil although soil was amended with sorbitan triolate, salicylic acid or histidine (Singer *et al.* 2007)

Much as the combination of organic and inorganic contaminants have shown negative effects, such as effect on plant growth and toxicity (Lin *et al*. 2006, Sun *et al.* 2011), Palmaroth *et al.* 2006 showed in a field based study that the removal of hydrocarbon by *Pinus sylvestris* and *Populus deltoides x wettsteinii* was enhanced in the presence of metals such as Zn, Pb and Cu. However, about 80% of the trees died due to toxicity. Cadmium was shown to improve the root and shoot accumulation of pyrene in *Zea mays* L., but the more important factor for pyrene dissipation was plant-promoted biodegradation in the rhizosphere (Zhang *et al.* 2009a). Zhang *et al*. (2011) showed that the presence of phenanthrene and pyrene at 50 or 250 mg kg^{-1} partially alleviated the toxicity of cadmium to *Juncus subsecundus*. Also 50 to 500 mg kg^{-1} of pyrene, increased shoot yields of *Zea mays* L. in the presence of Cu (Lin *et al.* 2008), suggesting the alleviation of toxicity of Cu to *Zea mays* L. by pyrene. In research carried out by Sun *et al.* (2011), although plant growth was affected, the presence of Cd, Pb and Cu reduced the uptake of benzo[*a*]pyrene in the ornamental plant- *Tagetes patula*. 2, 4 dichlorophenol also reduced the accumulation of zinc in the shoots of *Lolium perenne* L. (Chen *et al.* 2004) and low concentration of Cu and Cd (0.01 mg L^{-1}) increased the biodegradation of benzoate and 2-chlorophenol by 185 and 168% respectively (Kuo and Genthner 1996).

The issue of co-contamination could be addressed by using diverse plant communities. Plant diversity has shown to have an effect upon microbial community in their associated rhizosphere (Kowalchuk *et al.* 2002). For example, *Lolium perenne* L and *Medicago sativa* in combination increased the number of bacteria in the rhizosphere as well as the number of bacteria capable of degrading petroleum contamination (Kirk *et al.* 2005). Also when *Lolium perenne*, *Trifolium repens* and *Apium graveolens* were used in mixed culture in a PAH contaminated soil, the average amount of PAH remaining in soil was significantly lower than in monocultures although plant uptake contributed under 2% (Meng *et al.* 2011). However plant promoted biodegradation, which was the major pathway, increased with multiple species.

Since diversity of plant and bacteria community is mostly affected in polluted environment (Travis *et al.* 2008), it is necessary during phytoremediation, to choose plants with known capabilities of degrading or accumulating contaminants. For example, Batty and Anslow (2008) showed that the presence of Zn and pyrene significantly affected the growth of *B. juncea* and compromised its ability to accumulate Zn whereas *Festuca. arundinacea* showed no growth reduction in Zn and pyrene contaminated soil. This suggests that *F. arundinacea* could be used for remediation trials for mixed contaminated soils.

It is also important to select plants that complement each other rather than those that compete with each other. For example, when *Echinochloa crus-galli, Helianthus annuus* and *Abutilon avicennae* and *Aeschynomene indica* were used as mixed and mono-cultures in a 2,4,6-trinitrotoluene, Cd and Pb phytoremediation trial, trinitrotoulene was removed irrespective of mixed or monocultures. Moreover, more Cd was removed by mono-culture than mixed culture and there appeared to be competition as slower growth rate was reported in the mixed culture

(Lee *et al.* 2007). When different plant species interact, the normal response of a plant to a contaminant may change. For example, in a mixed culture of *Carex flava, Centaurea angustifolia* and *Salix caprea*, the negative effect of Zn in *Carex flava* was improved in the presence of *Salix caprea* (Koelbener *et al.* 2008). Very little phytoremediation trials on mixed pollutant has been carried out and examples are shown in Table 2.2.

Table 2.2: Few phytoremediation trials for mixed contaminants. All concentrations are maximum values

Pollutant	Soil concentration (mg kg^{-1})	Plant species	Growth condition	Amendment /Fertilizer	Measure of success	Reference
Pyrene, Cd	Pyrene- 100 Cd- 4.5	*Zea mays*	Glasshouse/ Spiked	Fertilized: NPK	Pyrene uptake stimulated by presence of Cd	Zhang *et al.* 2009b
Benzo[a]pyrene, Cu, Cd, Pb	B[a] P – 5 Cd- 50 Cu- 500 Pb- 3000	*Tagetes patula*	Glasshouse/ Spiked	None	Greater degradation of B[*a*]P in the presence of Cd	Sun *et al*. 2011
Pyrene, phenanthrene, Cd	Pyrene- 250 Phe- 250 Cd- 50	*Juncus subsecondus*	Glasshouse/ Spiked	Fertilized	Dissipation of PAH influenced by Cd.	Zhang *et al.* 2011

PCP, Cu	PCP- 100 Cu- 300	*Lolium perenne L*. *Raphanus sativus*	Greenhouse/ Spiked	Fertilized	Higher dissipation of PCP under 50 mg kg^{-1} with increasing Cu concentration.	Lin *et al*. 2006
Pyrene, Cu	Pyrene- 500 Cu- 400	*Zea mays L*	Greenhouse/ Spiked	Fertilized	Increasing concentration of pyrene alleviated inhibition of Cu to *Zea mays*. Also increasing concentration of Cu increased residual pyrene in soil	Lin *et al*. 2008

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3

Methodology

CHAPTER 3

Methodology

3.1 Soil localities and classification

Due to concerns about confidentiality, the exact location and full site details of study sites will not be mentioned. Two sites were surveyed, the first site was an old former vehicle service garage with diesel underground tanks in Great Shelford, Cambridgeshire undergoing clean up process at the time of visit while the other site, was a former refinery located at Swansea, South Wales under remediation by monitored natural attenuation. Both sites are redevelopment projects. Soil samples were collected in triplicates from different trial pits and points in Cambridgeshire and Swansea sites respectively and analyzed to understand the variety of contaminants present in contaminated sites. The soils used for the greenhouse plant trial experiments in Chapters 5 and 6 (Batch 1) and Chapter 7 (Batch 2) were agricultural topsoil purchased from Travis Birmingham, UK in February 2010 and are also included in Tables 3.2 and 3.4.

Figure 3.1: Sample site undergoing remedial process

3.2 Soil Sampling

Soil sample from Swansea and Cambridge sites were collected from selected sampling points and transferred into glass containers using a decontaminated hand trowel, sealed properly and kept in a box containing ice packs to maintain samples at 4 °C during transport to the laboratory. The samples were stored at 4 °C until analysis.

3.3 Characterization of soils from sites and soils used for planting

Top soil used for the present study was purchased from Travis Birmingham UK. All soils were air-dried at 20 ºC except those for PAH analysis before being ground and sieved through a 2 mm stainless steel sieve. Soils were stored in polythene bags for metal and other analysis or in glass bottles in cold rooms at 4 ºC for PAH analysis.

Soil pH: Approximately 10 g±0.1 of prepared soil sample was weighed and about 25 mLof deionized water was added, and left to stand for one hour to equilibrate (Blakemore *et al*. 1987) Soil pH in water was recorded using a potable combo probe (Hanna Instruments, Birdfordshire UK) calibrated using buffer solutions of pH 4.0 and 7.0 and pH 7.0 and 10 at 25 ºC (Table 3.1).

Electrical conductivity: Soil suspension prepared with soil and deionized water in 1:5 ratios (20 grams of soil and 100 mL of water) was allowed to stand for one hour. Soil electrical conductivity was analyzed using a potable combo probe (Hanna Instruments Bedfordshire UK).

Moisture content: about 10 g±0.01 g of soil was weighed into a crucible and placed in an oven at 105 ºC for 24hrs. Soil samples were placed in desiccators using tongs; allowed to cool and then weighed to a constant weight. The moisture content was expressed as a percentage.

$$\text{Moisture content} = (\text{mass of air} - \text{dried soil}) - (\text{mass of oven} - \text{dried soil}) \times 100$$

Table 3.1: pH, electrical conductivity and calculated moisture content for sample sites and two batches of top soil used in experiments described in Chapters 5, 6 and 7.

Soil	pH (In water)	Electrical conductivity (mS/cm)	Moisture content (%)
Topsoil batch 1 (planting trials)	6.2	640	0.29
Topsoil batch 2 (planting trials)	7.1	660	0.35
Cambridge	6.9	645	0.4
Swansea site 1	7.4	1795	0.84
Swansea site 2	6.6	1453	0.80

3.4 Plant and soil analysis using flame apart absorption spectrometry (FAAS)

All metal concentrations in the extraction methods described below were analyzed using AAnalyst 300 flame atomic absorbtion spectrophotometer (FAAS- PerkinElmer Instrument)

following a 5-point (1, 2, 3, 4 and 5 mg L^{-1}) calibration with standard solutions of Cu and (0.2, 0.4, 0.6, 0.8 and 1.0 mg L^{-1}) of Cr to be analyzed.

3.4.1 Analysis of plant samples for metal content

All plant samples (*Z. mays, B. juncea and M.* sativa, purchased from vegetable plant direct Sandford, UK) were rinsed thoroughly in deionized water to remove dust and sediments/soils contaminating their surfaces. Each plant was then divided into roots and shoots, carefully blotted dry, sealed in envelopes and dried in an oven at 65 ºC for 48 hours. After cooling in desiccators, plant was ground to a homogeneous sample and stored in a clean polythene bag prior to digestion. Sample was prepared for analysis by digesting 0.5 g of ground plant sample (or as described in individual experiments were plant growth was limited) in 5 mL of 30% HNO_3 (concentrated) and heated on a digestion block (DigiPREP MS-SCP SCIENCE) for 8hrs (up to 90 ºC). The resulting digest was made up to 15 mL with deionized water. All plant samples were analyzed for total Cu and Cr using FAAS. A reference standard (WEPAL-IPE-638 maize (plant) *Zea mays* L inorganic composition, Wageningen, Netherlands.) was used during batches of analysis to check the accuracy of the results and recovery was 89.13±2.232% and 82.73±1.903% for Cu and Cr respectively. The Translocation Factor (TF) was calculated as the ratio of root Cu/Cr concentrations to shoot Cu/Cr concentrations while the shoot and root Cr concentration factors were calculated as the ratio of Cr concentration in shoot or root of plant to the initial soil Cr concentration. The shoot/root accumulation of Cu/Cr was calculated as follows-

$$X = M \times C$$

Where

X= Shoot/root Cu or Cr accumulation (µg pot^{-1})

M= Shoot/root Cu or Cr concentration in mg kg^{-1}

C= Shoot/root dry weight of plant in kg

3.4.2 Analysis of soil samples for total metal concentration

Total recoverable Cu and Cr were extracted by Aqua regia method. About 3 g of ground oven dried soil was weighed and placed in a digestion tube. Twenty-three milli litre of concentrated HCl and 7 mL of concentrated HNO_3 were added to the soil, shaken and allowed to stand overnight. A condenser was placed over each tube and refluxed for 2 hours on a heating block (DigiPREP MS- SCP SCIENCE) at 80 °C. The tubes were allowed to cool and the resulting digest was filtered through a Whatman number 1 filter paper into a 100 mL volumetric flask. About 1 mL of 10% potassium chloride was added as an ionisation suppressant to the digest and made up to 100 mL with repeated washings of the digestion tube and filter paper. The resulting solution was stored at 4 °C until analysis by FAAS. A soil-certified reference standard (EnviroMAT-SCP SCIENCE) was used during batches of analysis to check the accuracy of results. The recovery for Cr and Cu were 94.92±2.742% and 98.83±0.427% respectively.

3.4.3 Water extractable metal in soils

The water-extractable metal content of soil was determined using methods described in Erica-andrea *et al*. (2010), where 5 g of prepared soil sample was shaken with 50 mL of deionized water in a 1:10 soil to water mixture for 2 hrs at room temperature. The solution was filtered through a Whatman number 1 filter paper and made up to 50 mL with deionized water. The water-extractable metal content was determined by FAAS.

Table 3.2: Total metal concentration for sample sites and two batches of top soil used in experiments described in Chapters 5, 6 and 7

Soil	**Cr (mg kg^{-1})**	**Cu (mg kg^{-1})**
Top soil batch 1	50.13 ±2.50	25.58 ±0.53
Top soil batch 2	16.55 ±0.99	53.24 ±1.02
Cambridge site	42.71 ±2.52	22.0 ±4.42
Swansea site 1	27.34 ±1.17	132.72 ±9.36
Swansea site 2	36.3 ±1.34	298.9 ±2.66

3.5 Soil PAH analysis by microwave extraction and gas chromatography-mass spectrometry (GC-MS)

3.5.1 Microwave extraction

Approximately 7 g of sodium sulphate (VWR Chemicals, Lutterworth UK) was added to 5 g of soil sample spiked with 40 µl of 500 ng.µl^{-1} recovery standard- *p*-terphenyl-*d14* (VWR Chemicals, Lutterworth UK) in microwave tubes. The blanks were treated in a similar way. This was followed by additions of 15 mL of hexane:acetone (2:1, v/v) solvent mixture and 5 mL triethylamine:acetone (4:1, v/v) mixture (Fischer chemical, Loughborough UK). The contents of the tubes were mixed using a vortex mixer (VWR collection) and shaken by inversion to dislodge solid material from the base. Extraction was carried out with the following conditions: temperature increased to 100 °C at 800 W for 12 minutes, held at 100 °C at 800 W for 10 minutes then cooled for 5 minutes. Following extraction, tube contents were mixed and allowed to settle. Clear extracts were transferred into vials and stored at 4 °C prior to clean-up and GC-MS analysis.

3.5.2 Clean up

PAH clean up was carried out using 2 g, 12 mL silica gel cartridges (Agilient, Wokingham UK). The silica gel cartridges were conditioned using 5 mL of hexane (Analytical grade VWR chemicals, Lutterworth UK). One ml of soil extract was filtered through the silica gel cartridge followed by the addition of 10 mL 1:1 hexane:dichloromethane mixture, and allowed to flow through the column until it reaches the frit. Sample blanks were also allowed to pass through the same process. The extract was concentrated by evaporation of the

dichloromethane under a stream of nitrogen and the residue was dissolved in hexane with a final volume of 1.0 mL for GC analysis.

3.5.3 GC-MS analysis

Concentrations of the 16 priority PAHs were analysed by an Agilent gas chromatography equipped with a mass spectrometer detector (Agilent Technologies 6890N). A HP 5MS fused silica capillary column with dimensions 30 m by 0.25 mm by 0.25 um film thickness was used. The GC-MS operating condition for USEPA method 8270D (mass range 35 to 500 amu, scan time: #1 sec/scan, initial temperature: 40 °C, held for 4 minutes, temperature program: 40 to 320 °C at 10 °C/min, final temperature: 320 °C, held for 2 minutes after benzo [g,h,i]perylene eluted, transfer line and injector temperature: 250 to 300 °C) was used with helium as a carrier gas at a constant flow rate of 30 cm/sec. The GC-MS was calibrated with RESTEK NJDEP EPH Aromatics Calibration Standard (2,000 µg ml^{-1} each of 16 Priority PAHs >98% purity in dichloromethane) internal standards mix (1,4-dichlorobenzene-*d4*, naphthalene-*d8*, acenaphthene-*d10*, phenanthrene-*d10*, chrysene-*d12* and perylene-*d12*) and surrogate standard, *p*-terphenyl-*d14* (Sigma chemical Co. UK). A six-point calibration standard of 50 pg $µl^{-1}$, 200 pg ul^{-1}, 500 pg $µl^{-1}$, 1000 pgul^{-1}, 5000 pg $µl^{-1}$ and 10000 pg $µl^{-1}$ was carried out. Quality controls were also set up with solvent blanks and matrix spikes controls. The concentration of each PAH in sample was determined by the MSD chemstation software based on the following calculation

$$x\ (pg.ul) = \frac{A(x) \times C(i) \times V(e) \times D}{A(i) \times RF(x) \times W(s)}$$

Where:

A(x) – area under the chromatogram of component x ($pg.\mu l^{-1}$)

A (i) – area under the chromatogram of the internal standard ($pg.\mu l^{-1}$)

C (i) –concentration of internal standard ($pg.\mu l^{-1}$)

W (s) – weight of soil sample extracted (g)

RF (x) – response factor of component of interest

V (e) – volume of extract (ml)

D –dilution factor, if further dilution is required

Table 3.3: Detection limits of GC-MS for 16 priority PAHs

Compounds	Detection limit ($pg.\mu l^{-1}$)
Naphthalene	0
Acenaphthylene	1.06
Acenaphthene	0
Fluorene	0.5
Phenanthrene	0
Anthracene	0.79
Fluoranthene	0.93
Pyrene	1.03
Benzo[*a*] anthracene	40.04
Chrysene	0
Benzo[*b*] fluoranthene	1.69

Benzo[*k*] fluoranthene	1.66
Benzo[*a*] pyrene	0.9
Indo [*1,2,3*] pyrene	0.67
Dibenzo[*a,h*] anthracene	0.26
Benzo [*g,h,i*] perylene	0.63

Table 3.4: Mean concentrations of 16 priority PAHs for sample sites and two batches of top soil used in experiments described in chapters 5, 6 and 7.

Compounds	Topsoil batch 1 (mg kg^{-1})	Topsoil batch 2 (mg kg^{-1})	Swansea 1 (mg kg^{-1})	Swansea 2 (mg kg^{-1})	Cambridge site (mg kg^{-1})
Naphthalene	N.D	N.D	N.D	N.D	0.17±0.01
Acenaphthylene	N.D	N.D	N.D	0.26±0.13	N.D
Acenaphthene	N.D	N.D	N.D	N.D	N.D
Fluorene	N.D	N.D	N.D	N.D	0.30±0.01
Phenanthrene	N.D	N.D	N.D	N.D	1.12±0.07
Anthracene	N.D	N.D	0.42±0.02	0.21±0.11	1.65±0.07
Fluoranthene	N.D	N.D	0.81±0.34	0.51±0.36	1.49±0.07
Pyrene	N.D	0.49±0.05	0.95±0.52	2.06±0.15	1.14±0.05
Benzo[*a*] anthracene	7.33±0.03	N.D	0.63	1.48±0.05	1.30±0.07
Chrysene	N.D	N.D	0.15±0.14	1.27±0.4	0.60±0.02
Benzo[*b*] fluoranthene	N.D	N.D	0.17±0.17	0.64±0.23	0.41±0.08
Benzo[*k*] fluoranthene	N.D	N.D	0.26±0.26	0.77±0.19	0.60±0.07
Benzo[*a*] pyrene	9.33±0.02	0.48±0.02	0.75±0.02	1.23±0.41	0.94±0.02
Indo [*1,2,3*]pyrene	N.D	N.D	0.67	0.95±0.29	N.D
Dibenzo[*a,h*] anthracene	N.D	N.D	N.D	N.D	N.D
Benzo [*g,h,i*] perylene	N.D	N.D	0.66	0.89±0.32	N.D

3.5.4 Experimental design and statistical analysis

The experimental layout for the greenhouse and germination studies was designed in a completely randomized design, where individual treatments are replicated 3 times as discussed for individual experiments in sections 4.1.1, 4.6.1, 5.2.2, 5.7.2, 6.1.2, 6.6.2, 7.2.2 and 7.7.2. ANOVA was used for the germination experiments instead of non-parametric tests because of the number of replicates and also because non-parametric tests are robust only when there are more than 3 replicates. One way ANOVA using the Minitab 15.0 statistical software was used for the analysis of data in all studies except for ageing experiments (Chapter 6) where two-way ANOVA with SPSS 20 was used to compare the effects of soil ageing and fresh soil within treatments during phytoremediation. The ANOVA is robust in analysis when data is normal, hence data that are not normally distributed were normalized using the log transformation when required and as discussed in individual chapters. Similarly, seed germination percentages were transformed using the arcsin conversion before analysis. This was done because percentages cannot be less than zero or more than 100 (have fixed limits), and data should be normally distributed and free to vary widely about the mean without imposed limits. When a significant difference is observed, multiple comparisons were made using Tukey Honestly Significant Difference (HSD). The mean of the the three sample replicates for each study was used for graphical representation and the standard error of mean was calculated with the following equation:

$$Standard\ error\ of\ mean\ = \frac{Standard\ deviation\ of\ mean}{\sqrt{n}}$$

Where, n= number of replicates

Some of the results in this study showed low standard error of means. Although the sampling error of mean should decrease or lower as the size of the random samples increases, it should be noted that as the variability on the treatments reduces, the sampling error reduces as well. QC procedures were undertaken for all analysis (see Section 3.4.1) and therefore there is no justification for not accepting the data despite a smaller error than might be expected with 3 replicates.

4

Single and joint toxicity of metal and PAH on early seedling growth of plants

4.0 Single and joint toxicity of Cu and pyrene on seedling growth of *L. perenne* using the water culture method- Introduction

Concerns regarding the toxicity effect of chemicals present in the environment have increased in recent years leading to additional efforts to provide an early evaluation method for their potential toxicity (Lee *et al*. 2000, Banni *et al*. 2009).

Copper (Cu) is a phytotoxic micronutrient when the concentration is above the macromolar level (Marschner 1995). The seed is well protected against various stresses, however after subsequent seedling emergence, they become stress-sensitive (Li *et al*. 2005). The toxicity effect of Cu on plants is first observed in the root while the translocation to the shoot is effectively restricted by large accumulation in the root. Therefore it is expected that rhizotoxicity will precede toxicity to shoot (Sheldon and Menzies 2005, Michaud *et al*. 2008,). Increasing concentration of Cu in plants induces oxidative stress, results in membrane damage and inhibits photosynthetic activity (Quartacci *et al*. 2000). Germination is an important stage in plant growth and is very sensitive to contaminants (Maila and Cloete, 2002) most especially heavy metal pollution (Jadia and Fulukar 2008).

Pyrene is one of the polycyclic aromatic hydrocarbons that have both mutagenic and tetragenic properties (Haritash and Kaushik 2009). They are persistent in the environment and their contamination is becoming prevalent due to increased industrialization (Srogi 2007). The effect of pyrene on plants includes morphological symptoms such as reduced growth and chlorosis, as well as physiological symptoms including oxidative stress induction, DNA damage and cell death (Alkio *et al.* 2005, Oguntimehin *et al.* 2010).

Although the individual toxicity of pyrene and Cu to plants has been investigated by researchers (Alkio *et al.* 2005, Li *et al*. 2005), pyrene and Cu often co-exist in the environment, particularly in contaminated situations. Recently, different researchers (Lin *et al.* 2008, Almeida *et al*. 2009) have tried to investigate the phytoremediation potential of plants to Cu and pyrene co-contaminated soil. However, the effect of contaminants on early plant growth needs to be investigated to better understand the early effects of co-

contamination. Hence the aim of this study is to determine the tolerance of *L. perenne* to co-contamination of Cu and pyrene by understanding their effect on germination as well as root and shoot length. The results of this research would provide valuable information for the application of *L. perenne* in Cu and pyrene co-contamination remediation.

4.1 Methods

4.1.1 Materials

All reagents used in this study are analytic grade. Seeds of *L. perenne* were commercially available from Vegetable Direct Seeds Co. Ltd, England. Pyrene and Cu_2SO_4 were supplied by VWR chemicals Lutterworth UK. Test solutions of pyrene were prepared by dissolving in 0.1% acetone and making up with deionized water while Cu_2SO_4 was prepared with deionized water only. Control treatments were also exposed to 0.1% acetone. Three replicates were set for each concentration.

4.1.2 Seed germination and root and shoot elongation tests.

Seeds of *L. perenne* were sterilized in 6% (v/v) hydrogen peroxide for 15 minutes and washed with tap water. Ten seeds of *L. perenne* were carefully placed on each petri dish and moistened with 3 mL of toxicants solution. The concentration of pyrene was set at 0, 1, 2, 3 and 4 mg L^{-1} and the tested concentration of Cu was 0, 2.15, 4.3, 8.6 and 12.9 mg L^{-1}. Petri dishes were sealed with Para film and allowed to germinate under daylight at 24 ±2 °C in the glasshouse for 9 days. First seed emergence was monitored on daily basis and germination percentage calculated on the 6^{th} day. A 1 mm radical emergence was considered as seed

germination. Seedling development was regarded as inhibited when the seed coat was visibly broken with the embryo not growing further. On the 9th day, germinated seeds were used for root and shoot length measurement. The Germination Rate Index (GI) was determined by the following formula,

GI = $\frac{G3}{3} + \frac{G6}{6} + \frac{G9}{9}$ (Ali 2007)

Where G3, G6 and G9 are germination percentages at 3, 6 and 9 days after initiation of germination

4.1.3 Statistical analysis

Statistical analysis including calculation of average values, standard error (S.E) was calculated by Microsoft office Excel 2007. One-way analysis of variance was carried out with Minitab 15. The shoot length results were log transformed prior to analysis. When a significant ($p< 0.05$) difference was observed between treatments, multiple comparisons were made by the Tukey test.

4.2 Results

4.2.1 Effects of Cu on seed germination

The final germination percentage reduced from 100 to 44% relative to control treatments as the concentration of Cu in solution increased from 2.15 to 12.9 mg L^{-1} (Figure 4.1). The final germination percentage of seeds of *L. perenne* on different concentrations of Cu showed no inhibition for the lowest concentration (2.15 mg L^{-1}) used in this study and a

slight but non- significant ($p>0.05$) 3% inhibition at 4.3 mg L^{-1} Cu^{2+} in solution. Germination was also inhibited by 37 and 50% for 8.6 and 12.9 mg L^{-1} culture medium respectively Relative to the control treatments, the average toxicity was highest for the highest concentration of Cu, and decreased to 3.7% as the concentration of Cu reduced to 4.3 mg L^{-1}. There was no total inhibition of seed germination in all concentration of Cu. One-way ANOVA showed that the inhibition of germination when the concentration of Cu in the growth medium was 2.15 and 4.3 mg L^{-1} were not significant ($p> 0.05$) but were significant when concentration increased to 8.6 and 12.9 mg L^{-1} cu.

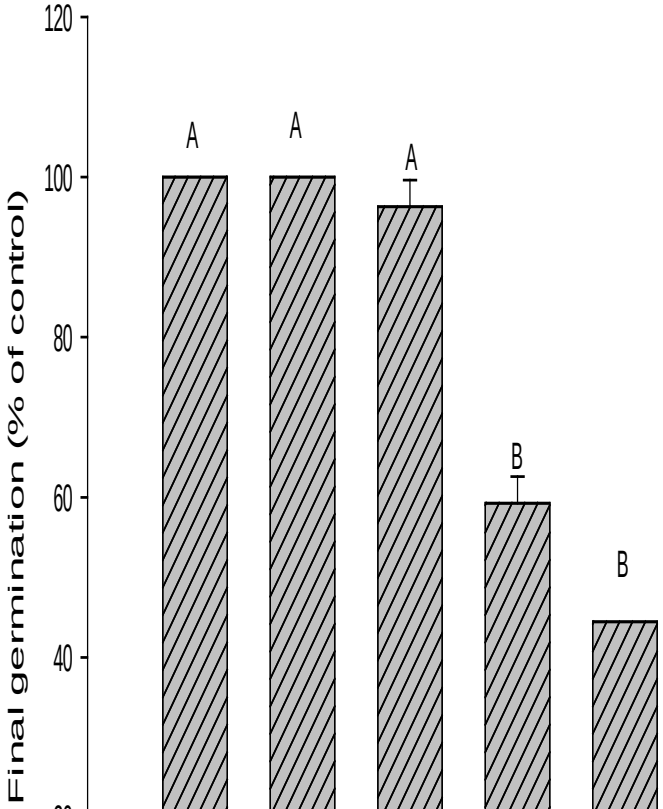

Figure 1**Figure 4.1:** Effect of Cu on final germination relative to control of seeds of *Lolium perenne*. Bars (mean ± SE, n= 3) followed by the different letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 4A.1

Over a 9-day germination period, the germination index (GI) significantly decreased with an increase in Cu concentration in solution. . Lower concentrations of Cu (2.15 and 4.3 mg L^{-1}) did not seem to affect the germination rate index, but there were significant differences a at 8.6 mg L^{-1} Cu or more.

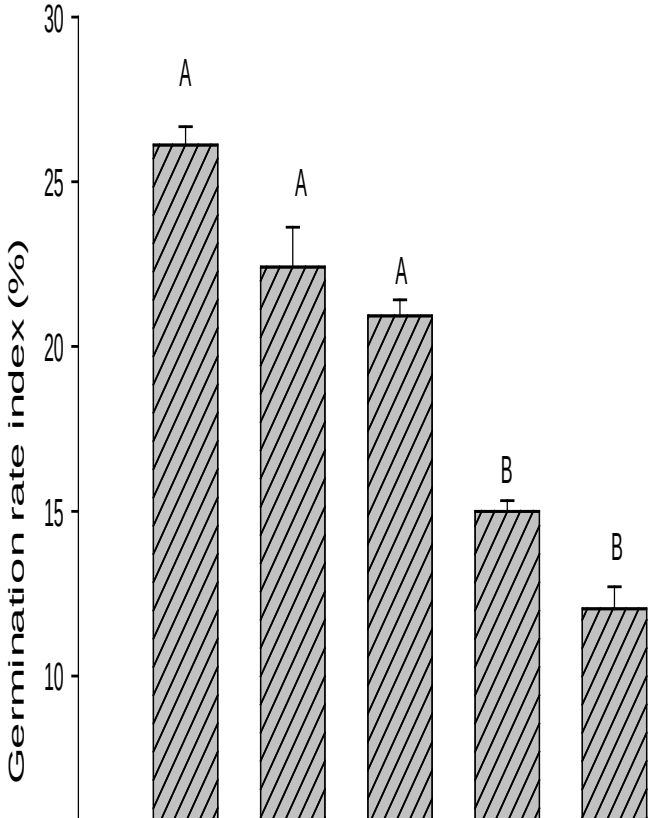

Figure 4.2: Effect of different concentrations of Cu on germination rate index relative to control of seeds of *L. perenne*. Different letters indicate a significant difference at P< 0.05 according to the Tukey- HSD. Appendix 4A.2

4.2.2 Effect of Cu on seedling growth of *L. perenne*

The effect of various Cu concentrations on the shoot and root length of *L. perenne* are shown in figures 4.3 and 4.4. The shoot length and root length in the present study was

expressed as a percentage of the growth of the control plants. Relative to control treatments, the shoot length of *L. perenne* was adversely affected by Cu and results showed a 19.2 and 39.6% significant ($p<0.05$) inhibition for 2.15 to 12.9 mg L^{-1} Cu contaminated media. There was a direct relationship between the increase in Cu concentration in solution and the severity of the response and the correlation coefficient reached -0.96. Similar results were observed for roots,. but the root length inhibition was higher than the shoot length inhibition. The present result showed that as the concentration of Cu in solution increased from 2.15 to 12.9 mg L^{-1}, the inhibition to the root length of *L. perenne* increased significantly ($p<0.05$) from 29 to 83.6% relative to control treatments. The reduction in the root length of *L. perenne* exposed to increasing concentration of Cu was ≥ 1.5 times that of the inhibition to the shoot.

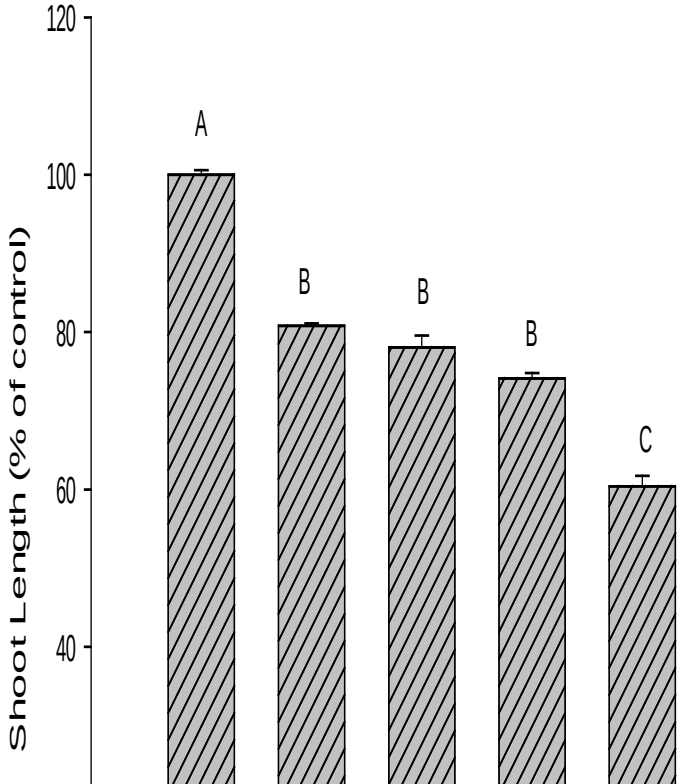

Figure 4.3: Effect of Cu on shoot length relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at $p < 0.05$ according to the Tukey- HSD. Appendix 4A.3

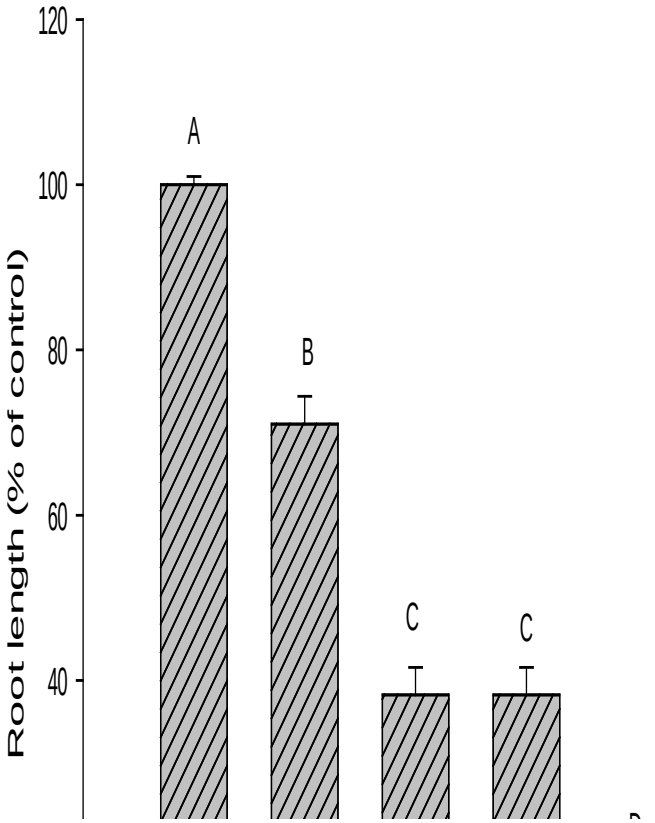

Figure 4.4: Effect of Cu on shoot length relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at p< 0.05 according to the Tukey- HSD. Appendix 4A.4

4.2.3 Effect of pyrene on seed germination

Pyrene seemed to have no effect on the germination rate of seeds of *L. perenne* (Figure 4.6). There were no significant differences with increasing concentration of pyrene in solution. The germination rate index over the 9-day period of germination was affected by

pyrene contamination and results in an 8.5-11.5% significant ($p<0.05$) inhibition as the pyrene concentration increased from 1-4mg L^{-1}.

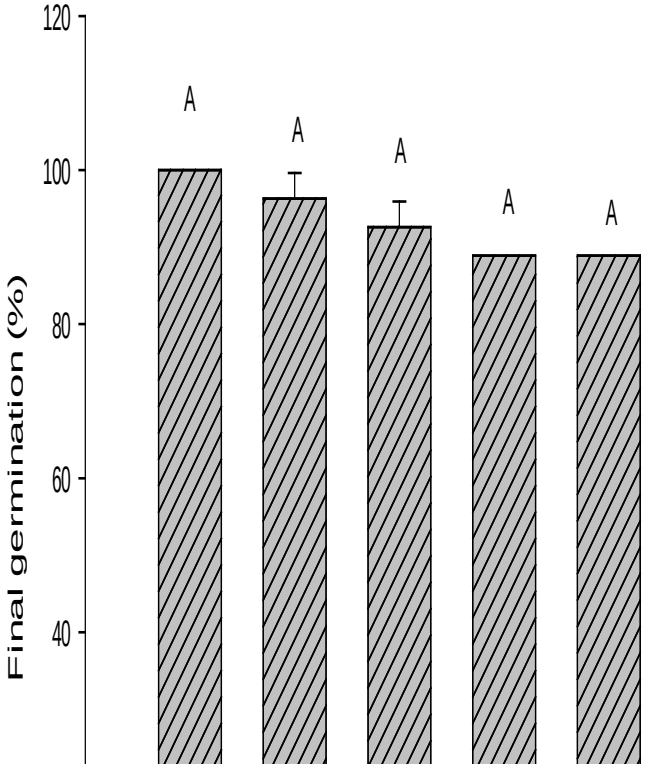

Figure 4.5: Effect of pyrene on final germination relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at $p< 0.05$ according to the Tukey- HSD. Appendix 4A.1

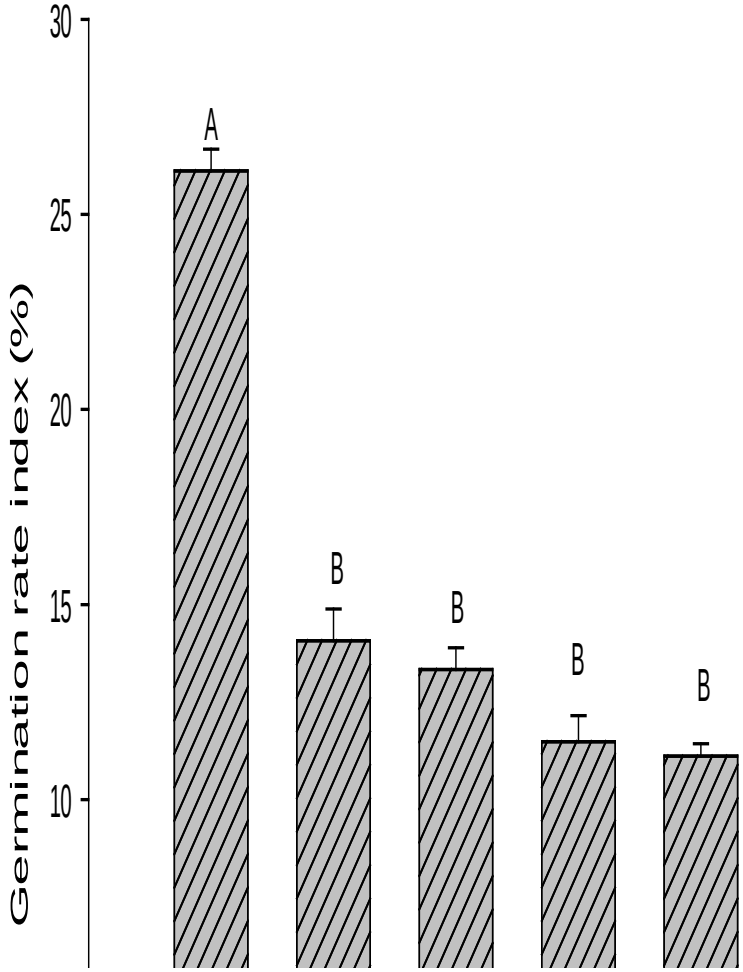

Figure 4.6: Effect of different concentrations of pyrene on germination rate index relative to control of seeds of *L. perenne*. Different letters indicate a significant difference at P< 0.05 according to the Tukey- HSD. Appendix 4A.2

4.2.4 Effect of pyrene on seedling growth of *L. perenne*

The shoot and root growth of *L. perenne* was inhibited by different concentrations of pyrene in the present study (Figures 4.7 and 4.8). Results showed that the shoot length of *L. perenne* decreased significantly ($p<0.05$) from 31 to 52% as the concentration of pyrene increased from 1 to 4 mg L^{-1}. . The correlation coefficient was used to understand the linear relationship between the concentration of pyrene and the inhibition of shoot length. The inhibition of the shoot length of *L. perenne* was negatively correlated with increasing concentration of pyrene in solution and the correlation coefficient reached -0.992.

The root length inhibition of *L. perenne* varied with increasing concentration of pyrene (Figure 4.8). As the concentration of pyrene in solution culture increased from 1 to 4 mg L^{-1}, the root length inhibition increased significantly ($p<0.05$) from 52 to 71% relative to control treatments. However, it was clear that as the concentration of pyrene in solution culture increased to 2 mg L^{-1}, the inhibition of the root length relative to control treatments remained at 66% and further increase in the concentration of pyrene to 3 and 4 mg L^{-1}, did not seem to further inhibit the root length ($p>0.05$).

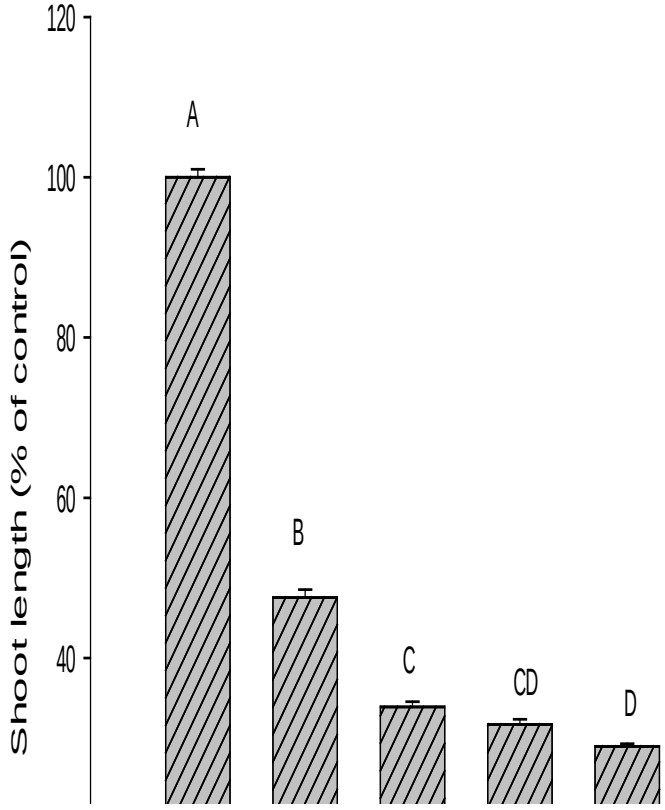

Figure 4.7: Effect of pyrene on shoot length relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at $p < 0.05$ according to the Tukey- HSD. Appendix 4A.3

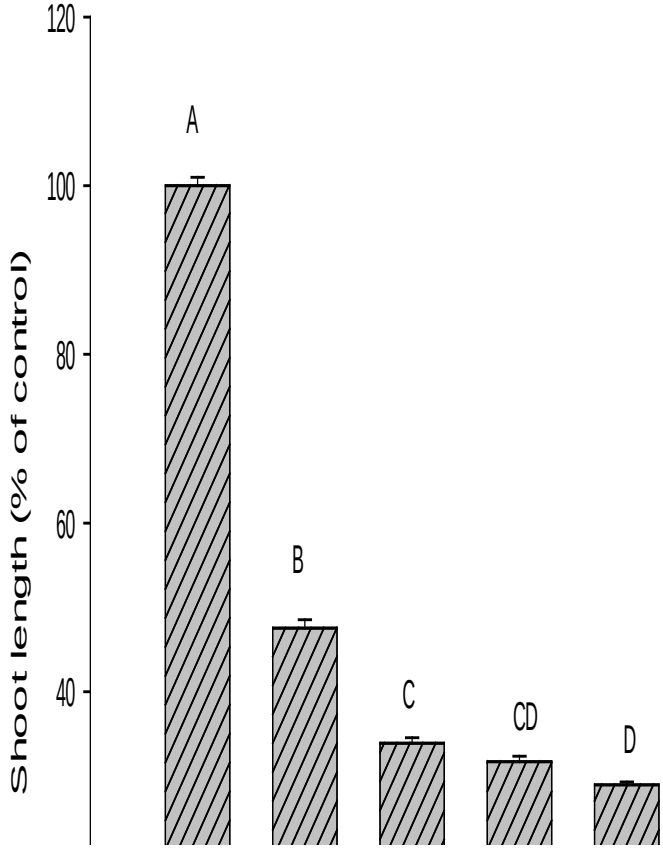

Figure 4.8: Effect of pyrene on root length relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at $p < 0.05$ according to the Tukey- HSD.

Appendix 4A.4

4.2.5 Joint effect of Cu and pyrene on seed germination

The joint toxicity of Cu and pyrene on the final germination percentage was different from that of single effects of Cu or pyrene. The final germination rate was inhibited more than in single contamination of Cu or pyrene. From Figure 4.9, it can be seen that when the concentration of Cu in solution remained at 2.15 mg L^{-1}, the final germination of *L. perenne* increased significantly ($p<0.05$) from 48 to 74% relative to control treatments with an increase in pyrene concentration from 1 to 4 mg L^{-1}. The relative toxicity based on final germination percentage reduced from 52 to 26%. Similarly, when the concentration of Cu in solution was increased to 4.3 and 8.6 mg L^{-1}, the final germination rate of *L. perenne* increased significantly ($p<0.05$) from 51 to 70% and 41 to 63% respectively as the concentration of pyrene in solution increased from 1 to 4 mg L^{-1}. The relative toxicity also reduced from 48 to 29% and 59 to 37% respectively. However at 12.9 mg L^{-1} fixed concentration of Cu, the final germination percentage of *L. perenne* reduced with increased concentration of pyrene. The present results showed a 48 to 33% reduction in final germination percentage as the concentration of pyrene increased from 1 to 4 mg L^{-1} which is a 51 to 66% increase in relative toxicity based on the final germination percentage.

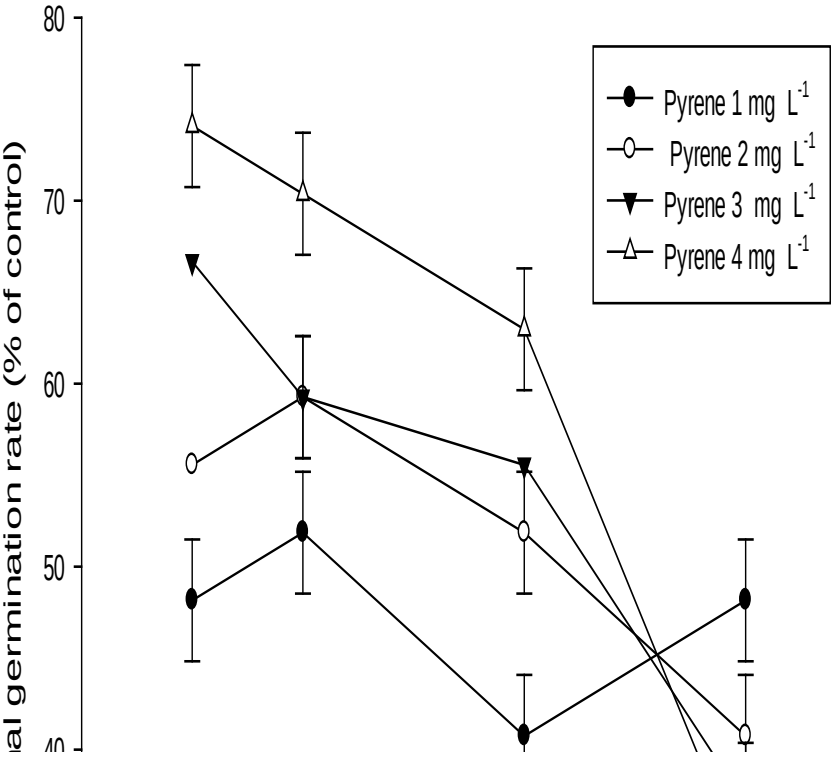

Figure 4.9: Joint effect of fixed Cu and pyrene on final germination rate of seeds of *L. perenne*. Error bars are standard error. Appendix 4A.1

Similarly, when the fixed concentration of pyrene remained at 1 and 2 mg L^{-1}, the final germination rate was similar with increasing concentration of Cu in solution. However, as the fixed concentration of pyrene increased to 3 and 4 mg L^{-1}, the effect of added Cu on final germination rate varied. The present results showed that when the fixed concentration of pyrene remained at 3 mg L^{-1} (Figure 4.10), the final germination rate was similar with the addition of 2.15, 4.3 and 8.6 mg $L^{-1}$of Cu, but was significantly decreased to 37% when 12.9 mg L^{-1} of Cu was added. Also when the concentration of pyrene increased to 4 mg L^{-1}, the final germination rate of *L. perenne* was similar when 2.15, 4.3 or 8.6 mg L^{-1} of Cu was added, while the addition of 12.9 mg L^{-1} of Cu significantly ($p<0.05$) decreased the germination rate to 30%.

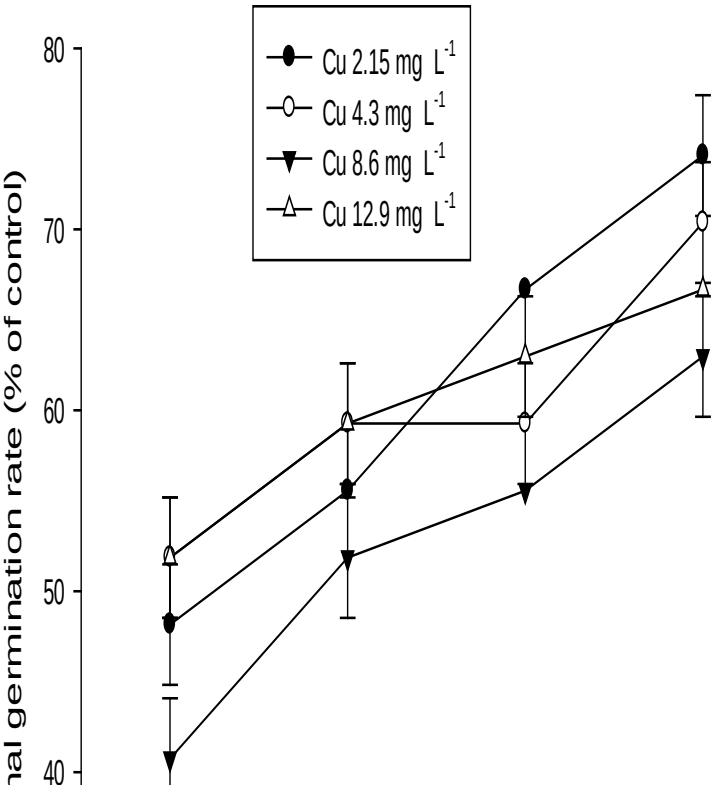

Figure 4.10: Joint effect of fixed pyrene and Cu on final germination rate of seeds of *L. perenne*. Error bars are standard error. Appendix 4A.1

4.2.6 Joint toxicity of Cu and pyrene on seedling growth of *L. perenne*

The nature of the joint effects of Cu and pyrene on shoot and root growth was strongly dependent on their concentrations in the mixture (Figure 4.11 and 4.11). The root growth in joint contamination was severely inhibited when compared to single concentration of Cu or pyrene. Roots were very stunted and there were no root hairs present. Compared to single contamination with 2.15 mg L^{-1} of Cu, the addition of 1, 2, 3 and 4 mg L^{-1} of pyrene significantly ($p<0.05$) decreased the shoot length of *L. perenne*. The present results showed that in solution containing only 2.15 mg L^{-1} of Cu, the shoot length of *L. perenne* remained at 85 mm while with the addition of 1, 2, 3 and 4 mg L^{-1} of pyrene, the shoot length decreased significantly ($p<0.05$) by over 58%. However, the increasing concentration of pyrene from 1 to 4 mg L^{-1} in the presence of 2.15 mg L^{-1} of Cu did not significantly ($p>0.05$) affect the shoot length. In contrast, when the concentration of Cu remained at 4.3 mg L^{-1}, the shoot length of *L. perenne* decreased to 78% relative to control treatments, while the addition of 1, 2, 3 and 4 mg L^{-1} of pyrene significantly decreased the shoot length to 34, 33, 31 and 30% respectively. At 8.6 mg L^{-1} fixed Cu concentration, the shoot length of *L. perenne* remained at 63 mm and decreased by over 65% with the addition of 1 to 4 mg L^{-1} of pyrene. From the present result, it is clear that increasing the concentration of pyrene in 4.3 and 8.6 mg L^{-1} fixed Cu concentrations did not significantly affect the shoot length of *L. perenne*. In contrast, when the fixed concentration of Cu increased to 12.9 mg L^{-1}, increasing the pyrene concentration to 4 mg L^{-1} significantly decreased the shoot length by 14% when compared to the addition of 1, 2 or 3 mg L^{-1} of pyrene.

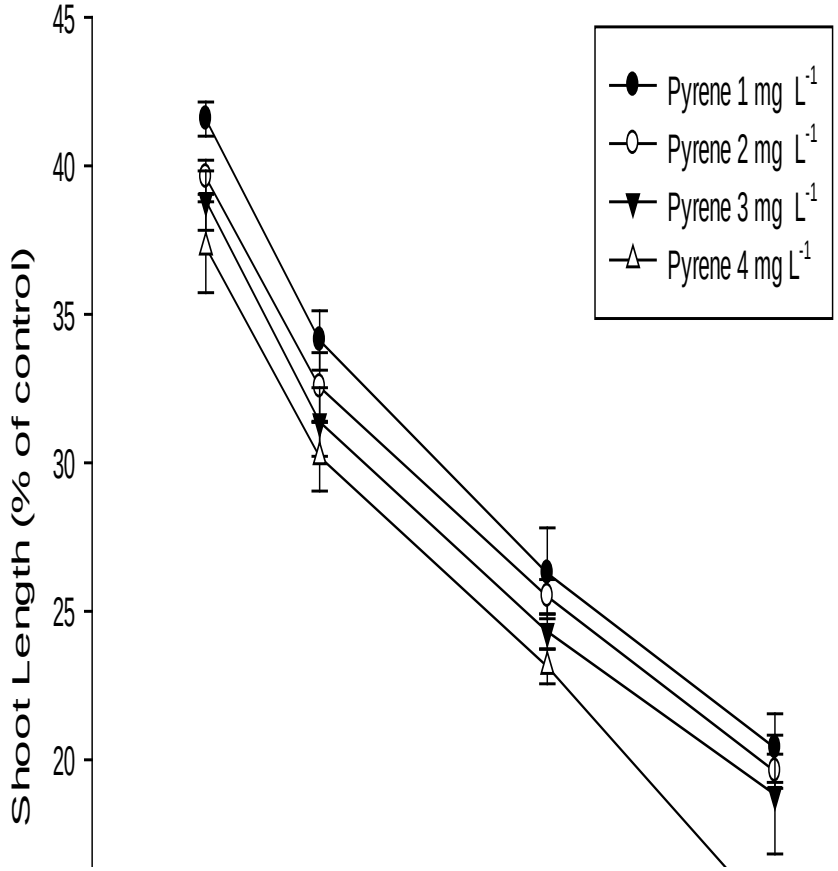

Figure 4.11: Joint effect of fixed Cu and pyrene on shoot length relative to control of seeds of *L. perenne.* Error bars are standard error. Appendix 4A.3

With fixed pyrene concentration, notable differences occurred. It is clear from the results that mixed application of Cu and pyrene significantly decreased the shoot length of *L. perenne* when compared to individual application of pyrene in all treatments. Figure 4.12 showed that for all fixed concentration of pyrene, increasing the concentration of added

Cu significantly reduced the shoot length of *L. perenne*. From the results, it was shown that when the concentration of pyrene was fixed at 1 mg L^{-1}, the addition of 2.15 to 12.9 mg L^{-1} of Cu significantly reduced the shoot length of *L. perenne* from 41.56 to 20.39% relative to control treatments. Similarly, at 2, 3 and 4 mg L^{-1} fixed concentration of pyrene, the addition of 2.15 to 12.9 mg L^{-1} of Cu significantly decreased the shoot length from 39.6 to19.6%, 38.8 to 18.8% and 37.25 to 14.50% respectively relative to control treatments.

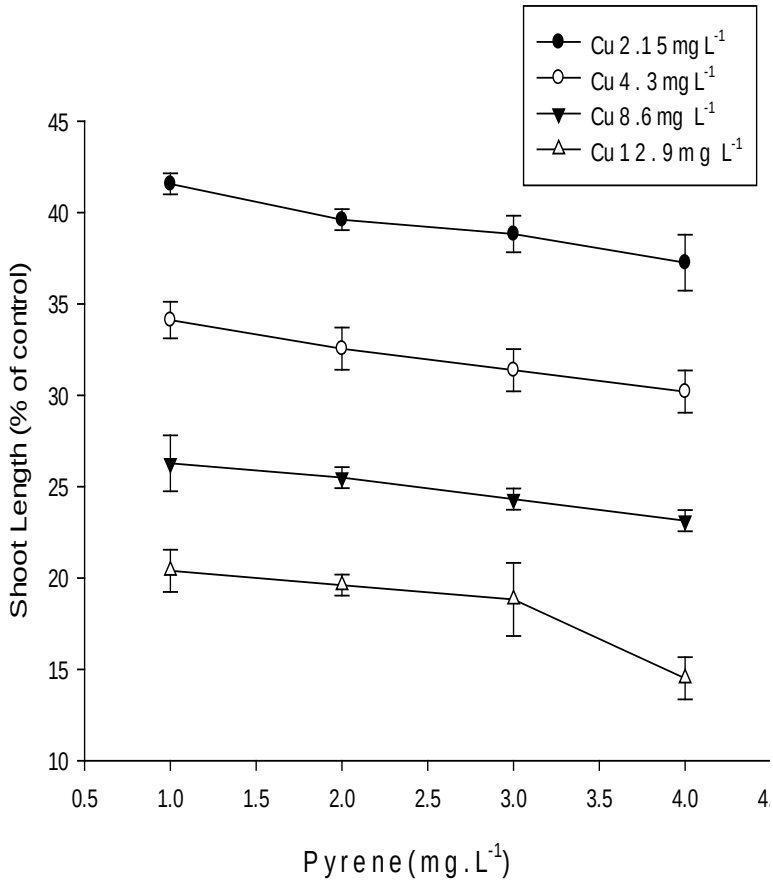

Figure 4.12: Joint effect of fixed pyrene and Cu on shoot length relative to control of seeds of *L. perenne.* Error bars are standard error. Appendix 4A.3

The root length inhibition of *L. perenne* varied with joint contamination. When the concentration of Cu in solution was fixed at 2.15 and 8.6 mg L^{-1}, the inhibition to the root length of *L. perenne* significantly increased respectively from 69 to 77% and 68 to 86.9% relative to control treatments as the concentration of pyrene increased from 1 to 4 mg L^{-1}. In contrast, when fixed Cu concentration in solution reached 4.3 and 12.9 mg L^{-1}, the inhibition to the root length was only significant compared to single Cu contaminated solution when the pyrene concentration reached 4 mg L^{-1}. Results showed an 85% and 97.3% inhibition relative to control treatments for 4.3 and 12.9 mg L^{-1} fixed Cu concentration respectively (Figure 4.13).

