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IMPORTANCE OF PLANTS AND MICROORGANISMS IN THE PHYTOREMEDIATION OF BROWNFIELD SITES

By

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ABSTRACT

Phytoremediation is an emerging green technology for the restoration of contaminated sites with various organic and inorganic contaminants. However, phytoremediation efficiency is limited by factors such as contaminant concentration, toxicity and bioavailability, plant choice and stress tolerance, and competence of indigenous microorganisms. A number of possible solutions have been proposed to overcome these limitations. The use of tolerant plant candidates, mixed plant communities and bioaugmentation with microbes and/or plant growth promoting bacteria (PGPB) have been proposed to suppress plant growth inhibition/phytotoxicity and enhance contaminant degradation through the rhizosphere effect but there is need for more research to understand their impact. This research assessed the impact of contaminant stress (diesel fuel, PAH; phenanthrene, fluoranthene and benzo[*a*]pyrene, and heavy metal) on selected plant species and microbial community structure, contribution of abiotic processes and rhizoremediation to PAH dissipation, and the impact of PGPB on plant growth and PAH dissipation. These objectives were achieved through greenhouse experiments with *M. sativa*, *F. arundinacea* and *L. perenne* on diesel fuel- and PAH-spiked soils. Diesel-fuel treatments had a negative impact on plant biomass yields while the single and mixed PAH treatments had stimulatory and inhibitory effects on plant biomass yields relative to the control. Diesel fuel and PAH dissipation from vegetated treatments was either comparable or slightly greater than that of the unvegetated controls in the different experiments. The presence of plants possibly inhibited PAH degradation in a few treatments. Hence volatilization and microbial degradation mainly contributed to contaminant dissipation. The impact of PGPB on plant biomass yield and PAH dissipation was inconsistent. PGPB inoculation significantly enhanced phenanthrene dissipation for *M. sativa-PAH* treatment and fluoranthene for *F. arundinacea-PAH+ HM* ($p<0.05$).

Contaminant treatments may affect plant growth and soil rhizosphere bacterial community with impact on contaminant dissipation. The findings of this research have emphasised the need for plant candidates with high stress tolerance to overcome growth inhibition from stresses during phytoremediation. While PGPB inoculation has the potential to enhance plant growth and phytoremediation, an understanding of factors that influence their impact such as inoculum size and method, survival and adaptation, plant-microbe interaction and other rhizosphere activities is crucial for the application of PGPB enhanced phytoremediation (PEP).

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CHAPTER 1

INTRODUCTION

The continued existence of life and biodiversity depends amongst other factors, on the quality of the soil; an important component of the ecosystem and the biosphere at large (Kaya, 2006). As the world population grows, the corresponding global demand for energy is resulting in an increase in industrialization, technological advancement, overexploitation of natural resources and release of pollutants into the environment (Glick, 2010). These pollutants include petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, heavy metals and salts, furans, dioxins and explosives (Singh and Jain, 2003; Jardine, 2006; Kaya, 2006; Gerhardt *et al*., 2009).

Although some of these compounds are naturally released to the environment, there has been a drastic increase in the inadvertent release of a variety of pollutants of anthropogenic origin into the environment in the last century (Singh and Jain, 2003; Jardine, 2006; Kaya, 2006; Gerhardt *et al*., 2009). The principal sources of widespread soil, air and ground water contamination with these pollutants are anthropogenic activities involving use of organic hydrocarbons, improper waste disposal, crude oil exploration, accidental spills and leaks of pollutants from storage facilities (Conte *et al*., 2005; Paria, 2008; Gerhardt *et al*., 2009; James and Strand, 2009; Mc Guinness and Dowling, 2009).

When pollutants enter the environment, their fate includes volatilization, biodegradation, bioaccumulation, leaching into groundwater and intra-soil processing. Factors such as soil properties, soil chemistry and environmental factors may determine the fate and behaviour of organic pollutants in the soil. The persistence of an organic compound is related amongst other things - to its hydrophobicity. Dissipation of pollutant from soil usually occurs in two

phases (preliminary short period of rapid loss and longer period of slower loss) which may be determined by pollutant properties such as hydrophobicity and affinity for organic matter. Intra-soil processing may cause sorption of pollutants to soil organic matter with the formation of residues with a decrease in bioavailability and degradation. The decrease in the availability compound over time is referred to as ageing (Hatzinger and Alexander, 1995; Jensen *et al*., 2006).

The ageing process may be determined by soil organic matter (nature and content), soil properties (pore size and structure) and pollutant properties (vapour pressure, octanol: water partition coefficient and water solubility). Sorption and pollutant entrapment are the two major processes involved in ageing. However, both processes may be enhanced by high soil organic matter and high pollutant concentration. Ageing may lead to the formation of a soil associated fractions which may desorbed (either be rapidly or slowly) or the formation of bound or non-extractable residues (Jones *et al*., 1996; Reid *et al*., 2000; Semple *et al*., 2001; Jensen *et al*., 2006). These processes facilitate the widespread occurrence, persistence and recalcitrance of pollutants (Conte *et al*., 2005; Paria, 2008; James and Strand, 2009).

Consequently, increasing concerns over the rising number of contaminated sites, growth in world population and the need for land for agriculture, infrastructure, housing and recreation have led to a pressing need for efficient contaminated land and groundwater remediation (Samanta *et al.*, 2002; Bamforth and Singleton, 2005). According to the Environmental Protection Agency report, as of May 2004, the United States had over 40,000 contaminated sites while some industrialised Western European countries have even more sites within a smaller area (Zhuang *et al*., 2007).

Initially, waste disposal into the environment was based on the assumption that pollutants will be absorbed. However, emphasis on the need for sustainable and efficient treatment technologies has resulted from the accumulation of toxic pollutants and deterioration of global environmental health, loss of biodiversity and water resources, ecosystem imbalance and impairment, poor vegetation development or complete habitat destruction and adverse health effects with increase in morbidity and mortality (Bamforth and Singleton, 2005; Conte *et al*., 2005; Escalante-Espinosa *et al*., 2005; Scullion, 2006; Paria, 2008; Glick 2010).

Physical and chemical remediation processes such as excavation, incineration and use of specialized landfills are characterized by high cost, low efficiency and a potential for secondary air and ground water contamination hence the search for more efficient, sustainable and eco-friendly remediation strategies and technologies (Singh and Jain, 2003, Scullion, 2006; Yan-Zheng *et al*., 2007; Mc Guinness and Dowling, 2009). A typical example of such remedial approach gaining increasing interests over the years is a bioremediation method called phytoremediation (Yan-Zheng *et al*., 2007). Phytoremediation involves the use of plants or plants and associated microbes to restore contaminated sites through processes such as stabilization, accumulation, extraction, volatilization, transpiration and degradation (Chaney *et al*., 1997; Glick, 2003; Morikawa and Erkin 2003; Singh and Jain, 2003). This concept is based on diverse detoxification mechanisms in plants for absorbing a variety of natural and man-made toxic compounds through their root system thus considered "*natural, solar-powered pump-and-treat systems"* that decrease contaminant concentrations in vegetated soils compared to unvegetated bulk soils (Escalante-Espinosa *et al*., 2005; Aken, 2008; Diab, 2008).

On the whole, phytoremediation offers the advantage of a cost-effective approach, solar-driven technology with minimal energy requirements, minimal environmental disruption, preservation of biological activity in soils, improvement of soil microbial diversity, long term applicability, application to a range of contaminants and public acceptance compared to the traditional methods (Morikawa and Erkin, 2003; Dheri, *et al*., 2007; Zhuang *et al*., 2007; Vangronsveld *et al*., 2009; Wenzel, 2009).

Of all the phytoremediation processes, rhizodegradation (rhizoremediation) is particularly effective for remediation of sites contaminated with organic compounds. It involves degradation as a result of plant-microbe interactions and co-metabolism in the rhizosphere driven by the release of nutrient-rich plant root exudates, enzymes and biosurfactants (Alkorta and Garibsu, 2001; Morikawa and Erkin, 2003; Barea *et al*., 2005; Dzantor, 2007; Gerhardt *et al*., 2009; Wenzel, 2009). Root exudates affect metabolic diversity of the soil microbial community in the rhizosphere with microbial populations up to 2-4 orders greater than those present in the bulk soils (Alkorta and Garbisu, 2001; Morikawa and Erkin, 2003; Gerhardt *et al*., 2009; Vangronsveld *et al*., 2009). Hence, phytoremediation efficiency depends on the successful establishment of plant-microbe interactions (Wenzel, 2009).

Efficient plant candidates for rhizoremediation of organic compounds are mainly grasses because of their extensive and widely branched root system and legumes for their nitrogen fixing ability (Barea *et al*., 2005; Merkl *et al*., 2005). Trees, particularly the *Salix* and *Poplar* species are attractive especially where pollutants are present at depths below the normal root zone of alternative species due to their extensive root system, high biomass and low-input cultivation (Kramer, 2005). Rhizospheric microbes of importance to rhizodegradation include degraders, plant growth promoting bacteria (PGPB), endophytes, nitrogen fixing bacteria,

arbuscular mycorhizal fungi (AMF) and solubilizing bacteria (Barea *et al*., 2005; Merkl *et al*., 2005).

Limitations of phytoremediation processes amongst others include bioavailability and ageing of contaminants, contaminant stress and phytotoxicity, biotic and abiotic stresses, and evapotranspiration of volatile organic pollutants resulting in air pollution (Gerhardt *et al*., 2009; Weyens *et al*., 2009). Despite these issues, a number of successful field trials have been documented for a range of hydrocarbons such as benzene, toluene, ethylbenzene and xylene (BTEX compounds), trichloroethylene (TCE), polychlorinated biphenyls (PCB) and trinitrotoluene (TNT) using plants with their associated indigenous soil microbes and endophytic microorganisms (Kuiper *et al*., 2004). Such successes have highlighted not only the lessons and research needs but the true potential and prospects of phytoremediation in the near future (Zhuang *et al.,* 2007; Gerhardt *et al*., 2009; Vangronsveld *et al*., 2009).

Diesel fuel and PAH contamination are quite common and studies have demonstrated the impact of contaminant type and concentration on plant growth, plant biomass yields and phytoremediation efficiency (Kamath *et al.,* 2004). As phytotoxicity and plant stresses limit phytoremediation successes, suggestions such as use of mixed plant communities rather than a single plant community has been proposed but results from such experiments have been conflicting (Huang *et al*., 2004a; Cheema *et al*., 2009; Gurska *et al*., 2009). Also noteworthy of mention is the need for studies on mixed contamination which would reflect real scenarios as most contaminated sites have a mixture of organic and /or inorganic contaminants (Gan *et al*., 2009). This has implication for plant biomass production, phytotoxicity, plant stress tolerance, soil microbial diversity, plant root exudation and rhizospheric interactions and ultimately phytoremediation efficiency.

The use of PGPB in pyhtoremediation also known as PGPB enhanced phytoremediation (PEP) is proposed as a cost-effective eco-friendly approach to enhance plant stress tolerance, biomass production and phytoremediation efficiency during contaminated land remediation. As there is little information in this area, extensive research would help enhance the potential of grasses, legumes and mixed plant communities and provide insights to plant growth promotion during rhizoremediation. A better understanding on the effect of contamination and plant stresses on microbial diversity would provide information on rhizosphere processes such as the complex interactions between pollutants, soil material, plant roots, and microorganisms in the rhizosphere (Vangronsfeld *et al*., 2009), identification of the major microbial community players and plant-microbe interaction (Kuiper *et al*., 2004; Dzantor, 2007; Haichar *et al*., 2008; Dennis *et al*., 2010). These findings would facilitate the exploitation of rhizodegradation potentials which may involve rhizoengineering and rhizosphere metabolomics driven approach.

Research aim and objectives

The aim of this research is to establish the potential of selected plant candidates in the phytoremediation of single and mixed contamination as well as the potential of PEP in order to optimise the application of phytoremediation. The aim was achieved with the following objectives:

1. To assess the level of PAH and heavy metal contamination in two selected sites in the United Kingdom.

2. To assess the rhizodegradation potential of selected plant candidates and mixed plant communities in petroleum- and PAH-contaminated soils

3. To assess the impact of contaminant treatments on plant biomass yields and soil bacterial community.

4. To assess the impact of plant growth promoting bacteria (PGPB) on plant biomass yield and rhizodegradation.

CHAPTER 2

LITERATURE REVIEW

2.1 Organic compounds

An organic compound can be defined as a compound with a hydrocarbon skeleton which may be substituted with other elements such as oxygen, sulphur and halogens (Bailey *et al*., 2002 and Paria, 2008). The substituted hydrocarbon may have multiple bonds in straight chains, branched chains or a combination of both structures. Organic compounds are naturally synthesized in living systems during metabolic reactions catalysed by organic substances called enzymes and can be degraded by degradative enzymes, hence "biodegradable". However, carbon compounds formed by geochemical processes (crude oil and coal) and industrial processes (dichlorodiphenyltrichloroethane; DDT and polyethylene) are not easily degraded hence their accumulation in the environment and adverse effects. As such these compounds are regarded as organic pollutants or contaminants (Bailey *et al*., 2002).

The widespread contamination of the biosphere with these compounds originates from two main sources; natural (biogenic and geochemical) and anthropogenic (Bamforth and Singleton, 2005). A toxic organic compound may exist naturally (e.g. polycyclic aromatic hydrocarbons from coal and petroleum) and natural activities such as volcanic eruptions and forest fires provide potential pathways by which these compounds are released. However, anthropogenic activities are primarily responsible for the release, presence, contamination and long-term persistence of organic contaminants in the environment with health and ecosystem implications. Such activities include incomplete combustion of coal, diesel and vegetation, petroleum spills and industrial processes leading to a high concentration of compounds such

as detergents, dyes, solvents and feedstock in wastes and effluents (Bamforth and Singleton, 2005; Scullion, 2006).

Sources of organic contaminants include coking sites, tar ponds and coal storage sites, landfills and garbage dumps, industrial leaks, oil spills, leaks from storage tanks, indiscriminate application of agrochemicals and improper disposal of household wastes, oils and antifreeze (Paria, 2008). A wide range of organic contaminants commonly found in the soil include chlorinated dioxins, dibenzofurans, polychlorinated aromatic hydrocarbons (PAHs), propellants, fuels, lubricants, herbicides and insecticides (Parales and Haddock, 2004; Bamforth and Singleton, 2005; Scullion, 2006).

USEPA classified some organic pollutants such as haloether (chlorophenylphenyl ethers, bromophenylphenyl ether, bis-(dichloroisopropyl) ether, bis-(chloroethoxy) methane) and polychlorinated diphenyl ethers as "toxic pollutants" and others such as PAHs, benzene, toluene, ethylbenzene and xylene (BTEX compounds), eldrin, DDT and aldrin are classed as "priority pollutants". This classification is based on toxicity, frequency of occurrence at contaminated sites and potential for human exposure (Xu *et al*., 2006).

2.2 Petroleum hydrocarbons

The term "total petroleum hydrocarbons" (TPHs) refers to the diverse hydrocarbon compounds and components that are found in crude oil and its products (Kamath *et al*., 2004; Urum *et al*., 2006; Bojes and Pope, 2007). Petroleum hydrocarbons in crude oil and other products are measured as total petroleum hydrocarbon content. TPHs are persistent, toxic and hazardous pollutants with the ability to bioaccumulate and bioconcentrate in food chains. Based on the physicochemical properties of hydrocarbons, TPHs may be divided into two

groups: gasoline range organics (GRO) and diesel range organics (DRO). The GRO consists of short chain alkanes ($C_6 - C_{10}$) with low boiling points (60-170°C) such as pentane, butane and BTEX compounds while DRO consists of longer chain alkanes ($C_{10} - C_{40}$) and hydrophobic aromatic compounds such as PAHs with high boiling points (>170°C) (Wang and Fingas, 2003; Kamath *et al*., 2004).

PAHs are important target components of concern because some are toxic, carcinogenic and mutagenic. The 16 priority PAHs are naphthalene, acenaphthene, acenaphthylene, anthracene, phenanthrene, fluorene, fluoranthene, benzo[*a*]anthracene, chrysene, pyrene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, dibenz[*a,h*])anthracene, benzo[*g,h,i*]perylene and indeno[*1,2,3-c,d*]pyrene and those considered to be carcinogenic include benzo[*a*]anthracene, chrysene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, dibenzo[*a,h*]anthracene and indeno[*1,2,3-c,d*]pyrene. The carcinogenic PAHs are characterised by a high molecular weight due to the number of aromatic rings, and lower vapour pressure and solubility constants unlike the non-carcinogenic PAHs (Bojes and Pope, 2007; Fingas, 2011).

Apart from PAHs other target analytes in crude oil and its products for oil spill studies include aliphatic hydrocarbons such as benzene and BTEX compounds (Fingas, 2011). The physiochemical properties of these compounds affect their behaviour and fate in the environment. While BTEX compounds are highly mobile and less hydrophobic, PAHs are hydrophobic and chemically stable hence persistent in the environment (Kamath *et al*., 2004; Vangronsfeld *et al*., 2009).

2.3 Potential for Environmental Contamination

Widespread contamination of the environment is attributed to an extensive use of organic and inorganic compounds in a wide range of anthropogenic activities (Gerhardt *et al*., 2009). Soil and groundwater contamination commonly results from the release of effluents containing various hazardous substances such as heavy metals and organics from oil refineries and industries such as chemical, electrical and electronics, electroplating metal, leather, mining and metallurgy, paint and dye, pesticide, pharmaceutical, paper and pulp industries (Paria, 2008). Also the products of these industries are present and used in every aspect of modern life leading to local (point source) or diffuse (non-point source) contamination with adverse effects (Mc Guinness and Dowling, 2009).

Due to close connection with the biosphere, groundwater is susceptible to contamination by organics such as petroleum hydrocarbon and halogenated organic compounds which strongly adsorb onto the soil matrix and may sparingly dissolve in water and persist in the environment. During large spillages, liquid organic compounds can easily percolate the soil to contaminate the groundwater and/or flow into surface waters (Paria, 2008). On a global scale, about 2 - 9 million metric tonnes/year of crude oil and hydrocarbons are released into the environment with a 7 x 10^8 gal/year crude oil input into the sea (Bailey *et al*., 2002). Prior to the 2010 Gulf of Mexico/British Petroleum spill, catastrophic spills have reduced due to the use of double-hulled tankers (with 9-10ft buffer area between hulls) and costly litigation from oil spills (Bailey *et al*., 2002). The Gulf of Mexico/British Petroleum spill considered one of the worst catastrophic spills was caused by explosion of an oil drill platform *Deep Water Horizon* off the coast of Louisiana and sinking of the oil rig causing eleven deaths in April 20, 2010 (Bozeman *et al*., 2011, Harlow *et al*., 2011 and Mariano *et al*., 2011). Huge volumes of crude oil estimated by the National Oceanic and Atmospheric Association (NOAA) to be

about 210,000 gallons leaked into the ocean each day from an uncapped well until it was completely sealed in September 19, 2010 (Muralidharan *et al*., 2011). For this reason, the Gulf of Mexico spill exceeded that of the well-reported Exxon Valdex spill which leaked 11 million gallons from tanker *Exxon Valdex* in Prince William Sound Alaska in 1989 (Bailey *et al*., 2002; Muralidharan *et al*., 2011). The spill covered over 88,000 square miles and spread to beaches, estuaries causing significant environmental damage and economic loss especially to the fishing industries and tourism around the US Gulf states. The estimated cost including clean up and compensation cost incurred by British Petroleum from the spill is over $40 billion (Bozeman *et al*., 2011; Muralidharan *et al*., 2011).

It was estimated that approximately 275 million tonnes of hazardous waste was generated in 1993 in the United States of America (Zeigler, 1993; Glick, 2010). Between 1983 and 2013, United States Environmental Protection (USEPA) listed 1694 uncontrolled waste sites on the National Priority List (NPL) with remedial action in place for 68% of NPL sites (USEPA, 2013). Between 2000 and 2007, 781 sites including 35 designated special sites were determined to be contaminated following site inspections in England and Wales at a cost of about £30 million (Environment Agency, 2009). The land use category of determined sites at the time of inspection may be derelict land, housing, commercial premises, industrial premises, agriculture, and park and recreation (Environment Agency, 2009).

2.3.1 Risk assessment of contaminated land

Land is said to be contaminated if there exists a contaminant source and a pathway (or pathways) for the contaminant to potentially affect a specified receptor. Hence contaminated land may pose risks to health and the environment that may be determined by a risk assessment. Risk assessment of contaminated land involves site characterisation and

description of land-use in relation to the planned use of the site, determination of ecological aspects and site-specific assessment for relative risk/damage determination (Oberg and Bergback, 2005; Jensen *et al*., 2006; Leo *et al*., 2008).

Historical land-use information may provide information about soil characteristics (pH, organic matter, soil type and the type of contaminants likely present). Defining the land-use and factors such as the likely human or wildlife receptors present, will determine the data collection and testing required and may help focus the risk assessment. The four main categories of land-use areas are: industrial, urban/residential, agricultural and nature. Each land-use category may have different requirements and soil functions (such as soil structure, supply of nutrients and ground water reservoir function). Site-specific assessment takes into consideration acceptable and unacceptable effect/damage in relation to land-use and the monitoring required. The concentration of contaminants and soil clean up targets, risk effects, time required for clean=up and the desired land-use will influence the selection of remediation strategies (for example, bioremediation, on site or off site techniques) if required (Jensen *et al*., 2006).

2.4 Health and Environmental Impacts of Organic Contamination

An increase in morbidity and about 40% of deaths worldwide has been attributed to environmental contamination (Glick, 2010) with toxic and hazardous compounds and priority pollutants as a major cause (Xu *et al*., 2006). The adverse effects of hazardous waste contamination include human health risks due to carcinogenic, teratogenic and mutagenic properties of some pollutants, poisoning of plants, animals and other life forms and ecological

imbalance (Bamforth and Singleton, 2005; Gao *et al*., 2007; Mc Guinness and Dowling, 2009).

Organic contaminants especially the persistent organic pollutants (POPs) are characterised by hydrophobicity and persistence, resulting in slow, long term release into the environment and causing a range of environmental and health impacts. In aquatic environments, organic pollutants such as pharmaceuticals originating from effluent of municipal wastewater treatment plants suppress growth and diversity of plankton, cyanobacteria and aquatic plants at concentrations as low as 60-100 μg L^{-1}. This results from the disruption of chemoreception which affects responses and signals for feeding and reproduction of aquatic life (Baileys *et al*., 2002). Following oil spills, all life forms are affected by crude oil and its toxicity is proportional to the percentage of aromatic fraction due to impacts of mutagenic and/or carcinogenic polycyclic aromatic hydrocarbons (Baileys *et al*., 2002; Antonio *et al*., 2011). Oil spills result in high mortality and pollution of key food sources affecting marine and terrestrial life, and human health (Banks *et al*., 2008; Al-Mailem *et al*., 2010).

Adverse effects on animals range from weakness and poor insulation capacity, eye irritation, physiological or endocrine disruption to death following adhesion to organisms and eggs or ingestion of oiled plants and animals (Baileys *et al*., 2002; Ornitz and Champ, 2002). In higher organisms in the food chain, exposure results in bioaccumulation in adipose tissues. In humans, these accumulated toxic organic contaminants can be vertically transmitted from mother to child in utero via the placenta or post-natally via breast milk (Samanta *et al*., 2002; McGuinness and Dowling 2009). Also, pharmaceutical mixtures present in water were shown to inhibit growth of human embryonic liver cells following long term exposure (Reinhold *et*

al., 2010). DDT, a widely used insecticide between 1940s and 1970s was banned in 2001 mainly for ecological reasons and chronic health effects (McGuinness and Dowling, 2009).

When plant foliage is coated with oil, it causes blockage of the stomatal pores leading to a rise in temperature stresses, reduced transpiration, and photosynthesis. These effects depend on the foliage surface area coated with oil, type and amount of oil spilled and hydrological conditions (tides and winds). Stomatal blockage may culminate in plant mortality if gas-exchange activity is not restored or new shoots sprout (Pezeshki *et al*., 2000). Ecosystem revival occurs within one to two years depending on the ocean activity on the shoreline to facilitate dispersal while spills in protected area or land such as wetlands, marshes and beaches require longer period ~50 years (Baileys *et al*., 2002; Ornitz and Champ, 2002).

Although production and use of POPs have either been restricted or banned due to toxicity and persistence, POPs and other organic compounds pose threat to all life-forms due to their presence in soil and groundwater which results in contamination of drinking water sources. Examples of organic contaminants in drinking water sources, their maximum contamination limits (MCL) and corresponding potential health effects as suggested by USEPA are shown in Table 2.1.

Table 2.1: Maximum contamination limits (MCL) of some organic contaminants in drinking water (Paria, 2008).

Contaminants	**MCL (mg L^{-1})**	**Health effects**	**Sources**
Benzene	0.005	Anaemia, thrombopenia, cancer	Effluent from industries, leaks from storage tanks and landfills
Benzo[*a*]pyrene (PAH)	0.0002	Reproductive difficulties and risk of cancer	Coal storage, leaching from linings of water storage tanks and distribution lines
Carbofuran	0.04	Anaemia, problems with the nervous and reproductive systems	Leaching of soil fumigant used on rice and alfalfa
Ethylbenzene	0.7	Liver and/or kidney problems	Discharge from petroleum refineries
Toluene	1	Problems with the nervous system and visceral organs	Discharge from petroleum refineries
Ethylene dibromide	0.00005	Problems with liver, stomach, reproductive system, or kidneys; increased risk of cancer	Discharge from petroleum refineries
1,1-Dichloroethylene	0.007	Liver problems	Discharge from industrial chemical factories

The adverse health and environmental effects of organic contaminants have increased attention and efforts on sustainable remediation technologies for contaminated land remediation as the physical and chemical remediation methods are expensive and inefficient (Gaskin and Bentham, 2010).

2.5 Traditional remediation techniques

In situ or ex situ treatment of contaminated land with organic compounds (crude oil or its derivatives) as well as inorganic compounds may be achieved by three broad approaches *viz*; physical (thermal, solidification, vapour extraction, electroremediation), chemical (oxidation, reduction, hydrolysis and solubilisation) and biological or bioremediation approaches (land farming, biopiling, composting and bioreactor). However, a combination of treatment processes from different approaches may be employed. Although *in situ* treatment minimizes ecosystem disruption, *ex situ* treatment allows for optimization of conditions required for remediation (Scullion, 2006; Gao *et al*., 2007; McGuinness and Dowling, 2009).

2.5.1 Physical processes

These include treatment processes such as incineration, vitrification, vapour extraction and air sparging, electro-remediation, excavation, solidification/stabilization and use of specialized landfills. In general, these processes allow removal of contaminants in a concentrated form from soil–water complex for further treatment. They are most effective for coarse-textured soil and may either facilitate biodegradation or actually lead to complete destruction of pollutants (Scullion, 2006).

1. Thermal treatment or incineration

This involves a single stage process using high temperatures usually >1000°C or a two stage process using a lower temperature of <600°C followed by combustion. Unlike the use of such high temperatures, the use of lower temperatures as in microwave heating and injection of steam or hot air at 100°C allow for vapour extraction with less damage to soil function. A thermal treatment process called vitrification, involves the use of temperatures above 1000°C allow inorganic pollutants to be trapped in a solid ceramic-like material. Also, cements may

be used to mix contaminated soils to form solid masses for land fill disposal or serve other uses. This form of thermal treatment is known as solidification. The disadvantages of these processes include air pollution, leaching of contaminants in liquid and gaseous states from landfills into ground water (Cunningham *et al*., 1995; Khan *et al*., 2004; Scullion, 2006).

2. Vapour extraction and air sparging treatments

These take advantage of the different phases in which some pollutants exist. These involve installation of vertical and/or horizontal wells, for the volatilization of volatile and semi-volatiles pollutants such as BTEX compounds and chlorobenzene into vapours or gases with air blowers. This is usually followed by adsorption onto activated carbon or oxidization. On the other hand, air sparging treatment partitions contaminants and facilitates biodegradation by increase in dissolved oxygen levels. These treatments are not appropriate for fine-textured soils and quite slow with organic soils (Scullion, 2006).

3. Soil washing

This treatment involves extraction of pollutants with solvents and separation of polluted solids based on size, density or surface chemistry to reduce the bulk of polluted solids requiring further treatment and save cost (Urum *et al*., 2003; Khan *et al*., 2004; Scullion, 2006). The target compounds include semi-volatile organic compounds, petroleum, heavy metals, PCBs, PAHs, and pesticides (Khan *et al*., 2004). Soil washing is commonly used in mining and mineral processing industries (Khan *et al*., 2004; Scullion, 2006).

4. Electroremediation/Electrokinetics

This is a method in which ionic pollutants such as metals, inorganic anions and polar organics are separated by migration through an electrical field for collection and further treatment (Jankaite and Vasarevicius, 2005). This has been found to be quite effective for clay soils polluted with metals but less effective on organic and carbonate rich media. It may also eliminate the need for excavation and hence minimise the release of toxic air emissions (Frick *et al.,* 1999; Kuiper *et al*., 2004; Scullion, 2006; Cameselle *et al*., 2013). Electroremediation has been combined with other remediation techniques such as bioremediation, chemical reduction/oxidation, thermal desorption and phytoremediation (Cameselle *et al*., 2013).

2.5.2 Chemical processes

These include oxidation, reduction, hydrolysis, solubilisation, dechlorination and pH manipulation. These approaches are mainly employed in treatment of polluted ground water and sometimes soil slurries to decrease toxicity, extract or immobilize pollutants. Chemical treatments are highly specific and applicable to different matrices with effective mixing. Added chemicals may further pollute soils if chemical reactions are incomplete. Chemical processes may not be effective for pollutants that are structurally similar to indigenous soil organic compounds (Frick *et al.,* 1999; Scullion, 2006).

1. Oxidation and reduction treatment

Oxidation treatment involves the use of ozone or hydrogen peroxide with iron as a catalyst (Fenton's reaction) to form hydroxyl radicals. It has been used for treatment of trichloroethylene (TCE) in soil water with sulphate free radical (SO_4-) at temperatures above

40°C and chelated ferrous iron were found to support persulphate decomposition and, therefore, TCE degradation. However research has shown that effectiveness of oxidation treatment especially for PAHs is affected by organic matter contents and soil porosity. Also it has been found that pollutants compete with indigenous soil carbon compounds for oxidants as in the case of TCE treatment (Cunnigham *et al*., 1995; Khan *et al*., 2004; Scullion, 2006). Reduction plays an important role in remediation by reducing toxic compounds to a less toxic state. A typical example is the reductive dechlorination of PCBs with alkali polyethylene glycol and the reduction of highly toxic chromium (VI) to less toxic chromium (III) by microbial action in the presence of organic carbon (Scullion, 2006).

2. Other chemical approaches

This may also involve addition of compounds such as liming materials, phosphate compounds and biosolids to increase binding sites and improve soil chemistry, pollutant extraction, metal desorption, reduce risks of water pollution and enhance vegetative cover to stabilize the soils (Frick *et al.,* 1999; Scullion, 2006). These are means of aiding pollutant behaviour and hence do not necessarily remediate the site. pH manipulation affects mobility of metals in contaminated sites and may adversely affect microbial physiology due to extreme pH levels. In the case of mixed contaminations with metal and organic compounds, a decrease in metal bioavailability may enhance microbial degradation of organics (Scullion, 2006; Batty and Dolan, 2013). An immobilization approach may be required to protect water, plants and other members of the food chain when a vast area is polluted (Frick *et al.,* 1999; Scullion, 2006).

According to Kuiper *et al*., (2004), remediation of contaminated sites in United States of America will cost about $ 1.7 trillion. The high cost of remediation sometimes leads to non-compliance which is cheaper and/or abandonment of a number of brownfields. In 2008, it was reported that 34% of known brownfields opted for the non-compliance option rather than incurring huge costs with physiocochemical remediation technologies (Mc-Guinness and Dowling, 2009). For the 2013 fiscal year, about $1.6 billion was committed to the clean up of sites by secured private parties and were billed an additional $93 million in oversight cost by EPA (USEPA, 2013). The remediation of most sites which takes many years usually commences over a year after site inspection. Between 2000 and 2007, 149 sites in England and Wales were reported by the local authorities as successfully remediated mainly by through excavation and off-site disposal of materials at £20.5 million and is estimated to increase to about £62 million for currently inspected sites. Other remedial solutions in use include containment, excavation and on-site disposal, physical and chemical treatments and to a less extent, *in situ* and *ex situ* bioremediation (Environment Agency, 2009).

Apart from high costs associated with the physico-chemical methods, other limitations include low efficiency, potential for secondary air and ground water contamination and other adverse effects on ecosystem and environmental health. These factors have led to the need for more efficient and eco-friendly alternative remediation strategies and techniques such as bioremediation which has gained increasing interest (Kuiper *et al*., 2004; Gao, *et al*., 2007; Robertson *et al*., 2010; Van Aken *et al*., 2010; Santos *et al*., 2011).

2.5.3 Bioremediation Approaches

Most persistent pollutants such as pesticides, pharmaceuticals and personal care products are man-made products however, microorganisms and plants have evolved to produce a diverse array of catabolic enzymes for their degradation due to structural similarity to some naturally occurring compounds (Singer *et al*., 2003, Scullion, 2006 and Reinhold *et al*., 2010). Bioremediation technologies exploit both genetic diversity and metabolic versatility of microorganisms and /or plants to reduce the concentration and/or toxicity of a pollutant" to less toxic materials, such as CO_2, methane, water, and inorganic salts (USEPA, 2001; Mc Guinness and Dowling, 2009). The benefits of bioremediation are mineralization, integration into biogeochemical cycles and biomass production (Mohsenzadeh *et al*., 2010 and Santos *et al*., 2011). Bioremediation may be grouped into microbial remediation (involving microbes) and phytoremediation (plant and associated microbes). Microbial remediation may be intrinsic or enhanced treatments and may be performed *in situ* or *ex situ* under aerobic or anaerobic conditions (Mc Guinness and Dowling, 2009).

Interestingly, bioremediation is listed as one of the top ten biotechnologies that improve human health (Daar *et al*., 2002; Van Aken *et al*., 2010) and is more attractive than the other traditional techniques due to the following advantages;

1. it usually involves *in situ* application without the need for excavation and transportation of contaminated soils hence less disturbances,

2. it offers low risk to the site and the environment,

3. it is cost-effective with a favourable cost-benefit ratio. The cost of bioremediation is usually 10-50% of the cost of physical and chemical methods,

4. it is less labour intensive,

5. it has a high level of acceptance by the public and

6. it involves lower carbon footprint

(Zhuang *et al*., 2007; Mc Guinness and Dowling, 2009; Santos *et al*, 2011; Van Aken *et al*., 2010).

Though the two main approaches to the treatment of petroleum-contaminated soil are microbial remediation and phytoremediation (Tang *et al*., 2010a), prior to the selection of a bioremediation treatment approach for any given site, a thorough study is important. This provides answers to parameters such as nature of the pollutants, soil structure and hydrogeology, nutritional state and microbial diversity on the site. In most cases, complete restoration of most contaminated sites usually with mixed pollutants involves a combination of treatment approaches over a long period of time (Kuiper *et al*., 2004).

2.5.3.1 Microbial Remediation

This includes natural attenuation, land farming, biopiling, composting and bioreactor. This may be applied *in situ*, on-site or in bioreactors depending on the treatment (Mc Guinness and Dowling, 2009).

1. Natural attenuation

It was previously referred to as the "*do nothing or hands off approach*" (Germida *et al*., 2002). This is because it mainly relies on the catabolic ability of the indigenous microbial

community to degrade pollutants on contaminated site hence it is the simplest bioremediation approach. Interestingly, the Arabian Gulf and many other areas have been found to be rich in hydrocarbon- and oil-utilizing bacteria which are involved in natural attenuation (Al-Mailem *et al*., 2010). Apart from degradation, natural attenuation may result in dispersion, dilution, sorption, volatilization and stabilization of contaminants. This approach has been successfully used for remediation of BTEX contaminated ground water, and sites contaminated with chlorinated hydrocarbons and methyl *tert*-butyl ether (MTBE) (Mulligan and Yong, 2004; Kuiper *et al*., 2004; Scow and Hicks, 2005). It is appropriate for sites with low environmental value and useful when time factor is not crucial since indigenous microbial communities have to adapt to the presence of xenobiotics. The limitations of this approach can include incomplete degradation of the pollutant, formation of more toxic compounds (for example, 1-napthol can be formed from naphthalene by aerobic bacteria), and catabolic incompetence of the indigenous microbial community (Kuiper *et al*., 2004). Heavy metals may only undergo immobilization or volatilization. Monitored natural attenuation involves monitoring natural degradation processes on site. This may involve the use of sampling wells to track the movement of pollutant, analyses of the concentration of pollutants, intermediate products and end product formation, electron acceptor utilization and toxicity, total DNA extraction, mRNA extraction, use of reporter bacteria (Kuiper *et al*., 2004; Declercq *et al*., 2012).

2. Land farming

This is applied on site as an above ground remediation technology that involves a solid phase approach of distributing petroleum polluted soil and nutrients in a thick layer approximately 1.5 m on to cultivated land. Hydrocarbon degaders are usually added to enhance degradation and microbial activity is stimulated by aeration, addition of water, nutrients and minerals.

Based on the degradation rates, more contaminated soil may be added at intervals to replenish hydrocarbon supply and maintain microbial activity (Megharaj *et al*., 2011). This technology is efficient and has been used for over 25 years by the petroleum industry. The lower molecular weight hydrocarbons are usually volatilized while the heavier hydrocarbons which are non-volatile are degraded by microorganisms. However, this technology requires a large amount of land and has a tendency to cause air pollution. It is also less efficient for heavy petroleum components and sites with over 50,000 ppm TPH content. Efficient remediation require an optimum temperature range of 25-40°C, moisture content of 18% and pH 6.5-7.5 (Khan *et al*., 2004).