The root inhibition of *L. perenne* when the concentration of pyrene was fixed with varying Cu concentration is shown in Figure 4.14. When the concentration of pyrene was fixed at 1 mg L^{-1} and 4 mg L^{-1}, the inhibition to the root length of *L. perenne* increased from 69 to 88% and 77.6 to 97.26% respectively relative to control treatments as the concentration of Cu increased from 2.15 to 12.9 mg L^{-1}. The increased root length inhibition was significant ($p<0.05$) when compared to single pyrene contaminated solution. At 2 and 3 mg L^{-1} fixed pyrene concentration, lower concentration of Cu did not seem to affect root length inhibition. However, when the concentration of pyrene increased to 12.9 mg L^{-1}, the root length of *L. perenne* was significantly inhibited by 92.9 and 95.6% respectively relative to control treatments.

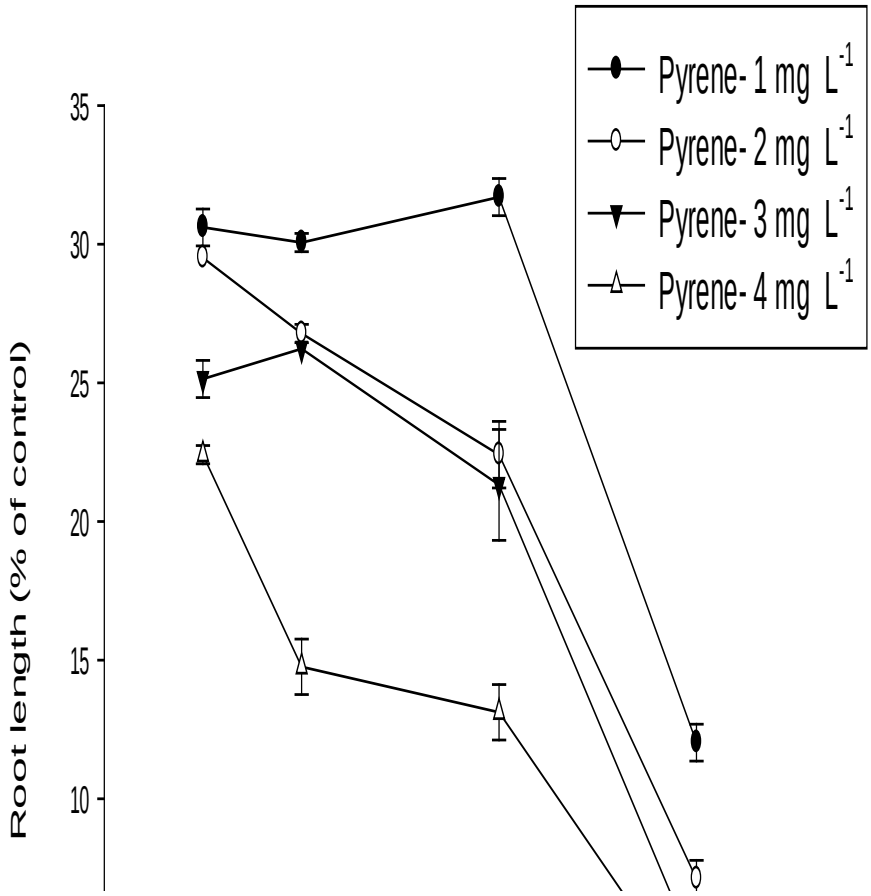

Figure 4.13: Joint effect of fixed Cu and pyrene on root length relative to control of seeds of *L. perenne*. Error bars are standard error. Appendix 4A.4

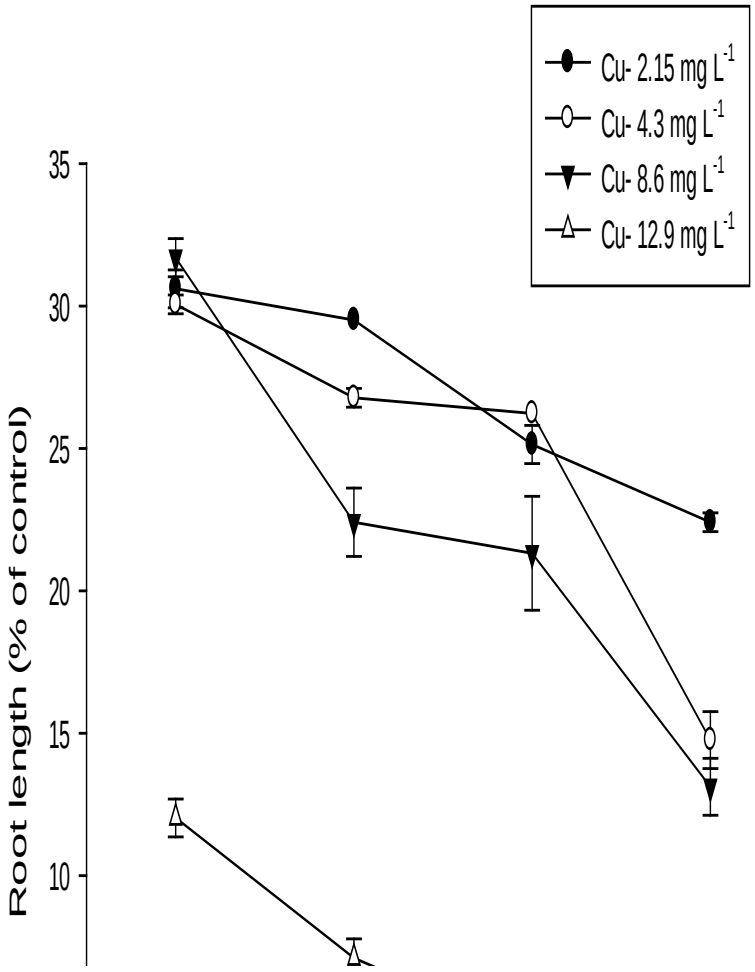

Figure 4.14: Joint effect of fixed pyrene and Cu on root length relative to control of seeds of *L. perenne*. Error bars are standard error. Appendix 4A.4

4.3 Discussion

4.3.1 Toxicity effect of single Cu

The incubation of seeds on filter paper soaked with metal or PAH is a common method that reduces the effects of other metals or PAHs that could be present in a natural soil as they may be synergistic or antagonistic to the effect of the metal or PAH in the present study (Munzuroglu and Geckil 2002). The effects of metals and PAHs on the development of plants can be understood by determining the germination characteristics of the seed. In the present study, Cu applied as $CuSO_4$ caused a decrease/delay in the germination of *L. perenne* only when the concentration of Cu in solution increased up to 8.6 mg L^{-1}. However there was no complete inhibition of germination even for the highest Cu concentration used (Figure 4.1). The present result is in line with previous studies on the effect of Cu on plants. For example, Cu was not as toxic to seed germination as it was to root and shoot growth (Munzuroglu and Geckil 2002, Li *et al*. 2005). The difference in the vigour between the control treatments and the increasing concentration of Cu was measured and shown to be significant in 2 ways. Firstly, the shoot length after 9 days of culture was significantly greater in control treatments than in increasing concentration of Cu (Figure 4.3). Secondly, the root length was also significantly lower in Cu treatments when compared to control treatments (Figure 4.4). At concentrations below 8.6 mg L^{-1} of Cu, the seed germination of *L. perenne* was similar to control treatments and only decreased when the concentration of Cu $\geq$ 8.6 mg L^{-1}. In low concentration, Cu acts like a micronutrient for the plant. Therefore when in low concentrations, it could accelerate the

germination of seeds while in high concentration, it can induce toxicity in a way that germination is inhibited.

The inhibition of root elongation is considered as the initial effects of the toxicity of metals to plants. In the present study, the increasing concentration of Cu in solution significantly decreased the root length of *L. perenne* (Figure 4.4). Previous reports have shown that the most important mechanism of Cu toxicity include- the penetration of Cu to cell membrane, blocking of receptors that help during photosynthesis as well as binding to receptors in the chloroplast (Stauber and Florenece 1987). In this study, there were no observed chloritic symptoms in the shoot of *L. perenne* over the 9-day period. As suggested by Arduini *et al*. (1994) and Muller *et al*. (2001), the primary route of Cu uptake is through the roots and this could be responsible for the decreased root length as observed in the present study. When this happens, cell division and elongation are inhibited in the root tip as well as the extension zones. Similar results have been observed by other researchers. For example, Singh *et al*. (2007) showed that after 14 days exposure of *Triticum aestivum* to 5, 25, 50 and 100 mg L^{-1} of Cu stress, the seed germination, root and shoot elongation and number of lateral roots were inhibited. Although the concentration of Cu used in their study was higher than in the present study, they however reported toxicity of Cu at 5 mg L^{-1} concentration. Also, Jiang (2001) and Doncheva (1998) showed that Cu is toxic to the morphology of chromosomes and that nuclei formation at the G_1/S transition points of the cell cycle is interrupted by Cu, preventing their entry into mitosis. When Cu affects the proliferation of the root meristem cells, root reduction occurs. Because this was carried out in solution culture, the reduction or

inhibition of root hair proliferation would not have affected the ability of *L. perenne* access to nutrients. However, if soil was the medium of growth, the root surface area is very important for uptake of nutrients and any significant reduction in root hair may affect plant growth (Sheldon and Menzies 2004). Yadav and Srivastava (1998) observed different types of mitotic aberration and concluded that Cd inhibits the mitotic index for plants such as *Hordeum vulgare* and *Setaria italic*.

4.3.2 Effectffect of single pyrene

According to Huang *et al*. (1996) and Ren *et al*. (1996), PAH phytotoxicity was shown to be a physiological toxicity. In this study, the germination rate of seeds of *L. perenne* was not inhibited by different concentrations of pyrene (Figure 4.5). This indicates that at the end of the germination tests (9 days), pyrene did not show any phytotoxic effect on seed germination. This is similar to work carried out by Henner *et al*. (1999) which showed that the presence of high molecular weight PAHs including phenanthrene and B[*a*]P were not phytotoxic (for germination) to *L. perenne* at the end of a 9-day germination tests. In contrast, the root and shoot lengths of *L perenne* were inhibited with increasing concentration of pyrene (Figures 4.7 and 4.9). This is in line with work carried out by Ahammed *et al*. (2012) which showed that pyrene exposure resulted in a dose-dependent increase in the inhibition of the shoot and root length of *S. lycopersicum*. Therefore there is a suggestion that the inhibition to the root and shoot length of *L. perenne* in the present study clearly indicates that *L. perenne* is sensitive to PAH stress. The toxicity of pyrene to *L. perenne* was dependent on the concentration of pyrene in solution. Although in the present study, it was more evident in the root systems of the plants. Because pyrene is a

hydrophobic compound and is likely to partition into lipid membrane (Edwards 1983), there is an expectation that it will accumulate in the membrane of the root systems first and only after saturation, will pyrene be moved up to the shoot. As the germination of *L. perenne* was not affected by the pyrene in solution, it could be assumed that the reduction in root length observed in the present study cannot be linked to delay in germination. Taiz and Zeiger (1991) showed that root growth is primarily due to cell expansion. Therefore it is possible that the cell expansion of *L. perenne* was affected and could be as a result of the inhibition of hormone action like auxin or interference with cellular metabolism.

4.3.3 Joint toxicity effect of Cu and pyrene

In the present study, it is clear that no matter the concentration of Cu or pyrene in the joint effect tests, the germination rate as well as the shoot and root length decreased when compared to single effect tests of Cu or pyrene. The exposure of plants to more than one contaminant could lead to interaction between the contaminants which affect the plants. This can be antagonistic, additive or synergistic. However, synergistic effects are found to be most common (Luo and Rimmer 1995, Wong and Chang 1991) and our results support this, showing synergistic effects of Cu and pyrene on the shoot and root length of *L. perenne.* When Cu concentration was fixed with increasing pyrene concentration or when pyrene was fixed with increasing concentration of Cu, the present results showed a synergistic effect for higher concentration of fixed Cu or pyrene concentration used in the present study. At 1 and 2 mg L^{-1} fixed pyrene concentration it was clear from the present result that only higher concentration of Cu (12.9 mg L^{-1}) had a synergistic effect on the root elongation of *L. perenne* (Figure 4.14). The possible reason is that pyrene could affect

the structure of the cell wall which could increase the possibility of Cu^{2+} to enter the root systems and go up to the shoot. Kang *et al*. (2010) showed that pyrene is always adsorbed onto the root cell walls prior to cell membrane penetration. It is recalcitrant to metabolism in roots, therefore it exhibits a stronger affinity for plant tissues, slowing up uptake from roots to shoot (Kang *et al*. 2010). When this happens there could be damage to the structure of the root cell wall. Liu *et al*. (2009) also observed synergistic effect of combined contamination of cypermethrin and Cu on *Brassica rapa* and was linked to plasma membrane and cell wall damage by cypermethrin resulting in enhanced Cu uptake. In the natural environment, synergistic interactions are very important because pollution is caused by more than one kind of contaminant. When considering the toxicity effects of the combined effects of multiple contaminants, the synergistic effect is the most important effect to protect against because it can result in enhanced toxicity effects.

Although synergistic effects were clear, the present study also showed evidence of antagonistic effects of Cu and pyrene on seed germination. Figures 4.9 and 4.10 showed that the joint contamination with Cu and pyrene had an antagonistic effect on the final germination rate of *L. perenne* at fixed Cu concentration. At low to high Cu concentration, increasing the concentration of pyrene increased the final germination rate, hence showing an antagonistic effect of pyrene under joint contamination. The germination of *L. perenne* could resist higher concentration of either pyrene or Cu than when jointly contaminated. This is obvious as the interactions of two groups of chemicals seem to change the mode of action of individual chemicals. For example, Chauhan and Gupta (2005) observed that the interactions of insecticides and herbicides changed the mode of action and resulted in induced ultrastructural

alterations whereas when the cells of *Allium cepa*, were exposed to individual compounds, there was no evidence of induced alterations. It could also be possible that the joint contamination of Cu and pyrene was dependent on the ratio of each compound as suggested by Liu *et al*. (2009). It was clear that varying the concentration of either Cu or pyrene significantly affected inhibition to shoot or root length as well as germination percentage of *L. perenne*. However, the solubility of pyrene in water is 0.14mg L^{-1} (Aoudia *et al*. 2010) which is less than the concentration used in this study. Due to the low solubility of pyrene, it was delivered in acetone to achieve concentration that allows for full scale doze response. It is clear that once toxicity test starts, bioaccumulation of the PAHs will begin and it becomes impractical to monitor PAH concentration as it is difficult to maintain stable exposure concentration due to evaporative losses (Ren *et al*. 1996). Pyrene could adsorb to the surface of the petri dishes used for the test or can undergo transformation during tests (Schrieber *et al.* 2008), leading to inhomogenous distribution of test chemicals with differing degrees of bioavailability (Tanneberger *et al*. 2010). This could cause varying differences in pyrene and their co-contamination in the toxicity tests with *L. perenne*. There is a possibility that the toxicity of pyrene to the seedling growth of *L. perenne* could be compromised by the difficulty of ensuring exposure concentration at the saturation level and keeping it constant during the test. Method improvement for this problem will require a process like passive dozing for maintaining the constant exposure condition for toxicity tests. Kwon *et al*. (2011) showed in their study that the calculated value of benzyl butyl phthalate in the medium using passive dozing method was similar to the measured free concentration, while when solvent (methanol and dimethylsulfoxide) was used, there were high discrepancies between the

nominal concentration and the measured free concentration as the nominal concentration exceeded the solubility of benzyl butyl phthalate in water.

4.4 Conclusion

In this study, the single and joint toxicity of Cu and pyrene on the shoot and root length as well as seed germination were investigated. The toxicity of pyrene, Cu and their combinations to *L. perenne* could have been influenced by the concentration of individual compounds in solution culture. Low concentrations of Cu did not affect the final germination percentage of *L. perenne*. However, all tested Cu concentration influenced the shoot and root length inhibition. The single application of pyrene up to 4 mg L^{-1} did not affect the germination percentage but decreased the shoot and root length of *L. perenne* over 9 days of culture. The joint toxicity of Cu and pyrene on *L. perenne* varied. Increasing concentration of pyrene showed an antagonistic effect on the germination rate under joint contamination while there was a synergistic effect on the shoot and root length under joint contamination. The joint toxicity was more dependent on the effect of pyrene than that of Cu. This suggests that in early toxicity assessment of contaminants on seed growth, the combined contamination of pyrene and Cu negatively affected the germination and seedling growth more than single contamination of either Cu or pyrene and highlights the effect of co-contamination during phytoremediation. The mixture toxicities of Cu and pyrene observed in this study provides some perspective of the implications of both contaminants during the early stages of plant growth. Such perspectives are not available from studies that consider single contaminants.

4.5 Effect of combined pollution of chromium and B[*a*]P on seed growth of *Lolium perenne*- Introduction

Part of this work has been published in chemosphere peer reviewed journal; Chigbo and Batty 2013.

Chromium (Cr) is abundant in the earth crusts and is ranked fourth out of 29 elements of biological importance (Subrahmanyam 2008). Due to industrial activities, substantial amount of Cr compounds are discharged in liquid, solid and gaseous waste into the environment causing significant adverse biological and ecological effects (Kotas and Stasicka 2000). Cr is toxic to plants, does not play any role in plant metabolism and its accumulation by plants can reduce growth, alter enzymatic functions as well as induce chlorosis in young leaves (Panda 2003). B[*a*]P on the other hand is a polycyclic aromatic hydrocarbon (PAH) that can be released into the environment during incomplete combustion or pyrolysis of organic materials (Carlo-Rojas and Lee 2009). It is a ubiquitous environmental pollutant used as a representative indication of total PAH level (Jagetia *et al.* 2003). Lower concentrations of B[*a*]P has shown to accelerate the speed of germination and photosynthesis (Diao *et al.* 2011). Increasing attention has been paid to the pollution problems associated with either Cr or B[*a*]P in recent years (Akinci and Akinci 2010, Diao *et al.* 2011). However, joint toxicity of Cr and B[*a*]P has been rarely studied.

Short term phytotoxicity tests which give clear information on inhibition, respiration and enzyme activation have always been carried out using seed germination and root elongation tests. They are suitable as stand-by test methods as well as a fast tool to evaluate the

ecological risks of xenobiotics (Wang *et al*. 2002). Hence, in view of the fact that the simultaneous occurrence of heavy metals and PAH are becoming more frequent, single and joint toxicity of Cr and B[*a*]P acting on *L. perenne* was investigated and the rate of germination, inhibition of shoot and root elongation which reflect the toxicity of hazardous chemicals were assessed.

4.6 Methods

4.6.1 Materials

All reagents used in this study are analytic grade. Seeds of *L. perenne* were commercially available from Vegetable direct seeds Co. Ltd, England. B[*a*]P and Potassium dichromate VI were supplied by VWR chemicals, Lutterworth UK. Test solutions of B[*a*]P were prepared by dissolving in 0.1% acetone and making up with deionized water while potassium dichromate VI was prepared with deionized water only. Control treatments were also exposed to 0.1% acetone. Three replicates were set for each concentration.

4.6.2 Seed germination and root and shoot elongation tests.

Seeds of *L. perenne* were sterilized in 6% (v/v) hydrogen peroxide for 15 minutes and washed with tap water. Ten seeds of *L perenne* were carefully placed on each petri dish and moistened with 3mL of toxicants solution. The concentration of B[*a*]P was set at 0, 1, 2, 3 and 4 mg L^{-1} and the tested concentration of Cr was 0, 2.35, 4.7, 9.4 and 14.1 mg L^{-1}. Petri dishes were sealed with Parafilm® and allowed to germinate under daylight at 24±2 °C in the glasshouse for 9 days. First seed emergence was monitored on daily basis and germination percentage

calculated on the 6th day. A 1 mm radical emergence was considered as seed germination. Seedling development was regarded as inhibited when the seed coat was visibly broken with the embryo not growing further. On the 9th day, germinated seeds were used for root and shoot length measurement. The Germination Rate Index (GI) was determined by the following formula,

$$GI = \frac{G3}{3} + \frac{G6}{6} + \frac{G9}{9}$$ (Ali 2007)

Where G3, G6 and G9 are germination percentages at 3, 6 and 9 days after initiation of germination

4.6.3 Statistical analysis

Statistical analysis including calculation of average values, standard error (S.E) was calculated by the Microsoft office Excel 2007. One-way analysis of variance was carried out with Minitab 15. The root length results were log transformed prior to analysis. When a significant ($p<0.05$) difference was observed between treatments, multiple comparisons were made by the Turkey test.

4.7 Results

4.7.1 Toxic effects of Cr on seed germination

The final germination percentage of seeds of *L. perenne* in medium containing different concentrations of Cr showed an inverse relationship. Figure 4.15 showed that the inhibition to final germination increased by 17 to 40% relative to control experiments as the concentration of Cr in the medium increased from 2.35 mg L^{-1} to 14.1 mg L^{-1}. However, inhibition at only 4.7 mg L^{-1}, 9.4 mg L^{-1} and 14.1 mg L^{-1} concentration of Cr showed significant differences ($p< 0.05$) when compared to control treatments. There was also a significant negative correlation ($r = -0.994$, $p< 0.05$) between the concentration of Cr and the final germination percentage.

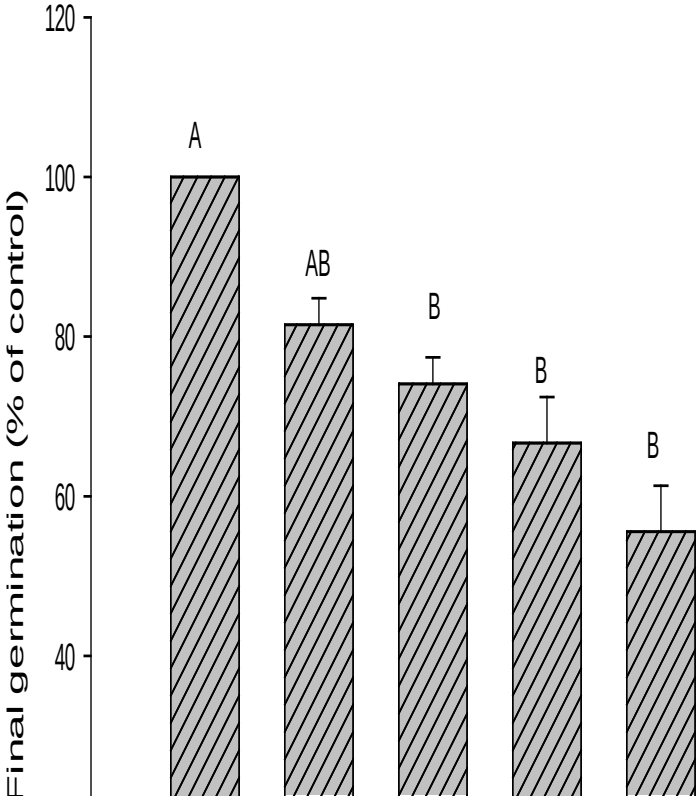

Figure 4.15: Toxic effect of Cr on final germination relative to control of seeds of *Lolium perenne.* Different letters indicate a significant difference at $p < 0.05$ according to the Tukey HSD. Appendix 4B.1

The absence of inhibition at lower concentration when compared to control treatments at the end of germination tests could mean that lower concentrations of Cr are not phytotoxic for germination.

Seeds in medium containing the lowest concentration of Cr showed signs of early breakage of seed coat that was absent in other treatments. In addition, all the concentrations of single Cr except at 14.1 mg L^{-1} were less toxic to seeds of *L. perenne* than all other mixed treatments when compared with control treatments. All other mixed treatments showed at least 2 to 4 orders of toxicity to seeds of *L. perenne* compared to the lowest concentration of Cr.

The germination rate index of seeds of *L. perenne* over the 9-day period reduced by 4 to 13% relative to control treatments as the concentration of Cr increased from 2.35 to 14.1 mg L^{-1}. Results showed no significant differences ($p > 0.05$) in germination rate index between the concentrations of Cr and control except when the Cr concentration reached 14.1 mg L^{-1}. Also there was only a significant difference ($p < 0.05$) in germination rate index between 2.35 mg L^{-1} of Cr and 14.1 mg L^{-1} of Cr, other increasing concentrations of Cr showed no significant differences ($p > 0.05$).

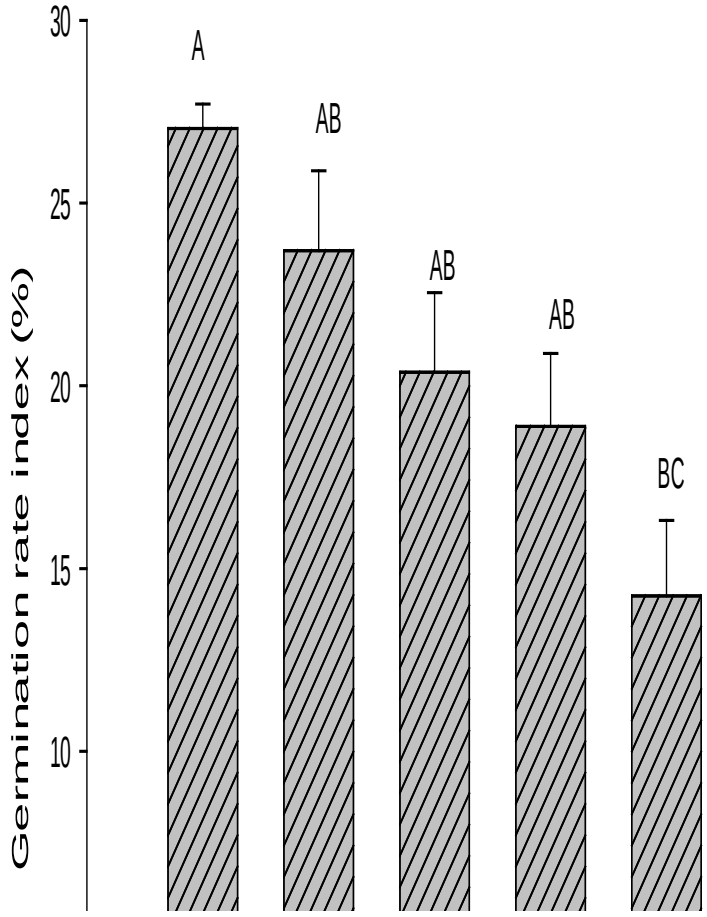

Figure 4.16 Toxic effect of different concentrations of Cr on germination rate index relative to control of seeds of *Lolium perenne*. Different letters indicate a significant difference at p< 0.05 according to the Tukey HSD**.** Appendix 4B.2

4.7.2 Toxic effect of Cr on seedling growth

Seedling establishment is the most sensitive to both physical and chemical adversities (Jeratha and Sahai 1982). The shoot length of seeds of *L. perenne* reduced for all concentration of Cr in media. The reduction in shoot elongation relative to control treatments increased from 19 to 40% as the concentration of Cr increased from 2.35 to 14.1 mg L^{-1}. Results obtained showed that the inhibition of shoot elongation with increased Cr (2.35 to 14.1 mg L^{-1}) , was significantly lower than control treatments. There was also a significant negative correlation ($r= -0.957$, $p< 0.05$) between shoot length inhibition and Cr concentration.

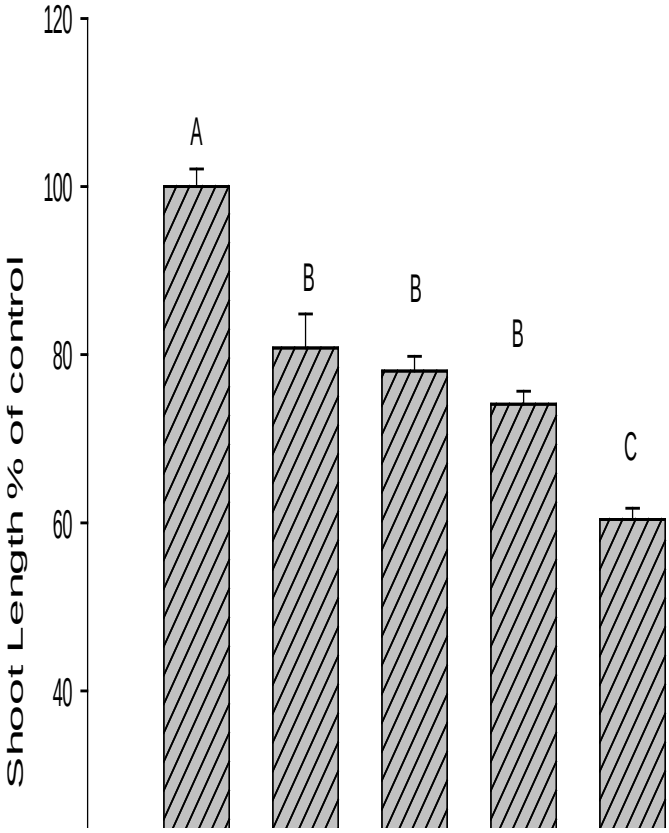

Figure 4.17: Toxic effect of Cr on shoot length relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at $p < 0.05$ according to the Tukey HSD. Appendix 4B.3

The root length of *L. perenne* was inhibited relative to control treatments for all concentrations of Cr. There was a 34 to 48% inhibition of roots relative to control treatments as the concentration of Cr increased from 2.35 to 14.1 mg L^{-1}. The root length of *L. perenne* in medium containing 2.35 mg L^{-1} Cr decreased when compared to control treatments and as the concentration of Cr increased to 4.7 mg L^{-1}, 9.4 mg L^{-1} and 14.1 mg L^{-1}, the root length of seeds in medium was also significantly ($p< 0.05$) inhibited relative to control treatments.

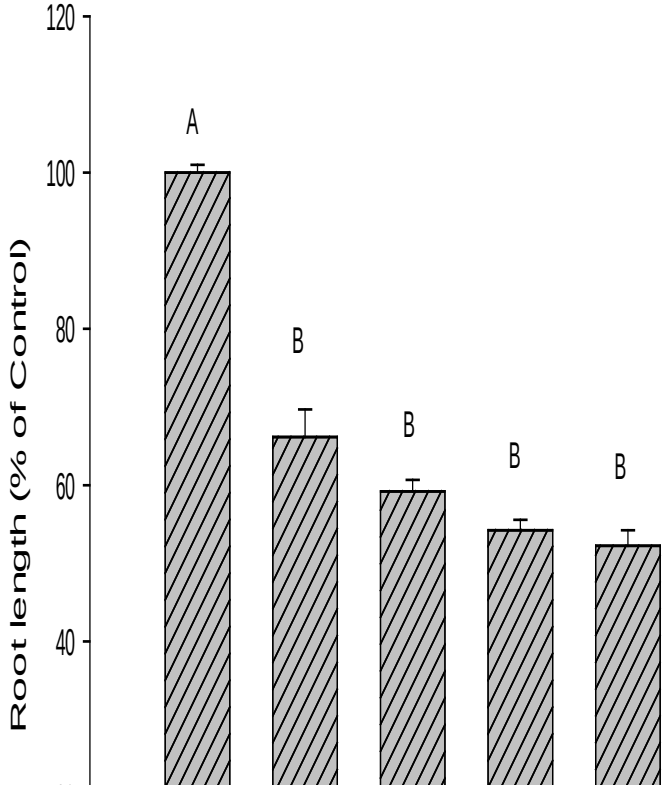

Figure 4.18: Toxic effect of Cr on root length relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at $p< 0.05$ according to the Tukey HSD. Appendix 4B.4

4.7.3 Effects of benzo [*a*] pyrene on seed germination

Single concentration of B [*a*] P when compared to control treatments caused stimulatory effects on the germination of seeds of *L. perenne*. The final germination percentage for the least concentration of B[*a*]P in culture medium was similar to control treatments. However, as the concentration of B[*a*]P in medium increased to 4 mg L^{-1}, germination stimulation relative to control increased significantly to 10%. Although one-way ANOVA showed no significant differences ($p < 0.05$) between lower B[*a*]P treatments and control treatments, the enhanced final germination as observed at 4 mg L^{-1} B[*a*]P concentration suggests that B[*a*]P and its degradation products could act as growth stimulators. B[*a*]P showed no toxicity to seeds ofl *L. perenne* based on final germination percentage but over the 9-day period, the rate of germination of perennial ryegrass was inhibited relative to control treatments. With the concentration of B[*a*]P at 1 mg L^{-1}, the rate of germination was reduced by 11% relative to control treatments and also reduced from 10 to 8% as the concentration of B[*a*]P increased from 2 mg L^{-1} to 4 mg L^{-1}. One-way ANOVA showed significant differences ($p < 0.05$) in germination rate index as B[*a*]P concentration increased from 1 mg L^{-1} to 4 mg L^{-1} (Figure 4.20).

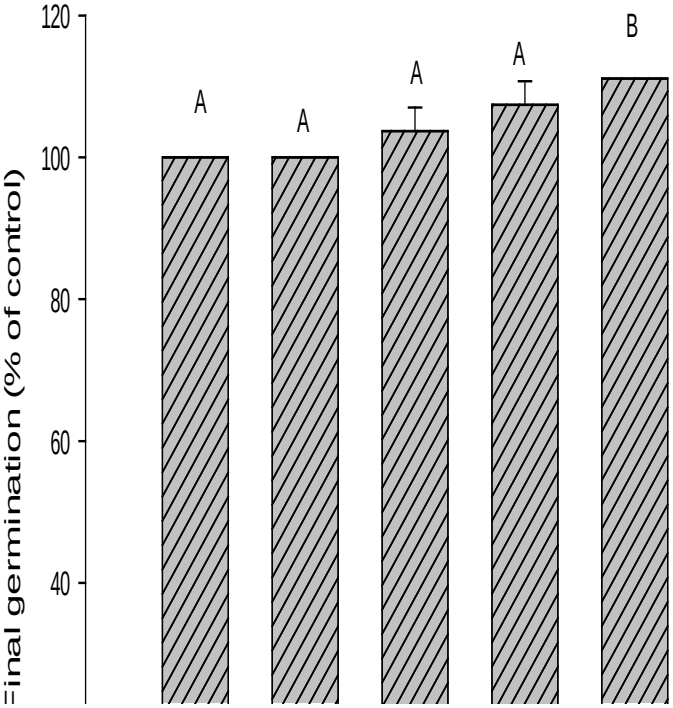

Figure 4.19 Stimulatory effect of B[*a*]P on final germination relative to control of seeds of *L. perenne.* Different letters indicate a significant difference at p< 0.05 according to the Tukey HSD. Appendix 4B.1

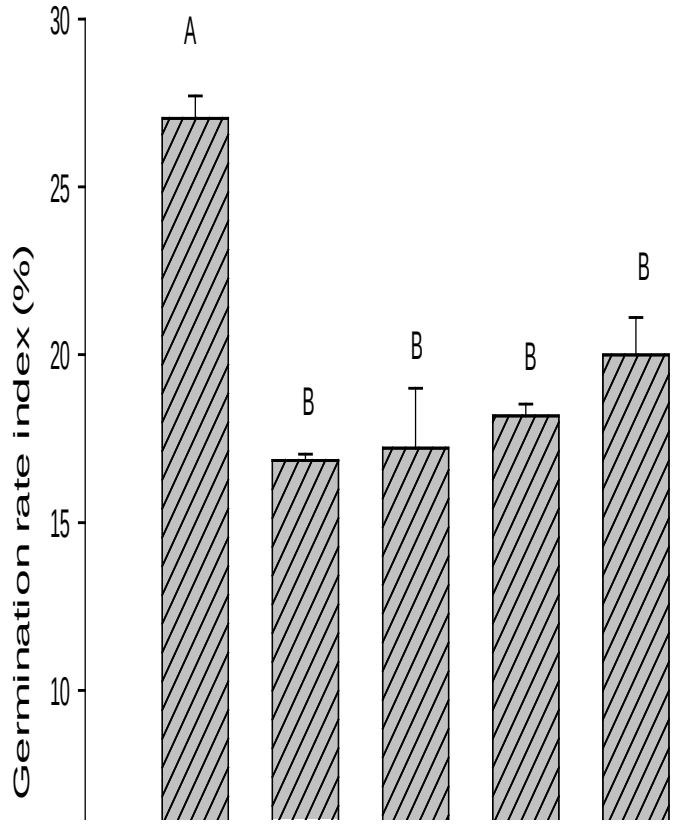

Figure 4.20: Toxic effect of different concentrations of B[*a*]P on germination rate index relative to control of seeds of *L. perenne*. Different letters indicate a significant difference at P< 0.05 according to the Tukey HSD. Appendix 4B.2

4.7.4 Toxic effect of B[*a*]P on seedling growth

B[*a*]P has an important influence on seedling growth of *L. perenne*. Figures 4.21 and 4.22 describes the changes in shoot and root growth of *L. perenne* under different B[*a*]P concentrations. As the concentration of B[*a*]P in medium increases from 1 mg L^{-1} to 4 mg L^{-1}, the shoot elongation of *L. perenne* increased from 1.25 to 10.41%, however this increase observed was non-significant when compared to control treatments. The root length elongation of *L. perenne* was inhibited by 12.5% at 1 mg L^{-1} of B[*a*]P concentration relative to control treatments, was similar to control treatments at 2 mg L^{-1} of B[*a*]P concentration, and was stimulated from 9 to 15% as the concentration of B[*a*]P increased from 3 mg L^{-1} to 4 mg L^{-1}. However, the increase in shoot and root length relative to control treatments were non significant ($p>0.05$). This could mean that B[*a*]P had no adverse effect on root and shoot development of *L. perenne*. The stimulating effect of B[*a*]P on *L. perenne* had a close correlation with the concentration of added B[*a*]P and the coefficient of correlation reached 0.983 and 0.984 for shoot and root length respectively.

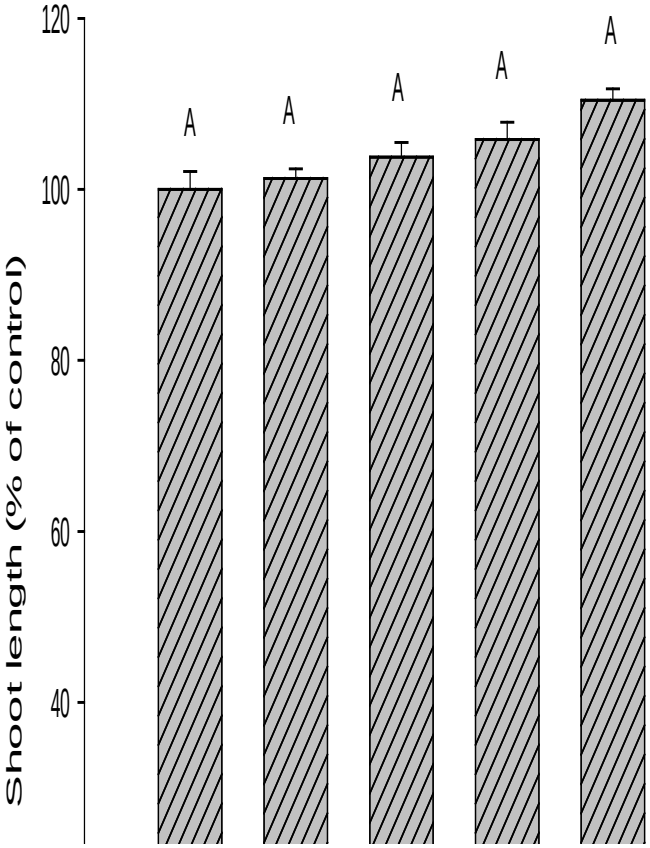

Figure 4.21: Toxic effect of B[a]P on shoot length relative to control of seeds of *L. perenne*. Different letters indicate a significant difference at $p < 0.05$ according to the Tukey HSD. Appendix 4B.3

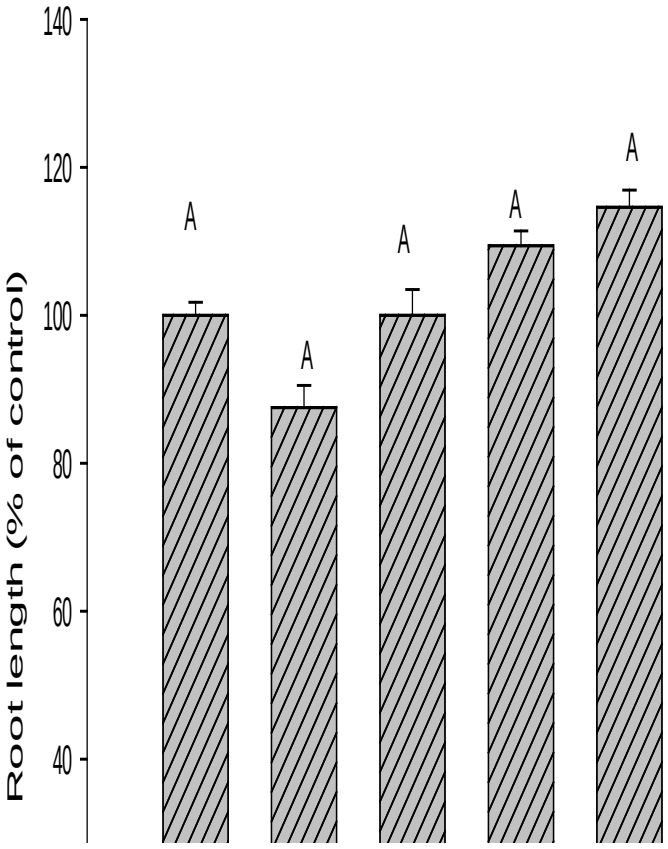

Figure 4.22: Effect of B[*a*]P on root length relative to control of seeds of *L. perenne*. Different letters indicate a significant difference at $p < 0.05$ according to the Tukey HSD. Appendix 4B.4

4.7.5 Joint toxic effect of chromium and B[*a*]P on seed germination

When B[*a*]P and Cr are mixed together, the effect on germination of seeds of *L. perenne* varies. There was an increased inhibition to the rate of germination and final germination percentage for all mixed treatments when compared to single treatment of B[*a*]P and Cr. All mixed concentration of Cr and B[*a*]P showed significant ($p < 0.05$) differences when compared with control treatments as well as single treatments of Cr or B[*a*]P. This is an evidence of enhanced inhibition of germination caused by mixed contamination. Figure 4.23 shows the effect of fixed Cr with varried B[*a*]P concentration on *L. perenne*. When Cr concentration remained 2.35 mg L^{-1} and 4.7 mg L^{-1}, the final germination percentage increased as the concentration of B[*a*]P increased from 1 to 4 mg L^{-1}. However, as the concentration of fixed Cr increased from 9.4 to 14.1 mg L^{-1}, the final germination percentage reduced. With concentration of Cr fixed at 2.35 and 4.7 mg L^{-1}, inhibition to final germination reduced non-significantly ($p>0.05$) from 53 to 37% and 50 to 33% respectively when the concentration of B[*a*]P increased from 1 to 4 mg L^{-1}. There was a 59 to 40% and 56 to 36% reduction in relative toxicity respectively at this time. When fixed Cr concentration increased to 9.4 and 14.1 mg L^{-1} and B[*a*]P concentration varied, inhibition to final germination increased from 33 to 63% and 63 to 70% respectively as the concentration of B[*a*]P increased from 1 to 4 mg L^{-1}. The relative toxicity based on final germination also increased from 37 to 70% and 70 to 78% respectively.

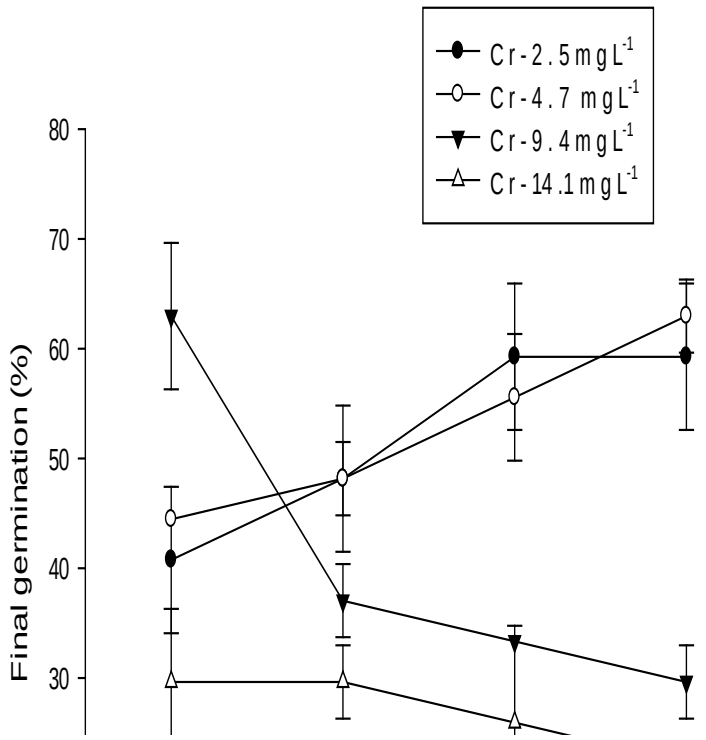

Figure 4.23: Joint effects of fixed Cr and B[*a*]P on final germination of *L. perenne*. Error bars are standard error Appendix 4B.1

When B[*a*]P remained the same and Cr concentration varied, the final germination percentage of seeds in media varied, with medium treated with 2, 3 and 4 mg L^{-1} fixed concentration of B[*a*]P showing similar trend.

Looking at figure 4.24, at 1 mg L^{-1} fixed concentration of B[*a*]P, inhibition to final germination percentage relative to control reduced from 53 to 33% as the concentration of Cr

increased from 2.35 to 9.4 mg L^{-1}, and increased to 63% as the concentration of Cr reached its maximum (14.1 mg L^{-1}). One-way ANOVA showed significant differences between final seed germination as the concentration of Cr in the mix increased from 9.4 to 14.1 mg L^{-1}.

When B[*a*]P concentration in medium remained 2 and 3 mg L^{-1}, the inhibition to final germination percentage relative to control treatments increased from 46 to 63% and 37 to 66% respectively as the concentration of Cr increased from 2.35 to 14.1 mg L^{-1}. Relative toxicity to seed of *L. perenne* based on germination increased from 52 to 70% and 41 to 74% respectively at this point. There were significant reductions in final germination percentage between 2.35 mg L^{-1} and 14.1 mg L^{-1} Cr concentration when B[*a*]P concentration remained 3 mg L^{-1}. With B[*a*]P fixed at 4 mg L^{-1} and Cr concentration varied, the final germination percentage of seeds of *L. perenne* increased for lower concentration of Cr. Inhibition to final germination relative to control treatment reduced from 37 to 33% as the concentration of Cr increased from 2.35 to 4.7 mg L^{-1}, and increased to 63% and a further 7% as the concentration of Cr increased to 9.4 mg L^{-1} and 14.1 mg L^{-1} respectively.

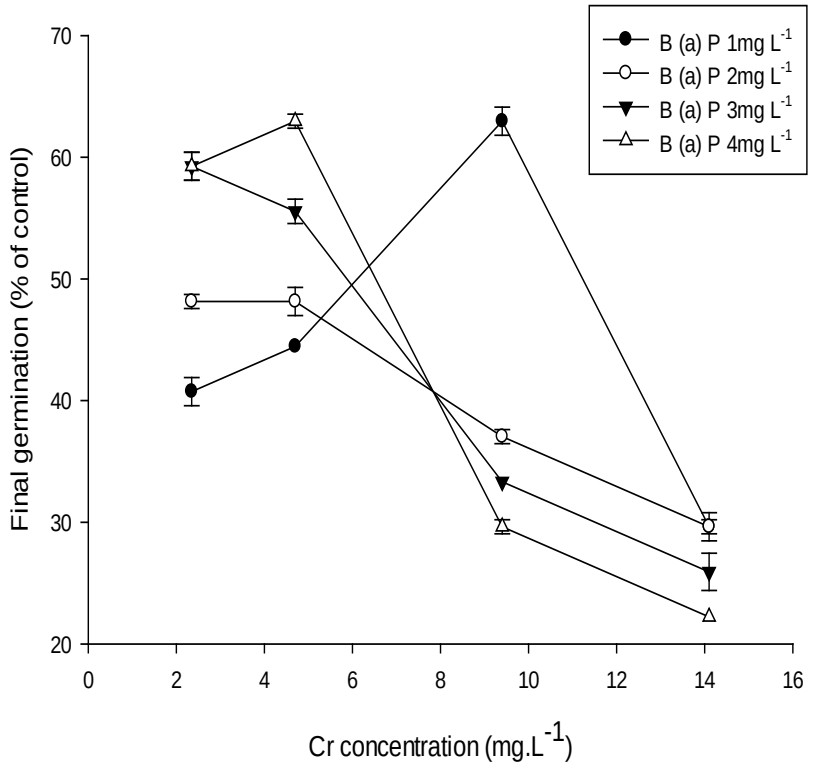

Figure 4.24: Joint effects of fixed B[*a*]P and Cr on final germination of *L. perenne*. Error bars are standard error. Appendix 4B. 1

Figure 4.25 shows the germination rate pattern for mixed Cr and B[*a*]P over the 9-day period of incubation. The variation in the rate of germination of *L. perenne* over the 9-day period was different when compared to final germination percentage for mixed Cr and B[*a*]P medium. Also there were different variations when Cr concentration is fixed with varied

concentration of B[*a*]P as well as when B[*a*]P concentration is fixed with varying concentration of Cr.

At 2.35 mg L^{-1} and 4.7 mg L^{-1} fixed Cr concentration, the germination rate index relative to control treatments reduced from 65 to 39% and 53 to 35% as the concentration of B[*a*]P increased from 1 to 4 mg L^{-1}. When the concentration of Cr increased to 9.4 mg L^{-1} and 14.1 mg L^{-1}, there was an increased reduction in germination rate index relative to control treatments. Results showed a 32 to 72% and 70 to 75% reduction relative to control treatments respectively as the concentration of B[*a*]P increased from 1 to 4 mg L^{-1}. Reductions in germination rates at fixed Cr concentration showed significant differences when compared with control treatments. Also there was significant increase ($p< 0.05$) in the rate of germination for 1 mg L^{-1} and 4 mg L^{-1} of B[*a*]P concentration when Cr concentration remained 9.4 mg L^{-1}.

Results varied when B[a]P concentrations were fixed. For example, When the concentration of B[*a*]P remained at 1 mg L^{-1}, reduction in germination rate index relative to control treatments reduced from 65 to 32% as the concentration of Cr increased from 2.35 mg L^{-1} to 9.4 mg L^{-1} and increased to 70% at 14.1 mg L^{-1} Cr concentration. However, as the concentration of B[*a*]P increased to 2, 3 and 4 mg L^{-1}, germination rate index followed a similar pattern. A slight increase (49 to 51%, 58 to 60%, and 61 to 65 % respectively) as the concentration of Cr increased from 2.35 to 4.7 mg L^{-1} and reduction (38 to 30%, 34 to 27% and 28 to 25% respectively) as concentration of Cr increased from 9.4 to 14.1 mg L^{-1}. The reductions in germination rate index compared to control treatments were statistically

significant ($p < 0.05$). When B[*a*]P remained at 2 mg L^{-1}, there were significant differences in germination rate as Cr concentration increased from 4.7 to 9.4 mg L^{-1}.

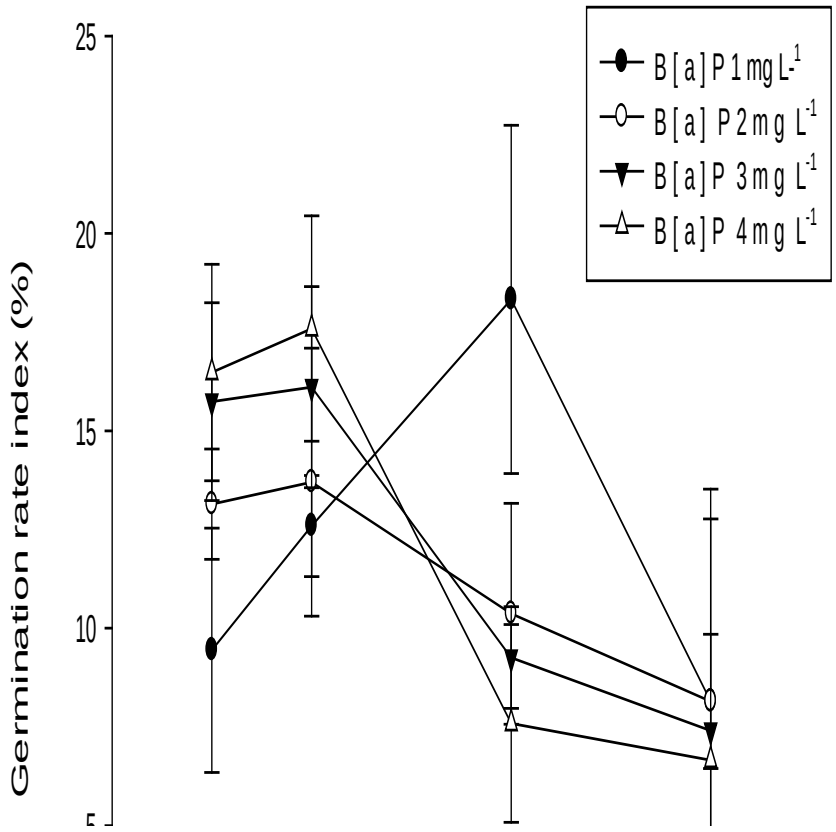

Figure 4.25: Joint effect of fixed B[*a*]P and Cr on Germination rate index of seeds of *L. perenne*. Error bars are standard error. Appendix 4B.2

4.7.6 Joint effect of Cr and B[*a*]P on seedling growth

The study on joint effect of B[*a*]P and Cr was performed on seedling growth and the shoot and root elongation was the end point of toxicity. The shoot and root of *l. perenne* was inhibited for all mixed treatments relative to control treatments as well as single treatments of B[*a*]P and Cr. Inhibition also varied with different concentration of joint contaminants.

Results from figures 4.26 and 4.27 showed that when the fixed concentration of Cr was 2.35 mg L^{-1}, inhibition to the shoot and root length of *L. perenne* significantly decreased ($p<0.05$) from 49 to 37% and 68 to 54% respectively, relative to control treatments as the concentration of B[*a*]P increased from 1 to 4 mg L^{-1}. Similar trends followed for 4.7, 9.4 and 14.1 mg L^{-1} fixed concentration of Cr with a 53 to 45%, 62 to 50% and 70 to 59% significant reduction ($p< 0.05$) in shoot length inhibition respectively and 71 to 56%, 76 to 63% and 83 to 72% for root length inhibition relative to control treatments as the concentration of B[*a*]P increased from 1 to 4mg L^{-1}. Results showed that the slight reduction in inhibition of shoot length as the concentration of B[*a*]P increased from 1 to 4 mg L^{-1} at 2.35 or 4.7 mg L^{-1} fixed Cr concentration was not significant. However, as the concentration of Cr increased to 9.4 and 14.1mg L^{-1}, there were significant differences in shoot length reduction between 1mg L^{-1} and 4 mg L^{-1} B[*a*]P concentration in mix.

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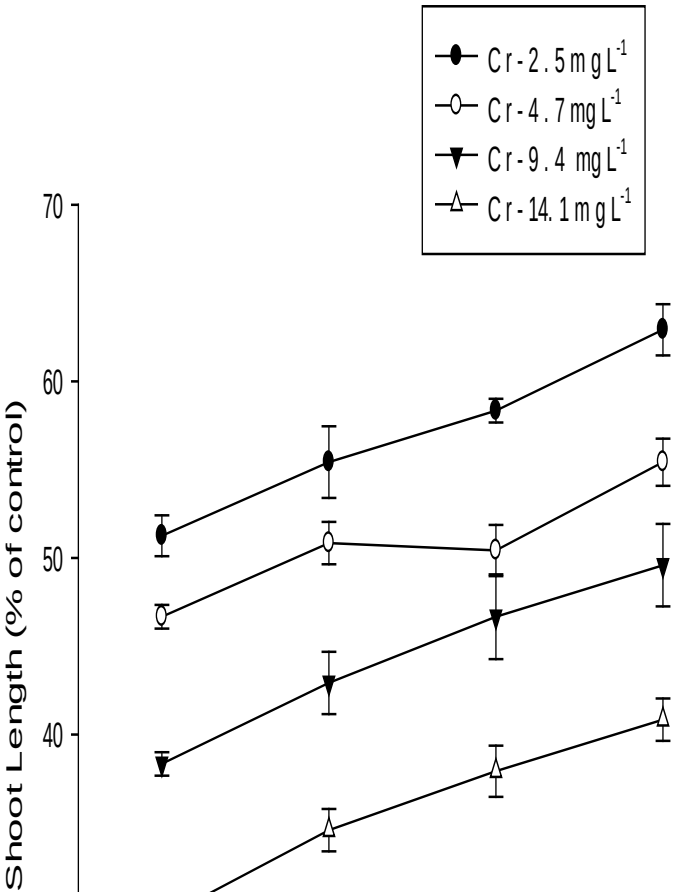


Figure 4.26: Joint effect of fixed Cr and B[*a*]P on shoot length relative to control of seeds of *L. perenne.* Error bars are standard error. Appendix 4B.3

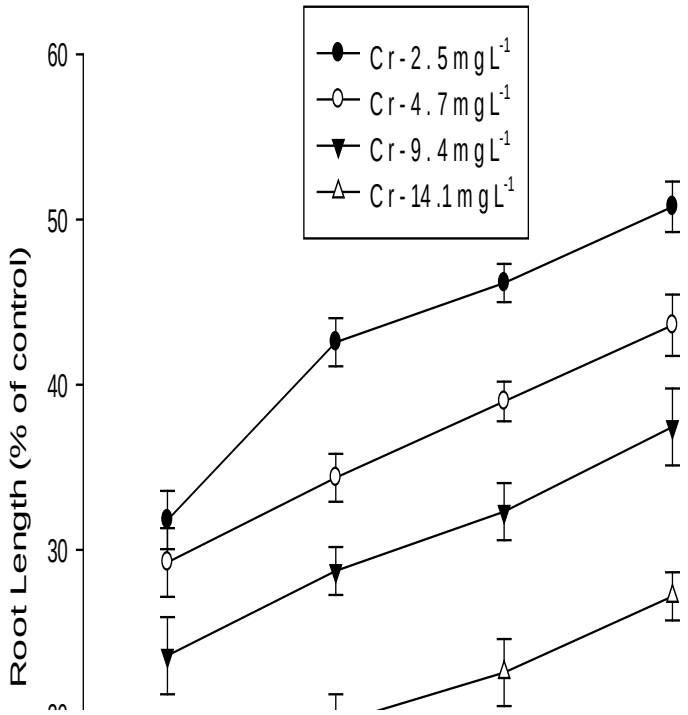


Figure 4.27: Joint effect of fixed Cr and B[*a*]P on root length relative to control of seeds of *L. perenne.* Error bars are standard error.. Appendix 4B.4

With fixed concentration of B[*a*]P, notable differences occurred. The inhibition to the shoot and root length of *L. perenne* increased as the concentration of Cr increased. Inhibition to the shoot length of *L. perenne* at 1 mg L^{-1} fixed B[*a*]P concentration significantly increased from 49 to 70% relative to control treatments as the concentration of Cr increased from 2.35 to 14.1 mg L^{-1}. Also there were 45 to 65%, 42 to 63% and 37 to 59% significant increases in shoot length inhibition relative to control treatments for 2, 3 and 4 mg L^{-1} fixed B[*a*]P concentration respectively, as the concentration of Cr increased from 2.35 to 14.1 mg L^{-1}.

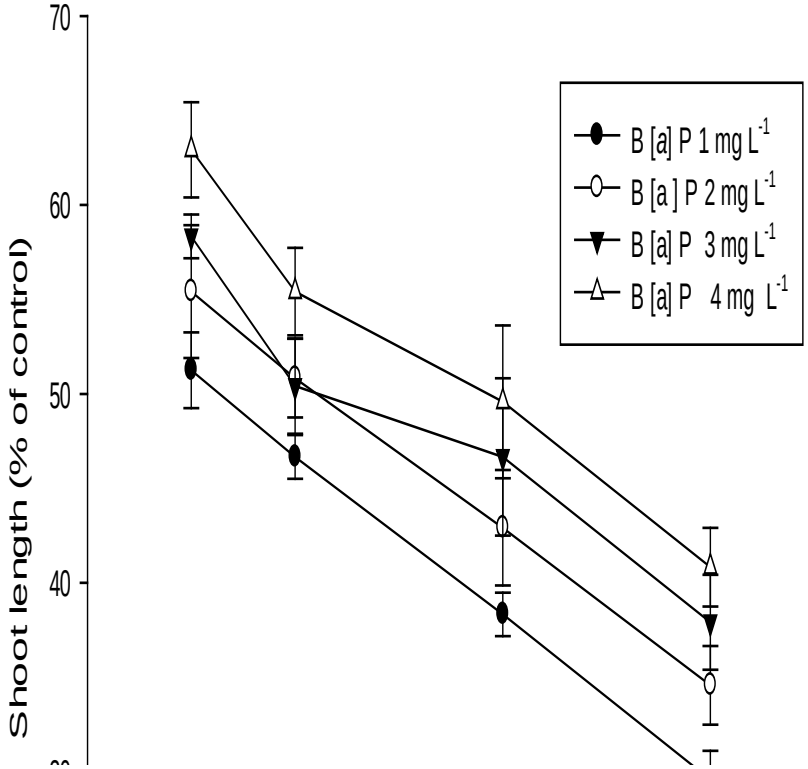


Figure 4.28: Joint effect of fixed B[*a*]P and Cr on shoot length relative to control of seeds of *L. perenne*. Error bars are standard error. Appendix 4B.3

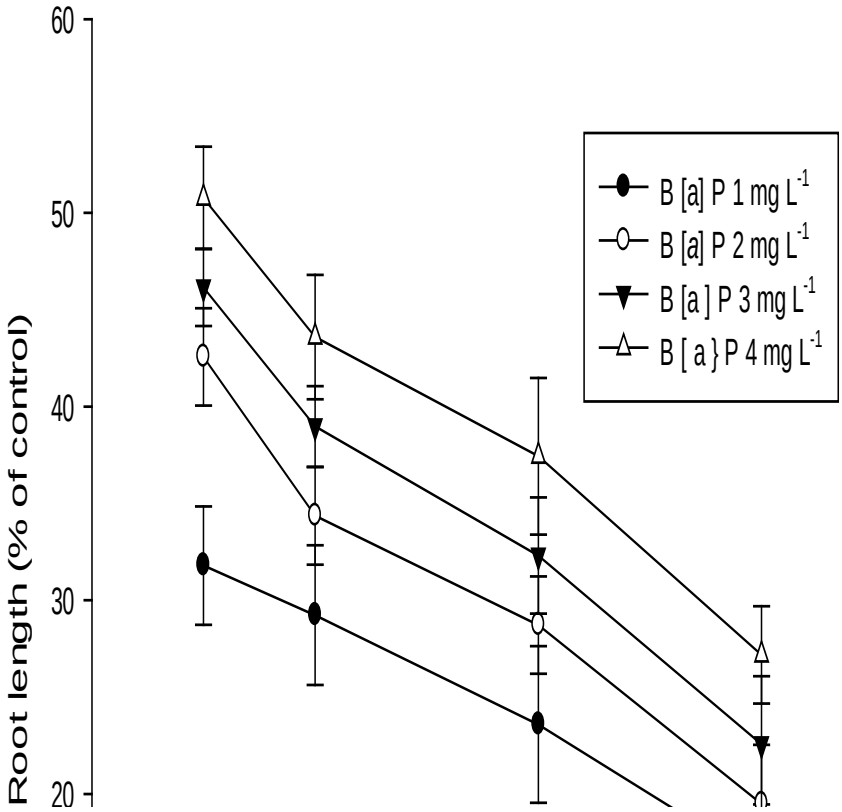


Figure 4.29: Joint effect of fixed B[*a*]P and Cr on root length relative to control of seeds of *L. perenne.* Error bars are standard error. Appendix 4B.4

4.8 Discussion

In toxicity assessment of higher plants, the experiment concerning seed germination, root elongation and shoot elongation at early stage are established methods (Cheng and Zhou 2002). In the present study, the three parameters as mentioned were measured and calculated in response to Cr and B[*a*]P toxicities.

From the present results, higher concentrations of Cr produced a significant reduction in the growth of *L. perenne*. The effects were mainly observed in roots and to a lesser extent, shoot length and seed germination as observed in Figures 4.15, 4.16 and 4.18. The reduction in shoot length with increasing concentration of Cr could be as a result of the binding of Cr to the root cell wall which will inhibit root cell division and elongation. It could also be due to the extension of cell cycle in roots as suggested by Woolhouse (1983). In the present study, the accumulation of Cr in roots of *L. perenne* was not studied; however some studies have shown that the inhibition of shoot growth with increasing concentration of Cr could be due to accumulation of Cr in the roots (Datta *et al*. 2011). The germination rate of *L. perenne* was only reduced when the concentration of Cr in medium was greater than 9.4 mg L^{-1}. This suggests that only higher concentration of Cr affected the germination rate at the present conditions. The inhibition of germination rate that was observed at only elevated Cr concentration could be as a result of depression of oxygen uptake and physiological disturbance in mobilization of reserve food materials of seeds (Agrawal *et al*. 1961) The observed inhibition to germination rate at elevated Cr concentration in the present study is supported by the work carried out by Bishoni (1993) who observed that the germination rate of *Pisum sativum* was not affected by 0.5 mM concentration of hexavalent chromium and

Akinci and Akinci (2010) who showed that the maximum germination of seeds of *Cucumuis melo* L. was not affected in 2.5 and 5 mg L^{-1} of Cr but was affected as the concentration of Cr increased to 10 mg L^{-1}.

The shoot elongation of *L. perenne* as observed in the present study was inhibited when the concentration of Cr remained 14.1 mg L^{-1} or more (Figure 4.17). This significant reduction could be linked to lesser nutrient and water transport to the above plant parts as suggested by Datta *et al*. (2011). Higher concentration of Cr can cause different toxicological effects and because hexavalent chromium is more water soluble and possesses greater oxidizing capacity, it is easily translocated in plants. Also the inhibitory effects of Cr on the growth of embryonic axis of germinating seeds can interfere with the emergence of healthy seedlings which are likely to have poorly developed root system resulting in reduced capacity of seedlings to absorb nutrients and water from soil.

From our results, it is clear that B[*a*]P was not toxic to seed germination or seedling growth of *L perenne.* At all concentrations of single B[*a*]P tested, there were no adverse effects on the seed final germination percentage or seedling root and shoot growth. However there was inhibition of germination rate over the 9-day period when B[*a*]P concentration increased. Our result is in line with the work of Ren *et al.* (1996) which showed that the efficiency and the rate of germination of *Brassica napus* was not affected by B[*a*]P. The reduction in the rate of germination could be as a result of delayed germination during the first days of incubation. Henner *et al.* (1999) showed that germination of seeds of *L. perenne* was delayed by PAHs including fluoranthene and naphthalene, Wang *et al*. (2011) showed that the germination of wheat was not sensitive to single pollution of B[*a*]P although soil was used as the medium

for germination and also work carried out by Sverdrup *et al*. (2007), showed that B[*a*]P did not affect seed emergence of *Brassica alba*, *Trifolium pratence* and *L. perenne*

Lower concentration of B[*a*]P seemed to stimulate the shoot growth of *L. perenne* (Figure 4.21). The stimulation in growth could be as a result of hormesis as explained by Calabrese (2005), which is an overcompensation in response to low levels of contaminants- a case where low dosage stimulates growth and high doses inhibits growth. Some works have reported stimulation of growth by low concentration of B[*a*]P. For example, Forrest *et al.* (1989) reported the accelerated growth of fern gametophytes under low concentration (0.1-0.32 μg ml^{-1}) of B[*a*]P. Soil has been used mostly as a medium for assessing the toxicity effects of B[*a*]P on seeds and seedlings. However, the results obtained with soils seem to be similar with the present study. For example, Sims and Overcash (1983) reported no adverse effects when *L. perenne*, *Triticum spp* and *Z. mays* were grown in soils with up to 1.2 mg $kg^{-1}$of B[*a*]P. Even when toxicity to growth (measured as the rate of production of leaves) was recorded, B[*a*]P was the least toxic on the growth of *Lemna gibba* when compared with other PAHs like pyrene, phenanthrene and fluoranthene (Huang *et al*. 1995).

The effects of Cr and B[*a*]P on higher plants have been documented (Huang *et al*. 1995, Huang *et al*. 1996, Peralta *et al.* 2001); however, and to our knowledge, no studies investigated the response of plants to mixed contaminant exposure. In the present study, seeds of *L. perenne* were exposed to varied concentrations B[*a*]P and Cr in combination and toxicities based on germination rate or shoot and root length were assessed. The reduction in the final germination percentage seemed to be significant as fixed Cr concentration increased

to 14.1 mg L^{-1}, which could mean that if concentration of fixed Cr further increases, the differences could become clearer. At the highest (4 mg L^{-1}) fixed concentration of B[*a*]P, the difference in final germination percentage at 9.4 and 14.1 mg L^{-1} Cr concentration was more significant than those with 2.35 and 4.7 mg L^{-1} Cr. From our results, it is difficult to ascertain which contaminant was more responsible for germination inhibition at fixed Cr concentration. However, with increased B[*a*]P concentration, Cr and *B*[*a*]*P* seemed to have significant synergistic effect on the final germination percentage of seeds of *L. perenne.* There is also suggestion from our results that at high concentration of B[*a*]P, the joint toxicity of Cr and B[*a*]P on final germination depends more on the toxicity of Cr.

Figures 4.26 and 4.27 showed that at low concentration of Cr, higher concentration of B[*a*]P had an antagonistic effect on shoot and root elongation of *L. perenne.* This could mean that with Cr in medium, the addition of high concentration of B[*a*]P could fix some part of Cr in the outer environment of the root of *L. perenne*, inhibiting some of the Cr and B[*a*]P from going to the shoot through the root system. This will reduce the toxic effects of Cr. Wang and Zhou (2005) reported antagonistic effect of chlorimuron-ethyl and cadmium at low cadmium concentration and suggested that when organic pollutants and metals are combined, the activities of heavy metals are reduced. The observed synergistic effect on root and shoot elongation of *L. perenne* at low B[*a*]P concentration and higher Cr concentration is in line with the work of Shuai *et al*. (2010) that reported a synergistic stimulatory effect of B[*a*]P and lead to dehydrogenase activity, which varied with time. It was suggested that it could be as a result of reduced bioavailability of contaminants with increase in time. The inhibitory effects of combined B[*a*]P and Cr on root and shoot length from our result further supports the work

of Zhou *et al*. (2004) that ecotoxicological effects of combined contamination were dependent on the concentration combination relationships of contaminants irrespective of other important factors like single concentration levels and natural characteristics. From our studies, the root length of *L. perenne* was more inhibited than the shoot length and germination percentage and could be more sensitive to the toxicity of mixed contamination of Cr and B[*a*]P. This is in line with the work of Wang and Zhou (2005) who observed that the joint toxicity of chlorimuron-ethyl (herbicide) with cadmium and copper on root elongation of *Triticum aestivum* were stronger than those of seed germination rate.

4.9 Conclusion

In this study, the single and joint effects of Cr and B[*a*]P on seed germination, elongation of root and shoot of *L. perenne* were investigated. It was shown in the single factor experiment of Cr or B[*a*]P and in the joint effects experiments of Cr and B[*a*]P that there were significant relationships between the concentration of pollutants and the elongation of root or shoot and germination rate of *L. perenne*.

Results showed that higher concentration of Cr inhibited the rate of germination of *L. perenne* while lower concentrations showed less or absence of inhibition. Increased concentration of B[*a*]P could slightly accelerate the germination rate of *L. perenne*. The joint effect of B[*a*]P and Cr could strongly inhibit the germination rate of *L. perenne*. Inhibition was more when compared to single pollution of Cr.

The shoot elongation of *L. perenne* was also inhibited for higher concentrations of Cr, whereas with single B[*a*]P contamination, the shoot elongation of *L. perenne* was closely

correlated with increasing B[*a*]P concentration suggesting stimulating effect of B[*a*]P on elongation of shoot of *L. perenne*. With mixed concentration of Cr and B[*a*]P, when the concentration of Cr is high with low concentration of B[*a*]P, Cr had an antagonistic effect with B[*a*]P on shoot elongation of *L. perenne*. While at low B[*a*]P concentration with high Cr concentration, Cr had a synergistic effect on shoot elongation of *L. perenne*.

Higher concentration of Cr inhibited the root elongation of *L. perenne*. There is also an indication that increasing concentration of B[*a*]P stimulated the root length of *L. perenne*. When the concentration of B[*a*]P is high with low Cr concentration, B[*a*]P had an antagonistic effect with Cr on root elongation of *L. perenne*. Also when the concentration of Cr is high with increasing concentration of B[*a*]P, Cr had a synergistic effect with B[*a*]P on root elongation of *L. perenne*.

The toxicity effect of Cr and B[*a*]P to seed germination or root and shoot elongation are root elongation > shoot elongation > germination rate. In order to assess the environmental risk of pollutants, more attention should be paid to long-term exposure of Cr and B[*a*]P to crops.

5

Phytoremediation of metal-PAH co-contaminated soils

5.1 Growth response of *Brassica juncea* in Cu-pyrene co-contaminated soil and the fate of contaminants- Introduction

Part of this work has been published in chemosphere peer reviewed journal; Chigbo et al. 2013.

The toxic effects of heavy metals and PAHs have been widely researched (Perronnet *et al.* 2000, Matitna *et al*. 2003, Kvesitadze *et al*. 2009) For example, the Environment Agency (2002) have set acceptable limits of land contamination which depends on the end use of the land. There are concerns of a potential negative impact of accumulated contaminants on human health as well as the environment; hence efforts have been stepped up in many countries to minimize the release of contaminants while applying remediation methods on already contaminated sites (Schnoor *et al*. 1995). In-situ remediation technology such as phytoremediation; the decontamination of pollutants using plants could be appealing to all countries (Kramer 2005). There are some promising results which show that phytoremediation could be an excellent alternative to chemical or mechanical methods in remediation of metal or PAH contamination (Lin *et al*. 2006). However the phytoremediation of co-contaminants (organics and inorganics) is poorly understood and this has become a problem since many soils are exposed to co-contamination (Zhang *et al*. 2011).

The co-contamination of soils could affect the process of phytoremediation. Different contaminants could interact with plants or with themselves and could affect the phytoremediation potential of plants (Almeida *et al*. 2008). The success of phytoremediation of organic contaminants depends on the influence of roots on degradation of the relevant

contaminant. In the presence of plant roots, certain changes occur, including changes in chemical characteristics as well as changes in microbial numbers and activity (Pilon-Smits 2005). Previous research has shown that the degradation of organic contaminants by plants could be negatively affected by heavy metals (Sandrin and Maier 2003). Heavy metals negatively or positively affect the root growth of plants, affecting the root enhanced dissipation of PAHs. It also directly affects the microbes in soil thereby affecting the degradation of organic contaminants (Lin *et al*. 2008). Compared to organic contaminants, heavy metals are not degradeable. However, a number of plants are able to take up and accumulate significant amounts of heavy metals from soil. The interaction of heavy metals and organic contaminants could affect metal uptake and accumulation by plants. For example, Lin *et al*. (2008) showed that in the presence of pyrene and 400 mg kg^{-1} Cu, the concentration and accumulation of Cu in *Z. mays* decreased when compared to the absence of pyrene. In contrast, the concentration of Zn in shoots of *B. juncea* grown in Zn-pyrene co-contaminated soil significantly increased relative to soil contaminated with Zn only (Batty and Anslow 2008). This shows that the effects of organic contaminants on phytoextraction of metals are complex in soils and could be related to certain factors including plant species and type of contaminant. Hence the objective of this study is to investigate the effect of co-contamination of Cu and pyrene on the growth of *B. juncea* and the fate of contaminants in plants and soil. Pyrene and Cu were used in this study because pyrene represents a class of organic compounds that are ever present in superfund sites while Cu is one of the priority contaminants. *B. juncea* was used because of its desirable characteristics. *B. juncea* is known to both accumulate and to tolerate high levels of heavy metals from polluted soils (Alvarez *et*

al. 2009). They also have desirable characteristics such as high shoot biomass, short life cycle and handling ease (Ariyakanon and Winaipanich 2006).

5.2 Methods

5.2.1 Soil spiking

Soil was spiked with pyrene by dissolving 250 and 500 mg of pyrene in 25 mL of acetone. The solution of acetone and pyrene was mixed with 250 g of soil as a portion and then mixed with 750 g of soil once the acetone had volatilized completely in the fume hood. 25 mL of acetone was also added to control and other soil treatments. 50 and 100 mg kg^{-1} of Cu was prepared by dissolving 0.126 and 0.251 g of $CuSO_4$ and added singly in pyrene spiked soils and fresh soils resulting in a total of 15 treatments. The spiked soil was thoroughly mixed by sieving and stored in a dark room for equilibration for 28 days before planting.

5.2.2 Experimental set up

The experimental layout was designed in a completely randomized design of 15 treatments with three replicates of each. Pots spiked with pyrene had treatments with no planting in order to observe non-plant facilitated dissipation of pyrene.

Table 5.1: Experimental layout

Treatments	Codes
Soil (with no addition of Cu) + *B. juncea*	C0P0
Soil + 250 mg kg^{-1} pyrene + *B. juncea*	P1
Soil + 250 mg kg^{-1} pyrene only	P1(N)
Soil + 500 mg kg^{-1} pyrene + *B. juncea*	P2
Soil + 500 mg kg^{-1} pyrene only	P2 (N)
Soil + 50 mg kg^{-1} Cu + *B. juncea*	C1
Soil + 100 mg kg^{-1} Cu + *B. juncea*	C2
Soil + 50 mg kg^{-1} Cu + 250 mg kg^{-1} pyrene + *B. juncea*	C1P1
Soil + 50 mg kg^{-1} Cu + 250 mg kg^{-1} pyrene only	C1P1 (N)
Soil + 50 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene + *B. juncea*	C1P2
Soil + 50 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene only	C1P2 (N)
Soil + 100 mg kg^{-1} Cu + 250 mg kg^{-1} pyrene + *B. juncea*	C2P1
Soil + 100 mg kg^{-1} Cu + 250 mg kg^{-1} pyrene only	C2P1 (N)
Soil + 100 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene + *B. juncea*	C2P2
Soil + 100 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene only	C2P2 (N)

5.2.3 Planting

Seeds of *B. juncea* were purchased from Vegetable Direct Seed Company in UK. Twenty seeds of *B. juncea* were sterilized in 6% v/v of hydrogen peroxide for 15 minutes, washed with tap water and soaked for 1 day. Sterilized seeds were sowed directly into 12.5 cm plastic pots containing prepared soils. After 10 days of germination, weaker seedlings were removed, leaving 5 seedlings with similar size in each pot. Pots were watered when required with tap water to maintain the soil moisture during plant growth and the leachates from all pots were collected using the tray and returned to the soil. Throughout the experiment, the pots were periodically repositioned to minimize edge effects. Soil was fertilized with N: K: micro nutrients fertilizer mixture (1 g kg^{-1}) containing 26% N, 26% K_2O, 0.013% B, 0.025% Cu, 0.05%, 0.05% Fe and 0.025% Mn.

After 65 days of growth, shoots were cut just above the soil surface and washed with deionized water. Each pot was then emptied and the roots were separated from the soil by washing with running tap water. The roots were then rinsed with deionized water 3 times to remove all soil particles. All samples were oven-dried to constant weight at 65 ºC for 72 hours. The dried samples were weighed to enable biomass calculations and used for plant analysis.

5.2.4 Analysis of plants and soil samples

Due to decreased root growth, the replicates of each treatment were merged together for analysis. Oven-dried plants were ground into small pieces using a coffee grinder (Krups, Italy). Approximately 0.3 g (for control and Cu treatments) and 0.1 g (Cu + pyrene

treatments) of shoot/root dry matter were digested according to methods used in section 3.4.1. Soils were also analyzed for total Cu concentration. Pyrene in soil samples was also analyzed using the Agilent GC-MS according to method used in Chapter 3. The average percentage recovery for surrogate was 78.99%.

5.2.5 Statistical analysis

All treatments were replicated three times. The means and standard error (SE) were calculated using Microsoft Office Excel 2007. The comparisons of shoot dry matter, Cu concentration, accumulation as well as soil residual pyrene were carried out by one-way analysis of variance using Minitab 15.0. The residual pyrene concentration results were log normalized prior to analysis. When a significant difference was observed between treatments, multiple comparisons were made by the Tukey HSD test.

5.3 Results

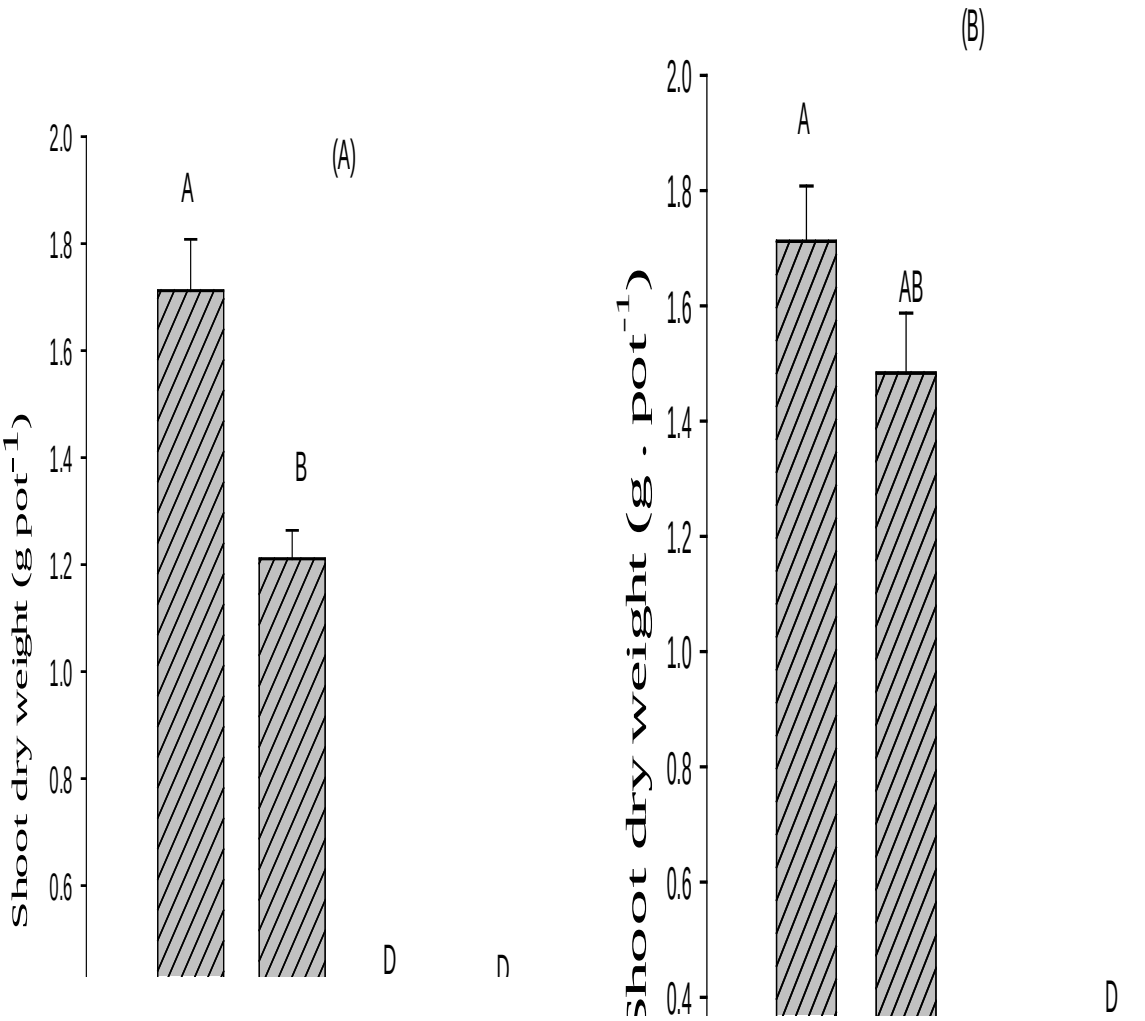
5.3.1 Growth response

The shoot and root biomass of *B. juncea* were affected by Cu and pyrene co-contamination. *B. juncea* showed visual signs of toxicity (chlorosis) in response to mixed contaminants and to single pyrene contamination. There was no visual evidence of toxicity to *B. juncea* to single Cu contamination.

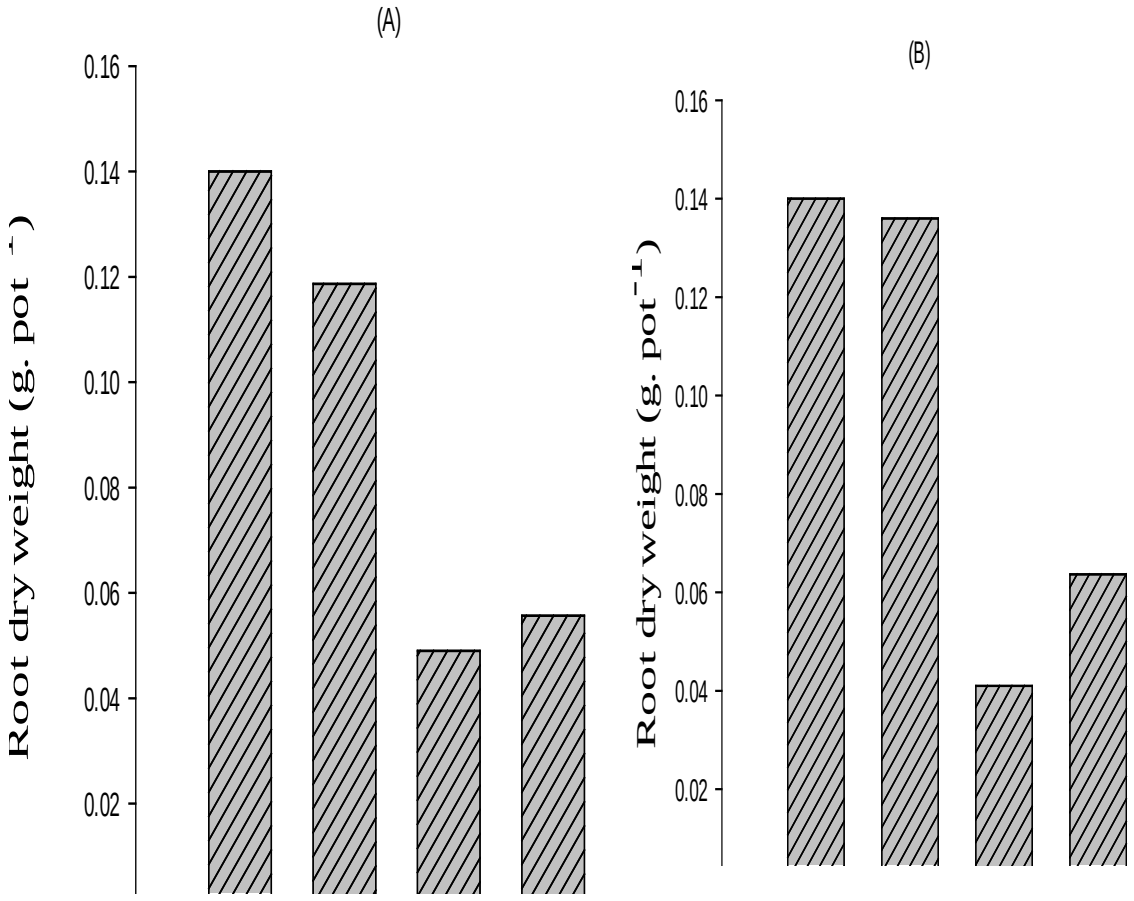
Figure 5.0: *Brassica juncea* after 21 days of sowing

Relative to control treatments, both single and mixed contamination of Cu and pyrene caused a decrease in the root and shoot dry weight of *B. juncea*. 50 mg kg-1 of Cu significantly decreased the shoot biomass of B. juncea by 29% and by 15% for the root biomass (Figures 5.1A and 5.2A). Shoot biomass was significantly reduced ($p<0.05$) in comparison to control treatments by 58 and 84% when exposed to 250 and 500 mg kg^{-1} pyrene respectively (Appendix 5A.1). Root biomass was also reduced by 20% and 57% upon exposure to the same concentration of pyrene (Appendix 5A.2). There was an 80% inhibition of shoot dry matter relative to control treatments when 50 mg of Cu was mixed with 250 mg of pyrene in 1 kg of soil and a further 4% significant ($p < 0.05$) inhibition when 500 mg of pyrene was added. Similar results were observed when 100 mg of Cu was mixed with 250 mg of pyrene. Results showed an 86% reduction in shoot dry weight relative to control treatments but a

slight reduction (1%) with the addition of 500 mg of pyrene. One-way ANOVA showed significant differences between the shoot dry weight of plants grown in Cu and pyrene spiked soil and the un-spiked soil. The shoot biomass tended to decrease under joint stress of Cu and pyrene and the effects were statistically significant ($P<0.05$) when compared with single Cu concentrations and control treatments. The result of the root biomass for mixed concentration of Cu and pyrene varied. Results showed a 65% reduction in root dry weight with 50 mg of Cu and 250 mg of pyrene in 1 kg of soil, and the addition of 500 mg of pyrene reduced the decrease in root dry weight by 5% in comparison with the addition of 250 mg pyrene. Similar results were observed in the treatment containing 100 mg of Cu and 250 mg or 500 mg kg^{-1} of pyrene. Results showed a 71% reduction in root dry weight when 250mg of pyrene was added and 16% improvement with the addition of 500 mg kg^{-1} of pyrene.



Figures 5.1a and b: Shoot dry weight (means ± SE, n= 3) of *B. Juncea* influenced by Cu and pyrene treatments after 65 days of growth. Bars that do not share a letter are significantly different (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene. Appendix 5A.1



Figures 5.2a and b: Root dry weight of *B. Juncea* influenced by Cu and Pyrene treatments after 65 days of growth. Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of Pyrene Appendix 5A.

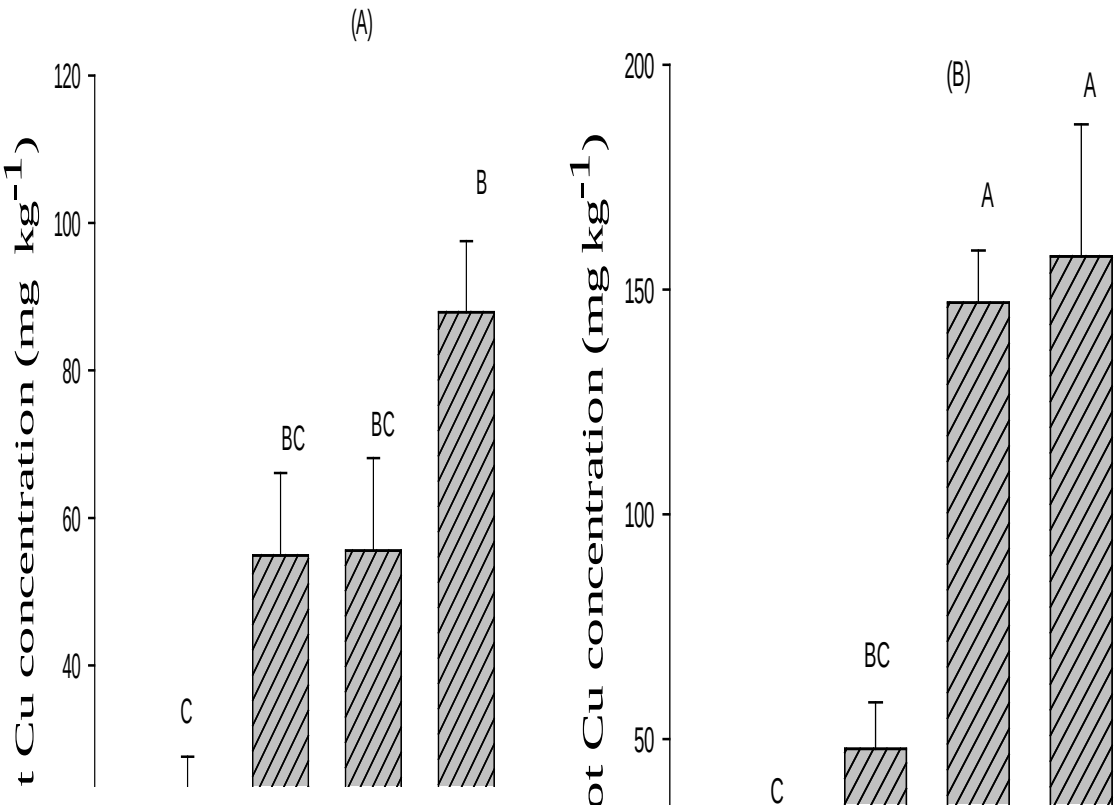
5.3.2 Cu concentration in *B. juncea*

In the absence of pyrene, the root Cu concentration in *B. juncea* increased with increasing concentration of soil Cu whereas the shoot Cu concentration decreased slightly with an increase

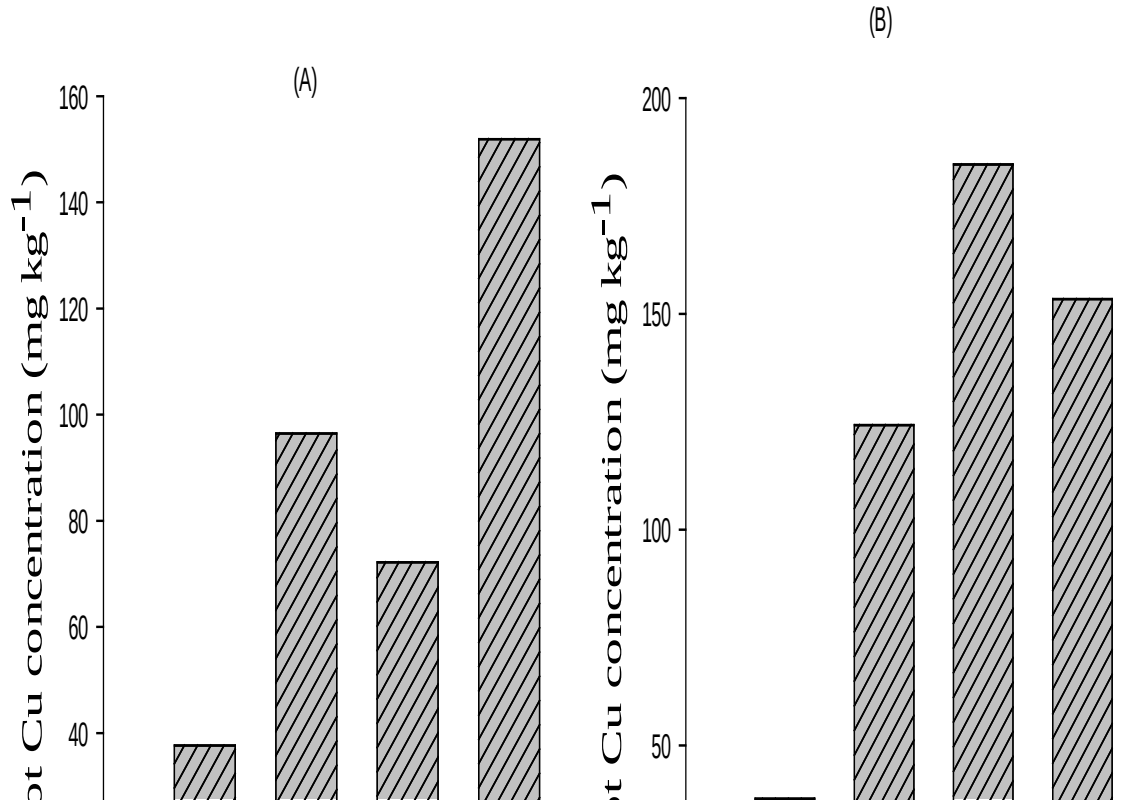
in soil Cu concentration (Figures 5.3A, 5.3B, 5.4A and 5.4B). As the concentration of Cu in soil increased from 50 to 100 mg kg^{-1}, the concentration of Cu in shoot and root of *B. juncea* decreased significantly ($P<0.05$) from 61 to 55% and increased from 61 to 70% relative to control treatments respectively. The ratio of shoot to root decreased from 0.57 for control treatments to 0.56 and 0.38 in 50 and 100 mg $Cu.kg^{-1}$ soil respectively.

The joint contamination with Cu and pyrene had a significant effect on Cu concentration in *B. juncea* and the interaction between pyrene and Cu seemed to be related to the extent of Cu concentration. For example in figure 5.3A and 5.3B, it was shown that in 50 mg kg^{-1} soil Cu concentration, the shoot Cu concentration in *B. juncea* in the presence of pyrene was not significantly different from that in the absence of pyrene. In contrast with 100 mg kg^{-1} soil Cu concentration, the concentration of Cu in shoot of *B. juncea* in the presence of pyrene was significantly ($P<0.05$) higher compared with the absence of pyrene. When soil Cu concentration remained at 50 mg $Cu.kg^{-1}$ with the addition of 250 mg of pyrene, the concentration of Cu in shoot of *B. juncea* was similar to that in 50 mg Cu only (Figure 5.3A). In contrast, when the concentration of pyrene increased to 500 mg, the concentration of Cu in shoot of *B. juncea* increased by 46% when compared to 50 mg Cu alone. The concentration of Cu in shoots of *B. juncea* in treatment with 100 mg $Cu.kg^{-1}$ with 250 and 500 mg of pyrene however increased significantly ($p<0.05$) by 68 and 70% relative to soil contaminated with 100 mg Cu only. The root concentration of Cu in *B. juncea* also varied with mixed contamination. Figure 5.4A showed that in 50 mg $Cu.kg^{-1}$ with 250 mg of pyrene, there was a 25% reduction in root Cu concentration when compared to soil contaminated with 50 mg $Cu.kg^{-1}$ only, and a 36% increase in root Cu concentration with the addition of 500 mg of pyrene. Whereas with the

increase in soil Cu concentration to 100 mg kg^{-1}, the addition of 250 and 500 mg of pyrene increased the root concentration of Cu by 33 and 19% respectively (Figure 5.4B).



Figures 5.3a and b: Shoot Cu concentration (means ± SE, n= 3) of *B. Juncea* influenced by Cu and pyrene treatments after 65 days of growth. Bars that do not share a letter are significantly different (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500mg kg^{-1} of pyrene. Appendix 5A.3

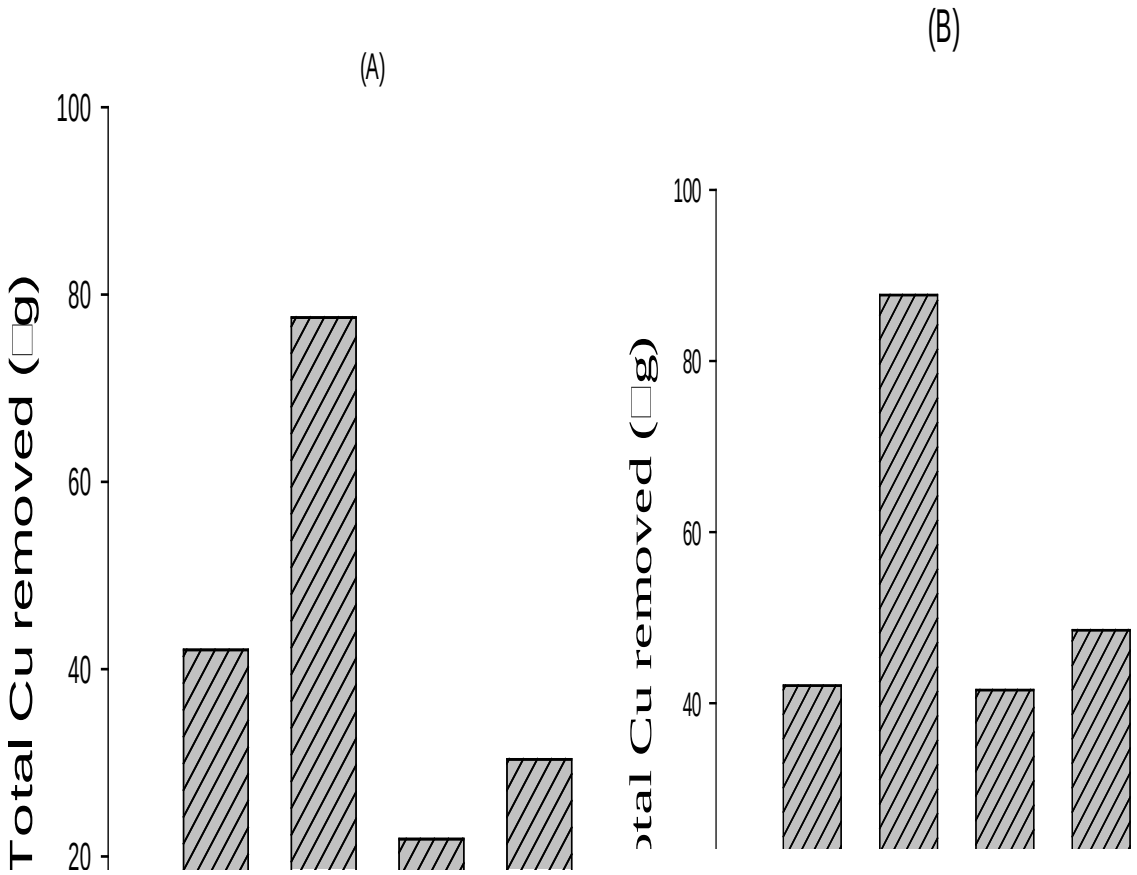


Figures 5.4a and b: Root Cu concentration of *B. Juncea* as influenced by Cu and Pyrene treatments after 65 days of growth. Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene**.** Appendix 5A.5

5.3.3 Total Cu accumulation in plants

Cu uptake and translocation in *B. juncea* depends largely on the Cu supply levels, the growth conditions as well as the stage of growth (Liao 2000). The total Cu accumulation in plant tissues as observed in our results increased with an increase in soil Cu concentration when soil was spiked with Cu alone. Relative to control treatments, the total Cu accumulated by *B. juncea* increased by 22% and 41% when the soil contaminant was 50 and 100 mg Cu kg^{-1} alone.

With the co-contamination of Cu and pyrene, the accumulation of Cu in tissue of *B. juncea* was drastically reduced. Result showed a 90% reduction in Cu accumulation by *B. juncea* in soil contaminated with a mix of 50 mg Cu kg^{-1} and 250 mg kg^{-1} of pyrene and with the addition of 500 mg kg^{-1} of pyrene, the total accumulation of Cu by *B. juncea* reduced slightly by a further 0.4% when compared to control treatments. A similar result was obtained with soil contaminated with 100 mg Cu kg^{-1} with varying concentration of pyrene. There was an 86.5% reduction in the total amount of Cu accumulated by *B. juncea* when 250 mg kg^{-1} of pyrene was added. However with the addition of 500 mg kg^{-1} of pyrene there was a 3% improvement in Cu accumulation.



Figures 5.5a and b: Total accumulation of Cu by *B. juncea* as affected by single and mixed contamination of Cu and Pyrene. Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of Pyrene.

5.3.4 Translocation factor (TF) of Cu

The translocation factor (TF) which indicates the internal metal transportation is the ratio of shoot to root metals (Deng *et al*. 2004). The TF (Table 5.2) showed that relative to control treatments there were reductions among the treatments without added pyrene and an increase with the addition of pyrene. It is clear from our results, that translocation increased with co-contamination. The rate at which Cu was translocated from the root to the shoot of *B. juncea*

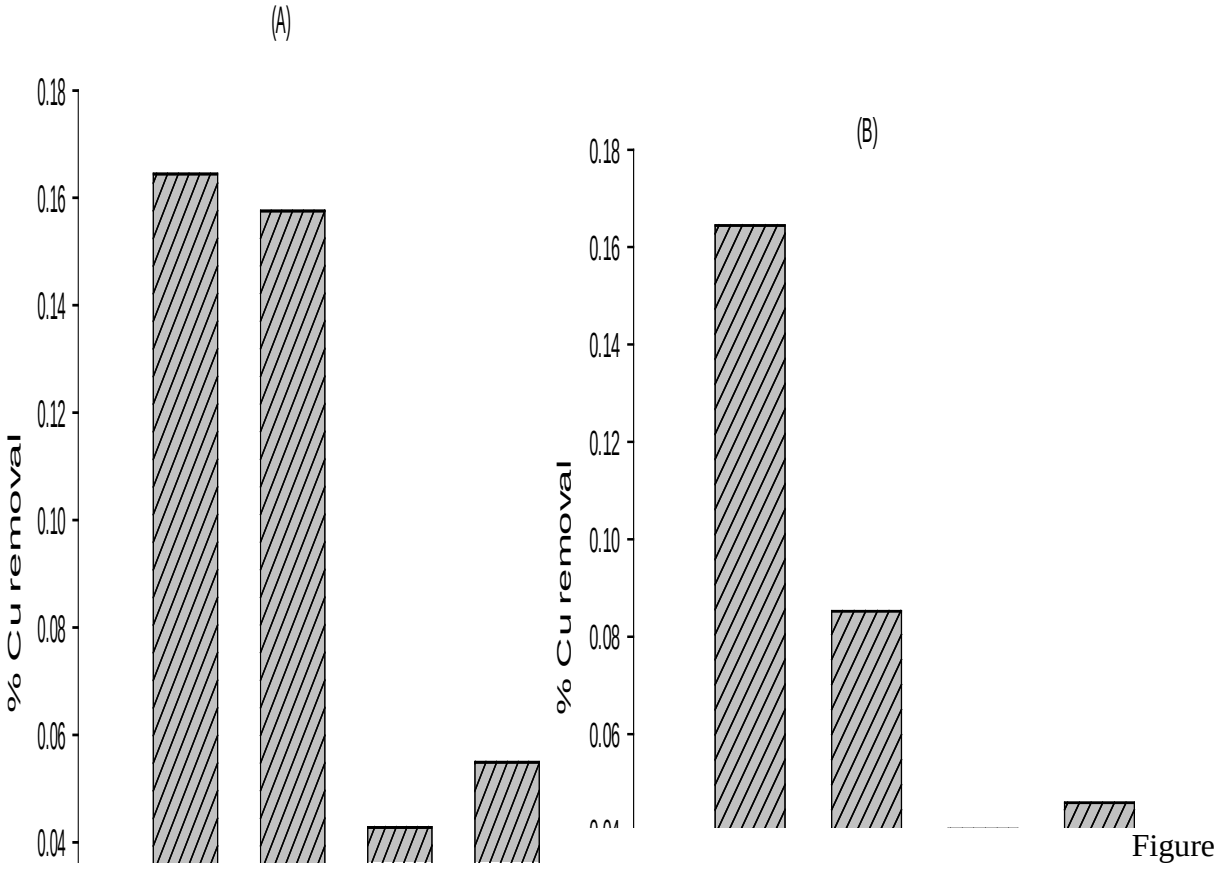
when 50 mg of Cu only was added to soil reduced by 0.24% relative to control treatments. With the addition of 250 mg of pyrene, the TF increased by a third compared to control treatments. However, there was only a 2% increase in TF when 500 mg of pyrene was added. With the application of 100 mg of Cu only, the rate at which Cu was translocated from root to shoot of *B. juncea* was reduced by a third when compared to treatments without Cu addition and increased by 20 and 50% when 250 and 500 mg of pyrene was added respectively.

Table 5.2: Root to shoot ratio and translocation factor of Cu as affected by single and mixed contamination

Treatments	Root to shoot ratio
C0	0.5717
C1	0.5693
C2	0.3851
C1P1	0.7699
C1P2	0.5788
C2P1	0.7965
C2P2	1.0254

5.3.5 Percentage removal of Cu

Over the period of planting, with the initial soil Cu concentration of 50 mg kg^{-1}, the percentage removal of Cu by *B. juncea* reduced slightly by 0.007% relative to control treatments and by 0.08% when the concentration of Cu in soil increased to 100mg kg^{-1} (Figures 5.6a and 5.6b). However, the addition of pyrene to soil caused a more significant reduction in the removal rate of Cu by *B. juncea* when compared to when Cu was the only soil contaminant. The results showed that at 50 and 100 mg kg^{-1} soil Cu, the addition of 250 mg kg^{-1} of pyrene reduced the rate of removal of Cu by 3.8 fold and 4.8 fold respectively. While there was a 2.9 and 3.58 fold reduction when soil pyrene concentration increased to 500 mg kg^{-1}. It was clear from the results that under Cu contamination, lower concentration of pyrene reduced the uptake of Cu by *B. juncea* which slightly increased with increased pyrene concentration.



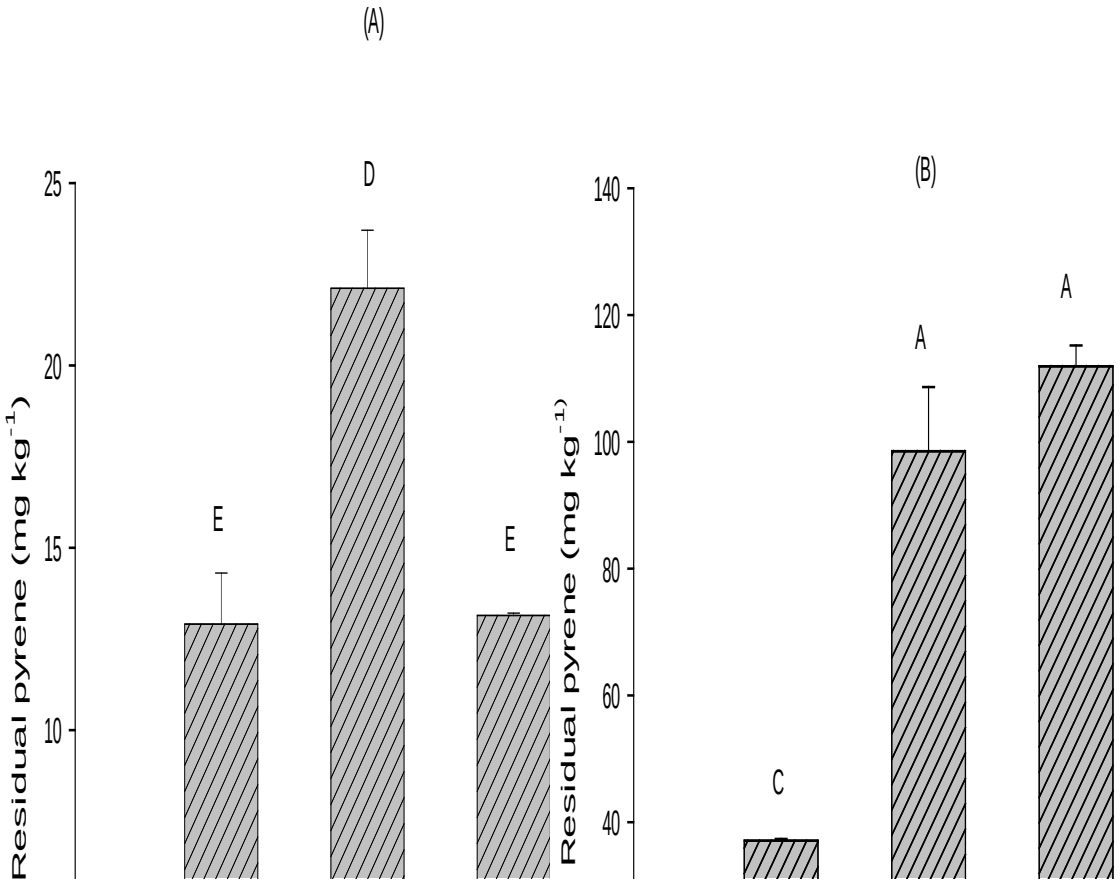
Figure

Figures 5.6a and b: Percentage removal of Cu by *B. Juncea* as influenced by Cu and Pyrene treatments after 65 days of growth. Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene.

5.3.6 Pyrene removal from soil

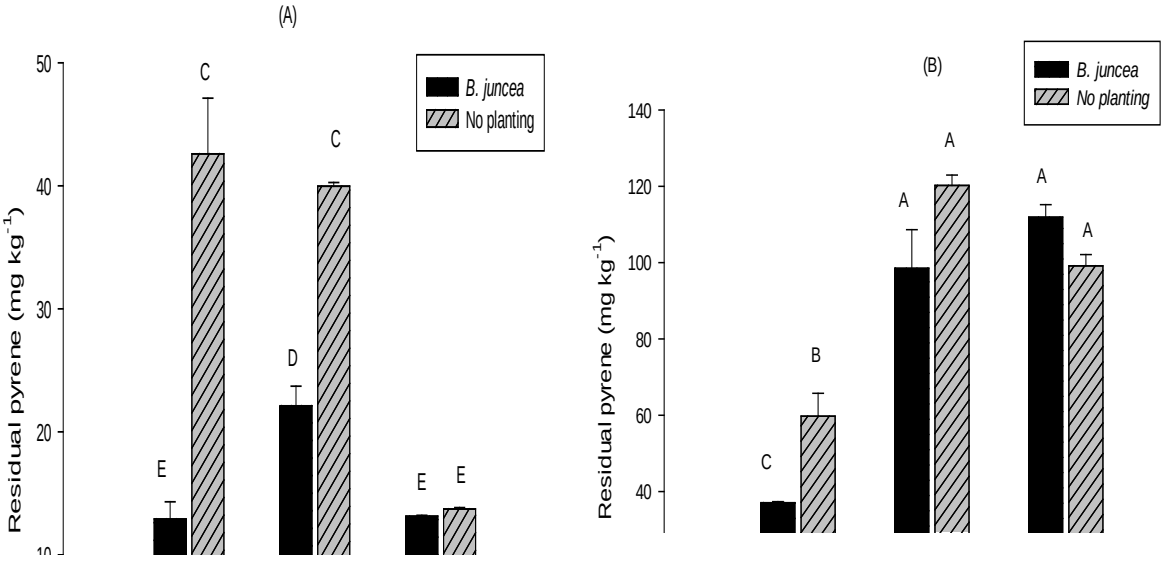
After 65 days of growth, the extractable pyrene decreased significantly ($p<0.05$) in soil planted with *B. juncea* as well as in the unplanted soil (Figure 5.7). This accounted for 90 to 94% of initial extractable pyrene in planted soil and 79 to 84% in unplanted soil for soil contaminated with pyrene alone. The percentage of pyrene removal was also influenced by the interaction of Cu, pyrene and planting/non planting treatments accounting for 67 to 89% and 65 to 92% for planted and unplanted soils respectively. The residual pyrene in soil planted with *B. juncea* was significantly ($p<0.05$) lower than in the unplanted soil when soil was contaminated with 250 and 500 mg kg^{-1} of pyrene. The results showed that in the presence of *B. juncea*, the extractable pyrene remained at 12.9 and 37.05 mg kg^{-1} for 250 and 500 mg kg^{-1} pyrene contaminated soil respectively. However in the soil without plants, the extractable pyrene increased to 42.59 and 59.75 mg kg^{-1} respectively.