3. Bioslurry/Bioreactor

This is an *ex situ* treatment technology that involves excavation of contaminated soil for treatment in a controlled closed bioreactor system with addition of water and specific degraders (Nano *et al*., 2003; Khan *et al*., 2004). This is carried out in three phases; the fill step (soil sieving, slurry preparation and reactor feeding), the react step (mixing and aeration) and the draw step (discharging and dewatering). The slurry formed is mixed to suspend the solids, increase aeration and contact between microbes and contaminants. As a result, degradation occurs at a fast rate ranging from less than a month to over 6 months. The slurry is dewatered for the soil to be disposed after the treatment process (Nano *et al*., 2003). Bioslurry is useful for soils and sediments contaminated with semi-volatile and volatile organic compounds, pesticides, explosives and PCB (Saponaro *et al*., 2002; Nano *et al*., 2003). Bioreactors are relatively simple, versatile and effective but the treatment process incurs extra costs from extensive site characterisation, excavation and dewatering (Khan *et al*., 2004).

4. Bioventing

This is an *in situ* treatment method that involves injection of air into the unsaturated or vadose zone of a contaminated media to increase microbial activity and optimize *in situ* biodegradation while avoiding the release of volatile compounds into the atmosphere (Khan *et al*., 2004; Scullion, 2006). This method is effective for a variety of petroleum products especially the mid weight products such as diesel unlike the lighter products which tend to volatilise easily and hence better suited for vapour extraction treatment. Bioventing technology is easy to set up and combine with other technologies with treatment achieved within a short term (6 -24 months) but it is not suitable for soils with high clay content or low permeability (Baker and Moore, 2000; Khan *et al*., 2004).

5. Biopiles

This is also known as biocells, bioheaps, biomounds, compost cells or heap pile bioremediation (Khan *et al*., 2004; Scullion, 2006). This treatment technology involves piling contaminated soils into piles or heaps about 2- 6m high and enhancing microbial activity by aeration through an underground system, addition of minerals, nutrients and water, pH and temperature control (Jorgensen *et al*., 2000; Khan *et al*., 2004; Scullion, 2006). To reduce leaching of contaminants into uncontaminated soils, treatment sites are covered with an impermeable liner. The heaps may also be covered to increase temperature and prevent evaporation, volatilisation and run off. Biopiles are similar to land farming but in the latter a larger area of land is required and aeration is achieved by agronomic practices such as tillage and ploughing. Biopiling is effective for the treatment of soils with organic contaminants such as petroleum hydrocarbon, chlorophenols, nitroaromatics and PAHs and pesticides with a

treatment period of 6-24 months (Jorgensen *et al*., 2000; Khan *et al*., 2004). Biopiles are easy to design but may not be effective for sites with TPH content of over 50,000 ppm and degradation rate may be affected by presence of high concentrations of heavy metals (Khan *et al*., 2004; Scullion, 2006).

2.5.3.2 Phytoremediation or Plant-mediated bioremediation

Phytoremediation, a new approach for the clean-up of contaminated sites, involves the use of plants or plants and their associated symbiotic microorganisms (Glick, 2003; Morikawa and Erkin, 2003). Germida *et al*. (2002) described phytoremediation as an ecological engineering that exploits the natural symbiotic relationships between plants and microorganisms. By different processes, phytoremediation could be used to detoxify, sequester, degrade or stabilize various environmental contaminants and possess the potential to be a sustainable waste management technology (Glick, 2003). Although the term "phytoremediation" derived from "*phyto*"-"plant" and "*remediation*" to "correct evil" was invoked in the 1980s, the remediation of radionuclide-contaminated soils with plants was investigated as early as the 1950s (Gerhardt *et al*., 2009). Interestingly, fundamental information about phytoremediation was compiled from a variety of research areas including constructed wetlands, oil spills, and agricultural plant accumulation of heavy metals (USEPA, 2000).

On close observation of the pathway used by plants to metabolize pesticides, Sandermann (1994) proposed the "*green liver concept*" suggesting the detoxification process which involves transformation, conjugation and sequestration in plants is quite similar to that of mammalian liver. This model describes the fate and disposition of organic compounds in

plants (Singh and Jain, 2003). Some conventional plants used in phytoremediation include grasses, sunflower, corn, hemp, flax, alfalfa, tobacco, willow, Indian mustard and poplar (Macek *et al*., 2009).

In the last decade there have been significant advances in modern phytoremediation following the elucidation of molecular and biochemical plant metabolism with respect to various chemical compounds (Morikawa and Erkin, 2003; Gao, *et al*., 2007). There are a number of advantages of phytoremediation compared to the physicochemical processes. It is a natural solar-driven clean-up technology with lower carbon foot print and is less labour intensive. The *in situ* nature of the process means minimal environmental disruption preserves the biological activity of the soils and the rhizosphere contributes to the microbial diversity of the soil. Phytoremediation is also a cost-effective approach typically 60-80% or even less costly than the conventional physicochemical methods of remediating contaminated soils and ground water (James and Strand, 2009; Alkorta and Garibisu, 2001; Mc Guinness and Dowling, 2009); for example, treatment of petroleum contaminated sites by phytoremediation costs about $\$162/m^3$ compared to $\$810/m^3$ for excavation and incineration (Zhang *et al*., 2010).

Other benefits of this technology include the fact that it is eco-friendly and accepted by the public as an attractive green biotechnology, it is a remedial strategy for a range of contaminants (organic and inorganic) present in soil, water and air through different processes (Cunningham *et al*., 1996, Morikawa and Erkin, 2003, Pilon-Smits, 2005, Zhuang *et al*., 2007, Macek *et al*., 2009 and Wenzel, 2009). It also facilitates site restoration, erosion control and biofuel production. On the other hand there are disadvantages of this technology. Contaminant concentration and toxicity affects vegetation and contaminants accumulated in

leaves may be reintroduced into the environment (Schnoor *et al*., 1995; Macek *et al*., 2000). As a result of phytoremediation, increased solubility of some contaminants may have greater adverse effects on the environment and the process of remediation may be slower than that of other technologies (Cunningham *et al*., 1996; Macek *et al*., 2000).

In developing countries where resources are lacking or insufficient, phytoremediation would be one of the few economically viable methods. Consequently, various researchers have focused on optimizing the processes of phytoremediation (James and Strand, 2009). For the above-mentioned advantages and need for optimization, investments in phytoremediation programs increased from $50 million in 1999 to $300 million in 2007(Van Aken *et al*., 2010). The fundamental processes of phytoremediation are described below:

1. Rhizodegradation

This is also known as rhizoremediation, phytostimulation, rhizosphere biodegradation, enhanced rhizosphere biodegradation and plant assisted biodegradation. This involves the breakdown of contaminants by microbial communities in the rhizosphere stimulated by rhizodeposition from plants (Alkorta and Garbisu, 2001; Germida *et al*., 2002; James and Strand, 2009) and co-metabolism of organic contaminants by diverse microbial populations in the rhizosphere (Cunningham *et al*., 1995; Chen *et al*., 2003; Tang *et al*., 2010a).

This process may be natural; involving the use of indigenous microbes or artificial when bioaugmentation with specific microbes is employed (Glick, 2010). Although plants and microorganisms are capable of degrading petroleum hydrocarbons and other organic compounds independently, degradation of petroleum hydrocarbons is primarily attributed to the plant-microbe interactions in the rhizosphere. Target compounds such as TPHs, PAHs,

pesticides and explosives possess high lipophilicity and hydrophobicity (high K_{OW}) hence low bioavailability for plant uptake and transformation and a high tendency of remaining in the roots (Macek *et al*, 2000; Alkorta and Garbisu, 2001). Thus, this phytoremediation approach is mainly exploited for the treatment of petroleum contaminated soils (Macek *et al*., 2000; Germida *et al*., 2002; Kamath *et al*., 2004; Tang, *et al*., 2010a,b). An increased attention to the potential and use of phytoremediation for restoration of soils contaminated with organics is attributed to the demonstration of an increased degradation rate of organic compounds in vegetated soil compared to unvegetated soils (Escalante-Espinosa *et al.,* 2005; Glick, 2010; Tang *et al*., 2010a,b; Zhang *et al*., 2010) (Table 2.2).

Table 2.2: Some reported cases of successful rhizodegradation of hydrocarbon contaminated soils.

Plant	**Microorganisms**	**Effects of plant-microbe synergy**	**Reference**
Cyperus laxus Lam.	*Bacillus cereus, Pseudomonas sp., Gordonia rubripertincta, Kocuria rosea, Arthrobacter oxydans, Bacillus subtilis, Micrococcus luteus, Penicillium janthinellum, Aspergillus terreus and Aspergillus carneus.*	55% TPH removal after 60 days and 90% TPH removal after 180 days	Escalante-Espinosa *et al*. (2005)
Gossypium hirsutum Linn	Effective microbial agents (*Acinetobacter radioresistens, Rhodococcus erythropolis*) PGPB (*Azospirillum brasilence*)	~ 55% TPH removal	Tang *et al*. (2010a)
Astragalus adsurgens	Rhizosphere microorganisms	~80% removal of diesel fuel with 60-70% removal of recalcitrant fraction over a period of 2 years.	Lin *et al*. (2008)
Lolium perenne, *Festuca arundinacea*, *Secale cereale*, *Hordeum vulgare*	PGPB - Pseudomonas strains, UW3 and UW4	PEP decreased TPH concentration from 130g/kg to ~50g/kg over 3 years	Gurska *et al*. (2009)
Viccia faba, Zea mays, and *Triticum aestivum*	Rhizosphere microorganisms	84.6%, 69.7% and 64.0% PAH reduction	Diab *et al*. (2008)
Lolium multiflorum	*Pseudomonas putida*	55% TPH removal	Kuiper *et al*. (2001) and Kuiper *et al*. (2004)
Alhaji cameleron L., Amaranthus retroflexus L., Convolvulus arvensis L., Chrozophora hierosolymitana Spreg., Noea mucronata L., Polygonum aviculare L.,	*Root associated fungi*	Decrease in TPH concentration from 5% to 0.9-1.2% over a 6 month period.	Mohsenzadeh *et al*. (2010)
Tripsacum dactyloides Panicum virgatum and *Carex stricta*	*Rhizosphere microbes*	70% TPH reduction after one year	Euliss *et al*. (2008)
Tall fescue	*Azospirillum brasilense* Cd *Enterbacter cloacae* CAL2 *Pseudomonas putida* UW3	55% removal of 16 priority PAHs within 120 days	Huang *et al*., (2004b)
Cyperus rotundus L.	Rhizospheric and endophytic microorganisms	42% TPH decrease over a period of 14 months	Diogo *et al*., (2010)
Lolium annual and *Strenotaphrum secundatum*	Indigenous microorganisms	42% and 50% TPH and PAH loss respectively at a crude oil spill site over 21 months.	

The key players of rhizodegradation of contaminants include the rhizosphere, rhizodeposits and root exudates, enzymes and soil microorganisms.

I. Rhizosphere

Lorenz Hiltner in 1904 first described the rhizosphere to include plant roots and their surrounding soil (Berg and Smalla, 2009 and Hartmann *et al*., 2009). The rhizophere may be better described as a complex dynamic microenvironment between 1-5 mm of the plant roots created by the interaction between diverse soil microbial communities and plant roots. Due to the complexity of the rhizosphere, it has been dubbed "hidden half" or "dark half" (Dzantor *et al*., 2007). The rhizosphere is made of three actively interacting components; the rhizosphere (soil), the rhizoplane and the plant root. The rhizosphere refers to soil that directly surrounds plant roots and receives exudates which influence soil microbiota while the rhizoplane refers to the root surface with its strongly adhering root particles. The root is taken as a component due to colonisation by unique microbes called endophytes. The presence of plant roots improves microbial biomass indirectly by its effect on carbon dioxide and oxygen concentrations, redox potential, moisture content, osmotic potential and soil pH (Lin *et al*., 2008). There are two interactions in the rhizosphere of importance; the detritus-based interaction and the living plant root interaction. The detritus-based interaction (dead plant material) determines the flow/fluxes of energy and nutrients while the living plant root interaction refers to the interaction between living plant roots and soil microbiota (Barea *et al*., 2005). Furthermore, the rhizosphere plays an important role in the flow, storage, maintenance and turnover of soil organic matter (SOM). The carbon from SOM originates mainly from the carbon flow, decomposed shoots, roots and litter. The flow of carbon is mediated in the rhizosphere by low molecular weight compounds such as carbohydrates,

amino acids and organic acids which are readily assimilated by soil microbes or degraded by extracellular enzymes prior to uptake. This carbon pool is important for soil microbial processes, microbial metabolism and physiology (Rangel-Castro *et al*., 2005).

II. Rhizodeposits and root exudates

Carbon containing compounds released from plant roots are collectively known as rhizodeposits. These include breakdown products of dead sloughed off root cells and tissues, mucilage, volatiles, lysates and exudates from damaged or living cells (Dennis *et al*., 2010). Plant root exudates consist of organic and inorganic substrates, low and high molecular weight organic acids (10-20 mM), sugars (90 mM) and amino acids (10-20 mM) (Singer *et al*., 2003; Dzantor *et al*., 2007) (Table 2.3). Mucilages (polysaccharide) which make up 2-12 % of rhizodeposits are secreted from the metabolically active border cells at the root cap. They also play a significant role in stimulating bacterial sporulation, suppression of pathogens, chemotaxis and competition (Dakora and Phillips, 2002; Berg and Smalla, 2009). Rhizodeposits influence rhizospheric microbial communities and their interactions such that their microbial populations are 5 to 100 times greater than those in the bulk soil (Narasimhan *et al*., 2003; Prosser *et al*., 2006). Consequently, the rhizosphere is characterized by a high microbial biomass (10^9-10^{12} per gram of soil) and highly intense and most diverse plant-microbe interactions. This effect of the plant exudates on microbial populations and activities in the rhizosphere is called the rhizosphere effect. The root exudate may also selectively stimulate proliferation of specific bacterial populations such as xenobiotic degraders and serve as pollutant analogues such as flavonoids or co-metabolites of organic pollutants. Previous research has shown that exudates containing phenols support the growth of PCB-degrading bacteria in the rhizosphere (Alkorta and Garbisu, 2001; Singer *et al*., 2003). Kamath *et al*.

(2004) showed that root exudates affect the activity and abundance of PAH degraders while a recent pioneering study by Cebron *et al*. (2011), showed that root exudates affect microbial diversity of PAH degraders.

Table 2.3: Rhizodeposits and their functional role in the rhizosphere. Compiled from Dakora and Phillips (2002), Germida *et al*. (2002) and Dennis *et al*. (2010).

Rhizodeposits released by plant roots	**Functions**
Root exudates	
Sugars: arabinose, fructose, galactose, glucose, maltose, mannose, mucilages, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose, deoxyribose	Carbon source.
Amino acids: α-alanine, β-alanine, γ-aminobuytric, α-aminodipic, arginine, asparagine, aspartic, citrulline, cystathionine, cysteine, cystine, deoxymugineic, 3-epihydromugineic, glutamine, glutamic, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, mugineic, ornithine, phenylalanine, pranine, proline, serine, threonine, tryptophan, tyrosine, valine	Nutrient source, chelators of poorly soluble mineral nutrients, chemoattractant signals to microbes.
Organic acids: Acetic, aconitic, ascorbic, aldonic, benzoic, butyric, caffeic, citric, *p*-coumaric, erythronic, ferulic, formic, fumaric, glutaric, glycolic, lactic, glyoxilic, malic, malonic, oxalacetic, oxalic, *p*-hydroxybenzoic, piscidic, propionic, succinic, syringic, tartaric, tetronic, valeric, vanillic	Nutrient source, chelators of poorly soluble mineral nutrients, chemoattractant signal molecules, soil acidifier, Aluminium detoxifiers, *Nod* gene inducers.
Fatty acids, sterols, alcohols, tannins and alkaloids	Carbon source.
Flavonones and nucleotides	Nutrient source.
Vitamins and growth factors: *p*-amino benezoic acid, biotin, choline, *N*-methyl nicotinic acid, niacin, pathothenic, thiamine, riboflavin, pyridoxine, pantothenate	Nutrient source, plant and microbial growth promoters.
Enyzmes Amylase, invertase, peroxidise, phenolase, acid/alkaline phosphatase, polygalacturonase, protease	Catalysts for phosphorus release from organic molecules.
Root border cells	Signalling for mitosis and gene expression regulation, release of chemoattractants, prevention of root cap infection and synthesis of defence molecules.

III. Soil microorganisms

Soil organisms which include bacteria and fungi are involved in a variety of symbiotic and saprophytic relationships which may be detrimental or beneficial to the plant. The detrimental microorganisms include agents of plant diseases while the beneficial microbes include decomposers of organic detritus, plant growth promoting rhizobacteria (PGPR) and microbial antagonists of root pathogens all promote plant growth and development. Other beneficial microbes include degraders, endophytes, nitrogen-fixing bacteria, phosphate solubilising bacteria and arbuscular mycorrhizal fungi (AMF) (Barea *et al*., 2005).

The activities of rhizospheric microorganisms affect the rooting patterns, supply of nutrients to plants and hence affect the quality and quantity of root exudates released (Barea *et al*., 2005). Soil microbial communities play important roles in the production of compounds that increase the rate of root exudation (Dzantor *et al*., 2007), improvement of tolerance to drought stresses, salinity and toxicity (Diogo *et al*., 2010), control of pathogens (by the production of hydrogen cyanide, siderophores and antibiotic production), soil enrichment and degradation processes (Narasimhan *et al*., 2003; Rangel-Castro *et al*., 2005; Prosser *et al*., 2006) and soil microaggregate formation (2-20 μm in diameter).

Due to the nutritional richness of the rhizosphere, bacterial populations characterized by diverse metabolic capabilities which facilitate degradation are up to 2-4 orders greater than those present in the bulk soils but little is known about their composition (Alkorta and Garbisu, 2001; Kuiper *et al*., 2004; James and Strand, 2009). The hydrophobic nature of petroleum hydrocarbons can adversely affect microbial communities due to disturbances to water, nutrient and oxygen availability leading to changes in microbial diversity and soil microbial processes over a long period. Studies have shown that although petroleum

hydrocarbon contamination may lead to an initial loss of bacterial diversity, it leads to the emergence of metabolically competent populations fit to survive in the new environment (Alonso-Gutiérrez *et al*., 2008).

Important groups of soil bacteria that degrade hydrocarbons include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Cycloclasticus, Bukholderias, Sphingomonas, Micrococcus, Cellulomonas, Dietzia*, *Gordonia* and *Mycobacterium* (Watanabe, 2001; Samanta *et al*., 2002; Alonso-Gutiérrez *et al*., 2008; Santos *et al*., 2011; Puškárová *et al*., 2013).

IV. Enzymes

Apart from the exudates secreted by plants into the rhizosphere, plants and soil microorganisms may also secrete enzymes (Table 2.4) that degrade organic contaminants following their release into soils (Wenzel, 2009). The phases of xenobiotic transformation in plants involve transformation, conjugation and compartmentation reactions. Oxygenation reaction is very important to increase water solubility and enhance conjugation of highly lipophylic compounds (Macek *et al*., 2000).

Table 2.4: Examples of enzymes involved in degradation of contaminants.

Enzyme	Catalytic action	Examples of sources	References
Nitroreductase	degrade various nitroaromatic compounds and ammunition wastes	*Comamonas* sp., *Pseudomonas putida* and *Populus* spp.	Alkorta and Garbisu, (2001); Singh and Jain (2003); Morikawa and Erkin, (2003); Gerhardt *et al*. (2009); Wenzel, (2009).
Dehalogenase	degrades organophosphate pesticides and chlorinated solvents, such as hexachloroethane	*Xanthobacter autotropicus, Sphingobium chlororphenolicum* and hybrid poplars	Morikawa and Erkin, (2003); Gerhardt *et al*. (2009).
Laccase	degrades lignin, dioxins and polychlorinated biphenyls (PCB).	bacteria, fungi, insects, and other higher plants	Morikawa and Erkin, (2003); Gerhardt *et al*. (2009).
Peroxidase	Involved in reductive dehalogenation of aliphatic hydrocarbons and fungal degradation of PAHs and dioxins.	*Phenerochaete chrysosporidium*, *Phenerochaete laevis* and *Medicago sativa*	Morikawa and Erkin (2003) and Gerhardt *et al*. (2009).
Nitrilase	cleaves cyanide groups from nitriles	willow and *Aspergillus niger*	Morikawa and Erkin (2003).
Cytochrome P450 monooxygenase	Catalyse addition of oxygen into steroids, fatty acids and xenobiotics; In plants, the P450s catalyse some reactions in the synthesis of flavonoids, alkaloids, hormones and detoxification of xenobiotics.	microorganisms, insects and plants	Macek *et al*. (2000); Singer *et al*. (2003); Gerhardt *et al*. (2009).

Other enzymes of importance in phytoremediation include carboxylesterases, transferases such as the gluthathione S-transferases (Macek *et al*., 2000), oxygenases, phosphatases and dioxygenases (Gerhardt *et al*., 2009).

2. Phytodegradation

This is also known as phytotransformation. It exploits the metabolic capabilities of plants to degrade or transform organic (PAHs and TPHs) and inorganic (atmospheric nitrogen oxide and sulphur oxides) pollutants, internally through metabolic processes or externally by extracellular enzymes. Direct phytoremediation (*in planta*) as is the case in phytotransformation involves processes such as uptake and diffusion from the roots to the truck and leaves for transformation and sequestration as in phytodegradation (Cunningham *et al*., 1995; James and Strand, 2009). The uptake of organics is usually determined by factors such as octanol-water partition coefficient (Log K_{OW}), acidity constant, (pka) and concentration (Alkorta and Garibsu, 2001; Morikawa and Erkin, 2003; Dzantor, 2007; Wenzel, 2009). There is little evidence that plants directly degrade petroleum hydrocarbons but enzymes of plant origin have been found to transform contaminants in soil and sediments. These include dehalogenase, nitroreductase, peroxidase, laccase and nitrilase (Germida *et al*., 2002).

3. Phytostabilization

This involves concentration and containment of certain heavy metals and organic compounds in the root zone by adsorption and precipitation. Rhizospheric processes and soil additives facilitate precipitation and immobilization of soil contaminants preventing them from contaminating other ecosystem compartments such as the bulk soil, ground water and food chain. Arbuscal mycorrhizal fungi are particularly useful as they sequester heavy metals by chelation and adsorption (Morikawa and Erkin, 2003; Barea *et al*., 2005; Wenzel, 2009). Plant uptake and accumulation of petroleum hydrocarbon by this mechanism is not efficient (for example 2-8% and <2% accumulation of benzene in alfalfa shoots and roots respectively)

hence it may be employed to prevent migration of contaminant by erosion, leaching and dispersion. It would also allow incorporation of organic contaminants into humic materials. The latter involves binding contaminants into soil organic matter by plant enzymes or increase in soil organic matter content by humification which accounted for the mineralization of four PAHs in soils planted with deep-rooted prairie grasses (Germida *et al*., 2002).

4. Phytovolatization or rhizovolatilization

This volatilizes pollutants through metabolic reactions of plants and associated rhizospheric organisms, followed by the translocation to the stomata and in some cases the stem tissues and bark for release into the atmosphere. However, volatilized compounds in the atmosphere may be degraded or oxidized in the presence of hydroxyl radical. This is mainly used for contaminants treated by conventional air sparging such as BTEX, TCE, vinyl chloride, and carbon tetrachloride with a Henry constant value K_H >10 atm m^3 air but not suitable for compounds like phenol which has a Henry constant value K_H <10 atm m^3 air thus a relatively low volatility (Kamath *et al*., 2004). Examples of phytovolatilization applications include volatilization of trichloroethylene by poplar trees, methyltertiary butyl ether (MTBE) by eucalyptus and selenium by Indian mustard (Alkorta and Garibsu, 2001, Morikawa and Erkin, 2003, Dzantor, 2007 and Wenzel, 2009). This mechanism has raised concerns due to its potential for air pollution but it is not the main dissipation pathway used by most pollutants (USEPA, 2000 and Germida *et al*., 2002).

5. Evapotranspiration

This process is not involved in degradation but a form of containment useful in hydraulic control of groundwater by evaporation and vapourisation of water at the stomata of plants.

Contaminant containment is by plume capture through the formation of a cone of depression by plant roots within the aquifer to prevent off-site migration and downward migration of contaminants (Kamath *et al*., 2004). Evapotranspiration or vegetative cover systems were initial proposed for the arid and semi-arid region but may be applicable to other regions with climate and other factors in consideration (USEPA, 2000). Examples of plants involved in this process include phreatophyte trees such as poplar, eucalyptus, and river cedar which can transpire 200-1100 litres of water/day due to their deep roots (Morikawa and Erkin, 2003).

6. Phytoaccumulation

This is also known as phytoextraction or phytomining and is mainly used for sites with inorganic contaminants but may be useful for an integrated remediation approach for sites (soils, sediments, sludges and to a lesser extent water) co-contaminated with petroleum hydrocarbons and inorganic pollutants especially heavy metals as commonly encountered (Schnoor *et al*., 1995, Cunningham *et al*., 1996 and USEPA, 2000). This treatment approach involves extraction of inorganic contaminants from soil and water by plant roots and their translocation to plant shoots followed by plant harvest for disposal or recycling since the mechanism involves concentration or accumulation rather than degradation. It is applicable to metals: silver, cadmium, cobalt, chromium, copper, mercury, manganese, molybdenum, nickel, lead and zinc and metalloids such as arsenic and selenium (USEPA, 2000 and Zhang *et al*., 2010).

There are over 400 known species of hyper-accumulator plants present throughout the plant kingdom including metal hyper-accumulators that can accumulate over 1.0% manganese, 0.1% (copper, cobalt, lead, nickel and zinc) and 0.01% cadmium of leaf dry matter. The mechanism of uptake may be similar to that used for the uptake of metals or nutrients required

for example as co-factors for enzymatic activities. Following the uptake process, they are stored in vacuoles to protect the plant against their toxic effects. Such plants have been exploited at the Chernobyl nuclear power plant accident site in Ukraine for heavy metal remediation (Morikawa and Erkin, 2003; Macek *et al*., 2009; Wenzel, 2009).

Most hyper-accumulator plants are unsuitable for phytoremediation processes due to their low biomass formation which is of importance (Macek *et al*., 2009). Plant biomass production especially the root system has impact on the rhizosphere activity, microbial biomass and metabolism (Lin *et al*., 2008). Another important criterion for selection of suitable candidates is the effectiveness of accumulation. As for contaminated water, water plants, micro-algae, root filters and immobilized bacteria are suitable candidates (Macek *et al*., 2009). Like phytoaccummulation, rhizofiltration is also a concentration technology but involves root accumulation and subsequent harvest in hydroponic media. This is mainly useful for treating contaminated water and was demonstrated by the US Department of Energy on sites with radionuclides (USEPA, 2000). Advantages include the use of harvest plants as a resource; for example biomass containing the essential nutrient selenium can serve as animal feed, while disadvantages include the slow growth rate, small biomass and shallow root systems of metal accumulators, phytotoxic effect of metals and need for harvest and recycling of plant biomass (USEPA, 2000).

Other processes used in phytoremediation include the use of artificial wetlands and lagoon systems and use of transgenic plants (genetic engineering) to suit phytoremediation needs (Macek *et al*., 2009; Zhang *et al*., 2010; Megharaj *et al*., 2011; Jadeja and Batty, 2013).

2.6 Plant Growth Promoting Bacteria/Rhizobacteria (PGPB/PGPR)

The PGPB are of two groups: symbiotic bacteria and free-living bacteria. They are characterized by root colonization, survival and multiplication in the root atmosphere and enhancement of plant growth despite competition with other soil microorganisms. PGPB are involved in the synthesis of specific compounds, uptake of nutrients and prevention of plant diseases. Consequently, the PGPB are important in the biological control of plant pathogens, nutrient cycling and seed growth. Typical examples are members of the genera *Pseudomonas*, *Bacillus* and *Azospirillum* (Barea *et al*., 2005). Apart from the nitrogen-fixing activity of the latter, they are known to secrete an auxin-type phytohormone with effects on root morphology and also enhances nutrient uptake. As biocontrol agents, PGPB may release antibiotics and antifungal factors that suppress saprophytic growth of pathogens and root infections or trigger an "induced systemic resistance" (ISR) in plant. Typical examples with a combination of both mechanisms are *Rhizobacteria* and filamentous fungi *Trichoderma.* The former releases phenazine while the latter releases antibiotic gliotin, gliovirin, peptabiols and a battery of lytic enzymes such as chitinases, glucanases and proteases (Barea *et al*., 2005).

2.6.1 Mechanism of growth promotion by PGPB and potentials in phytoremediation

When plants are under stress, ethylene, a plant hormone, is produced and suppresses plant growth; however the PGPB are known to promote growth. PGPB promote plant growth by degrading amino-cyclopropane carboxylic acid (ACC), the precursor to ethylene by the synthesis of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) (Zhuang *et al*., 2007).

According to Zhuang *et al*., (2007) the combination of PGPB and plants for phytoremediation may not be effective but the use of specific degraders has been found to be effective in a multi

process phytoremediation system (MPPS) for soils contaminated with total petroleum hydrocarbon (TPHs). However, recent studies by Gurska *et al.* (2009) have shown PGPB enhanced phytoremediation (PEP) is effective in the remediation of TPH-contaminated soils. Beneficial microbes adapted to the rhizosphere in form of bio-inoculants may be introduced into plants by coating seeds with bacteria but successful root colonization varies amongst bacterial species and strains (Kuiper *et al*., 2004). Kuiper *et al*. (2001) hypothesized that following successful root colonization, the adapted bacteria becomes integrated into the indigenous bacterial community, dispersed by the growing root system and enhance degradation of xenobiotics. A specific plant-microbial pair facilitated the successful degradation of naphthalene, prevented phytoxicity to the grass seeds and transported the bacteria beyond layers the roots can reach (Kuiper *et al*., 2004).

As the understanding of plant-microbe-contaminant interaction improves, rhizospheric and endophytic PGPB are increasingly exploited for enhanced plant growth and stress tolerance especially contaminant stress for efficient degradation and prevention of soil erosion during in phytoremediation approaches such as rhizodegradation of organic compounds including petroleum hydrocarbons (Glick, 2010; De-Bashan *et al*., 2012). The full exploitation of PGPB technology will depend on regulations that support use of genetically engineered bacteria after assuring the scientific community and the public of their safety with research findings in the future (Glick, 2010).

2.7 Limitations of Phytoremediation Processes and Possible Solutions

Before selecting a phytoremediation approach, technical consideration such as suitability and effectiveness of any technique for the given site, establishment and survival of plants,

possibility of remediation within an acceptable time frame, evaluation of remediation success and identification of a contingency plan in case it is unsuccessful must be considered (USEPA, 2000; Pilon-Smits, 2005). Although phytoremediation has been shown to be an efficient and cost-effective approach to the restoration of contaminated sites with various organic and inorganic pollutants, it is faced with some limitations. These include high concentration and toxicity of contaminants, bioavailability of contaminants, incompetence of plants and indigenous microorganisms, low nutrient levels and poor soil conditions (Schnoor *et al*., 1995; Semple *et al*., 2003; Hosokawa *et al*., 2009; Vangronsveld *et al*., 2009; Vazquez *et al*., 2009; Wenzel *et al*., 2009; Perelo *et al*., 2010; Van Aken *et al*., 2010).

i. **Plant candidate**: From the perspective of the plants, phytoremediation is mainly suited for shallow contamination with compounds of moderate hydrophobicity within the reach of plant roots. The autotrophic metabolism of plants means they may lack the required biochemical pathways for the complete degradation of some recalcitrant compounds resulting to the release of some toxic intermediate metabolites into the soil, atmosphere and food chain (Van Aken *et al*., 2010). According to Tang *et al*. (2010a), the order of effectiveness for rhizoremediation of TPH is tall fescue>ryegrass>alfalfa>cotton. These inherent limitations do not only emphasize the need for careful selection of plant candidates but also gave rise to the idea of using fast growing well adapted plants with deep rooting systems (Kuiper *et al*., 2004), transgenic plants with bacterial or mammalian genes and the use of genetically modified bacteria with more efficient degradative capabilities for remediation (Kuiper *et al*., 2004; Van Aken *et al*., 2010). Another strategy is the use of a combination of plants and biodegradative bacteria with plant growth promoting property which bind to the roots or colonize internal tissues. This has been successfully exploited in greenhouse as well as field trials for soil

contaminated with petroleum products, PAHs and halogenated compounds (Glick, 2010). Selected plants candidates such as grasses and legumes must be able to thrive on contaminated soils. Some of the desirable features required include a fibrous root system due to the large surface area of the rhizoplane to be colonized by soil microbes, high biomass production (Escalante-Espinosa *et al*., 2005). Grasses have fibrous root systems which form a dense and extensive rhizosphere for microbial colonization, proliferation and degradation. On the other hand, legumes are capable of stimulating metabolically diverse microbes in addition to their nitrogen fixing ability which is crucial in contaminated soils, usually nutrient deficient (Adam and Duncan, 2002 and Hall *et al*., 2011).

ii. **Plant growth rate and root systems**: Plant growth rate is slow hence phytoremediation processes may take several years unlike excavation and disposal or incineration that take weeks to months. As a result, it may not be appropriate for sites with acute risks to humans and other ecological components (Schnoor *et al*., 1995). Also, plant root depth varies with species, soil and climatic condition. This is an important limitation as the roots need to be in contact with the pollutants by root elongation or movement of contaminated media within plant reach for phytoremediation to take place. This may be achieved by the use of agricultural equipment for deep ploughing to bring soils 2-3 feet deep within 8-10 inches within the reach of grasses or the irrigation of trees and grasses with contaminated water. But this may lead to emission of volatile organic compounds hence a need for risk assessment. The maximum root depth and target contaminants for Indian mustard, grasses, and poplar is 12 inches (metals), 48 inches (organics) and 15 feet (metals, organics and chlorinated solvents), respectively (USEPA, 2002).

iii. **Contaminant concentration and toxicity**: The most appropriate sites for phytoremediation are those with widespread, low to moderate contaminant concentration within the root zone (USEPA, 2000). However, most contaminated soils are faced with the problems of toxicity due to mixed contamination and high concentrations, poor soil condition, nutrient deficiency and water stress which together makes it difficult to support vegetation. Depending on the type of contaminants and the concentration inhibition of plant growth due to toxicity can vary. In the case of petroleum contaminated soils, the most important factor that affects rhizoremediation is the TPH content (Tang *et al*., 2010b). TPH concentration determines the diversity and abundance of the total microbial community especially the hydrocarbon degraders thus, a high concentration of TPH influences rhizoremediation due to toxicity to microbes and plants and effects on growth and biomass production (Tang, *et al*., 2010a,b). A study by Gurska *et al*. (2009) on ryegrass and PGPB in a site with 13% TPH content showed a 61.5% degradation rate over a period of 3 years while Huang *et al*. (2005) showed at 5% TPH content, 90% of all TPH fractions were removed. More recently, Tang *et al*. (2010b) showed that the optimum TPH concentration that will facilitate rhizoremediation with minimal effects to plants and associating microbial populations is 5%. From the results of these studies, it is evident that TPH concentration should be considered as crucial to the success of rhizoremediation. For sites with high concentration, an integrated or tiered remedial approach (short term *ex situ* techniques followed by *in situ* phytoremediation) may be most appropriate (USEPA, 2000).

iv. **Competence of indigenous microbes**: Similarly, contaminated sites may have low numbers of autochthonous organisms capable of degrading the target contaminant or even lack microbes with the required enzymatic machinery especially in sites with high

concentration of pollutants. This may lead to slow or incomplete degradation of recalcitrant compounds (Hosokawa *et al*., 2009; Vazquez *et al*., 2009). The degradation rate may be only between 9.1-20% higher than that of the control soil (Tang *et al*., 2010a) hence the need for bioaugmentation which refers to addition of pollutant-degrading bacteria whether wild or genetically modified strains with desirable enzymes to supplement indigenous microbial communities for a more efficient rhizoremediation (Hosokawa *et al*., 2009; Vazquez *et al*., 2009; Tang *et al*., 2010b). A number of studies have shown that bioaugmentation with PGPB and/or effective microbial agents (consortia) improved degradation rates of organic contaminants such as TPH (Escalante-Espinosa *et al*., 2005; Lin *et al*., 2008; Tang *et al*., 2010b).

Successful adaptation and survival of inoculants depends on environmental conditions, soil properties, protozoa predation, competition with autochthonous organisms present hence sometimes this treatment may not be effective *in situ* especially when the inoculants are phylogenetically distant from the autochthonous organisms. TetraZyme; a seed culture was found to enhance degradation of oil on the coast of Japan sea after a heavy spill from a Russian tanker Nakhodka in 1997 (Hosokawa *et al*., 2009). Due to the fact that the proliferation of inoculants is affected by chemical structure and concentration of the pollutants, bioavailability, microbial community and physical environment, a number of studies on bioaugmentation treatment have concluded that it would be more appropriate to use autochthonous organisms from the contaminated site as inoculants. This is called the autochthonous bioaugmentation or the ABA technology or reinnoculation. Also, the use of recombinant strains of autochthonous organisms rather than exogenous organisms has prospects in phytoremediation. But like other GMOs, bioaugmentation with genetically engineered organisms (plants and microbes)

has not been accepted by the public despite their great potential (Hosokawa *et al*., 2009; Vazquez *et al*., 2009).

v. **Nutrient deficiency**: Nutrient concentrations especially nitrogen and phosphorus are also crucial to the success of phytoremediation because plant and microbial growth rates and degradation are affected due to resource competition. Hence this is addressed by a treatment process termed biostimulation which involves appropriate application of nutrients to a contaminated site or the use of nutrient fixing organisms (Vazquez *et al*., 2009). According to Chaineau *et al* (2005) biostimulation may stimulate the growth of soil bacteria, but it may not necessarily enhance the rate or efficiency of phytoremediation of crude oil contaminated sites. However, a study by Tang *et al*., (2010a) showed that fertilizer application had beneficial effects on remediation as increase in tall fescue biomass was shown with biostimulation with urea (<20 g/Nm^2) but a decrease in biomass was noticed with an increase from 20 to 30 g/Nm^2 due to salt toxicity. Statistical analyses showed a positive correlation between fertilizer application and TPH degradation rates but only moderate fertilizer application is recommended for plant growth and enhanced TPH degradation.