Figures 5.7A and 5.7B show the effect of Cu on pyrene dissipation in soil planted with *B. juncea*. Results show that the addition of Cu to pyrene contaminated soil seemed to increase the residual pyrene concentration in soil. The addition of 50 mg kg^{-1} of Cu to 250 mg kg^{-1} pyrene contaminated soil significantly increased the residual pyrene from 12.9 to 22.11 mg kg^{-1}. The dissipation of pyrene appeared unaffected by the plants in soil co-contaminated with 250 mg kg^{-1} of pyrene and 100mg kg^{-1} of Cu. When the initial soil pyrene concentration increased to 500 mg kg^{-1}, the application of 50 and 100 mg kg^{-1} of Cu significantly increased the residual pyrene concentration in soil. It was observed that pyrene concentration in the absence of Cu was 37.05 mg kg^{-1} for 500 mg kg^{-1} pyrene while in the presence of 50 and 100 mg kg^{-1} of Cu, the residual soil pyrene concentration increased to 98.48 and 111.9 mg kg^{-1}.



Figures 5.7a and b: Residual pyrene in planted soil as affected by Cu and pyrene after 65 days of planting. Bars that do not share a letter are significantly different (Tukey HSD $p \leq 0.05$). Treatments C1and C2 represents 50 and 100 mg Cu kg^{-1}; P1and P2 represents 250 and 500 mg kg^{-1} of pyrene. Appendix A.6

There was an interesting result for pyrene dissipation under co-contamination in the presence and absence of plants (Figures 5.8A and 5.8B). For example, the residual pyrene in soil co-contaminated with 50 mg kg^{-1} of Cu and 250 mg kg^{-1} of pyrene was significantly higher in planted soil than in non-planted soil. The residual pyrene remained 22.11 mg kg^{-1} in planted soil and increased by 44% in non-planted soil. However, for a higher concentration of Cu (100 mg kg^{-1}), there was no significant difference in the residual pyrene in soils between the planted and non- planted soil.



Figures 5.8a and b: Residual pyrene in planted and non planted soil after 65 days. Bars that do not share a letter are significantly different (Tukey HSD $p \leq 0.05$). Treatments C1and C2 represents 50 and 100 mg Cu kg^{-1}; P1and P2 represents 250 and 500 mg kg^{-1} of pyrene Appendix A7

5.4 Discussion

5.4.1 Interaction of Cu and pyrene affecting plant biomass

The higher shoot dry matter yield obtained under conditions of higher Cu concentration in soil could be due to stimulation of growth of *B. juncea* by Cu. This is supported by the work of Gardea-Toresday *et al*. (2004) which observed a 4.2% increase in shoot dry matter yield of *Convolvulus arvensis* as Cu concentration in medium increased from 20 mg L^{-1} to 40 mg L^{-1}. The increased growth of *B. juncea* as observed in our result could also be related to the effects Cu has on various macronutrient contents (N, P, K, Na, Ca and Mg). For example, Manivasagaperumal *et al*. (2011) suggested that lower concentration of Cu (50 mg kg^{-1}) increased the nutrient (N, P, K, Na, Ca and Mg) contents of *Vigna radiata* L., notably an increase in leaf nitrogen, however they observed a gradual decline in dry matter production in higher Cu concentrations (100 to 250 mg kg^{-1}). Cu (2008) based on their result suggested that the threshold of Cu^{2+} mobilization to *B. juncea* might be listed at around 30 mg kg^{-1} and severe effects can be seen with the contents higher than 50 mg kg^{-1}. This was in contrast with our findings which showed no serious effect of Cu to *B. juncea* at 100 mg kg^{-1} soil Cu concentration probably due to nitrogen applied as fertilizers to enhance growth. Wu *et al.* (2004a) showed that nitrogen supplied as urea at 200 and 400 mg kg^{-1} increased the shoot yield of *B. juncea* in 70 mg kg^{-1} total Cu contaminated soil and similar result was observed for *Pteris vitata* on arsenic (As) contaminated soil where nitrogen fertilizers increased plant biomass at higher As concentration (Liao *et al*. 2007).

It could be suggested that the presence of pyrene had an inhibitive effect on the growth of *B. juncea*. The shoot and root dry weight of *B. juncea* decreased with increasing concentration of pyrene. This is supported by the work of Fan *et al.* (2008) which showed the inhibition of root and shoot biomass of *Medicago sativa* with an increase in soil pyrene concentration up to 500 mg kg^{-1} over a 60-day period. Also Gao and Zhu (2004) showed that the root and shoot dry weights of 12 plant species including *Brassica chinensis* L. were consistently lower in PAH treated soils when compared to un-spiked control soils. The reduction in dry matter yield of plants grown in PAH polluted soil could be as a result of inherent toxicity of PAHs which affects the plants indirectly. Reilley *et al*. (1996) showed that PAHs might affect the plant indirectly by reducing water and nutrient availability to plants in polluted soil leading to reduction in dry matter production. Plants are very susceptible to low molecular weight hydrocarbons and they have been shown to penetrate cell membranes and inhibiting plant growth (Jackson *et al*. 1997), but there are reports of uptake of higher molecular weight hydrocarbons like pyrene (Gao and Zhu 2004). Higher shoot biomass indicates good plant health but does not necessarily indicate enhanced remediation efficiency (Banks *et al*. 2003).

An interactive effect of Cu and pyrene on the growth of *B. juncea* was observed in this present study, causing a reduction in plant growth at both 250 and 500 mg kg^{-1} pyrene concentration. This was in contrast to reports of alleviation of metal toxicity by PAH in other studies. For example Lin *et al* (2008) observed an increase in shoot yield of *Zea mays* with Cu and pyrene co-contamination, whereas our results showed that the yield of both root and shoot of *B. juncea* decreased with the increase of soil pyrene and Cu concentration. This suggests a synergistic effect of metals and PAH in co-contaminated soil and is supported by Zhang *et al.*

(2009) which showed that pyrene did not alleviate the toxicity Cd to *Z. mays*. Our results coupled with other works (Lin *et al*. 2008, Zhang *et al*. 2011) suggests that growth response of plants to joint toxicity of metal and organic pollutants is dependent on certain factors including plant species.

5.4.2 The ability of Cu uptake and accumulation by *B. juncea*

Results presented in this study suggest that the root uptake of Cu intensified with an increase in soil Cu concentration whereas shoot uptake reduced. A 61 to 70% increase in root Cu concentration (Figure 5.4) and a 61 to 55% reduction (Figure 5.3) in shoot concentration as the concentration of soil Cu increased from 50 to 100 mg kg^{-1} was reported. The reduction in shoot concentration could be as a result of dilution effect of the increase in yield caused by the addition of N and P as fertilizers. This was supported by Wu *et al*. (2004a) which showed that added N, P and K fertilizer reduced Cu shoot concentration of *B. juncea* in 70 mg kg^{-1} Cu soil contamination due to increased yield. Cattani *et al*. (2006) also showed that there was no significant difference between the Cu shoot concentration of the polluted vineyard soil with 183 mg kg^{-1} total Cu soil concentration and the unpolluted forest soil with 18.4 mg kg^{-1} total Cu soil concentration whereas the Cu concentration of the root in the vineyard soil was significantly four times greater than the forest soil. Poschenrieder *et al*. (2001) in their study on the accumulation of Cu in root and shoot of 32 plant species on soil with a wide range (30 to 18,500 mg kg^{-1}) of Cu suggested that plants must be able to mobilize and absorb soil Cu or to tolerate low plant Cu concentrations by high Cu-use efficiency in low Cu contaminated soils whereas in soils with higher Cu concentration, plants require either or both a strong exclusion capacity and a good tissue tolerance of high Cu concentration. The restriction of Cu transport to the shoot

seemed a common feature in most plants with Cu resistance and was reported by Baker (1981) for many metallophytes. Co-contamination with PAH can change the extent of Cu uptake by plants or change the Cu solubility. For example, Mucha *et al*. (2005) observed that plants can release organic compounds which may complex metals and therefore change the availability of metals. It is possible that pyrene might control the release of Cu ligands that are capable of forming bioavailable Cu complexes. Dissolved metals in soils are present as free ions or as complexes with organic or inorganic ligands. Increased metal availability, especially an increased uptake by plants in the presence of metal complexes has been found (Degryse *et al*. 2006). Alternatively as explained by Alkio *et al.* (2005), PAH may passively penetrate the root cell membranes without any carrier which can therefore facilitate the penetration of metal or metal complexes into the cell. For example, Gao and Zhu (2004) observed that the root accumulation of pyrene by *Brassica rapa* increased with an increment in soil pyrene concentration and in lightly and heavily spiked soil with 8.01 and 489 mg kg^{-1} initial concentration respectively, pyrene concentration in roots reached 5.12 and 428 mg kg^{-1} respectively. The penetration of pyrene to root cell membranes could be the reason for the observed increase in shoot and root concentration of Cu with the addition of pyrene in our results. Lin *et al*. (2008) observed a reduction in the concentration of Cu in *Z. mays* with Cu-pyrene co-contamination. Their result contradicts our own findings probably due to differences in plant species. For example, Hinsinger (2001) has shown that plants have significant influence on the mobility of Cu as a result of the changes in the rhizosphere. These include changes in ion concentrations, values of redox potential as well the concentration of the root exudates and

results have shown that tomato plants were significantly more sensitive to toxic Cu levels in the soil than *Brassica napus* (Chaignon *et al*. 2002).

It was clear from our results that whether soil Cu was present in single or mixed contamination, higher Cu concentrations were always observed in the root. This was supported by the work of Brun *et al*. (2001) which showed that root Cu concentration is the best indicator of Cu contamination as it is the most sensitive. Lwasaki *et al*. (1990) reported that more than 60% of root Cu was bound to the root cell walls and that such adsorption of Cu in the root apoplasm may result in some protection of the root against Cu toxicity. However, apoplasmic Cu was not determined in our present experiment and it is not possible to know whether the elevated root Cu concentration reported here concerns mostly apoplasmic Cu only or both apoplasmic and symplasmic Cu. In any case, *B. juncea* appeared capable of restricting the shoot translocation of Cu.

The Cu removal efficiency by *B. juncea* was both relative to biomass production and tissue concentration. The observed increase in total Cu accumulation with increasing soil Cu concentration (Figures 5.5A and 5.5B) could be as a result of increased plant biomass caused by fertilizer application and is supported by other research. For example, Xie *et al*. (2011) showed that the total Cu accumulation in *Z. mays* at 100 mg kg^{-1} soil Cu concentration significantly increased with the application of either NPK or NP fertilizers. They observed that the fertilizers increased plant biomass which is a factor in accumulation of Cu. Our results showed that the increase in shoot dry matter yield compensated for the decrease in shoot Cu concentration.

With Cu and pyrene co-contamination, the phytoextraction of Cu by *B. juncea* is limited and it could be that pyrene altered the way *B. juncea* influenced Cu sorption and solubility. This could be as a result of the influence on plant growth as observed in the present study where co-contamination reduced shoot dry weight. Baek *et al*. (2006) observed that TNT significantly affected Cd uptake as a result of reduction in growth of *Rumex crispus*. Chen *et al*. (2004) observed a slight decrease in the accumulation of Cu by *Lolium perenne* in Cu-2,4-dichlorophenol co-contaminated soil and suggested that it contributed to the improved activation of Cu in planted soils. When pyrene was co-contaminated with cadmium, Zhang *et al*. (2009) suggested that the reduction in cadmium uptake with increased pyrene concentration could be as a result of the competition for adsorption between the co-existent pyrene and cadmium. In contrast, Almeida *et al.* (2009a) observed an increased accumulation of Cu by roots of *Halimione portulacoides* with the addition of PAHs. They suggested that PAHs could have altered the way the plants influenced Cu solubility and sorption. These assumptions were however restricted to hydroponics. It is interesting to observe similar changes from our soil experiment with these hydroponic experiments. While hydroponics is important for screening interesting properties in plants, there is evidence suggesting that plants respond in a different way when growth takes place in soil or hydroponics (Fitz and Wenzel 2002, Mehrag 2005). However, Moreno-Jimenez *et al.* (2010) in their work showed that the concentration of arsenic plants shoot and roots increased correspondingly with the arsenic dose either hydroponically or in soil. This suggests that soil values and hydroponics can be compared and the effects can be interpolated.

5.4.3 The potential of phytoremediation of Cu for combined contamination of Cu and pyrene

The phytoremediation of heavy metals from contaminated soils could require the phytoextraction of metals from contaminated soils by plants and for this to be possible, high metal uptake and TF are very important characteristics for plants used (Sun *et al*. 2011).

The rate of translocation within plants depends on the metal and plant species concerned (Deng *et al*. 2004). Our results showed that in soil contaminated with Cu only, the metal concentration in *B. juncea* was largely retained in the root while with the addition of 500 mg pyrene to 100mg Cu contaminated soil, the shoot of *B. juncea* retained more Cu than the root. It was clear that when soil was contaminated with Cu only, the more abundant the metal in the soil, the lower the TF of the metal. Deng *et al*. (2004) showed that the more abundant the Pb, Zn, Cu and Cd in sediments the less the translocation factors in plants including *Leersia hexandra*. The general trend in figures 5.3 and 5.4 showed that the concentration of Cu in root tissues is greater than in shoot of *B. juncea* although there is an exception at high concentration of Cu in combination with high pyrene concentration. There is a suggestion of a high plant availability of the substrate metal as well as its limited mobility as soon as it is inside the plant. Previous research (Cardwell *et al.* 2002, Fitzgerald *et al.* 2003) showed that the concentration of heavy metals in the root tissues of freshwater macrophytes from polluted areas usually contained higher concentrations of most metals relative to above ground parts. It was clear from our results that at higher soil Cu concentration with increased pyrene concentration (500 mg kg^{-1}), the TF was over 100% suggesting an increased phytoextracting efficiency of *B. juncea* for Cu in highly Cu-pyrene polluted soils. The efficient Cu transfer from root to shoot could be as a result of the

differences in the concentration of Cu and pyrene in the growth media. This is another important result in this experiment. The removal of Cu by plants in contaminated soils depends on the exposure time. Juang *et al.* (2011) showed that in short exposure time, the total Cu removed by *Medicago sativa* increased with increased Cu concentration in solution. However, for a longer exposure time, the maximum total Cu was removed for the lowest Cu concentration in solution. This could provide an explanation for our results where *B. juncea* was less efficient in removing Cu from soil contaminated with 100 mg kg^{-1} Cu over a 65-day period than soil with 50 mg kg^{-1} Cu. It could also be that *B. juncea* is capable of removing Cu at lower concentrations. Mokhtar *et al.* (2011) observed that *Eichornia crassipis* was capable of removing higher amount of Cu when the concentration of Cu was low.

5.4.4 Interaction of Cu and pyrene on pyrene removal form soil

The dissipation of pyrene in planted soil was greater than in non-planted soil except in co-contaminated soils which showed similar rates of dissipation (Figures 5.8A and 5.8B). This shows the benefit of vegetation in pyrene contaminated soils. This result is in agreement with other research. For example Lin *et al.* (2008) showed that the residual pyrene in soil planted with *Z. mays* was significantly lower than in non-planted soil with the initial pyrene concentration up to 500 mg kg^{-1}. Zhang *et al*. (2009b) also observed similar results with the use of *Z. mays*. In their research, planting reduced the extractable pyrene in soil by 21 to 31% while non-planting reduced soil pyrene by 12 to 27%. The increased dissipation of PAHs in soil has been widely researched and is mostly attributed to biotic and abiotic factors including increased biodegradation; primarily due to increased soil microbial activity (Kaimi *et al*. 2006), photodegradation, minimal plant uptake (He *et al.* 2005 and Nakamura *et al*.2004),

volatilization, leaching and incorporation into soil organic material (Gao *et al.* 2006). In the present study the roles of biotic and abiotic factors were not separated and the abiotic losses such as photodegradation, volatilization as well as leaching could have been high. It is important to note that leaching was reduced by collecting extra water in trays placed under the pots. Pyrene uptake by *B. juncea* was not investigated in the present study because in previous studies, plant uptake of PAHs has been found to be low and the uptake of pyrene could have occurred from air instead of the soil (Gao and Zhu 2004). The high removal rate of pyrene in the presence of *B. juncea* in pyrene only contaminated soil could also be related to the rhizospheric microbes that plays an important role in degradation of organics. For example Sun *et al*. (2010) showed that the dissipation of pyrene was higher in soil amended with root exudates than in soil with growing root of *L. perenne* releasing organic substances. This shows that the contribution of plants to the dissipation of PAH through processes such as degradation and immobilization highly depends on the processes that occur in the rhizosphere (Pilon-Smits 2005).

A negative effect of Cu on dissipation of pyrene was observed mainly in higher (100 mg kg^{-1}) concentration of Cu in the present study (Figures 5.7A and 5.7B). An interactive effect of heavy metals and PAHs on the degradation of PAHs can either cause a negative or positive effect depending on the type and concentration of both PAHs and heavy metals (Khan *et al*. 2009). For example, Khan *et al*. (2009) showed that Pb can increase the dissipation rate of pyrene in Pb- pyrene co-contaminated soil and an enhanced bacterial community was detected in soil while Baath (1989) reported that the presence of metals including Cd inhibited a broad range of microbial processes. The phytoremediation of organic compounds is highly based on

the efficient degradation of organics by plant roots with the formation of bound residues and from our results the increased concentration of residual pyrene in the presence of higher concentration of Cu could be linked to change in microbial activity as well as the composition of the microbes. Roberts *et al*. (1998) showed that when 0.1 to 2.0 mg L^{-1} of total metal was added to TNT-contaminated soil, the degradation rates of TNT decreased as a result of an increase in the time it takes for microbes to acclimatize to the new environment. The presence of Cu could have been the most important abiotic factor that affected the dissipation of pyrene in the present study. It is clear that Cu-tolerant or resistant microbes are favored in Cu-contaminated soil and they persist as long as they can withstand Cu toxicity (Sokhn *et al*. 2001). 100 mg Cu kg^{-1} could have reduced microbial activity as a result of the direct toxicity effect of Cu at the cellular level or maybe the enzymes that help in degradation of pyrene intermediates were inhibited. However, the residual pyrene in soil decreased even at 100 mg Cu kg^{-1} when compared initial soil concentration. This implies that even at higher Cu concentration, highly adapted Cu-resistant microbes could have enhanced degradation of pyrene.

There was also an important result observed in non-planted soils under co-contamination. The present study showed that in the presence of a high concentration (100 mg kg^{-1}) of Cu, the residual pyrene in planted soil was similar to that in non-planted soil. This implies that the presence of *B. juncea* did not affect the dissipation of pyrene in higher Cu co-contaminated soil and therefore there could be a suggestion that the phytoremediation of PAH in heavy metal co-contaminated soil is quite different to that in single PAH contaminated soil. As suggested by Olsen *et al*. (2003), the distinction in the quality or quantity of nutrients released

by plants root exudates as well as dead roots could lead to differences in microbial PAH degradation. These differences could be either positive or negative, however in the present study, there seemed to be an opposing effect on PAH dissipation. Lin *et al*. (2008) and Zhang *et al*. (2009) further showed that the degradation rate of pyrene in high Cu or Cd co-contamination in the presence of plants were significantly less compared to non-planted co-contaminated soils. Although their results were not similar to the present study, our result and theirs showed that plants did not enhance dissipation in heavy metal and PAH co-contaminated soil.

5.5 Conclusion

Phytoremediation is promising as a treatment technology for co-contaminated sites. The present study explored the potential of using plants for remediation of Cu and pyrene co-contaminated soils.

The growth of *B. juncea* was inhibited by the co-contamination of Cu and pyrene. Due to the decreased plant growth, the accumulation and removal percentage of Cu were inhibited. However, in the present study, the concentration of Cu in shoots of *B. juncea* increased with co- contamination which could be a positive effect of the interaction of Cu and pyrene. It was observed that *B. juncea* removed an average of 0.157 and 0.08% of total Cu for 50 and 100 mg kg^{-1} soil Cu contaminations by plant uptake, but the ability of Cu phytoextraction was halved under co-contamination of pyrene.

The dissipation of pyrene was enhanced by vegetation only in single pyrene contaminated soil. Co-contamination of pyrene and Cu inhibited the dissipation of pyrene which was clear

with increased Cu load. This could suggest that the changes in microbial numbers and activities as well as the root physiology under Cu stress were not favorable to the dissipation of pyrene.

It is difficult for *B. juncea* to grow normally under co-contamination of Cu and pyrene, but from a predictive perspective, if the growth of *B. juncea* could be enhanced, there are great chances of enhancing phytoextraction in Cu and pyrene co-contaminated soils and thereby effectively treat sites with these types of contamination.

5.6 Phytoremediation of Cr-B[*a*]P co-contaminated soils using *Zea mays*- Introduction

Heavy metal (HM) and polycyclic aromatic hydrocarbon (PAH) contamination have been a concern within the environment over recent years (Sun *et al*. 2011). Due to rapid urbanization and industrialization in developed and developing countries, these contaminants can be released into the environment (Watts *et al*. 2006).

PAHs are formed by incomplete combustion or pyrolysis of organic matter and are deposed from the atmosphere into the soil (Shen *et al*. 2006). They are recalcitrant, carcinogenic or mutagenic contaminants and their bioaccumulation tendency in the food chain is a concern (Jian *et al*. 2004). The accumulation of HMs and PAHs in the soil is becoming a significant environmental problem due to their potential impacts on soil health and the implication for food safety and human health risk (Khan *et al*. 2008).

Phytoremediation is a technology that uses plants to sequester metal and organic contaminants (Sun *et al*. 2008). This occurs through extraction, degradation, assimilation, metabolization or detoxification. Several studies have shown that plants can help in the removal of organic contaminants through various mechanisms such as volatilization, re-deposition on plant's leaves, sorption from direct contact with soil and degradation (Wild *et al* 2004, Lin *et al.* 2008).

Recently, research has addressed the uptake and accumulation of HM by plants in HM contaminated soils (Zayed *et al*. 1998, Babu *et al.* 2013) or the plant-enhanced dissipation of PAH in PAH contaminated soil (Li *et al*. 2006, Khan *et al.* 2013). For example, Liu *et al*.

(2008) in their study showed that *Acrasis rosea* accumulated up to 100 mg kg^{-1} of Cd in shoots when soil was contaminated with Cd alone. In addition, phytoremediation trials for organic contaminants have resulted in increased dissipation of PAH (Watts *et al* 2006, Xu *et al*. 2009) and PCB (Lin *et al*. 2006). However this does not often reflect real world situations where complex industrial processes and extended histories of multiple land use create sites containing mixed pollutants. Few papers have analyzed the combined uptake of HM as well as the dissipation of PAH in co-contaminated soil during phytoremediation. Therefore the aims of this study were to- (1) to examine the growth response of *Z. mays* to single Cr or B[*a*]P and co-contaminated Cr- B[*a*]P soils. (2) to understand the uptake, accumulation and translocation of Cr by *Z. mays*; (3) to understand the role of *Z. mays* in dissipation of B[a]P. B[*a*]P was chosen as the representative PAH because it is classified as a priority contaminant by the US Environmental Protection Agency because of it carcinogenic potential (Juhasz and Naidu 2000) whereas Cr is an important environment pollutant because of its widespread industrial use (Shanker *et al*. 2005). Results obtained from this study will provide more knowledge on the phytoremediation potential of *Z. mays* in Cr- B[*a*]P co-contaminated soils.

5.7 Methods

5.7.1 Soil spiking

Soil was spiked with B[*a*]P by dissolving 1, 5 and 10 mg of B[*a*]P in 25 mL of acetone. The solution of acetone and B[*a*]P was transferred into 250 g of soil as a portion and then mixed with 750 g of soil once the acetone had volatilized completely in the fume hood. 25 mL of acetone was also added to control and other soil treatments. 50 and 100 mg kg^{-1} of Cr was prepared by dissolving 0.141 and 0.282 g of $K_2Cr_2O_7$ and added singly in B[*a*]P spiked soils and fresh soils. The spiked soil was thoroughly mixed by sieving and stored in a dark room for equilibration for 28 days before planting.

5.7.2 Experimental set up

The experimental layout was designed in a completely randomized design of 21 treatments with three replicates of each. Pots spiked with B[*a*]P had treatments with no planting in order to observe non-plant facilitated dissipation of B[*a*]P.

5.7.3 Planting

Plastic pots of 12.5 cm in height were used for the present study. One kilogram of each spiked soil was placed in each pot. One seedling of *Z. mays* with uniform size of about 3 to 4 cm, 3 leaves and about 3 weeks old was transferred into each pot. Pots were watered when required with tap water to maintain the soil moisture during plant growth and the leachates from all pots were collected using the tray and returned to the soil. Throughout the experiment, the pots were periodically repositioned to minimize edge effects. After 60 days of growth, shoots

were cut just above the soil surface and washed with deionized water. Each pot was then emptied and the roots were separated from the soil by washing with running tap water. The roots were then rinsed with deionized water 3 times to remove all soil particles. All samples were oven-dried to constant weight at 65 ºC for 72 hours. The dried samples were weighed to enable biomass calculations and used for plant analysis.

5.7.4 Analysis of plants and soil samples

Oven-dried plants were ground into small pieces using a coffee grinder (Krups, Italy). Approximately 0.3 g and 0.1 g of shoot and root dry matter respectively were digested using 5 mL of 30% HNO_3 and placed on a heating block (section 3.4.1). Digested plant samples were then analyzed for total Cr using FAAS. B[*a*]P concentration in soil samples was analyzed using the Agilent GC-MS (sections 3.5.1, 3.5.2 and 3.5.3). The average percentage recovery for surrogate was 76%.

5.7.5 Statistical analysis

All treatments were replicated three times. The means and standard error (SE) were calculated using Microsoft Office Excel 2007. The comparisons of shoot dry matter, Cr concentration, accumulation as well as soil residual B[*a*]P were carried out by one-way analysis of variance using Minitab 15.0. The root accumulation results were log normalized. When a significant difference was observed between treatments, multiple comparisons were made by the Tukey HSD test.

5.8 Results

5.8.1 Plant growth and biomass

After 60 days of exposure to Cr, B[*a*]P or a combination of B[*a*]P and Cr, no visible toxic symptoms were observed for *Z. mays* in all treatments. Compared to control treatments, all concentrations of individual or combined pollutants had no significant effect on root and shoot biomass ($p>0.05$). Although results were not statistically significant, 1 and 5 mg kg^{-1} of B[*a*]P seemed to slightly increas the shoot dry weight of *Z. mays* by 4.6 and 14.4% respectively relative to control treatments while 10 mg kg^{-1} reduced the shoot biomass by 38.6%. Increasing concentration of Cr in soil reduced the shoot dry weight of *Z. mays*. Under conditions of 50 and 100 mg Cr kg^{-1} soil concentration, the shoots dry weight relative to control treatments decreased by 13 and 25% respectively (Table 5.3).

The presence of Cr did not seem to affect the growth of *Z. mays* under co-contamination of Cr and B[*a*]P. For example, when 50 mg of Cr was combined with 1 and 5 mg of B[*a*]P, the 11 and 31% shoot dry weight reduction observed when compared to shoot biomass in soil contaminated with 1 and 5 mg of B[*a*]P alone was not significant. Similar results were observed when 100 mg Cr was combined with 1 and 5 mg of B[*a*]P.

Similarly, the root biomass of *Z. mays* did not seem to be affected for all single and mixed contamination of Cr and/or B[*a*]P ($p=0.05$).The maximum root biomass was observed when the concentration of Cr in soil was 100mg kg^{-1} and root biomass was higher than when concentration in soil was 50 mg kg^{-1}.

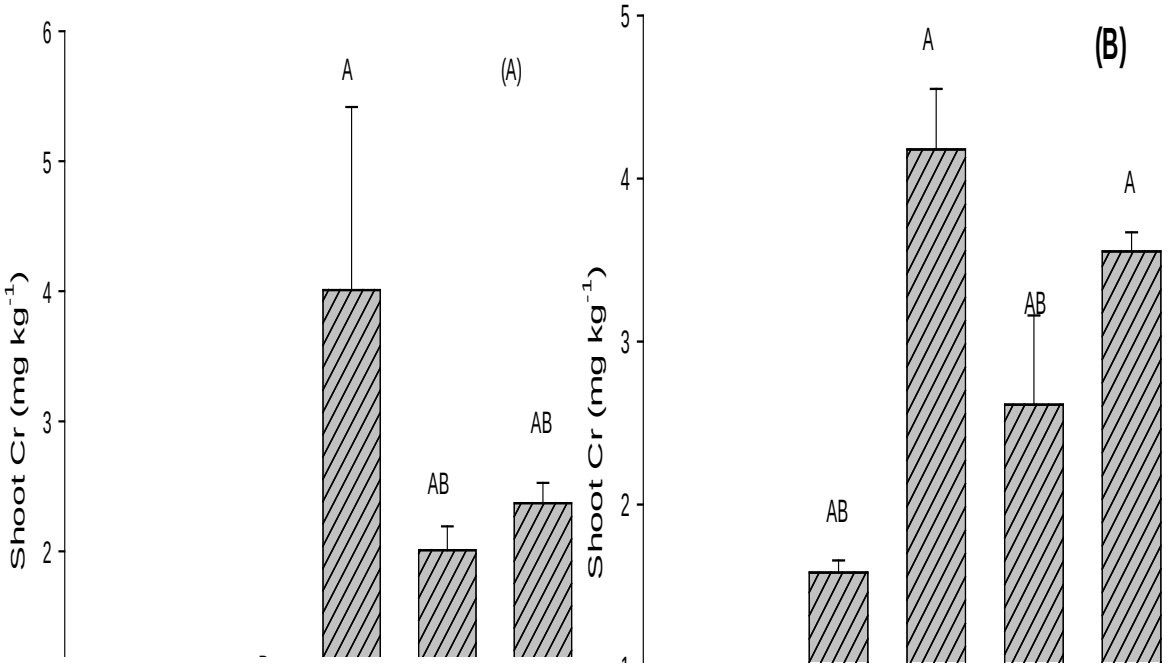
Table 5.3: Plant dry matter yield of *Z. mays* as affected by co- contamination of B[*a*]P and Cr after 60 days of growth. Values are mean ± SE. Different letters within a column indicate a significant difference (1-way ANOVA, Tukey HSD, P< 0.05). Appendix 5B.1 and 5B.2

Cr added (mg kg^{-1})	B[*a*]P added (mg kg^{-1})	Shoot dry weight (g)	Root dry weight (g)
0	0	2.76 ±0.35[a]	0.23 ±0.03[a]
50	0	2.41 ±0.06 [a]	0.20 ±0.06 [a]
100	0	2.07 ±0.18[a]	0.50 ±0.00[a]
0	1	2.90 ±0.10 [a]	0.30 ± 0.06 [a]
50	1	2.57 ±1.02 [a]	0.33 ±0.09[a]
100	1	2.27 ±0.13 [a]	0.30 ±0.00 [a]
0	5	3.20 ±0.43 [a]	0.40 ±0.06 [a]
50	5	2.23 ±0.26 [a]	0.37 ±0.09[a]
100	5	2.47 ±0.09 [a]	0.37 ±0.03 [a]
0	10	1.70 ±0.78 [a]	0.23 ±0.07 [a]
50	10	2.23 ±0.18 [a]	0.33 ±0.07 [a]
100	10	1.23 ±0.15[a]	0.27 ±0.03 [a]

5.8.2 Cr concentration in plant tissues of *Z. mays* exposed to Cr and B[*a*]P

The concentration of Cr in plant tissues was affected by Cr and B[*a*]P treatments and significant interactions were detected. The concentrations of Cr in different plant tissues increased with an increase in soil Cr and increased further with B[*a*]P additions except in roots which decreased with B[*a*]P addition (Figures 5.9A and 5.9B). When 50 and 100 mg Cr was added to soil, the shoot Cr concentrations were 0.67 and 1.58 mg kg^{-1}. This represents a 41 and 75% increase in shoot concentration of Cr relative to control treatments. The addition of 1 mg kg^{-1} of B[*a*]P to 50 and 100 mg kg^{-1} of Cr significantly ($p<0.05$) increased the concentration of Cr in shoot of *Z. mays* by 90%. The combined treatment of 10 mg B[*a*]P with

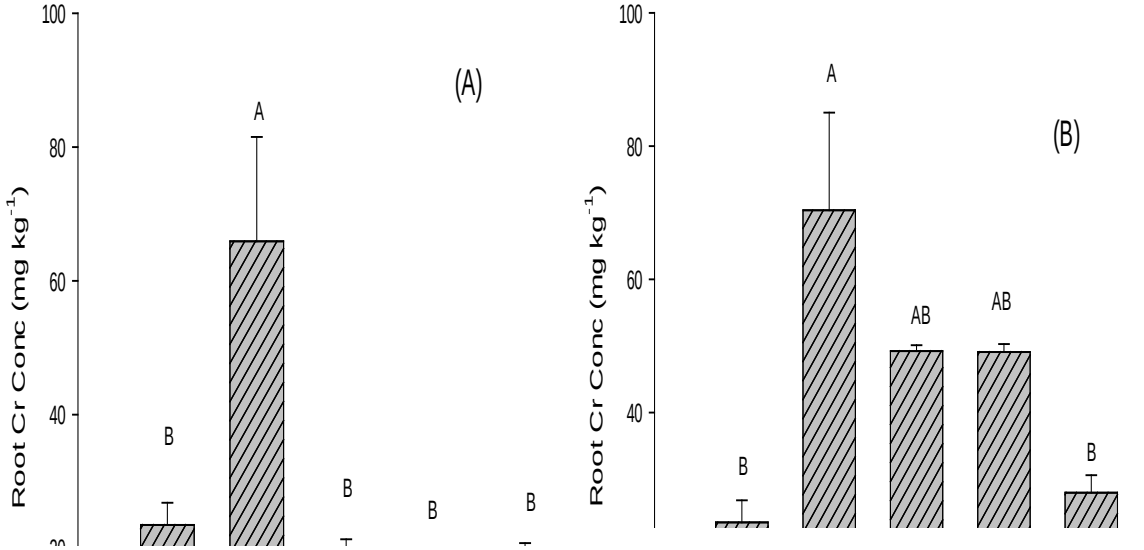
100mg Cr also significantly ($p<0.05$) increased the shoot concentration of *Z. mays* by 88% when compared with control treatments. Figure 5.9A shows that the addition of 5 mg kg^{-1} of B[*a*]P to 50 mg kg^{-1} Cr caused the lowest shoot Cr concentration for mixed contaminants. The larger error bar observed for treatment CIBI relates to the variable growth performance in plants.



Figures 5.9a and b: Cr concentration in shoot of *Z. mays* as a function of various concentrations of Cr and B[*a*]P. Bars indicate (means ± SE, n= 3). Bars that do not share a letter are significantly different (Tukey HSD, $p \leq 0.05$). Treatments C0, C1and C2 represents 0, 50 and 100 mg Cr kg^{-1}; B0, B1, B2 and B3 represents 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P. Appendix 5B.3

The amount and distribution of Cr uptake in the root of *Z. mays* are shown in figures 5.10A and B. The concentration of Cr in roots increased with an increase in soil Cr concentration. As the concentration of Cr in soil increased from 50 to 100 mg kg^{-1}, the Cr in roots of *Z. mays* increased significantly from 65.8 to 70 mg kg^{-1}. Co-contamination of Cr and B[*a*]P seemed to

reduce root Cr concentration compared to single contamination of Cr (Figures 5.10A and 5.10B). The co- contamination of 10 mg kg^{-1} of B[*a*]P with 100 mg kg^{-1} of Cr significantly ($p<0.05$) reduced the root concentration of *Z. mays* by 60% relative to single treatment with 100 mg kg^{-1} of Cr while 1 and 5 mg kg^{-1} of B[*a*]P did not have any effect statistically. Also the root concentration of Cu under co-contamination of 1, 5 and 10 mg kg^{-1} of B[*a*]P with 50 mg kg^{-1} of Cr reduced significantly by 74, 78 and 71% respectively compared to single treatment with 50 mg kg^{-1} of Cr.

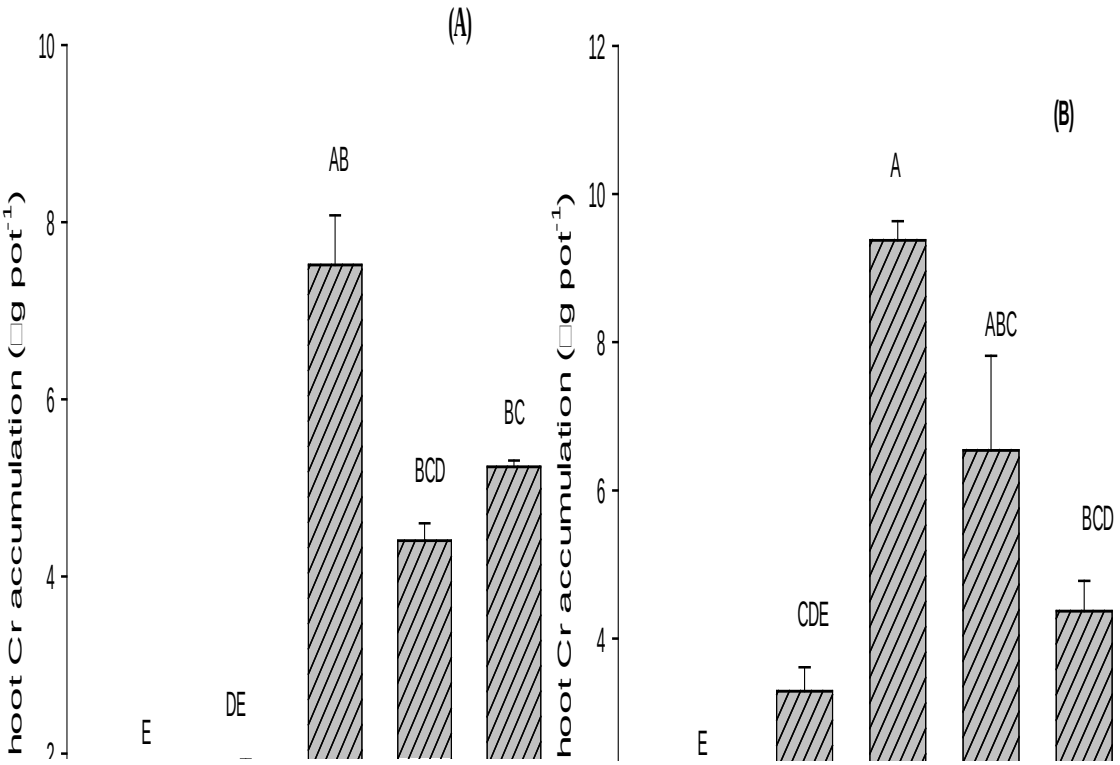


Figures 5.10a and b: Cr concentration in root of *Z. mays* as a function of various concentrations of Cr and B[*a*]P. Bars indicate (means ± SE, n= 3). Bars that do not share a letter are significantly different (Tukey HSD, $p \leq 0.05$). Treatments C0, C1and C2 represents 0, 50 and 100 mg Cr kg^{-1}; B0, B1, B2 and B3 represents 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P. Appendix 5B.5

5.8.3 Cr accumulation in plant tissues of *Z. mays* under joint stress of Cr and B[*a*]P

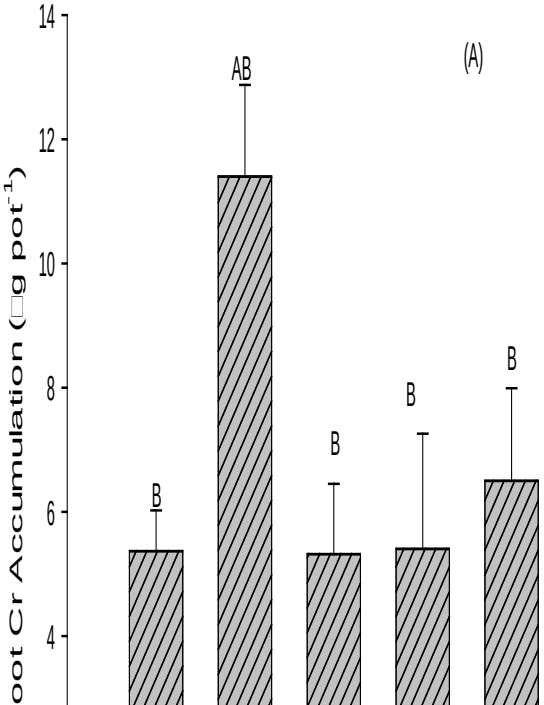
The accumulation of Cr by *Z. mays* is relatively low in soils contaminated by Cr (Figures 5.11A and 5.11B). The average Cr in shoots of *Z. mays* was less than 4 µg pot^{-1} even when the concentration of Cr in soil reached 100 mg kg^{-1}. Our result showed that as the initial soil Cr concentration increased from 50 to 100 mg kg^{-1} the total accumulation of Cr in shoots of *Z. mays* increased from 1.63 to 3.29 µg pot^{-1}.

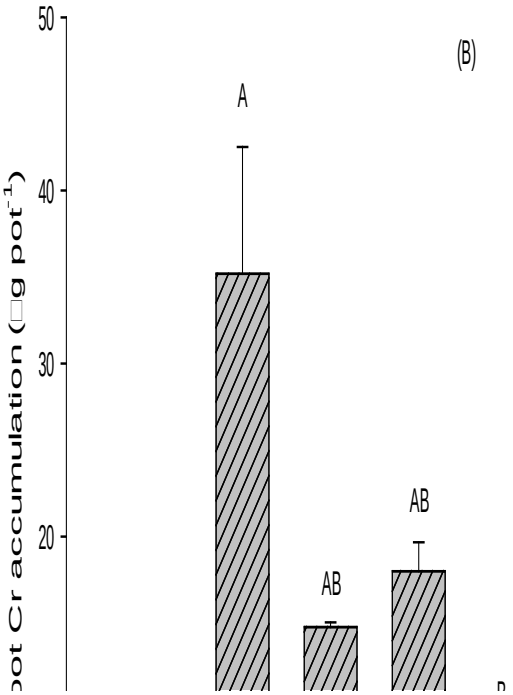
An interactive effect of Cr and B[*a*]P on plant accumulation of Cr in shoot ($P < 0.05$) and root ($P < 0.05$) was observed. It is interesting to note that that the addition of B[*a*]P significantly ($P < 0.05$) increased Cr accumulation in shoot of *Z. mays* relative to control treatments and significantly reduced root Cr concentrations at 100 mg kg^{-1} soil Cr concentration. When the concentration of Cr remained at 50 mg kg^{-1}, the addition of 1, 5 and 10 mg kg^{-1} B[*a*]P significantly ($p<0.05$) increased the accumulation of Cr in shoots of *Z. mays* by 88, 79 and 82% respectively relative to control treatments. Similarly when the concentration of Cr in soil remained at 100 mg kg^{-1}, the addition of 1, 5 and 10 mg kg^{-1} B[*a*]P significantly increased the accumulation of Cr in shoots of *Z. mays* by 90, 86 and 79% respectively relative to control treatments. Lower concentrations of B[*a*]P in co- contaminated soil seemed to increase Cr accumulation more than higher B[*a*]P concentrations.


(A)
10
8
6
4
2
hoot Cr accumulation (□g pot-1)
E
DE
AB
BCD
BC
(B)
12
10
8
6
4
hoot Cr accumulation (□g pot-1)
E
CDE
A
ABC
BCD

Figures 5.11a and b: Cr accumulation in shoot of *Z. mays* as a function of various concentrations of Cr and B[*a*]P. Bars indicate (means ± SE, n= 3). Bars that do not share a letter are significantly different (Tukey HSD, $p \leq 0.05$). Treatments C0, C1and C2 represents 0, 50 and 100 mg Cr kg^{-1}; B0, B1, B2 and B3 represents 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P. Appendix 5B.4

The accumulation of Cr within roots of *Z. mays* increased with increasing concentration of Cr in single Cr contaminated soil and decreased with co-contamination with B[*a*]P. Figures 5.12A and B show that as the concentration of Cr in soil increased from 50 to 100 mg kg^{-1}, the root accumulation of Cr increased from 11.39 to 35.18 µg.pot^{-1}. Results were only significant ($P < 0.05$) at 100 mg kg^{-1} soil Cr concentration. Co-contamination of 50 mg kg^{-1} Cr with 1, 5 and 10 mg kg^{-1} B[*a*]P did not seem to affect root accumulation of Cr when compared to control treatments. Also, co-contamination of 100 mg kg^{-1} soil Cr with B[*a*]P significantly ($p<0.05$) increased the root Cr accumulation from 14.76 to 17.98 µg.pot^{-1} as the concentration of B[*a*]P increased from 1 to 5 mg kg^{-1}. In contrast, root accumulation reduced to 7.8 µg.pot^{-1} at 10 mg kg^{-1} B[*a*]P soil concentration.





Figures 5.12a and b: Cr accumulation in root of *Z. mays* as a function of various concentrations of Cr and B[*a*]P. Bars indicate (means ± SE, n= 3). Bars that do not share a letter are significantly different (Tukey HSD, $p \leq 0.05$). Treatments C0, C1and C2 represents 0, 50 and 100 mg Cr kg^{-1}; B0, B1, B2 and B3 represents 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P. Appendix 5B.6

5.8.4 Bioconcentration and Translocation Factors

The shoot concentration factors (SCF) and root concentration factors (RCF), defined as the compartment concentration ratio of Cr in plant to that of soil, are used to evaluate the plant

accumulation capacity. The interactive effect of Cr and B[*a*]P on the SCFs and RCFs is shown in Table 5.4. The RCFs were much higher than the SCFs under both single Cr exposure as well as when B[*a*]P was added. With single contamination of soil at 50 mg kg^{-1} of Cr, the SCF reduced by 0.008 and increased significantly by 79% with co-contamination with 1mg kg^{-1} B[*a*]P. As the soil Cr concentration increased to 100 mg kg^{-1}, the SCF were similar to control treatment as well as with the addition of 1, 5 and 10 mg kg^{-1} of B[*a*]P. The RCF for plants in 50 mg kg^{-1} Cr contaminated soils remained at 0.77 and reduced significantly from 0.178 to 0.167 with co- contamination of 1 and 5 mg kg^{-1} B[*a*]P. At 100 mg kg^{-1} soil Cr contamination, the RCF was lower when compared to 50 mg kg^{-1} and did not differ with co-contamination with 1, 5 and 10 mg kg^{-1} of B[*a*]P.

The TF under single contamination was less than 100% and increased with co-contamination (Table 5.4). With single contamination of 50 mg kg^{-1} Cr, the TF was 1.14% which increased by 1.4% as the concentration of Cr in soil increased to 100 mg kg^{-1}. Relative to single contamination of 50 mg kg^{-1} Cr, co-contamination of 50 mg kg^{-1} Cr with 1, 5 and 10 mg kg^{-1} of B[*a*]P increased the TF by 20, 13 and 11% respectively. Similarly, the TF for co-contamination of 100 mg kg^{-1} Cr and 1, 5 and 10 mg kg^{-1} B[*a*]P also increased by 5.98, 2.8 and 10.3% respectively when compared to single treatments with 100 mg kg^{-1} Cr.

Table 5.4: Translocation Factors of Cr as affected by single and co- contamination of Cr and B[*a*]P Appendix 5B.7 and 5B.8

Cr added (mg kg^{-1})	**B[*a*]P added (mg kg^{-1})**	**TF**	**SCF**	**RCF**
0	0	0.02±0.008	0.008±0.005	0.477±0.094

50	0	0.01±0.003	0.008±0.002	0.777±0.189
100	0	0.03±0.007	0.013±0.000	0.563±0.126
50	1	0.21±0.043	0.041±0.013	0.178±0.306
100	1	0.15±0.023	0.033±0.004	0.385±0.215
50	5	0.12±0.014	0.023±0.002	0.167±0.360
100	5	0.09±0.009	0.020±0.004	0.383±0.017
50	10	0.05±0.012	0.027±0.003	0.218±0.012
100	10	0.12±0.008	0.026±0.001	0.208±0.218

5.8.5 B[*a*]P dissipation

After 60 days of planting with *Z. mays*, the residual concentration of B[*a*]P in soil for single B[*a*]P contaminated soil ranged from 0.59 to 3.32 mg kg^{-1}, while in co-contaminated soil treatments, the residual B[*a*]P concentration ranged from 0.61 to 5.82 mg kg^{-1}.

Figure 5.13 shows that the residual B[*a*]P concentration decreased for 1, 5 and 10 mg kg^{-1} single B[*a*]P contaminated soil when compared to zero time concentration, with 10 mg kg^{-1} having the highest concentration of B[*a*]P in soil after planting.

After 60 days of planting in 1 mg kg^{-1} B[*a*]P contaminated soil, the residual B[*a*]P remained at 0.59 mg kg^{-1}. Co-contamination with 50 or 100 mg kg^{-1} of Cr did not seem to affect the dissipation of B[*a*]P in soil ($P \geq 0.05$). In contrast, when the concentration of B[*a*]P in soil increased to 5 and 10 mg kg^{-1}, the residual B[*a*]P concentration in soil after planting remained at 1.81 and 3.31 mg kg^{-1} respectively while co-contamination of 5 mg kg^{-1} of B[*a*]P with 50 and 100 mg kg^{-1} of Cr significantly decreased the dissipation rate of B[*a*]P concentration by 26 and 30% respectively.

When planted and non-planted soils are compared, the residual B[*a*]P concentration in soils contaminated with B[*a*]P alone seemed not to be affected by planting except in 10 mg kg^{-1} B[*a*]P contaminated soil. As shown in figure 5.13, the presence of plants only significantly decreased the residual B[*a*]P concentration from 5.49 to 3.32 mg kg^{-1} in 10 mg kg^{-1} B[*a*]P contaminated soil when compared to non-planted soil.

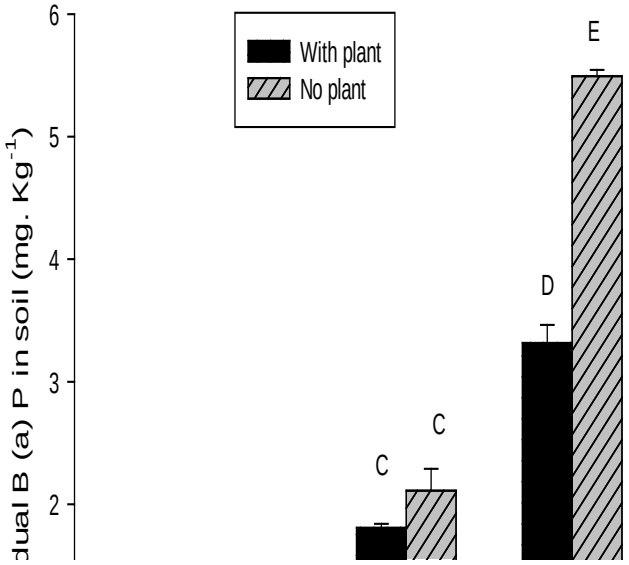
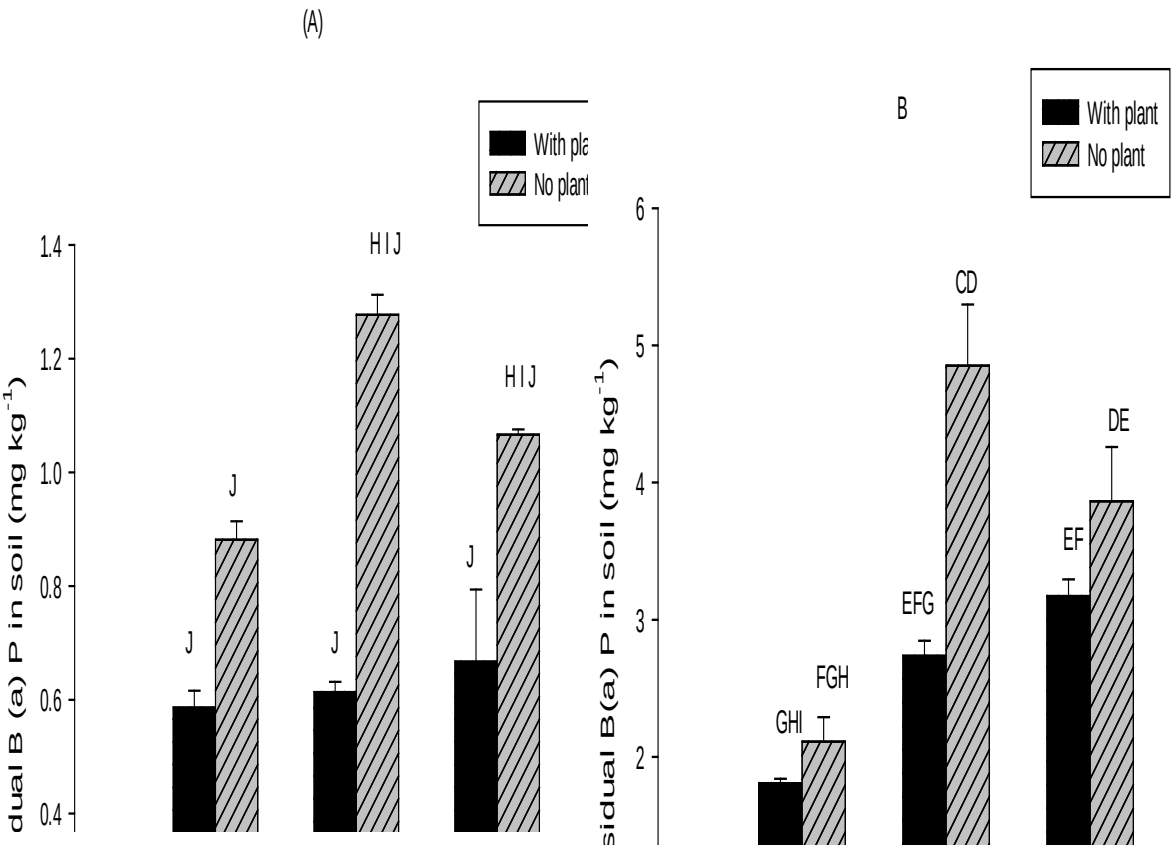
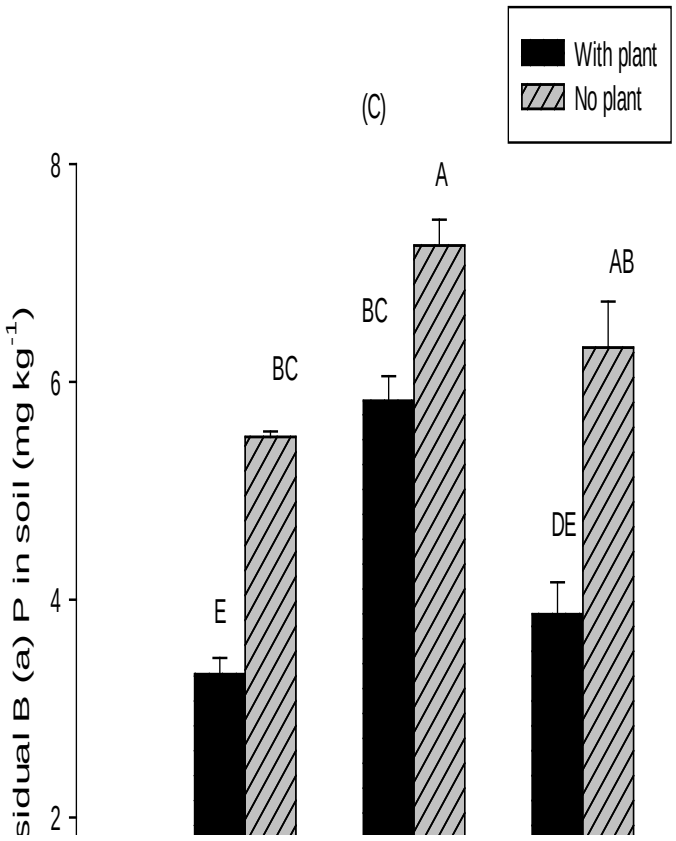


Figure 5.13: Residual B[*a*]P concentration in planted and non-planted soil after 6o days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different (Tukey HSD, $p \leq 0.05$). Treatments B1, B2and B2 represents 1, 5 and 10 mg Cu kg^{-1} of B[*a*]P Appendix 5B.9

Under co-contamination, the effect of planting on B[*a*]P dissipation varied. Planting did not significantly affect the dissipation of B[*a*]P in soils co-contaminated with 1 mg kg^{-1} of B[*a*]P and Cr (Figure 5.14A). In contrast there seemed to be a significant effect of planting on B[*a*]P dissipation in soil co-contaminated with 5 and 10 mg kg^{-1} B[*a*]P. It is clear from figure

5.14C that when the concentration of B[*a*]P remained at 10 mg kg^{-1}, co-contamination with 50 and 100 mg kg^{-1} Cr significantly decreased the residual B[*a*]P concentration from 6.31 to 3.87 mg kg^{-1} and 7.25 to 5.82 mg kg^{-1} respectively in the presence of plants. Also after planting in 50 mg kg^{-1} Cr + 5 mg kg^{-1} co-contaminated soil, the residual B[*a*]P significantly decreased by 44% when compared to non-planted soil.





Figures 5.14 (A, B and C): comparison of residual B[*a*]P concentration in single and co-contaminated soil. Means that do not share a letter are significantly different (Tukey HSD, $p \leq 0.05$).Treatments C1and C2 represents 50 and 100 mg Cr kg^{-1}; B1, B2 and B3 represents 1, 5 and 10 mg kg^{-1} of B[*a*]P. Appendix 5B.9

5.9 Discussion

5.9.1 Interaction of Cr and B[*a*]P influencing plant growth

From our results, the reduction in shoot biomass with increasing Cr concentration was not significantly different from the control treatments. Also the root biomass was not negatively affected by increasing concentrations of Cr. This is a demonstration of plant tolerance to Cr as suggested by (Gardea-Torresday *et al*. 2004). Their research showed no significant difference between the root elongation of *Convolvulus arvensis* exposed to 20, 40 and 80 mg L^{-1} of Cr. Chromium is thought to alter plant membrane systems with the primary toxic effect being membrane damage and chlorophyll biosynthesis reduction due to high oxidative potential of Cr (Vajpayee *et al*. 2000). However, the physiological and metabolic response is not understood properly. Davies *et al*. (2001) suggested that Cr acts principally on plant roots, thereby causing intense growth inhibition. There were also contrasting reports on biomass of plants as affected by Cr. For example, Han *et al*. (2004) observed an increase in biomass of *Brassica juncea* up to 100 mg kg^{-1} of Cr whereas the yield of *Hordeum vulgare* . *L* was markedly decreased by Cr in soil (Wyszkowski and Radziemska 2010). It could be suggested that the phytotoxicity of Cr is dependent on plant species. The phytotoxicity threshold concentration for *B. juncea* in Cr contaminated soil was 6.2 mg kg^{-1} (Bolan *et al*. 2003), 5.9 mg kg^{-1} for *Z. mays* grown in sludge amended soil (Mortvedt and Giordano 1975) and 185 mg kg^{-1} for *Pisum sativum* grown in Cr (VI) sand medium (Bishnoi *et al*. 1993).

B[*a*]P did not seem to affect the dry matter yield of *Z. mays* in the present study. Although shoot biomass was more inhibited as the concentration of B[*a*]P reached 10 mg kg^{-1}, *Z. mays*

was capable of withstanding an addition of 10 mg kg^{-1} of B[*a*]P in soil and survive until the end of the assay.

. Plants showed better growth in B[*a*]P treatment alone than when in a mixture of Cr and B[*a*]P, except for 50 mg Cr + 10 mg B[*a*]P treatment which showed higher biomass than 10 mg B[*a*]P single contamination. The combination of Cr and B[*a*]P exerted an antagonistic effect on plant growth and performance, although results were not significant. This is similar to results obtained by Sun *et al.* (2011) where shoot mass of *Tagetes patula* tended to decrease non-significantly under joint stress of heavy metals (Pb and Cd) and B[*a*]P relative to single B[*a*]P treatments. Based on the non-significant results observed between, B[*a*]P, Cr and control treatments, it could be suggested that *Z. mays* may be considered to be species of greater biomass potential for phytoremediation of B[*a*]P and Cr co-contaminated sites.

5.9.2 Cr concentration and accumulation as affected by co-contamination

The observed higher concentration of Cr in *Z. mays* (Figures 5.9A and B, 5.10A and B) could be as a result of toxicity of Cr which may damage the plasmalemma (Pandev *et al*. 2009). This damage results in an increased uptake of Cr. Vazquez *et al*. (1987) suggested that the primary toxicity of Cr (VI) to *Phaseolus vulgaris* is membrane damage due to high oxidative power. They also suggested that when Cr is taken up by roots, it interacts with the organic matter and is reduced to trivalent Cr which is located in the vacuoles. Precipitation of Cr in root cell vacuoles results in low translocation of Cr from roots into shoots with minimal injury. This maybe a natural toxicity response of the plant since the accumulation of Cr in the vacuoles of root cells renders it less toxic assumedly due to it being away from the sites of

photosynthesis (Shankar *et al.* 2005). Zayed *et al*. (1998) observed that the roots of many vegetable plants including *Brassica oleracea* converted Cr (VI) to Cr (III). Our results confirm the observation of Zayed *et al*. (1998) that the translocation of Cr from roots to shoots was limited. Even at higher soil Cr concentration (100 mg kg^{-1}), the uptake of Cr by *Z. mays* remained low (Figure 5.9B). This could be related to the strong binding of Cr to solid-phase components in the soil where Cr (VI) is reduced to Cr (III). Similar results were observed for *B. juncea* at 50, 100, 250 and 500 mg kg^{-1} soil Cr concentration (Han *et al*. 2004).

The increased concentration of Cr in shoot of *Z. mays* in co-contamination of B[*a*]P and Cr compared to single contamination of Cr could be as a result of low accumulation of Cr by roots. When the concentration of Cr is low, Cr has higher transfer mobility from root to shoots and when the roots take up higher concentration of Cr from soils, the rate of transfer from root to shoot decreases (Han *et al*. 2004). Probably, the interactive effect of Cr and B[*a*]P reduced the amount of Cr taken up by the root of *Z. mays* leading to an increased translocation of Cr to the shoots. Sun *et al.* (2011) observed similar results with B[*a*]P and cadmium (Cd), where a high amount of Cd was translocated from root to shoot of *T. patula* under co-contamination of B[*a*]P and Cd. Although their results were not compared to single contamination with Cd alone, other research on Cd accumulation by *T. patula* on single Cd contamination showed lower translocation of Cd from root to shoot and lower amounts of Cd in shoot compared to roots (Bareen and Nazir 2010, Liu *et al*. 2011). Organic contaminants are mainly adsorbed onto organic matter within the soil, and are linked to the soil surfaces by either hydrogen or other chemical bonds (Xing and Pignatello 1998). Co- contamination of B[*a*]P could therefore, affect the activation potential of Cr in the soil changing the extent of interaction

with organic matter. For example, Chen *et al*. (2004) showed that co- contamination of 2,4-dichlorophenol affected the behavior of heavy metals (Zn and Cu) in soil organic matter resulting in a change in their activation potential. In their research, soluble plus exchangeable Cu and Zn increased from 2.8 to 3.1 mg kg^{-1} and from 13.9 to 16.2 mg kg^{-1} respectively with co-contamination of 2,4-dichlorophenol when compared to single contamination of Cu or Zn. Also co-contamination of 2,4-dichlorophenol with Zn and Cu slightly decreased the water soluble Cu and Zn while the bound Cu and Zn increased.

5.9.3 Phytoremediation potential of *Z. mays* for combined pollution of Cr and B[*a*]P

As shown in Table 5.4, the shoot concentration factor of *Z. mays* under single contamination of Cr increased from 0.008 to 0.013 when the concentration of Cr in soil increased from 50 mg kg^{-1} to 100 mg kg^{-1}. This suggests that the concentration factor of Cr in *Z. mays* increased with increased loading level in Cr contaminated soil at the levels used in the present study. It therefore implies that the uptake efficiency of Cr in single Cr contaminated soil increases at higher concentrations. Similar results were obtained by Han *et al*. (2004) for both Cr (III) and Cr (VI) treated soil where SCF increased from 0.5 to 1.5 and 1.5 to 3 as soil Cr concentration reached 2000 and 500 mg kg^{-1} respectively. For hyperaccumulator plants, TF is typically greater than 1 (Tappero *et al*. 2007). Our results with single and mixed contamination of Cr showed that *Z. mays* accumulated only small amount of Cr. With single contamination of 50 and 100 mg kg^{-1}, it was obvious that Cr was slowly translocated within the plant from the root to the shoot. This was evident as the TF for *Z. mays* reached highest value of 0.025 for 100 mg kg^{-1} single soil Cr concentration (Table 5.4). This result is in agreement with other studies which also reported highest Cr accumulation in roots (Zayed *et al*. 1998, Gheju *et al*. 2009).

The translocation factor under co-contamination of Cr and B[*a*]P showed a remarkable increase especially for 50 mg kg^{-1} soil Cr co-contaminated with B[a]P. The TF for treatment C1B1 reached 0.21 while treatments C1B2 and C1B3 also reached 0.15 and 0.12 respectively. For plants to be able to hyperaccumulate Cr from soil, the plants must be efficient in a series of procedures including solubilization of Cr in soil, absorption of soluble Cr as well as translocation and detoxification of absorbed Cr within the plant (Hossner *et al*. 1998). Although the detailed mechanism of Cr translocation varies from one plant species to another (Yu *et al*. 2008), there are reports that Fe-deficient and P-deficient plants have better Cr translocation rates from roots to shoot (Bonet *et al*. 1991). It is possible that the antagonistic effect of the combination of Cr and B[*a*]P on the growth of *Z. mays* could have an effect on P and Fe content and as such could be a reason for increased translocation of Cr from root to shoot under co- contamination. From our results, it does not mean that higher TF of Cr in mixed contamination relative to single Cr contamination demonstrated better phytoaccumulation of Cr from soil. The bioconcentration factor (BCF) suggests that single contaminants had higher BCF and Cr removal rate over the course of study. It seemed that co-contamination of Cr and B[*a*]P stabilized more Cr in soil compared to single treatments and therefore probably allowed only lesser accumulation in roots. It could also be possible that the toxic and mobile hexavalent Cr was reduced to a stable Cr (III). Barnhart (1997) suggested that even if Cr is added to the soil in its mobile form, it tends to be converted to the trivalent oxide when in contact with the natural environment. The low root uptake of Cr (Figures 5.12A and B) is perhaps related to the partial conversion of Cr (VI) to Cr (III) where in the presence of oxidizing Cr (VI), is hydrolyzed and immobilized instead of getting converted to

labile Cr (III). Mishara *et al.* (1994) observed similar trends with *Z. mays* under single Cr soil contamination. Co-contamination of Cr and B[*a*]P could have been more toxic to the root of *Z. mays*, therefore inhibiting root growth and leading to less root Cr uptake from the soil. This is evident as the present study showed that the root growth of *Z. mays* in soil treatments with a mix of 100 mg kg^{-1} Cr and 1, 5 or 10 mg kg^{-1} B[*a*]P reduced when compared to single treatments of 100 mg kg^{-1} Cr. Similar results were observed by other researchers. For example, Mukharji and Roy (1978) suggested that Cr (VI) acts principally on roots resulting in intense growth inhibitions while Wang *et al*. (2011) concluded in their research that the joint action of combined pollution of B[*a*]P and Pb to the root growth of *Triticum spp* was mainly antagonistic inhibitory effect. Rooting patterns could also be one of the reasons why dicots transported more Cr to shoots than monocots (Cary *et al*. 1977, Sampanapanish *et al*. 2006). *Z. mays* is a monocot and this could be the reason why the removal rate of Cr from soil in the present study remained low both in single and mixed contamination.

5.9.4 B[*a*]P dissipation in soil

The interaction of HM and PAH in co-contaminated soil may cause either positive or negative effects on the growth of plants, the uptake of compounds as well as directly affecting the microbial consortium thereby inhibiting or enhancing the dissipation of PAH (Lin *et al*. 2008). In the present study, co-contamination of Cr and B[*a*]P did not seem to affect the root growth of *Z. mays*. Therefore it is possible that a direct effect on B[*a*]P degrading indigenous microbes could have inhibited the dissipation of B[*a*]P in co-contaminated soils when compared to single B[*a*]P contaminated soil (Figure 5.13). Knight *et al*. (1997a) suggested that co-contamination can affect a broad range of microbial processes in soil including,

methane metabolism, nitrogen conversion and other reductive processes and that the result is a direct or indirect degradation of organic contaminants.

Also, in this study, the role of planting and non-planting was studied. It was obvious that cultivation with *Z. mays* did not affect the dissipation of lower concentration (1 mg kg^{-1}) of B[*a*]P in single and co-contaminated soils. However, in 10 mg kg^{-1} B[*a*]P single and co-contaminated soil, about 24% and 29% of B[*a*]P was dissipated with the help of plants respectively. Several processes could have enhanced the dissipation of B[*a*]P in the present study even in non-planted soil. It is known that B[*a*]P is a semi-volatile compound and could evaporate partly from soil due to increased temperature (Wild and Jones 1993). Also because the soil used for the present study was not sterile, it could also be possible that biodegradation by indigenous B[*a*]P degrading microorganisms present in contaminated soil took place. The biodegradation of B[*a*]P by indigenous microbes is however a long-term solution to contaminated land treatment (Keck *et al*. 1989). The similar dissipation rate observed in planted and non-planted soil at lower B[*a*]P concentration could be due to the competition between *Z. mays* and microorganism for nitrogen and other nutrients in soil. When this happens, the dissipation of B[*a*]P from the rhizosphere of *Z. mays* could be impeded.

In higher B[*a*]P co-contaminated soil (10 mg kg^{-1}), the presence of plants most likely enhanced the dissipation rate of B[*a*]P when compared to non-planted soil. As explained by Wilcock *et al*. (1996), the half life of B[*a*]P in soil is in the region of 100 days to 14.6 years. Therefore it is important to note the observed 42 to 63% increase in single B[*a*]P contaminated soil as well as a 17 to 46% dissipation rate in co-contaminated soil over a 60 day greenhouse study. It is important to further study the microorganisms that enhanced the

dissipation of B[*a*]P in soil as well as other processes that significantly decreased the half life of B[*a*]P. The soil used for the present study and the environmental conditions seemed to be favorable for biodegradation. These include lower pH and constant greenhouse temperature.

Gao *et al*. (2008) suggested that the mechanisms of PAH dissipation in soil are through processes such as leaching, volatilization, biodegradation, incorporation of pollutant into soil organic matter and plant uptake. However, Reilley *et al*. (1996) reported no trace of 4 to 5 ring PAHs in leachates in planted and non-planted soil, while Trapp *et al*. (1990) observed negligible or non-significant results for phytovolatilization and plant metabolism. Therefore the dissipation of PAHs will most likely come from plant direct accumulation and biodegradation (Gao *et al*. 2008, Lin *et al*. 2008). In the present study, plant direct accumulation was not studied, therefore the enhanced dissipation of B[*a*]P in 10 mg kg^{-1} single B[*a*]P contaminated soil and 50 mg kg^{-1} Cr+ 10 mg kg^{-1} B[*a*]P and 100 mg kg^{-1} Cr+ 10 mg kg^{-1} B[*a*]P co-contaminated soil in the presence of plants could be as a result of plant promoted dissipaton.

5.10 Conclusion

The present study explores the phytoremediation potential of *Z. mays* in Cr and B[*a*]P co-contaminated soils. The growth of *Z. mays* was not significantly affected by either single or mixed contamination. However, under co-contamination of Cr-B[*a*]P treatments, the addition of B[*a*]P could affect the uptake and accumulation of Cr. *Z. mays* can accumulate Cr in co-contaminated soil while there was a low efficient accumulation in single contaminated soil. The concentration of Cr in plant shoot was elevated in the presence of B[*a*]P whereas the root

concentration decreased. However the concentration of Cr in shoots were lower than in roots and the TFs were lower than 1.0 for all treatments with plants grown in co-contaminated soils having higher TFs than single contaminated soil.

The dissipation of B[*a*]P was inhibited in the presence of Cr in co-contaminated soils. There was no evidence of plant enhanced dissipation of B[*a*]P in lower B[*a*]P single or co-contaminated soils whereas in 10 mg kg^{-1} B[*a*]P single and co-contaminated soils, *Z. mays* can enhance the dissipation of B[*a*]P. The evidence of plant enhanced dissipation of B[*a*]P could be due to plant promoted microbial degradation. Hence *Z. mays* could be used for phytoremediation of Cr-B[*a*]P co-contaminated soil but only where concentrations are higher.

6

Phytoremediation of metal-PAH co-contaminated soils: Comparing freshly spiked soils with aged soil

6.0 Phytoremediation potential of *B. juncea* in Cu-pyrene co-contaminated soil: Comparing freshly spiked soil with aged soil- Introduction

Part of this work has been published in journal of environmental management; Chigbo and Batty 2013.

Phytoremediation of contaminated soils, sediments as well as ground water have been shown to be economically and ecologically appropriate clean up procedures (Olsen *et al*. 2008, Kathi and Khan 2011). Plants can affect the fate of contaminants including metals and PAH in different ways. For example, the plant can help in volatilization with respect to PAHs, contaminants could be sorbed in the roots, taken up into plants or degraded (Marr *et al*. 2006, Olsen *et al*. 2008).

Recently, the impact of heavy metals and PAH on soil have been a concern due to their persistence in soil as well as their effects on the security of food chains (Huang *et al*. 2004, Kidd *et al*. 2007). However, the physicochemical property of the soil such as ageing affects the metal fractions and PAH in soil and this largely determines their availability in the soil (Jelali and Khanlari 2008, Nouri *et al*. 2011).

Ageing of soil will most likely have a significant influence on the mobility and the bioavailability of heavy metals (Chaignon and Hisinger 2003, Anxiang *et al*. 2009) and PAHs (Li *et al*. 2008). In natural soils, most contaminants are shown to be less toxic than in fresh soils used for laboratory experiments (Alexander 2000). Ageing of metals in soil has been identified as one of the factors that determine their availability in soil and subsequent uptake by plants during phytoremediation (Ma *et al*. 2006). The fate of metals in soils depends on the

retention capacity of the soil. For example metals tend to be adsorbed in soils with high organic matter Voegelin *et al*. (2003) and Whittinghall and Hobbie (2011) suggested that the ageing affects the rate of soil organic matter decomposition. Also, Lu *et al*. (2009) observed that the exchangeable Cu decreased with increase in ageing time whereas the residual Cu increased. This could subsequently influence uptake by plants and growth during phytoremediation.

PAHs in soil are less toxic to plants in the short term than monoaromatics (Henner *et al*. 1997) Therefore it is expected that if plant growth can be successfully established and maintained in contaminated soils, PAH dissipation can be increased (Binet *et al*. 2000). Most studies have shown that the translocation of PAH from root to shoot or their volatilization from leaves are negligible and should not be a concern during phytoremediation of fresh or aged soil (Qiu *et al*. 1997). Although it is believed that the longer the contaminants stay in soil, the lower the contaminant mobility and bioavailability, few studies have investigated whether the residence time of metals (Elhers and Luthy 2003, Martinez *et al*. 2003) or PAHs (Hamdi *et al*. 2012) affects their bioavailability and subsequent removal by plants. Presently, no study has investigated the role of aging in co-contaminated soils. Dissipation studies have only tried to investigate losses from freshly spiked PAH or PAH+metal contaminated soils (Binet *et al*. 2000, Lin *et al.* 2008) whereas Smith *et al*. (2011) and Ahn *et al.* (2005) tried to investigate the dissipation of PAH in aged PAH only contaminated soil.

Therefore the aim of this study is to compare the phytoremediation potential of *B. juncea* in freshly spiked and aged Cu, pyrene or Cu and pyrene co-contaminated soil. This study will investigate whether uptake of Cu by plants is affected by soil ageing, whether plants increase

the dissipation of PAHs and to understand the differences between dissipation in freshly spiked soil and in aged soil.

6.1 Methods

6.1.1 Soil Spiking and ageing process

Soil was spiked as discussed is section 5.2.1. The soil used for ageing study was stored in sealed bags in the dark for 8 months prior to planting.

6.1.2 Experimental set up

The experimental layout was designed in a completely randomized design of 30 treatments with three replicates of each for freshly spiked soil and aged soil. Pots spiked with pyrene had treatments with no planting in order to observe non-plant facilitated dissipation of pyrene. Twenty seeds of *B. juncea* were sterilized in 6% v/v of hydrogen peroxide for 15 minutes, washed with tap water and soaked for 1 day. Sterilized seeds were sowed directly into 12.5 cm plastic pots containing prepared soils. After 10 days of germination, weaker seedlings were removed, leaving 5 seedlings with similar size in each pot. Pots were watered when required with tap water to maintain the soil moisture during plant growth and the leachates from all pots were collected using the tray and returned to the soil. Throughout the experiment, the pots were periodically repositioned to minimize edge effects. Soil was fertilized with N: K: micro nutrients fertilizer mixture (1 g kg^{-1}) containing 26% N, 26% K_2O, 0.013% B, 0.025% Cu, 0.05%, 0.05% Fe and 0.025% Mn. After 65 days of growth, shoots were cut just above the soil surface and washed with deionized water. Each pot was then

emptied and the roots were separated from the soil by washing with running tap water. The roots were then rinsed with deionized water 3 times to remove all soil particles. All samples were oven-dried to constant weight at 65 ºC for 72 hours. The dried samples were weighed to enable biomass calculations and used for plant analysis.

6.1.3 Analysis of plants and soil samples

Due to decreased root growth in freshly spiked soil, the replicates of each treatment were merged together for analysis. Oven-dried plants were ground into small pieces using a coffee grinder (Krups, Italy). Approximately 0.3 g (for control and Cu treatments) and 0.1 g (Cu + pyrene treatments) of shoot/root dry matter for freshly spiked soil while 0.3 g for shoot and 0.1 g for root in aged soil were digested using 5 mL of 30% HNO_3 and placed on a heating block (Section 3.4.1). Digested plant samples were then analysed for total Cu using FAAS. The pyrene in soil samples was analysed using the Agilent GC-MS as described in sections 3.5.1, 3.5.2 and 3.5.3. The average percentage recovery for surrogate was 78.99% and 103.59% for freshly spiked and aged soil respectively.

6.1.4 Statistical analysis

All treatments were replicated three times. The means and standard error (SE) were calculated using Microsoft Office Excel 2007. The comparisons of shoot dry matter, Cu concentration, accumulation as well as soil residual pyrene were carried out by two way analysis of variance using SPSS 20. The results for pyrene dissipation rate was transformed with arcsin while the shoot accumulation was log transformed prior to analysis.When a significant difference was observed between treatments, multiple comparisons were made by the Tukey HSD test.

6.2 Results

6.2.1 Effect of aging on vegetation growth

The yield of *B. juncea* was higher in aged contaminated soil when compared to freshly contaminated soil in the present study (Table 6.1). The effect of Cu, pyrene or their combinations on yield of *B. juncea* varied when compared to control treatments. The present result showed that in fresh soil contaminated with 250 and 500 mg kg^{-1} pyrene alone, the shoot biomass decreased by 76 and 93% respectively when compared to aged soil. The root biomass in fresh soil also decreased by 90.2 and 85% respectively when compared to aged soil. It is clear that in freshly spiked soil, the shoot biomass decreased as the concentration of pyrene increased from 250 to 500 mg kg^{-1} while it increased with increase in pyrene concentration in aged soil.

In 50 and 100 mg kg^{-1} Cu contaminated soil, the shoot biomass in fresh soil after 60 days of planting was significantly lowered by 56 and 73% respectively when compared to aged soil. Similarly, the root biomass in freshly spiked soil decreased by 72 and 88% when compared to root biomass in aged soil.

The effect of co-contamination of Cu and pyrene on shoot and root biomass of *B. juncea* varied (Table 6.1). For example when the concentration of Cu remained at 50 mg kg^{-1}, co-contamination with 250 mg kg^{-1} of pyrene in freshly spiked soil significantly lowered the shoot biomass of *B. juncea* from 1.21 to 0.33 g, while in aged soil the shoot biomass significantly increased from 2.78 to 3.10 g. Also in comparison to aged soil, the shoot biomass was significantly lowered by 83 and 51% in soil freshly co-contaminated with 100mg

kg^{-1}Cu + 250 mg kg^{-1} pyrene and 100 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene respectively. The root biomass was lowered by 86.4 and 52.3%.

Table 6.1: Shoot and root biomass (mean±SE, n=3) of *B. juncea* influenced by Cu or pyrene after 65 days of planting. Different letters indicate a significant difference between fresh and aged soil (Tukey HSD $p \leq 0.05$). Appendix 6A.1

Cu added (mg kg^{-1})	**Pyrene added (mg kg^{-1})**	**Shoot dry matter (g)**		**Root dry matter (g)**	
		Fresh soil	Aged soil	Fresh soil	Aged soil
0	0	1.71±0.06^{a}	6.11±0.1^{b}	0.14	1.43±0.07
0	250	0.71±0.15^{bc}	2.98±0.05^{d}	0.11	0.72±0.06
0	500	0.27±0.04^{de}	4.01±0.05^{f}	0.06	0.76±0.09
50	0	1.21±0.03^{a}	2.78±0.07^{b}	0.12	0.42±0.03
50	250	0.33±0.01^{c}	3.10±0.01^{d}	0.05	0.94±0.01
50	500	0.26±0.06^{e}	2.21±0.03^{f}	0.06	0.47±0.06
100	0	1.48±0.06^{b}	5.53±0.09^{c}	0.14	1.13±0.04
100	250	0.24±0.01^{d}	1.45±0.09^{e}	0.04	0.30±0.05
100	500	0.26±0.04^{c}	0.52±0.05^{a}	0.06	0.13±0.01

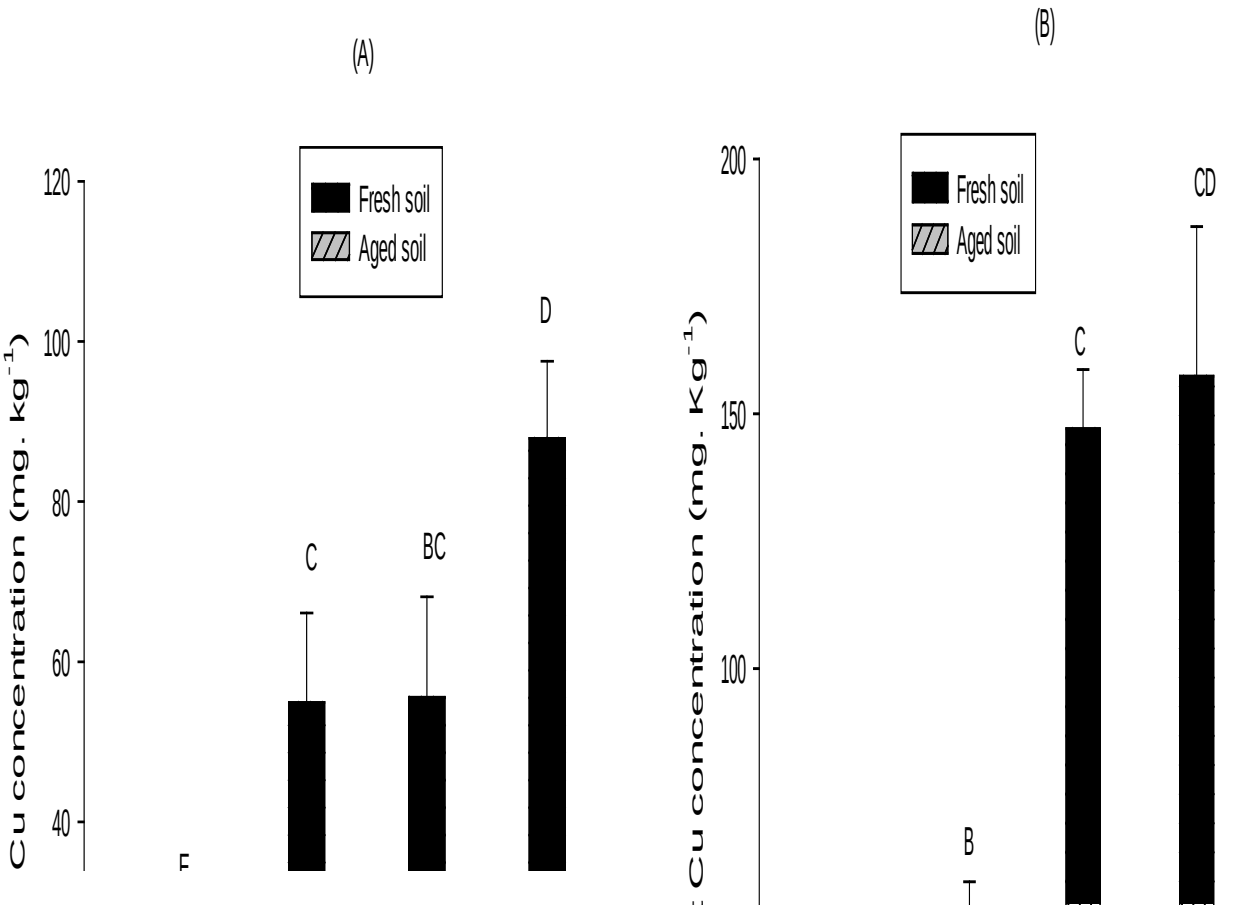
6.2.2 Effect of ageing on Cu concentration in B. juncea

The shoot Cu concentration in fresh and aged soil varied significantly and is shown in figures 6.1A and 6.1B. In aged soil, the addition of lower concentration of pyrene (250 mg kg^{-1}) significantly suppressed Cu uptake whereas in freshly contaminated soil, the addition of pyrene significantly increased the uptake of Cu. It was shown that in freshly contaminated

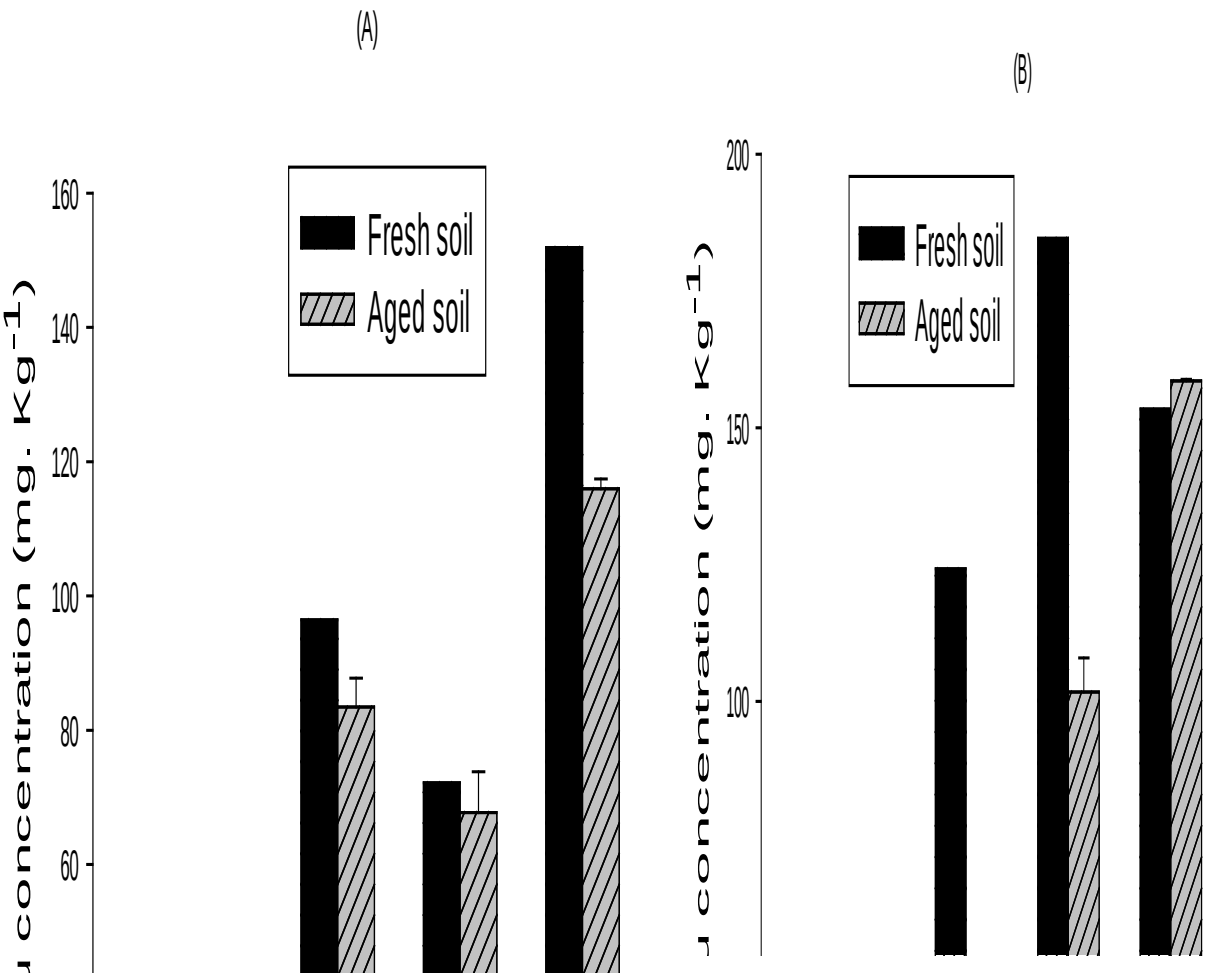
soil, when the concentration of Cu remained at 50 mg kg^{-1}, the shoot Cu concentration was 54.9 mg kg^{-1} while the addition of 500 mg kg^{-1} of pyrene significantly increased the shoot Cu concentration to 87.9 mg kg^{-1}. In contrast, when the concentration of Cu remained 50 mg kg^{-1} in aged soil, the addition of 250 and 500 mg kg^{-1} pyrene significantly decreased the shoot Cu concentration from 8.09 to 1.77 and 1.42 mg kg^{-1} respectively (Figure 6.1A). When the concentration of Cu in soil increased to 100 mg kg^{-1}, the addition of 250 and 500 mg of pyrene in aged and fresh soil significantly increased the Cu uptake. Result showed that the plant Cu uptake reached 147 and 157 mg kg^{-1} in freshly contaminated soils with the addition of 250 and 500 mg kg^{-1} pyrene respectively and 9.36 and 24.6 mg kg^{-1} in aged soil.

The Cu concentration in the roots of *B. juncea* varied in freshly spiked soil and aged soil. As the concentration of Cu increased from 50 to 100 mg kg^{-1}, the root Cu concentration increased from 96 to 124 mg kg^{-1} in freshly spiked soil and decreased from 83.4 to 52 mg kg^{-1} in aged soil. In the present study, the root dry weight of *B. juncea* in freshly spiked soil was merged together due to poor growth hence the root Cu concentration in aged and freshly spiked soil could not be compared statistically. However, co-contamination with Cu and pyrene with freshly spiked or aged soils showed a similar trend, although the root Cu concentration was always higher in freshly spiked soil than in aged soil. For example, when the concentration of Cu remained at 50 mg kg^{-1}, co-contamination with 250 mg kg^{-1} of pyrene decreased the root Cu concentration from 96 to 72 mg kg^{-1} for freshly spiked soil and from 83.4 to 67 mg kg^{-1} in aged soil, while the addition of 500mg kg^{-1} pyrene increased the root Cu concentration to 151 and 115 mg kg^{-1} respectively (Figure 6.2A). Also, when the concentration of Cu in soil increased to 100 mg kg^{-1}, co-contamination with 250 mg and 500 mg kg^{-1} of pyrene increased

the root Cu concentration from 124 to 184 and 153 mg kg^{-1} and 52 to 101 and 158 mg kg^{-1} for freshly contaminated soil and aged soil respectively (Figure 6.2B).



Figures 6.1 A and B: Shoot Cu concentration (mean ± SE, n= 3) of B. juncea in freshly and aged soil after 65 days of planting. Different letters indicate a significant difference between fresh and aged soil for each treatment (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene. Appendix 6A.3

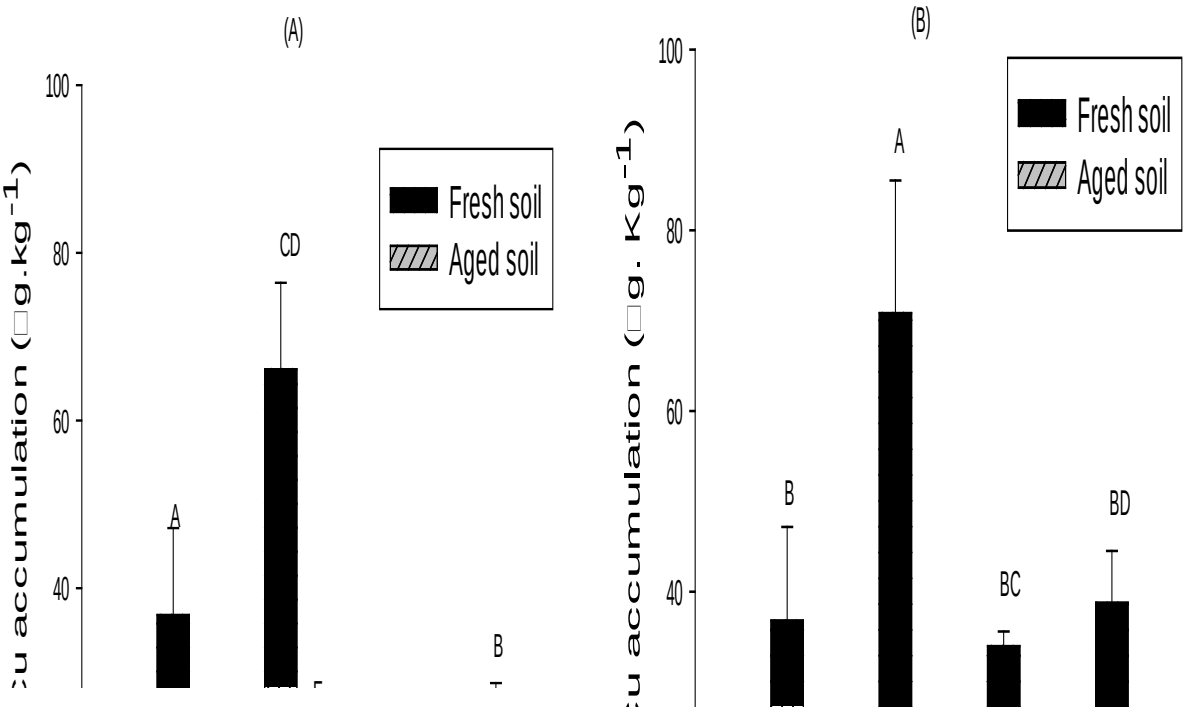


Figures 6.2 A and B: Root Cu concentration in freshly and aged soil after 65 days of planting. Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene. Appendix 6A.5

6.2.3 Effect of ageing on Cu accumulation

In freshly and aged contaminated soil, the shoot Cu accumulation significantly increased with increased Cu concentration in soil. Although in freshly spiked soil, there was no significant difference in shoot accumulation as the soil Cu concentration increased from 50 to 100 mg kg^{-1}, in aged soil as the concentration of Cu in soil increased from 50 to 100 mg kg^{-1} the shoot Cu accumulation significantly decreased from 22.59 to 8.87 µg kg^{-1}. The shoot Cu accumulation in fresh soil was about 3 times and 9 times that in aged soil for 50 and 100 mg kg^{-1} soil Cu contamination respectively.

Co-contamination of 50 and 100 mg kg^{-1} of Cu with 250 and 500 mg kg^{-1} of pyrene in aged soil significantly decreased the shoot Cu accumulation from 5.48 to 3.16 µg kg^{-1} and 13.61 to 12.60 µg kg^{-1} respectively (Figures 6.2A and B). In contrast, co-contamination of 50 and 100 mg kg^{-1} Cu with 250 and 500 mg kg^{-1} pyrene in freshly spiked soils significantly increased the shoot Cu accumulation from 18.3 to 21.9 µg kg^{-1} and 33.9 to 38.74 µg kg^{-1} respectively. It was clear that the shoot Cu accumulation under co-contamination in fresh soil was always about 3 times or more when compared to that in aged soil.



Figures 6.3 A and B: Shoot Cu accumulation (means ± SE, n= 3) in freshly and aged soil after 65 days of planting. Different letters indicate a significant difference between fresh and aged soil for each treatment (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene. Appendix 6A.4

6.2.4 Total Cu removal from soil and Translocation Factor

The concentration of Cu removed by *B. juncea* in the present study varied when freshly contaminated soil was compared with aged soil (Table 6.2). In single Cu contaminated soil, the total concentration of Cu removed from soil was higher in freshly spiked soil than in aged soil. However, under co-contamination, *B. juncea* in aged soil seemed to remove more Cu from soil than from freshly contaminated soil. The total Cu removed from freshly contaminated soil and aged soil increased from 58.1 to 68.3 µg kg^{-1} and 77.5 to 87 µg kg^{-1} respectively as the concentration of Cu increased from 50 to 100 mg kg^{-1}. This represents a 25 and 21.5% reduction in Cu removal in aged soil when compared to freshly spiked soil.

A contrasting result was observed under co-contamination. At 50 mg kg^{-1} soil Cu concentration, the total Cu removed from soil remained at 21.8 and 30.3 µg kg^{-1} when soil was freshly co-contaminated with 250 and 500 mg kg^{-1} of pyrene. In aged soil however, *B. juncea* removed over 3 times as much Cu from 50 mg kg^{-1} Cu + 250 mg kg^{-1} of pyrene co-contaminated soil and about twice as much in 50 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene when compared to freshly contaminated soil (Table 6.2). The Cu removal by *B. juncea* in soil co-contaminated with 100 mg kg^{-1} of Cu with 250 or 500 mg kg^{-1} of pyrene increased from 41.5 to 48.5 µg kg^{-1} in freshly spiked soil and decreased from 44 to 33.8 µg kg^{-1} in aged soil. Although the concentration of Cu removed from soil was higher in aged co-contaminated soil than in freshly spiked, it seemed that most of the Cu was retained in the root as suggested by the TF. The present result showed that the TF under freshly co-contaminated soil was always higher than in aged soil (Table 6.2). For example, in freshly co-contaminated soil, the highest shoot to root ratio reached 1.025 for 100 mg kg^{-1} Cu+ 500 mg kg^{-1} pyrene, while it was 0.15

for same treatment in aged soil. Similarly, in single Cu contaminated soil, the highest TF was 0.569 for freshly contaminated soil and 0.097 when soil was aged.

Table 6.2: Total Cu removed (mean±SE, n=3) by *B.juncea* and Translocation Factor (TF) after 65 days of planting in freshly spiked and aged soil. Appendix 6A.7 and A.8

Cu added (mg kg^{-1})	**Pyrene added(mg kg^{-1})**	**Total Cu removed (µg)**		**Translocation Factor (TF)**	
		Fresh soil	Aged soil	Fresh soil	Aged soil
0	0	42.05	21.22±3.09	0.57	0.04
50	0	77.53	58.18±2.50	0.57	0.097
50	250	21.84	69.33±10.37	0.77	0.026
50	500	30.37	57.78±6.97	0.58	0.012
100	0	87.68	68.31±1.79	0.39	0.03
100	250	41.52	44.77±7.68	0.80	0.09
100	500	48.51	33.81±1.55	1.03	0.15

6.2.5 Pyrene levels in fresh and aged soil at zero time

Table 6.3 shows the concentration of pyrene added to soils and the levels found at zerotime. The concentration of pyrene decreased over the 8 months with 250 mg kg^{-1} and 500 mg kg^{-1} pyrene dropping to 46 and 35% of its original concentration respectively. Co-contamination of Cu and pyrene also showed similar reductions over the period of ageing. When 50 mg kg^{-1} of Cu was added to 250 and 500 mg kg^{-1} of pyrene contaminated soil, the concentration of

pyrene at zerotime in aged contaminated soil decreased by approximately 36 and 34% respectively, while the addition of 100 mg kg^{-1} of Cu to 250 and 500 mg kg^{-1} of pyrene contaminated soil reduced the pyrene concentration in aged soil by 44 and 35% respectively.

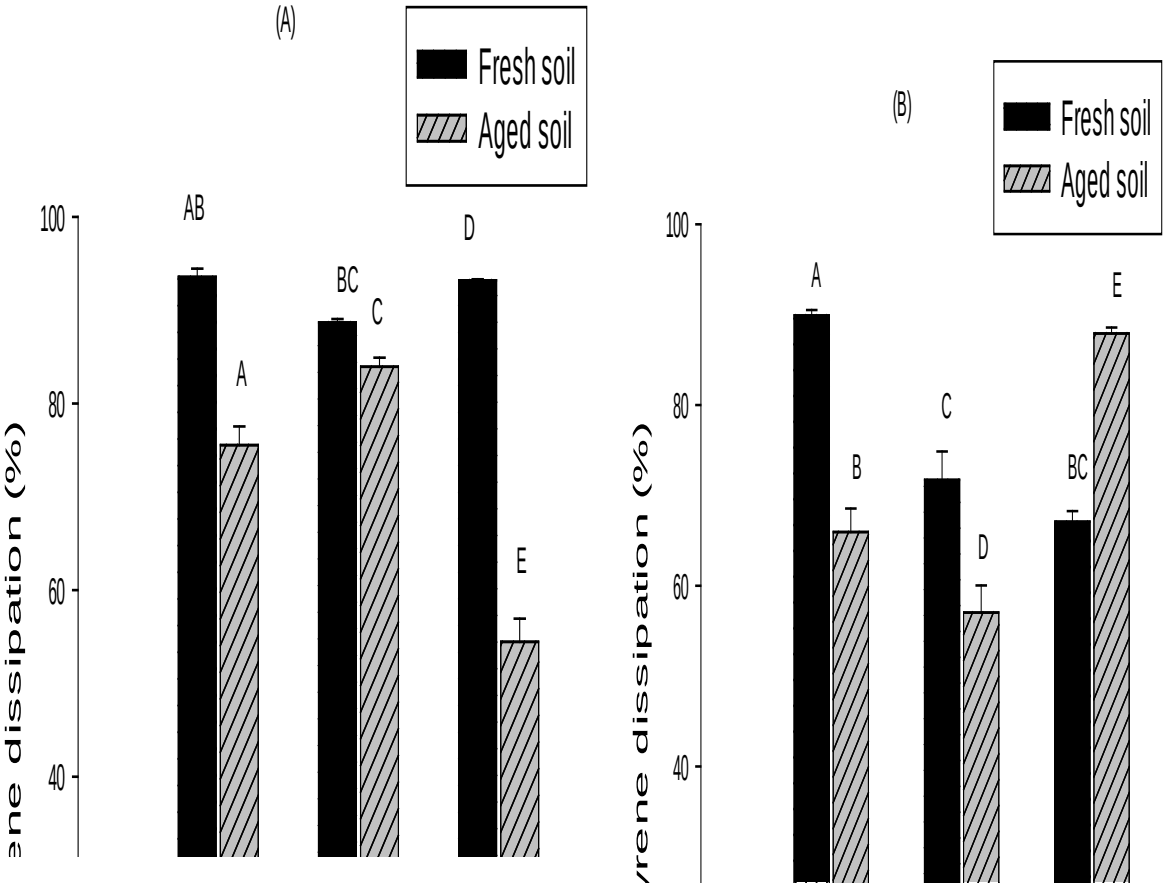
Table 6.3: Pyrene level in freshly spiked soils and aged soils (8 months) Appendix 6A.10

Cu added (mg kg^{-1})	**Pyrene added (mg kg^{-1})**	**Freshly spiked soil (mg kg^{-1})**	**Aged soil (mg kg^{-1})**
0	250	201.82 ±5.63	108.63 ± 8.88
50	250	194.66 ±9.31	123.22 ±11.05
100	250	193.29 ±3.45	107.55 ±0.90
0	500	369.48 ±25.3	219.32 ±14.43
50	500	348.67 ±4.3	228.09 ±21.14
100	500	339.96 ±3.11	237.87 ±9.76

6.2.6 Effect of ageing on residual pyrene concentration in soil

After 65 days of planting, the soil was quantitatively analyzed for pyrene concentration. The concentration of pyrene in freshly spiked and aged soil significantly decreased over the period of planting (Figures 6.4A and 6.4B). The dissipation of pyrene with planting seemed to vary between freshly spiked soils and aged soils. The present result showed that the dissipation rate of pyrene in freshly contaminated soil was from 67.1 to 93.6% whereas in aged soil the dissipation rate varied from 54 to 87.9%. There were significant differences between the removal rates of pyrene in freshly contaminated soil when compared to aged soil in the

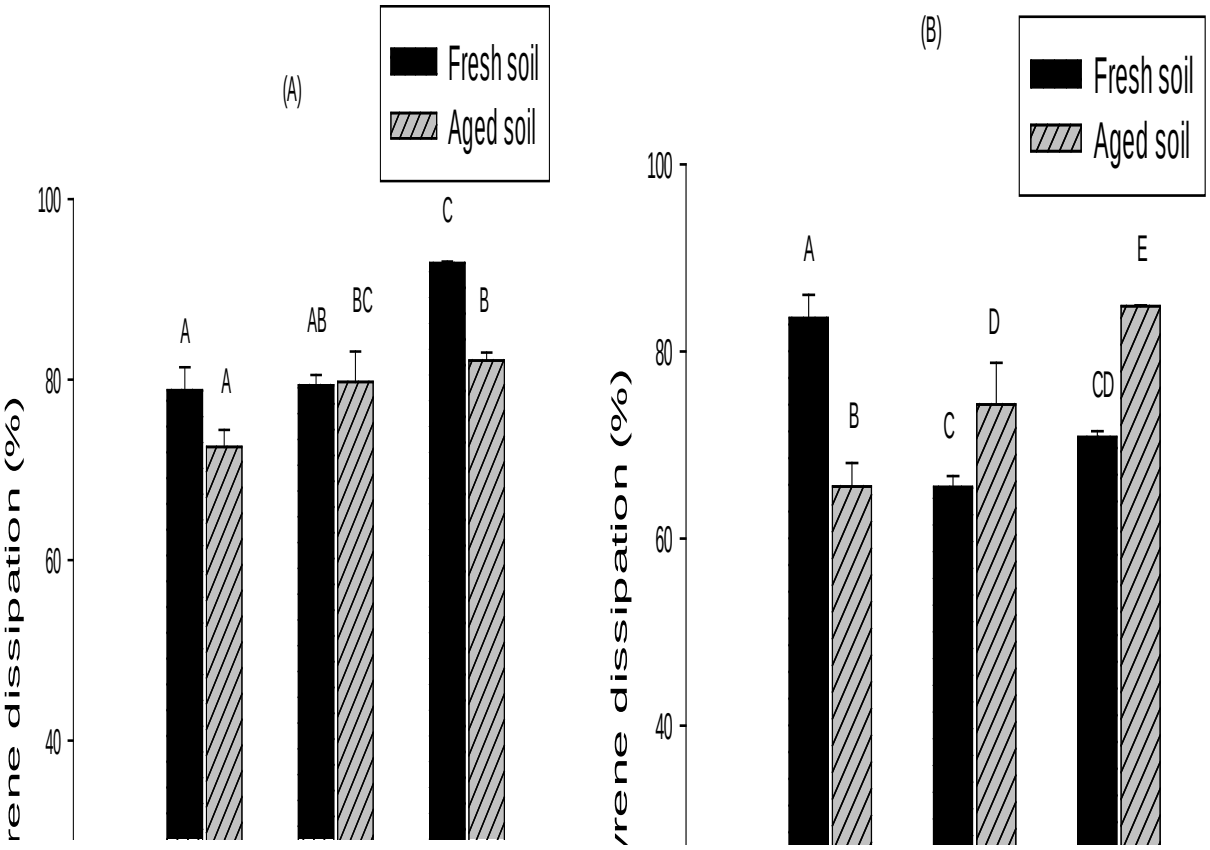
presence of plants, except for 250 mg kg^{-1} pyrene and 50 mg kg^{-1} Cu + 250 mg kg^{-1} pyrene. When the concentration of pyrene remained at 250 mg kg^{-1}, the dissipation rate in freshly contaminated and aged soil was similar. Co-contamination with 50 mg kg^{-1} Cu did not seem to affect the dissipation rate, while co-contamination with 100 mg kg^{-1} Cu significantly decreased the dissipation of pyrene by 39% when aged soil was compared to freshly spiked soils. There were contrasting results when the concentration of pyrene increased to 500 mg kg^{-1}. Compared to freshly spiked soils, the dissipation rate of pyrene after planting in aged soil decreased by 24%. . There were similar results when soil was co-contaminated with 50 mg kg^{-1} Cu. The dissipation rate decreased by 14% when aged soil was compared to freshly spiked soils.. However, the dissipation rate of pyrene increased in aged soil when compared to freshly contaminated soil in 100 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene. Results showed that the dissipation rate of pyrene increased by 21% when aged soil was compared to freshly contaminated soil (Figure 6.4B).



Figures 6.4 A and B: Pyrene dissipation (mean ± SE, n= 3) in freshly and aged soil after 65 days of planting. Different letters indicate a significant difference between fresh and aged soil for each treatment (Tukey HSD $p \leq 0.05$). Treatments, C1and C2 represent 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene. Appendix 6A.12

6.2.7 Residual pyrene concentration in unplanted soil

Pyrene was highly dissipated in fresh and aged soil with or without planting. However, in freshly contaminated soil the presence of *B. juncea* significantly decreased the pyrene concentration after planting when compared to soils with no planting. On the other hand, there was no significant effect of *B. juncea* on pyrene dissipation in aged soil. The present study showed that in C1P2 and C2P1 aged soil the dissipation of pyrene in the absence of plants was about 17 and 28% higher than in planted soil. There seemed to be an increased dissipation rate of pyrene in freshly contaminated soil than when the soil was aged in the absence of plants (figures 6.5A and 6.5B). The present results showed that in freshly spiked soil, the dissipation rate ranged from 65.5 to 92.9% for single and mixed contaminated soil, while in aged soil, it ranged from 65.5 to 84.8%. With the exception of 250 mg kg^{-1} of pyrene and 50 mg kg^{-1} Cu + 250 mg kg^{-1} pyrene contaminated soil, all other treatments showed significant differences when freshly spiked soil was compared to aged soil. For example, when the concentration of pyrene in soil remained at 250 mg kg^{-1}, the dissipation rate of pyrene in freshly spiked and aged soil in the absence of plants was similar. Also, co-contamination with 50 mg kg^{-1} of Cu did not affect the dissipation of pyrene, while the addition of 100 mg kg^{-1} of Cu significantly decreased pyrene dissipation to 92.9 and 82.1% for freshly contaminated and aged soil respectively. In contrast, when the concentration of pyrene in soil remained at 500 mg kg^{-1}, the dissipation rate of pyrene decreased to 83.6 and 65.6% for freshly spiked and aged soil while co-contamination with 50 mg kg^{-1} and 100 mg kg^{-1} of Cu significantly increased ($p \leq 0.05$) pyrene dissipation to 74.3% and 84.8% respectively for freshly and aged soil (Figure 6.5B).



Figures 6.5 A and B: Pyrene dissipation (mean ± SE, n= 3) in freshly and aged non planted soil. Different letters indicate a significant difference between fresh and aged soil for each treatment (Tukey HSD $p \leq 0.05$). Treatments, C1and C2 represent 50 and 100 mg Cu kg^{-1}; P1 and P2 represent 250 and 500 mg kg^{-1} of pyrene. Appendix 6A.13

6.3 Discussion

6.3.1 Ageing effect on biomass and accumulation of Cu in *B. juncea*

The dry biomass of *B. juncea* significantly increased in aged soil for all treatments when compared to freshly contaminated soil (Table 6.1). This could be as a result of the prolonged ageing period of 8 months used in the present study. It could be possible that due to ageing, Cu toxicity was reduced. Other studies have reported similar results. For example Anxiang *et al.* (2009) showed that in 500 mg kg^{-1} Cu contaminated soil, an increase in incubation (ageing) period significantly increased the dry biomass of *Triticum aesitivum*.

Although the dry biomass increased in aged soil relative to freshly contaminated soil, the concentration and accumulation of Cu in shoot was always lower (Figures 6.2A and B, 6.3A and B). This could be related to the availability of Cu in soil. Although this was not analyzed in the present study, Pederson *et al.* (2000) showed that Cu accumulation in a 12-week aged soil was always lower than those in soils that were aged for shorter periods. Although their result was not significant due to the short ageing period (1 to 12 weeks), the bioavailability of Cu was assumed to be the reason for the observed reduction in Cu accumulation. The differences in Cu accumulation in shoots of *B. juncea* could be related to the absorbed Cu in the root parts and was reflected in the plant biomass for freshly and aged contaminated soil (Table 6.1). In this study, *B. juncea* in freshly contaminated soil grew less than in aged soil while accumulating more Cu. Similar accumulation patterns have been observed in many other species. For example, Umebese and Motojo (2008) showed that the uptake of higher concentration of Cu by *Ceratophyllum demersum* significantly decreased the shoot biomass.

The translocation of Cu from the root to the shoot of *B. juncea* varied with freshly spiked soil and aged soil (Table 6.2). In freshly spiked soil, although the root Cu concentration was always higher than the shoot Cu concentration except for 100 mg kg^{-1} Cu + 500 mg kg^{-1} pyrene, the fraction translocated to the shoot was high when compared to aged soil. It could be that in aged soil, the bioavailability of Cu was reduced and more soil Cu was bound into the more stable fractions (Anxiang *et al*. 2009). This could make *B. juncea* resistant to Cu co-contamination by immobilizing Cu outside or inside the roots thereby preventing excess translocation to the shoots (Brun *et al.* 2001).

6.3.2 Pyrene concentration in treated soil at zero time

Table 6.3 shows the pyrene levels in freshly spiked soil and aged soil at zero time (before planting). It was clear from the results that pyrene concentration in aged soil decreased more than in freshly spiked soil for all treatments. The observed loss of pyrene over time was not due to the procedure used for extraction from soil. Some reports show that the efficiency of extraction is affected in aged soil since compounds are bound to the soil matrix. However, from the present study, the recovery of pyrene was consistent in freshly spiked soil as well as in aged soil. It was clear from the results that the stabilization time affected the dissipation of pyrene prior to planting. Normally, as the molecular weight of PAHs increases, dissipation is less due to the reduction in the saturation vapor pressure (Smith *et al.* 2011). However, since pyrene has a lower molecular weight, dissipation could be evident as shown in the present study. The dissipation observed in the present study could be either through breakdown by microbes present in soil with possibilities of other abiotic losses like irreversible sorption unto soil (Smith *et al*. 2011) or through volatilization. However, since the spiked soil was

stabilized by storing spiked samples in the dark before greenhouse planting, it is less likely that volatilization could remain a mechanism for pyrene dissipation. Sun *et al.* (2010) could not attribute any mechanism for the loss of pyrene and phenanthrene from a freshly spiked barren soil. This loss was so rapid that there was no significant difference when planted and unplanted soils were compared.