Nutrient deficiency may also be addressed by an integrated phytoremediation system which may involve inoculation with phosphorus- and iron-solubilizing bacteria, pollutant resistant mycorrhizal fungi, co-cropping of different species to support the growth of diverse microbial communities at the rhizosphere for example, growth of legumes to support to nitrogen-fixing bacteria and metal resistant PGPR, also the use of metal extraction crops such as willow and rhizodegradation plants will be useful for mixed contaminated sites (Wenzel *et al*., 2009).

vi. **Bioavailability**: This is perhaps the most important limitation of bioremediation as a whole. Bioavailability can be defined as the fraction of a compound that is readily available for uptake, utilization and transformation by living organisms from its immediate surroundings (Vangronsveld *et al*., 2009; Wenzel *et al*., 2009). This depends on the chemistry of the compound (solubility, diffusion and mass transport to niches with degraders) and soil factors (porosity, water content and diffusivity). The solubility of an organic compound is affected by soil properties, soil composition (mineral, clay and organic matter content), pH, redox potential and the presence of sloughed-off cells, mucilage and root debris. The bioavailability of organics is affected by the octanol-water partition coefficient K_{OW}, high chemical stability, vapour pressure which indicates the rate of pollutant volatilization in dry soils and Henry's constant which measures the volatilization potential in wet and flooded soils (Vangronsveld *et al*., 2009). Over time, organic pollutants tend to bind to mineral and organic matter in the soil matrix, absorb into humic complexes and pores, becoming less available for uptake and utilization by living organisms. This process is called "ageing" (Hatzinger and Alexander 1995; Semple *et al*., 2003).

However, bioavailability is enhanced naturally by roots which improve the mass flow and diffusion of water and pollutants through the creation of pores, exudation of biosurfactants, induction of mass flow towards the rhizosphere and transportation of dissolved pollutants and microbes to niches of diverse microbial communities and activity. Some microorganisms increase bioavailability by chemotaxis and exudation of biosurfactants such as rhamnolipids which have been used in soil washing for soils with crude oil (Wenzel *et al*., 2009). Promising approaches include selection of plants and microbes with the ability to exude biosurfactants which solubilise and transport organic

pollutants and engineering of plant-microbe systems with degradative genes such as *Rhizobium tropici* strain expressing 1,9a dioxygenase. Although this has not been carried out in field experiments due to ecological concerns, the use of biological containment systems to restrict changes to the rhizosphere may limit the risks (Wenzel *et al*., 2009).

A number of studies reviewed by Wenzel *et al.* (2009) showed that contaminants in freshly spiked soils are more bioavailable compared to those aged soils during phytoremediation. Bioaugmentation with degrader strains in soils with poor bioavailability may not enhance degradation but it may be facilitated by inducing nutritional bias towards the inoculants. However this involves selection, breeding and engineering plants to exude specific substrates (Wenzel *et al*., 2009). Also root exudates profiling to determine the quantity and chemical composition of exudates as well as their microbial utilization patterns are also crucial for the creation of nutritional bias and enhancement of degradation (Narasimham *et al*., 2003).

The combination of phytoremediation and electroremediation has also been proposed as a solution to some limitations of phytoremediation. Coupled phytoremediation-electroremediation technology involves the growing plants on contaminated soil to which low intensity electric field is applied. The electric field enhances remediation of contaminants by improving bioavailability through the processes of desorption and transportation of the contaminants (Cameselle *et al*., 2013).

2.8 Advances and Potential of Phytoremediation

Despite these limitations and challenges encountered, especially during field studies, phytoremediation has great potential yet to be fully exploited and this research area is still at

its infancy. However, increasing research in this area is gradually leading to advancement through experiments which involve the use of *in vitro* cultures such as hydroponic cultures, differentiated embryonic cultures, hairy root cultures, shooty teratomas and tissue cultures in comparison with natural plants to improve phytoremediation efficiency. These studies provide the advantage of standard laboratory conditions, rapid growth unaffected by weather and climate, and low cost of analysis (Macek *et al*., 2000; Gerhardt *et al*., 2009; Ali *et al*., 2013).

Moreover, the application of genetic engineering in the degradation of xenobiotics has been investigated since the early 1980s when first experimentation was carried out on microorganisms. Over the years, research has shown the possibility of over-expressing certain genes or insertion of genes of microbial or mammalian origin with degradative or accumulative ability into plants (pollution-removing GM plants) with desired agronomic characteristics to improve the efficiency of phytoremediation (Macek *et al*., 2009). An example is the insertion of the cytochrome P450 genes to improve plants' natural ability to withstand allelochemicals of natural origin and enhance degradation of toxic volatile compounds including trichloroethylene, benzene and chloroform. Typical examples of transgenic plants that have been designed include those expressing bacterial pentaerythritol tetranitrate reductase and bacterial biphenyl-chlorophenyl dioxygnase for the degradation of explosives and PCB respectively (Macek *et al*., 2009; Van Aken *et al*., 2010).

In the case of heavy metal accumulation, genetic manipulation targeting proteins involved in metal homoestasis such as metallothionins, phytochetin and gluthathione have been achieved by over-expressing gluthathione synthetase, gamma-glutamylcysteine synthetase and phytochelatin synthase (Macek *et al*., 2009; Ali *et al*., 2013). Also the formation of fusion

proteins by insertion of additional metal-binding domain with high affinity for heavy metals has been successful for cadmium, zinc and nickel (Macek *et al*., 2009; Ali *et al*., 2013).

Other possibilities include use of transgenic plants with features such as pest resistance, production of insect pheromones and improved root colonization, and the use of genetically modified symbiotic bacteria and/or arbuscular mycorhizal fungi for improved performance in the rhizosphere (Cherian and Oliveira 2005; Macek *et al*., 2009; Maestri and Marmiroli, 2011).

Noteworthy are problems with the use of genetically modified organisms (GMOs) such as legislative barriers, concerns about gene flow, poor survival rate when introduced into contaminated soils thus the need for better adapted strains such as root colonisers (Macek *et al*., 2009). However, the introduction of transgenes into chloroplast DNA and use of conditional lethal genes are possible approaches to addressing horizontal gene transfer (Van Aken *et al*., 2010).

In summary, the successes and efficiencies of phytoremediation greatly depend on the root and shoot systems of plant candidates, rate of root proliferation, presence of root activities that facilitate establishment of diverse and complex microbial communities and interactions in the rhizosphere (Wenzel, 2009). For these reasons, research on phytoremediation processes are now focused amongst other factors, on selection, traditional breeding, genetic engineering to increase pollutant tolerance, root and shoot biomass, root structure, pollutant uptake properties and degradation capabilities, genetic engineering of microbes, soil management and improvement of bioavailability (Wenzel, 2009). There are great prospects for the use of GM plants in the phytoremediation of agrochemical, industrial and accidental contamination but first there is a need to tackle legislative barriers and gain public acceptance through

further research and proper awareness (Macek *et al*., 2009, Van Aken *et al*., 2010; Ali *et al*., 2013).

CHAPTER 3

MATERIALS AND METHODS

The general methodology for determining the phytoremediation efficiency of selected plant candidates grown spiked soils and the effect of contaminants on plant biomass yield and soil microbial diversity investigated in this thesis consists of the following major steps; analysis of PAH and heavy metal concentration in two selected brownfield sites, greenhouse experiment on diesel-contaminated soil, greenhouse experiment on single and mixed PAH-contaminated soils and effects on soil microbial diversity, greenhouse experiment on mixed PAH and heavy metal-contaminated soils with PGPR inoculation. The design of the greenhouse experiments including the selected PAH and plant candidates are detailed in the experimental chapters.

3.1 Preparation of glassware

All glassware were soaked overnight with detergent, washed thoroughly and rinsed with distilled water. The glassware were then dried in an oven and rinsed with acetone before use. Glassware and syringes for organic analysis were rinsed with dichloromethane twenty times to avoid contamination and carryover of samples before and after use.

3.2 Soil properties of experimental soil

A sandy loam soil sourced from a commercial supplier (Travis Perkins, United Kingdom) was used for the greenhouse experiments. The soil properties assessed are detailed below.

3.2.1 pH and Conductivity

A soil suspension was prepared with soil and deionized water in 1:5 ratio (20 g of soil and 100 mL of water) and allowed to stand for one hour. Soil pH and electrical conductivity were measured using a portable combination probe (Hanna Instruments, United Kingdom) calibrated in accordance with the manufacturer's instructions.

3.2.2 Moisture content

About 10 g ± 0.001 g soil was weighed into a clean pre-weighed tared porcelain crucible and placed in an oven at 105°C overnight. The sample was then placed in a desiccator using tongs, allowed to cool and then weighed to a constant weight. The moisture content is expressed as a percentage via the following algorithm.

Moisture content = [(mass of air-dried soil – mass of oven-dried soil)/mass of air-dried soil] x 100 (Watts and Lyndsay, 1996).

3.2.3 Organic matter content by loss on ignition analysis

A clean dry porcelain crucible was placed in an oven at 100 °C for an hour then allowed to cool before taking the weight of the crucible (W1). About 5 g of 2 mm sieved soil was weighed in the pre-weighed crucible (W2) and dried in an oven at 105 °C for 24 hours. The pre-ignition weight after oven drying at 105 °C was measured and calculated (DW_{105}). The crucible was placed in the oven at 550 °C for 4 hours. The post ignition weight was taken and calculated as DW_{550} after heating the soil at 550 °C (Heiri *et al*., 2001 and Ribeiro *et al*., 2011). Organic matter is calculated as the loss on ignition at 550°C divided by two.

Loss on ignition (LOI_{550}) expressed as a percentage = $\{(DW_{105} - DW_{550})/ DW_{105})\} * 100$

Where;

DW_{105} is the dry weight of sample heated at 105°C

DW_{550} is the dry weight of sample heated at 550°C

Organic matter content = LOI_{550} /2

All samples were analysed in triplicate and the results are shown in table 3.1.

Table 3.1: Soil properties of experimental soil. Mean values ± SE (n=3).

Soil parameters	
pH	7.5
Conductivity (µS)	1450
Moisture content (%)	0.801±0.0314
Organic matter (%)	6.19

3.3 General method for preparation of experimental soil

Soils were air-dried in clean plastic trays and later sieved with a clean 2 mm sieve. About 250 g of sieved soil (25% of soil for planting in each pot) was spiked with contaminants (diesel/PAH/heavy metal) and mixed thoroughly to achieve homogeneity in a fume hood. The spiked soils were mixed with about 750 g of unspiked soil to make about 1 kg of soil. All spiked soils of the same group were mixed thoroughly and sieved through a 2 mm sieve for homogenization and then stored in separate labelled zip lock bags in the dark for four weeks. About 1 kg of spiked soil was placed in *Desch* plant plastic pots with the dimensions 13.5 cm; diameter, 11.7 cm height and a capacity of 1.12 liters with *Sankey* saucers to fit 13-15cm pot size (LBS horticulture, UK).

3.3.1 Soil sampling and transplantation of plant seedlings

Soil samples were taken from all treatment groups to assess the initial contaminant concentration and microbial diversity. Four-week old seedlings of *Medicago sativa*, *Festuca arundinacea* and *Lolium perenne* in perlites were sourced from a commercial supplier (Vegetable Plants Direct, United Kingdom). Excess compost on each perlite was gently removed from the seedling roots and the seedlings transplanted into spiked soils. Growth parameters for the green house experiment included; temperature of 25-28°C during the day, 15-20°C at night and 16 hours light/8 hours dark. Plants were watered with equal volumes of

water as required by the plants. The plant seedlings were thinned after two weeks to 20 seedlings per pot.

3.4 Plant harvest and soil sampling after greenhouse experiment

Following a growth period of 65 days, all plants were uprooted and shaken to remove loosely adhering soils. Plant roots were washed gently with water to remove rhizosphere soil and the excess water blotted off roots with clean dry tissue paper. Plant shoots were separated from roots with a clean pair of scissors and placed into pre-weighed and labelled envelopes. Envelopes containing plant shoots and roots were oven-dried at 65°C for 48 hours and weighed using a weighing balance (Mettler, UK). About 10 g of soil samples were collected from plant rhizosphere into glass tubes for contaminant concentration analysis and stored at 4°C while about 2 g of soil samples were collected into sterile tubes for microbial diversity analysis.

3.5 Organic contaminant analysis

3.5.1 Microwave extraction

Sodium sulphate (7g) was added to soil samples (5g) in microwave extraction tubes. This was followed by addition of 20 mL of 1:1 hexane: acetone solvent mixture for diesel spiked soils and for PAH spiked soil, 15 mL of 2:1 hexane: acetone mixture and 5 mL 1:4 triethylamine: acetone mixture. For diesel- and PAH-spiked soils, 40 µL of 500 ng μL^{-1} surrogate standard; ortho terphenyl and *p*-terphenyl- *d14* respectively were added. The tube contents were mixed using a vortex mixer (VWR collection, UK) and shaken by inversion to dislodge solid material from the base. Microwave extraction was carried out with the following conditions:

temperature ramp to 100°C at 800Watts for 12 minutes, hold at 100°C at 800 Watts for 10 minutes then cool for 5 minutes. Following the extraction, tube contents were mixed and allowed to settle. Clear extracts were transferred into 20 mL glass tubes and stored at 4°C prior to sample clean up by silica gel solid phase extraction (SPE).

3.5.2 SPE and sample preparation

SPE HF Mega BE-SI 2gm 12 mL cartridges (Agilent, UK) were conditioned with 5 mL of hexane which was allowed to flow until the remaining hexane in the column was just above the column frit. The collected hexane was discarded, 1 mL of sample extract added and eluted with 10 mL of 1:1 hexane: dichloromethane (DCM) mixture. The eluant was collected in a clean 20 mL glass vial. The eluant was concentrated to a final volume of 1 mL under a gentle stream of nitrogen gas. Samples were prepared in 2 mL vials (Agilent, UK) by adding a semi-volatile internal standard mix containing acenaphthene-*d10*, chrysene-*d12*, 1,4-dichlorobenzene-*d4*, naphthalene-*d8*, perylene-*d12* and phenanthrene-*d10* in DCM to each concentrated sample extract from PAH spiked soils for GC-MS analysis. Samples for TPH analysis were analysed by GC-FID.

3.5.3 PAH analysis by gas chromatography mass spectrophotometry (GC-MS)

PAH concentrations were determined using an Agilent gas chromatograph-mass selective detector (Agilent technologies 6890N Network GC System)**.** A HP 5MS fused silica capillary column of dimensions 30 m X 0.25 mm i.d. X 0.25 µm film thickness was used. The GC-MS operating conditions for USEPA method 8270D (mass range 35-500 amu, scan time: #1 sec/scan, initial temperature: 40°C, held for 4 min, temperature program: 40-320°C at 10 °C/min, final temperature: 320 °C, held for 2 min after benzo[*g,h,i*]perylene eluted, transfer line and injector temperature: 250-300°C) were used with helium as a carrier gas at a constant

flow rate of 30 cm sec^{-1}. The GC-MS was calibrated with RESTEK NJDEP EPH 10/08 Rev.2 Aromatics Calibration Standard (2,000 µg mL^{-1} each of acenaphthene, acenaphthylene, anthracenebenzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h.i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenzo[*a,h*]anthracene, fluoranthene, fluorene, indeno[*1,2,3-cd*]pyrene, 2-methylnaphthalene, naphthalene, phenanthrene, pyrene and 1,2,3-trimethylbenzene in methylene chloride). The calibration points were 50, 100, 500, 1000, 2000, 5000, 10,000 pg µL $^{-1}$. An internal standard mix (1, 4-dichlorobenzene-*d4*, naphthalene-*d8*, acenaphthene-*d10*, phenanthrene-*d10*, chrysene-*d12* and perylene-*d12*) was used for calibration while *p*-terphenyl-*d14* was used as the surrogate standard. Quality controls were also set up with solvent blanks, matrix spikes controls. The GC-MS detection limits for the following compounds; naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-c,d*]pyrene**,** dibenzo[*a,h*]anthracene and benzo[*g,h,i*]perylene are 0.24, 1.06, 0.37, 0.50, 0.44, 0.79, 0.93, 1.03, 40.05, 0.50, 1.69, 1.66, 0.91, 0.67, 0.29 and 0.63 pg/µL respectively. The surrogate standard was added to the soil samples to be extracted while the internal standard mix was added to cleaned up samples analysing by GC-MS. Quality controls were also set up with solvent blanks and matrix spikes. Percentage recovery for surrogate standard, *p*-terphenyl was 46.04 - 93.3%.

3.5.4 TPH analysis by Gas chromatography-flame ionization detector (GC-FID)

The GC-MS operating condition for USEPA method 8270D were used as prescribed by NJDEP EPH 10/08 methodology. Aliphatic standard mixture 1 mL/ampule (NJDEP EPH 10/08 Rev.2 Aliphatics Calibration Standard) containing 2,000 µg mL^{-1} each of *n*-nonane (C9), *n*-decane (C10), *n*-dodecane (C12), *n*-tetradecane (C14), *n*-hexadecane (C16), *n*-

octadecane (C18), *n*-eicosane (C20), *n*-heneicosane (C21), *n*-docosane (C22), *n*-tetracosane (C24), *n*-hexacosane (C26), *n*-octacosane (C28) and *n*-triacontane (C30) in hexane : carbon disulfide (80:20), and surrogate standards ortho-terphenyl and chlorooctadecane were used to prepare calibration standards for a five-point calibration curve (20 ng uL^{-1}, 100 ng uL^{-1}, 250 ng uL^{-1}, 500 ng uL^{-1} and 1000 ng uL^{-1}). External standard calibration was used for the analysis of the diesel spiked soils. The integration of the collective peak areas from the baseline included the unresolved mixture "hump" areas. Unadjusted TPH concentration was calculated from the equations below using the total peak areas excluding those of the surrogate standards as prescribed in the Extractable Petroleum Hydrocarbon (EPH) methodology by New Jersey Department of Environmental Protection (NJDEP EPH 10/08). The concentration of the target or diesel PAH analytes were not determined by GC-MS, hence all the concentration values reported are for the collective concentration of unadjusted TPH as no data adjustment (subtraction of the concentration of the target or diesel PAH analytes) were made.

3.5.4.1 External calibration

The calculation of the TPH concentration was determined with the concentration of the carbon ranges and the corresponding area response. The range calibration factor was calculated from the carbon range concentration and the total peak area of the range (Appendices 5.1 and 5.2).

Concentration of hydrocarbon range or TPH (ug kg^{-1}) = {(Ax)(D)(V_t)}/ range CF(W_d)

Where Ax is the response for the analyte or hydrocarbon range, Vt is the volume of the extract in uL, D = dilution factor, CF is the calibration factor and W_d is the dry weight of the solid extracted (g).

Calibration factor (CF) = area of peak/concentration injected (ng μL^{-1})

Carbon range calibration factor (CRCF) = summed area of peak in range /total concentration injected (ng μL^{-1}).

The areas for the surrogates were subtracted from the area summation of the TPH area. The percentage relative standard deviation (%RSD) of the calibration factor is expected to be less than or equal to 25% over the working calibration range.

%RSD = standard deviation of 5 range CFs /mean of 5 range CFs

As the %RSD for some of carbon ranges were >25%, external calibration was then carried out using linear regression analysis.

TPH calibration standards for the five concentration levels analysed and the area responses were used to plot a calibration curve. The correlation coefficient (r) of the resultant calibration curve of greater than or equal to 0.99 was required.

The TPH concentration was calculated with the equation below:

The concentration of the analyte or hydrocarbon range (ug kg^{-1})

$C= \{(Ax-b)/a\} * \{(Vt * D)/ W_d\}$

Where Ax is the response for the analyte or hydrocarbon range, a is the slope, b is the intercept, Vt is the volume of the extract in uL, D = dilution factor, and W_d is the dry weight of the solid extracted (g). Areas of the surrogate standards were not included when calculating the range concentration.

Dilution factor = {sample volume (mL) + diluent volume (mL)}/sample extract volume

3.5.5 Integration of chromatograms

Peak areas for PAH and TPH were determined via manual integration of chromatograms the on MSD Chemstation Data analysis user interface (D.03.00.611 Copyright © Agilent Technologies 1989-2006).

3.6 Heavy metal analysis

3.6.1 Extraction by Aqua Regia method

Three gram of ground oven-dried soil was weighed and placed in a 15 mL digestion tube. 23 mL of concentrated hydrochloric acid and 7 mL of concentrated nitric acid were added to the soil, agitated and allowed to stand overnight. A condenser was placed over each tube and refluxed for 2 hours on a DigiPREP MS (SCP SCIENCE) heating block 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. 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 for chromium, copper and lead.

3.6.2 Heavy metal analysis by flame atomic absorbance spectrophotometer

Acid digests were analysed on AAnalyst 300 atomic absorption spectrophotometer (PerkinElmer Instrument) following calibration with standard solutions (Fluka, UK) of individual metals (Cr, Cu and Pb) to be analysed, in accordance to the manufacturer's instructions. Percentage recovery of Cr, Cu and Pb following acid digestion and analysis of certified soil reference material SS-2 (*Enviro*MAT SCP Science, UK) were 99.88%, 99.45% and 102.10% respectively.

3.7 Statistical analysis

Averages and standard errors of plant heights, plant shoot and root weights were calculated on Microsoft Excel 2007 while Analysis of variance (ANOVA) analyses at a level of significance of 0.05 were carried out with SPSS 20. For treatments with statistically significant differences ($p < 0.05$), multiple comparisons were made with the Tukey Honest Significant Difference (HSD) post-hoc test.

CHAPTER 4
PRESENCE OF PRIORITY PAHs, CHROMIUM, COPPER AND LEAD IN TWO SELECTED BROWNFIELD SITES IN THE UNITED KINGDOM

Abstract

In recent years, concerns about adverse effects of increasing land contamination paralleled with the increasing demand for land has emphasized need for sustainable remediation strategies. Soil samples were collected from two brownfield sites, in Saltley, Birmingham and Swansea, South Wales, United Kingdom and analysed to establish the extent of contamination by priority polycyclic aromatic hydrocarbon compounds. The Saltley site was determined to have a total PAH concentration of 41.0 mg kg^{-1} with high concentrations of phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene and benzo[*a*]pyrene, total benzo[*a*]pyrene equivalent value of 6.0 mg kg^{-1} and total carcinogenic PAH of 19.0 mg kg^{-1}. At the Swansea site total PAH concentration ranged from 5.0 – 85.0 mg kg^{-1} with pyrene, fluoranthene, benzo[*a*]anthracene, chrysene, benzo[*a*]pyrene as the predominant PAHs, total benzo[*a*]pyrene equivalent value of 0.9 – 2.0 mg kg^{-1} and total carcinogenic PAH concentration of 2.6 – 11.0 mg kg^{-1}. Heavy metal analysis on the soil samples showed most samples with concentrations above the ambient background concentrations for urban/industrial area. Both sites had a mixture of PAH and heavy metal contamination in varying concentrations with implication for the selection of efficient remedial strategies.

4.1 Background

Most contaminated sites contain a mixture of organic and/or inorganic compounds of anthropogenic origin. Typical examples of organic compounds include petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, solvents, while inorganic include radionuclides, metals and salts (Gerhardt *et al*., 2009). PAHs and heavy metals are commonly found as co-contaminants with adverse health effects (Baileys *et al*., 2002; Glick, 2010; Antonio *et al*., 2011).

Although PAHs are naturally occurring compounds, prolonged use of fuels, industrial emissions, car exhausts, incineration and atmospheric deposition has made PAHs ubiquitous environmental contaminants (Wild *et al*., 1990; USEPA, 2008). Atmospheric deposition of combustion-derived PAHs on soil surfaces is quite common with concentrations varying with proximity to point sources and soil factors (Wild *et al*., 1990; Nam *et al*., 2009). Hence high PAH concentrations are found in urban soils, roadside soils and even higher concentrations in contaminated sites such as old gas works worldwide including in the United Kingdom (Wild and Jones, 1995; Marusenko *et al*., 2011).

Densely populated areas in the United Kingdom such as the South, Midlands and some parts of Northern England have greater inputs of PAH emission compared to less densely populated areas (Nam *et al*., 2008). Another factor that affects PAH abundance and distribution is the climatic zone which in turn affects soil organic matter and microbial degradation (Nam *et al*., 2009). Natural soils of the UK are high in organic matter hence have great impact on the fate, persistence and longevity of PAHs characterised by hydrophobic and lipophilic properties (Nam *et al*., 2008; Marusenko *et al*., 2011; Cachada *et al*., 2012). Note worthily, PAHs in soils have a half life ranging from 0.2->3 years for acenapthene, to 1-9 years for

benzo[*g,h,i*]perylene but even so, are more readily degraded compared to PCB and dioxins (Environment Agency, 2007a,b). Human health risk assessment of PAH-contaminated soil is based on a benzo[*a*]pyrene equivalent value calculated with the PAH toxic equivalent factors (TEFs) approach recommended by several organizations (Health Protection Agency, 2010; Cachada *et al*., 2012; Jennings, 2012).

Considering metal contaminants, geogenic processes which involve weathering and mineralisation during soil formation naturally result in the presence of metals at background concentrations in soils. Other sources of metals in soil are anthropogenic activities such as mining, gasification/liquefaction, fuel processing and traffic emissions (Bradley *et al*., 1994; Wild and Jones, 1995; Environment Agency, 2007; Nadal, *et al*., 2007; Nadal, *et al.*, 2009).

The increasing number of contaminated sites is paralleled by an increase in land demands for purposes such as agriculture, housing, recreation and infrastructure (Roy *et al*., 2005; Environment Agency, 2006; USEPA, 2008; Luo *et al.,* 2009). To tackle adverse effects of contamination while meeting land demands, many countries promote remediation of derelict sites known as brownfields for redevelopment instead of using greenfields (agricultural land) (Environment Agency, 2003; Roy *et al*., 2005; Environment Agency, 2006; USEPA, 2008; Luo *et al.,* 2009). For example, the United States Environment Protection Agency's brownfield program provides grants for environmental assessment, remediation, job training activities and redevelopments (USEPA, 2008).

In the United Kingdom, new developments on brownfield land increased from a national average of 56% in 1997 to 70% in 2005 and the target was 75% for 2010 (UK Task force, 2005). Despite challenges of brownfield redevelopment such as environmental liability concerns, financial barriers, remediation considerations and reuse planning compared to other

real estate development projects there has been a number of successful brownfield redevelopment projects (USEPA, 2008). However, the presence of mixed contaminants on such sites still poses technical and economic challenges for remediation (Roy *et al*., 2005). The co-contamination of sites with PAHs and heavy metals is quite common as studies have shown that organic pollutants (PAHs) and heavy metal contamination are related and may also provide information on the source of contamination (Cachada *et al*., 2012). Consequently, contaminated land remediation usually involves a combination of treatment approaches. Despite the success of traditional solutions there is now a greater emphasis on the need for more sustainable and cost-effective alternative technologies. This has drawn more attention to bioremediation approaches such as phytoremediation which have significant remediation potential (Glick, 2003; Van Hamme *et al*., 2003; Kuiper *et al*., 2004; Scullion, 2006; Gao, *et al*., 2007; Glick, *et al*., 2007; McGuinness and Dowling, 2009).

This study was undertaken to determine the extent of PAH and heavy metal contamination in typical brownfield sites in the United Kingdom. The results would be useful in selecting PAH compounds to test the potential of selected plant candidates for remediation.

4.2 Methods

4.2.1 *Site details*

Two contaminated sites proposed for redevelopment projects were surveyed. The first site was an old derelict gas work station located in Saltley, Birmingham undergoing preliminary investigations at the time of visit while the other site, was a former oil refinery premises located at Swansea, South Wales under remediation by biopiling and monitored natural attenuation (see Section 2.5.3.1). At the Swansea site, wood chips were added to the biopiles

and mixed periodically by a tractor to facilitate aeration for microbial degradation (Figure 4.1). There were three biopiles at the Swansea site; top soil biopile, NSR biopile and Rhead biopile with top soil biopile said to be the least contaminated. Also present at the Swansea site were wastes from pipeline and sludge (Figure 4.2) being treated using an equipment for oil recovery (Figure 4.3). For reasons of confidentiality, the exact locations and full site details cannot be mentioned. As cameras were not allowed on the Saltley site, only photographs of Swansea site were taken. Figures 4.1-4.4 show photos of the Swansea site.

Figure 4.1: Tractor mixing soils on biopiles to facilitate aeration.

Figure 4.2: Sludge on Swansea site.

Figure 4.3: Equipment for oil recovery on Swansea site.

Figure 4.4: Running water contaminated by petroleum on Swansea site.

4.2.2 *Soil sample collection*

Soil samples were randomly collected in triplicate from a trial pit at Saltley site, and from biopiles ("top soil biopile", "NSR biopile" and "Rhead biopile") and wastes (pipeline and sludge) at the Swansea site. Grab samples were collected from a depth of 0 to 1 m and 1-2 m from soils dug out from sampling pit while surface samples were randomly collected from biopiles and sludge. All samples were collected in 60 mL glass jars with Teflon lined caps using a decontaminated hand trowel and were stored in a cold box of 4°C in the dark (Pies *et al*., 2007). The samples were transported to the laboratory at the University of Birmingham and stored in a freezer at 4°C.

All sampling equipment was decontaminated before and between sampling events and waste products generated properly disposed of. This was achieved by the following procedure; brushing and rinsing with tap water to remove gross contamination, washing with a soap

solution, rinsing with water, rinsing with methanol, rinsing with water then wrapping in aluminium foil to avoid contamination.

4.2.3 *Soil sampling and analysis*

Only soil samples from the 1-2 m depth at the Saltley site were analysed as the samples taken from the 0 to 1 m depth were mainly coal. Samples were homogenized by sieving with a 2 mm sieve. The pH and electrical conductivity of the samples were measured as described in section 3.2.1 of Chapter 3. Soil samples were extracted by microwave extraction (Chapter 3) and analysed for PAH concentrations of the 16 priority PAHs by GC-MS as described in Chapter 3. Heavy metal concentration was assessed by AAS following acid digestion as described in Chapter 3.

4.2.4 Calculations

i. Total PAH (tPAH or ΣPAHs) is the sum of the 16 priority PAHs analysed.

ii. Total carcinogenic PAH (cPAH or Σcarc. PAHs) is the sum of all the carcinogenic PAHs; benzo[*a*]pyrene, benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, chrysene, dibenzo[*a,h*]anthracene and indeno[*1,2,3-cd*]pyrene.

iii. The percentage of the carcinogenic PAHs to the total PAHs is given as Σcarc. PAHs/ΣPAHs*100.

iv. The total benzo[*a*]pyrene equivalent value was calculated as total equivalent concentration as benzo[a]pyrene, using the toxicity equivalent (TE) for each PAH (Table 4.1), and the formula below

Total benzo[*a*]pyrene equivalent value (B[*a*]P-TE) = $\Sigma_i C_i$ X TE_i

where: C_i – concentration of the respective PAHs μg kg^{-1}

TE_i – the toxicity equivalent of the corresponding PAHs.

Table 4.1: PAH compounds and their toxic equivalent factors (TEFs) (Tsai *et al.*, 2004).

PAH	**TEFs**
Naphthalene	0.001
Acenaphthylene	0.001
Acenaphthene	0.001
Fluorene	0.001
Phenanthrene	0.001
Anthracene	0.01
Fluoranthene	0.001
Pyrene	0.001
Benzo[*a*]anthracene	0.1
Chrysene	0.01
Benzo[*b*]fluoranthene	0.1
Benzo[*k*]fluoranthene	0.1
Benzo[*a*]pyrene	1
Indo[*1,2,3*]pyrene	0.1
Dibenzo[*a,h*]anthracene	1
Benzo[*g,h,i*]perylene	0.01

4.3 Results

4.3.1 Soil properties

The electrical conductivities of soil samples from Saltley, Swansea top soil, NSR, Rhead biopile, pipeline and sludge are 1580, 644, 1795, 2103, 254 and 1453 μs cm^{-1} respectively while pH values were generally circum-neutral. Slightly acidic values were found (6.1 and 6.6) at Swansea Rhead biopile and sludge (Table 4.2).

Table 4.2: pH and electrical conductivity measurements.

Site	**Sampling site**	**pH**	**Conductivity ($\mu s\ cm^{-1}$)**
1. Saltley	Trial pit	7.1	1580
2. Swansea	Top soil	7.4	644
	NSR biopile	7.2	1800.
	Rhead biopile	6.1	2103
	Pipeline waste	7.7	254
	Sludge	6.6	1450

4.3.2 PAH concentration

4.3.2.1 Saltley site

Saltley site had a tPAH of 41.6 mg kg^{-1}, B[*a*]P-TE of 5.79 mg kg^{-1}, and cPAH of 19.43 mg kg^{-1}. The predominant compounds include phenanthrene (3.12 mg kg^{-1} , anthracene (3.64 mg kg^{-1}, fluoranthrene, (6.75 mg kg^{-1}), pyrene (5.56 mg kg^{-1}), benzo[*a*]anthracene (3.67 mg kg^{-1}), benzo[*b*]fluoranthene (2.57 mg kg^{-1}), benzo[*a*]pyrene (4.02 mg kg^{-1}), indeno[*1,2,3-c,d*]pyrene (2.47 mg kg^{-1}), and benzo [*g,h,i*] perylene (2.52 mg kg^{-1}) as shown in Table 4.3.

Table 4.3: Mean concentrations of 16 priority PAH compounds, total carcinogenic PAH, total PAH and benzo[*a*]pyrene equivalent value. (Average ± SE, n=3). ND - Not detected/below detection limit.

PAH ($mg.kg^{-1}$)	**Saltley site**	**South Wales top soil biopile**	**NSR biopile**	**Rhead biopile**	**Pipeline Waste**	**Sludge**
Naphthalene	0.21±0.140	ND	ND	ND	0.167±0.0998	2.65±0.116
Acenaphthylene	0.27±0.134	ND	ND	0.255±0.129	ND	4.10±0.0302
Acenaphthene	ND	ND	ND	ND	ND	4.98±0.0504
Fluorene	ND	ND	ND	ND	0.301±0.0134	14.90±0.300
Phenanthrene	3.12±1.09	ND	ND	ND	1.12±0.0679	37.3±0.0598
Anthracene	3.64±1.11	0.418±0.0157	0.391±0.0488	0.213±0.107	1.65±0.0709	2.26±0.0436
Fluoranthene	6.75±2.31	0.814±0.359	0.408±0.131	0.513±0.364	1.49±0.0725	2.00±0.0932
Pyrene	5.56±1.83	0.952±0.519	1.34±0.286	2.06±0.149	1.14±0.0543	6.01±0.0937
Benzo[*a*] anthracene	3.67±1.14	0.628±0.0021 2	1.631±0.152	1.48±0.0476	1.30±0.070	1.75±0.0563
Chrysene	3.74±1.10	0.149±0.137	1.19±0.211	1.27±0.402	0.600±0.0183	4.23±0.125
Benzo [*b*] fluoranthene	2.57±0.684	0.166±0.166	0.641±0.144	0.642±0.229	0.411±0.0840	1.23±0.196
Benzo[*k*] fluoranthene	2.41±0.575	0.259±0.259	0.803±0.155	0.766±0.192	0.597±0.0701	1.23±0.101
Benzo[*a*]pyrene	4.02±1.18	0.748±0.0182	0.972±0.112	1.23±0.412	0.935±0.0194	1.44±0.283
Indeno[*1,2,3*]c,d pyrene	2.47±0.744	0.675±0.0040 1	0.803±0.153	0.945±0.285	ND	0.952±0.260
Dibenzo[*a,h*] anthracene	0.55±0.273	ND	ND	ND	ND	ND
Benzo[*g,h,i*] perylene	2.52±0.754	0.663±0.0018 9	0.763±0.239	0.887±0.315	ND	0.900±0.238
cPAH	**19.4**	**2.63**	**6.04**	**6.55**	**3.85**	**10.82**
B[*a*]P-TE	**5.79**	**0.940**	**1.38**	**1.86**	**1.19**	**2.10**
tPAH	**41.6**	**5.47**	**8.93**	**10.5**	**9.72**	**84.9**

4.3.2.2 Swansea site

4.3.2.2.1 *Sludge*

Swansea sludge displayed a cPAH of 10.8 mg kg^{-1} (12.7% of tPAH) and a high concentration of all analytes (>1 mg kg^{-1}) with the exception of indeno[*1,2,3-c,d*]pyrene, benzo[*g,h,i*]perylene and dibenzo[*a,h*]anthracene which were 0.95, 0.90 and <detection limit respectively (Appendix 4.1).

4.3.2.2.2 *Biopiles and pipeline waste*

Swansea site biopiles and wastes had a tPAH ranging from 5.47 – 10.5 mg kg^{-1}, B[*a*]P-TE of 0.94- 1.86 mg kg^{-1} and cPAH of 2.63 – 6.55 mg kg^{-1} (Table 4.2). Rhead biopile displayed a cPAH of 6.55 mg kg^{-1} (62.3% of tPAH). Interestingly, the B[*a*]P-TE value of Swansea sludge (2.10 mg kg^{-1}) and Rhead biopile (1.86 mg kg^{-1}) were comparable despite the difference in tPAH concentration of 84.9 and 10.5 mg kg^{-1} respectively. NSR biopile and pipeline waste had a cPAH of 6.04 mg kg^{-1} (70.4% of tPAH) and 3.85 mg kg^{-1} (39.6% of tPAH) with a corresponding B[*a*]P-TE value of 1.2 and 1.1 respectively. Top soil biopile had the lowest cPAH concentration (2.63 mg kg^{-1}) and a corresponding B[*a*]P-TE of 0.940 mg kg^{-1} (Table 4.3).

Naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and dibenzo[*a,h*] anthracene were not detected in top soil and NSR biopiles, while the concentration of the other compounds were less than 1 mg kg^{-1} except for pyrene and benzo[*a*]anthracene which were 1.34 and 1.63 mg kg^{-1} in NSR biopile.

Table 4.3: Mean concentrations of 16 priority PAH compounds, total carcinogenic PAH, total PAH and benzo[*a*]pyrene equivalent value. (Average ± SE, n=3). ND - Not detected/below detection limit.

PAH ($mg.kg^{-1}$)	**Saltley site**	**South Wales top soil biopile**	**NSR biopile**	**Rhead biopile**	**Pipeline Waste**	**Sludge**
Naphthalene	0.21±0.140	ND	ND	ND	0.167±0.0998	2.65±0.116
Acenaphthylene	0.27±0.134	ND	ND	0.255±0.129	ND	4.10±0.0302
Acenaphthene	ND	ND	ND	ND	ND	4.98±0.0504
Fluorene	ND	ND	ND	ND	0.301±0.0134	14.90±0.300
Phenanthrene	3.12±1.09	ND	ND	ND	1.12±0.0679	37.3±0.0598
Anthracene	3.64±1.11	0.418±0.0157	0.391±0.0488	0.213±0.107	1.65±0.0709	2.26±0.0436
Fluoranthene	6.75±2.31	0.814±0.359	0.408±0.131	0.513±0.364	1.49±0.0725	2.00±0.0932
Pyrene	5.56±1.83	0.952±0.519	1.34±0.286	2.06±0.149	1.14±0.0543	6.01±0.0937
Benzo[*a*] anthracene	3.67±1.14	0.628±0.0021 2	1.631±0.152	1.48±0.0476	1.30±0.070	1.75±0.0563
Chrysene	3.74±1.10	0.149±0.137	1.19±0.211	1.27±0.402	0.600±0.0183	4.23±0.125
Benzo [*b*] fluoranthene	2.57±0.684	0.166±0.166	0.641±0.144	0.642±0.229	0.411±0.0840	1.23±0.196
Benzo[*k*] fluoranthene	2.41±0.575	0.259±0.259	0.803±0.155	0.766±0.192	0.597±0.0701	1.23±0.101
Benzo[*a*]pyrene	4.02±1.18	0.748±0.0182	0.972±0.112	1.23±0.412	0.935±0.0194	1.44±0.283
Indeno[*1,2,3*]c,d pyrene	2.47±0.744	0.675±0.0040 1	0.803±0.153	0.945±0.285	ND	0.952±0.260
Dibenzo[*a,h*] anthracene	0.55±0.273	ND	ND	ND	ND	ND
Benzo[*g,h,i*] perylene	2.52±0.754	0.663±0.0018 9	0.763±0.239	0.887±0.315	ND	0.900±0.238
cPAH	**19.4**	**2.63**	**6.04**	**6.55**	**3.85**	**10.82**
B[*a*]P-TE	**5.79**	**0.940**	**1.38**	**1.86**	**1.19**	**2.10**
tPAH	**41.6**	**5.47**	**8.93**	**10.5**	**9.72**	**84.9**

4.3.3 Heavy metal concentration

Saltley site

The chromium, copper and lead mean concentrations displayed by the Saltey site were 72.5±3.90, 186±17.5 and 132±25.0 mg kg^{-1} respectively. The mean chromium concentration for Saltley site (72.5 mg kg^{-1}) was within the mean ambient background soil concentration of 51.6-86.0 mg kg^{-1} (Environment Agency, 2007), while lead concentration (132±25.0 mg kg^{-1}) was slightly above the upper limit of the mean ambient background soil concentration; 78.8-131 mg kg^{-1} (Environment Agency, 2007). The copper concentration (186±17.5 mg kg^{-1}) was above the mean ambient background soil concentration of 30.9-51.5 mg kg^{-1} (Environment Agency, 2007).

Swansea site

Rhead biopile had the highest concentration of chromium with 37.6 mg kg^{-1} while pipeline waste had the lowest with 11.3 mg kg^{-1}. The concentration of copper was highest in the sludge with 392 mg kg^{-1} while pipeline had the lowest with 38.5 mg kg^{-1}. The highest lead concentration was present in Rhead biopile with 239 mg kg^{-1} and lowest in pipeline waste with 23.4 mg kg^{-1}. Pipeline waste was found to contain the lowest concentrations of Cu, Cr and Pb compared to the other biopiles and sludge.

In general, the chromium concentrations were below the mean ambient background soil concentration of 51.6-86.0 mg kg^{-1}. Apart from the copper concentration of the pipeline wastes (38.5 mg kg^{-1}), those of the other site samples ranging from 123.4 mg kg^{-1} for Rhead biopile and 392 mg kg^{-1} for sludge were above the mean ambient background soil concentration of 30.9-51.5 mg kg^{-1} (Environment Agency, 2007). Also, the lead concentration

for Swansea top soil (225 mg kg^{-1}) and Rhead biopile (239 mg kg^{-1}) were above the mean ambient background soil concentration of 78.8-131 mg kg^{-1} (Environment Agency, 2007).

Table 4.4: Mean concentrations (mg kg^{-1}) of Cr, Cu and Pb following AAS analysis (Average±SE, n=3).The mean ambient background soil concentrations of Cr, Cu and Pb at urban/industrial sites in the United Kingdom are 51.6-86.0, 30.9-51.5 and 78.8-131 mg kg^{-1} respectively (Environment Agency, 2007).

Site	**Chromium**	**Copper**	**Lead**
1. Saltley, Birmingham	72.5±3.89	186±17.5	132±25.0
2. Swansea, South Wales			
i. Top soil	28.5±3.00	142±12.3	200±26.0
ii. NSR biopile	26.2±0.530	206±3.88	103±10.5
iii. Rhead biopile	37.6±4.52	123±15.7	300±68.2
iv. Pipeline	11.3±0.250	38.5±0.660	23.4±7.53
v. Sludge	35.0±1.99	392±8.40	122.2±27.1

4.4 Discussion

4.4.1 *PAH concentration at Saltley and Swansea*

The presence and the concentrations of PAHs in Saltley and Swansea sites could be attributed to the activities leading to contamination with coal and crude oil respectively on the site based on the site information provided. Furthermore, as both sites are located in industrial areas contamination may be attributed to industrial emission and other sources such as car exhausts, incineration and atmospheric deposition (Wild *et al*., 1990). Also, between the early 19^{th} century and early – mid 20^{th} century, there were intense industrial activities in the United Kingdom based on coal burning while wood burning for domestic heating was quite common (Nam *et al*., 2008). A variety of other land uses caused contamination in the United Kingdom such as railway land, engineering works, gas and coke works, chemical works, power stations

and refineries (Environment Agency, 2002). The most common contaminants identified on contaminated land sites in the UK were metals, metalloids and organic compounds. In England the energy and waste industries were identified as the greatest source of pollution of contaminated land while for that of Wales is attributed to the deposit of ash (Environment Agency, 2009).

Between 1846 and 1996, there were 1774 oil refineries or sites (3842 hectares) while between 1958 and 1996 there were 601 sites (3443 hectares) involved in bulk storage of crude oil and petroleum products in England. Also there were 13716 (29,117 hectares) and 424 (5176 hectares) gas works, coke works and other coal carbonisation plant sites from 1846-1996 and 1958-1996 respectively in England (Environment Agency, 2002). Consequently, it is very likely that some PAHs contaminations in both sites may have been caused during the industrial era although fresh contaminations due to combustion, traffic emission, atmospheric deposition and oil spillage (in the case of the Swansea site) may have occurred in the recent years (Nam *et al*., 2008).

From Table 4.3, Saltley soil (41.6 mg kg^{-1}) and Swansea sludge (84.9 mg kg^{-1}) had tPAH concentrations above the expected mean ambient background PAH concentrations at urban/industrial sites in the UK (11.2 mg kg^{-1}) while those of NSR biopile (8.58 mg kg^{-1}), Rhead biopile (10.5 mg kg^{-1}) and pipeline (9.72 mg kg^{-1}) were comparable to the expected value. The mean ambient background PAH concentrations at urban/industrial sites in the UK is based on the United Kingdom Soil and Herbage Pollutant survey of 122 rural areas. The mean ambient background PAH concentrations of rural soils in the UK, England, Northern Ireland, Scotland and Wales are 2.24, 1.90, 0.730, 1.30 and 8.94 mg kg^{-1} (Environment Agency, 2007).

The highest tPAH concentration (84.9 mg kg^{-1}) displayed by samples from Swansea sludge in comparison to the other samples may be explained by the fact that high concentration of organic contaminants as well as toxic metals are usually expected in sludge with the highest PAH concentration in waste products of refinery process (Kuriakose and Manjooran, 1994). Also our finding of the lowest tPAH concentration in the top soil biopile agrees with the site manager's assessment of the PAH levels.

PAH profiles

The predominance of fluoranthene, pyrene, chrysene, benzo[*a*]pyrene, benzo[*b*]fluoranthene and benzo[*g,h.i*]perylene (Table 4.3) in both sites agrees with source apportionment studies of PAH profiles in UK soils dominated by fluoranthene, pyrene, phenanthrene, chrysene, benzo[*b*]fluoranthrene, benzo[*k*]fluoranthrene, and benzo[*a*]pyrene irrespective of the area whether rural, urban or industrial (Wild and Jones, 1995; Environment Agency, 2007). Also there is an expected presence of medium molecular weight PAHs such as fluoranthene and pyrene at very high concentrations in anthropogenic sites (Nadal *et al*., 2011).

The PAH profile and high fluoranthene concentration in Saltley agrees with the finding of a study by Cousins *et al*. (1997) where contemporary surface soil samples collected from 46 locations widely distributed over the United Kingdom were analysed for 12 PAH compounds (anthracene, acenaphthene, fluorene, fluoranthene, phenanthrene, chrysene, benzo[*a*]anthracene, benzo[*b*]fluoranthrene, benzo[*k*]fluoranthrene, benzo[*a*]pyrene, dibenzo[*a,h*]anthracene and benzo [*g,h,i*]perylene).

The low PAH concentration of low molecular weight PAH (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene and fluoranthene) confirms greater susceptibility to microbial degradation than higher molecular weight PAHs which exhibit greater environmental persistence. This is as a result of the decrease in solubility and increase in hydrophobicity as molecular weight increases (Bojes and Pope, 2007). Hence, microbial degradation and volatilization may account for the low concentrations of some lower molecular weight PAHs compared to higher molecular weight PAHs (Table 4.3) with the exception of the Saltley and Swansea sludge samples where contaminant toxicity and bioavailability may affect microbial degradation. Other limiting factors of microbial degradation such as aeration, nutrient levels (especially nitrogen and phosphorus) may have had an impact on the PAH concentration and profile.

Risk to human health

Saltley with B[*a*]P-TE value of 5.79 mg kg^{-1} and South Wales (top soil, NSR biopile, Rhead biopile, pipeline and sludge with B[*a*]P-TE values 0.94, 1.38, 1.86, 1.19 and 2.10 mg kg^{-1} respectively) both exceeded the soil clean up target levels (SCTL) for industrial direct exposure of 0.6 mg kg^{-1} (CCME, 2010) and median value of 0.7 mg kg^{-1} for B[*a*]P-TE (FDEP, 2005 and Jennings, 2012). However, concentrations of the non-carcinogenic PAHs in both sites were below SCTL values for industrial/commercial direct exposure. Background concentration of PAHs in both sites must be compared with SCTL for remediation but soil PAH concentration between 1-3 mg kg^{-1} are considered to be in the upper range (WHO, 2000). Consequently, based on the tPAH, cPAH and B[*a*]P-TE values there may be an indication of the risk of both sites to human health depending on the proposed land use. The

risk assessment and the proposed redevelopment projects may determine and the need for remediation.

4.4.2 *Heavy metal concentration at Saltley and Swansea sites*

On the whole, varying contaminant concentrations may be attributed to site location and activities at different stages in extraction, refining and manufacturing processes. As previously mentioned, the industrial revolution in the United Kingdom also led to an increase in metal concentrations in soils (Environment Agency, 2007). Most metals are strongly retained in soils with minimal losses by erosion and leaching as such any soil metal concentration is the result of cumulative additions and those derived by geogenic processes on parent rocks (Environment Agency, 2007). This may account for the presence and concentrations of Cr, Cu and Pb (Table 4.4) above the mean ambient background concentrations (Environment Agency, 2007) in both sites.

It is important to note that although the use of biopile as a remedial strategy at the Swansea site may be efficient for PAH contaminated soils, an alternative strategy would be required for the heavy metal remediation as reflected by high copper and lead concentration in the top soil biopile (Table 4.4) (Scullion, 2006). On the whole, remediation strategies are still required on both sites to achieve concentrations below mean ambient background concentrations (Environment Agency, 2007). Some soil samples from both sites had Cr, Cu and Pb concentrations above the given ambient background concentrations. However, it would also be important to use bioavailable concentrations of the heavy metals in assessing health risks and monitoring remediation success.

4.5 Conclusion

The presence of the PAHs and heavy metals at Saltley and Swansea sites may be attributed to activities during the industrial era in the United Kingdom as well as recent anthropogenic activities such as crude oil processing, combustion, incineration and traffic emissions. However, the hydrophobic nature of PAHs which facilitates adsorption to soil organic matter content may have affected their bioavailability, persistence and fate over the years. The presence of metabolically diverse soil microbes may have been involved in natural attenuation and biopiling, however effective remediation may be affected by a number of factors such as concentration of contaminants present and bioavailability. PAHs were present in varying concentration in the soil samples collected from both sites with history of industrial activities. The PAH profile were in agreement with those of other PAH-contaminated soils in the UK and anthropogenic soils in general. Generally, concentrations of the total PAH, copper and lead in both sites were above expected mean ambient background concentrations at industrial sites in the United Kingdom (Environment Agency, 2007 and Jennings, 2012). Based on the results, phenanthrene, fluoranthrene, benzo[*a*]pyrene and lead were selected as contaminants for greenhouse experiments in Chapters 6 and 8.

CHAPTER 5

EFFECT OF DIFFERENT DIESEL TREATMENTS ON GROWTH OF SINGLE AND MIXED PLANT COMMUNITIES AND PETROLEUM HYDROCARBON DISSIPATION

Abstract

Phytotoxicity has impacts on plant growth and phytoremediation success. The use of mixed plant communities has been proposed to address phytotoxicity while improving plant stress tolerance and contaminant degradation. However, there has been conflicting findings on the use of mixed plant community. This study was designed to assess the impact of diesel treatments on plant growth and TPH dissipation in single and mixed plant communities. The growth of *M. sativa*, *L. perenne* and *F. arundinacea* were inhibited on diesel-spiked soils. The biomass yield was greater for plant roots compared to plant shoots especially for *F. arundinacea* and *L. perenne*. There was a significant decrease in the root biomass yield of *M. sativa*, *L. perenne*, *F. arundinacea* and *M. sativa* + *L. perenne*. The highest TPH dissipation of 81, 69 and 72 % was displayed by *L. perenne* in the treatments with 102000,151000 and 320000 μg kg^{-1} TPH, respectively. However, TPH dissipation was generally comparable for the vegetated and unvegetated soil and were not significantly different ($p>0.05$) for the different plants and treatments. Phytotoxic effects of diesel may have an impact on plant growth, tolerance and rhizoremediation. Mixed plants may enhance rhizoremediation of TPH-contaminated soils but factors such as plant species, TPH concentration and soil properties affect the exploitation of the benefits of individual plants.

5.1 Background

Global industrialization and high energy demands over the past two centuries have led to widespread contamination of the environment with organic and inorganic compounds (Gaskin *et al*., 2010). As petroleum is the largest global energy source, the majority of global contamination with petroleum hydrocarbons is attributed to accidental spills, leaks from storage tanks and pipelines and illegal waste disposal (Margesin *et al*., 2007; Cai *et al*., 2010; Afzal *et al*., 2011).

The adverse health effects of contaminated land and increase in the number of brownfield sites have emphasised the need for eco-friendly and cost-effective remediation strategies such as phytoremediation (Kuiper *et al*., 2004; Gao, *et al*., 2007; Glick, *et al*., 2007; Mc Guinness and Dowling, 2009). Studies have shown that during rhizoremediation, plant roots enhance degradation of petroleum hydrocarbon by stimulating microbial metabolic activities, improving aeration and increasing water infiltration (Hou *et al*., 2001; Lin *et al*., 2008). As such efficient rhizoremediation depends on successful plant root growth and distribution in contaminated soils. Root growth however, may be affected by the presence of contaminants and soil factors such as temperature, moisture, nutrient content, porosity and oxygen levels (Hou *et al*., 2001).

The total petroleum hydrocarbon (TPH) content is the most important factor that limits rhizoremediation of petroleum-contaminated soils as it affects soil properties, diversity, abundance and activity of soil microbes, plant growth and establishment, plant biomass yield, stress tolerance, plant-microbe interaction with an overall impact on degradation (Hou *et al*., 2001; Lapinskien *et al*., 2006; Kechavarzi *et al*., 2007; Tang, *et al*., 2010a). TPH content significantly affects hydrocarbon removal rate which varies amongst plant families down to

species and genotype level (Kulakow *et al*., 2000; Hou *et al*., 2001; Minai-Tehrani *et al*., 2007; Lin *et al*., 2008). Tang *et al*., (2010a) showed that the optimum TPH concentration that will facilitate rhizoremediation with minimal effects to plants and associating microbial populations is 5% w/w. Some studies have demonstrated that many plants are able to grow and survive in oil contaminated soils with moderate contamination (10% w/w oil) (Radwan *et al*., 1995) while an ecotoxicology study by Lapinskien *et al*., (2006) reported that soils with >3% w/w diesel are toxic.

A few recent studies have reported the potential of mixed plant communities and biodegradative bacteria with plant growth promoting property instead of single plant communities to enhance plant tolerance and TPH removal during rhizoremediation (Kamath *et al*., 2004; Nedunuri *et al*., 2010). It is expected that in a mixed plant community, there should be a cumulative benefit from the combination of different root types and the root exudate profiles resulting in the proliferation of more diverse microbial communities compared to single plant communities (Cheema *et al*., 2010). However, few studies on the rhizoremediation of petroleum-contaminated soils have been carried out using mixed plant communities with conflicting findings on their impact on biomass yield and contaminant dissipation (Phillips *et al*., 2006; Phillips *et al*., 2009; Cheema *et al*., 2010). A study by Phillips *et al*. (2006) reported that the single-species grass treatment with *Festuca rubra* (TPH loss by 50% after 4.5 months) displayed greater TPH degradation than the mixed plant treatment and control. Also, Phillips *et al*. (2009) reported that the use of mixed plant community with *Thinopyrum ponticum* (tall wheat grass), *Elymus angustus* (altai wild rye) and *M.sativa* did not result in a cumulative degradation during the first growing season while the single plant treatments displayed up to 54% TPH degradation. However, at the end of the second growing season, TPH level of all the treatments were comparable. They concluded

that the use of mixed plant community with legumes such as *M. sativa* may inhibit degradation of contaminants during phytoremediation. They attributed this to the fact that the selective stimulation and proliferation of degraders by *Medicago sativa* does not instantly result in an increased degradation. There is little knowledge on whether the suppressive effect of *M. sativa* on degradation extends over the initial adaptation period. On the other hand, Cheema *et al*. (2010) reported a higher PAH dissipation in mixed plant treatment compared to single plant treatment with *Festuca arundinacea*, *Lolium perenne*, *Medicago sativa* and *Brassica napus* as supported by other studies (Cheema *et al*., 2009; Gurska *et al*., 2009; Meng *et al*., 2011; Sun *et al*., 2011). These conflicting findings have resulted in the need for further study on the potential of mixed plant community to enhance plant tolerance and biomass yields during phytoremediation.

This study was undertaken to assess the impact of diesel treatments on plant root and shoot biomass yields during rhizoremediation by *Festuca arundinacea*, *Lolium perenne*, *Medicago sativa* and a mixed plant community of *M. sativa* and *L. perenne* used in previous greenhouse and field studies (Huang *et al*., 2004; Cheema *et al*., 2009; Gurska *et al*., 2009; Tang *et al*., 2010b). Also, phytoremediation potential of the single and mixed plant community was assessed.

5.2 Hypotheses

1. Different diesel treatment concentrations will affect growth and biomass yields of selected plants relative to controls.
2. TPH loss from the different diesel treatments will vary between different plant treatments.

3. TPH loss for the diesel treatments with mixed plant community will be greater than that for single plant community.

5.3 Methods

5.3.1 *Soil preparation and experimental design*

Diesel was obtained commercially from a gas station in Birmingham, United Kingdom. About 250 g of air-dried and sieved soil (25% of soil for planting in each pot) was spiked with diesel at 0.5%, 1% and 2% w/w (Table 5.1) and mixed thoroughly to achieve homogeneity in a fume hood. The spiked soils were mixed with about 750 g of unspiked soil to make about 1 kg of soil. Following the spiking, soils were homogenized, stored and later dispensed for planting as described in the Section 3.2.3. The control treatment assessed contribution made by abiotic processes and soil microbes to TPH dissipation. Plant seedlings in pertiles were transplanted into the spiked and control soils. Soil sample collection and transplantation were conducted as discussed in Section 3.2.4. As TPH loss by microbial degradation and volatilization is expected during the storage period, soil samples were collected during seedling transplantation to measure the initial TPH concentration as described in Section 3.5.4.

Table 5.1: Greenhouse experiment with diesel contaminated soil. Initial TPH concentrations were Treatment 1; 102000±2870, Treatment 2; 151000±15900, Treatment 3; 320000±160000 µg kg^{-1} (Appendix 5.1-5.5).

Diesel Treatment	***M. sativa***	***L. perenne***	***Festuca arundinacea***	***M. sativa* + *L. perenne***	**Unplanted Control**
Control	X3	X3	X3	X3	X3
1	X3	X3	X3	X3	X3
2	X3	X3	X3	X3	X3
3	X3	X3	X3	X3	X3

Plant shoot heights were measured at intervals of two weeks. The plants were harvested and soil samples collected and stored as detailed in Section 3.4. TPH was extracted from soil by microwave extraction method. Sample extracts were concentrated over a gentle stream of Nitrogen gas to 1 mL of sample and followed by sample clean up by solid phase extraction without fractionation as described in Section 3.5.2. Standards and concentrated samples were sent for analysis by EPH methodology (version 3.0) by New Jersey Department of Environmental Protection (NJDEP EPH 10/08) on GC–FID Perkin-Elmer Autosystem XL at the School of Chemistry, University of Sheffield, United Kingdom with details of the method described in Chapter 3.

5.4 Results

5.4.1 Plant response to diesel-spiked soils

No plant death was observed for any treatments (0.5, 1 and 2% w/w diesel) throughout the growth period but other signs of phytotoxicity such as yellowing of leaves and stunted growth

were observed compared to control plants for all plants in all treatments. Figure 5.1a and b show photographs of plants taken during the greenhouse experiment.

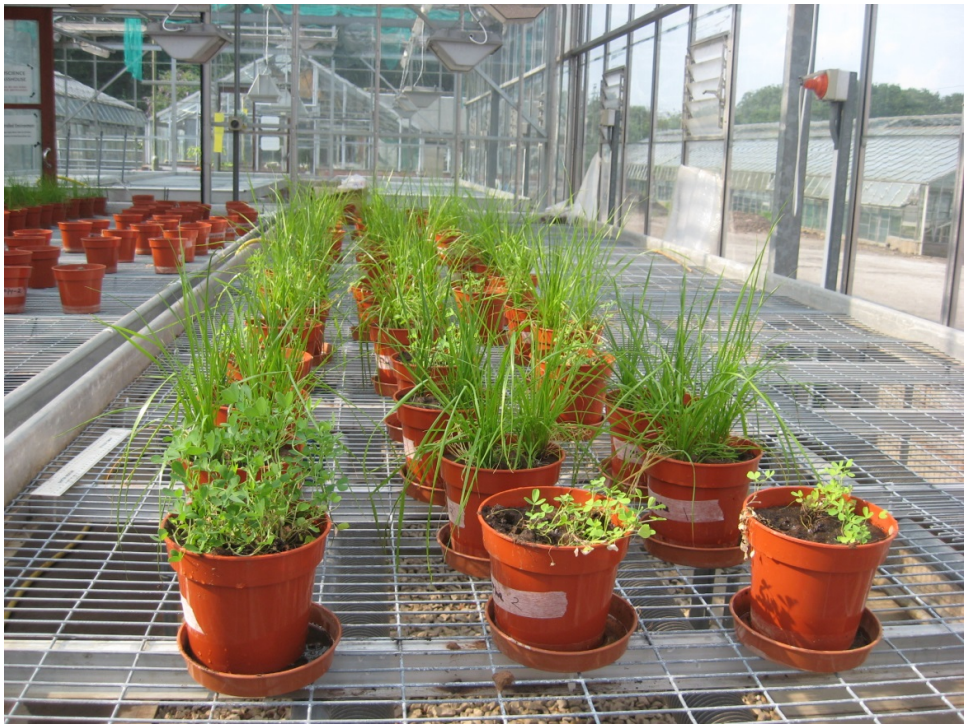

Figure 5.1a: Photographs of *M. sativa*, *F. arundinacea* and *L. perenne* during greenhouse experiment with contaminated soil

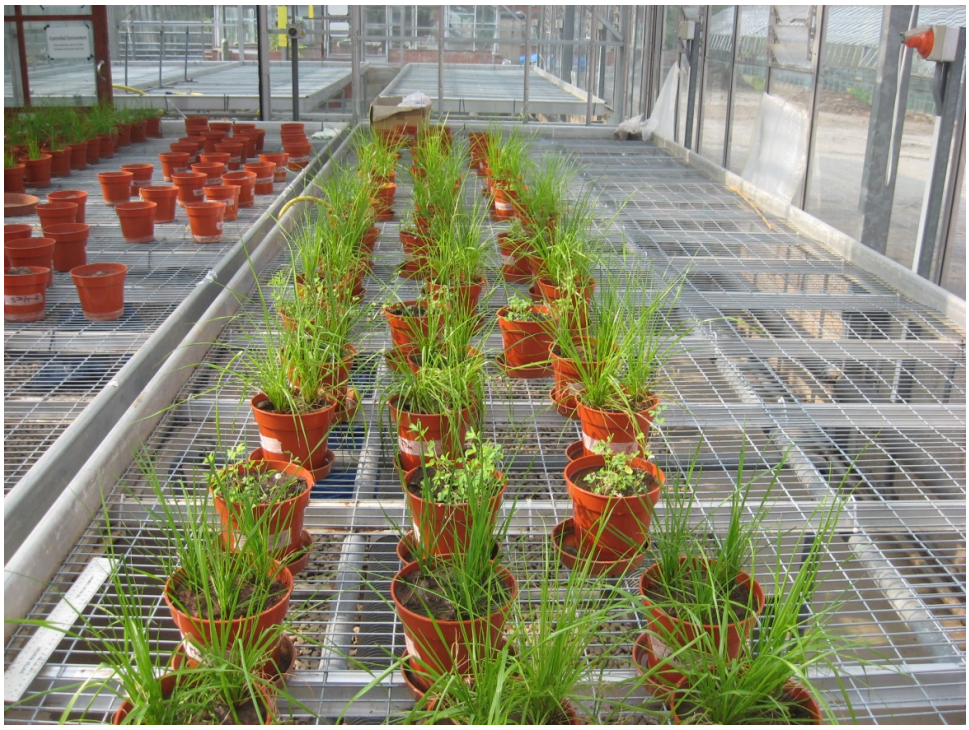

Figure 5.1b: Photographs of *M. sativa*, *F. arundinacea* and *L. perenne* during greenhouse experiment with contaminated soil.

5.4.1.1 Impact of diesel treatments on plant height

The diesel treatments affected plant growth compared to those of control plants over the growth period irrespective of plant type as displayed by the shoot height (Appendix 5.7). The impact of diesel on plant growth was greatest for those grown in diesel-spiked soils with 320000 µg kg^{-1} compared to control plants. Figures 5.2, 5.3 and 5.4 show the impact of diesel treatments on *M. sativa*, *F. arundinacea* and *L. perenne* shoot height, respectively. The plant heights for treatments 1, 2 and 3 decreased in comparison to their control plants by 20, 39 and 46% for *M. sativa*, 24, 43 and 50% for *F. arundinacea* and 23, 29 and 38% for *L. perenne* respectively after 57 days.

In general, there was an inverse relationship between diesel treatment concentration and plant height hence average plant shoot height (cm) was in this order; 320000 µg kg^{-1} < 151000 µg kg^{-1} < 102000 µg kg^{-1} < control. The impact of diesel treatment on the growth of *F. arundinacea* and *L. perenne* was similar to that of *M. sativa* except that it was observed that treatment with 102000 µg kg^{-1} may have had a stimulatory effect on *F. arundinacea* and *L. perenne* growth at the early stage compared to those of the control and other treatments. The result of two-way ANOVA showed diesel treatments and growth period significantly affected the shoot height of *M. sativa, L. perenne and F. arundinacea* ($p<0.05$).

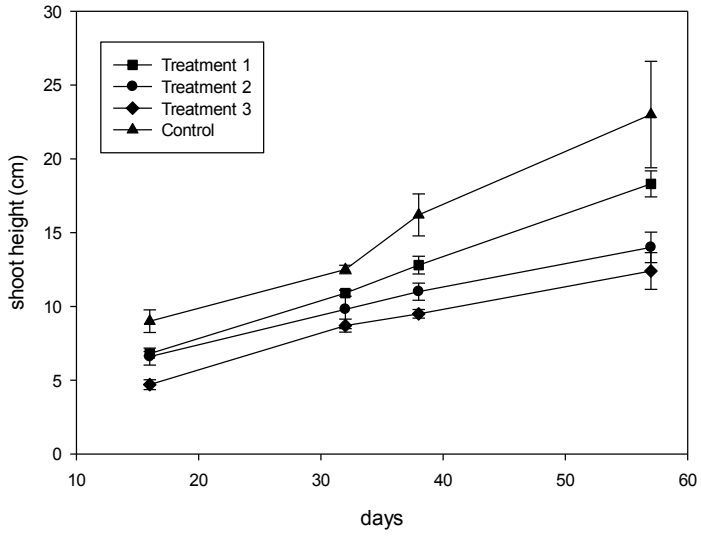

Figure 5.2: Effect of diesel treatments on *M. sativa* shoot height (Average values ± SE, n=3). (Treatment 1; 102000±2870, Treatment 2; 151000±15900, Treatment 3; 320000±160000 µg kg^{-1}).

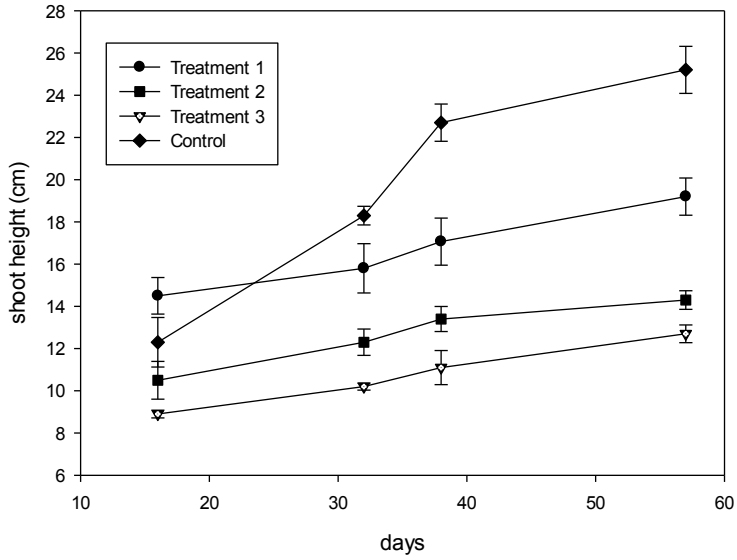

Figure 5.3: Effect of diesel treatments on F. arundinacea shoot heights. (Average values ± SE, n=3). (Treatment 1; 102000±2870, Treatment 2; 151000±15900, Treatment 3; 320000±160000 µg kg^{-1}).

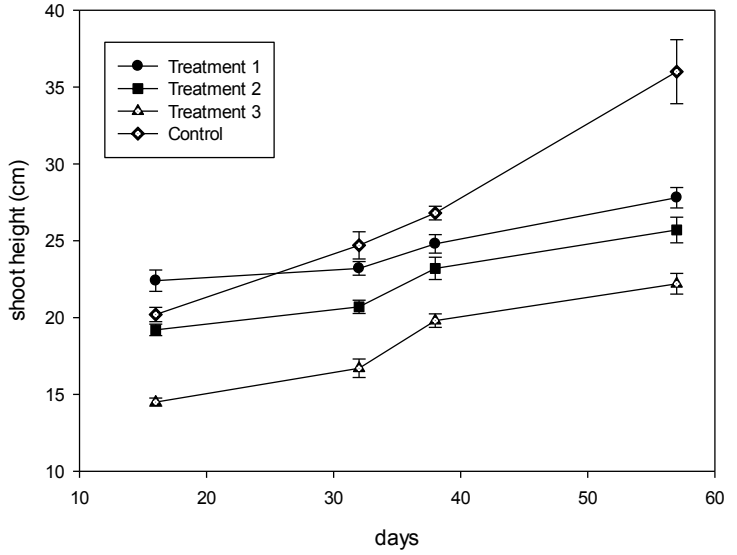

Figure 5.4: Effect of diesel treatments of *L. perenne* shoot height. (Average values ± SE, n=3). (Treatment 1; 102000±2870, Treatment 2; 151000±15900, Treatment 3; 320000±160000 µg kg^{-1}).

5.4.1.2 Impact of diesel treatments on plant biomass

The diesel treatments also affected plant biomass yield (shoot and root dry weights) irrespective of plant species compared to those of controls after the growth period (Table 5.2

and Appendix 5.8). Also biomass yield was generally greater for plant roots compared to plant shoots especially for *F. arundinacea* and *L. perenne*. A decrease in biomass yield was observed across the treatments for all the plants. The biomass yield decrease was greatest in the 319600 μg kg^{-1} treatment and lowest for the 102000 μg kg^{-1} treatment (Table 5.2). The greatest decrease in biomass yield amongst the plant monoculture was observed for *M. sativa* (shoot biomass; 44-73% and root biomass; 81-90%) followed by *F. arundinacea* (shoot biomass; 41-64% and root biomass; 27-74%) and *L. perenne* (shoot biomass; 28-50% and root biomass 45-74%). As for the mixed plant community (*M. sativa* + *L. perenne*), the decrease in shoot and root biomass was 13-62% and 14-86% respectively. There was a decrease in the root/shoot ratio of the plants from the different treatments in comparison to their controls (Table 5.2).

The impact of diesel treatments on *M. sativa* root biomass was statistically significant ($p<0.05$). Tukey test showed that biomass yield for the 151000 μg kg^{-1} treatment was significantly different from those of the control group. There was a significant antagonistic effect and inverse relationship between *F. arundinacea* shoot and root biomass yield and increase in diesel concentration. Results from Tukey post-hoc test showed that *F. arundinacea* root and shoot biomass of treatments with 151000 μg kg^{-1} and 320000 μg kg^{-1} were significantly different from those of the control group ($p < 0.05$). For *L. perenne*, the impact of diesel treatments on biomass yield was statistically significant for shoot biomass ($p < 0.05$) with treatments with 151000 μg kg^{-1} and 320000 μg kg^{-1} but significantly different from those of the control based on the Tukey post-hoc test. The effect of diesel treatment was not significant for root biomass ($p> 0.05$) however, there was a substantial difference in mean root biomass. Also there was a statistically significant relationship between the treatments and mixed plant biomass yields ($p < 0.05$) (Appendix 5.8). Tukey post-hoc test revealed shoot and

root biomass from treatments with 150500 μg kg^{-1} and 320000 μg kg^{-1} were significantly different from those of the control and treatment with 102000 μg kg^{-1}. The biomass yield of the mixed plant was greater than those of the monoculture of *M. sativa* but less those of the monoculture of *L. perenne*.

Table 5.2: Shoot and root biomass of M. sativa, L. perenne and F. arundinacea following a 60 day growth period in different diesel treatments. (Average values ± SE, n=3). (Treatment 1; 102000±2870, Treatment 2; 151000±15900, Treatment 3; 320000±160000 μg kg^{-1}).

Plant	Diesel Treatment	Shoot biomass (g)	Decrease in shoot biomass yield (%)	Root biomass (g)	Decrease in root biomass yield (%)	Root/ shoot ratio
M. sativa	Control	1.97±0.72		6.73±4.60		3.41
	1	1.10±0.31	44	1.27±0.43	81	1.15
	2	0.67±0.09	66	0.57±0.13	92	0.85
	3	0.53±0.12	73	0.7±0.15	90	1.32
L. perenne	Control	4.37±0.38		16.77±4.96		3.84
	1	3.17±0.30	28	11.97±3.77	27	3.78
	2	2.77±0.24	37	5.87±0.58	65	2.12
	3	2.20±0.15	50	4.33±0.50	74	1.97
F. arundinacea	Control	3.97±0.58		8.20±0.42		2.07
	1	2.33±0.33	41	4.50±1.47	45	1.93
	2	1.57±0.32	61	2.30±0.44	72	1.46
	3	1.43±0.09	64	2.13±0.34	74	1.49
Mixed plants	Control	4.50±0.36		19.83± 2.16		4.41
	1	3.90±0.46	13	16.97± 2.95	14	4.35
	2	2.20±0.35	51	4.13±0.96	79	1.88
	3	1.73±0.13	62	2.80±0.66	86	1.62

5.4.1.2 Impact of plant choice and TPH concentration on dissipation

Among the selected plants, the highest TPH dissipation were displayed by *L. perenne* (81% at 102000 μg kg^{-1}, 69% at 151000 μg kg^{-1} and 72% at 320000 μg kg^{-1}) while the lowest were

displayed by *M. sativa* (52% at 102000 μg kg^{-1} and 56%; 151000 μg kg^{-1}) and mixed plant (57%; 320000 μg kg^{-1}) (Table 5.3). Results from a two-way ANOVA revealed that TPH dissipation did not significantly differ across the treatments, plant species and unplanted controls ($p>0.05$) (Appendix 5.9). The unplanted control displayed greater TPH dissipation than some plants for the treatment at 102000 μg kg^{-1}; *F. arundinacea* and *M. sativa*, the treatment at 151000 μg kg^{-1}; *F. arundinacea*, *M. sativa* and mixed plant and at the treatment at 320000 μg kg^{-1}; *M. sativa*, *L. perenne*, *F. arundinacea* and mixed plant.

TPH dissipation for the mixed plants from treatments with 102000 μg kg^{-1} and 151000 μg kg^{-1} were comparable to that of the unplanted control but greater than those of *M. sativa* and *F. arundinacea*. As for treatment with 320000 μg kg^{-1}, dissipation for the mixed plant was comparable to that of *M. sativa* but less than those for *L. perenne*, *F. arundinacea* and the unplanted control (Table 5.3).

Table 5.3: Total petroleum hydrocarbon dissipation by plant. (Average values ± SE, n=3). Initial TPH concentrations (µg kg^{-1}): Treatment 1; 102000±2870, Treatment 2; 151000±15900, Treatment 3; 320000±160000 µg kg^{-1}.

Diesel Treatment	**Plant**	**Mean extractable TPH (µg kg^{-1})**	**Mean Dissipation (%)**
1	Unplanted control	23100±2870	77
	M. sativa	48400±9680	52
	L. perenne	19100±4770	81
	F. arundinacea	25100±13800	75
	Mixed plant	22000±9020	78
2	Unplanted control	57100±8840	62
	M. sativa	65500±11700	56
	L. perenne	46200±3470	69
	F. arundinacea	62900±1650	58
	Mixed plant	58400±3530	61
3	Unplanted control	77700±35000	83
	M. sativa	133000±13300	58
	L. perenne	90400±11900	72
	F. arundinacea	96300±13600	70
	Mixed plant	138000±52800	57

5.5 Discussion

5.5.1 Impact of diesel treatment on plant growth

Although no plant death was recorded in this study, the stunted growth and sign of chlorosis displayed compared to the control plants (Figures 5.2, 5.3 and 5.4) are similar to the findings of Agamuthu *et al*. (2010), where *Jatropha curcas* grown in soil contaminated with 1 and 2.5 % w/w waste lubricating oil and organic wastes displayed yellowing, stunted growth and plant death as signs of phytotoxicity. Our findings are also supported by previous studies on tropical legumes and grasses grown on soil contaminated with 5% (w/w) heavy crude oil for

180 days (Merkl *et al*., 2004; Merkl *et al*., 2005) and *L. perenne and T. repens* grown on soil with 12000 mg diesel kg^{-1} for 30 days (Barrutia *et al*., 2011).

The stunted growth and decreased biomass yield is attributed to phytotoxic effects mainly from the uptake of small molecular weight volatile hydrocarbon and dissolved diesel causing early stress and inhibiting plant establishment, root elongation and viability over the growth period (Henner *et al*., 1999; Kechavarzi *et al.*, 2007). Also petroleum hydrocarbons affect the pattern and quantity of plant growth regulators (PGRs) produced by plant roots. Consequently this affects plant development and senescence with plant relative growth found to be higher for plants in contaminated soils compared to those in uncontaminated soils (Merkl *et al*., 2005).

The lower growth inhibition for *F. arundinacea* and *L. perenne* compared to *M. sativa* may be due to differences in stress tolerance as supported by previous studies (Henner *et al*., 1999; Adam and Duncan 1999; Olson *et al*., 2007). A phytotoxicity study with pure PAHs, coking soil and gas work soils by Henner *et al*. (1999) on a range of native plant species showed *L. perenne* and maize to be most tolerant to hydrocarbons compared to other plant candidates including the legumes; *M. sativa* and *T. repens*. Furthermore, previous studies by Kaimi *et al*. (2007), Zhang *et al*. (2010) and Barrutia *et al*. (2011) have also shown that *L. perenne* is highly tolerant to diesel contamination. Barrutia *et al*. (2011) reported that *L. perenne* was more tolerant than *T. repens* following a five-month greenhouse experiment with diesel-spiked soil (12,000 mg diesel kg^{-1}). The difference in tolerance is attributed to the difference in the root systems and morphology between grasses and legumes (Adam and Duncan, 2002; Barrutia *et al*., 2011; Hall *et al*., 2011). Unlike, *M. sativa*, which has tap roots and less biomass, the extensive fibrous root systems of *F. arundinacea* and *L. perenne* are

characterised by the presence of a sheath with an extensive surface area for microbial colonisation and increased penetration ability. These facilitate better soil aeration and bioavailability of contaminants for biodegradation and hence reduce phytotoxicity (Hall *et al*., 2011; Wang *et al*., 2011).

The difference in tolerance between *L. perenne* and *T. repens* despite the similarities in their root system may be attributed to differences in rhizosphere activities such as microbial colonization and plant-microbe interactions (Hall *et al*., 2011; Wang *et al*., 2011). An early but brief stimulatory growth effect of the 102000 μg kg^{-1} treatment on *F. arundinacea* and *L. perenne* compared to their controls (Figures 5.3 and 5.4) as supported by reports by Adam and Duncan (1999) and Gaskin *et al*. (2008), may be attributed to plant tolerance, carbon/energy input from diesel and low phytotoxic effect, as discussed above. This may also be due to the remnant pertiles serving as a protective barrier between the emerging roots and diesel-spiked soil compared to the other treatments although this has not been suggested in previous literature. However, the decrease in growth rate compared to that of the control may be explained by cumulative effect of the hydrocarbons as root exposure and elongation into the diesel-spiked soil increases over the growth period.

The greatest impact of diesel on the biomass yield for the 320000 μg kg^{-1} treatment and the least impact for the 102000 μg kg^{-1} treatment (decrease in plant biomass yield with increasing diesel concentration in the treatments) confirms that phytotoxicity is dependent on hydrocarbon levels amongst other factors (Kirk *et al*., 2002). Also the phytotoxic effect of diesel as reflected by the decrease in biomass of *M. sativa*, *F. arundinacea*, *L. perenne* and the mixed plant are similar to findings of Merkl *et al*. (2005). Merkl *et al*. (2005) assessed the phytoremediation potential of some tropical grasses and legumes on petroleum-contaminated

soils with 5% w/w heavy crude oil and observed a significant decrease in plant biomass compared to those of plants grown in uncontaminated soils after 180 days. However, Gaskin *et al*. (2008) reported an increased root biomass of *Cymbopogon ambiguus* in the presence of diesel/oil contamination (0.5% and 1% w/w) compared to their controls after 12 weeks growth but reported a decreased shoot biomass production compared to the control. The contradictory finding on plant biomass yield may be as a result of differences in factors such as the actual initial TPH concentration, plant species, number of seedlings, soil type, indigenous microbes and, rhizosphere interaction and activity.

Furthermore, the decrease in root to shoot ratio in the diesel treatments compared to the control is also due to phytototoxic effects of diesel over the 60 day growth period. This disagrees with the findings of Merkl *et al*. (2005), Kaimi *et al*. (2007) and Gaskin *et al*. (2008) of an increase in root to shoot ratio in diesel treatments. However, these studies were conducted for a longer period (84-180 days). A study by Kulakow *et al*. (2006) reported higher root/shoot ratio and high relative root-length densities for plants under stressed conditions compared to their controls. They also reported a slow biomass yield increase between 60 and 180 days but later observed a ten-fold increase in above ground growth and root biomass with shorter but thicker roots especially for grasses. By 180 days, the biomass from the contaminated soil was about three times those observed for plants in the uncontaminated soil at 60 days. *L. perenne and F. arundinacea* had the highest root-length densities and were followed by *Dactylis glomerata* (orchard grass) amongst 29 species including grasses and legumes (Kulakow *et al*., 2006). A 60 day growth period was used in this study as soils freshly spiked with contaminants are readily subjected to loss by volatilization and microbial degradation (Smith *et al*., 2009; Wenzel, 2009) and maximum TPH removal rate is observed at 60 days (Escalante-Espinosa *et al*., 2005).

In the mixed plant treatment, *L. perenne* was more dominant than *M. sativa* as a result of the more extensive root systems with an impact on the contaminant tolerance and biomass yield compared to the monoculture with *M. sativa*. This is similar to the findings of a study by Meng *et al*. (2011) where *L. perenne* was more dominant than *T. repens* in a mixed plant culture. The phytotoxic effects of diesel had a greater impact on the physiology and growth of *M. sativa* compared to *L. perenne* in the mono-and mixed- culture in this study. Additional information such as the soil quality index may be useful in comparing plant tolerance and capacity for the maintenance of the rhizosphere conditions and interactions (Barrutia *et al*., 2011).

5.5.2 TPH *dissipation*

The contribution of volatilization and microbial degradation as compared to rhizoremediation to the overall TPH dissipation are evident from the comparable dissipation between the unvegetated control and vegetated soil in this study. Our study involved the use of grasses (*L. perenne* and *T. repens*) and legume (*M. sativa*) without inoculation with hydrocarbon degraders or plant growth promoting bacteria to enhance phytoremediation. The finding of a comparable dissipation in both vegetated and unvegetated treatments is supported in the literature by a comparable TPH degradation from highly contaminated petroleum sludge (TPH >35 g kg^{-1}) for planted treatments with *Cynodon dactylon* (68%) and *Festuca arundinacea* (62%) and unplanted treatment (57%) after one year (Hutchinson *et al*., 2001). Kulakow *et al*. (2006) also reported a comparable TPH concentration in planted soil (18119 mg kg^{-1}) and unplanted soils (19,400 mg kg^{-1}) following a 180 day experiment. To this end, Tang *et al*, (2010b) explained that the presence of plants roots had less impact on microbial

degradation in TPH-contaminated soils than soil properties. Some studies have reported that presence of plants may not necessarily enhance contaminant dissipation as a result of an inhibition of degrading microorganisms (Liste and Alexander, 2000) and catabolic repression by root extracts (Louvel *et al*., 2011).

On the contrary, some studies have reported a significantly higher TPH dissipation from vegetated soil compared to unvegetated soil (Banks *et al*., 1999; Merkl *et al*., 2005; Escalante-Espinosa *et al*., 2005; Huang *et al*., 2005; Gurska *et al*., 2009; Gaskin and Bentham 2010). Merkl *et al*. (2005) used tropical grasses (*Brachiaria brizantha*, *Cyperus aggregatus*, *Eleusine indica*) and legumes (*Calopogonium mucunoides*, *Centrosema brasilianum*, *Stylosanthes capitata)* with 5% (w/w) heavy crude oil for 180 days and reported a lower concentration of saturated hydrocarbon in soils with *B. brizantha* and *C. aggregatus* compared to unplanted soil. Escalante-Espinosa *et al*. (2005) used *Cyperus laxus* Lam. and a microbial consortium with TPH at 5 g kg^{-1} dry perlites for a 180 day greenhouse experiment. The perlite was spiked with hydrocarbon extracted from weathered soil to improve bioavailability while improving soil condition and plant growth. The presence of *Cyperus laxus* Lam. improved removal of aromatic and asphaltenes/polar fractions compared to the unvegetated controls. The maximum TPH removal for the inoculated plants (0.51 mg g^{-1}) and non-innoculated plants (0.29 mg g^{-1}) was observed at 60 days and decreased to < 0.1 mg g^{-1} at 180 days. They concluded that the maximum dissipation takes place before flowering and hence concluded that phytoremediation activity is linked with plant life cycle. In a three year field study by Gurska *et al*. (2009), a decrease in TPH from 130 g kg^{-1} to 50 g kg^{-1} in vegetated soil with *L. perenne*, *F. arundinacea*, *Secale cereale* and *Hordeum vulgare* compared to unvegetated control was attributed to plant seed treatment with PGPB (*Pseudomonas* strains UW3 and UW4). Also, Gaskin and Bentham (2010) reported a significantly lower residual TPH concentration for

soils with Australian native grasses (*Cymbopogon ambiguus*, *Brachiaria decumbens* and *Microlaena stipoides*) compared to unplanted controls following a 100 day experiment on soil contaminated with 60:40 diesel oil mixture at 1% w/w concentration. The conflicting results may be attributed to factors such as soil properties, plant species, indigenous microbial population, contaminant concentration, plant-microbe interaction.

The comparable dissipation in vegetated and unvegetated soil may question successful establishment of plant-microbe interactions in the rhizosphere and impacts of other factors on degradation (Barea *et al*., 2005; Dzantor, 2007; Gerhardt *et al*., 2009; Wenzel, 2009). A two year field study conducted with *Astragalus adsurgens* (Erect milkvetch) and degraders isolated from an aged oil contaminated soil as inoculants to evaluate the impact of plant-microbe synergy in the remediation of diesel-contaminated soil (>10 g kg^{-1}), reported an improved TPH loss of 13-30% higher than that of plant alone (Lin *et al*., 2008). They also observed that plant-microbe synergy was significantly affected by the diesel concentrations (0-5000 mg kg^{-1}). Hence the impact of diesel hydrocarbon on microbial diversity, plant health and plant-microbial interaction cannot be excluded in this study. Also such plant-induced stimulation and changes in microbial population and diversity are usually plant-specific (Kirk *et al*., 2005; Euliss *et al*., 2008).

Noteworthy of mention is the possible difference in the concentration and composition of hydrocarbon ranges/groups (aliphatics and aromatics) in the residual TPH despite the comparable dissipation from the different plants and treatments at the end of the greenhouse experiment. Escalante-Espinosa *et al*. (2005) reported >95% and 55% dissipation of aliphatic and aromatic fractions, respectively following greenhouse experiment with *Cyperus laxus* Lam and a microbial consortium. The concentration of residual hydrocarbon fraction may

have an impact on the recovery of soil health. This refers to the capacity of the soil to perform its function. Physical, chemical and biological soil properties serve as indicators of soil health (Barrutia *et al*., 2011). Barrutia *et al*. (2011) reported that *L. perenne* exhibited a faster recovery of soil health in terms of its rhizosphere microbial community than *T. repens* despite the comparable concentration of n-alkanes. They concluded that the physiological status of a plant and its tolerance determines the adverse impact of diesel contamination on rhizosphere microbial populations and the recovery of soil health. Consequently, the comparable dissipation displayed by the selected plants in the different diesel treatments may not indicate equal tolerance to diesel contamination.

5.5.2.1 *Dissipation by plant by treatments*

TPH dissipation for all the different plants and treatments were not significantly different ($p>0.05$) and agrees with the finding of Kulakow *et al*. (2006) using 29 species for the rhizoremediation of petroleum-contaminated soils who also found no differences between species. High TPH content may suppress TPH dissipation due to toxicity as reported by Tang *et al*. (2010a) who reported 5% w/w crude oil as the optimum TPH concentration that may result to minimal phytotoxic effect. TPH concentration is an important factor that affects the abundance of total bacteria and that of specific hydrocarbon degraders (Kaimi *et al*., 2006). Factors that may have contributed to our findings include the impact of hydrocarbons on root exudation and compositions, selection and proliferation of metabolically diverse microbial communities, and other activities in the rhizosphere (Adam and Duncan, 2002; Tang *et al*, 2010b; Hall *et al*., 2011).

The highest TPH dissipation although not significantly different displayed by *L. perenne* in all the treatments confirms previous findings of Tang *et al*, (2010b), which showed that *L. perenne* and *F. arundinacea* were better candidates for rhizoremediation of TPH-contaminated soil than *M. sativa* and *Gossypium hirsutum*. Kaimi *et al*., (2006) also reported an enhanced biodegradation of diesel-contaminated soil (1.8% w/w) by *L. perenne*. As previously discussed, this is attributed to high tolerance, extensive rooting systems and high biomass of *L. perenne* and *F. arundinacea* which facilitate aeration, microbial proliferation and biodegradation (Hall *et al*., 2011; Wang *et al*., 2011). Also rooting intensity (mg root kg^{-1} soil) and root development are known to be crucial for high TPH loss and phytoremediation potential respectively (Hou *et al*., 2001; Tang *et al*., 2010a).

5.5.2.2 *TPH loss in soils with monoculture versus mixed culture*

The higher dissipation for the mixed plants in comparison to those for monocultures of *M. sativa* and *F. arundinacea* in treatments 1 and 2 were not significantly different but may be attributed to beneficial interaction between both plants and their microbial communities. This is supported by studies by Cheema *et al*. (2009), Gurska *et al*. (2009), Meng *et al*. (2011) and Sun *et al*. (2011) which reported mixed plant communities enhanced hydrocarbon degradation. Sheng-wang *et al*. (2008) also observed a significantly enhanced PAH (pyrene and phenanthrene) dissipation for mixed cropping with *M. sativa* and *Brassica campestris* as compared to single cropping cultivation. They attributed the enhanced dissipation under mixed cropping to mainly plant-microbe interaction but also mentioned plant accumulation and plant metabolism as other mechanisms of enhancement. Wei and Pan (2010) reported that

a mixed plants cultivation with *M. sativa* and *B. campestris* stimulated plant-microbe interaction.

Apart from an enhanced PAH dissipation by combined plants, Xu *et al*. (2006) also reported an improved soil structure which facilitates the transport of water, nutrients as well as dispersal of microbes for efficient degradation at depths. Another possible explanation for the enhanced dissipation is that root interaction modifies the root physiology in terms of enzyme activity, exudation and longevity hence stimulating degradation by root derived enzymes and rhizosphere microorganisms. Also the interacting roots may affect root surface properties or rhizosphere soil properties which affect contaminant bioavailability and soil structure (Joner and Leyval 2003; Xu *et al*., 2006; Cheema *et al*., 2010). Sun *et al*. (2011) concluded that mixed planting with *M. sativa* and *F. arundinacea* enhanced PAH degradation by stimulating microbial activities and increasing soil dehydrogenase and urease activity in the soil.

The greater TPH dissipation from the 320000 µg kg^{-1} treatment for the monoculture compared to that of the mixed culture is supported by the findings of Phillips *et al*. (2006) and Phillips *et al*. (2009). Phillips *et al*. (2006) reported that the use of mixed plant community with legumes such as *M. sativa* may inhibit contaminant degradation of contaminants due to its selective stimulation and proliferation of degraders which may not immediately result in an increased degradation. Also, Phillips *et al*. (2009) showed a monoculture had 54% TPH decrease compared to control and mixed plant culture with *L. perenne*, *M. sativa* and *Triticum aestivum*. Based on their findings, they concluded that the use of mixed plants may deter phytoremediation efficiency. Aside from the phytotoxic effects of diesel hydrocarbons, mixed plant culture may have impacted on the plant-plant interactions, plant-microbe interaction as well as other rhizosphere activities hence the low TPH dissipation. Also a study by Li *et al*.

(2013) reported an increased copy number of biphenyl dioxygenase genes, total bacteria counts and dehydrogenase activity in mixed cropping soil with *M. sativa* and *F. arundinacea* however, the single plant culture with *F. arundinacea* displayed the highest PCB removal after five months followed by the mixed plants. They attributed their findings to the greater biomass of *F. arundinacea* which probably facilitated the dissipation of PCB. They also found that there was no correlation between the abundance of biphenyl dioxygenase genes and the extent of degradation probably due to a variety of pathways and inducers involved in PCB degradation.

The comparable dissipation for both vegetated and unvegetated soils and the conflicting outcome for the mixed plant treatment may be attributed to factors such as plant choice, TPH concentration and toxicity, plant-plant interaction, plant-microbe interaction, soil properties, nutrient level and rhizosphere activities (Li *et al*., 2013).

5.6 Conclusion

M. sativa, *F. arundinacea* and *L. perenne* were able to grow and establish on diesel spiked soils however, yellowing of leaves, stunted growth and decrease in biomass production compared to the control groups were noticed as signs of phytotoxicity and plant stress. *L. perenne* was found to be most tolerant to the presence of diesel, next was *F. arundinacea* while *M. sativa* was the least tolerant with significant inhibitory effects on plant growth and biomass yield. Plant stress and phytotoxicity from petroleum significantly affected plant growth and biomass yields.

The highest TPH dissipation was displayed by *L. perenne* in all the treatments; however, TPH dissipation was generally comparable and not significantly different for all the vegetated and

unvegetated soil as reported by some previous studies. Also, the significant effect of the diesel treatments on plant growth and in other words plant health may have affected root exudation with a resultant impact on the stimulation and proliferation of microbes, plant-microbe interaction and microbial degradation in the rhizosphere. These are the most important features that determine rhizoremediation success. Other important factors include plant choice, TPH concentration, soil properties, nutrient level and plant nutrient uptake mechanisms and rhizosphere activities (Li *et al*., 2013). Hence the plant selection and the full exploitation of the benefits of plants for phytoremediation whether in single or mixed communities may rely on the proper understanding of the individual site variables and conditions.

CHAPTER 6

EFFECT OF SINGLE AND MIXED PAH CONTAMINATION ON *M. sativa*, *L. perenne* and *F. arundinacea* BIOMASS YIELD AND PAH DISSIPATION DURING PHYTOREMEDIATION

Abstract

PAH contaminated sites usually have a mixture of PAHs of varying concentrations. While many studies have focused on the use of rhizoremediation for single contaminant remediation, studies on mixed contaminants are few. The presence of mixed PAHs may affect PAH dissipation differently to contamination with a single PAH. This study investigated the effect of single and mixed PAH contamination on plant biomass yield and PAH dissipation. *M. sativa*, *L. perenne* and *F. arundinacea* were selected for greenhouse experiments with soils spiked with phenanthrene, fluoranthene and benzo[*a*]pyrene in single and mixed treatments. The single PAH treatment had higher stimulatory effect (80-240%) on *M. sativa* root biomass yield in comparison with the mixed PAH treatment (40%). The single PAH treatment displayed a higher stimulatory effect on root biomass yield of *L. perenne* (30-210%) and *F. arundinacea* (45-170%), while there was a decrease in root biomass yield of 0.7% and 4.2% respectively in the mixed PAH treatment. In comparison to the unplanted control, *L. perenne* had the highest dissipation for phenanthrene (1.13%), fluoranthene (5.86%) in the single PAH treatment as well as fluoranthene in the mixed PAH treatment (4.35%). *F. arundinacea* had the highest benzo[*a*]pyrene dissipation for both the single PAH (48%) and mixed PAH treatments (21%). The presence of mixed PAH contaminants influenced dissipation of the individual compounds. In some cases, the presence of plants showed no evidence of rhizoremediation suggesting no enhancement of PAH dissipation. Successful

rhizoremediation of PAH-contaminated soils therefore appears dependent on plant choice, successful plant establishment and survival, plant tolerance and phytostimulation.

6.1 Background

Recent years have seen a marked increase in research on phytoremediation as a promising eco-friendly remediation technology. This has been driven by reports of enhanced biodegradation of organic compounds including PAH in the presence of plants compared to unplanted soils (Siciliano *et al*., 2003; Xu *et al*., 2006; Olson *et al*., 2007; Wu *et al*., 2011). Unlike inorganic compounds for which direct plant uptake and accumulation (also known as phytoextraction /phytoaccumulation) is the main phytoremediation process, this has not been identified as one of the main pathways for PAH dissipation. Studies have shown that phytoextraction/phytoaccumulation of PAH is usually negligible and that the principal phytoremediation process involved in the dissipation of PAH and other organics is rhizoremediation (Kaimi *et al.*, 2006; Cheema *et al.*, 2010; Lu *et al*., 2011; Meng *et al*., 2011). Increased dissipation in vegetated soils has been attributed to rhizospheric effects through root exudation, which improves soil condition, stimulates microbial activity and improves their metabolic capability (Kirk *et al*., 2005; Cheema *et al.*, 2010). Other benefits include facilitation of soil aeration, water infiltration and bioavailability (Kaimi *et al*., 2006; Hamdi *et al*., 2012).

Apart from microbial degradation and rhizoremediation, there are other important PAH dissipation pathways. These include abiotic processes such as volatilization, leaching with irrigation and absorption to soil fractions (Kaimi *et al.*, 2006). Smith *et al*. (2011) attributed the substantial loss of low molecular weight PAHs including phenanthrene and fluoranthene

from soils amended with pure PAH to volatilization and microbial degradation in comparison to coal tar amended soils. They also showed a significant but comparable reduction of PAH levels in planted and unplanted soil during greenhouse experiments. The residual PAH levels in planted and unplanted soils for pure PAH-spiked soils suggested no evidence of phytoremediation. A similiar study by Sun *et al*. (2010) demonstrated a substantial abiotic loss of phenanthrene and pyrene from sterile freshly spiked soil. PAH losses in planted soils and unplanted controls were similar.

Grasses and legumes have been identified as candidates for efficient rhizoremediation of organic compounds (Kuiper *et al*., 2004; Gerhardt *et al*., 2009; Gurska *et al*., 2009; Vangronsveld *et al*., 2009). A study comparing PAH dissipation among 18 plant species representing eight families (*Asclepiadaceae*, *Asteraceae*, *Fabaceae*, *Geraniaceae*, *Lamiaceae*, *Poaceae*, *Polygonaceae*, and *Verbenaceae*) showed *Poaceae* was the most effective of the tested families while *L. perenne* was the most effective plant species (Olson *et al*., 2007). Rhizoremediation potentials and prospects for restoring contaminated land have however, been based mainly on greenhouse experiments and a few field trials with spiked soils and contaminated soils (Schwitzguebel *et al*., 2002; Trapp *et al*., 2006; Gerhardt *et al*., 2009; Gurska *et al*., 2009; Vangronsveld *et al*., 2009). Some studies have suggested that PAH in freshly spiked soils will behave differently from those in aged contaminated soils (Smith *et al*., 2011). Interestingly a few studies have shown that presence of plants may not necessarily enhance PAH dissipation. This was explained by an inhibition of PAH degrading microorganisms (Liste and Alexander, 2000) and catabolite repression by root extracts (Louvel *et al*., 2011). Perelo *et al*. (2010) also stated that aeration by plant root may inhibit reducing condition required for some reactions such as reductive dechlorination.

Studies have shown the adverse effects of PAH on seed germination in freshly spiked soil and aged soils (Henner *et al*., 1999). Such germination studies however are unable to predict the successful growth and establishment of plant species in PAH contaminated soils (Smith *et al*., 2006). Phytotoxicity is mainly attributed to volatile components which when lost from soil by weathering, volatilization and biodegradation, allow for plant establishment and growth (Henner *et al*., 1999). Biomass yields in PAH contaminated soils have been found to be lower than those from control soils (Cheema *et al*., 2010). There is a correlation between plant biomass yield and PAH degradation (Parrish *et al*., 2004). Grasses have been shown to have better biomass yield than legumes on PAH-spiked soils and soils with aged PAH (Smith *et al*., 2006). Some studies have shown that legumes are more tolerant to the presence of PAH than grasses (Lee *et al*., 2008; Hall *et al*, 2011) while some studies suggest the opposite (Cheema *et al*., 2010).

Studies have confirmed the role of plants in shaping and sustaining soil microbial diversity through rhizospheric effect as well as inducing metabolic pathways in contaminated sites (Singer *et al*., 2003; Dzantor *et al*., 2007; Mackova *et al*., 2009). As root exudates compositions vary with plant species, there would be variation in the diversity and activities of stimulated microbes, plant-microbe interactions and utilization of carbon sources including contaminants (Diogo *et al*., 2010).

All PAH contaminated sites have mixed PAH present but early studies have focused on single contaminant remediation. Recent phytoremediation studies are shifting towards mixed contamination remediation to reflect real site remediation scenarios (Gan *et al*., 2009). This study investigated the effect of single PAH and mixed PAH on plant biomass yield, PAH dissipation and microbial diversity. *M. sativa*, *L. perenne* and *F. arundinacea* were selected

for this study based on their rhizoremediation efficiency identified in previous studies (Kaimi *et al.*, 2006; Cheema *et al.*, 2010; Lu *et al*., 2011). The PAH studied were phenanthrene, fluoranthene and benzo[*a*]pyrene based upon their presence and concentrations in existing contaminated UK sites including a disused gas works site in Saltley, Birmingham and an old refinery site in Swansea, Wales.

6.2 Hypotheses

1. Single PAH and mixed PAH treatments will affect biomass yields and PAH dissipation for selected plants.
2. Following the greenhouse experiments, residual PAH concentrations of the different treatments will differ between vegetated soils and non-vegetated soils.

6.3 Methods

6.3.1 Soil preparation and experimental design

A sandy loam soil sourced from a commercial supplier (Travis Perkins, United Kingdom) was air-dried and sieved through a 2 mm sieve. Phenanthrene (>98% purity), fluoranthene (>98% purity) and benzo[*a*]pyrene (>96% purity) were obtained from VWR, UK. Soils for the single PAH experiment were prepared by spiking about 250 g of air-dried and sieved soils (25% of soil for planting in each pot) with phenanthrene (~300 mg), fluoranthene (~200 mg) and benzo[*a*]pyrene (~5 mg) dissolved in 20 mL of acetone in a fume hood. For the mixed PAH experiments, about 250 g of soil was spiked with all three compounds (phenanthrene; 300 mg, fluoranthene; 200 mg, benzo[*a*]pyrene; 5 mg) dissolved in 20 mL of acetone (Fisher, UK). The soils were mixed in glass beakers with a spatula. All spiking was carried out in triplicate.

The spiked soils were allowed to air-dry in a fume hood for three days before adding about 750 g of unspiked soil. Following the spiking, soils were homogenized, stored and later dispensed for planting as described in Section 3.2.3.

Abiotic controls were set up using unplanted spiked soil with 30 mL of formalin added every 7 days to inhibit microbial growth and activity. Abiotic controls assessed contributions made by abiotic processes while controls without formalin assessed contributions of abiotic processes as well as microbial degradation to PAH dissipation**.** Plant seedlings in pertiles were transplanted into the spiked and control soils and soil samples collected and stored as discussed in Section 3.2.4. Soil samples were extracted by microwave extraction and analysed for PAH content by GC-MS as described in Section 3.5. Further soil samples (~2 g) were collected in sterile tubes for microbial diversity analysis (Section 7.3.2).

Table 6.1: Greenhouse experiment on single and mixed PAH contamination. Initial PAH concentration in the single PAH treatment were: phenanthrene; 222±40.6 mg kg^{-1}, fluoranthene; 104±18.6 mg kg^{-1}, benzo[*a*]pyrene; 2.08±0.208 mg kg^{-1}. Those for the mixed PAH treatment were: phenanthrene; 254±42.2 mg kg^{-1}, fluoranthene; 153±17.7 mg kg^{-1}, benzo[*a*]pyrene; 2.65±0.560 mg kg^{-1} (mean ± SE, number of replicate = 6) (Appendix 6.1).

Treatments	***M. sativa***	***L. perenne***	***F. arundinacea***	**Unplanted control**	**Abiotic control**
Control	X3	X3	X3	X3	X3
PHE	X3	X3	X3	X3	X3
FLU	X3	X3	X3	X3	X3
B[*a*]P	X3	X3	X3	X3	X3
PHE+FLU+ B[*a*]P	X3	X3	X3	X3	X3

6.3.2.1 Dissipation Calculations

The difference between the mean initial PAH concentration (n=6) and mean residual concentration for the treatments (n=3) at the end of the greenhouse experiments represents the concentration dissipated. This was used for the calculation of the overall percentage dissipation, proportion of overall dissipation attributable to plants and proportion of overall dissipation attributable to plants and microbes expressed as a percentage.

- Percentage dissipation= concentration dissipated / initial concentration * 100
- Proportion of overall dissipation attributable to plants (%) = (dissipation from plant experiment -dissipation for unplanted control) / initial concentration * 100
- Proportion of overall dissipation attributable to plants and microbes (%) = (dissipation from plant experiment -dissipation for abiotic control) / initial concentration * 100

6.4 Results

6.4.1 Impacts on Plant Biomass Yield

6.4.1.1 *M. sativa* biomass

The results show that the treatments had a stimulatory effect on *M. sativa* shoot and root biomass compared to the control plants. For shoot biomass, there was 190, 180, 190 and 110% yield increase following the phenanthrene (Phe), fluoranthene (Flu), benzo[*a*]pyrene (B[*a*]P) and mixed PAH treatments respectively compared to the control. *M. sativa* root biomass on the other hand had 240, 160, 80 and 40% yield increase in the Phe, Flu, B[*a*]P and PAH Mix treatments respectively compared to the control (Table 6.2, Appendix 6.2). The lowest shoot and root biomass yield was seen in the mixed PAH treatment with the highest shoot and root biomass yields seen in the B[*a*]P and Phe treatments respectively. Statistical

analysis by one-way ANOVA showed that the relationship between the single and mixed PAH treatments and *M. sativa* shoot and root biomass yield were not significant ($p>0.05$) (Appendices 6.2 and 6.3).

Table 6.2: Shoot and root biomass of *M. sativa* grown on soils with single PAH and mixed PAH treatments. (Average values ± SE, n=3).

M. sativa	**Treatments**	**Average biomass yield (g)**	**Biomass increase relative to the control (%)**
Shoot	Control	1.10±0.208	
	Phenanthrene	3.13±1.13	190
	Fluoranthene	3.03±0.751	180
	Benzo[*a*]pyrene	3.23±0.291	190
	Mixed PAH	2.33±0.219	110
Root	Control	0.70±0.100	
	Phenanthrene	2.40±1.21	240
	Fluoranthene	1.80±0.709	160
	Benzo[*a*]pyrene	1.23±0.417	76
	Mixed PAH	1.00±0.0577	43

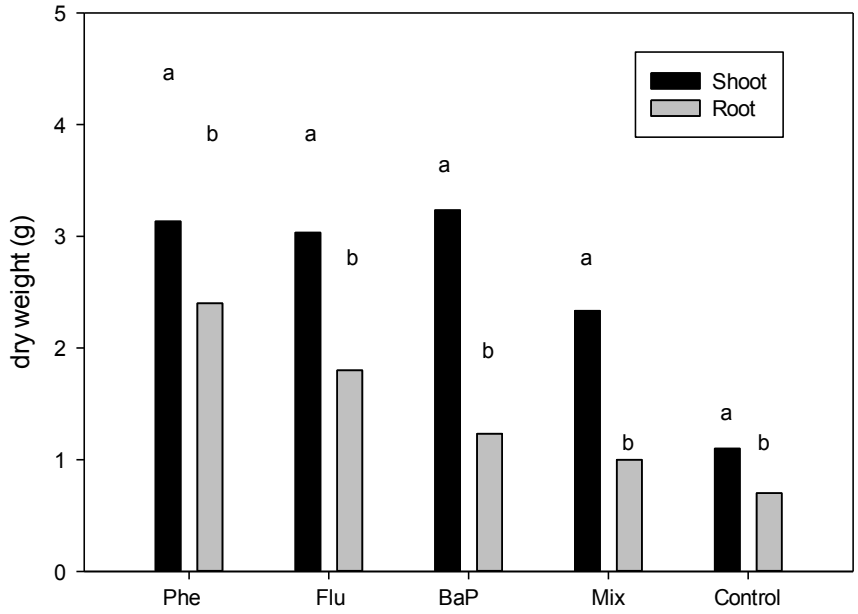

Figure 6.1: Shoot and root biomass of *M. sativa* grown on soils with phenanthrene (Phe), fluoranthene (Flu), benzo[*a*]pyrene (B[*a*]P) and phenanthrene + fluoranthene + benzo[*a*]pyrene (Mixed PAH) after 65 days of growth. Error bars represent standard deviation of three sampled pots. Different letters indicate a significant difference (p=0.05).

6.4.1.2. *L. perenne* biomass

In general, the results show greater stimulatory effect on *L. perenne* root biomass yield compared to those for shoot biomass. The treatments had a generally weak inhibitory effect on shoot biomass yield. This is evident from the decreases in biomasses by 5, 6, and 8% for the Phe, Flu and mixed PAH treatments respectively compared to that of the control plant. There was however, a stimulatory effect (3% increase) on biomass yield for *L. perenne* shoot yield under B[*a*]P treatment compared to the control plant. Root biomass increases were 210, 30 and 30% for the Phe, Flu and B[*a*]P treatments respectively while *L. perenne* under the mixed PAH treatment displayed a decrease in root biomass by 0.7% compared to the control (Table 6.3). Despite the stimulatory effect of the treatments on *L. perenne* root biomass and

inhibitory effect on the shoot biomass, these effects were not statistically significant for shoot biomass and root biomass ($p>0.05$) (Appendices 6.2 and 6.3).

Table 6.3: Shoot and root biomass of *L. perenne* grown on soils with single PAH and mixed PAH treatments. (Average values ± SE, n=3).