6.3.3 Pyrene dissipation in planted and non-planted soil

There have been many research papers that have examined spiking agricultural soil with pure PAHs or with heavy metal mixtures and comparing their dissipation rate in planted and unplanted soils. Different plant species have been tested in pot experiments. Some of these pot experiments have been successful, showing a clear phytoremediation effect in freshly spiked soil (Wang *et al.* 2008, Zhang *et al.* 2009, Wang *et al*. 2012). However, there have been suggestions that freshly spiked soils may not behave in the same way as aged soil. The significant variability in physical properties between fresh soils and aged soils is a factor which produces different conditions resulting in varied PAH degradation (Joner *et al*. 2004). They observed in their study that in old soil that was contaminated with creosote, the 3, 4 and 5 ringed PAHs were more available due to the disturbance of the soil which lead to improved aeration of soil during preparation for planting. When this happens, the effect the plant has on dissipation of PAH could be affected. The present study tried to be objective by stabilizing the freshly spiked soil and aged soil for 4 weeks and 8 months respectively following soil spiking to reduce the effects of soil disturbance. The removal of pyrene in planted soil was higher in freshly contaminated soil when compared to aged soil in pyrene only contaminated soil (Figure 6.4A and B). Also when a lower concentration of pyrene was co-contaminated with

Cu, the residual pyrene in soil tended to increase. This could be due to the increased availability of pyrene in freshly spiked soil than in aged soil. Although this was not studied, Smith *et al*. (2011) suggested that the stabilization time prior to planting can affect the bioavailability of PAH thereby increasing degradation of PAHs for soils with lower stabilization time. Also, PAHs in aged soils are less bioavailable because they are sequestered and stored in the organic matter which makes them less assessable to microbes leading to slow degradation or diffusion (Alexander 2000). Under co-contamination of Cu with higher concentration of pyrene (500 mg kg^{-1}), the removal rate of pyrene in freshly spiked soil seemed to be lower than in aged soil. This could be due to the modified root morphology of *B. juncea* in the present study. It was obvious from Table 6.1 that the root biomass of *B. juncea* in freshly spiked soil was lower than in aged soil. Also, the death of plant root in freshly spiked soil due to the toxicity of Cu and also the direct toxicity of Cu on microbes could have led to the variability in PAH degradation, thereby diminishing the effects on microbial PAH degradation (Sokhn *et al*. 2001, Olsen *et al*. 2003).

When planted and non-planted soils were compared for freshly spiked and aged soil the result varied. In freshly contaminated soils, there was a strong evidence of phytoremediation in all single and co-contaminated soils except when pyrene was co-contaminated with 100 mg kg^{-1} of Cu; whereas in aged soil there was no evidence of phytoremediation in all treatments. In aged soil treatments, the removal of pyrene in non-planted soil was either similar or was significantly higher than in planted soil. For effective phytoremediation of pyrene, the roots of *B. juncea* do not only need to increase the pyrene bioavailability by increasing microbial activity, but must increase the contact time between the microbe and pyrene (Smith *et al*.

2011). It could be that in the present study, the roots of *B. juncea* did not increase the bioavailability of pyrene in aged soil. Although significant differences existed in the root biomass of *B. juncea* in aged soil and was always significantly larger than in freshly spiked soil (Table 6.1), it would be expected that this would contribute highly to pyrene dissipation. Despite this, there was no significant difference in pyrene dissipation in aged planted soil and non-planted soil. This suggests that pyrene dissipation in aged soils cannot be related to the plant biomass which is further evidence that factors other than planting such as bioavailability and bulk microbial degradation (Mueller and Shann 2005) had a greater impact on pyrene dissipation. The absence of pyrene dissipation with planting in aged soil is not unique to this study. For example, *L. perenne* (Binet *et al*. 2000) and *Panicum virgatum* (Chen *et al*. 2003a) have shown the ability of plant to increase the dissipation of PAH in soil while other studies have shown no positive effect of planting on PAH dissipation including pyrene (Checkol *et al*. 2002). The decreased dissipation of pyrene in aged soil in the presence of *B. juncea* in this study is in line with the work carried out by Olexa *et al*. (2000) which showed that *L. perenne* decreased the dissipation of pyrene when compared to soils with no planting.

6.4 Conclusion

This study showed that phytoextraction of Cu and removal of pyrene was much greater in freshly spiked soil than in aged soil. In freshly spiked soil, although plant biomass was inhibited due to the toxicity of Cu, the concentration and accumulation of Cu as well as the translocation from root to shoot was increased when compared to aged soil. The total removal of Cu or the phytoextraction potential of *B. juncea* for Cu co-contaminated was enhanced in freshly spiked soil.

The residual pyrene concentration decreased in all treatments for planted and non- planted soil in freshly spiked soil as well as in aged soil. However, there was an evidence of plant enhanced dissipation in freshly spiked soil, while in aged soil there was no evidence of dissipation of pyrene in planted soil. In some aged soil treatments, pyrene dissipation in non-planted soil was greater than in planted soil. Since most contaminants in natural soil has a long residence time, it could be assumed that phytoremediation of Cu and pyrene co-contaminated soil with *B. juncea* may not be a feasible approach for contaminant removal in aged soil. This shows that although freshly spiked soils can be used in greenhouse studies to understand the mechanism of heavy metal and PAH removal from soils, care should be taken when comparing the removal rates in real life scenarios

6.5 Effect of ageing on the phytoremediation potential of *Z. mays* in chromium and B[*a*]P co-contaminated soil- Introduction

The environmental contamination of soils by heavy metals and PAHs are practically inevitable due to natural and industrial activities. Heavy metal toxicity and their accumulation potential in soils is a major concern (Naqvi and Rizvi 2000). Metals occur from different sources in the environment and most occur naturally in compounds in soils (Adriano 2001). Anthropogenic sources, including refining, steel production, and combustion of coal and oil contribute greatly to the natural concentration of metals in soil (Adriano 2001) and hence metals are important environmental pollutant because of widespread industrial use (Shanker *et al*. 2005). They can be very toxic and cause severe phytotoxic symptoms including membrane damage, distortion in metabolic activity and growth inhibition (Panda and Choudhury 2005). For example, the role of Cr has been studied intensively and most studies reported inhibition to plant growth (Samantarey *et al.* 2001, Samantaray 2002, Panda 2007). The exact effect could depend on several factors including plant species, soil types and Cr concentration (Vernay *et al*. 2008). At the cellular level, Cr (VI) causes severe damage to cell membranes due to its strong oxidizing nature (Shahandeh and Hossner 2000, Mei *et al*. 2002).

The toxicity of polycyclic aromatic hydrocarbons (PAH) has been studied extensively (Henner *et al.* 1997, Smith *et al*. 2006) and their major removal pathways in the soil include leaching, volatilization, biodegradation, accumulation by plants or irreversible sorption (Reilley *et al*. 1996). PAH with three or more rings are generally more hydrophobic in nature and are hardly leached or volatilized from the soil (Robinson *et al*. 2003). Consequently, in vegetated soils, PAH are adsorbed to the roots of plants while translocation to the shoot is less

likely. The root zone of plants provides a good environment for degradation of organic compounds by providing a biologically active soil region that enhances microbial activity and contaminant availability (Wenzel 2009). In un-vegetated soils, the dissipation of PAH occurs rapidly initially and reduces with time for non-volatile and recalcitrant compounds (Sims and Overcash 1983). With ageing, PAH sorb slowly to soil organic matter and hence their bioavailabilities are impacted (Hatzinger and Alexander 1995). For example Tang *et al*. (1998) showed that PAHs such as phenanthrene, anthracene, fluoranthene and pyrene were less available to bacteria and anthracene to wheat and barley in aged contaminated soil. The remediation of soil contaminated by metals or PAH is not only considered because of environmental problems, since the preservation of agricultural productivity is also important (Soleimani *et al*. 2010).

The effect of ageing on the phytoremediation of PAH-contaminated soil has been studied and shown that ageing affects their bioavailability during remediation (Hatzinger and Alexander 1995, Nedunuri *et al*. 2000, Robinson *et al*. 2003). Also PAH dissipation studies have mostly tried to investigate losses in freshly spiked soil (Robinson *et al*. 2003). However, little or no research has been carried out on aged metal and PAH co-contaminated soils. Also, in chapter 5 there were evidence of interactions of Cr and B[*a*]P during phytoremediation of co-contaminated soils. Therefore, the aim of this study is to compare the role of *Z. mays* during phytoremediation of aged and freshly spiked single Cr or B[*a*]P and Cr + B[*a*]P co-contaminated soil.

6.6 Methods

6.6.1 Soil spiking and ageing process

Soil was spiked as discussed in section 5.7.1. The soil used for ageing study was stored in sealed bags in the dark for 8 months prior to planting.

6.6.2 Experimental set up

The experimental layout was designed in a completely randomized design of 42 treatments with three replicates of each for freshly spiked soil and aged soil. Pots spiked with B[*a*]P had treatments with no planting in order to observe non-plant facilitated dissipation of B[*a*]P. One seedling of *Z. mays* with uniform size of about 3 to 4 cm, 3 leaves and about 3 weeks old was transferred into each pot. Pots were watered when required, with tap water to maintain the soil moisture during plant growth and the leachates from all pots were collected using the tray and returned to the soil. Throughout the experiment, the pots were periodically repositioned to minimize edge effects. After 60 days of growth, shoots were cut just above the soil surface and washed with deionized water. Each pot was then emptied and the roots were separated from the soil by washing with running tap water. The roots were then rinsed with deionized water three times to remove all soil particles. All samples were oven-dried to constant weight at 65 ºC for 72 hours. The dried samples were weighed to enable biomass calculations and used for plant analysis.

6.6.3 Analysis of plants and soil samples

Oven-dried plants were ground into small pieces using a coffee grinder (Krups, Italy). Approximately 0.3 g and 0.1 g of shoot and root dry matter respectively were digested using 5ml of 30% HNO_3 and placed on a heating block (Section 3.4.1). Digested plant samples were then analyzed for total Cr using FAAS. The translocation factor (TF) and the soil pH were analyzed as described in chapter 3. B[*a*]P concentration in soil samples was analyzed using the Agilent GC-MS as described in sections 3.5.1, 3.5.2 and 3.5.3. The average recovery for surrogate was 76% for freshly spiked soil and 73% for aged soil.

6.6.4 Statistical analysis

All treatments were replicated three times and the means and standard error (SE) were calculated. The comparisons of shoot dry matter, Cr concentration, accumulation as well as soil residual pyrene were carried out by two-way ANOVA using SPSS 20. When a significant difference was observed between treatments, multiple comparisons were made by the Tukey HSD test.

6.7 Results

6.7.1 Plant biomass

The shoot biomass of *Z. mays* in aged and freshly contaminated soil varied significantly (Table 6.4). Under single B[*a*]P contamination, the shoot biomass of *Z. mays* seemed to decrease in aged soil compared with freshly spiked soil. Table 6.4 showed that the shoot biomass of *Z. mays* decreased by more than 46% for single B[*a*]P contaminated soil when

aged soil was compared to freshly spiked soil. In contrast, aged Cr or Cr + B[*a*]P contaminated soil affected the shoot biomass in a different way. There seemed to be an increased shoot biomass in aged soil compared with freshly spiked soil. In soil contaminated with 100 mg kg^{-1} Cr or 100 mg kg^{-1} Cr + 10 mg kg^{-1} B[*a*]P, ageing significantly ($P<0.05$) increased the shoot biomass by over 50 and 100% respectively. Also in the present result, it was clear that co-contamination led to significantly lower shoot biomass when compared to single Cr contamination in aged soil but not in freshly spiked soil. The root biomass for all single and co-contaminated soil was significantly lower in freshly contaminated soil when compared to aged soil (Table 6.4). In soil freshly spiked with single Cr or B[*a*]P, the root biomass of *Z. mays* significantly ($P<0.05$) decreased by over 50% when compared to aged contaminated soil, whereas under co-contamination of Cr and B[*a*]P, the root biomass in freshly spiked soil was significantly ($P<0.05$) lower by over 40% when compared to aged soil.

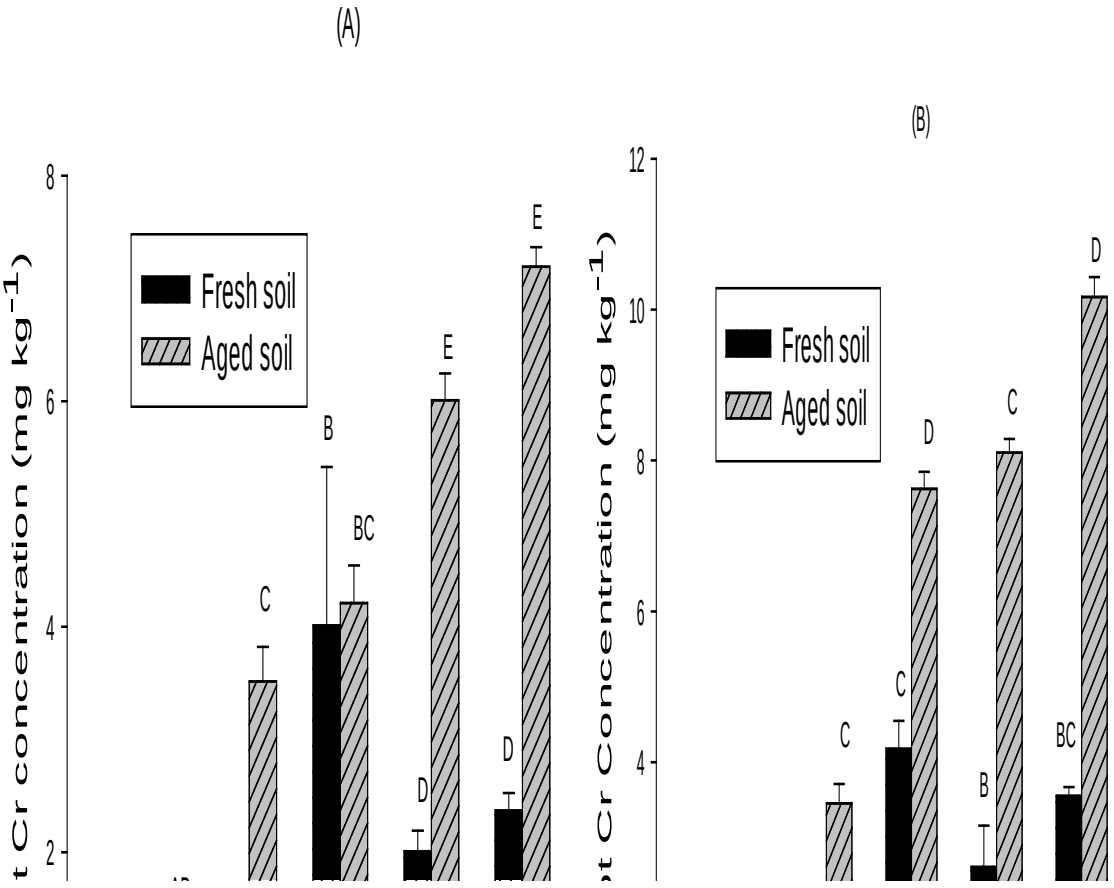
Table 6.4: Shoot and root biomass (mean±SE, n=3) of *B. juncea* influenced by Cu or pyrene after 65 days of planting. Different letters indicate a significant difference between fresh and aged soil (Tukey HSD $p \leq 0.05$). Appendix 6B.1 and 6B.2

Cr added (mg kg^{-1})	**B[*a*]P added (mg kg^{-1})**	**Shoot dry matter (g)**		**Root dry matter (g)**	
		Fresh soil	Aged soil	Fresh soil	Aged soil
0	0	2.77 ± 0.35^{a}	1.37 ± 0.03^{b}	0.23 ± 0.03^{a}	0.62 ± 0.03^{b}
0	1	2.90 ± 0.1^{a}	1.57 ± 0.17^{b}	0.30 ± 0.06^{a}	0.69 ± 0.06^{b}
0	5	3.23 ± 0.43^{a}	1.47 ± 0.18^{b}	0.40 ± 0.06^{a}	0.88 ± 0.01^{c}
0	10	1.70 ± 0.78^{b}	1.90 ± 0.06^{ab}	0.23 ± 0.07^{a}	0.64 ± 0.01^{b}
50	0	2.40 ± 0.06^{c}	2.50 ± 0.06^{cd}	0.20 ± 0.06^{a}	0.64 ± 0.01^{b}
50	1	2.57 ± 1.02^{c}	2.53 ± 0.15^{bc}	0.33 ± 0.09^{b}	0.59 ± 0.04^{a}

50	5	2.23±0.26^{b}	2.77±0.15^{b}	0.37±0.09^{ab}	0.83±0.02^{a}
50	10	2.23±0.18^{c}	2.60±0.12^{c}	0.33±0.07^{a}	0.76±0.02^{ab}
100	0	2.07±0.18^{a}	3.47±0.2^{b}	0.50±0.00^{a}	1.37±0.08^{d}
100	1	2.27±0.13^{c}	3.10±0.17^{cd}	0.30±0.00^{a}	0.82±0.01^{b}
100	5	2.47±0.09^{cd}	2.13±0.15^{c}	0.37±0.03^{a}	0.70±0.04^{c}
100	10	1.23±0.15^{e}	2.80±0.1^{d}	0.27±0.03^{a}	0.88±0.03^{b}

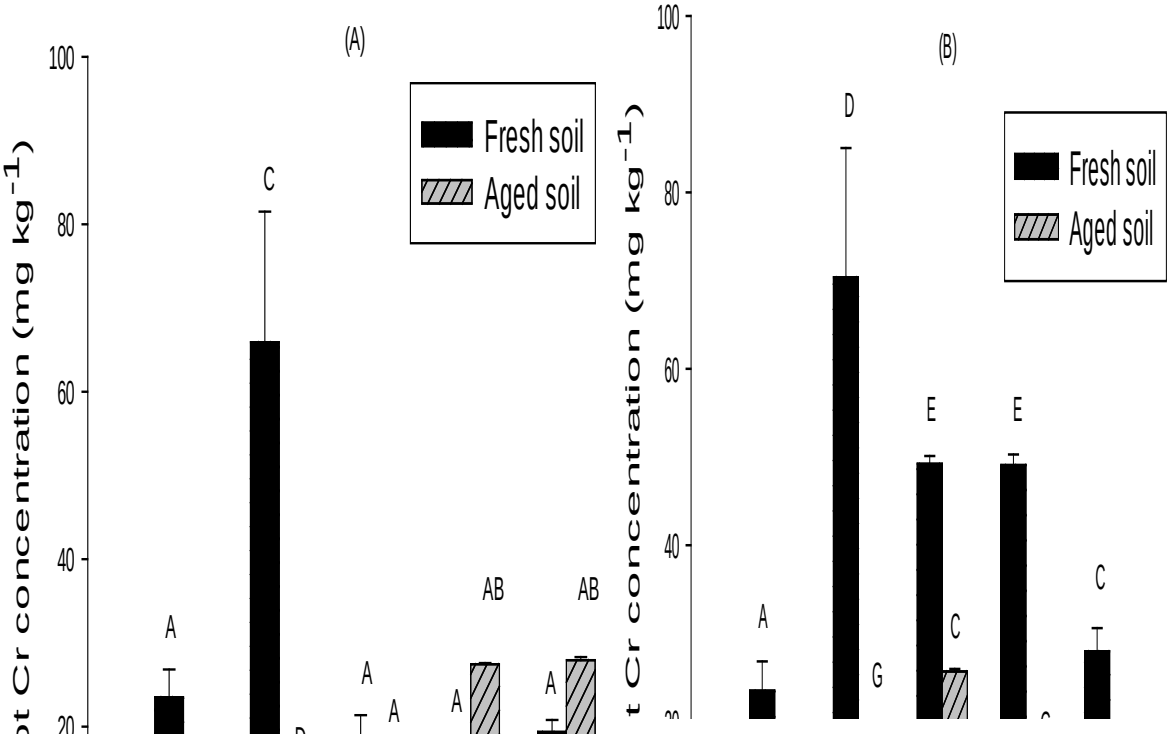
6.7.2 Plant Cr concentration in freshly spiked and aged soil

The shoot Cr concentration was affected by single contamination of Cr, co-contamination and ageing (Figure 6.6A). The shoot Cr concentration was always higher in *Z. mays* planted in aged soil than in freshly spiked soil for single or co-contamination experiments. The present result showed that in freshly spiked soils, the shoot Cr concentration was significantly lower by 81 and 54% for 50 and 100 mg kg^{-1} Cr contaminated soil respectively when compared to aged soil. A similar trend was observed for aged and freshly spiked co-contaminated soil. Figure 6.6A and B showed that in co-contaminated soils, the shoot Cr concentration in aged soils was higher than in freshly spiked soils. For example, in freshly spiked soils, the shoot Cr concentration remained 4 and 2 mg kg^{-1} for soils co-contaminated by 50 mg kg^{-1} Cr with 5 or 10 mg kg^{-1} B[*a*]P whereas in aged soil it increased to 6 and 7 mg kg^{-1} respectively (Figure 6.6A). Similarly, in soil freshly co-contaminated with 100 mg kg^{-1} Cr and 1, 5 and 10 mg kg^{-1} B[*a*]P, the shoot Cr concentration was about 4.1, 2.6 and 3.6 mg kg^{-1} respectively; whereas in aged soil the shoot Cr concentration increased by over 50%



Figures 6.6A and B: Shoot Cr concentration (mean ± SE, n= 3) in freshly and aged soil after 60 days of planting. Different letters indicate a significant difference between fresh and aged soil for each treatment (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cr kg^{-1}; B0, B1 and B2 and B3 represent 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P. Appendix 6B.3

The root Cr concentration over 60 days of planting varied between freshly spiked soil and aged soil (Figures 6.7A and B). In soils freshly spiked with Cr alone, the root Cr concentration was greater than in aged soil. In 50 and 100 mg kg^{-1} Cr contaminated soil, the root Cr concentration in aged soil was about 83 and 75% less than that in freshly spiked soil. The result was different in co-contaminated soils. For example, in soil co-contaminated with B[a]P and 50 mg kg^{-1} Cr, there seemed to be no significant difference in root Cr concentration between freshly spiked soil and aged soil (Figure 6.7A), whereas in 100 mg kg^{-1} Cr co-contaminated soil with 1 and 5 mg kg^{-1} B[*a*]P, the root Cr concentration in aged soil was lower by 48 and 70% respectively when compared to freshly spiked soil (Figure 6.7B).



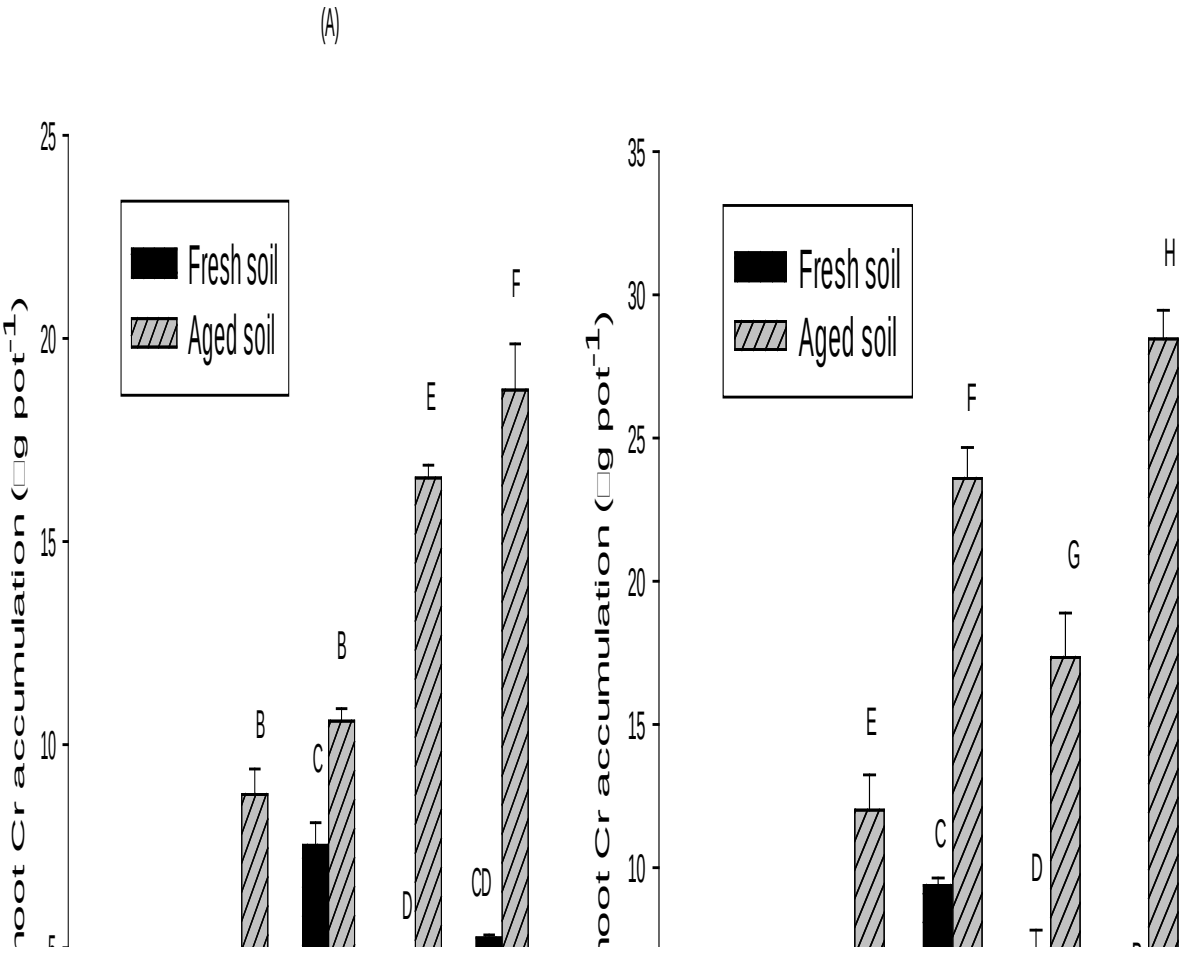
Figures 6.7A and B: Root Cr concentration (mean ± SE, n= 3) in freshly and aged soil after 60 days of planting. Different letters indicate a significant difference between fresh and aged soil for each treatment (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cr kg^{-1}; B0, B1 and B2 and B3 represent 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P Appendix 6B.5

6.7.3 Cr accumulation

In aged soil, the shoot accumulation of Cr over 60 days of planting was higher than in freshly spiked soil for single Cr or co-contamination (Figure 6.8A and B).

In aged soil, when the initial spiked Cr concentration in soil remained 50 and 100 mg kg^{-1}, the shoot Cr accumulation remained 8.76 and 12 µg pot^{-1}. However in freshly spiked soil, it decreased to 1.63 and 3.28 µg pot^{-1} representing an 81 and 73% reduction respectively. Similarly, under co-contamination, the shoot Cr accumulation in freshly spiked soil was significantly lower than in aged soil. The present result shows that the shoot Cr accumulation in soil freshly co-contaminated with 50 mg kg^{-1} of Cr and B[*a*]P was lower by over 29% when compared to aged soil and over 60% for 100 mg kg^{-1} Cr co-contaminated with B[*a*]P.

A contrasting result was observed when freshly spiked soils and aged soils were compared for higher initial Cr contaminated soils. As shown in figure 6.8B, at 100 mg kg^{-1} initial Cr concentration, the shoot accumulation of Cr in *Z. mays* remained 12 µg pot^{-1} and co-contamination with 10 mg kg^{-1} B[*a*]P significantly increased the shoot Cr accumulation to 28.5 µg pot^{-1} in aged soil. However, in freshly spiked soil, there was no significant difference between the shoot Cr accumulation in 100 mg kg^{-1} Cr contaminated soil and 100 mg kg^{-1} Cr + 10 mg kg^{-1} B[*a*]P co-contaminated soil.



Figures 6.8 A and B: Shoot Cr accumulation (mean ± SE, n= 3) in freshly and aged soil after 60 days of planting. Different letters indicate a significant difference between fresh and aged soil (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cr kg^{-1}; B0, B1 and B2 and B3 represent 0, 1, 5 and 10 mg kg^{-1} of B (a) P. Appendix 6B.4

6.7.4 Soil pH, total Cr removal and Translocation Factor (TF)

From table 6.5, it can be seen that soil ageing did not significantly affect the total removal of Cr by *Z. mays* in single Cr contaminated soil. Under co-contamination, the total Cr removed by the plant was significantly lower in freshly spiked soils when compared to aged soil. For example, in soil freshly spiked with 50 mg kg^{-1} Cr and 1, 5 or 10 mg kg^{-1} B[*a*]P, the total Cr removed by the plants was about 36, 75 and 70% lower than in aged soil. Similarly, in soil freshly spiked with 100 mg kg^{-1} Cr and 1, 5 and 10 mg kg^{-1} B[*a*]P, the total Cr removed by the plant was over 45% lower than in aged soil.

The TF was always higher and the pH slightly lower in aged soil for all single or co-contaminated treatments than in freshly spiked soil. It was also observed that in freshly spiked soil, when the concentration of Cr remained at 50 mg kg^{-1}, the TF remained at 0.01 and increased to 0.02, 0.14 and 0.12 with 1, 5 and 10 mg kg^{-1} B[a]P co-contamination. However in aged soil, when the Cr concentration remained at 50 mg kg^{-1}, the TF remained at 0.3 and decreased to ≤0.25 with B[*a*]P co-contamination.

Table 6.5: Total Cr removed (mean ±SE, n=3) by *Z .mays* and Translocation Factor (TF) after 60 days of planting in freshly spiked and aged contaminated soil. Appendix 6B.8

Cr added (mg kg^{-1})	B (a) P added (mg kg^{-1})	Fresh soil			Aged soil		
		Total Cr (µg)	pH	TF	Total Cr (µg)	pH	TF
0	0	6.29±0.54	6.2	0.02	3.98±0.20	6.2	0.37
50	0	13.03±1.53	6.3	0.01	16.12±0.54	5.6	0.31
50	1	12.83±1.56	6.3	0.21	20.27±0.73	5.8	0.26
50	5	9.80±2.07	6.3	0.15	39.42±0.77	5.9	0.22
50	10	11.74±1.58	6.2	0.12	39.80±1.43	5.8	0.26
100	0	38.47±6.95	6.2	0.03	36.02±1.6	5.9	0.20
100	1	24.14±0.07	6.4	0.09	44.69±1.39	5.8	0.30
100	5	24.53±3.03	6.4	0.05	27.57±1.79	5.7	0.55
100	10	11.65±0.80	6.4	0.13	40.74±0.94	5.6	0.73

6.7.5 B[*a*]P concentration in soil at zero time

Due to different incubation periods of soil prior to planting, the zero-time B[*a*]P concentration in freshly spiked and aged soil was compared. Table 6.6 shows that the zero-time B[*a*]P concentration after 8 months of ageing was always lower than in freshly spiked soil.

For single B[*a*]P contaminated soil, the present result showed a 22.5, 20.8 and 32.6% decrease in zero-time B[*a*]P concentration in aged soil compared to freshly spiked soil. Similar results were observed for soils co-contaminated with B[*a*]P and Cr. For example, in freshly spiked soil, the zero-time B[*a*]P concentration remained at 1.14, 3.46 and 8.15 mg kg^{-1} for soil co-contaminated with 50 mg kg^{-1} Cr and 1, 5 and 10 mg kg^{-1} B[*a*]P respectively and decreased by over 28% in aged soil. Also in 100 mg kg^{-1} Cr co-contaminated soil, the zero-time B[*a*]P concentration decreased by over 46% in aged soil compared to freshly spiked soil.

Table 6.6: Zero-time concentration (mean± SE, n=3) of B[*a*]P in freshly and aged soil. Appendix 6B.9

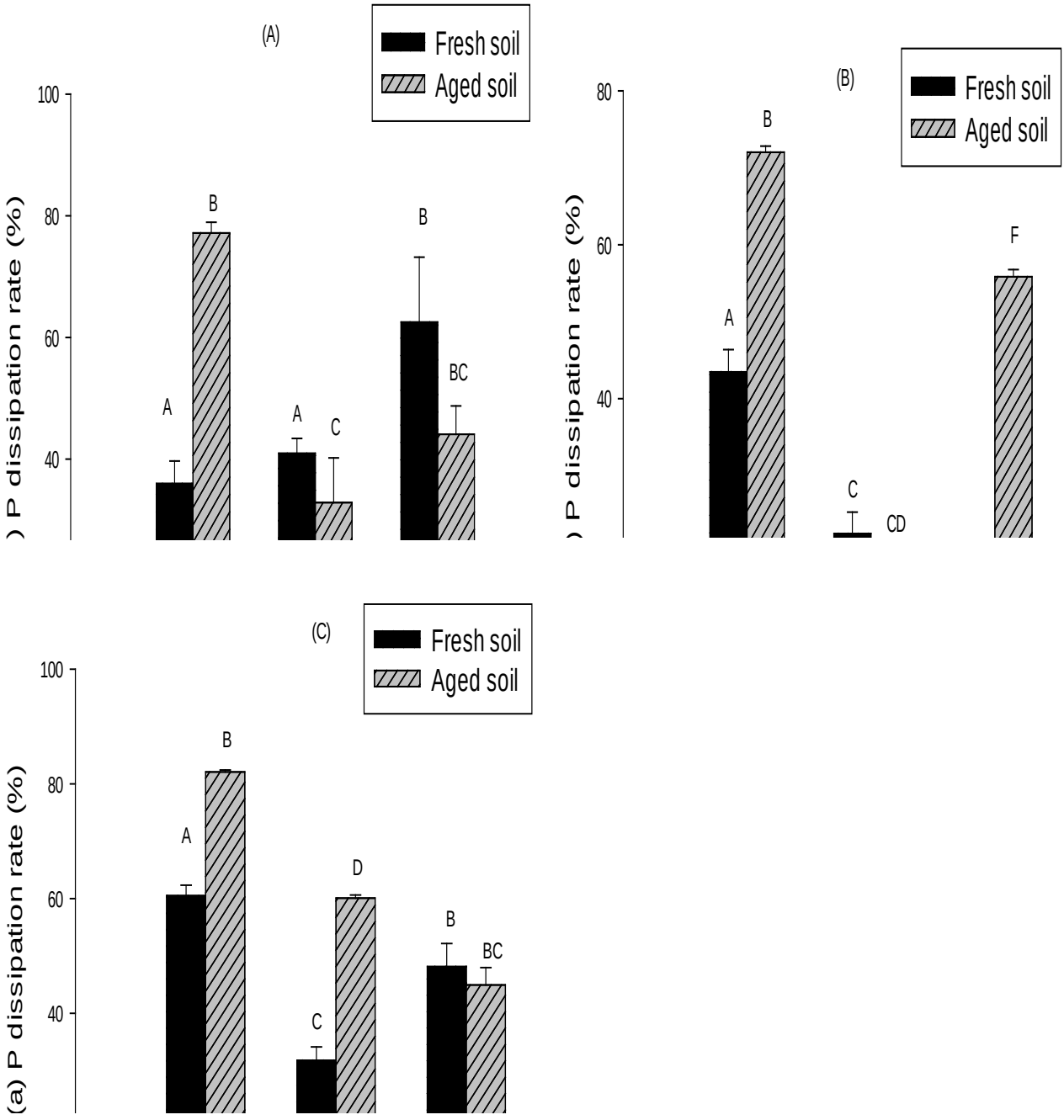
Cr added (mg kg^{-1})	**B[*a*]P added (mg kg^{-1})**	**Freshly spiked soil (mg kg^{-1})**	**Aged soil (mg kg^{-1})**
0	1	1.03±0.10	0.79±0.04
0	5	3.43±0.14	2.72±0.08
0	10	8.86±0.04	5.97±0.11
50	1	1.14±0.08	0.64±0.07
50	5	3.46±0.06	2.39±0.02
50	10	8.15±0.07	5.85±0.08
100	1	1.20±0.01	0.64±0.02
100	5	3.82±0.31	2.36±0.01
100	10	6.57±0.08	5.87±0.14

6.7.6 B[*a*]P dissipation

The dissipation rate of B[*a*]P in planted soil seemed to be significantly higher in aged soil than in freshly spiked soil for single and co-contaminated soils (Figures 6.9A, B and C). The present results show that when soil was spiked with 1, 5 or 10 mg kg^{-1} B[*a*]P, the dissipation rate of B[*a*]P in planted soil was lower by over 19% when freshly spiked soil was compared to aged soil. Also in co-contaminated soils, similar results were obtained. The dissipation rate was lower by over 31% when freshly spiked soil was compared to aged soil.

Contrasting results were observed in non-planted soil for single B[*a*]P and co-contaminated soil. In single contaminated soil, the dissipation of B[*a*]P in the absence of plants was significantly lower in aged soil than in freshly spiked soil. The present result showed over 20% reduction in dissipation rate when aged soil was compared to freshly spiked soil for 5 and 10 mg kg^{-1} B[*a*]P contaminated soil. In co-contaminated soil, there seemed to be no significant difference between the dissipation rates of B[*a*]P in aged or freshly spiked soil in the absence of plants. Only 100 mg kg Cr^{-1} + 10 mg kg^{-1} B[*a*]P freshly spiked soil significantly showed a 30% reduction in B[*a*]P dissipation rate when compared to aged spiked soil.

There was also an important result observed in the present study. For example, in the presence of plants, the dissipation of B[*a*]P in soil freshly spiked with 1 mg kg^{-1} B[*a*]P remained 0.59 mg kg^{-1}, while co-contamination with Cr did not seem to affect the dissipation of B[*a*]P. However, in aged soil, co-contamination of 1 mg kg^{-1} B[*a*]P with Cr significantly lowered the dissipation rate of B[*a*]P when compared to single B[*a*]P contaminated soil.



Figures 6.9 A, B and C: B[*a*]P dissipation (mean ± SE, n= 3) in freshly and aged soil after 60 days of planting. Different letters indicate a significant difference between fresh and aged soil (Tukey HSD $p \leq 0.05$). Treatments C0, C1and C2 represent 0, 50 and 100 mg Cr kg^{-1}; B0, B1 and B2 and B3 represent 0, 1, 5 and 10 mg kg^{-1} of B[*a*]P Appemdix 6B.12

6.8 Discussion

6.8.1 Plant biomass and Cr accumulation

The ageing process of spiked soil increased the Cr translocation from root to shoots of *Z. mays* (Table 6.5). During the ageing process of the soil, metal availability, and toxicity as well as solubilization, can be modified (Lock and Janssen 2001, Jalali and Khanlari 2008 and, Zapusek and Lestan 2009). Although as noted by Lin and Xing (2008), the phytotoxicity of metals cannot be explained only by the dissolution of metals ions, particles can aggregate and bind to the root surface and can cause damage to the vascular cells by penetration into the epidermal and cortical cells. From the present result, it was clear that co-contamination significantly decreased the shoot biomass when compared to single Cr contamination in aged soil but not in freshly spiked soil (Table 6.4). Therefore it can be assumed that the ageing process over 8 months combines the effect of metallic ion dissolution as well as particle aggregation and penetration. In solely Cr-contaminated soils, freshly spiked soils had a lower shoot to root ratio when compared to aged soils, but the accumulation of Cr in plant tissue was higher (Figures 6.8A and B). This indicates poor metal translocation efficiency from root to shoot which may be linked to the reduction of Cr (VI) in roots and retaining in the cell walls and vacuoles of *Z. mays* (Lytle *et al*. 1998). It is possible that the Cr (VI) had been transformed to a more stable Cr (III) and then adsorbed on soil. In co-contaminated soils, both the shoot to root ratio and total accumulation was higher in aged soil than in freshly spiked soil. This could be the influence of ageing on the translocation efficiency. Also there could be an effect of B[*a*]P on Cr release and transfer in co-contaminated soils. Some authors have reported the effect of organic matter on metal release transfer and toxicity in soils (Bermudez

et al. 2010). The presence of organic matter could modify both the metal uptake as well as phytotoxicity by changing the concentration of free metal through speciation (Shahid *et al.* 2011). Spiking Cr in soil for several months could also have changed the solubility of Cr by reduction of pH as observed in the present study (Table 6.5). This is shown by the increase of Cr transfer in shoot of *Z. mays* after the ageing period. It is known that Cr availability is highest in soils with lower pH and as suggested by Lock and Janssen (2003), the difference in Cr uptake and toxicity between aged and freshly spiked soil could be related to the pH and the cation exchange capacity. These differences will be greater if the soil used has a low cation exchange capacity. If the soil cation exchange capacity is high, a greater fraction of Cr will be absorbed immediately after spiking which will result in smaller ageing effect. The decrease in soil pH after ageing could probably be due to oxidation processes that are enhanced by high temperature (Lacal *et al*. 2003)

6.8.2 B[*a*]P dissipation in freshly spiked and aged soil

The efficiency of extraction of PAH from soils is influenced by different factors including soil moisture content, ageing and soil texture (Fischer *et al*. 1994, Letellier *et al*. 1999). Although these characteristics might not be similar in aged and fresh soil, however in the present study the extraction efficiency for B[*a*]P added was similar. That is about 73 and 75% for freshly spiked and aged soil respectively. The dissipation of B[*a*]P in long-term contaminated soil has already been considered to be slow and a long term process (Bossat and Bertha 1986). For example, Wilcock *et al*. (1996) suggested that the half life of B[*a*]P in soil ranges from about 100 days to 14 years. However, in the present study, it was clear that the dissipation rate of B[*a*]P in aged soil was always higher than in freshly spiked soil for both single and co-

contaminated soil over the 60 days of glasshouse plant trial (Figures 6.9A, B and C). It is possible that the soil properties appeared to enhance the dissipation of B[*a*]P. The soil pH was slightly lower in aged soils than in freshly spiked soils after 60 days of planting (Table 6.5) and according to Fu *et al.* (2012), a relatively lower soil pH may be conducive for the degradation of B[*a*]P. It is known that B[*a*]P is a semi-volatile PAH and can evaporate from soil during glasshouse study. Although, this study did not try to ascertain the direct method of dissipation, it was clear that planting *Z. mays* played a major role in the dissipation of B[*a*]P in aged co-contaminated soils. As observed in the present study, there seemed to be no significant difference between the dissipation of B[*a*]P in aged or freshly spiked co-contaminated soil in the absence of plants whereas when *Z. mays* was planted for 60 days, the dissipation rate of B[*a*]P in aged soil increased when compared to freshly spiked soils (Figure 6.9A, B and C). Also the difference in B[*a*]P concentration between unplanted and planted soil reflects the enhanced dissipation of B[*a*]P by *Z. mays*. Soil pH is very important during the study of B[*a*]P dissipation from soil. When the soil pH is low, B[*a*]P dissipation could be favored (Fu *et al.* 2012). In the present study, soil ageing as well as planting with *Z. mays* influenced the soil pH (Table 6.5). Some studies have shown that planting Z. mays increased the soil pH slightly (Marschner and Romheld 1983) or did not affect soil pH (Ruark *et al.* 2012). Therefore how planting affects the soil pH may be related to the soil types.

The major reason for the lower concentration of B[*a*]P in aged soil than in freshly spiked soil are most likely photo-oxidation, evaporation and microbial degradation during the ageing period (Huang *et al*. 2004). Since microorganisms play an important role during the dissipation of PAHs (Andrew and John 2000), it is possible that due to ageing, the microbial

numbers and communities increased. Although ageing limits the bioavailability of PAHs in soil (Morrison *et al*. 2000, Cunliffe and Kertesz 2006,), the rapid dissipation of B[*a*]P in aged soil when compared to freshly spiked soil in the presence of *Z. mays* suggests that other factors such as microbial degradation or evaporation were more prominent. For example, most studies on aged contaminated soil showed that although bioavailability of PAH were affected, the native microbes enhanced the dissipation of the PAH due to their long stay in the soil and also because variety of pollutant degrader niches are filled from within, degradation of contaminants can even occur without inoculation (Cunliffe and Kertesz 2006).

6.9 Conclusion

In this study, planting of *Z. mays* enhanced the remediation of Cr and B[*a*]P in aged co-contaminated soil than in freshly contaminated soil. The biomass of *Z. mays* was lower in freshly contaminated soil when compared to aged soil. Similarly the Cr concentration, accumulation and TF were higher in aged soil when compared to freshly spiked soil.

There was evidence of B[*a*]P dissipation in all treatments (planted, non-planted, freshly spiked and aged soils). In aged soil, planting with *Z. mays* for 60 days enhanced the dissipation of B[*a*]P more than in freshly spiked soil. Whereas in non-planted soils, the dissipation of B[*a*]P was either lower or not significantly different when aged soil was compared with freshly spiked soil. This study shows that there was evidence of enhanced simultaneous removal of Cr and dissipation of B[*a*]P by *Z. mays* in aged contaminated soil and could be proposed for phytoremediation of Cr and B[*a*]P aged co-contaminated soils. Therefore, although this study does not inform us with realistic time frames for remediation of

contaminants, it could be possible that some long-term Cr and B[*a*]P contaminated soils could be remedied with plants.

7

Chelate assisted phytoremediation of metal-PAH co-contaminated soils.

7.1 Chelate-assisted phytoremediation of Cu-pyrene contaminated soil using *Z. mays*- Introduction

Heavy metals including Cu can affect the way land is used in the future because of their non-biodegradable nature. They can cause varying toxicities to plants and as such could affect vegetation growth (Bell *et al*. 1991). PAH including pyrene has also become a problem to the soil environment as a result of processes including wastewater irrigation and industrial activities (Shi *et al*. 2005). High concentrations of Cu in the environment pose a risk to plant species by reducing plant growth and photosynthesis as well as inducing oxidative stress (Schill *et al.* 2003, Gunawardana *et al*. 2011). Pyrene on the other hand is photomutagenic and since the simultaneous exposure to light and pyrene by humans is inevitable, there is a threat to human health (Yan *et al.* 2004). Therefore a robust and economical technology for treatment of these pollutants is required and phytoremediation may have the potential to fully remediate soils contaminated with Cu and pyrene.

Various studies have shown the role plants play in uptake of metals (Ebbs and Kochian 1998, Chen and Cutright 2001) as well as in the remediation of soil contaminated with organic contaminants (Binnet *et al.* 2000, Wang *et al*. 2012). However, the ability of plants to remediate PAH has been low, partly due to their recalcitrant nature or low solubility in soil (Ke *et al.* 2003). On one hand, this could be beneficial as the toxicity of PAH to plants is decreased while on the other hand it could pose a long term problem since the PAH will remain in soil and not biodegrade.

A large number of studies have been carried out on uptake of metals with the help of chelates such as ethylenediaminetetraacetic acid (EDTA), citric acid, nitriliotriacetic acid (NTA) or their combinations (Nowack *et al*. 2006, Jean *et al*. 2008). EDTA is a synthetic chelating agent that is not biodegradable in soil (Wasay *et al.* 1998). EDTA plays an important role in phytoextraction of metals from soil by complexing the metals and increasing their concentration in shoot of plants. Citric acid on the other hand is a natural low molecular weight organic acid that is biodegradable (Jean *et al*. 2008). These chelates have high affinity for metals and are able to increase their bioavailability in soil. This helps to increase the uptake of these metals to the upper part of plants during phytoremediation. Similarly, various studies have shown the role of chelates including humic acid in facilitating the degradation of PAHs in soil directly or indirectly by stimulating microbial activity (Ke *et al*. 2003). However, very few studies have investigated the role of chelates during phytoremediation of PAH and heavy metal co-contaminated soils.

The aim of the present study was to understand the role of two chelating agents - a synthetic chelate (EDTA) and a naturally occurring organic acid (citric acid) and their combinations on the degradation of pyrene and the concurrent phytoextraction of Cu by *Z. mays* in a co-contaminated soil. *Z. mays* was chosen because of its high biomass production and ability to tolerate higher concentrations of heavy metals including Cu (Wuana and Okieimen, 2010).

7.2 Methods

7.2.1 Soil spiking

Soil was initially spiked with pyrene by dissolving 100 mg pyrene in 25 mL of acetone. The solution of acetone and pyrene was added to 250 g of soilas a portion and then 750 g of unspiked soil was added once the acetone had volatilized completely in the fume hood to make up to 1 kg. 25 mL of acetone was also added to control and other soil treatments. 50 mg kg^{-1} of Cu was prepared by dissolving 0.126 g of $CuSO_4$ and was added singly to pyrene spiked soils and fresh soils resulting in a total of 16 treatments. The spiked soil was thoroughly mixed by sieving and was stored in a dark room for equilibration for 28 days before planting.

7.2.2 Experimental set up

The experimental layout was designed in a completerly randomized design of 16 treatments with three replicates of each. Experiment included pots with no plants in order to observe non-plant facilitated dissipation of pyrene.

7.2.3 Planting

Plastic pots of 12.5 cm in height were used for the present study. One kilogram of each spiked soil was placed in each pot. One seedling of *Z. mays* with uniform size of about 3 to 4 cm, 3 leaves and about 3 weeks old were transferred into each pot. The chelates used in the present study were EDTA, citric acid and a combined addition of EDTA and citric acid. The chelates were applied after 15 days of transplanting the *Z. mays* in order to allow for acclimatization.

All the chelates were divided into three parts and applied each week for 3 weeks. Treatments included the control soil (without application of chelate), 0.146 g kg^{-1} of EDTA, 3 g kg^{-1} of citric acid and 0.146 g kg^{-1} EDTA + 3 g kg^{-1} citric acid applied as solutions to each soil surface at doses of 48.6 mg kg^{-1} and 1 g kg^{-1} for EDTA and citric acid respectively for three weeks to reduce the effect of the chelates on plant growth, and as suggested by Wenzel *et al*. (2003), split applications were more effective. Saucers were placed beneath the pots to collect potential leachates during the plant trial. After 60 days of growth, plants were harvested by cutting shoots just above the soil surface and washed with deionized water. Each pot was then emptied and the roots were separated from the soil by washing with running tap water. The roots were rinsed with deionized water 3 times to remove all soil particles. All samples were oven- dried to constant weight at 65 ºC for 72 hours. The dried samples were weighed to enable biomass calculations and used for plant analysis.

7.2.4 Analysis of plants and soil samples

Oven-dried plants were ground into small pieces using a coffee grinder (Krups, Italy). Approximately 0.2 g for shoot and 0.1 g for root dry matter were digested using 5 mL of 30% HNO_3 and placed on a heating block (Section 3.4.1). Digested plant samples were then analyzed for total Cu using FAAS. Soluble Cu in soil was analyzed as described in section 3.4.3. Pyrene concentration in soil samples was analyzed using the Agilent GC-MS as described in sections 3.5.1, 3.5.2 and 3.5.3. The average percentage recovery for surrogate was 86.9%.

7.2.5 Statistical analysis

All treatments were replicated three times. The mean and standard error (SE) of each treatment was calculated using Microsoft Office Excel 2007. The comparisons of shoot dry matter, Cu concentration, accumulation as well as soil residual pyrene were carried out by one-way analysis of variance using Minitab 15.0. When a significant difference was observed between treatments, multiple comparisons were made by the Tukey HSD test.

7.3 Results

7.3.1 Effects of EDTA and citric acid on growth of *Z. mays*

In the absence of chelates, *Z. mays* planted in Cu polluted soil showed normal development and no visual symptoms of toxicity to Cu. Compared to the treatments with no chelates, the addition of 0.146 g kg^{-1} soil EDTA significantly inhibited ($p<0.05$) plant growth in soil contaminated with Cu only (Figures 7.1 and 7.2). A 43% significant reduction ($p<0.05$) in shoot dry matter with the addition of EDTA was observed. 3 $g.kg^{-1}$ soil citric acid or a combination of citric acid and EDTA did not significantly reduce shoot dry matter yield. Plants were slightly chlorotic and visibly stunted with EDTA application at the end of the experiment. They also appeared to wilt on day 1 and 2 when EDTA was added. However, the addition of citric acid appeared to be less toxic to *Z. mays* relative to EDTA or a combination of both.

The growth of *Z. mays* in soil only spiked with pyrene was not affected by the addition of chelates except in the case of citric acid which significantly reduced the shoot dry matter by

44%. Addition of EDTA or a combination of the EDTA and citric acid slightly reduced plant shoot dry matter although results were not significant. Irrespective of the slight reduction in shoot dry matter yield, all plants survived in pots and showed no visual toxicity symptoms.

Co-contamination with Cu and pyrene caused significant inhibitory effects on *Z. mays* (Figure 7.1). Shoot dry matter yield significantly decreased by 47% relative to control treatments (no contamination and no chelates-appndix 7A.1). The combined application of EDTA and citric acid promoted the growth of *Z. mays*. With a 41% significant increase in the shoot dry matter yield when compared to polluted soil treatments with no chelates application. The single application of either EDTA or citric acid did not seem to have an effect on the shoot dry matter yield of *Z. mays*, with the apparent 16% increase and 6% reduction observed not showing any statistical significance.

The application of chelates including EDTA, citric acid and EDTA + citric acid to pyrene and Cu + pyrene co-contaminated soil did not significantly ($p>0.05$) affect the root biomass of *Z. mays* relative to control treatments. However EDTA and EDTA + citric acid significantly reduced the root biomass of *Z. mays* from 0.433 to 0.233 g pot^{-1} in single Cucontaminated soil. The effect of citric acid on root growth in Cu contaminated soils was not significant ($p>0.05$) when compared to treatments with no chelates, nonetheless relative to EDTA or EDTA + citric acid, single application of citric acid significantly increased root biomass of *Z. mays*.

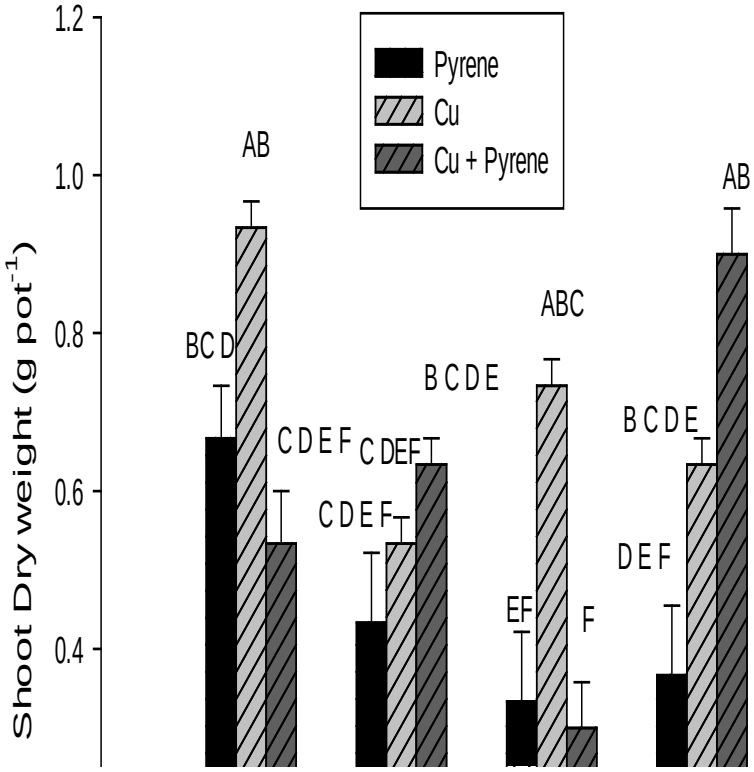


Figure 7.1: Effects of chemical amendments and pollutant combination on shoot dry weight of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share a letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7A.1

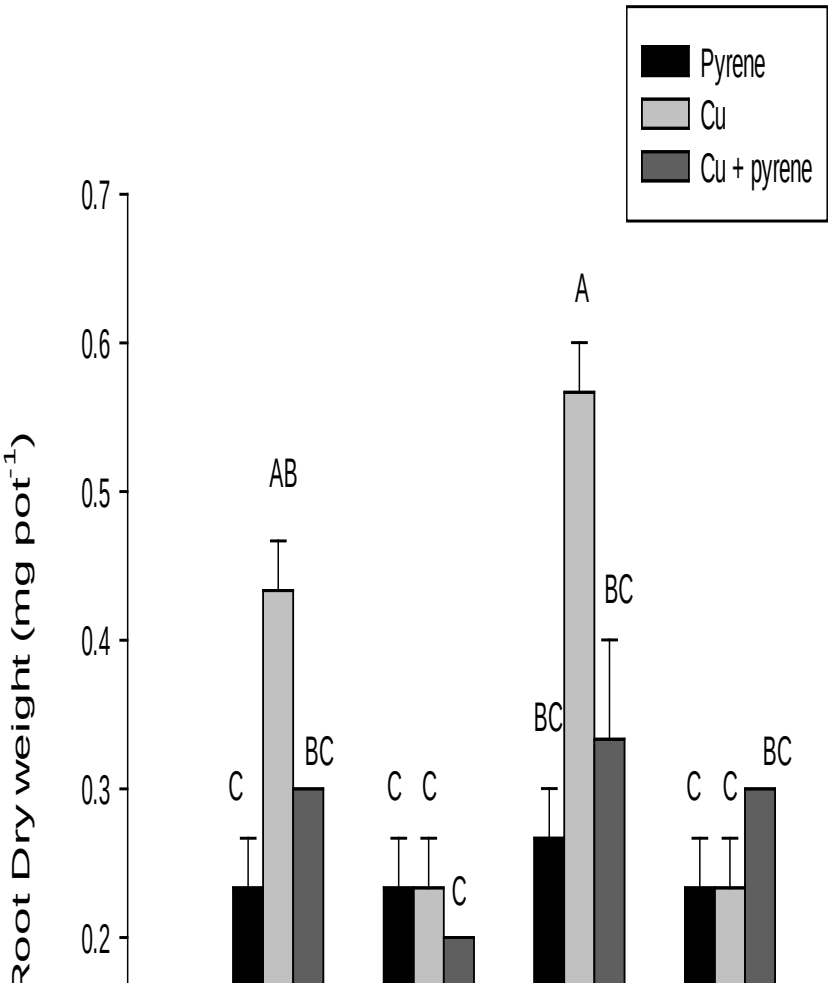


Figure 7.2: Effects of chemical amendments and pollutant combination on root dry weight of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7A.2

7.3.2 Effects of EDTA and citric acid on shoot and root Cu concentration

After 60 days, the average Cu concentration in the shoots of *Z. mays* under single Cu soil contamination with no amendment was 20 mg kg^{-1} (Figure 7.3). The application of EDTA significantly ($p< 0.05$) elevated shoot Cu concentration to 53.8 mg kg^{-1}. Citric acid on its own and a combination of citric acid and EDTA did not affect shoot Cu concentration in our present study. Results showed that concentrations remained at 16.9 and 17.7 mg kg^{-1} respectively. The application of EDTA to single Cu contaminated soil also resulted in enhanced root Cu concentration (Figure 7.4). The mean root concentration of Cu in comparison with control pots (metal with no amendment) increased significantly ($p<0.05$) by 45%. Citric acid and EDTA+ citric acid caused a slight inhibitory effect on root Cu concentration although results were not significant.