Lolium perenne	**Treatments**	**Average biomass yield (g)**	**Biomass increase relative to the control (%)**
Shoot	Control	5.47±0.317	
	Phenanthrene	5.20±0.361	- 5
	Fluoranthene	5.17±0.868	- 6
	Benzo[*a*]pyrene	5.63±0.664	3
	Mixed PAH	5.03±0.167	- 8
Root	Control	8.73±2.28	
	Phenanthrene	27.0±8.07	209
	Fluoranthene	11.1±1.82	27
	Benzo[*a*]pyrene	11.3±1.89	30
	Mixed PAH	8.67±0.84	-1

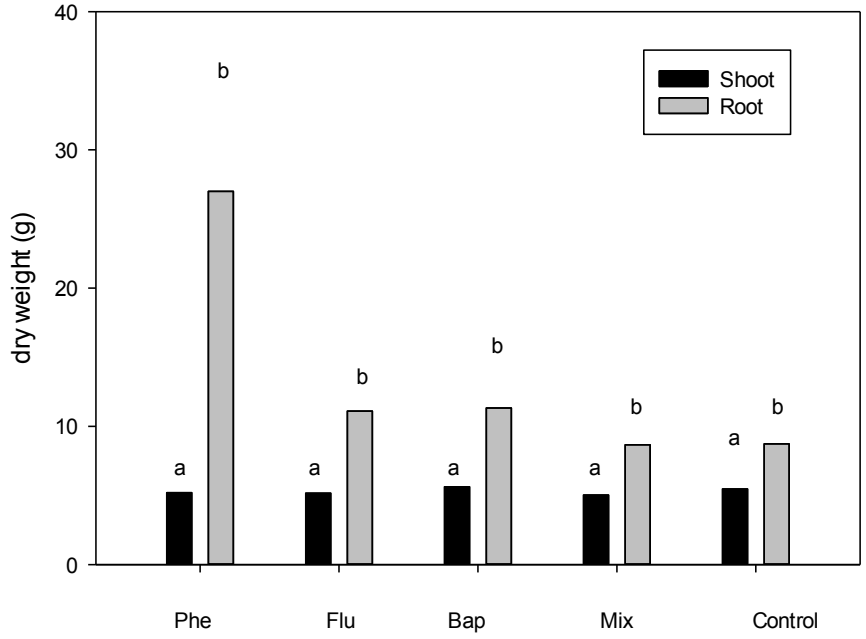

Figure 6.2: Shoot and root biomass of *L. perenne* grown on soils with phenanthrene (Phe), fluoranthene (Flu), benzo[*a*]pyrene (B[*a*]P) and phenanthrene + fluoranthene + benzo[*a*]pyrene (mixed PAH) after 65 days of growth. Error bars represent standard deviation of three sampled pots. Different letters indicate a significant difference (shoot biomass p >0.05 and root biomass; p > 0.05, n=3).

6.4.1.3. *F. arundinacea* biomass

There was a decrease in *F. arundinacea* shoot biomass yield by 7% and 12% for Phe and PAH Mix treatments respectively while an increase in shoot biomass yield by 7% and 2% was observed for Phe and B[*a*]P treatments respectively. As for *F. arundinacea* root biomass; 170%, 86% and 45% yield increase was observed in the Phe, Flu and B[*a*]P treatments respectively compared to the control (Table 6.4). A root biomass decrease of 4% was seen in *F. arundinacea* root after the mixed PAH treatment. One-way ANOVA showed that the relationship between the treatments and *F. arundinacea* shoot biomass was not significant ($p>0.05$) while that for *F. arundinacea* root biomass ($p<0.01$) was significant (Appendices 6.2 and 6.3).

Table 6.4: Shoot and root biomass of *F. arundinacea* grown on soils with single PAH and mixed PAH treatments. (Average values ± SE, n=3).

F. arundinacea	**Treatments**	**Average biomass yield (g)**	**Biomass increase relative to the control (%)**
Shoot	Control	4.57±0.202	
	Phenanthrene	4.23±0.285	-7
	Fluoranthene	4.90±0.10	7
	Benzo[*a*]pyrene	4.67±0.371	2
	Mixed PAH	4.03±0.384	-12
Root	Control	4.73±0.589	
	Phenanthrene	13.0±2.14	175
	Fluoranthene	8.80±1.31	86
	Benzo[*a*]pyrene	6.87±1.02	45
	Mixed PAH	4.53±0.32	-4

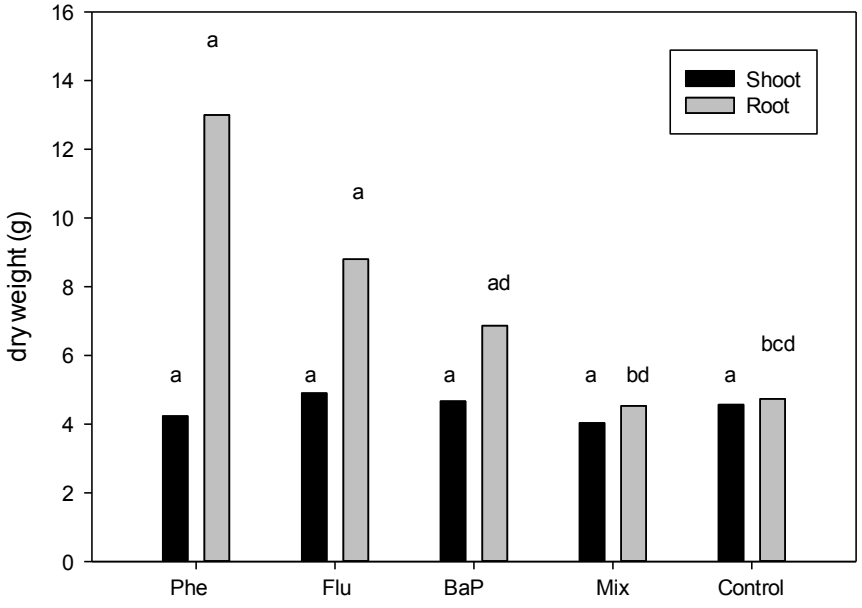

Figure 6.3: Shoot and root biomasses of *F. arundinacea* grown on soils with phenanthrene (Phe), fluoranthene (Flu), benzo[*a*]pyrene (B[*a*]P) and phenanthrene + fluoranthene + benzo[*a*]pyrene (mixed PAH) after 65 days of growth. Error bars represent standard deviation of three sampled pots. Different letters indicate a significant difference at $p < 0.05$ according to Tukey's HSD test (shoot biomass $p > 0.05$ and root biomass; $p < 0.01$, n=3). Tukey test was carried out for multiple comparisons where there is statistically significant difference for ANOVA ($p < 0.05$).

6.4.2. PAH residual concentration and dissipation in single and mixed PAH treatments

Over the course of the greenhouse experiments, the initial concentrations in the single PAH treatment, phenanthrene; 222±40.6 mg kg^{-1}, fluoranthene; 104±18.6 mg kg^{-1}, benzo[*a*]pyrene; 2.08±0.208 mg kg^{-1} and those for the mixed PAH treatment, phenanthrene; 254±42.2 mg kg^{-1}, fluoranthene; 153±17.7 mg kg^{-1}, benzo[*a*]pyrene; 2.65±0.560 mg kg^{-1} decreased in the planted soil as well as the unplanted control. The PAH loss was greater in planted soils compared to unplanted controls for benzo[*a*]pyrene; but for phenanthrene and fluoranthene, dissipation was slightly greater or equal to the unplanted controls. PAH dissipation by *M. sativa*, *L. perenne* and *F. arundinacea* plants during the phytoremediation experiment varied between compounds. Among the single PAH treatments, *L. perenne* had the highest PAH dissipation for phenanthrene (99%) while *F. arundinacea* had the highest dissipation for fluoranthene (99%) and benzo[*a*]pyrene (72%). In the mixed PAH experiments, *F. arundinacea* had the highest dissipation for phenanthrene (99%) and benzo[*a*]pyrene (71%) while *L. perenne* had the highest dissipation for fluoranthene (99%). Noteworthy of mention is that the dissipation of phenanthrene from *F. arundinacea* was the same as that of the unplanted control. Residual concentrations and dissipation figures for phenanthrene, fluoranthene and benzo[*a*]pyrene in single and mixed contaminations with *M. sativa*, *F. arundinacea* and *L. perenne* following a 65 day growth period are shown in Tables 6.5 and 6.7 (Appendix 6.4).

Table 6.5: Residual concentrations and percentage dissipation of PAH in single treatments with phenanthrene, fluoranthene and benzo[*a*]pyrene following rhizoremediation by *M. sativa*, *F. arundinacea* and *L. perenne*. (Average values ± SE, n=3). Initial PAH concentration in the single PAH treatment were: phenanthrene; 222±40.6 mg kg^{-1}, fluoranthene; 104±18.6 mg kg^{-1}, benzo[*a*]pyrene; 2.08±0.208 mg kg^{-1} (mean ± SE, number of replicate = 6) (Appendix 6.1).

Single PAH treatment	**Plant/control**	**Mean residual concentration (mg kg^{-1})**	**Amount dissipated (mg kg^{-1})**	**Percentage Dissipation**
Phenanthrene	*M. sativa*	3.62±2.88	219	98
	L. perenne	1.53±0.0918	221	99
	F. arundinacea	1.99±0.0885	220	99
	Abiotic control	70.7±0.740	152	68
	Unplanted control	4.05±2.01	218	98
Fluoranthene	*M. sativa*	7.51±0.488	96.5	93
	L. perenne	1.06±0.146	103	99
	F. arundinacea	0.830±0.294	103	99
	Abiotic control	61.7±3.91	42.2	41
	Unplanted control	6.92±1.69	97.0	93
Benzo[*a*]pyrene	*M. sativa*	1.59±0.132	0.494	24
	L. perenne	1.92±0.434	0.158	8
	F. arundinacea	0.579±0.123	1.50	72
	Abiotic control	1.79±0.190	0.290	14
	Unplanted control	1.58±0.320	0.496	24

Table 6.6: Contribution of plant root-enhanced dissipation and plant-microbe dissipation in single contaminations with phenanthrene, fluoranthene and benzo[*a*]pyrene following rhizoremediation by *M. sativa*, *F. arundinacea* and *L. perenne.* (Average values ± SE, n=3).

[a] difference between dissipation from plant experiment and unplanted control expressed as a percentage of initial concentration; [b] difference between dissipation from planted experiment and abiotic control expressed as a percentage of initial concentration; * Asterisked (negative) values represent percentage inhibition.

PAH	**Plant**	**Proportion of overall dissipation attributable to plants (%)[a]**	**Proportion of overall dissipation attributable to plants and microbes (%)[b]**
Phenanthrene	*M. sativa*	0.190	30
	L. perenne	1.13	31
	F. arundinacea	0.930	31
Fluoranthene	*M. sativa*	-0.570*	52
	L. perenne	5.86	58
	F. arundinacea	5.64	59
Benzo[*a*]pyrene	*M. sativa*	-0.480*	10
	L. perenne	-16.4*	-6*
	F. arundinacea	48.1	58

Table 6.7: Residual concentrations and percentage dissipation of PAH in mixed contamination experiment with phenanthrene, fluoranthene and benzo[*a*]pyrene following rhizoremediation by *M. sativa*, *F. arundinacea* and *L. perenne*. (Average values ± SE, n=3). Initial concentrations of soils with mixed PAH were: phenanthrene; 254±42.2 mg kg^{-1}, fluoranthene; 153±17.7 mg kg^{-1}, benzo[*a*]pyrene; 2.65±0.560 mg kg^{-1} (mean ± SE, number of replicate = 6) (Appendix 6.1).

Mixed PAH Treatment	**Plant/control**	**Mean residual concentration (mg kg^{-1})**	**Amount dissipated (mg kg^{-1})**	**Percentage dissipation**
Phenanthrene	*M. sativa*	36.2±29.1	218	86
	L. perenne	2.10±0.260	252	99
	F. arundinacea	1.74±0.400	252	99
	Abiotic control	85.4±4.03	169	66
	Unplanted control	1.78±0.350	252	99
Fluoranthene	*M. sativa*	22.4±5.10	131	85
	L. perenne	1.87±0.190	151	98
	F. arundinacea	3.71±1.54	149	98
	Abiotic control	59.3±3.18	93.8	61
	Unplanted control	8.54±2.21	145	94
Benzo[*a*]pyrene	*M. sativa*	1.66±0.130	0.990	37
	L. perenne	2.15±0.0600	0.500	19
	F. arundinacea	0.780±0.01	1.87	71
	Abiotic control	1.39±0.100	1.26	48
	Unplanted control	1.33±0.120	1.32	50

Table 6.8: Contribution of plant root-enhanced dissipation and plant-microbe dissipation in mixed contaminations with phenanthrene, fluoranthene and benzo[*a*]pyrene following rhizoremediation by *M. sativa*, *F. arundinacea* and *L. perenne*. [a] difference between dissipation from plant experiment and unplanted control expressed as a percentage of initial concentration; [b] difference between dissipation from planted experiment and abiotic control expressed as a percentage of initial concentration; * Asterisked (negative) values represent percentage inhibition.

PAH	**Plant**	**Proportion of overall dissipation attributable to plants (%)[a]**	**Proportion of overall dissipation attributable to plants and microbes (%)[b]**
Phenanthrene	*M. sativa*	-13.6*	19
	L. perenne	-0.130*	33
	F. arundinacea	0.02	33
Fluoranthene	*M. sativa*	-9.05*	24
	L. perenne	4.35	38
	F. arundinacea	3.15	36
Benzo[*a*]pyrene	*M. sativa*	-12.5*	-11*
	L. perenne	-30.9*	-29.1*
	F. arundinacea	20.8	22.6

6.4.2.1 Single PAH treatment with phenanthrene, fluoranthene and benzo[*a*]pyrene

Phenanthrene

The mean residual concentration of phenanthrene in the single Phe treatment was highest in the abiotic control (70.7±1.29 mg kg^{-1}) and lowest in the *L. perenne* treatment (1.53±0.918 mg kg^{-1}). The amounts of phenanthrene dissipated amongst the plant candidates tested as well as the unplanted control were comparable with the other treatments, *L. perenne* (221 mg kg^{-1}), *F. arundinacea* (220 mg kg^{-1}), *M. sativa* (219 mg kg^{-1}) and control (218 mg kg^{-1}). The abiotic control however, dissipated 152 mg kg^{-1} of phenanthrene (Table 6.5). The percentage contribution of root exudate-enhanced degradation and rhizoremediation to phenanthrene

dissipation ranged from 0.19-1.1% and 30-31% respectively (Table 6.6). Based on the abiotic control, the contribution of abiotic processes such as volatilization to phenanthrene dissipation was calculated as 68% of the total dissipation observed. Using one-way ANOVA, it was observed that treatments significantly affected the residual concentration of phenanthrene ($p<0.05$). Application of the Tukey post-hoc test showed that only the residual concentrations of phenanthrene from the abiotic control and *M. sativa* were significantly different from each other ($p<0.05$) (Appendix 6.5).

Fluoranthene

The mean residual concentration of fluoranthene was highest for the abiotic control (61.7±3.91 mg kg^{-1}) and lowest for *F. arundinacea* (0.83±0.294 mg kg^{-1}) with a corresponding dissipation of 42.0 mg kg^{-1} (41%) and 103 mg kg^{-1} (99%). The unplanted control had a mean residual concentration of 6.92±1.69 mg kg^{-1} with 97.0 mg kg^{-1} (93%) dissipation (Table 6.5). Root exudate-enhanced fluoranthene degradation accounted for -0.57-5.86% while rhizoremediation accounted for 52-58 % of total dissipation (Table 6.6). The contribution of abiotic processes to overall fluoranthene dissipation was 41%. The results from a one-way ANOVA showed that residual concentrations of fluoranthene were significantly affected by the treatments ($p < 0.01$). A Tukey post-hoc test however, showed that the plant treatments were not significantly different ($p>0.05$) from each other.

Benzo[a]pyrene

For the benzo[*a*]pyrene treatment, the lowest mean residual concentration was observed for *F. arundinacea* with 0.579±0.123 mg kg^{-1} with a corresponding dissipation loss of 1.50 mg kg^{-1} (72%), while the highest mean residual concentration was observed from *L. perenne* with 1.92±0.434 mg kg^{-1} with a corresponding 0.158 mg kg^{-1} (8%) dissipation (Table 6.5).

ANOVA showed that there was a statistically significant relationship ($p< 0.05$) between the residual concentration of B[*a*]P and the treatments. A Tukey post-hoc test reveals that the residual concentrations for *L. perenne* and *F. arundinacea* were significantly different from each other. Interestingly the mean residual concentration for *L. perenne* (1.92 mg kg^{-1}) was higher than that of the unplanted control (1.58 mg kg^{-1}). Rhizoremediation accounted for -6.3, 9.6 and 58% of B[*a*]P dissipation by *L. perenne*, *M. sativa* and *F. arundinacea* respectively (Table 6.6).

6.4.2.2 Mixed PAH treatment with phenanthrene, fluoranthene and benzo[*a*]pyrene

The PAH dissipation for *M. sativa*, *F. arundinacea* and *L. perenne* varied between compounds, phenanthrene (86-99%), fluoranthene (85-99%) and benzo[*a*]pyrene (37-71%). The mean residual concentration of phenanthrene was highest for the abiotic control 85.4±4.03 mg kg^{-1} with 169 mg kg^{-1} (66%) dissipated and lowest for *F. arundinacea* with 1.74±0.40 mg kg^{-1} as residual concentration and 252 mg kg^{-1} (99%) dissipated. The dissipation in the unplanted control, *F. arundinacea* and *L. perenne* treatments were all equal (252 mg kg^{-1}) as shown in Table 6.7

Phenanthrene

Root exudate-enhanced dissipation and rhizoremediation accounted for -13.6-0.02 to 5.9% and 19-33 % phenanthrene dissipation respectively (Table 6.8). The results from a one-way ANOVA showed that residual concentration of phenanthrene in the different treatments were significantly different ($p <0.01$). A Tukey post-hoc test showed that the treatment with *M. sativa* differed significantly from those involving *F. arundinacea* and *L. perenne*.

Fluoranthene

Fluoranthene dissipation was quite similar for the *M. sativa* treatment (131 mg kg^{-1}) and the abiotic control (94 mg kg^{-1}). Also the findings for the *F. arundinacea* treatment (149 mg kg^{-1}) were similar to those for the *L. perenne* treatment (151 mg kg^{-1}) as shown in Table 6.7. The results from a one-way ANOVA showed that residual concentration of fluoranthene were significantly affected by the PAH treatments likely due to microbial activity ($p < 0.01$).Tukey HSD test revealed that the residual concentration for *M. sativa* was significantly different from those of the unplanted control, abiotic control, *F. arundinacea* and *L. perenne*.

Benzo[a]pyrene

B[*a*]P dissipation in the mixed PAH treatment, was greatest for *F. arundinacea* (1.87 mg kg^{-1}; 71%) and lowest for *L. perenne* (0.5 mg kg^{-1}; 19%) as shown in Table 6.7. One-way ANOVA showed that there was a statistically significant relationship ($p < 0.01$) between the residual concentration of B[*a*]P and the treatments. A Tukey post-hoc test showed that B[*a*]P dissipation in the *F. arundinacea* and *L. perenne* treatments were significantly different. Also the dissipation for benzo[*a*]pyrene in soils planted with *M. sativa* were higher than those planted with *L. perenne* in both the single (*M. sativa*; 24%, *L. perenne*; 8%) and mixed PAH (*M. sativa*; 37%, *L. perenne*; 19%) treatments (Tables 6.5 and 6.7).

6.5 Discussion

6.5.1 Plant Biomass Experiment Data

Among the plant candidates, root and shoot biomass yield was greatest for *L. perenne* and least for *M. sativa* after the greenhouse experiment. This is expected as *L. perenne* has a

fibrous root system characterised by the presence of a sheath around its root while *M. sativa* has tap roots (Hall *et al*., 2011).

The substantial increase in *M. sativa* root and shoot biomass across all treatments compared to the plant control is an indication of its tolerance to single and mixed PAH contaminants at the given concentrations as well as the stimulatory effect of the treatment on biomass yield. This is contrary to the findings of a study by Cheema *et al*., (2010) which showed a 35% reduction in *M. sativa* biomass compared to that of the control in soils spiked with phenanthrene (200 mg kg^{-1}) and pyrene (199 mg kg^{-1}).

In the case of *L. perenne* and *F. arundinacea*, biomass yield was greater for roots than shoots as shown in Figures 6.2 and 6.3 respectively. The inhibitory effect of the single and mixed PAH treatments on *L. perenne* and *F. arundinacea* shoot biomass as well as the decrease in *L. perenne* and *F. arundinacea* root biomass for the mixed PAH treatment may be attributed to phytotoxicity from single or synergistic effects of the PAH compounds, and nutrient imbalance. With regards to phytotoxicity, Reilley *et al.* (1996) suggested that PAH have an indirect adverse impact on water and nutrient supply to plants, thereby causing a decrease in biomass yield even though plants may not exhibit apparent signs of stress or toxicity as in this study. These findings are also supported by a study by Cheema *et al*. (2010) on the degradation of phenanthrene and pyrene using *M. sativa*, *F. arundinacea*, *L. perenne* and rape seed in single and mixed plant cultivation. Also the number of metabolically capable degraders and/or the rate of degradation may have affected the level of phytotoxicity with effects on plant growth as suggested by Kechavarzi *et al*. (2007).

Stimulation of biomass yield in the B[*a*]P treatment across the plants (Figures 6.1-6.3) agrees with the findings of Sun *et al*. (2011). They reported that ≤ 10 mg kg^{-1} of B[*a*]P facilitated plant growth and increased biomass yield compared to the control.

6.5.2 PAH dissipation

The results of this study suggest that PAH dissipation pathways include degradation by soil microorganisms, rhizoremediation, as well as abiotic processes. All of these processes are known mechanisms of PAH dissipation. Another possible mechanism is that of plant uptake and accumulation but as this has been reported elsewhere to be negligible (Kaimi *et al*, 2006; Cheema *et al.*, 2010; Lu *et al*., 2011; Meng *et al*., 2011), this process was not assessed in this study. The presence of viable microbes and extracellular enzymes in the abiotic control may not be excluded as complete maintenance of abiotic control is difficult (Margesin *et al*., 2000; Kaimi *et al*., 2006).

6.5.2.1 Phenanthrene and fluoranthene dissipation

Interestingly there was comparable phenanthrene and fluoranthene dissipation across the *F. arundinacea* and *L. perenne* single treatments but this exceeded that seen in the controls while that of *M. sativa* was similar to the unplanted control (Table 6.5). The increase in dissipation observed for phenanthrene in planted soil treatments compared to the unplanted control (Table 6.5) supports the findings of previous studies showing that the presence of vegetation stimulates PAH degradation (Olson *et al*., 2007; Hall *et al.*, 2011). A study by Cheema *et al*., (2010) also showed that rape seed, *L. perenne*, *F. arundinacea* and *M. sativa* enhanced phenanthrene dissipation. Low molecular weight PAH such as phenanthrene and naphthalene

are easily volatilized and/or biodegraded in comparison to high molecular weight PAH which tend to persist in the environment (Cheema *et al*., 2010). Lu *et al*. (2011) reported that phenanthrene and pyrene degradation followed the order: near rhizosphere > root compartment > far rhizosphere soil zones of mangrove *Kandelia candel* (L.) Druce. They also found that the contribution of direct plant uptake and accumulation to PAH dissipation was low compared to rhizoremediation which was the principal mechanism of dissipation. It is important to note that the most important pathway for the loss for phenanthrene which is a 2-ringed PAH of low molecular weight (178.23 g mol^{-1}), vapour pressure (18 mPa) and solubility in water at 25° C of 1.18 mg L^{-1} (Sun *et al*., 2010; Smith *et al*., 2011) is probably volatilization. This is supported by the findings of Sun *et al*., (2010) with regards to a substantial abiotic loss of phenanthrene (83.4%) and pyrene (57.2%) from freshly spiked sterile soil. In comparison to the unplanted control it is evident that phenanthrene dissipation occurred principally by both abiotic processes and microbial degradation.

The presence of plants and their association with soil microbes was observed to have more impact on the dissipation of fluoranthene than phenanthrene (Table 6.6). Our findings are consistent with those of Xu *et al*. (2006) and Lee *et al*. (2008) who showed significant effects of plants on pyrene dissipation compared to phenanthrene dissipation. During greenhouse experiments, it is difficult to distinguish the various mechanisms contributing to PAH dissipation. Volatilization however is less likely to be a PAH dissipation mechanism for PAH with 3 or more rings and low vapour pressures in this case fluoranthene and especially for benzo[*a*]pyrene dissipation. As such microbial degradation and rhizoremediation become relatively more important mechanisms for the dissipation of high molecular weight PAH. The mechanisms of microbial degradation include mineralization, co-metabolic degradation and non-specific radical oxidation (Smith *et al*., 2011). Also microbial degradation would depend

on the metabolic capacity of the soil microbes, the concentration, type and availability of PAH compound as well as the presence of other carbon and energy sources (Toyama *et al*., 2011).

A study by Smith *et al*. (2011) showed greater losses of low molecular weight PAH including phenanthrene and fluoranthene and no losses for high molecular weight PAH from soils amended with pure PAH in comparison to coal tar amended soils. Their study also showed a substantial reduction of PAH levels in soil amended with pure PAH over a 12 week growth period with *F. arundinacea*, *F. rubra*, *L. perenne, T. pratense* and *T. repens*. Also there was markedly reduced loss of fluoranthene compared to phenanthrene. They suggested the losses were mainly attributed to microbial degradation of readily available PAH in spiked soils and irreversible sorption unto soil but also acknowledged that volatilization contributed to the dissipation of low molecular weight PAH.

6.5.2.2 Benzo[*a*]pyrene dissipation

Higher dissipation for benzo[*a*]pyrene in soils with *M. sativa* compared to *L. perenne* in both single and mixed PAH treatments (Tables 6.6-6.8) were mainly attributed to indigenous PAH degraders present in the soil and irreversible sorption. Surprisingly, the presence of the plants had an inhibitory effect on benzo[*a*]pyrene dissipation in comparison to the unplanted control as appears the case for fluoranthene dissipation by *M. sativa*, and phenanthrene dissipation in the mixed treatment (Table 6.5-6.8). The presence of plants is expected to facilitate selection of PAH degraders by the rhizosphere effect as previously mentioned. As the presence of plants does not always necessarily improve PAH dissipation (Liste and Alexander, 2000) this reduced PAH dissipation in planted soil compared to unplanted control (Table 6.6 and 6.8) may be explained by an inhibition of PAH degrading microorganisms. This is supported by a

suggestion by Olson *et al*. (2007) who observed that the residual PAH in planted soil (852 mg kg^{-1}) was higher than that of the unplanted soil (542 mg kg^{-1}). Kamath *et al*. (2004) showed that some root extracts, sugars and amino acids inhibited the expression of a degradative gene (*nah*G) required for naphthalene degradation by *Pseudomonas fluorescens* HK44. Likewise, root extracts such as pyruvate, glucose and acetate have been shown to repress phenanthrene degradation activity of *Pseudomonas* sp. by catabolite repression (Louvel *et al*., 2011).

Other possible explanations include the fact that chemical properties such as low water solubility as well as high organic carbon-water (K_{OC}) and octanol-water partition coefficients (K_{OW}) of B[*a*]P may cause the formation of non-extractable bound residues due to absorption to soil organic matter thereby decreasing bioavailability (Kaimi *et al*., 2006; Hamdi *et al*., 2012). It is important to note that even with the presence of root exudates which usually contain surfactants expected to increase bioavailability and facilitate PAH dissipation (Qui *et al*., 1997), this could be quite complex for high molecular weight PAH like B[*a*]P as other factors such as soil properties are important. Liste and Alexander (2000) showed that soybean rhizosphere contained more phenanthrene than unplanted soil. Their possible explanations included slow PAH biodegradation and low bioavailability due to higher organic matter in planted soil but their most probable explanation was that the compound was mobilized from the bulk soil to the rhizosphere as the PAH concentration in sterile *F. arundinacea* and wheat rhizosphere in their study was 4-5 fold greater than initial concentrations. Their study showed that plants are important in the movement of hydrophobic compounds from bulk soil to the rhizosphere zone rich in diverse metabolic activity. Furthermore, if B[*a*]P is mobilised, the degree of mobilization from contaminated soil to the roots is affected by its high K_{OW} and hence partitions to the epidermis of roots in high concentrations based on an increase in lipophilicity (Cheema *et al*., 2010). As such if soil samples collected contained root hairs

especially when the soil is extensively covered with roots as was the case in this study, a high concentration could be observed.

6.5.2.3 Dissipation by plant species

6.5.2.4 PAH dissipation in single PAH versus mixed PAH treatments and biomass yields

Among the plant treatments, treatment with *F. arundinacea* was found to be more efficient at fluoranthene and B[*a*]P dissipation while *L. perenne* treatment was more efficient at phenanthrene dissipation in both single and mixed PAH treatments (Tables 6.5-6.8). The *M. sativa* treatment however, was more efficient at dissipating B[*a*]P than the *L. perenne* treatment as previously discussed.

Regardless of the compound, there was higher root-enhanced dissipation in single PAH treatments compared to the mixed PAH treatment as shown in tables 6.6 and 6.9. For example, a 48% and 21% difference in B[*a*]P dissipation compared to the unplanted control in single and mixed PAH treatments by *F. arundinacea* respectively. This is greater than the slight increase in dissipation of 5 and 6 ringed PAH in planted soils reported by Soleimani *et al.* (2010). They suggested that high molecular weight-PAH are broken down by co-metabolism resulting from a rhizosphere effect involving a symbiotic plant-microbe association. The difference suggests that the presence of mixed PAH affects plant microbe interactions and co-metabolism. This clearly shows that the presence of other compounds affects the dissipation of each compound. In the mixed PAH treatments the plants as well as soil microbes may have been affected by the synergistic phytotoxic effect of all three compounds present in comparison to that arising in the single PAH treatments. Apart from phytotoxicity, other important factors that may contribute to this difference in dissipation

between the single and mixed treatments, is the presence of simpler carbon sources including root exudates, phenanthrene, and fluoranthene compared to B[*a*]P, that are likely to trigger catabolic repression.

PAH contamination and the resulting degradation products may cause phytotoxic effects such as inhibition of photosynthesis and other physiological processes that may affect plant establishment, growth, and biomass yield, and that are important in PAH degradation (Oguntimehin *et al*., 2010). Root biomass yield is also affected by contaminant concentration and toxicity (Kechavarzi *et al.*, 2007). The root-enhanced dissipation of each plant may be related to the biomass yield which revealed *M. sativa*, *L. perenne* and *F. arundinacea* root biomass yields in single treatments to be higher than those observed in the mixed PAH treatment. A decrease in root biomass as a result of the toxicity of contaminants results in a decrease in the rhizosphere area and a corresponding decrease in stimulation and proliferation of diverse microbial populations required for biodegradation (Merkl *et al*., 2005; Tang, *et al*., 2010). As phytoremediation efficiency depends on the successful establishment of plant-microbe interactions (Wenzel, 2009), root exudation and rhizosphere effect would be affected by the type, number and concentration of PAH present. Ultimately different factors such as soil properties, plant type, rhizosphere effect, plant-microbe interaction, contaminant type and concentration, and plant stress may be responsible for the differences observed here and elsewhere in PAH dissipation between both single and mixed PAH experiments (Dakora and Phillips, 2002; Haichar *et al.*, 2008; Berg and Smalla, 2009; Wenzel *et al*., 2009).

6.6 Conclusion

Both single and mixed PAH treatments affected all the plant biomass yields. However, the yields for the mixed PAH contamination revealed a greater effect on biomass compared to those for the single PAH treatment. The single and mixed PAH treatment had stimulatory/inhibitory effects on biomass yield changes (both increases and decreases) during the experiments. This however varied from one plant species to another with *F. arundinacea* and *L. perenne* (both grasses) having similar effects especially with respect to root and shoot biomass. These effects are also likely to differ with the type of compound, their concentrations and toxicities. Of particular interest is the root biomass yield of selected plants which affect the rhizosphere area and microbial communities as well as contaminant dissipation. There are other factors that can affect the entire process such as presence of alternative carbon sources, nutrient levels, soil properties, plant (type and age), climate, agricultural practices and growth with other plants.

The differences in PAH dissipation observed between different treatments in this study is in agreement with the findings of previous studies which have shown that low molecular weight PAH are more easily dissipated than high molecular weight PAH (Lee *et al*., 2008). Differences in dissipation also vary with plant candidates but *L. perenne* had the highest dissipation rate for phenanthrene and fluoranthene in the single treatment and fluoranthene in the mixed treatment. *F. arundinacea* displayed the highest benzo[*a*]pyrene dissipation in comparison to the unplanted control for the single PAH and mixed PAH treatment. The presence of mixed contaminants was found to affect the dissipation of the individual compounds in the planted experiments. In comparison with the single PAH contamination, mixed PAH contamination resulted in a comparable or decreased PAH dissipation in some treatments or an increased PAH dissipation in others.

There was a slight increase in phenanthrene dissipation in planted soils compared to unplanted soils. A greater difference was found for fluoranthene dissipation in the *L. perenne* and *F. arundinacea* experiments and a substantial increase was observed for benzo[*a*]pyrene dissipation in the *F. arundinacea* experiment. Rhizoremediation efficiency was greater for *F. arundinacea* and *L. perenne* compared to *M. sativa*. Contrary to our expectation of an enhanced degradation, the presence of plants may inhibit dissipation of PAH compared to unplanted control possibly by suppression of PAH degraders and/or catabolic repression as simpler carbon and energy sources are released from plant roots into the rhizosphere.

CHAPTER 7

EFFECT OF PAH CONTAMINATION AND PLANT SPECIES ON MICROBIAL COMMUNITY STRUCTURE DURING PHYTOREMEDIATION

Abstract

The presence of contaminants in soil results in a decrease in species richness but there may be stimulation of specific groups with significant impact on soil microbial community. Consequently, studies have shown that bacterial communities in contaminated and pristine soils are quite different. Plants, soil properties, and environmental factors may also cause changes in microbial community. However, there are few reports on the impact of plants on the bacterial community structure of PAH-contaminated soils during rhizoremediation. This study was designed to assess the effect of single and mixed PAH treatments, and plant species on the bacterial community structure during a greenhouse experiment. ANOSIM analysis revealed that PAH treatments significantly affected the bacterial community across the selected plants with TRF 133 bp and 135 bp mainly contributing to dissimilarity. Plant choice significantly affected the soil bacterial community in the PAH-spiked soils. Pairwise tests revealed that *M. sativa* and *F. arundinacea* had significantly different bacterial communities with TRFs 133 and 135 bp accounting for the major contribution to dissimilarity. The 133 bp TRF had a higher relative abundance (12.30) in *M. sativa* while the 135 bp TRF had a higher relative abundance (18.62) in *F. arundinacea.* An overall decrease in the average number of TRFs indicates a decrease in soil bacterial diversity. The highest mean TRFs were observed for *M. sativa* in the phenanthrene treatment and *F. arundinacea* for the benzo[*a*]pyrene and

PAH mix treatments. Stress tolerance of suitable plant candidates for PAH-contaminated land remediation will influence soil microbial communities which determine remediation outcome.

7.1 Background

The soil is a repository of microbial diversity with bacterial communities (autotrophs and heterotrophs) of up to 10 billion viable culturable cells/g dry soil of different species (Trevors, 1996; Torsvik and Ovreas 2002). Factors that may influence microbial diversity include soil structure-particle size, type and amount of available organic substrates, nutrient levels, competitive interactions and pH (Torsvik and Ovreas 2002). Apart from the vital role of soil microbes in ecosystem processes such as decomposition and nutrient cycling (Dubey *et al.*, 2006; Maron *et al*., 2011), they degrade contaminants and hence are of importance in the remediation of contaminated sites and restoration of the ecosystem (Dubey *et al*., 2006).

Contaminated soils may contain a range of both organic and inorganic contaminants of varying concentration and toxicity with significant impact on soil microbial community. Contaminants may have stimulatory, inhibitory or neutral effect on soil microbiota compared to controls (Trevors, 1996; Kozdroj and van Elsas, 2001). Contaminant toxicity results in a decrease in species richness but there may also be selection and stimulation of specific groups of organisms capable of using a particular contaminant as a substrate (Liu *et al*., 2009). As such, a high relative density of specialist microbes is observed with a resultant decrease in biodiversity in contaminated soils (Trevors, 1996; Kozdroj and van Elsas, 2001). Consequently, studies have shown that bacteria communities in contaminated and pristine soils differ significantly (Liu *et al*., 2009). Changes in microbial diversity due to the presence of contaminants may serve as indicators of stresses (Liao and Xie, 2007), but there is limited

information in this regard (Frey *et al.*, 2006 and Liao and Xie, 2007, Liu *et al*., 2009 and Maron *et al.,* 2011).

Apart from the presence of contaminants, other factors with significant impact on soil microbial community structure and diversity include plant community, soil properties, restricted dispersal of soil microbes and environmental determinants (Kennedy *et al*., 2004, Cebron *et al*., 2011; Pritchina *et al*., 2011 and Ding *et al*., 2013). However, soil chemistry has been identified as more significant than the plant species in shaping rhizosphere bacteria community (Kennedy *et al*., 2004; Pritchina *et al*., 2011).

Rhizoremediation exploits the ability of plants to enhance contaminant degradation by stimulating the proliferation of catabolically diverse soil microorganisms through the rhizosphere effect (Siciliano *et al*., 2003; Singer *et al*., 2003; Xu *et al*., 2006; Dzantor *et al*., 2007; Olson *et al*., 2007; Wu *et al*., 2008; Mackova *et al*., 2009). However, some studies have reported that plants have no effect or an inhibitory effect on contaminant degradation (Liste and Alexander, 2000; Kamath *et al*., 2004; Olson *et al*., 2007; Perelo *et al*., 2010; Louvel *et al*., 2011). Furthermore as discussed in Chapter 6, this study found that plants did not enhance PAH dissipation in some cases. These conflicting findings raise important questions about the impact of plant on microbial communities and the rhizoremediation outcomes for contaminated sites.

There are reports on the soil microbial community structure associated with different environmental pollutants, including PAHs (Liao and Xie, 2007; Tian, *et al*., 2008; Liu *et al*., 2009; Zhou *et al*., 2009; Zhang *et al*., 2010) but the impact of plants on the bacterial communities of PAH-contaminated soils during rhizoremediation is poorly described (Siciliano *et al*., 2003; Vinas *et al*., 2005; Cebron *et al*., 2009).

Also most studies have focused on single PAH contamination rather than mixed PAH contamination, which reflects real scenarios in PAH-contaminated sites (Gan *et al*., 2009) this may have implications for the selection and suppression of microbial groups, microbial community structure, plant-microbe interactions and ultimately rhizoremediation success. An understanding of the impact of plants and contaminants on microbial community structure, density and diversity would provide insights into the microbial groups of importance in rhizoremediation (Cebron *et al*., 2009). This study was designed to assess the effect of single and mixed PAH treatments, and plant species on microbial community structure before and after a 65 day rhizoremediation greenhouse experiment. This study is an extension of Chapter 6 titled "Effect of single and mixed PAH contamination on *M. sativa*, *L. perenne* and *F. arundinacea* biomass yield and PAH dissipation during phytoremediation". The findings of this Chapter and those of Chapter 6 will be linked (as appropriate).

7.2 Hypotheses

1. The presence of contaminants, PAH treatments, vegetation and time will affect bacterial community structure.

2. There will be a relationship between the microbial communities of the PAH treatments and control treatment.

7.3 Methods

7.3.1 Experimental design

The greenhouse experimental design and soil sample collection is described in Section 6.3.1 (Soil preparation and experimental design).

7.3.2 Microbial diversity analysis

7.3.2.1 *DNA extraction*

DNA was extracted using *PowerSoil* DNA isolation kit (MO BIO laboratories, Inc. USA). Soil sample (~0.25 g) was added to the PowerBead tubes and gently vortexed to mix the contents. Solution C1 (60 µL) containing Sodium dodecyl sulphate was added to the Powerbead tubes containing soil samples and briefly mixed inverting several times and then vortexed for 5-10 minutes. PowerBead tubes were then centrifuged at 10,000 x *g* for 30 seconds at room temperature. The supernatant formed was transferred into a clean 2 mL collection tube. Solution C2 (250 µL) was added to the supernatant and vortexed for 5 seconds. The supernatant was then incubated at 4 °C for 5 minutes and centrifuged at room temperature for 1 minute at 10,000 x *g*. The supernatant (~600 µL) was transferred to a clean 2 mL collection tube to which 200 µL of solution C3 was added. The mixture was briefly vortexed, incubated at 4°C for 5 minutes and then centrifuged at room temperature for 1 minute at 10,000 x *g*. The supernatant (750 µL) was transferred into a clean 2 mL collection tube; 1.2 mL of Solution C4 was added and then vortexed for 5 seconds. of The supernatant (~675 µL) was loaded onto a spin filter and centrifuged at 10,000 x *g* for 1 minute at room temperature. The elutant was discarded before adding another 675 µL of supernatant onto the spin filter and centrifuging at 10,000 x *g* for 1 minute at room temperature. The remaining supernatant was loaded onto the spin filter and centrifuged as described previously. Solution C5 (was 500 µL) added onto the spin filter and centrifuged at room temperature for 30 seconds at 10,000 x *g*. The flow through in the 2 mL collection tube was discarded and the tube was centrifuged for 1 minute at 10,000 x *g*. The spin filter was carefully placed in a clean 2 mL collection tube after which 100 µL of solution C6 was added to the centre of the white

filter membrane. The tube was centrifuged at room temperature for 30 seconds at 10,000 x *g*. The spin filter was discarded while the solution containing the collected DNA was stored at -20 °C.

7.3.2.2 *PCR amplification*

Extracted DNA (~10 ng) was PCR amplified by a single reaction for universal bacterial 16S rRNA with primers 63F-VIC labelled and 1087R (Table 7.1; Singh *et al*., 2006).The lyophilised primer pellets (Applied Biosystems UK) were reconstituted in 1mM Tris-Hydrochloride (pH 8.0)/0.01mM Ethylenediaminetetraacetic acid (Sigma, UK) in aliquots and used at a final concentration of 200 nM. Briefly, all PCRs were performed in a final volume of 50 µL containing 47 µL of Megamix (Microzone Limited, UK), 1 µL forward primer (10µM), 1 µL of reverse primer (10µM) and 1µL of template DNA. PCR was performed on a QuantaBiotech Qcycler II (United Kingdom) and the cycle consisted of an initial denaturation step for 3 min at 95 °C, followed by 30 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 60 s, and elongation at 72 °C for 60 s. The last cycle was followed by a final 10 min extension period at 72 °C. PCR products were visualised with Ethidium bromide (0.5µg/mL) staining on a 1% (w/v) agarose gel using UV radiation.

Table 7.1: PCR primers for Eubacterial 16S rRNA gene amplification (Singh *et al*., 2006).

16S rRNA gene Primer	**Fluorescent label**	**Sequence (5' to 3')**
63f	None	AGGCCTAACACATGCAAGTC
1087r	VIC (green)	CTCGTTGCGGGACTTACCCC

7.3.2.3 *Amplicon digestion and Terminal Restriction Fragment (TRF) detection*

Prior to digestion, PCR products were purified using a *GenElute* PCR clean-up kit (Sigma–Aldrich, UK) according to the manufacturer's instructions. Purified PCR product (~500 ng) was digested with 10 U (1uL of 10 units μL^{-1}) of *Hha* I and 2 µl of 10X restriction enzyme buffer in a final volume of 20 µL containing 0.2 µL of acetylated bovine serum albumin (BSA; 0.1 µg μL^{-1}; Promega, UK) and sterile deionized water. Samples were mixed by pipetting then incubated at 37 °C for 3 hours followed by 15 minutes at 95 °C to inactivate the enzyme. After digestion, 1 µL of each sample were mixed with 10 µL of 50 µL LIZ-labelled GS500 (600 internal size standard) and 950 µL of Hidi-formamide (Applied Biosystems, UK) and denatured at 95 °C for 5 min, then chilled on ice for 5 minutes (Macdonald *et al*., 2010). Fragment size analysis was undertaken using an Applied Biosystems 3730 DNA analyser.

7.3.2.4 *Data analysis*

Bacterial TRFLP profiles were produced using GeneMarker software (version 1.60; SoftGenetics LLC, USA). Fragment analysis was performed between 50 and 600 base pair (bp), which was within the linear range of the internal size standard. All terminal restriction fragment (TRFs) with fluorescence units less than 50 bp were discarded from the data analysis to avoid detection of primers and undigested PCR products. For community analysis, two TRFs that were separated from one another by >1 bp were considered as distinct TRFs. The TRFLP community profile expressed in relative abundance was used for statistical analysis. The relative abundance of TRFs in a profile was calculated as a proportion of the total peak height of all the TRFs in a profile. TRFs with relative abundance above 0.5% were used for statistical analysis. All plots were derived using Bray-Curtis resemblance matrices

generated using the TRFLP data (Macdonald *et al*., 2010; Hilton *et al*., 2013). The similarity matrix is shown in Appendix 7.1.

7.3.2.5 *Statistical analysis*

The relative abundance data was analysed on PRIMER, Version 6 (Primer–E) for resemblance using analysis of similarity (ANOSIM) and non-metric multi-dimensional scaling (NMDS). ANOSIM is a permutation-based statistical test, similar to univariate ANOVA, which tests difference between sample groups of treatments. It reveals the degree of dissimilarity between sample groups (global *R*) and the level of significance (P). Global *R* ranges between +1 and -1; positive R values indicate greater dissimilarity between groups than within groups, whilst negative R values indicate greater dissimilarity within the group than between groups. Permutation tests were used to generate significance values. The level of significance is analogous to the univariate p-value where 0.1%, 1% and 5% are equivalent to the conventional P-statistic, P=0.001, P=0.01 and P=0.05 respectively (Macdonald *et al*., 2010). Similarity Percentage- species contribution (SIMPER) was used to assess the relative contribution (%) of each TRF to the similarity matrix structure (Macdonald *et al*., 2010; Hilton *et al*., 2013).

7.4 Results

7.4.1 Impact of contamination on bacterial community structure

NMDS plot of the TRFLP data revealed similarities in the composition of the PAH-contaminated soil and control soils (Figures 7.1and 7.2). ANOSIM analysis revealed that overall, PAH contamination did not have significant effect on the bacterial communities in

vegetated and unvegetated soil (R=-0.045; p=0.64). However, the contaminant type (single and mixed PAH) significantly affected bacterial community across the plant species but were still related (R=0.291; p=0.001). Pairwise test revealed all the PAH treatments to be significantly different from each other in terms of their bacterial community except those of phenanthrene and benzo[*a*]pyrene treatments (R= -0.046; p=0.687). Table 7.2 show results from SIMPER analysis with the TRFs that contributed most to the differences in vegetated PAH treatments (Appendix 7.2).

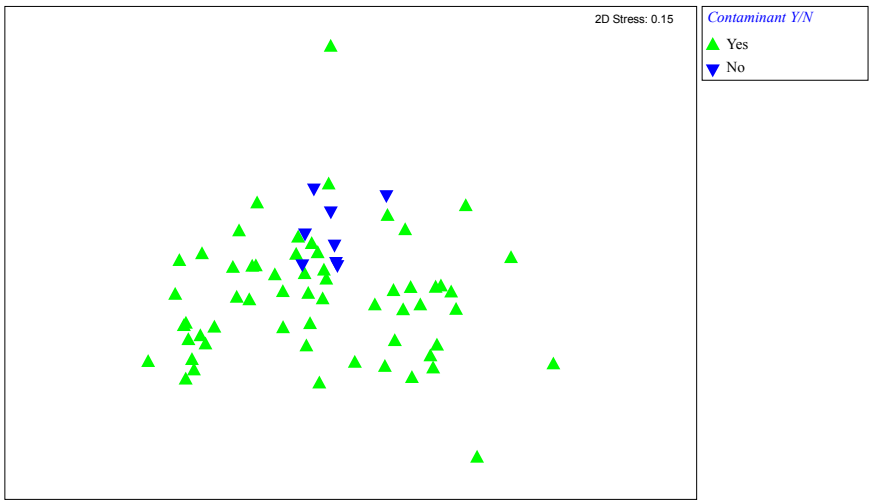

Figure 7.1: NMDS plot representing relative ordination of bacterial T-RFLP profiles across PAH-contaminated soils and control soil.

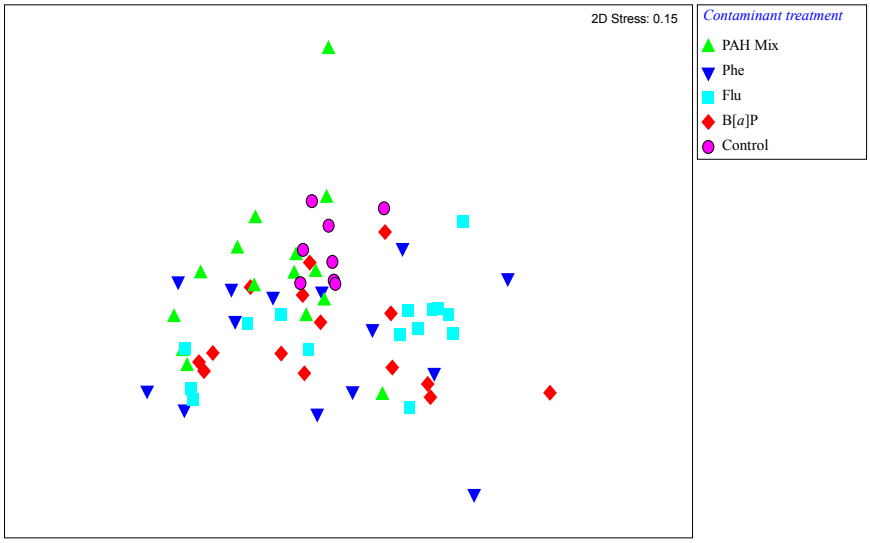

Figure 7.2: NMDS plot representing relative ordination of bacterial T-RFLP profiles across PAH treatments and control.

Table 7.2: Results from SIMPER analysis showing TRFs contributing to dissimilarity between PAH treatments. The table shows only the first five TRFs with the highest contributions to dissimilarity (Appendix 7.2).

PAH treatments compared	**TRFs contributing to dissimilarity**	**Percentage contribution to dissimilarity (%)**
Phe/ Flu	133	12.43
	135	5.48
	524	5.28
	531	5.18
	100	4.7
Flu/B[*a*]P	133	10.93
	135	7.8
	529	5.99
	531	5.48
	528	5.1
Phe/PAH mix	133	11.82
	135	11.30
	524	5.25
	100	5.14
	528	4.15
Flu/PAH mix	135	11.13
	133	7.66
	100	6.11
	531	6.01
	528	5.74
B[*a*]P/PAH mix	135	10.95
	133	9.59
	100	5.21
	529	5.15
	531	3.96

7.4.2 Impact of the presence of vegetation on bacterial community structure

NMDS with ANOSIM analysis revealed the presence of vegetation significantly affected the soil bacterial community in PAH-spiked soils (R=0.488; p=0.001). This is clearly depicted in Figure 7.3 where the bacterial community of the unvegetated control soil formed a cluster but also had a slight overlap with those of the vegetated soils, indicating some similarities. Based on the SIMPER analysis, 133 and 135 bp TRFs accounted for the most difference in bacterial community structure between vegetated soils and unvegetated control soils. The 133bp TRF

had a higher relative abundance (11.89) in vegetated soils while the 135 bp TRF had a higher relative abundance (22.16) in unvegetated soil.

Also, the plant species had a significant effect on the rhizosphere bacterial community but with less dissimilarity between the plant species (R= 0.077; p=0.029). However, pairwise tests revealed that only *M. sativa* and *F. arundinacea* had significantly different bacterial communities (R=0.119; p=0.015). This is also observed by the dispersion of the TRF data of *M. sativa* and *F. arundinacea* in Figure 7.3. SIMPER analysis revealed that the TRF that contributed most to differences between the rhizosphere bacterial communities of *M. sativa* and *F. arundinacea* were 133 and 135 bp with 9.51% and 9.03% contribution to dissimilarity respectively. The 133bp TRF had a higher relative abundance (12.30) in *M. sativa* while the 135 bp TRF had a higher relative abundance (18.62) in *F. arundinacea* (Appendix 7.3).

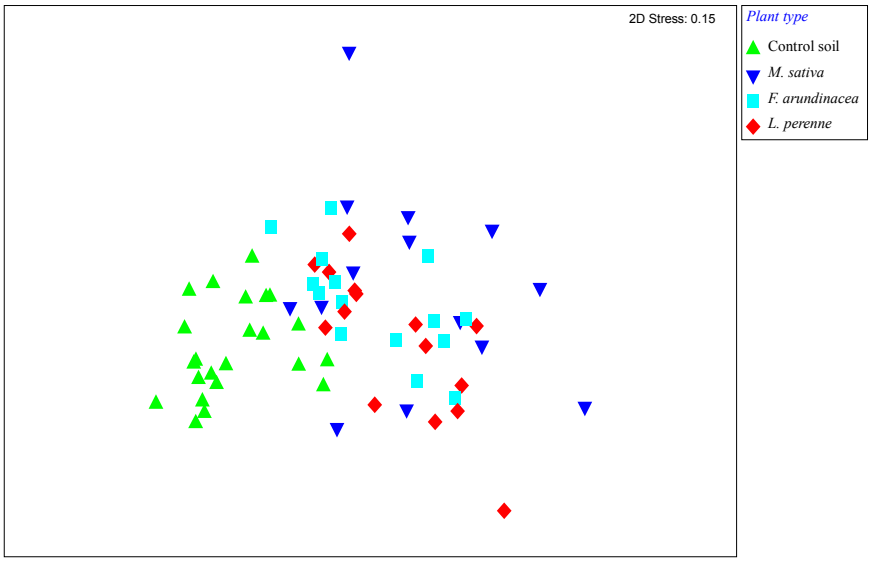

Figure 7.3: NMDS plot representing relative ordination of bacterial TRFLP profiles across rhizosphere (vegetated) and control (unvegetated) soil spiked with PAH.

7.4.3 Impact of time on rhizosphere bacterial community subjected to PAH contamination

There was a decrease in the average number of TRFs at the end of the 65 day-greenhouse experiment as shown in Table 7.3. *M. sativa* had the greatest average number (n=3) of TRFs in the phenanthrene treatment while *F. arundinacea* had the greatest average number of TRFs in the benzo[*a*]pyrene and PAH mix treatments.

ANOSIM test based on the factors; plant type and time, revealed that the initial and final of bacterial communities were significantly different for the unplanted soils (R=0.907; p=0.001) and planted soil (R=0.465; p=0.001) (Appendix 7.4). The difference between the initial and final bacterial communities of the unplanted control treatment is clearly reflected by the global R (0.907) depicted by the two separate clusters in Figure 7.4a. The TRF communities of the initial unplanted control soil clustered close together with little dispersion while that of those of the final unplanted control soil showed considerably more dispersion, suggesting the communities were different. Figure 7.4b clearly depicts the divergence of microbial communities of the initial and final single and mixed PAH treatments.

From the NMDS plot with combination of plant type and time, the TRF communities of *L. perenne* and *F. arundinacea* clustered closer together while *M. sativa* showed great dispersion (Figure 7.5). This suggests that the microbial communities of *L. perenne* and *F. arundinacea* were more similar to each other than to that of *M. sativa*. Pairwise comparison showed that the bacterial community structure of all plant and control treatments were significantly different to each other but final bacterial community structure of *L. perenne* and *F. arundinacea* were not significantly different. SIMPER analysis revealed that the TRF that contributed most to differences between the initial and final unplanted control in were 133,

245,135, 406 and 134 bp while TRFs 133, 135, 246, 473, 527 and 529 bp were important in contributing to the dissimilarity between the initial unplanted and final planted treatments (Table 7.4) .

A multivariate ANOSIM test carried out with contaminant type, plant type and time gave an R value of 0.605 and a significance level of 0.001. A summary of the results from the SIMPER test are shown in Table 7.5. The TRF 133bp was found to be most important with the highest relative abundance across all the plant-PAH treatments. TRF 133 bp also accounted for the greatest dissimilarity in most of the treatments except in the case of *F. arundinacea*-phenanthrene treatment with 524 bp, *F. arundinacea- and M. sativa*-fluoranthene treatments with 246 bp as the most important TRF contributing to dissimilarity (Table 7.5). Also the SIMPER test revealed that the average abundance of the TRF 133bp was higher in all final PAH treatments compared to the initial PAH treatments (Appendix 7.5).

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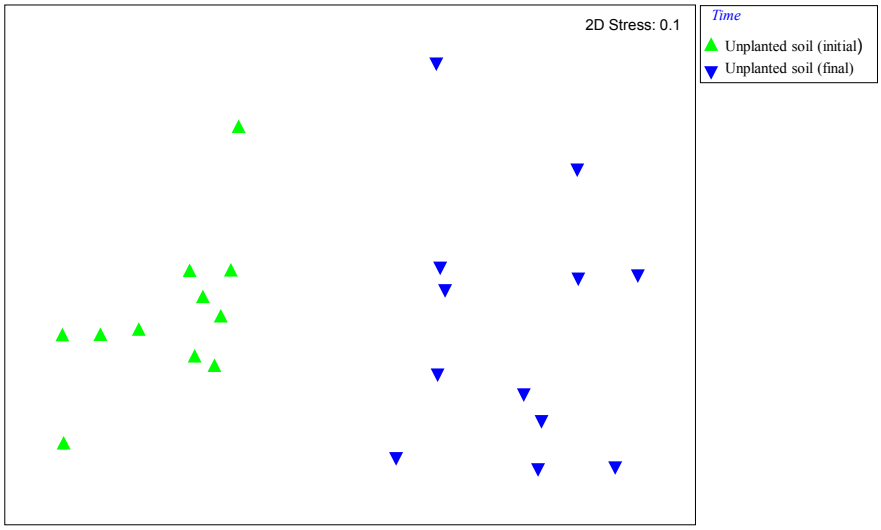
A)



B)

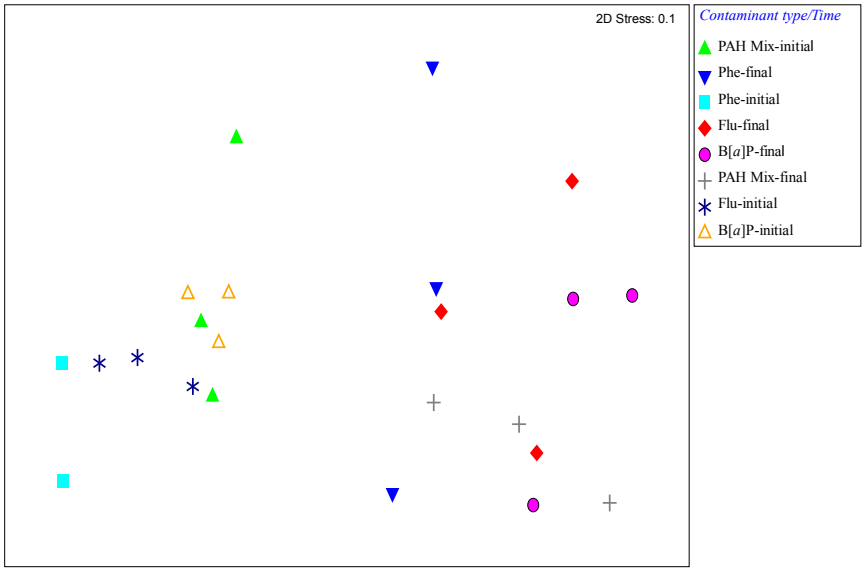


Figure 7.4: NMDS plot representing relative ordination of initial and final bacterial TRFLP profiles across unplanted PAH-spiked soil. The analysis is based on (A) time and (B) contaminant type and time.

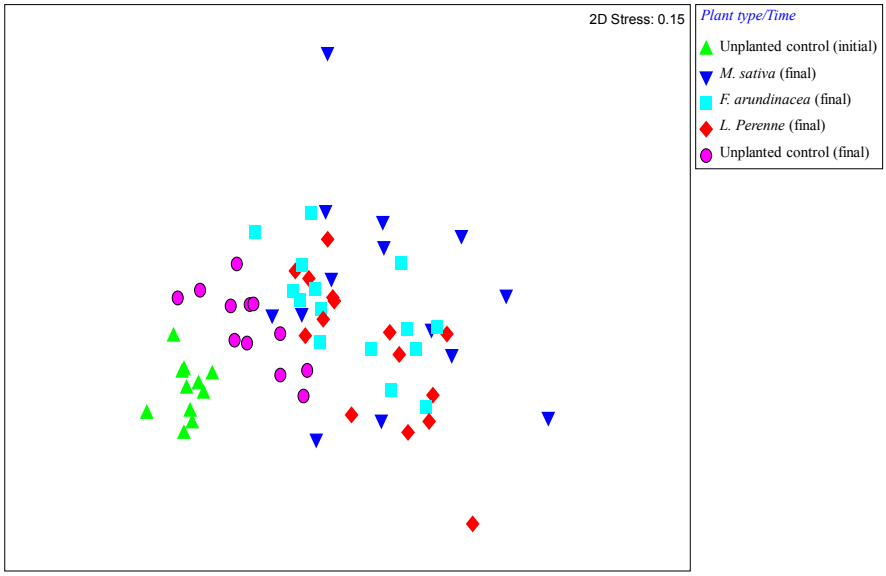


Figure 7.5: NMDS plot representing relative ordination of initial and final bacterial TRFLP profiles across planted and unplanted PAH-spiked soil.

Table 7.3: Average TRFs (n=3) at days 0 and 60 in PAH treatments for planted and unplanted control. A total of 87 TRFs ranging from 52 to 531 base pairs were found in the TRFLP data. Only TRFs with peak area ≥ 0.5 were summed (Appendix 7.1).

PAH	**Treatment**	**Average TRFs at day 0**	**Average TRFs after 65 days**
Phenanthrene	Unplanted control	36.0±1.0	31.0±2.65
	M. sativa		32.7±3.33
	L. perenne		25.7±0.67
	F. arundinacea		30.3±0.88
Fluoranthrene	Unplanted control	35.0±0.58	27.7±1.45
	M. sativa		29.7±0.33
	L. perenne		27.7±1.86
	F. arundinacea		29.7±0.33
Benzo[*a*]pyrene	Unplanted control	34.0±1.15	30.3±1.45
	M. sativa		28.7±2.96
	L. perenne		26.7±2.33
	F. arundinacea		29.0±1.53
PAH mix	Unplanted control	35.3±0.88	31.0±0.58
	M. sativa		26.3±1.86
	L. perenne		30.3±1.20
	F. arundinacea		31.7±0.67

Table 7.4: Results from SIMPER analysis showing TRFs contributing to dissimilarity between initial and final bacterial community of plant treatments and control. A multivariate ANOSIM was carried with the factors, contaminant type, plant type and time. The table shows the three most common TRFs (Appendix 7.5).

Treatments compared	**TRFs contributing to dissimilarity**	**Percentage contribution to dissimilarity (%)**
Initial/final unplanted soil	133	14.33
	245	8.44
	135	5.13
Initial/final *M. sativa*	133	11.1
	135	7.78
	246	6.49
Initial/final *L. perenne*	133	14.46
	246	6.88
	135	6.18
Initial/final *F. arundinacea*	133	7.70
	246	7.34
	135	6.03
Final *M. sativa*/ *L. perenne*	133	10.64
	135	7.85
	473	4.38
Final *M. sativa*/ *F. arundinacea*	133	9.51
	135	9.03
	529	4.69
Final unplanted soil/*M. sativa*	135	11.87
	133	7.54
	527	4.58
Final unplanted soil/*L. perenne*	135	10.60b
	133	8.26
	524	5.22
Final unplanted soil/*F. arundinacea*	135	9.34
	524	8.95
	133	6.51

Table 7.5: Results from SIMPER analysis showing TRFs contributing to dissimilarity between initial and final rhizosphere bacterial community of the PAH treatment. The Table shows the three most common TRFs. A multivariate ANOSIM was carried with the factors-contaminant type, plant type and time.

PAH treatment	**Plant treatment compared (initial and final)**	**Average dissimilarity**	**TRFs and percentage contribution to dissimilarity (%)**
Phenanthrene	*M. sativa*	35.19	133 (13.01), 246 (7.41), 406 (4.31)
	F. arundinacea	34.03	524 (8.24), 246 (7.63), 133 (6.57)
	L. perenne	42.82	133 (20.35), 246 (6.99), 135 (5.62)
Fluoranthene	*M. sativa*	40.88	246 (7.93), 133 (7.46), 135 (6.93)
	F. arundinacea	37.18	246 (8.40), 135 (6.98), 531 (5.71)
	L. perenne	37.26	133 (8.97), 246 (8.75), 135 (7.25)
Benzo[*a*]pyrene	*M. sativa*	37.42	133 (16.37), 135 (9.85), 529 (6.51)
	F. arundinacea	32.57	133 (10.98), 135 (8.32), 246 (7.18)
	L. perenne	33.50	133 (15.51), 135 (6.79), 246 (5.07)
PAH Mix	*M. sativa*	36.99	133 (10.43), 135 (7.91), 341 (5.91)
	F. arundinacea	29.14	133 (11.19), 100 (6.04), 524 (5.92)
	L. perenne	28.90	133 (13.64), 246 (5.64), 524 (5.34)

7.5 Discussion

Impact of PAH contamination on bacterial community structure

These results show that there was no significant overall effect of the presence of contamination on bacterial community structure. This is supported by the finding of Macdonald *et al*., (2010) following amendment of soils with zinc at different concentration levels (p=0.13). However, Figure 7.1 clearly shows a separate clustering of the communities of contaminated soil and the control soils. Interestingly, the ANOSIM results revealed that for specific contaminants, the bacterial community structures were significantly different between the contaminated (single and mixed) and control treatments. This agrees with Liu *et al*., (2009), who reported significantly different microbial community structures between

contaminated and pristine soil, with hydrocarbon degraders well-established in oil-contaminated soils.

Furthermore, there was a change in the bacterial community structure of both planted and unplanted single and mixed PAH treatments. This is supported by previous studies using planted soils (Kirk *et al*., 2005; Juhanson *et al*., 2007; Ma *et al*., 2010; Liu *et al*., 2014; Wei *et al*., 2014) and unplanted soils (Tian *et al*., 2008; Muckian *et al*., 2009; Zhou *et al*., 2009; Zhang *et al*., 2010; Zhang *et al*., 2011; Sun *et al*., 2012). Zhang *et al*. (2011) reported a change in microbial community structure of unplanted leachate-contaminated soil amended with phenanthrene with the dominance of groups such as *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*. Muckian *et al*. (2009) also found a significant change in soil microbial community structure in response of phenanthrene and fluoranthene amendment in the absence of plants. Rhizosphere communities of *Lactuca sativa* var. Tango (lettuce), *Cucurbita pepo* var. Black beauty (zucchini) and *Cucurbita pepo var.* Howden (pumpkin) responded to high PAH-contaminated soil (30,000 ppm) from a former manufactured gas plant (Pritchina *et al*., 2011). However, clustering was thought to be mainly driven by contaminant type rather than plant type (Pritchina *et al*., 2011). The changes in soil microbial community in both single and mixed PAH treatments in this study may have resulted from contaminant toxicity to soil microbes and plants. For the planted treatments, PAH contamination cause phytotoxicity with impacts on plant growth and physiology, root exudation and plant-microbe interaction which in turn affect soil microbial community (Cebron *et al*., 2011; Pritchina *et al*., 2011). The impact of the single and mixed PAH treatment on plant biomass yield is discussed in Section 6.4.

The TRF 133 and 135 bp which mainly contributed to dissimilarity between the PAH treatments may be attributed to the selective proliferation of PAH degraders driven by the PAH compounds and plant species. The TRFs are likely from important hydrocarbon degraders groups such as *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes* and *Firmicutes* important in PAH degradation (Alonso-Gutiérrez *et al*., 2008). Typical examples include members of the genus *Pseudomonas, Enterobacter, Bacillus, Arthrobacter, Acinetobacter*, *Alcanivorax*, *Shewanella*, *Vibrio*, *Pseudoalteromonas* and *Marinomonas* and *Sphingomonas* previously isolated from PAH-contaminated soil (Alonso-Gutiérrez *et al*., 2008; Puskarova *et al*., 2013). Puskarova *et al*. (2013) isolated *Pseudomonas putida*, *Arthrobacter oxydans*, *Sphingomonas* sp. and *S. paucimobilis* with PAHs-degrading abilities and prospects in bioremediation.

Impact of the presence of vegetation on bacterial community structure

The finding of an impact of plant presence and type on the bacterial community structure is supported by previous studies using both single and mixed plant treatments exposed to PAH contaminated soils (Siciliano *et al*., 2001; Cebron *et al*., 2009; Liu *et al*., 2014). Liu *et al*., (2014) reported that the microbial community structure in the rhizosphere of fire phoenix significantly changed and there was an increase in microbial diversity compared to unplanted aged PAH contaminated soils at 60, 120 and 150 days. The aged PAH soil (70.80-79.81mg kg^{-1}) contained fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene and dibenzo[*a,h*]anthracene. They also reported that the bacterial communities of the planted and unplanted soils formed separate clusters and concluded that vegetation could change bacterial community structure and diversity. DGGE

analysis revealed that plant roots selectively stimulated the growth of PAH degrader, *Gordonia* sp. and strains of uncultured gamma and beta proteobacteria. Juhanson *et al*., (2007) also reported a change in microbial community and an increase in the hydrocarbon degraders in hydrocarbon-contaminated soils (300 mg kg^{-1}) with mixed plant culture of *L. perenne, Poa pratensis* (Kentucky bluegrass), *Festuca rubra* (red fescue) and *Festuca ovina* (blue fescue) in comparsion to the unplanted controls. Another recent study by Wei *et al*. (2014) reported rhizodeposition by *Apium graveolens* (celery) significantly stimulated a change in microbial community in pyrene spiked soils (50 mg kg^{-1}) compared to control soils.

Siciliano *et al*. (2001) and Cebron *et al*. (2009) proposed that when plants are exposed to contaminants, they selectively enhance proliferation of less diverse but catabolically versatile degraders to suppress contaminant toxicity and promote rhizoremediation in the rhizosphere. The root exudates make up 10-20% of the carbon assimilated during photosynthesis and hence modify the biophysical microenvironment of the rhizosphere (Singer *et al*., 2003; Dzantor *et al*., 2007). This high carbon and organic matter input results in a 4-100 fold increase in rhizosphere bacterial density and increases bioavailability of PAH (Cebron *et al*., 2009; Wei *et al*., 2014). Therefore, a change in microbial community structure and diversity in planted soils is attributed to the root exudation which indirectly reduces contaminant toxicity, improve heterogeneity of the rhizosphere and promote proliferation of rhizosphere microbes with a positive impact on microbial activity and degradation of PAH (Euliss *et al*., 2008; Liu *et al*., 2014). Root exudate composition and quantities vary with plant species, and hence can account for plant-specific rhizosphere communities (Kirk *et al*., 2005, Dzantor *et al*., 2007; Euliss *et al*., 2008; Cebron *et al*., 2011; Pritchina *et al*., 2011).

The finding of significantly different rhizosphere bacterial communities of *M. sativa* and *F. arundinacea* in the PAH treatment is supported by Kirk *et al*. (2005). They reported that *L. perenne* stimulates general proliferation of soil microbes and increase microbial activity while *M. sativa* promotes selective proliferation of hydrocarbon degraders. Also, Pritchina *et al*. (2011) found that the communities of *Lactuca sativa* var. Tango, *Cucurbita pepo* var. Black beauty and *Cucurbita pepo var.* Howden grown in PAH-contaminated soil were similar to one another but different to that of *Triticum aestivum* (wheat). This may also be explained by the fact that they belong to different families; *M. sativa* - *Fabaceae* (legume) family while *L. perenne* – *Poaceae* (grass) family. Also as root exudate composition varies with plant, rhizosphere activities such as selection of microbial population, microbial colonization and plant-microbe interactions may also account for the difference in the microbial community structure of *M. sativa* and *F. arundinacea* (Dennis *et al*., 2010; Hall *et al*., 2011; Wang *et al*., 2011). The differences in the selection of microbial population may be evident from a higher relative abundance of the TRF 133 in *M. sativa* while the TRF 135 was more abundant in *F. arundinacea.* Previous studies have shown that legumes may be better at selecting PAH catabolic communities which may facilitate PAH degradation than grasses (Phillips *et al*., 2006; Hall *et al*., 2011). However, with reference to the findings in Chapter 6 (Tables 6.5 and 6.7), phenanthrene and fluoranthrene dissipation were greater for *F. arundinacea* and *L. perenne* compared to *M. sativa* while benzo[*a*]pyrene dissipation for *M. sativa* was greater than that of *L. perenne* for the single and mixed PAH treatments.

Impact of time on rhizosphere bacterial community subjected to PAH contamination

Generally a decrease in the mean number of TRFs at the end of the experimental period (65 days) was observed compared to the mean TRF observed at day 0 (Table 7.3). This finding is supported by previous reports of decreasing microbial diversity following PAH contamination (Sun *et al*., 2010; Zhang *et al*., 2010). Sun *et al*. (2010) reported a decrease in the diversity of nitrogen-fixing bacteria following naphathalene, pyrene and fluorene contaminations at different doses (1, 10 and 100 mg kg^{-1}) after 24 days. Zhang *et al*. (2010) reported that different levels of PAH contamination (299.4-2777 μg kg^{-1}) affected microbial community by the selection of PAH degraders such as members of β-*proteobacteria* and *Firmicutes* and suppression of certain groups of bacteria.

Time was found to impact on the bacteria community structure, as reflected by changes in the abundance of the TRFs in the initial and final soil samples from PAH treatments after 65 days; this agrees with a number of previous studies (Juhanson *et al*., 2007; Zhou *et al*., 2009; Sun *et al*., 2010). Juhanson *et al*. (2007) reported temporal variation in rhizosphere microbial community composition during a three-year field study with hydrocarbon contaminated land, while the study by Zhang *et al.* (2011) reported changes in microbial community structure of planted soils after 30 days. Vinas *et al*. (2005) also reported temporal changes in bacterial community of an unplanted PAH-creosote contaminated soil (8000 mg kg^{-1} total petroleum hydrocarbon) after 200 days.

The change in microbial community structure of the planted and unplanted PAH treatments after 65 days may have been driven by the impact of PAH concentration and toxicity of the PAH compounds and degradation products to soil microbes, leading to a period of adaption with the suppression and emergence of bacterial groups (Trevors, 1996; Kozdroj and van

Elsas, 2001; Liao and Xie, 2007; Liu *et al*., 2009). For planted treatments, the profile and quantity of root exudates are affected by the age of the plant and the presence of contaminants. Such a change in root exudate composition would have an impact on microbial community structure (Singer *et al*., 2003; Xu *et al*., 2006; Dzantor *et al*., 2007; Olson *et al*., 2007; Wu *et al*., 2008; Mackova *et al*., 2009; Diogo *et al*., 2010).

7.6 Conclusion

The presence of contaminants did not significantly affect the bacterial community structure but specific contaminant treatments (single and mixed), the plant type (*M. sativa*, *L. perenne* and *F. arundinacea*) and time had a significant impact on the bacterial community structure. The impact of PAH contamination on soil bacterial community may be attributed to contaminant toxicity to soil microbes and/or plants. The similar bacterial community structure for phenanthrene and benzo[*a*]pyrene treatments may be attributed to the selective proliferation of similar bacterial groups (Liu *et al*., 2009). However, the impact of volatilization on phenanthrene dissipation due to its low molecular weight and sorption of benzo[*a*]pyrene resulting in a decrease in the bioavailability and minimal effect on bacterial community structure may not be excluded (Smith *et al*., 2011; Hamdi *et al*., 2012).

A decrease in mean number of TRFs at the end of the greenhouse experiment indicated a decrease in diversity as a result of PAH contamination (Sun *et al*., 2010; Zhang *et al*., 2010). The TRFs 133 and 135 bp common to all the PAH treatments are likely to be from PAH degraders of the groups *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* and *Firmicutes* (Alonso-Gutiérrez *et al*., 2008; Puskarova *et al*., 2013).

The significant impact of plant presence and type on the bacterial community of the PAH treatments may be attributed to the ability of plants to tailor root exudates for the selectively proliferation of PAH degraders to suppress toxicity and promote rhizoremediation (Singer *et al*., 2003; Dzantor *et al*., 2007; Siciliano *et al*., 2001; Cebron *et al*., 2009). The rhizosphere bacterial communities of *M. sativa* and *F. arundinacea* were significantly different, as previously reported (Kirk *et al*., 2005; Pritchina *et al*., 2011). This may also explain the difference in PAH dissipation for *M. sativa* and *F. arundinacea* as discussed in Section 6.5.2.3.