The application of EDTA or citric acid to soil co-contaminated with Cu and pyrene, did not significantly increase shoot Cu concentration. Without chelate application, the shoot concentration of Cu was 29.8 mg kg^{-1} and with the application of EDTA or citric acid, the shoot Cu concentration remained 26 mg kg^{-1} or 29.1 mg kg^{-1} respectively. As shown in Figure 7.3, the control plants were more efficient in the uptake of Cu compared to plants treated with EDTA or citric acid. However, the combination of EDTA and citric acid was significant and resulted in the highest shoot Cu concentration of *Z. mays* in Cu + pyrene contaminated soil. The total shoot Cu concentration was thus significantly ($p<0.05$) enhanced by the exogenous provision of a combination of EDTA and citric acid and was about 1.7 times the concentration in the control (metal + PAH without amendment). The effects of chelates on the root concentration of Cu varied (Figure 7.4). When compared to control treatments, the differences

in root concentration when soil was treated with EDTA + citric acid were negligible in Cu + pyrene contaminated soil. However, there was a 1.77 and 1.98 fold significant reduction in root Cu concentration of *Z. mays* when single application of EDTA or citric acid was applied.

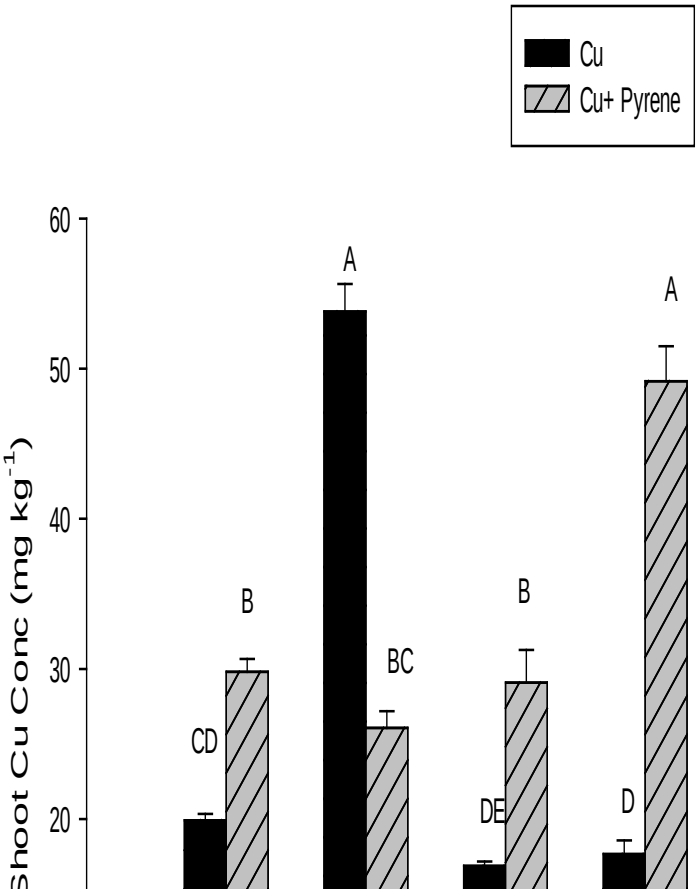


Figure 7.3: Effects of chemical amendments pollutant combination on Cu concentration in shoot of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7A.3

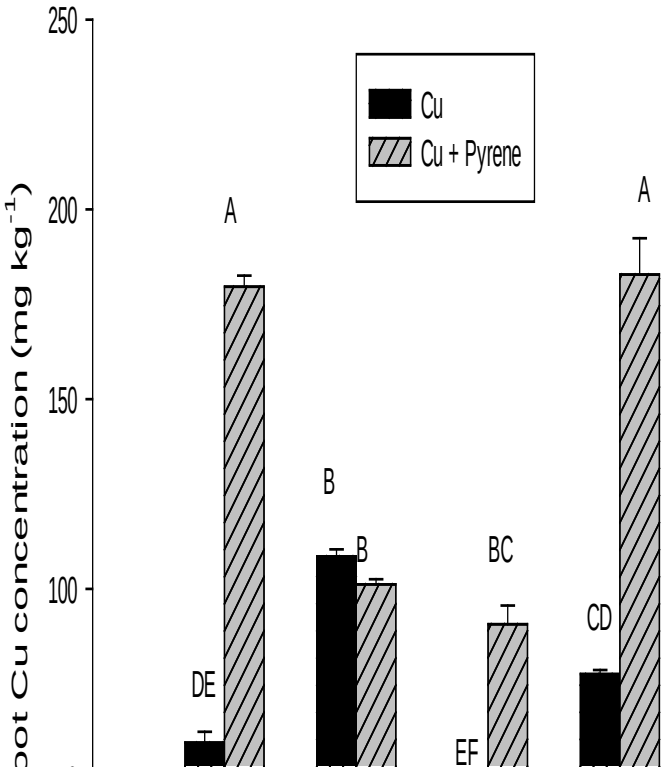


Figure 7.4: Effects of chemical amendments and pollutant combination on Cu concentration in root of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7A.5

7.3.3 Effect of EDTA and citric acid on shoot and root Cu accumulation

For single Cu soil contamination, the mean accumulation of Cu in the shoot of *Z. mays* increased with the application of EDTA and reduced with the combined application of EDTA and citric acid (Figure 7.5). Without chelates (control), *Z. mays* accumulated 18.6 µg pot^{-1} of Cu. The increase in Cu accumulation in *Z. mays* shoot as compared to control pots where EDTA was applied was significant ($P<0.05$), increasing by 35%. Although EDTA was more effective in enhancing shoot accumulation of Cu under single soil Cu contamination, citric acid did not seem to affect Cu shoot accumulation. Results showed that Cu accumulation in the shoot of *Z. mays* remained at 12.4 µg pot^{-1}. When EDTA and citric acid were combined, the shoot accumulation of Cu significantly reduced 1.66-fold when compared to control treatments (no chelates). Under co-contamination of Cu and pyrene, the combined application of EDTA and citric acid seemed to affect shoot accumulation of Cu. Results showed a significant ($p<0.05$) 2.77-fold increase when EDTA and citric acid were added together to co-contaminated soils. Single application of EDTA or citric acid did not affect the shoot accumulation of Cu in co-contaminated soils.

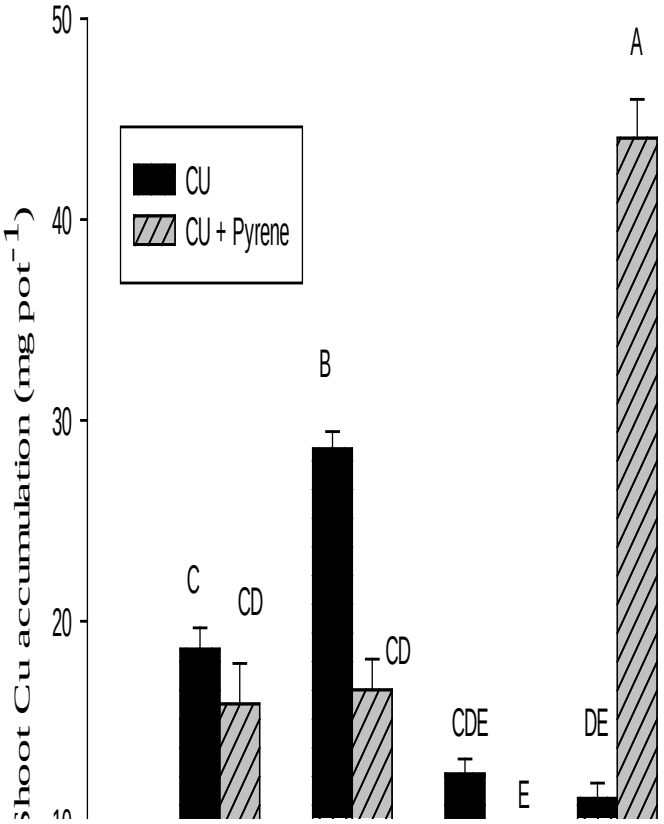


Figure 7.5: Effects of chemical amendments and pollutant combination on Cu accumulation in shoot of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7A.4

Under single soil Cu contamination, all applied chelates in single and combination did not seem to affect the accumulation of Cu in the roots of *Z. mays*.

However, in co-contaminated soils, the application of chelates (EDTA or citric acid) significantly reduced the Cu accumulation in the roots of *Z. mays* (figure 7.6) The effect of EDTA on root accumulation of Cu was more prominent, and was significant when compared to combined treatment of EDTA + citric acid or treatments with no chelates. In the case of citric acid application, the accumulation of Cu in root reduced to 29.59 µg pot^{-1} while the combined application of EDTA and citric acid did not seem to have any effect on root Cu accumulation.

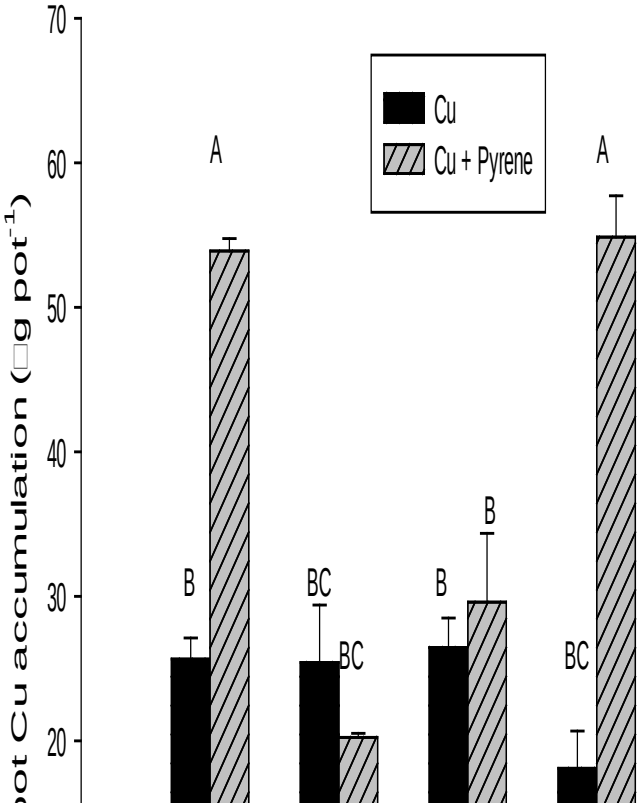


Figure 7.6: Effects of chemical amendments and pollutant combination on Cu accumulation in root of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7A.6

7.3.4 Effect of EDTA and citric acid on Cu translocation

Cu translocation from the root to shoot of *Z. mays* was affected by amendments. The addition of EDTA resulted in significantly ($p<0.05$) higher translocation ratios of Cu after 60 days of planting in single Cu contaminated soil (Figure 7.7). Our results showed that the translocation of Cu from root to shoot reached 0.495 with EDTA application and had increased by 2.36-fold when compared to control treatments (metal with no chelates). The translocation of Cu with the application of citric acid was less efficient when compared to EDTA but nevertheless had significantly increased from 0.209 to 0.363. Combined EDTA and citric acid did not seem to affect the translocation of Cu under single soil Cu contamination.

When soil was co-contaminated with Cu and pyrene, the translocation factor for Cu reached 0.119 without chelate application. Single application of EDTA or citric acid and a combination of EDTA and citric acid dramatically increased Cu translocation without any severe toxicity symptoms being observed. Results showed a 2.15-fold significant ($p<0.05$) increase with the single application of EDTA while citric acid and the combination of EDTA and citric acid significantly ($p<0.05$) increased the translocation of Cu from root to shoot by up to 2.79 and 1.96-fold respectively when compared to co-contaminated soil without the application of chelates.

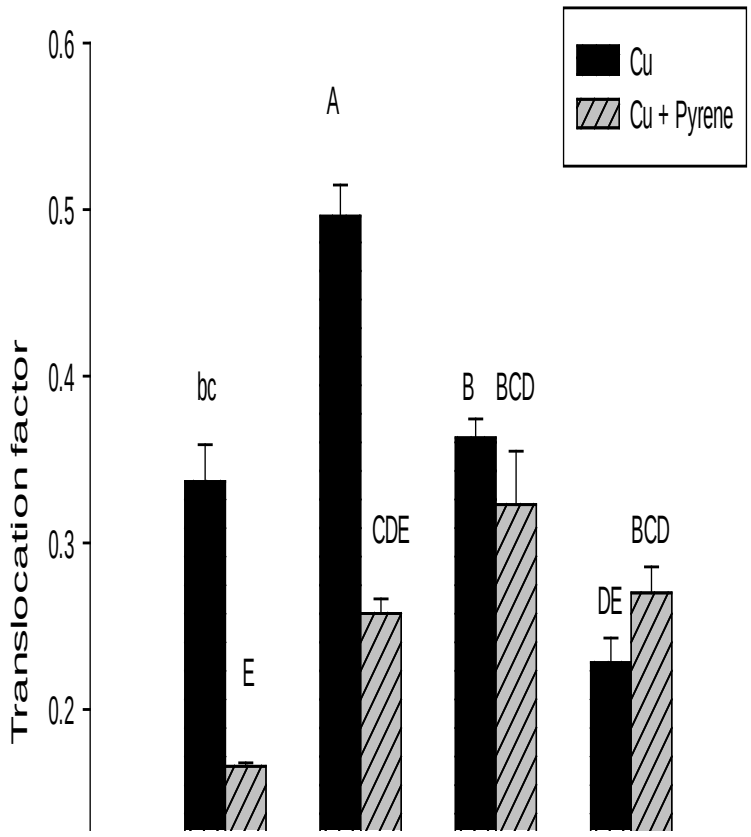


Figure 7.7: Effects of chemical amendments and pollutant combination on translocation of Cu from root to shoot of *Z. mays* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different in each contaminant group based on Tukey HSD ($p \leq 0.05$). Appendix 7A.7

7.3.5 Effects of EDTA and citric acid on solubility of Cu in soil

Water extractable Cu is equivalent to the Cu ion in solution that can be taken up directly by plants (Schramel *et al*. 2000). The concentration of water soluble Cu in soil was examined to assess the relative efficiency of EDTA, citric acid or a combination of both in enhancing Cu solubilization from single Cu and Cu-pyrene co-contaminated soils. From our results, the addition of EDTA significantly increased the water extractable Cu in single Cu contaminated soil relative to contaminated soil with no chelates (Figure 7.8). This significant increase was not noticed in Cu-pyrene co-contaminated soils. The application of EDTA increased the water extractable Cu from 0.73 to 1.84 mg kg^{-1} in single Cu contaminated soil. Citric acid did not significantly ($p<0.05$) affect the concentration of soluble Cu in soils spiked with Cu alone or in Cu-pyrene co-contaminated soils. The Cu mobilized by EDTA in single Cu contaminated soil was to a significant ($p<0.05$) extent higher than citric acid or a combination of citric acid and EDTA. It was observed that the combined application of EDTA and citric acid to single Cu contaminated soil did not significantly ($p>0.05$) affect Cu solubility when compared to single Cu contaminated soil with no chelates. However, when soil was co-contaminated with pyrene, the addition of combined EDTA and citric acid significantly ($p<0.05$) increased the concentration of soluble Cu from 0.396 to 2.12 mg kg^{-1}.

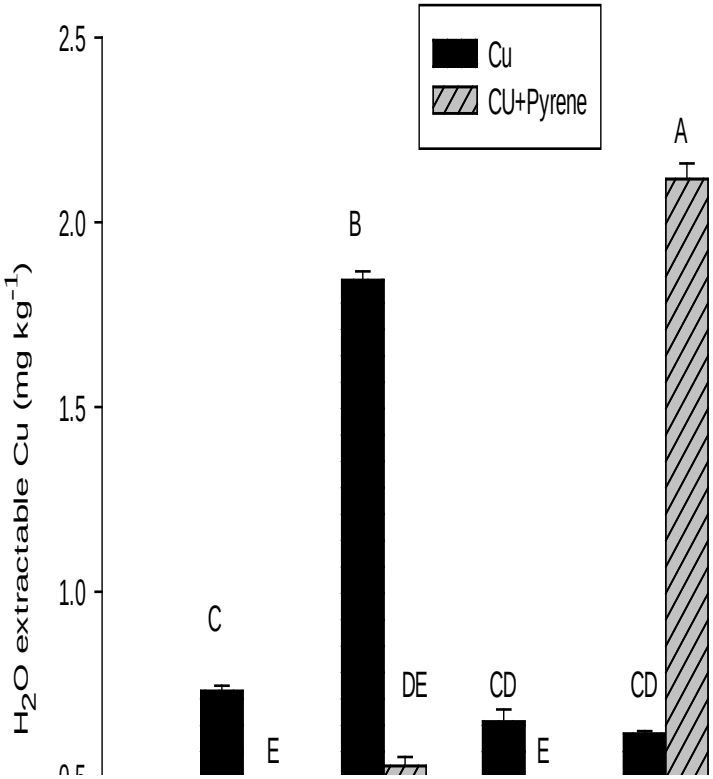


Figure 7.8: Effects of chemical amendments and pollutant combination on H_2O extractable Cu in soil after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significant based on Tukey HSD ($p \leq 0.05$). Appendix 7A.8

7.3.6 Effect of EDTA and citric acid on total removal of Cu by *Z. mays* from contaminated soils

In Table 7.2, the total amount of Cu removed from soils in each treatment as well as the effectiveness of each chelate is shown. EDTA removed higher amount of Cu than citric acid or EDTA + citric acid treatment in soil contaminated with only Cu. Our results showed that with the application of EDTA, the net removal of Cu from soils increased from 44.26 to 53.99 µg .

When soil was co-contaminated with Cu and pyrene, the efficiency of chelates applied varied. Single application of EDTA or citric acid were ineffective for the removal of Cu in soils, while EDTA + citric acid treatment significantly ($p<0.05$) increased the total removal of Cu from 69.76 to 98.9 µg .

The effectiveness of each chelate was evaluated by the ratio of removal of Cu by each chelate to the removal in non-treated pots and results showed that the net removal of Cu by EDTA in single Cu contaminated soils increased by a factor of 0.34 and 0.56 when compared to citric acid and EDTA + citric acid treatments. In soils co-contaminated with Cu and pyrene, the net removal of Cu by EDTA + citric acid treatment increased by a factor of 0.89 and 0.87 when compared to single applications of EDTA and citric acid.

Table 7.2- Total plant removal of Cu per treatment

Treatments	**50 mg kg^{-1} Cu**		**50 mg kg^{-1} Cu + 100 mg kg^{-1} pyrene**	
	Total Cu removal (µg)	Cu removal ratio	Total Cu removal (µg)	Cu removal ratio
0.146 g kg^{-1} EDTA	53.99±2.36	1.22	36.81±1.68	0.53
3 g kg^{-1} citric acid	38.84±2.36	0.88	38.19±6.16	0.55
0.146 g kg^{-1} EDTA+3 g kg^{-1} citric acid	29.28±3.11	0.66	98.90±1.15	1.42
No chelates	44.26±2.29		69.00±2.31	

7.3.7 Effect of EDTA or citric acid on residual pyrene concentration in soil

After 60 days of planting, the residual pyrene in soil decreased for all treatments including soils with no planting (Figure 7.10). However, soil without planting had lower dissipation rate when compared to other treatments for both single and co-contaminated soil. When soil was contaminated with pyrene only, all the chelates applied significantly ($p<0.05$) decreased the residual pyrene in soil when compared to no application of chelates (Figure 7.9). The results showed that the application of citric acid significantly decreased the residual pyrene from 20.09 to 7.46 mg kg^{-1}. Correspondingly, EDTA and EDTA + citric acid also significantly decreased the residual pyrene concentration from 20.09 to 13.06 mg kg^{-1} and 12.61 mg kg^{-1} respectively.

In Cu + pyrene co-contaminated soil, the effect of applied chelates varied. EDTA did not seem to enhance the dissipation of pyrene when compared to planted soil without the application of chelates (Figure 7.9). The soil residual pyrene concentration remained at 23.25 mg kg^{-1} representing a 69% dissipation of pyrene in soil over 60 days of planting. Interestingly, the application of citric acid and EDTA + citric acid significantly decreased the residual pyrene concentration from 15.5 to 7.69 and 10.61 mg kg^{-1} respectively when compared to planted soil without the application of chelates.

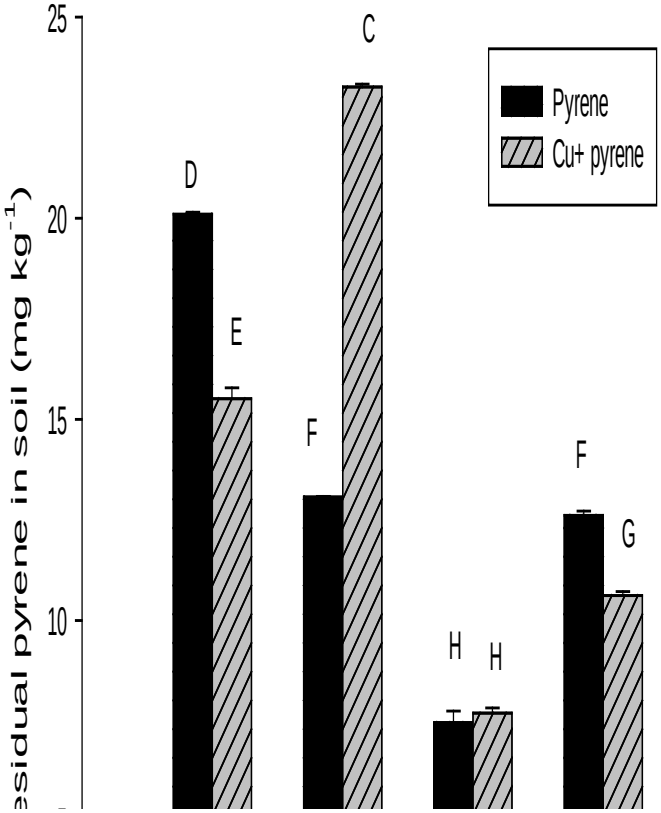


Figure 7.9: Effects of chemical amendments and pollutant combination on residual pyrene concentration in soil after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are not significantly different based on Tukey HSD ($p \leq 0.05$). Appendix A.9

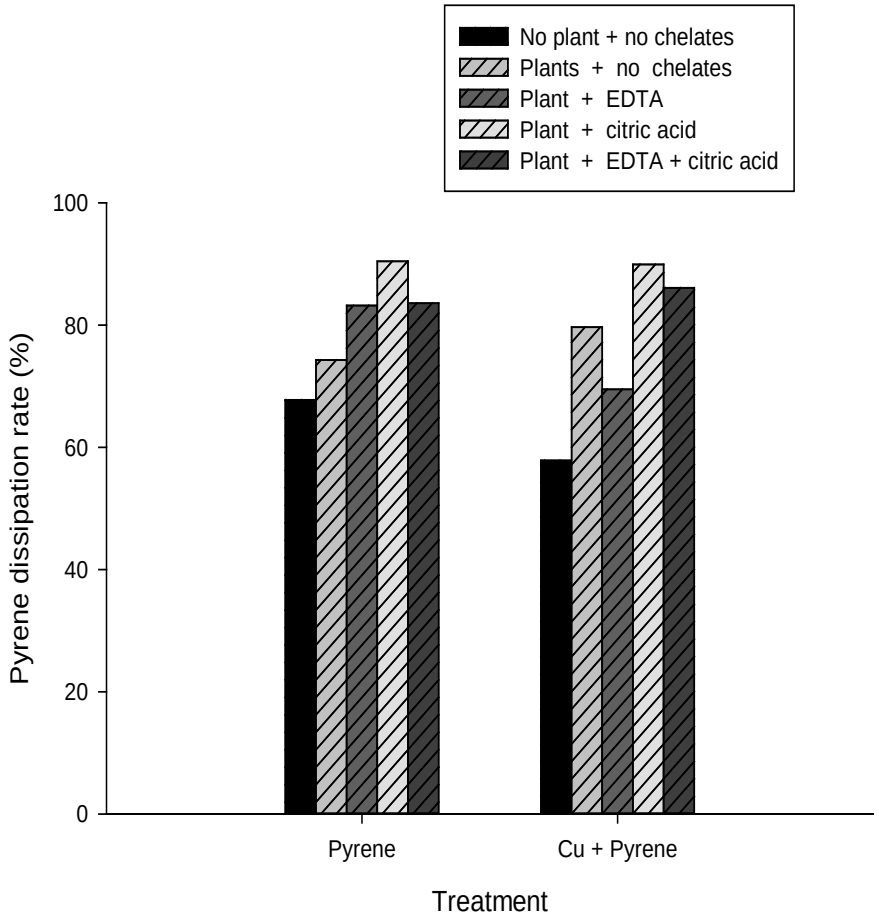


Figure 7.10: The dissipation rate of pyrene under different treatments after 60 days of planting

7.4 Discussion

7.4.1 Plant growth

From our results, without the application of chelates, Cu did not affect the growth of *Z. mays* while pyrene and a combination of pyrene and Cu significantly decreased the growth of *Z. mays* after 60 days of planting (Appendix 7A.1). This is similar to the work of Zhang *et al*. (2009a) which showed that the addition of pyrene up to 100 mg kg^{-1} as well as its co-contamination with cadmium decreased the root and shoot dry matter of *Z. mays* when compared to un-spiked soils. Visual symptoms of toxicity like wilting and chlorosis were observed in *Z. mays* leaves growing in single soil Cu contaminated soil treated with chelates. However relative to control (Cu contamination without chelates), figure 1 showed that only EDTA application had an effect on dry matter. The reduction in plant growth after EDTA treatment is possibly due to the toxicity of EDTA itself and the metal-EDTA complexes (Vassil *et al*. 1998, Chen and Cutright 2001). Obviously, the comparatively low biomass reduction observed with EDTA in the present study could be due to the lower concentration of EDTA used (0.146 g kg^{-1}). The application of citric acid or a combination of citric acid and EDTA did not affect plant growth under single Cu soil contamination. Since adequate concentrations of natural low molecular weight organic acids (NLMWOA) - including citric acid have the ability to detoxify intracellular heavy metals through binding (Lee *et al*. 1977), the concentration of citric acid applied to the contaminated soil was most probably sufficient to detoxify the intracellular Cu and hence limit plant growth inhibition. Similar results were observed by Evangelou *et al*. (2006) where dry matter production of *Nicotiana tabacum* was not affected by the application of citric acid up to 62.5 mmol kg^{-1}.

In this study, the application of EDTA or citric acid alone or in combination did not significantly affect the growth of *Z. mays* in pyrene contaminated soil. As shown in Figure 7.1, citric acid and EDTA caused a slight but non-significant decrease in shoot dry matter of *Z. mays*. This non- significant decrease in shoot dry matter may have been as a result of the toxic effect the pyrene contaminated soil already had on the growth of *Z. mays*. It is also possible that the concentrations of chelates applied may not have been enough to cause a more significant toxicity effect on the *Z. mays* than the one caused by pyrene contamination.

On the contrary, under co-contamination of Cu and pyrene, the combination of EDTA and citric acid promoted the growth of *Z. mays*. There was a significant ($p<0.05$) increase in shoot dry matter of *Z. mays*, indicating that a combination of EDTA and citric acid at the present concentration could alleviate the growth inhibition caused by pyrene and Cu co-contamination and increase plant tolerance to adverse environmental conditions. Gunawardana *et al*. (2011) observed that sulfate and citric acid treatments significantly increase biomass yield of *L. perenne* although it also enhanced accumulation of Cu. It is likely that when *Z. mays* is exposed over a longer period to EDTA + citric acid, reduction in biomass of *Z. mays* could be observed. However it is also expected that the longer *Z. mays* absorbs Cu, the higher the amount of Cu is extracted. Therefore, high biomass production by plants treated with chelates that are less phytotoxic could be better suited for removal of Cu from soil as will be discussed later.

7.4.2 Concentration and accumulation of Cu

Chemically enhanced phytoextraction has been proposed as an effective approach for the removal of heavy metal from soils using plants (Blaylock *et al*. 1997, Liphadzi *et al*. 2003). Several chelating agents such as EDTA, citric acid, EDDS and salicylic acid have been tested for their ability to mobilize and increase the accumulation of heavy metals (Luo *et al*. 2005, Turgut *et al*. 2004, Yang *et al*. 2011). The solubility of Cu in soil dramatically increases with the addition of some chelating agents including EDTA (Tandy *et al*. 2004) and although Cu uptake by plant shoots remained minimal, Blaylock *et al*. (1997) reported increased concentration of Cu to 1000 mg kg^{-1} DW in *B. juncea* shoots when 2.5 mmol kg^{-1} of EDTA was applied. In our study, the highest concentration and accumulation of Cu reached 53.8mg kg^{-1} DW and 28.57 µg pot^{-1} respectively in *Z. mays* shoots after the application of EDTA to soil contaminated with only Cu. This is about three times the concentration or twice the accumulation observed in plants without the application of chelates, with the application of 3 g kg^{-1} of citric acid or the combination of citric acid and EDTA. The low enhancement factor observed could be due to the low concentration of EDTA used as well as low concentration of Cu. Similar results were observed by Wu *et al.* (2004b) where 3 mmol kg^{-1} of EDTA significantly enhanced shoot uptake of Cu. When EDTA is applied to the soil, its initial action is to complex soluble metals in the soil solution. This reduces the activity of the free metals while the dissolution of bound metal ions begins to compensate for the shift in equilibrium (Blaylock *et al*. 1997). For example, if EDTA is added in an appropriate amount, almost all the soluble Cu will be complexed as Cu-EDTA. In the present study, the application of 0.146g kg^{-1} of EDTA to single Cu contaminated soil increased the H_2O extractable Cu from 0.73 to

1.84 mg kg^{-1}. Among the chelates evaluated in this study, EDTA appeared to most effectively solubilize soil-bound Cu and maintain a high soluble Cu concentration in single Cu contaminated soil. Under co-contamination, the combined application of EDTA and citric acid was more effective than single application of EDTA or citric acid. Soluble metals are potentially bioavailable and can either be taken up by plants, leached or dissolved by the soil exchange sites (Kim and Li 2010). The shoot accumulation of Cu was always directly proportional to the amount of soluble Cu in soil and correlation reached 0.935. This could be an important factor in Cu uptake. It is possible that EDTA destroyed the physiological barriers in roots which control the uptake and translocation of metals (Luo *et al*. 2005). The plasma membrane surrounding the root plays a role in the formation of these barriers whereas zinc and calcium ions are involved in stabilizing the plasma membrane (Kaszuba and Hunt 1990). Therefore EDTA in single Cu-contaminated soil may induce Cu-EDTA uptake and accumulation by removal of the stabilizing zinc and calcium ions from the plasma membrane. When this happens, a rapid equilibrium of soil solution with the xylem sap occurs and as soon as the solutes are in the sap, Cu-EDTA would flow through the transportation stream and shoot accumulation increases (Vassil *et al.* 1998). As EDTA has a more negative charge than the Cu cation, metal complexation by EDTA is likely to reduce metal sorption to cation exchange sites in plants (Wenger *et al*. 2003). This will probably reduce Cu retention in the root tissues, allowing greater translocation of metals to shoots. The effectiveness of EDTA in enhancing the shoot accumulation of Cu in single Cu contaminated soil was significantly higher than that of citric acid and a combination of citric acid and EDTA (Figure 7.5). Under single soil Cu contamination, the addition of citric acid seemed not to enhance the uptake of

Cu in shoot of *Z. mays*. This is similar to the result of study carried out by Luo *et al*. (2005) who observed that 5.0 mmol kg^{-1} of citric acid did not significantly increase the concentration of Cu in *Z. mays*. Also Wu *et al*. (2003) observed that low molecular weight organic acids including citric acid had a very small effect on the concentration of Cu, Zn, Cd and Pb in shoot of *B. juncea* when compared to EDTA. The non-increase of Cu in shoot of *Z. mays* with the application of citric acid could be as a result of the lower stability of the metal complexes formed and also because citric acid is weak and biodegradable. Probably, due to the biodegradation of organic acids like citric acid, it is possible that the pH of soil will increase as a result of consumption of H^+ from carboxylic acid and liberation of OH^- and CO_2 (Gramss *et al*. 2004). This results in a lack of complexing agents and as such the bioavailability of Cu is decreased.

Interestingly, it was clear that EDTA enhanced Cu uptake by *Z. mays* under single soil Cu contamination while it had no effect on Cu and pyrene co-contaminated soils (Figure 7.5). Our results also showed that after the application of EDTA, *Z. mays* suffered from more severe phytotoxicity under single Cu contaminated soil than in Cu-pyrene co-contaminated soils. It is possible that the root of *Z. mays* in single Cu contaminated soil would experience heavier physiological damages which could lead to subsequent breakdown of the root exclusion mechanism causing indiscriminate uptake of Cu by plants. This assumption is consistent with the fact that enhancement of Cu concentrations in the shoots of *Z. mays* were more pronounced in single Cu contaminated soil than when Cu and pyrene were combined. Vassil *et al*. (1998) suggested that a threshold of EDTA is required to induce the accumulation of metals in plant shoots. For example Blaylock *et al*.

(1997) observed that the concentration of EDTA required for an increased accumulation of Pb in shoots of *B. juncea* containing 600 mg of Pb in 1 kg of soil was about 1 to 5 mmol kg^{-1}. At this concentration, EDTA could damage the membrane of the root cells which controls the translocation of solutes (Meers *et al*. 2009). In the present study, under co-contamination of Cu and pyrene, the increased accumulation of Cu in *Z. mays* as observed under single Cu soil contamination was not found in EDTA treatment up to 0.146 g kg^{-1}. Probably under co-contamination of Cu and pyrene, less than 0.146 g kg^{-1} of EDTA application was insufficient to break down plant uptake barriers under the conditions of our present study. This observation was consistent with the observation that EDTA was less toxic to *Z. mays* under Cu and pyrene mixed contaminated soil than in single Cu contaminated soil.

The effects of amendment combinations on contaminated soil can be synergistic or antagonistic (Gunawardana *et al*. 2010). Under co- contamination with Cu and pyrene, the enhancement of Cu accumulation with EDTA + citric acid was obvious and interestingly, biomass yield was not decreased (Figure 7.1). Therefore a combination of EDTA and citric acid could be considered as viable amendments for enhancing Cu phytoextraction from metal-PAH contaminated soil. The shoot Cu concentration as well as the water extractable Cu with the application of EDTA + citric acid was also the highest of any treatment under co-contamination at the end of the experiment (Figures 7.3 and 7.8). This increase could be attributed to the synergistic effect of citric acid or EDTA which increases ligand availability in solution through a potentially different mode of action and uptake pathway (Gunawardana *et al*. 2010). In metal mixtures, different researchers observed a reduced effect of individual

amendments when compared to mixed amendments. For example, Gunawardana *et al*. (2010) and Gunawardana *et al*. (2011) showed that the combination of rhamnolipd + EDDS or rhamnolipid + citric acid + EDDS was more efficient in increasing shoot Cu, Cd and Pb concentration as opposed to single applications. Although the present study contain a mixture of metal and PAH, similar results were observed.

7.4.3 Phytoremediation potential

The success of phytoremediation is dependent on shoot biomass as well as shoots Cu concentration (Jiang *et al*. 2004). The potential effectiveness of each plant with chelate application was evaluated by the total amount of Cu removed from the soil. Our results showed that EDTA was more efficient than citric acid or EDTA + citric acid when soil was contaminated with Cu alone. Sinhal *et al*. (2010) showed in their research that although both citric acid and EDTA enhanced phytoextraction of Zn, Cu, Pb and Cd, EDTA was more efficient during phytoextraction. In co- contaminated soils, the combined application of EDTA and citric acid was more efficient compared to citric acid, EDTA or control (no chelates) treatments and is supported by Yang *et al*. (2011) which suggested that combined treatments of EDTA, cysteine and tween-80 was more promising application to improve the phytoremediation of heavy metals under Cd-PAH mixed contaminated soil situations. In addition to total metal content, the translocation factor (Figure 7.7) needs to be considered in order to evaluate the ability of an accumulator to accumulate and transport heavy metals in plants. Metal translocation is expressed as the ratio of the metal level in the shoots to that in the roots (Marchiol *et al*. 2004, Gunawardana *et al*. 2010). In the present study, it indicates the ability of chelates to affect the transfer of Cu from root to shoot.

It was found that EDTA significantly enhanced the translocation of Cu in Cu contaminated soil but not in co-contaminated soils containing Cu and pyrene. The combination of EDTA and citric acid significantly reduced the translocation of Cu in single Cu contaminated soils, but the shoot to root efficiency increased in soils co-contaminated with Cu and pyrene. Higher translocation as observed with EDTA in single Cu contaminated soils could be as a result of reduced metal binding to root tissues (Blaylock *et al*. 1997). Romkens *et al*. (2002) suggested that when Cu is complexed with an amendment, Cu would be more easily reallocated to harvestable plant tissues than free metal ions. Relatively stable Cu complexes are readily absorbed by roots and transported to above ground parts due to the higher affinity of EDTA to Cu (Degryse *et al*. 2006). This complexation could decrease the binding of free metal ions to negatively charged carboxyl groups in the xylem cell walls (Wenger *et al.* 2003). In co-contaminated soils (Figure 7.7), the enhancement of translocation with EDTA was less than in single Cu contaminated soil. It could be that the interactions of Cu and pyrene with EDTA resulted in reduced Cu transport through the plant parts. Luo *et al*. (2005) showed that when metal and metal-EDTA complexes are simultaneously present in solution, they effectively compete for uptake, therefore reducing Cu transport rate to shoots. Also pyrene has been shown to be able to accumulate in shoots of plants from direct translocation from roots (Gao and Zhu 2004). Therefore, increased competition for uptake in the presence of pyrene, Cu and Cu-EDTA complex could have caused the slight reduction observed.

As shown in Figure 7.7, treatment with EDTA + citric acid under combined contamination of Cu and pyrene had a significantly higher TF than control treatments. This is possibly because additional chemicals sharply increased the concentration of Cu in plant shoots while there was

no corresponding increase in plant roots. Our results revealed that in all treatments, the plant roots were more affected by chemicals during the phytoextraction of Cu.

7.4.4 Pyrene dissipation

High molecular weight PAH including pyrene has often not been successfully dissipated in contaminated soil. The prospect of improving the dissipation has often been challenging.

At the end of the plant trial, the residual pyrene concentration in single or co-contaminated soils was highly decreased and reached 57.8 and 67.7% respectively even without the application of chelates or plant growth (Figure 7.10). The dissipation of pyrene in single pyrene or Cu-pyrene co-contaminated soils was significantly higher in planted soils than in unplanted soils. This suggests that the root system of *Z. mays* and probably other physiological characteristics of *Z. mays* played an important role in pyrene dissipation in Cu-pyrene contaminated soils.

The role of chelates in the removal of pyrene in soil in the present study is shown in figure 7.9. It could be seen that in single and co-contaminated soils, all tested chelates significantly increased the dissipation of pyrene except EDTA which did not enhance pyrene dissipation in co-contaminated soil. Also, citric acid had a more significant effect when compared to EDTA or combined application of EDTA and citric acid. The dissipation rate of pyrene with the application of citric acid reached 90% at the end of the plant trial (Figure 7.10). It could be that citric acid provided more nutrients for indigenous microbes to proliferate and biodegrade the pyrene in soil thereby increasing the biodegradation rate (Andrew *et al*. 2007). It could also be explained that when the root of *Z. mays* exude organic compounds, the solubility of

PAH may be influenced indirectly through the effects on the activity of the microbes in soil (Marschner *et al*. 1995). The concentration of the chelates including citric acid in final soil was not analyzed and it could be possible that co-metabolism of the citric acid with pyrene occurred to improve biodegradation of pyrene (Wei *et al*. 2009). The dissipation of pyrene with the application of chelates in single as well as co-contaminated soils could be associated with the effects of chelates on physico-chemical processes including contact between micro-organisms and PAH. Wei *et al*. (2009) suggested that variations in pH values caused by low molecular weight organic acids (LMWOAs) hardly had any effect on PAH degradation and therefore concluded that contact between PAH and micro-organisms was highly related to PAH biodegradation. The present result of this study showed that citric acid was more effective in enhancing pyrene dissipation than EDTA or EDTA + citric acid in both single and mixed contaminated soil and this reflects the result of previous works which showed that organic acids influence the activities of enzymes that help in the degradation of PAH like laccases and manganese peroxidase (Eibes *et al.* 2005, Ting *et al*. 2011). In contrast, when Fentons reagent was used for treatment of PAH contaminated soil, the removal of PAH increased with the application of EDTA than citric acid (Venny *et al*. 2012). It is clear that EDTA-Fe^{3+} complex formed during the treatment was stronger than that of citric acid and could explain why EDTA enhanced PAH removal more than citric acid.

7.5 Conclusion

The present study showed that *Z. mays* could be very effective in phytoextraction of Cu and dissipation of pyrene in Cu-pyrene co-contaminated soil with the help of chelates. Of all the chelates used in the present study, EDTA was more effective in the removal of Cu from single

Cu contaminated soil at the concentration used in the trial, whereas the combined application of EDTA and citric acid had the most effective improvement in Cu uptake in Cu + pyrene co-contaminated soil.

The effectiveness of the applied chelates in the dissipation of pyrene varied in the present study. In single pyrene contaminated soil, all the applied chelates were effective in decreasing the residual pyrene in soils with the help of *Z. mays*. However, citric acid was more effective when compared to EDTA or citric acid + EDTA. When soil was co-contaminated with Cu and pyrene, only citric acid and EDTA + citric acid was effective in the dissipation of pyrene. EDTA was completely ineffective in co-contaminated soil and deserves a further study.

It can be proposed from results of the present study that during the phytoremediation of Cu and pyrene co-contaminated soil, the combined treatment of EDTA + citric acid will best suit the phytoextraction of Cu as well as the dissipation of pyrene. Although citric acid was more effective than EDTA + citric acid in the dissipation of pyrene in co-contaminated soil, the difference in dissipation rate was only over 3%. Also, because citric acid did not enhance the uptake of Cu in co-contaminated soil, the combined treatment of EDTA + citric acid which enhanced both the uptake of Cu as well as the dissipation of pyrene will be the preferred alternative.

7.6 The effect of EDTA and citric acid on phytoremediation of Cr and B[*a*]P co-contaminated soil- Introduction

Part of this work has been published in environmental science and pollution research peer reviewed journal; Chigbo and Batty 2013.

Phytoremediation which is the ability of plants to remediate or sequester contaminants from the environment is an emerging technology that can be used for treatment of heavy metal and PAH contaminated soil (Turgut *et al*. 2004). For example, phytoextraction and rhyzodegradation are the two types of phytoremediation that can be applied to heavy metal and PAH contaminated soil (Zhang *et al*. 2011). During phytoextraction, the plant plays the primary role by taking up heavy metals into the root and translocating to the upper parts whereas in rhizodegradation, the plants play a secondary role in dissipation of PAH through the release of root exudates that promote the growth and activity of microbes in the rhizosphere.

The efficiency of treating heavy metal and PAH contaminated soil however depends on the availability of these contaminants for uptake and degradation, respectively. Although some of these contaminants have low bioavailability in sites, there is a need to meet stringent cleanup targets. This has led to several studies on different chelates that increase the bioavailability of contaminants (Evangelou *et al*. 2007). EDTA is a synthetic chelating agent that is efficient in complexing metals and increasing their concentration in the upper parts of plants. However, they are persistent in the environment (Nowack 2002) and can lead to uncontrolled leaching in the soil which can affect the underground water (Meers *et al*. 2005). Barona *et al*. (2001)

observed that Pb, Ni and Zn were more mobile after the application of EDTA because the metal studied became weakly adsorbed to the soil, increasing the possibilities of leaching. Biodegradable compounds like low molecular weight organic acids (LMWOAs) including citric acids are natural strong ligands that are capable of forming complexes with heavy metals. They are produced by plants and microbes and are less phytotoxic (Jones 1998). In terms of organic contaminant dissipation, they play an important role by increasing the water solubility and therefore enhance the bioavailability of hydrophobic compounds (Gao *et al*. 2007). Several studies have shown that chelating compounds such as EDTA and citric acid can increase the availability of metals and therefore enhance phytoextraction (Huang *et al*. 1997, Chen *et al*. 2003b). For example, 1.0 g kg^{-1} EDTA and 2 mmol kg^{-1} citric acid were reported to be effective in increasing Pb and Cd concentration respectively in *Zea mays* and *Pisum sativum* (Huang *et al*. 1997). Whereas many studies have reported the effects of chelating agents on the extraction of heavy metals from soil, there has been little research on the role of chelates during phytoremediation of heavy metal and PAH co-contaminated soil.

The aim of this work is to determine the effect of synthetic and natural occurring chelating agents (EDTA and citric acid, respectively) on the phytoremediation of soils co-contaminated with heavy metals and PAH. Cr and B[*a*]P are chosen as representative of heavy metal and PAH contaminants that are present in multi contaminated soils which are rarely studied (Jean *et a*l. 2008, Sun *et al*. 2011). *M. sativa* was chosen as the plant candidate due to its high biomass, extensive root system and tolerance to heavy metals and PAHs (Fan *et al*. 2008).

7.7 Methods

7.7.1 Soil Spiking

Soil was initially spiked with B[*a*]P by dissolving 10 mg B[*a*]P in 25mL of acetone. The solution of acetone and pyrene was added to 250 g of soil as a portion and 750 g of unspiked soil to make up to 1 kg once the acetone had volatilized completely in the fume hood. 25 mL of acetone was also added to control and other soil treatments. 50 mg kg^{-1} of Cr was prepared by dissolving 0.187 g of $K_2Cr_20_7$ and was added singly to pyrene-spiked soils and fresh soils resulting in a total of 16 treatments. The spiked soil was thoroughly mixed by sieving and was stored in a dark room for equilibration for 28 days before planting.

7.7.2 Experimental set up

The experimental layout was designed in a completely randomized design of 16 treatments with three replicates of each. Experiment included pots with no plants in order to observe non-plant facilitated dissipation of pyrene.

7.7.3 Planting

Plastic pots of 12.5 cm in height were used for the present study. One kilogram of each spiked soil was placed in each pot. Ten seedlings of *M. sativa* with uniform size of about 3 to 4 cm and about one month old were transferred into each pot. The chelates used in the present study were EDTA, citric acid and a combined addition of EDTA and citric acid. The chelates were applied after 15 days of transplanting the *M. sativa* in order to allow for acclimatization. All the chelates were divided into three parts and applied each week for 3 weeks. Treatments

included the control soil (without application of chelate), 0.146 g kg^{-1} of EDTA, 3 g kg^{-1} of citric acid and 0.146 g kg^{-1} EDTA + 3 g kg^{-1} citric acid applied as solutions to each soil surface at doses of 48.6 mg kg^{-1} and 1 g kg^{-1} for EDTA and citric acid respectively for three weeks to reduce the effect of the chelates on plant growth as explained in section 7.2.4, split applications were more effective. Saucers were placed beneath the pots to collect potential leachates during the plant trial. After 60 days of growth, plants were harvested by cutting shoots just above the soil surface and washed with deionized water. Each pot was then emptied and the roots were separated from the soil by washing with running tap water. The roots were then rinsed with deionized water 3 times to remove all soil particles. All samples were oven- dried to a constant weight at 65 ºC for 72 hours. The dried samples were weighed to enable biomass calculations and used for plant analysis.

Figure 7.10: *M. sativa* after 14 days of planting

7.7.4 Analysis of plants and soil samples

Oven-dried plants were ground into small pieces using a coffee grinder (Krups, Italy). Approximately 0.2 g for shoot and root dry matter were digested using 5 mL of 30% HNO_3 and placed on a heating block (Section 3.4.1). Digested plant samples were then analyzed for total Cr using FAAS. Soluble Cr in soil was analyzed by method described in section 3.4.2.

B[*a*]P concentration in soil samples was analyzed using the Agilent GC-MS as described in sections 3.5.1, 3.5.2 and 3.5.3. The average percentage recovery for surrogate was 102%

7.7.5 Statistical analysis

All treatments were replicated three times and the mean and standard error (SE) of each treatment was calculated. The comparisons of shoot dry matter, Cr concentration, accumulation as well as soil residual B[*a*]P were carried out by one-way analysis of variance using Minitab 15 . The shoot Cu accumulation and total Cu accumulation results were log normalized while soil residual B[a]P concentration was normalized using box cox. When a significant difference was observed between treatments, multiple comparisons were made by the Tukey HSD test.

7.8 Results

7.8.1 Effects of EDTA and citric acid on growth of *M. sativa*

At harvest, all plants were in growth stage without flowering and no seeds were generated. Some chlorosis of leaves was found when EDTA and EDTA + citric acid were applied to 50 mg kg^{-1} Cr and 50 mg kg^{-1} Cr + 10 mg kg^{-1} B[*a*]P contaminated soils under experimental conditions at day 60. Compared to the control treatments (Cr contamination with no chelates), the shoot dry weight of *M. sativa* decreased significantly ($p<0.05$) from 0.67 to 0.3 g pot^{-1} when EDTA + citric acid was applied to soil contaminated with Cr alone (Figure 7.11). The combined application of EDTA and citric acid to Cr contaminated soil significantly affected the shoot biomass of *M. sativa* more than the application of citric acid only.

The single application of EDTA, citric acid or the combined application of EDTA and citric acid respectively did not seem to affect the shoot biomass of *M. sativa* when soil was contaminated with B[*a*]P or Cr + B[*a*]P.

The effect of chelates on the root biomass of *M. sativa* varied and is shown in Figure 7.12. Compared to the dry weight of control roots, the addition of EDTA or citric acid alone, or the combined application of EDTA and citric acid did not significantly affect the root biomass of *M. sativa* in B[*a*]P or Cr + B[*a*]P contaminated soil. However, in Cr contaminated soil, citric acid and EDTA + citric acid significantly ($p<0.05$) reduced the root biomass of *M. sativa* from 0.39 to 0.2 and 0.12 g pot^{-1} respectively.

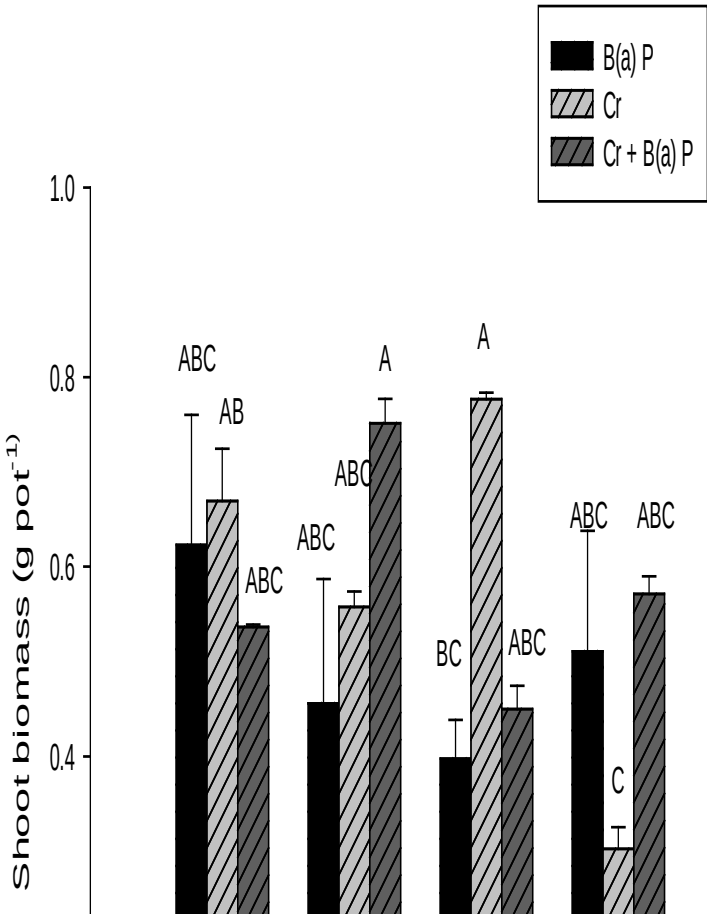


Figure 7.11: Effects of chemical amendments and combination on shoot dry weight of *M. sativa* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7B.1

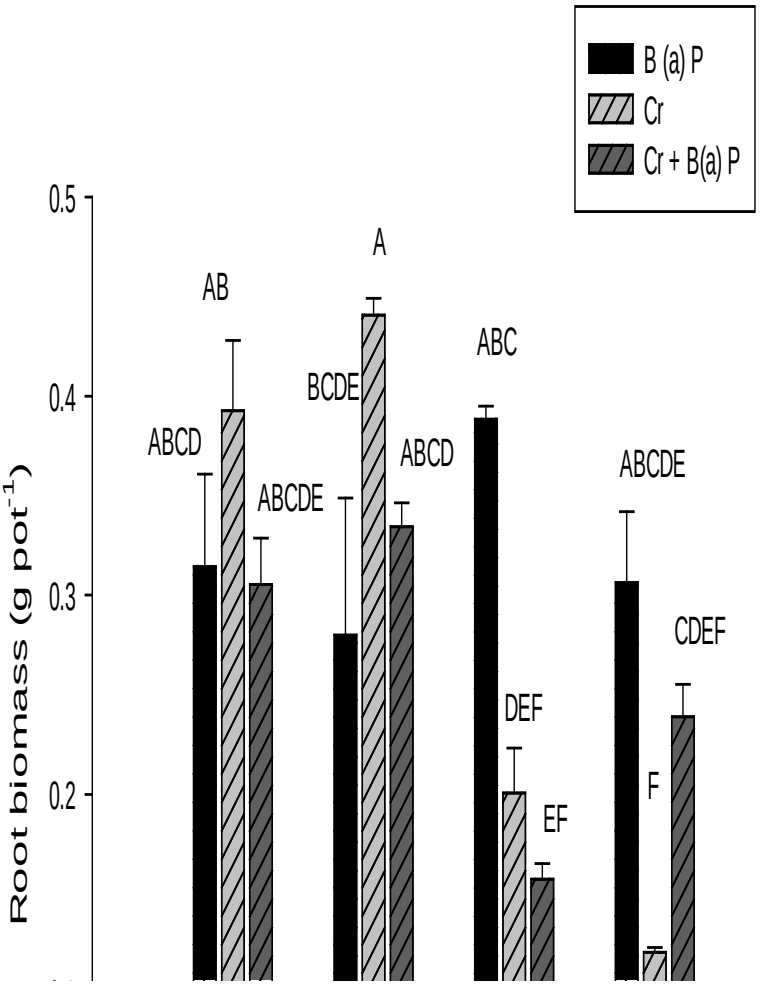


Figure 7.12: Effects of chemical amendments and combination on root dry weight of *M. sativa* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7B.2

7.8.2 Effect of EDTA and citric acid on soluble Cr concentration

For Cr phytoextraction, Cr must be available and be absorbed by the roots. Bioavailability depends on the solubility of Cr in soil solution. It was clear that in single or co-contamination of Cr and B[*a*]P, EDTA, citric acid or the combined application of EDTA and citric acid significantly increased the soluble concentration of Cr in soil (Table 7.3). Results showed that the application of EDTA, citric acid or EDTA + citric acid significantly ($p<0.05$) increased the soluble Cr concentration from 2.45 mg kg^{-1} to 4.09, 5.03 and 3.59 mg kg^{-1} respectively. Citric acid was however more efficient in increasing the soluble Cr in solution than other applied chelates. In Cr + B[*a*]P co-contaminated soils, the application of EDTA, citric acid and EDTA + citric acid also significantly increased the soluble Cr concentration from 1.733 mg kg^{-1} to 4.64, 3.51 and 4.79 mg kg^{-1}. The effect of EDTA and EDTA + citric acid was similar, but more efficient than citric acid in increasing the soluble Cr concentration in co-contaminated soils.

Table 7.3: Soluble Cr concentration in soils (figures are mean values ± SE). Appendix 7B.9

Treatment	**50 mg kg^{-1} Cr**	**50 mg kg^{-1} Cr + 10 mg kg^{-1} B[*a*]P**
	Soluble Cr concentration (mg kg^{-1})	
EDTA	4.09±0.20	4.64±0.07
Citric acid	5.03±0.04	3.51±0.06
EDTA + citric acid	3.59±0.15	4.79±0.12
No chelate	2.45±0.09	1.73±0.02

7.8.3 Effects of EDTA and citric acid on shoot and root Cr concentration

The Cr concentration in shoot of *M. sativa* in soil contaminated with 50 mg kg^{-1} Cr was not significant when compared to that in uncontaminated soil. However, the co-contamination of soil with 50 mg kg^{-1} Cr and 10 mg kg^{-1} B[*a*]P significantly increased the concentration of shoot Cr from 15.02 to 18.91 mg kg^{-1} in the present study, suggesting a synergistic effect of Cr and B[*a*]P co-contamination.

The concentration of Cr in shoot of *M. sativa* was markedly enhanced with the application of citric acid or a combination of citric acid and EDTA in Cr or Cr + B[*a*]P contaminated soils (Figure 7.13). EDTA was only effective in Cr + B[*a*]P co-contaminated soils. After 60 days of planting, the application of citric acid to soil contaminated with Cr alone significantly ($p<0.05$) increased the shoot concentration of *M. sativa* from 18.91 to 42.79 mg kg^{-1}. The combined application of EDTA and citric acid was more effective and increased the shoot Cr concentration 3.41-fold relative to control treatments. The application of EDTA in the present study did not enhance the concentration of Cr in shoot of *M. sativa* when soil was contaminated with Cr alone.

The effects of chelates in soil co-contaminated with Cr and B[*a*]P varied. EDTA seemed to be more effective than citric acid in enhancing the concentration of Cr in shoots of *M. sativa* (Figure 7.13). The application of EDTA significantly ($p<0.05$) increased the shoot Cr concentration from 35.44 to 53.29 mg kg^{-1}. Citric acid was ineffective in increasing the shoot Cr concentration of *M. sativa*. However the combined application of EDTA and citric acid dramatically increased the shoot concentration from 35.44 to 102.81 mg kg^{-1}. The present

result suggests that the combined application of EDTA and citric acid was more effective than single applications of either EDTA or citric acid to single Cu contaminated soil as well as Cr + B[*a*]P co-contaminated soils.

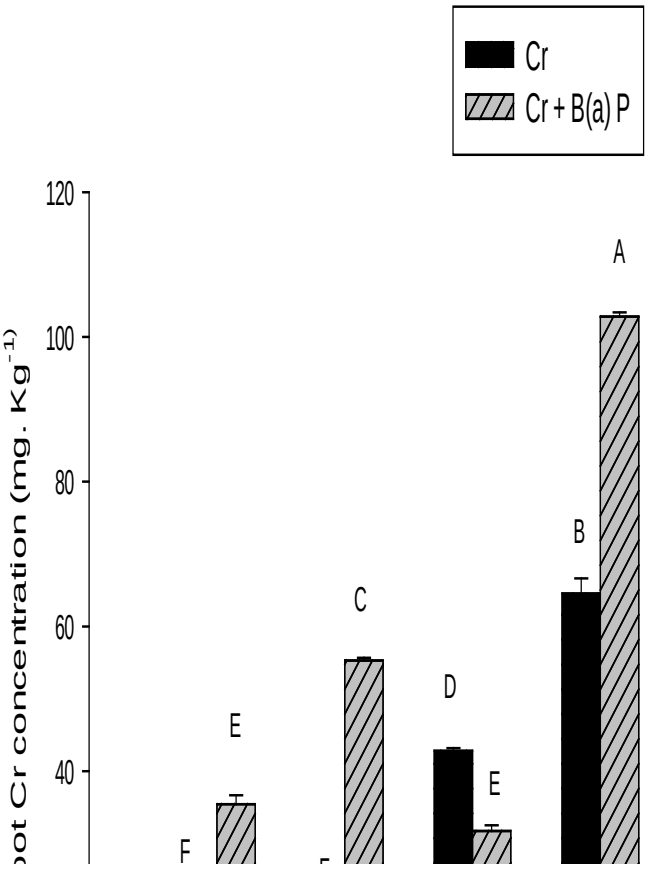


Figure 7.13: Effects of chemical amendments and combination on shoot Cr concentration of *M. sativa* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7B.3

The root Cr concentrations of *M. sativa* in soil contaminated with Cr or Cr + B[*a*]P were significantly ($p<0.05$) higher than in uncontaminated soils. As shown in Figure 7.14, EDTA did not affect the root Cr concentration of *M. sativa* when soil was contaminated with Cr alone but in Cr + B[*a*]P co-contaminated soils, the application of EDTA significantly reduced the root concentration of Cr from 42.07 to 20.37 mg kg^{-1}. When soil was contaminated with Cr alone, the application of citric acid or the combined application of EDTA and citric acid significantly ($p<0.05$) increased the root Cr concentration from 26.7 to 39 and 43.77 mg kg^{-1} respectively while in Cr + B[*a*]P co- contaminated soil, neither EDTA nor citric acid had any significant effect on root Cr concentration relative to control treatments.

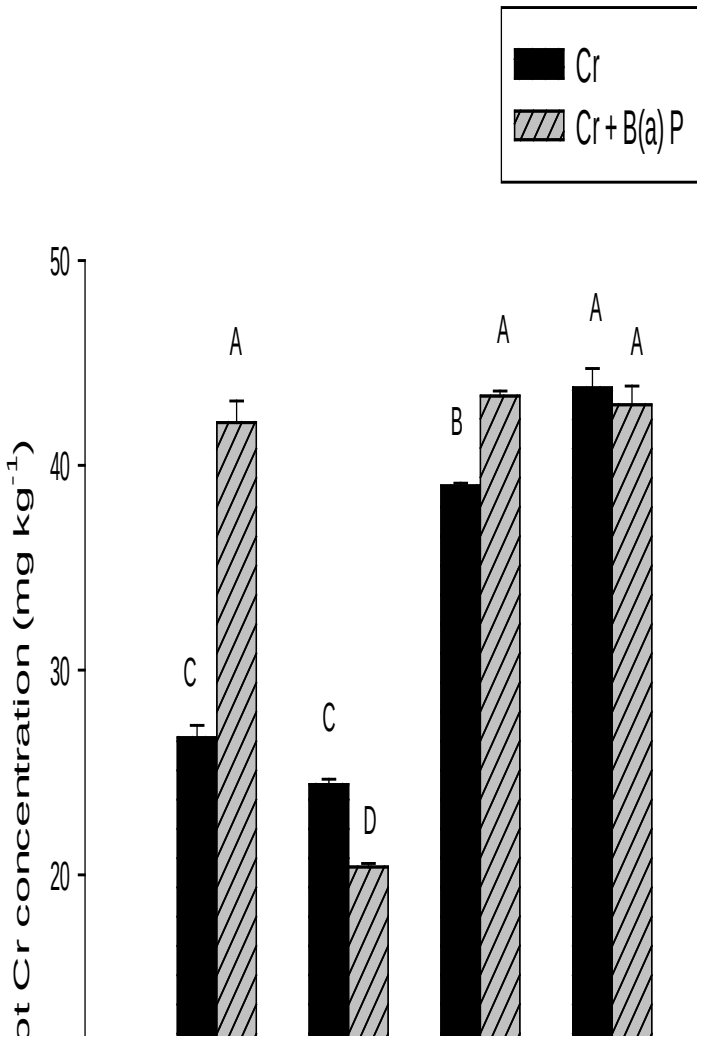


Figure 7.14: Effects of chemical amendments and combination on root Cr concentration of *M. sativa* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7B.5

7.8.4 Effect of EDTA and citric acid on shoot and root Cr accumulation

The average Cr concentration in shoot of *M. sativa* as affected by different chelates varied. The shoot Cr accumulation was significantly ($p<0.05$) increased with contamination of Cr as well as co- contamination with Cr and B[*a*]P (Figure 7.15)

In soils contaminated with only Cr, the application of citric acid or EDTA + citric acid significantly enhanced the shoot accumulation of Cr. Taking dry matter reduction and heavy metal absorption into consideration, the combined application of EDTA and citric acid was however less effective when compared with single application of citric acid. The present results showed that when only citric acid was added to soil contaminated with Cr, the shoot accumulation of Cr increased from 12.61 to 33.2 µg pot^{-1}. The combined application of EDTA and citric acid increased the shoot accumulation of Cr slightly but significantly from 12.61 to 19.45 µg pot^{-1}. EDTA was not effective in increasing the shoot accumulation of Cr and as shown in Figure 7.15

In Cr + B[*a*]P co-contaminated soils, all chelates applied to soil significantly affected the accumulation of Cr in shoot of *M. sativa*. The application of EDTA and EDTA + citric acid significantly increased the shoot accumulation of Cr while citric acid significantly reduced the shoot accumulation of Cr (Figure 7.15). The present result showed that EDTA increased the shoot accumulation of Cr from 19.01 to 41.53 µg pot^{-1} and when EDTA and citric acid were combined, the shoot accumulation of Cr increased by 3.09 fold. The control plants were more effective in the uptake of Cr by *M. sativa* than when citric acid was added to Cr + B[*a*]P co-

contaminated soils. Results showed a significant ($p<0.05$) reduction in shoot accumulation from 19.01 to 14.28 µg pot^{-1}.

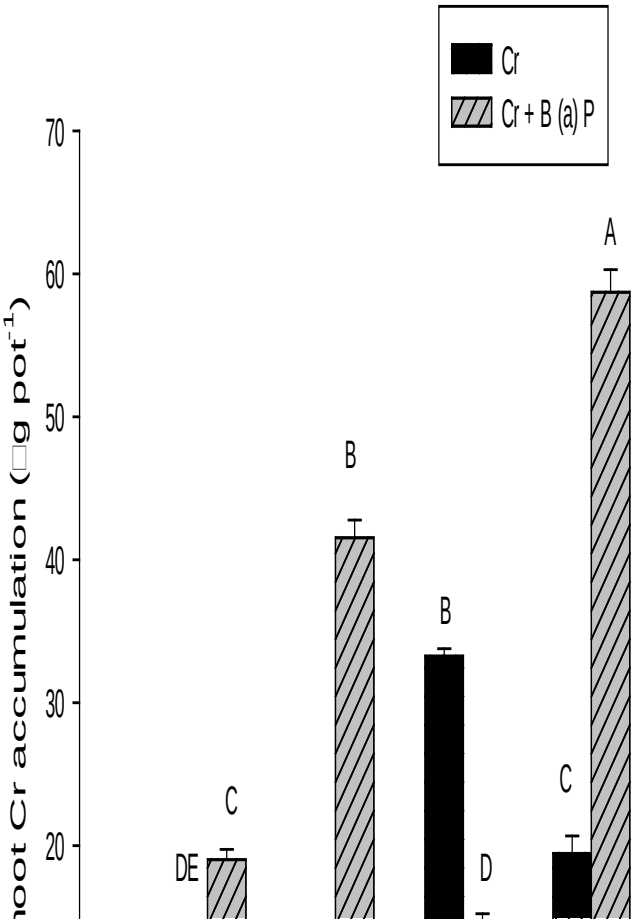


Figure 7.15: Effects of chemical amendments and combination on the accumulation of Cr in shoot of *M. sativa* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p\leq0.05$). Appendix 7B.4

In soils contaminated with Cr alone, the application of EDTA or citric acid did not affect the root Cr accumulation of *M. sativa*. The root accumulation remained at 10.75 and 7.82 µg pot^{-1} when EDTA and citric acid were applied respectively. However, the combined application of EDTA and citric acid significantly reduced the root accumulation of Cr from 10.45 to 5.28 µg pot^{-1}.

In soils co-contaminated with Cr and B[*a*]P, the application of EDTA and citric acid significantly ($p<0.05$) reduced the root accumulation of Cr. Results showed that EDTA significantly reduced the root accumulation of *M. sativa* from 12.87 to 6.81 µg pot^{-1}, while citric acid reduced the root accumulation to 6.82 µg pot^{-1}. The effectiveness of EDTA or citric acid in root accumulation of Cr was similar ($p\geq0.05$). The root accumulation of Cr when EDTA and citric acid were combined was statistically similar to control treatments and remained at 10.29 µg pot^{-1}.

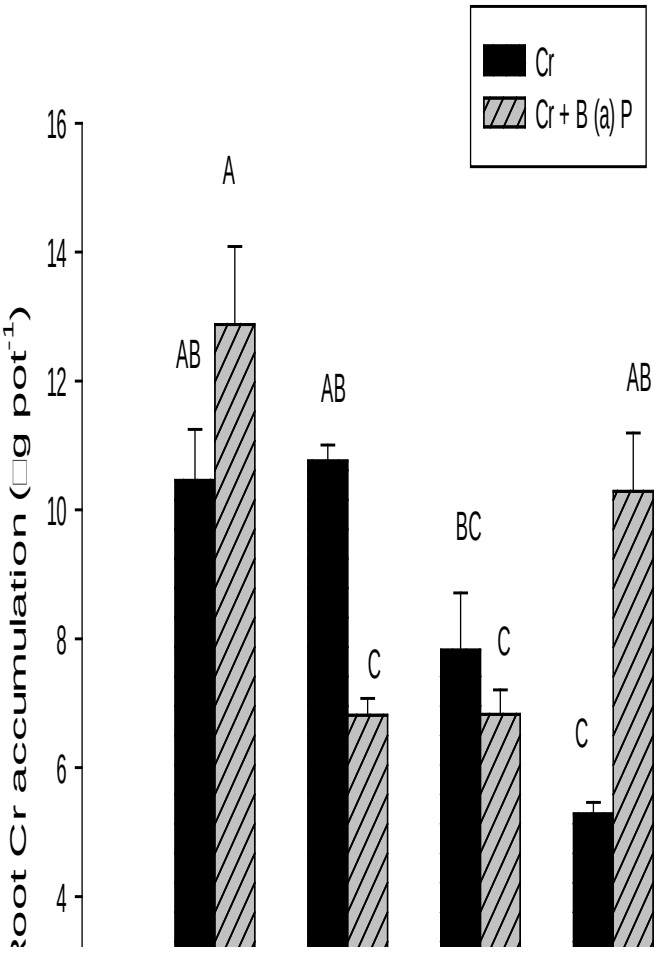


Figure 7.16: Effects of chemical amendments and combination on the accumulation of Cr in roots of *M. sativa* after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly based on Tukey HSD ($p \leq 0.05$). Appendix 7B.6

7.8.5 Effects of EDTA and citric acid on the translocation and bioconcentration factors of Cr

In this study, the presence of Cr in shoot of *M. sativa* in all treatments suggests that translocation from root to shoot has taken place. The TF of Cr showed no significant difference with the application of EDTA in single Cr contaminated soil while citric acid and citric acid + EDTA significantly increased the TF in single Cr contaminated soil Table 7.4). After the application of citric acid or the combined application of citric acid and EDTA to single Cr contaminated soil, the TF of Cr increased from 0.7 to 1.09 and 1.473 respectively.

In mixed contaminated soil, the single application of citric acid did not seem to affect the TF of Cr in *M. sativa* whereas EDTA and a combined application of EDTA and citric acid significantly increased the TF of Cr. Our results showed that EDTA and EDTA + citric acid significantly increased the TF of Cr from 0.84 to 2.71 and 2.39 respectively.

The Bioconcentration Factor (BF) is the ratio of the metal concentration in the plant to the metal concentration in the soil. According to Zhou and Song (2004), hyperaccumulators have BCF values that are greater than one. Single factor ANOVA showed that the single application of EDTA did not affect the BCF of Cr in *M. sativa* (Table 7.4). However, similar to TF, citric acid significantly increased the BCF from 0.67 to 1.20 in soil contaminated with Cr only. The combined application of EDTA and citric acid also enhanced the BCF in single and mixed contaminated soil in the present study. EDTA + citric acid significantly ($p<0.05$) increased the BCF from 0.67 to 1.59 for single Cr contaminated soil and 1.13 to 2.13 for Cr + B[*a*]P co-contaminated soil.

Table 7.4: Translocation facor (TF) and Bioconcentration factors of Cr (values are mean ± SE, n=3). Appendix 7B.7 and 7B.8

Treatments	50 mg kg^{-1} Cr		50 mg kg^{-1} Cr + 10 mg kg^{-1} B[*a*]P	
	TF	BCF	TF	BCF
EDTA	0.721±0.038	0.617±0.007	2.714±0.018	1.110±0.007
Citric acid	1.097±0.013	1.203±0.004	0.732±0.021	1.101±0.011
EDTA+ citric acid	1..474±0.018	1.593±0.044	2.395±0.041	2.137±0.021
No chelates	0.710±0.029	0.671±0.005	0.845±0..051	1.137±0.003

7.8.6 Effect of EDTA and citric acid on total removal of Cr by *M. sativa* from contaminated soils

The effectiveness of each chelate applied to single or mixed contaminated soil was evaluated by the ratio of removal of Cr by each chelate to the removal in soils with no application of chelates. Citric acid was the only efficient chelate that helped in the removal of Cr in single Cr contaminated soil. The present results showed that when citric acid was applied to single Cr contaminated soil, the removal of Cr increased significantly ($p<0.05$) from 23.07 to 41.07 µg . The ratio of removal reached 1.78 and when compared to EDTA or EDTA + citric acid application, showed an increase of 0.89 and 0.71 respectively (Table 7.5).

In co-contaminated soils, results showed that EDTA and the combined treatment of EDTA and citric acid were the only effective chelates for Cr removal from soil. However the combined application of EDTA and citric acid was more effective than single application of EDTA. There was a significant increase from 31.89 to 48.34 and 69.01 µg with the application of EDTA and EDTA + citric acid respectively. Similarly the ratio of removal reached 1.51 and 2.16 and was more than twice when compared to citric acid.

Table 7.5: Plant removal of Cr per treatment (values are mean ± SE, n=3).

Treatments	50 mg kg^{-1} Cr		50 mg kg^{-1} Cr + 10 mg kg^{-1} B[*a*]P	
	Total Cr removal (µg)	Cr removal Ratio	Total Cr removal (µg)	Cr removal ratioRatio
EDTA	20.56±0.54	0.89	48.34±1.04	1.51
Citric acid	41.07±0.70	1.78	21.11±0.63	0.66
EDTA+ citric acid	24.74±1.08	1.07	69.01±1.23	2.16
No chelates	23.07±1.07		31.89±0.85	

7.8.7 Effect of EDTA and citric acid on B[*a*]P concentration in soil

The concentration of B[*a*]P in soil decreased for all treatments including soils with no planting and no chelate application (Figure 7.18). The initial concentration of B[*a*]P in spiked soil for single and co-contaminated soils remained approximately 10 mg kg^{-1} (Appendix 7B.11). The percentage removal of B[*a*]P reached 52% for single B[*a*]P contaminated soil and 56% for Cr + B[*a*]P co-contaminated soils (Figure 7.18). The dissipation of B[*a*]P in single B[*a*]P contaminated soil was effective even without planting while in co-contaminated soils, it was related to the application of either EDTA or EDTA+citric acid. The present result showed that the application of EDTA or citric acid in single B[*a*]P contaminated soil significantly ($p \leq 0.05$) decreased the residual concentration of B[*a*]P in soil from 5.86 to 4.97 and 5.02 mg kg^{-1} when compared to planted soil without the application of chelates (Figure

7.17). Similarly, in Cr+B[*a*]P co-contaminated soils, the application of EDTA or EDTA+citric acid significantly decreased the B[*a*]P concentration in final soil from 5.09 to 4.64 and 4.49 mg kg^{-1} respectively.

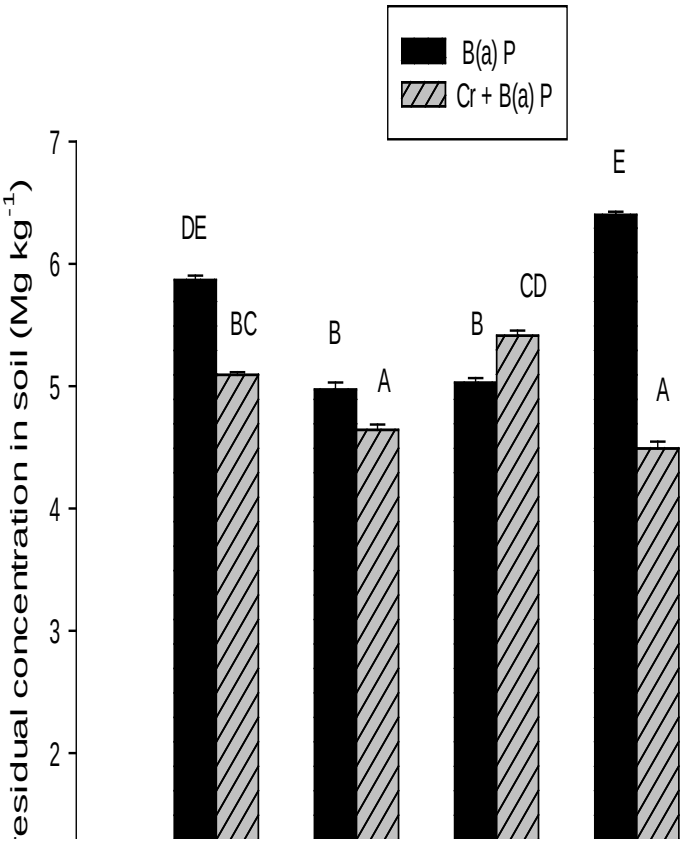


Figure 7.17: Effects of chemical amendments and pollutant combination on residual B[*a*]P concentration in soil after 60 days. Bars (means ± SE, n= 3) that do not share the same letter are significantly different based on Tukey HSD ($p \leq 0.05$). Appendix 7B.10

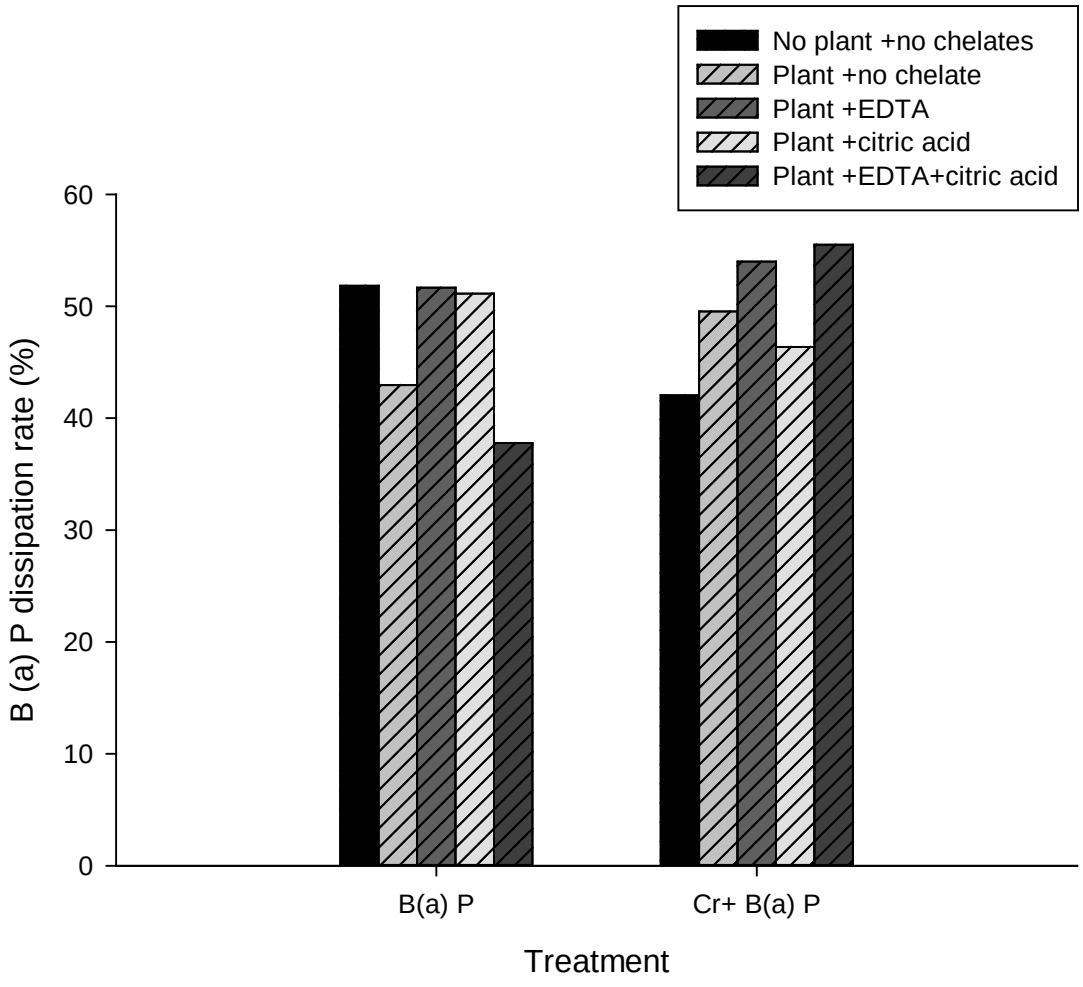


Figure 7.18: B[*a*]P dissipation rate (%) in soils after 60 days.

7.9 Discussion

7.9.1 Effect of EDTA and citric acid on plant biomass

In the present study, only the combined amendment of EDTA and citric acid significantly reduced the shoot biomass of *M. sativa* and correspondingly the root biomass in soil contaminated with Cr alone, while amendments in single or combination did not affect the shoot biomass of *M. sativa* in B[*a*]P or Cr + B[*a*]P contaminated soils (Figure 7.11). The reduction in biomass in single Cr contaminated soil when EDTA and citric acid was added could be related to the enhanced metal concentration in shoot tissues of *M. sativa* in Cr contaminated soils (Figure 7.13). The observed toxicity symptoms in plants are always associated with an increased chemical concentration in solution, an increased metal accumulation as well as enhanced metal translocation (Gunawardan *et al*. 2010). Similar results were observed by Gunawardan *et al*. (2010) where amendment combinations of rhamnolipid + EDDS and rhamnolipid + sulfate significantly reduced the shoot biomass of *L. perenne* as a result of increased metal concentration. Correspondingly, poor root growth would be expected to reduce water and ion uptake (Lepp, 2005) resulting in deficiencies of essential nutrients (Boussama *et al*. 1999). Once chelates are introduced to soils, they tend to form complexes to a varying degree with metals present in solution and when the cations simultaneously compete for amendments, the free metal and amendments concentration in the solution may be affected, hence creating an imbalance of chemical components in the solution. This leads to various toxicities since the free metal ion and the unbound amendments are known to be more toxic to plants (Vassil *et al*. 1998).

7.9.2 Effect of EDTA and citric acid on concentration and accumulation of Cr

The effect of EDTA on the shoot Cr concentration in single Cr contaminated soils was negligible when compared to control treatments (Figure 7.13). This could be as a result of the minor effect the concentration of EDTA used in the present study has on Cr carrier. Targut *et al*. (2004) found that chelates may affect the selectivity of metals by forming a chelate-metal complex that alters the uptake rate of the metal. It could also be that since 0.146 g kg^{-1} EDTA amendment exhibited similar shoot and root concentration with control, this dosage may not have been sufficient for overcoming the limited transfer of Cr through the root wall. Lombi *et al.* (2001) and Madrid *et al.* (2003) reported that EDTA was very effective at mobilizing metals through the soil but they observed that the ability to improve root to shoot translocation was metal specific. This was similar to our results as EDTA effectively enhanced the water extractable Cr concentration in soil without improving shoot accumulation (Table 7.3).

Under co-contamination with Cr and B[*a*]P, there was a prominent increase in Cr concentration and accumulation with the application of EDTA. It could be that under co-contamination, since EDTA enhanced solubility of Cr (Table 7.3), it could have also enhanced the absorption of the Cr- EDTA complex by *M. sativa*. The size and molecular weight of a contaminant may play a role in the ability of a plant to take up a contaminant. Anderson *et al*. (1993) observed that plant roots usually favor the uptake of small and low molecular weight compounds, whereas larger and higher molecular weight compounds seem to be excluded from the roots. Results in the present study indicate that due to the application of EDTA in Cr + B[*a*]P co-contaminated soils, the root Cr concentration of *M. sativa*

decreased remarkably when compared to control treatments (Figure 7.14). This could be because Cr easily reacts with EDTA before uptake and forms a Cr-EDTA complex which is more stable and has a higher molecular weight than Cr only. Therefore the amount available to the plant root will highly depend on the competition between the Cr-EDTA complex, as well as the cation exchange sites of the root cell wall (Yu and Gu 2008). Also the competition for Cr or B[*a*]P uptake by *M. sativa* could also affect the amount of Cr available to the plant root.

The application of citric acid to soil contaminated with Cr alone significantly increased the shoot and root concentration of Cr in *M. sativa.* Citric acid however was more efficient compared to amendment with EDTA alone (Figures 7.13 and 7.14). According to Marschner *et al*. (1986) and Samet *et al*. (2001), the predominant theory for metal-chelate uptake is the split uptake or the free metal ion mechanism where only free metals are absorbed by plant roots. Complexing agents are divided into weak, moderate and high, depending on the metal complex formation and according to Schowanek *et al.* (1997), EDTA is rated as a high complexing agent. It is therefore expected that the metal complex formed with EDTA will yield less free metal ion than the metal–citrate complex. The resulting effect is a lower enhancement in metal uptake by EDTA when compared to citric acid. Although recent research has shown EDTA to be more effective in enhancing metal uptake than citric acid (Turgut *et al*. 2004, Sinhal *et al*. 2010), research by Jean *et al*. (2008) showed that although 2 mmol kg^{-1} of EDTA and 5 mmol kg^{-1} of citric acid enhanced the concentration of Cr in shoot of *Datura innoxia,* citric acid alone seemed to be more efficient. The effectiveness of citric acid compared to EDTA in soil contaminated with Cr in this study could also be as a result of

differences in concentration of chelates applied. Although citric acid was effective in enhancing shoot Cr concentration and accumulation in Cr contaminated soil, under co-contamination it was ineffective in increasing shoot Cr concentration and significantly reduced the shoot accumulation relative to control treatments. This was surprising since the mobile fraction of Cr increased from 1.73 to 3.53 mg kg^{-1} (Table 7.3). In contrast, the root Cr concentration was not reduced. This reduction in shoot Cr uptake could be attributed to the competition with the plants' own metal-binding agents in the presence of B[*a*]P which causes the Cr to diffuse downwards to the root system of the plant. Similar results were observed by Robinson *et al*. (1999), where the addition of nitrilotriacetic acid (NTA) significantly reduced the plant nickel uptake with no reduction in biomass yield.

The enhancement of Cr concentration and accumulation with EDTA + citric acid were significant. The shoot Cr concentration was highest when compared to any treatment as well as the shoot accumulation except in single Cr contaminated soil. The remarkable increase in shoot concentration with EDTA + citric acid in soil contaminated with Cr alone and Cr + B[*a*]P could be attributed to the synergistic actions of EDTA and citric acid on the uptake pathways and in the present study, the combined treatments negatively affected plant biomass only in soil contaminated with Cr only. This effectively reduced the shoot accumulation in soil contaminated with Cr alone (Figure 7.15). The relatively small increase in Cr accumulation in the shoot of *M. sativa* when soil was amended with a combination of EDTA and citric acid in single Cr contaminated soil may suggest a low phytotoxic threshold of *M. sativa* for Cr under mixed amendment in soil contaminated with Cr alone. This could be related to the observed toxic effect EDTA + citric acid had on shoot biomass of *M. sativa*

(Figure 7.11) possibly because the roots' physiological barriers could be damaged by the toxicity of high concentrations of Cr in soil solution and as suggested by Schaider *et al*. (2006), when there is a break in the root endodermis, there could be enhanced uptake of metals to roots and subsequent transfer to shoots through bypass flow.

7.9.3 Phytoremediation potential

The process of phytoextraction with chelates is based on the fact that chelate application to soil will significantly enhance metal accumulation as well as the translocation of heavy metals from soil to shoots (Grabisu and Alkorta 2001, Ruley *et al*. 2006). Soil to plant translocation ratio is also very important during phytoextraction. For an effective phytoextraction of metals with amendments, the amendment must in addition to mobilization of metals into soil solution, enhance uptake of metals by plant roots and translocation to shoots (Tsetimi and Okieimen 2011). In the present study, the bioaccumulation and translocation factors shown in Table 7.4were used to evaluate the effectiveness of *M. sativa* in metal accumulation and translocation. EDTA has been proven to be very efficient in enhancing the uptake of metals in metal contaminated soil including Cr. For example, Jean *et al*. (2008) showed that EDTA was efficient in the uptake of Cr by *D. innoxia*. However, in the present study under single Cr contaminated soil, the uptake and accumulation of Cr was not enhanced with the addition of EDTA. The translocation of Cr from root to shoot of *M. sativa* as well as the BCF was also not enhanced with the application of EDTA. However in Cr + B[*a*]P co-contaminated soil, although EDTA increased the accumulation and TF of Cr, the BCF was not significantly enhanced. The high Cr contents in the root and the poor translocation to the shoots could be because Cr was retained in the cation exchange sites or it was immobilized in the vacuoles of

the root cells. This could have made Cr less toxic to plants as observed in plant biomass yield. It is important to note that the application of EDTA resulted in a significant increase in soluble Cr concentration in single Cr contaminated soil (Table 7.3). However, it did not significantly increase Cr removal by plants in single Cr contaminated soil. This could render a larger fraction of soil Cr vulnerable to loss processes which could potentially harm the environment.

The effect of citric acid on the TF (Table 7.4) showed that more Cr translocated to the shoot of *M. sativa* in Cr contaminated soils. Also the BCF increased with the application of citric acid in single Cr contaminated soils whereas in Cr + B[*a*]P co-contaminated soils, the application of citric acid did not have any significant effect on TF as well as the BCF. The combined application of EDTA and citric acid seemed to be effective in enhancing TF as well as BCF in both single Cr and co-contaminated soils. The higher TF and BCF values when soil was amended with citric acid in single Cr contaminated soil or EDTA+ citric acid in single and co-contaminated soils indicate that with the amendments, *M. sativa* could move and distribute more Cr. It may be related to the fact that the application of chelates enhances shoot accumulation by reducing Cr affinity for the binding site in the cell walls of *M. sativa*. This is in contrast to the work carried out by Qu *et al*. (2011) which showed lower TF values of heavy metals including Cr in *M. sativa* when soil was amended with a combined application of sodium hydrogen phosphate and citric acid.

7.9.4 Residual B[*a*]P in soil

The result of this study show that the dissipation of PAH compound such as B[*a*]P in co-contaminated soils can be increased in the presence of chelates. Citric acid and EDTA are strong chelating agents of polyvalent ion such as Cr^{6+}. They are capable of extracting some of the Cr in the soil into the aqueous phase and when this happens it can affect the concentration of B[*a*]P in various ways. For example, any macromolecule that is bound to the soil may be released with any sorbed B[*a*]P into the aqueous phase. Also the removal of Cr with the help of the present chelates can cause the soil organic matter to become less constrained thereby increasing the diffusion rate of B[*a*]P (Yang *et al*. 2001). This supports the present study which showed that the application of EDTA or EDTA + citric acid had the highest dissipation rate of B[*a*]P in co-contaminated soils reaching 54 and 56% respectively. This decrease in the residual B[*a*]P concentration after amendment with EDTA or EDTA + citric acid (Figure 7.17) could also be as a result of the desorption of B[*a*]P which could enhance the bioavailable B[*a*]P in contaminated soil. When the bioavailable B[*a*]P is enhanced, it becomes easier for microorganisms to degrade them. In most cases the limited bioavailability of PAH can prevent the full exploitation of the degradation potential of microbes in soil. This is because when PAH is not available, it means that they are either less soluble in water or they bind strongly to the soil matrix (Harms and Bosma 1997). Therefore since the bioavailable fraction of the B[*a*]P is only available to the microorganisms, the rate of degradation will depend on the mass transfer of B[*a*]P from the soil to the aqueous phase and in the present result, it is likely that EDTA + citric acid could have had more ability to desorb B[*a*]P from co-contaminated soil. This is similar to the work carried out by Bach *et al*. (2005) where

TWEEN 80 which is a biostimulant increased the bioavailable PAH in sediments for degradation by microorganisms. The amendment of co-contaminated soil with only EDTA enhanced the disspation of B[*a*]P with the help of *M. sativa*, which shows the importance of inorganic chelates as limiting factors of B[*a*]P dissipation in co-contaminated soils, whereas the combined application of inorganic chelates (EDTA) and organic chelates (citric acid) increased the rate of B[*a*]P dissipation more than the individual application of each chelate (Figure 7.17). This is similar to the work of Bach *et al.* (2005) showed that the combined application of inorganic and organic nutrients enhanced PAH degradation more than single application and could suggest that amending Cr + B[*a*]P co-contaminated soil with a combined application of inorganic chelate (EDTA) and organic chelate (citric acid) could increase the dissipation of B[*a*]P.

7.10 Conclusion

The result of this study show that aqueous solution of EDTA and citric acid can be applied in single Cr or B[*a*]P and co- contaminated soils in the presence of *M. sativa*, to efficiently remove Cr or B[*a*]P in single contaminated soil or simultaneously remove Cr and B[*a*]P in co-contaminated soils.

Organic acids such as citric acid have been proposed as responsible chelates for enhancing the translocation of metals from plant root to shoots. Chelates have limited binding capacity and their molecules can only carry a restricted amount of ions depending on the number of binding sites. For example, since citric acid enhanced Cr solubility in single Cr contaminated soil and increased the amount of Cr that was absorbed by roots and translocated to shoots in

the present study, it could be preferred to the application of EDTA or EDTA+ citric acid for single Cr contaminated soil. Since citric acid is biodegradable, increased leaching during plant trial will cause less environmental risk than EDTA amended soils.

In co-contaminated soils, the present study show that single application of EDTA or EDTA + citric acid could be regarded as an efficient chelate candidate for the simultaneous phytoextraction of Cr and dissipation of B[*a*]P by *M. sativa*. The increase in TF as well as the metal extraction ratio following the application of EDTA or EDTA + citric acid provides a basis for further detailed study in the ability of *M. sativa* to simultaneously accumulate Cr and remove B[*a*]P in the presence of EDTA or EDTA + citric acid.

General discussion, conclusions and approaches for further studies

8.1 Summary

Phytoremediation is an emerging technology that uses various plants to treat contaminated soils in different ways. It is widely accepted for treatment of metals or PAH contaminated soils. However, there are still challenges for the use of plants in remediation of co-contaminated soils (Lin *et al*. 2008). Commonly, the interaction of metals and PAH in co-contaminated soils could affect the bioavailability of metals and/or PAH and thereby limiting the potential of remediation with plants. The growth of pants can also be affected by the interactions of metals or PAH in co-contaminated soils. These effects can be antagonistic or synergistic (Lin *et al*. 2006). Successful applications of phytoremediation have been recorded for individual contaminants or mixes of those within the same group. There is little information on the ability of plants to remediate both organic and inorganic compounds within contaminated soils

The overall aim of this thesis was to determine whether phytoremediation could be applied to co-contaminated soils. Cu, Cr, pyrene and B[a]P were used as model contaminants. The overall aim was achieved by understanding the effects of contaminants on germination of seeds, early and latter seedling growth, the role of amendments, and the effect of soil ageing in four distinct but complementary chapters.

- Chapter 4 assessed the effect of Cu and pyrene, and Cr and B[*a*]P on the early seedling growth of *L. perenne* using growth media.
- Chapter 5 assessed the role of *B. juncea* or *Z. mays* as model plants for the remediation of soils co-contaminated with Cu and pyrene, or Cr and B[*a*]P respectively.

- Chapter 6 compared freshly spiked soils and aged soils of Cu and pyrene, or Cr and B[*a*]P contaminated soils using *B. juncea* and *Z. mays* respectively as model plants.
- Chapter 7 assessed the role of amendments (EDTA and citric acid) during the phytoremediation of soils contaminated with Cu and pyrene, or Cr and B[*a*]P using *M. sativa* and *Z. mays* as model plants respectively.

8.2 Seed germination and seedling growth in growth media

Growth medium spiked with Cu, pyrene, Cr and B[*a*]P were chosen as model contaminants and seeds of *L. perenne* were used to evaluate the response of seeds during early growth stage to co-contamination. Single Cu or Cr contamination significantly inhibited the germination rate as well as the early seedling growth of *L. perenne* (Figures 4.1, 4.4, 4.5, 4.17, 4.20 and 4.21). Single contamination of pyrene or B[*a*]P did not show any negative effect on seed germination, however pyrene showed significant inhibition on early seedling growth whereas B[*a*]P enhanced seedling growth.

Co-contamination of Cu and pyrene or Cr and B[*a*]P significantly inhibited the germination rate as well as the seedling growth when compared to single contamination of Cr, Cu, pyrene or B[*a*]P. In Cu and pyrene co-contaminated growth medium, over 48% of seeds failed to germinate and over 58% and 68% of shoot and root length of *L. perenne* respectively were inhibited relative to single treatments. Similarly, over 35% of seeds failed to germinate in Cr and B[*a*]P co-contaminated growth medium and over 37% and 56% of shoot and root length respectively were inhibited. There were also significant relationships when Cu and pyrene, or Cr and B[*a*]P were co-contaminated in solution. Joint contamination of Cu and pyrene had a

significant synergistic effect on the shoot and root elongation of *L. perenne* and a significant antagonistic effect on final germination rate. In contrast, the joint contamination of Cr and B[*a*]P had a significant antagonistic effect on shoot and root elongation as well as the final germination rate of *L. perenne*. This suggests that different contaminants affect the early seedling stage of plants in different ways. In both studies, the root elongation was the most sensitive to the toxicity of the co-contaminants.

8.3 Effect of co-contaminated soils

In plant glasshouse trial study, the growth of *B. juncea* was inhibited by fresh Cu-pyrene co-contamination. There was no visual evidence of toxicity of Cu to plants in soils contaminated with Cu alone. However, a noticeable visible toxicity effect of pyrene or Cu-pyrene contamination on *B. juncea* was observed (Figure 5.1A and B). Although the shoot Cu concentration and TF under co-contamination of Cu and pyrene were enhanced (Figure 5.3A and B, Table 5.2), but due to the reduced growth of *B. juncea* as seen in Figures 5.1A and 5.1B, the amount of Cu removed under co-contamination was severely inhibited. In contrast, the single or co-contamination of Cr and B[*a*]P did not affect the plant growth of *Z. mays.* However, the removal of Cr by *Z. mays* in soils co-contaminated with Cr and B[*a*]P increased by over 79% (Figures 5.11A and B) and the rate of translocation of Cr from root to shoot of *Z. mays* also increased with co-contamination (Table 5.4).

The dissipation of pyrene in single or co-contaminated fresh soils was significantly decreased with or without planting, but planting of *B. juncea* accounted for higher dissipation of pyrene reaching 90 to 94% of zero-time pyrene concentration. The presence of Cu in soil decreased

the dissipation of pyrene in planted soil to an extent that at 100 mg kg^{-1} Cu, there was no evidence of plant enhanced pyrene dissipation (Figure 5.8A and B). On the other hand, planting of *Z. mays* did not enhance the dissipation of lower (1 and 5 mg kg^{-1}) B[*a*]P co-contaminated soils but only in soils co-contaminated with 10 mg kg^{-1} B[*a*]P or more.

8.4 Effect of soil ageing

Soil ageing affected the plant biomass, metal accumulation and PAH dissipation in different ways. *B. juncea* and *Z. mays* grew better in aged Cu-pyrene and Cr-B[*a*]P co-contaminated soils respectively (Tables 6.1 and 6.4). The amount of Cr removed by *Z. mays* was higher (>29%) in aged soil than in freshly spiked Cr-B[*a*]P co-contaminated soils (Table 6.5) while the Cu accumulated by *B. juncea* was significantly reduced in aged soil when compared to freshly spiked soils for all treatments except for lower Cu-pyrene co-contaminated soils (Table 6.2).

It was clear from the results (Tables 6.3 and 6.6), that ageing influenced the dissipation rates of pyrene and B[*a*]P in co-contaminated soils. However, as shown in figures 6.4A and B, and 6.5A and B, planting *B. juncea* in aged Cu-pyrene co-contaminated soils did not affect the dissipation rate of pyrene whereas planting *Z. mays* in aged Cr-B[*a*]P co-contaminated soils helped in the dissipation (>31%) of B[*a*]P than in freshly spiked soils (Figures 6.9A, B and C).

8.5 Effect of amendments

Organic and inorganic chelates were studied to understand the efficiency of their application during phytoremediation of co-contaminated soils.

In Cu-pyrene co-contaminated soils, the application of citric acid or EDTA negatively affected the growth of *Z. mays* while the combined application of EDTA and citric acid improved the growth of *Z. mays* by over 41% (Figure 7.1). In contrast, EDTA and/or citric acid did not seem to affect the growth of *M. sativa* in Cr-B[*a*]P co-contaminated soils. This suggests that the phytoremediation of co-contaminated soils with the help of ammendments depended on the contaminant mix and the type of plant usedOf all the chelates used, the combined application of EDTA and citric acid seemed to be the most effective chelate application for both Cu-pyrene and Cr-B[*a*]P co-contaminated soils. In Cr-B[*a*]P or Cu-pyrene co-contaminated soils, the application of EDTA+ citric acid in the presence of *M. sativa* and *Z. mays* respectively increased the shoot Cr and Cu concentration by over 100% (Figures 7.13 and 7.3) and also enhanced the dissipation of B[*a*]P or pyrene by over 11% and 31% respectively (Figures 7.17 and 7.9). Over 69 µg of Cr and about 98.9 µg of Cu were removed by *M. sativa* and *Z. mays* respectively with the help of combined application of EDTA and citric acid over the planting period. This represented about 3 times the amount of Cr removed with citric acid application or about twice the amount of Cr removed with EDTA application (Table 7.5) and over twice the amount of Cu removed with the help of EDTA or citric acid (Table 7.2).

There was an important observation with single Cr or Cu contaminated soil. For example in soils contaminated with Cr alone, citric acid helped in the phytoextraction of more Cr than EDTA or EDTA+ citric acid. This is in contrast with Cr-B[*a*]P co-contaminated soil, where plants grown with the application of citric acid did not show any evidence of phytoextraction. Similarly, in soils contaminated with Cu alone, the application of EDTA enhanced the phytoextraction of Cu whereas in Cu-pyrene co-contaminated soils, the application of EDTA inhibited the phytoextraction of Cu (Table 7.2).

8.6 Soil PAH partitioning

The chemical activity of the PAH which includes the concentration of PAH in the aqueous phase (pore water) of the soil is one of the indications of PAH bioavailability (Reichenberg and Mayer 2006). In this study, the two PAHs used (pyrene and B[*a*]P) have different characteristics and different aqueous solubility (Wick *et al* 2011), hence their bioavailability and degradation potential will vary. The pyrene concentration in pore water fraction of soil was always higher than that of B[*a*]P. At high concentration of pyrene used in this study (500 mg kg^{-1}), the soil gets more saturated than at lower concentrations and closer to the solubility of pyrene in water (Appendix 9A). There would be some potential for pyrene to come out as solid phase and limit bioavailability and degradation. When this happens, pyrene may be less available for microbial degradation. In contrast, the B[*a*]P concentrations used n this study seemed not be saturated. Even at 10 mg kg^{-1}, the calculated concentration of B[*a*]P in pore water was below the solubility of B[*a*]P in water. This could be as a result of the low concentration of B[*a*]P used in this study. It is however known that higher ring PAHs including B[*a*]P are usually adsorbed into the soil phase (Jiries *et al*. 2000) while lower

molecular weight PAHs like pyrene are removed faster from soils because of their higher solubility. Therefore, it is important when looking at research, to consider the organic matter and clay percentages of the soil used in the experiment as well as pre treatment (ie sieving size) prior to the treatment additions. Sorptions to organic matter and clays may occur as the soil age and the microbial communities will undoubtedly change as the soil shifts from anaerobic to aerobic (Wick *et al*. 2011) thereby affecting the dissipation of PAHs.

8.7 Phytoremediation potential

Although plants used in this study showed evidence of phytoremediation of co-contaminated soils, there are many different economic drivers that can determine whether it can be applied to the field. The technologies that are often preferred are those that are also cost effective and take less time. Conventional clean up activities for contaminated soils may be the cause of external effects such as green house gas emission from heavy duty machinery powered by diesel fuel (Suer and Anderson-skold 2011). With this in mind, the recent superfund green remediation strategy of USEPA stipulates that green remediation factors will be considered in evaluation of the economic efficiency of remediation projects (EPA 2010), and phytoremediation have been suggested as a cost effective approach. However, it is clear from our study why implementation of phytoremediation remains a problem. Tables 8.1 to 8.3 shows the time it will take the plants used in this study to completely remove the contaminants in soil. The practical implementation of phytoremediation has been constrained by the expectation that site remediation should be achieved in time that is comparable to other clean up technologies. Our study seem to suggest that the phytoremediation of co-

contaminated soils at the concentration used for this study will take so many years for complete removal of metals but with less than a year for PAH treatment for fresh contaminated and aged soil- it is clear that natural processes also helped in the dissipation of PAH in soils. It is shown in Tables 8.1 and 8.2 that *B. juncea* will likely help in the dissipation of all pyrene in freshly and aged soil in 96 days and 119 days respectively. However, it will require over 524 years (fresh soil) and 1308 years (aged soil) for it to completely remove all the Cu at the rate applied in the present study. Similarly, *Z. mays* will take about 949 years for complete removal of Cr in co-contaminated soils (Table 8.2). The application of EDTA and citric acid to co-contaminated soils enhanced the removal of metals and PAH used in this study (Table 8.3). This is evident as the number of years it will take *Z. mays* or *M. sativa* to completely remove contaminants decreased. But there is a problem with the management of the side effects related to the addition of chelates which includes metal leaching. The cost of additional liners for containing the pollutants will increase the cost of phytoremediation. It is clear that the effect of combined chelates remained a factor in the reduction of Cu or Cr and as it will take *Z. mays* or *M. sativa* about 187 and 140 years respectively to clean up soil with the application of chelates, or longer without chelates application, this time would have been less of a constraint if phytoremediation could be combined with a profit making operation. However, the Cu or Cr biomass of plants would have to be deposited and cannot be used for energy production. Metals have a proven effect on the enzymes responsible for the breakdown of biomass particles and whether they stimulate or inhibit biogas production depends on the total metal concentration, the chemical form and the process related effect (Chen *et al*. 2008). Cr and Cu however have shown

tendencies to reduce biogas yield during digestion (Wong and Cheung 1995). This will greatly increase the cost of phytoremediation. In relation to this study, it is not possible to draw a conclusion on the economic justification of phytoremediation, say as compared with other conventional clean up studies because there will be so many variables which were not analyzed in this study (like cost, energy production and CO_2 abatement), however, the length of time it takes for remediation deserves attention. The factors of economics cannot be ruled out from selection of any remedial action. There may be cost savings up front in the implementation of phytoremediation compared to other conventional alternatives that are engineering intensive. A lower initial cost may however be overshadowed by the cost associated with the long term needs of phytoremediation- in this case, the requirement of over 400 years (without application of chelates) or over 100 years (with chelates) of monitoring and maintenance. As such, the total cost of phytoremediation may exceed the cost of alternative technologies. Although this study showed that phytoremediation can be applied to co-contaminated sites, it is clear that new technologies find it difficult to enter the market, and based on the long time it will take for complete treatment of co-contaminated soils with plants in this study, it will be difficult to see it changing.

Table 8.1: Biomass yield (g kg^{-1}) and average exraction of metal and PAH by *B. juncea* and *Z. mays* in fresh co-contaminated soils

Plants	Soil Cu conc.(mg kg^{-1})	Soil pyrene conc. (mg kg^{-1})	Soil Cr conc. (mg kg^{-1})	Soil B[*a*]P conc. (mg kg^{-1})	Biomass(g)	Cu removal(µg kg^{-1})	Cr removal (µg kg^{-1})	Metal Clean up time (y)[c]	Pyrene removal (%)	B[*a*]P removal (%)	PAH Clean up time (d)[c]
B. juncea	50	250			0.33	18.3		487	88.66		73
	50	500			0.25	21.91		406	71.7		91
	100	250			0.24	33.96		524	93.20		70
	100	500			0.26	38.75		460	67.06		97
Z. mays			50	1	2.57		7.52	1093		46.70	128
			50	5	2.23		4.4	1868		22.87	262
			50	10	2.23		5.23	1572		37.63	159
			100	1	2.27		9.38	1752		31.09	193
			100	5	2.47		6.54	2514		15.84	379
			100	10	1.23		4.37	3762		62.87	95

[C] Calculation based on 1 kg of soil and assuming linear extrapolation

Table 8.2: Biomass yield (g kg^{-1}) and average exraction of metal and PAH by *B. juncea* and *Z. mays* in aged soils

Plants	Cu conc. (mg kg^{-1})	pyrene conc. (mg kg^{-1})	Cr conc. (mg kg^{-1})	B[*a*]P conc. (mg kg^{-1})	Biomass(g)	Cu removal (µg kg^{-1})	Cr removal (µg kg^{-1})	Metal Clean up time (y) [c]	Pyrene removal (%)	B[*a*]P removal (%)	PAH Clean up time (d) [c]
B. juncea	50	250			3.1	5.49		1622	83.91		77
	50	500			2.21	3.16		2818	57.02		114
	100	250			1.45	13.61		1308	54.45		119
	100	500			0.52	12.6		1413	87.89		74
Z. mays			50	1	2.53		10.57	778		32.86	183
			50	5	2.77		16.56	496		18.48	325
			50	10	2.6		18.72	439		60.08	100
			100	1	3.1		23.58	697		44.06	136
			100	5	2.13		17.33	949		35.83	167
			100	10	2.8		28.45	578		44.93	134

[C] Calculation based on 1 kg of soil and assuming linear extrapolation

Table 8.3: Biomass yield (g kg^{-1}) and average exraction of metal and PAH by *Z. mays and M. sativa* with the application of combined chelates in co-contaminated soils

Fresh soil	**Cu conc. (mg kg^{-1})**	**pyrene conc. (mg kg^{-1})**	**Cr conc .(mg kg^{-1})**	**B[*a*]P conc. (mg kg^{-1})**	**Biomass(g)**	**Cu removal (µg kg^{-1})**	**Cr removal (µg kg^{-1})**	**Metal Clean up time (y) [c]**	**Pyrene removal (%)**	**B[*a*]P removal (%)**	**PAH Clean up time (d) [c]**
Z. mays	50	100			0.9	44.05		187	86		70
M. sativa			50	10	0.338		58.718	140		55	109

[C] Calculation based on 1 kg of soil and assuming linear extrapolation

8.6 Conclusions

The investigation on the phytoremediation potential of co-contaminated soils with different plant species, soils with contrasting properties, range of metal and PAH concentrations and presence of organic and inorganic chelates in this study represented a rigorous assessment of this area of research. The degree to which these tests could explain the ability of remediating co-contaminated soils with plants appeared to be metal-PAH specific with the metal-PAH interactions influencing the way different plants simultaneously removes metals and dissipates PAHs from soils.

From the study, it was clear that phytoremediation could be applied to co-contaminated soils, but length of time it takes for soil treatment could prohibit the commercialization of this technology.

8.7 Approaches to further studies

The findings of the research presented in this thesis suggest further studies in the following areas

- The root and shoot uptake of pyrene or B[a]P during remediation should be studied. Although it is not a direct mechanism of PAH removal, however, would it have influenced remediation?
- Does root morphology impact upon metal uptake and PAH dissipation? Different plant species have different root systems, would this alter metal uptake and PAH dissipation in co-contaminated soils?

- Does soil microbial activity affect metal and PAH availabilities and hence there removal from soil? If so, are there specific communities associated with plant species that can be identified?
- Does plant diversity affect metal uptake as well as PAH dissipation? Will they compete for growth within co-contaminated soils, or will they complement each other?
- lower concentrations of pyrene or B[*a*]P at the level of their respective aqueous solubilities during the germination of and early seedling growth of plants should be studied.

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Appendix

Appendix 4A: Results for Cu and pyrene germination experiment

4A. 1Final germination percentage

4A. 2 Germination rate index

4A. 3 Shoot length

4A. 4 Root length

Appendix 4B Results for Cr and B[*a*]P germination experiment (Chapter 4)

4B.1 Final germination percentage

4B.2 Germination rate index

4B.3 Shoot length

4B.4 Root length

Appendix 5A: Results for Cu and pyrene co-contaminated soil experiment (Chapter 5)

5A.1 Shoot dry weight

5A.2 Root dry weight

5A.3 Shoot Cu concentration

5A.4 Shoot Cu accumulation

5A. 5 Root Cu concentration

5A. 6 Residual pyrene concentration in final soil (with planting)

5A. 7 Residual pyrene concentration in final soil (no planting)

5A. 8 Zero time pyrene concentration in soil

Appendix 5B: Results for Cr and co-contaminated soil experiment (Chapter 5)

5B.1 Shoot biomass

5B.2 Root biomass

5B.3 Shoot Cr concentration

5B.4 Shoot Cr accumulation

5B.5 Root Cr concentration

5B.6 Root Cr accumulation

5B. 7 Shoot concentration factor

5B. 8 Root concentration factor

5B. 9 Residual B[a]P concentration in final soil (after planting)

5B. 10 Residual B[a]P concentration in final soil (no planting)

Appendix 6A: Results for ageing experiment (Chapter 6)

6A.1 Shoot biomass

6A.2 Root biomass

6A.3 Shoot Cu concentration

6A.4 Shoot Cu accumulation

6A.5 Root Cu concentration

6A.6 Root Cu accumulation

6A.7 Total Cu

6A.8 Translocation factor

6A.9 Final soil residual pyrene (with plant)

6A.10 Zero time pyrene concentration

6A.11 Final soil residual pyrene (no planting)

6A.12 Final soil percentage dissipation of pyrene (with planting)

6A.13 Final soil percentage dissipation of pyrene (without planting)

Appendix 6B: Results for Cr + B[*a*]P ageing experiment (Chapter 6)

6B.1 Shoot biomass

6B.2 Root biomass

6B.3 Shoot Cr concentration

6B.4 Shoot Cr accumulation

6B.5 Root Cr concentration

6B.6 Root Cr accumulation

6B. 7 Total Cr

6B. 8 Translocation factor

6B. 9 Zero time B[*a*]P concentration in soil

6B.10 Residual B[a]P concentration in final soil (with plant)

6B.11 Residual B[a]P concentration in final soil (No planting)

6B.12 B[a]P dissipation (%) in planted soil

6B.13 B[a]P dissipation (%) in non planted soil

Appendix 7A: Results for Cu+ Pyrene with chelate application (Chapter 7)

7A.1 Shoot biomass

7A.2 Root biomass

7A.3 Shoot Cu concentration

7A.4 Shoot Cu accumulation

7A.5 Root Cu concentration

7A.6 Root Cu accumulation

7A.7 Translocation factor

7A.8 Water extractable Cu

7A.9 Residual pyrene in soil

7A.10 Zero time Soil Pyrene concentration

Appendix 7B: Results for Cr+ B[a]P with chelate application

7B.1 Shoot Biomass

7B.2 Root Biomass

7B.3 Shoot Cr concentration

7B.4 Shoot Cr Accumulation

7B.5 Root Cr concentration

7B.6 Root accumulation

7B.7 Translocation factor

7B.8 Bioconcentration factor

7B.9 Soluble Cr in soil

7B.10 Residual soil B[a]P concentration

7B.11 Initial Soil B[a]P

Appendix 8A:

8A.1 Detection limits of GC-MS for 16 priority PAHs

Appendix 9A: Soil Partitioning calculation