Changes in the TRF abundance after 60 days may have been driven by changes in the soil chemistry from the presence of PAH and PAH degradation products continuously influencing root exudation and shaping the bacterial community structure over time (Diogo *et al*., 2010).

Soil bacterial community structure was influenced by plant species through the rhizosphere effect and possibly phytotoxicity with impact on overall plant health (Section 6.5.1). This may account for the comparable PAH dissipation in the planted and unplanted treatment (see Table 6.7) as discussed in Section 6.5.2 despite the difference in TRF abundance. Rhizoremediation outcome may be affected by microbial community structure, which in turn may be influence by a number of factors such as the contaminant and plant stress tolerance. These have implication for selection of rhizoremediation candidates for PAH-contaminated sites

CHAPTER 8

EFFECT OF PLANT GROWTH PROMOTING BACTERIA; *Pseudomonas Putida* UW4 ON PLANT BIOMASS YIELD AND PHYTOREMEDIATION OF MIXED PAH-CONTAMINATED SOIL

Abstract

Plant growth promoting bacteria (PGPB) are used extensively in agriculture to improve plant growth and plant tolerance however, PGPB application in phytoremediation is a relatively new technology. Few studies have reported the impact of PGPB on plants in the presence of organic toxicants and metals. This study investigated the effect of *Ps. putida* UW4 on plant biomass yield and phytoremediation efficiency in two different treatments; "*PAH"* treatment spiked with phenanthrene; 300 mg kg^{-1}, fluoranthene; 200 mg kg^{-1} and benzo[*a*]pyrene; 5 mg kg^{-1}, and "*PAH+HM"* treatment ('*PAH*' treatments + 100 mg of Pb per kg). *M. sativa*, *F. arundinacea*, *L. perenne* and mixed plants (*L. perenne* and *F. arundinacea*) were selected for thegreenhouse experiment. Following 60 day growth period, the contaminant treatments had both stimulatory and inhibitory effects on plant biomass yields but these effects were not significant. PGPB inoculation significantly enhanced biomass yields of mixed plants for the PAH+HM+PGPB treatment. PGPB significantly enhanced dissipation of phenanthrene and fluoranthene for *M. sativa*-PAH+PGPB treatment and fluoranthene for *F. arundinacea*-PAH+HM+PGPB treatment. In most cases, PAH dissipation in the mixed plant treatment was equal or lower than those of the single plant treatment and higher in a few cases. PGPB innoculation may have the potential to improve plant tolerance and enhance phytoremediation. However, this may be dependent on achieving and maintaining a high biomass of metabolically active inoculum throughout the remediation process. Plant choice,

PGPB inoculum, plant-microbe specificity and type of contaminants are important factors to consider.

8.1 Background

Phytoremediation efficiency is limited by factors such as contaminant stress, nutrient deficiency and phytotoxicity (Wenzel *et al*., 2009). Plant stress response is characterized by an elevated "stress" ethylene production, which inhibits plant growth and biomass yield through senescence, chlorosis and abscission (Wenzel, 2009; Cheng *et al*., 2012). Although genetic engineering may confer plants with stress tolerance and enhance growth in contaminated environments, the use of plant growth promoting rhizobacteria (PGPB or PGBR) (Kloepper *et al*., 1980) has been proposed as an alternative approach (Cheng *et al*., 2012).

PGPB include species of *Azotobacter, Azospirillum, Bacillus* and *Pseudomonas*. The growth promoting ability of PGPB was traditionally explained by the production of phytohormones such as indoleacetic acid (IAA) which stimulate root elongation. Better insights regarding the mechanism of plant growth promotion have been provided, following the recent discovery of some 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing PGPB which suppress stress ethylene levels (Glick *et al*., 1995; Burd *et al*., 1998). Other mechanisms used by PGPB include production of antibiotics, fungicidal compounds, enzymes and siderophores, asymbiotic nitrogen fixation, and solubilisation of nutrients and mineral phosphates (Lugtenberg and Dekkers, 1999; Barea *et al*., 2005; Zhuang *et al*., 2007; Gholami and Nezarat, 2009; Glick, 2010). Some PGPB also have a phytoextraction ability which promotes plant growth under heavy metal toxicity (Burd *et al*., 1998; So-Yeon and Cho, 2009). These

different traits may be used by PGPB to facilitate growth and development at various stages during the life cycle of the plant (Glick, 2010).

As plant-microbe interactions may be specific, successful PGPB colonization and establishment may vary with plant species, bacterial species and strains despite the root colonization efficiency of PGPB (Kuiper *et al*., 2004). Plant-microbe interactions are usually initiated by plants through the release of signals in their root exudates recognised by soil microbes which respond by initiating colonization (Nihorimbere *et al*., 2011). Successful root colonization by PGPB may also be affected by phosphorus, nitrogen, iron and oxygen availability (Sorenson *et al*., 2001). Hence PGPB competence would depend on their ability to survive and proliferate in the presence of indigenous microbes in plant rhizosphere (Nihorimbere *et al*., 2011).

Studies have shown that successful root colonization by PGPB have significantly increased seed germination, seedling growth, plant yield and nutrient uptake (De-Bashan *et al.,* 2004; Gholami and Nezarat, 2009). Strains of *Ps. putida* and *Pseudomonas fluorescens* increased root and shoot elongation in *Brassica napus* (canola), *Triticum spp.* (wheat) and *Solanum spp* (potatoes) (Gholami and Nezarat, 2009). *Pseudomanas putida* UW4 isolated from the rhizosphere of *Phragmites australis* (common reed) (Glick, 2003, 2004; Hontzeas *et al*., 2004) produces siderophores, indoleacetic acid and ACC deaminase (Patten and Glick, 2002; Nihorimbere *et al*., 2011). Also, *Pseudomans putida* UW4 inoculation improved growth of *Cucumis sativus* (cucumber) (Gamalero *et al*., 2010) and Canola (Cheng *et al*., 2007; Cheng *et al*., 2012) under salinity stress. Furthermore, studies on PGPB have reported protection from growth inhibition caused by flooding, phytopathogens and drought (Glick, 2010).

There is however, little information on the relatively recent approach to contaminant remediation, PGPB enhanced phytoremediation (PEP) (Gurska *et al*., 2009; Glick, 2010; De-Bashan *et al*., 2011). There are a few reports on the effect of PGPB on plant tolerance and growth in the presence of organic toxicants (Glick, 2003; Huang *et al*., 2004; Huang *et al*., 2005; Reed and Glick, 2005; Gurska *et al*., 2009) and metals (Burd *et al*., 1998; Glick, 2003; Reed and Glick, 2005). *Pseudomans putida* UW3, *Pseudomans putida* UW4 (Greenberg *et al*., 2006; 2007) *Enterobacter cloacae* CAL2 and *Azospirillum brasilense* Cd (Huang *et al*., 2004a; Huang *et al*., 2004b) improved biomass yields, increased stress tolerance and enhanced total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) dissipation during phytoremediation. There are, however, few reports on the effect of PGPB on plant biomass yields and phytoremediation of mixed contamination with organic and inorganic compounds (De-Bashan *et al*., 2011). PAHs and heavy metals are important co-contaminants with adverse effects on plant biodiversity and health. PAHs can affect the phytoremediation of heavy metals and *vice versa,* consequently, contaminant type is important in selecting remedial strategies for contaminated sites (Gerhardt *et al*., 2009).

This study was designed to determine the effect of *Pseudomanas putida* UW4 on plant biomass yields and phytoremediation of mixed PAH (phenanthrene, fluoranthene and benzo[*a*]pyrene) and heavy metal (HM) (lead) contamination; *M. sativa*, *F. arundinacea*, *L. perenne* and mixed plant community of *L. perenne* and *F. arundinacea* were selected for this study.

8.2 Hypotheses

1. Different contaminant groups (PAH, PAH+HM and HM), will affect plant biomass yields and PAH dissipation.

2. Addition of PGPB in the PAH and PAH+HM treatments will improve plant biomass yield and PAH dissipation.
3. PAH dissipation will be higher in mixed plant community (*F. arundinacea* and *L. perenne*) with PGPB in comparison with single plant cultures when treated with PGPB.

8.3 Methods

8.3.1 Soil preparation and experimental design

Treatment groups and controls for this experiment were PAH, PAH +PGPB, PAH + HM, PAH+HM+PGPB, HM, unplanted treatment controls, plant controls (Table 8.1). A sandy loam soil sourced from a commercial supplier (Travis Perkins, United Kingdom) was air-dried and sieved through a 2 mm sieve. Phenanthrene (>98% purity), fluoranthene (>98% purity) and benzo[*a*]pyrene (>96% purity) were obtained from VWR, UK. About 250 g of air-dried and sieved soils (25% of soil for planting in each pot) was spiked with phenanthrene (~300 mg), fluoranthene (~200 mg) and benzo[*a*]pyrene (~5 mg) dissolved in 20 mL of acetone in a fume hood. The soils were mixed in glass beakers with a spatula. The spiked soils were allowed to air-dry in a fume hood for three days before adding about 750 g of unspiked soil. For the PAH+HM contaminant group, 100 mg of lead (lead sulphate) dissolved in 20 mL of water was used to further spike the PAH spiked soils, air-dried for 24 hours before adding 750 g of unspiked soil. Finally for the HM contaminant group, 100 mg of lead dissolved in 20 mL of water was used to spike about 250 g air-dried and sieved soils, mixed and air-dried for 24 hours before adding 750 g of unspiked soil. Following the spiking, soils were homogenized, stored and later dispensed for planting as described in Section 3.2.3.

The control treatment assessed contributions made by abiotic processes and soil microbes to PAH dissipation. Plant seedlings in pertiles were transplanted into the spiked and control soils and soil samples collected as discussed in Section 3.2.4. The soil samples were extracted and PAH concentration assessed by GC-MS as described in Chapter 3.

Table 8.1: Greenhouse experimental design to assess the effect of PGPB inoculation on the phytoremediation of co-contaminated soil. PAH1 - phenanthrene (300 mg kg^{-1}); PAH2 - fluoranthene (200 mg kg^{-1}) and PAH3 - B[*a*]P (5 mg kg^{-1}); HM- Lead (100 mg kg^{-1}) PGPB- plant growth promoting bacteria. Initial PAH concentration in the *PAH* treatments were: phenanthrene; 250±18.9 mg kg^{-1}, fluoranthene; 72.0±5.36 mg kg^{-1}, benzo[*a*]pyrene; 3.01±0.20 mg kg^{-1}. Those for the *PAH+HM* treatment were: phenanthrene; 234±17.5 mg kg^{-1}, fluoranthene; 70.8±4.46 mg kg^{-1} and benzo[*a*]pyrene; 2.29±0.19 mg kg^{-1} (mean ± SE, n = 6) (Appendix 8.1).

Treatments	***M. sativa***	***L. perenne***	***F. arundinacea***	***L. perenne* + *F. arundinacea***	**Unplanted control**
Control	X3	X3	X3	X3	X3
HM	X3	X3	X3	X3	X3
PAH123	X3	X3	X3	X3	X3
PAH123+PGPB	X3	X3	X3	X3	X3
PAH123+HM	X3	X3	X3	X3	X3
PAH123+HM+PGPB	X3	X3	X3	X3	X3

8.3.2 Preparation of inoculum

8.3.2.1 ***Source of bacterial strain***: Bacterial strain *Enterobacter cloacae* UW4 (reclassified as *Ps. putida* UW4 based on 16S rDNA sequence analyses and metabolic activity) isolated from the rhizosphere of common reeds (Glick, 1995) was obtained from Dr. B.R. Glick, Department of Biology, University of Waterloo, Canada.

8.3.2.2 ***Media preparation and inoculation*:** Nutrient agar (NA) and nutrient broth (NB) obtained from Sigma, UK were prepared according to manufacturer's instructions and

sterilized at 121°C for 15 minutes in an autoclave (Boxer laboratory equipment, ware 01920/468727/8, UK). Sterile agar was poured into sterile Petri dishes and allowed to set. *Ps. putida* UW4 was sub-cultured on NA agar plates by the plate streaking technique and incubated at 28°C for 24-48 hours (Figure 8.1). Pure and isolated colonies were aseptically taken and inoculated into 25 mL fresh NB in serum bottle and incubated at 28°C and 150 rpm for 24-48 hours. The bacterial suspension was sub-cultured into 250 mL fresh NB in conical flasks. The inoculated NB were placed on a mechanical shaker at 150 rpm and incubated at 28°C for 3 days. Following the incubation period, the bacterial culture was dispensed into sterile falcon tubes (50 mL) and centrifuged at 5000 rpm for 15 minutes at 7°C. The supernatant was discarded after which harvested cell pellets were rinsed thrice with sterile distilled water and re-suspended in 125 mL of sterile distilled water.

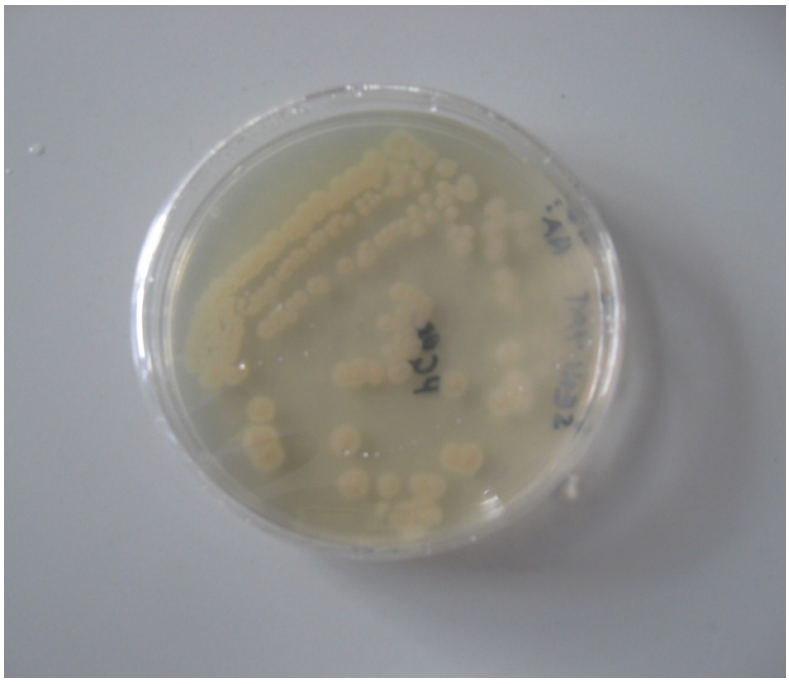


Figure 8.1: Colonies of *Ps. putida* UW4 on nutrient agar.

8.3.2.3 ***Colony forming units*****:** The number of viable cells was assessed by the plate count method. One mL of stock bacterial suspension was added to 9 mL sterile distilled water and mixed by pipetting. This was serially diluted in tubes containing 9 mL sterile distilled water until 10^{-6} dilution was achieved. 0.1 mL of dilutions 10^{-4}, 10^{-5} and10^{-6} were sub-cultured on NA plates and incubated in an incubator (Raven 2, UK) at 28°C for 24 hours. Colonies were counted manually in each quadrant and the total colony forming unit per mL was calculated. Total colony forming units (CFU mL^{-1}) = number of colonies per plate X reciprocal of final dilution factor (Figure 8.2).

Total colony forming unit = 296 X 10^{7} CFU mL^{-1}

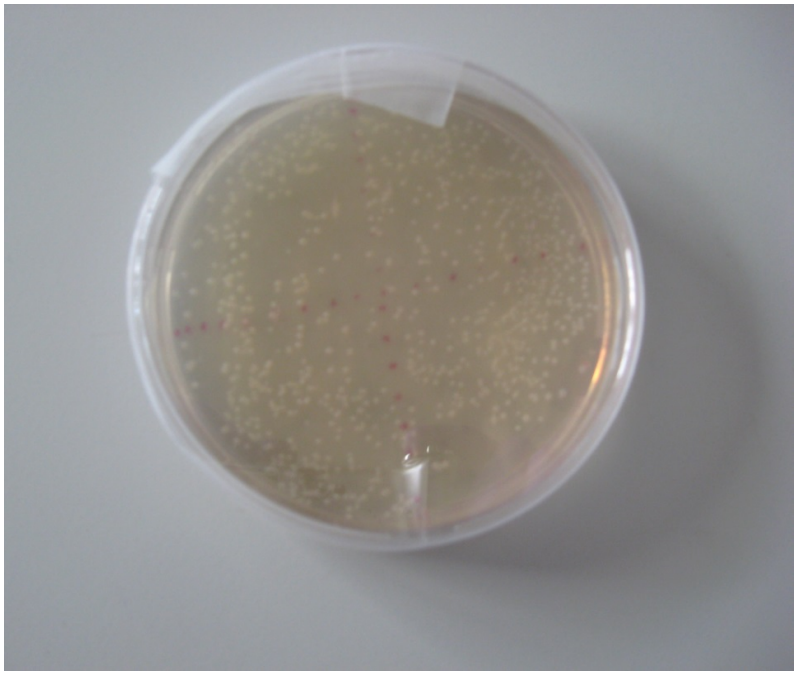


Figure 8.2: Colonies of *Ps. putida* UW4 on nutrient agar following serial dilution of bacterial stock solution to10^{-6}.

8.3.2.4 ***Absorbance of bacterial suspension*:** The absorbance of the bacterial suspension was measured using Jenway ultraviolet/visible light spectrophotometer (model 68000 Com1: 6507060) in accordance with the manufacturer's instructions. About 3 mL of sterile NB was dispensed into a clean cuvette (10 x 10 x 45 mm, polystyrene) and placed into the blank slot of spectrophotometer. This was followed by placing another cuvette containing 3 mL of bacterial suspension in the sample slot for analysis. Absorbance of the bacterial suspension was measured at 600 nm.

8.3.3 Plant seedling inoculation

Two weeks after transplantation, plant seedlings were inoculated in the rhizosphere soil by adding 5 mL of *Ps. putida* UW4 suspension. (Total colony forming unit = 296 X 10^7 CFU mL^{-1}; absorbance at 600 nm = 2.57). Soils were not watered for the first 48 hours to allow bacterial colonization and to prevent leaching of bacterial suspension.

Following the growth period (65 days), soil samples and plant biomass were collected, processed and stored as described in Section 3.4. Soil samples were extracted by microwave extraction and analysed for PAH content by GC-MS as described in Section 3.5. Initial PAH concentration in the *PAH* treatments were: phenanthrene; 250±18.9 mg kg^{-1}, fluoranthene; 72.0±5.36 mg kg^{-1}, benzo[*a*]pyrene; 3.01±0.20 mg kg^{-1}. Those for the *PAH+HM* treatment were: phenanthrene; 234±17.5 mg kg^{-1}, fluoranthene; 70.8±4.46 mg kg^{-1} and benzo[*a*]pyrene; 2.29±0.19 mg kg^{-1} (mean ± SE, n = 6).

8.3.4 Calculations

Percentage increase in plant biomass relative to the control = Difference in plant biomass from PAH+PGPB and PAH-PGPB treatment/plant biomass for PAH-PGPB x 100

Total concentration of PAH dissipated (mg kg^{-1}) = mean initial PAH in spiked soil (mg kg^{-1}) – mean residual PAH (mg kg^{-1})

Percentage dissipation= Total concentration of PAH dissipated/ mean initial PAH in spiked soil X 100.

8.4 Results

8.4.1 Impact of contaminant type on plant biomass yield

Plant biomass yields varied across the contaminant treatments (PAH, PAH+HM, and HM) as shown in Table 8.2. In comparison to plant control, an increased shoot biomass yield was observed for all plants except *M. sativa* which displayed a 9-14% decrease for all treatments.

On the other hand, decreased root biomass were observed for *M. sativa* in the PAH (9%) and HM (17%) treatments, *L. perenne* for PAH+HM (33%) and HM (4%) treatments, *F. arundinacea* for PAH (32%) and HM (40%) treatments and mixed plants for HM (13%) treatments (Table 8.2).

Table 8.2: Plant biomass yield from contaminant treatment groups (Average values ± SE, n=3). Positive and negative values indicate percentage increase and decrease in biomass respectively in comparison to control. Values sharing the same letter are not statistically significant following analysis by a one-way ANOVA ($p<0.05$) (Appendix 8.2).

Plant	**Treatment**	**Average shoot biomass yield (g)**	**Biomass increase relative to control (%)**	**Average root biomass yield (g)**	**Biomass increase relative to control (%)**
M. sativa	PAH	4.11±0.114a	-9	3.22±0.122a	-9
	PAH+HM	4.04±0.382a	-10	3.60±0.117a	1
	HM	3.87±0.154a	-14	2.96±0.425a	-17
	Control	4.51±0.433a		3.55±0.218a	
L. perenne	PAH	4.80±0.200a	12	10.4±3.74a	9
	PAH+HM	4.83±0.328a	12	6.37±0.982a	-33
	HM	5.10±0.289a	19	9.20±1.96a	-4
	Control	4.30±0.305a		9.57±3.57a	
F. arundinacea	PAH	3.20±0.153a	6	2.70±0.288a	-32
	PAH+HM	3.00±0.153a	1	4.73±1.42a	19
	HM	3.03±0.202a	0	2.40±0.305a	-40
	Control	3.03±0.0881a		3.97±1.45a	
L. perenne + *F. arundinacea*	PAH	4.47±0.409a	8	3.30±0.435ab	9
	PAH+HM	3.03±0.202b	-27	2.63±0.176a	-13
	HM	4.57±0.272a	11	4.90±0.346b	62
	Control	4.13±0.296ab		3.03±0.0333a	

8.4.2 Impact of PGPB on plant biomass yield from different treatments

The results show that PGPB did not significantly enhance plant biomass yields in both *PAH+PGPB* and *PAH+HM+PGPB* treatments (Tables 8.3 and 8.4, and Appendix 8.4).

Table 8.3: Plant biomass yield from PAH treatment with PGPB inoculation and PAH treatment without PGPB inoculation (Average values ± SE, n=3) (Appendix 8.2). Values for the PAH and PAH+PGPB groups for each plant biomass sharing the same letter are not statistically significant following analysis by a one-way ANOVA (p>0.05).

Plant	**Treatment**	**Average shoot biomass yield (g)**	**Average root biomass yield (g)**
M. sativa	PAH	4.11±0.114a	3.22±0.121a
	PAH+PGPB	4.82±0.529a	3.56±0.279a
	Control	4.51±0.433a	3.55±0.218a
L. perenne	PAH	4.80±0.200a	10.40±3.74a
	PAH+PGPB	5.17±0.120a	17.20± 3.41a
	Control	4.30±0.306a	9.57±3.57a
F. arundinacea	PAH	3.20±0.153a	2.70±0.289a
	PAH+PGPB	3.73±0.233a	4.27±0.338a
	Control	3.03±0.0882a	3.97±1.45a
L. perenne + *F. arundinacea*	PAH	4.47±0.409a	3.30±0.435a
	PAH+PGPB	5.40±0.404a	4.63±0.959a
	Control	4.13±0.296a	3.03±0.0333a

8.4.2.1 *PAH+PGPB treatment*

Increased *M. sativa* shoot (17%) and root (11%) biomass yields for the PAH+PGPB treatment compared to those of the PAH-PGPB (Table 8.5) were not statistically significant (p>0.05). Also, PGPB did not significantly enhance the biomass yields of *L. perenne*, *F. arundinacea* and mixed plants in the PAH+PGPB treatment (p>0.05). The mixed plant community displayed a 21% shoot biomass increase and 40% root biomass increase for PAH+PGPB treatment (p>0.05).

8.4.2.2 *PAH+HM+PGPB treatment*

Based on a two-way ANOVA and Tukey post-hoc test, changes in *M. sativa* biomass yield (33% shoot biomass yield increase and 4% root biomass yield decrease) for the PAH+HM+PGPB treatment (Table 8.4) were not statistically significant (p>0.05). Also the

biomass yield increase for *F. arundinacea* shoot (2-21%) and *L. perenne* root (8-101%) were not significant.

On the other hand, PGPB had a significant impact ($p < 0.05$) on shoot biomass for mixed plant community of the PAH+HM+PGPB treatments. Tukey post-hoc test revealed that mixed plants shoot biomass yields for the PAH+HM and PAH+HM+PGPB treatments were not significantly different from each other but interestingly, those of the PAH+HM+PGPB treatment were not different to those of the control. However the shoot biomass for the PAH+HM treatment was significantly different to those of the control.

Table 8.4: Plant biomass yield from PAH+HM spiked soils with PGPB inoculation and PAH+HM spiked soils without PGPB inoculation (Average values ± SE, n=3) (Appendix 8.2). Values for the PAH+HM and PAH+HM+PGPB groups for each plant biomass sharing the same letter are not statistically significant following a one-way ANOVA ($p<0.05$).

Plant	**Treatment**	**Average shoot biomass yield (g)**	**Average root biomass yield (g)**
M. sativa	PAH+HM	4.05±0.382a	3.60±0.117a
	PAH+HM+PGPB	5.39±0.442a	3.45±0.410a
	Control	4.51±0.433a	3.55±0.218a
L. perenne	PAH+HM	4.83±0.328a	6.37±0.982a
	PAH+HM+PGPB	4.93±0.338a	12.83±3.37a
	Control	4.30±0.305a	9.57±3.57a
F. arundinacea	PAH+HM	3.00±0.153a	4.73±1.426a
	PAH+HM+PGPB	3.10±0.200a	5.10±0.503a
	Control	3.03±0.0881a	3.97±1.452a
L. perenne + *F. arundinacea*	PAH+HM	3.03±0.203a	2.63±0.176a
	PAH+HM+PGPB	3.67±0.120ab	3.63±0.736a
	Control	4.13±0.296b	3.03±0.0333a

8.4.3 Effect of lead on plant biomass yield

During the green house experiments, some plants in the PAH+HM treatment displayed senescence of leaves. Also, decreased biomass yields were displayed for *F. arundinacea* shoot, *L. perenne* root, mixed plants shoot and root compared to those of the PAH treatment and controls. An increased biomass yield was displayed for *M. sativa* shoot and root, *L. perenne* shoot and *F. arundinacea* root (Table 8.5). These increase and decrease in biomass yield observed for the PAH+HM with PGPB inoculation and PAH+HM without PGPB inoculation were not significantly different ($p > 0.05$) (Appendix 8.2).

Table 8.5: Plant biomass yield increase from PAH and PAH+HM treatment groups with PGPB inoculation relative to treatments without PGPB inoculation (Average values ± SE, n=3) (Appendix 8.2).* Asterisked (negative) values represent percentage inhibition.

Plant	**Treatment**	**Increase in shoot biomass relative to that from treatment without PGPB (%)**	**Increase in root biomass relative to that from treatment without PGPB (%)**
M. sativa	PAH+PGPB	17	11
	PAH+HM+PGPB	33	-4*
L. perenne	PAH+PGPB	8	65
	PAH+HM+PGPB	2	101
F. arundinacea	PAH+PGPB	17	58
	PAH+HM+PGPB	3	8
L. perenne + *F. arundinacea*	PAH+PGPB	21	40
	PAH+HM+PGPB	21	38

8.4.4 Impact of PGPB on PAH dissipation

PGPB mainly had a stimulatory effect and in a few cases an inhibitory effect on PAH dissipation but the impact was not significant (Figures 8.3-8.6 and Appendix 8.3).

8.4.4.1 *No Enhancement/inhibition of PAH Dissipation by PGPB*

PAH dissipation for PAH+PGPB and PAH+HM+PGPB treatments were somewhat similar across the selected plant candidates (Figures 8.4-8.6). Furthermore, PGPB had no impact on phenanthrene dissipation (0%) for mixed plants in the PAH+PGPB treatment. Interestingly, there was a decreased phenanthrene dissipation for *F. arundinacea* (2%), benzo[*a*]pyrene dissipation for *L. perenne* (2%) and mixed plants (9%) in the PAH+PGPB treatment in comparison to the PAH-PGPB treatments. Also, there was a decreased phenanthrene dissipation (4%) for *M. sativa* for the PAH+HM+PGPB treatment in comparison to the PAH+HM-PGPB treatment.

8.4.4.2 *Enhancement of PAH Dissipation by PGPB*

A stimulatory effect on dissipation was displayed for phenanthrene (*M. sativa*-PAH+PGPB; 25% and *M. sativa*-PAH+HM+PGPB treatment; 34%, mixed plants-PAH+HM+PGPB treatment; 1%). Enhanced fluoranthene dissipation was also displayed for the PAH+PGPB treatment (*M. sativa*; 34%, *L. perenne*; 6%, *F. arundinacea*; 10% and mixed plants; 5%) and for the PAH+HM+PGPB treatment (*M. sativa*; 48%, *L. perenne*; 1%, *F. arundinacea*; 24% and mixed plants; 12%). There was also an increased benzo[*a*]pyrene dissipation for the PAH+PGPB treatment (*M. sativa*; 2%, *F. arundinacea*; 10% and *L. perenne*; 13%) and for the PAH+HM+PGPB treatment (*F. arundinacea*; 7% and mixed plants; 16%).

PAH dissipation was significantly different for all the plants of the different treatments while the impact of PGPB was significantly different only for *M. sativa*-PAH treatment and *F. arundinacea*-PAH+HM-PGPB treatment ($p < 0.05$) (Appendix 8.6). Based on a Tukey post-hoc test, the impact of PGPB on PAH dissipation was significantly different for phenanthrene and fluoranthene for *M. sativa* (PAH-PGPB versus PAH+PGPB treatment) and phenanthrene

for *M. sativa* and fluoranthene for *F. arundinacea* (PAH+HM-PGPB versus PAH+HM+PGPB treatments).

8.4.5 *PAH Dissipation: PAH+PGPB versus PAH-PGPB*

8.4.5.1 *Phenanthrene*

The highest phenanthrene dissipation (99%) was observed for both *L. perenne* and mixed plant community in the PAH+PGPB treatments. The lowest phenanthrene dissipation; 73% and 96% was observed for *M. sativa* in the PAH-PGPB treatment, and *F. arundinacea* in the PAH+PGPB treatment, respectively.

8.4.5.2 *Fluoranthene and benzo[a]pyrene*

The highest (86%) and lowest (57%) fluoranthene dissipation was displayed by *L. perenne*/mixed plants and *M. sativa* respectively in the PAH-PGPB treatment. *L. perenne* displayed the highest fluoranthene dissipation (92%) while *F. arundinacea* displayed the lowest fluoranthene dissipation (76%) for the PAH+PGPB treatments.

For the PAH-PGPB treatment, *L. perenne* and mixed plants displayed the highest (69%) and lowest (53%) benzo[*a*]pyrene dissipation respectively. An equal benzo[*a*]pyrene dissipation (67%) was observed for *M. sativa* and *L. perenne* while the lowest dissipation of 44% was observed for the mixed plant community for the PAH+PGPB treatment.

8.4.6 PAH Dissipation: PAH+HM+PGPB versus PAH+HM-PGPB

8.4.6.1 *Phenanthrene*

The highest and lowest phenanthrene dissipation for the PAH+HM-PGPB treatment were observed for *L. perenne* (99%) and *M. sativa* (76%) respectively. For the PAH+HM+PGPB treatment, *L. perenne* displayed the highest phenanthrene dissipation (99%) while the lowest dissipation (72%) was displayed by *M. sativa*.

8.4.6.2 *Fluoranthene and benzo[a]pyrene*

For fluoranthene, *L. perenne* displayed the highest dissipation (92%) while *F. arundinacea* displayed the lowest dissipation (42%) in the PAH+HM-PGPB treatment. As for fluoranthene dissipation in the PAH+HM+PGPB treatment, *L. perenne* displayed the highest (93%) while *F. arundinacea* displayed the lowest (66%) dissipation. The highest benzo[*a*]pyrene dissipation was displayed by *M. sativa* (62%) and mixed plants (65%) in the PAH+HM-PGPB and PAH+HM+PGPB treatments respectively. *F. arundinacea* displayed the lowest benzo[*a*]pyrene dissipation of 36% and 43% in the PAH+HM-PGPB and PAH+HM+PGPB treatments respectively.

8.4.7 PAH Dissipation: *Single plant versus mixed plant treatments*

Phenanthrene and fluoranthene dissipation displayed by the mixed plants for the PAH+PGPB treatment (99% and 91% respectively) and benzo[*a*]pyrene dissipation (65%) for the PAH+HM+PGPB were greater compared to single plant treatments of either plant. Phenanthrene dissipation for *L. perenne* (99%) was higher than that of the mixed plants (97%)

in the PAH+HM-PGPB treatment. Also benzo[*a*]pyrene dissipation for *L. perenne* (69%) was higher than that of the mixed plants (53%) in the PAH-PGPB treatment. An equal fluoranthene dissipation displayed by *L. perenne* and mixed plants (86%) was higher in comparison to *F. arundinacea* (66%) in the PAH-PGPB treatment.

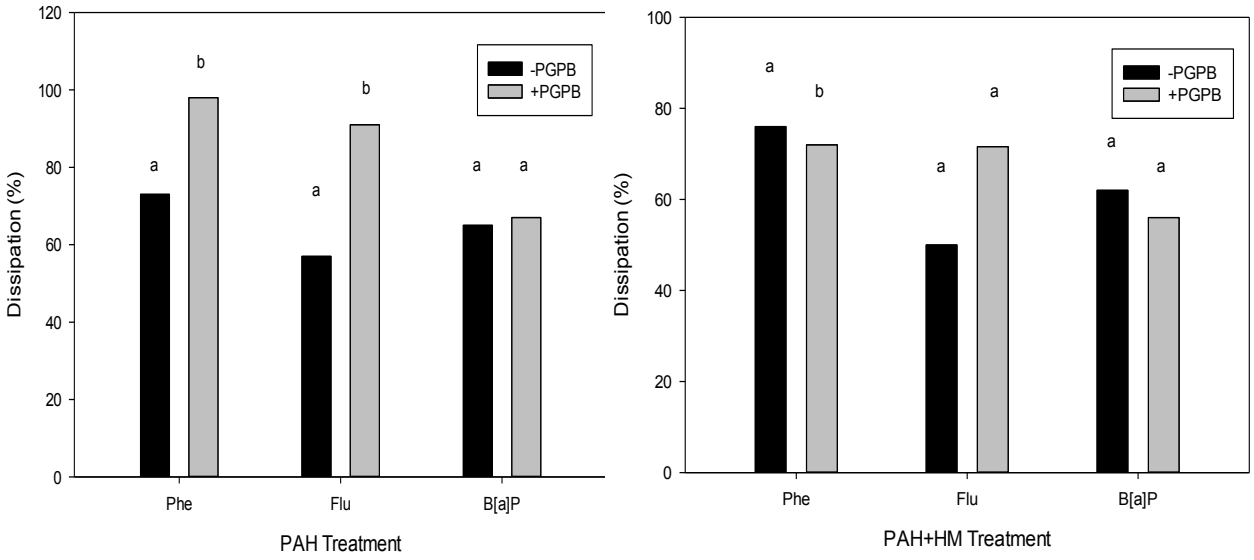


Figure 8.3: Effect of PGPB addition on PAH dissipation from PAH and PAH+HM treatments with *M. sativa*. (Bars represent mean dissipation (%) ± SE, n = 3). +PGPB and – PGPB indicate plant grown in treatments with and without PGPB respectively. Bars with different letters indicate significantly different PAH dissipation in each PAH ($p < 0.05$).

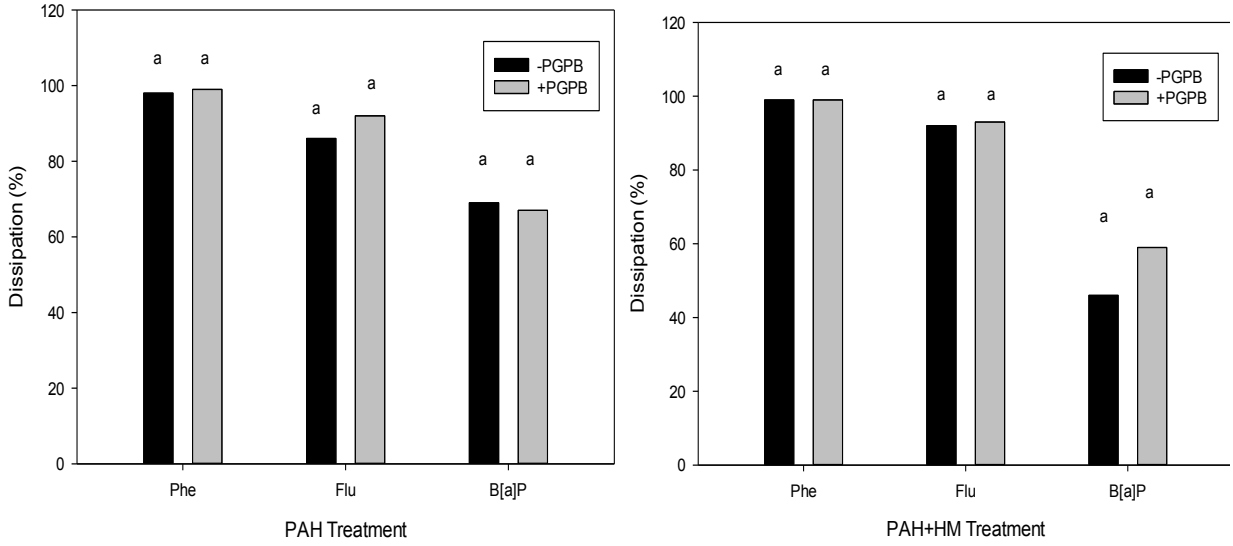


Figure 8.4: Effect of PGPB addition on PAH dissipation from PAH and PAH+HM treatments with *L. perenne*. (Bars represent mean dissipation (%) ± SE, n = 3). +PGPB and – PGPB indicate plant grown in treatments with and without addition of PGPB respectively. Bars with different letters indicate significantly different PAH dissipations ($p < 0.05$).

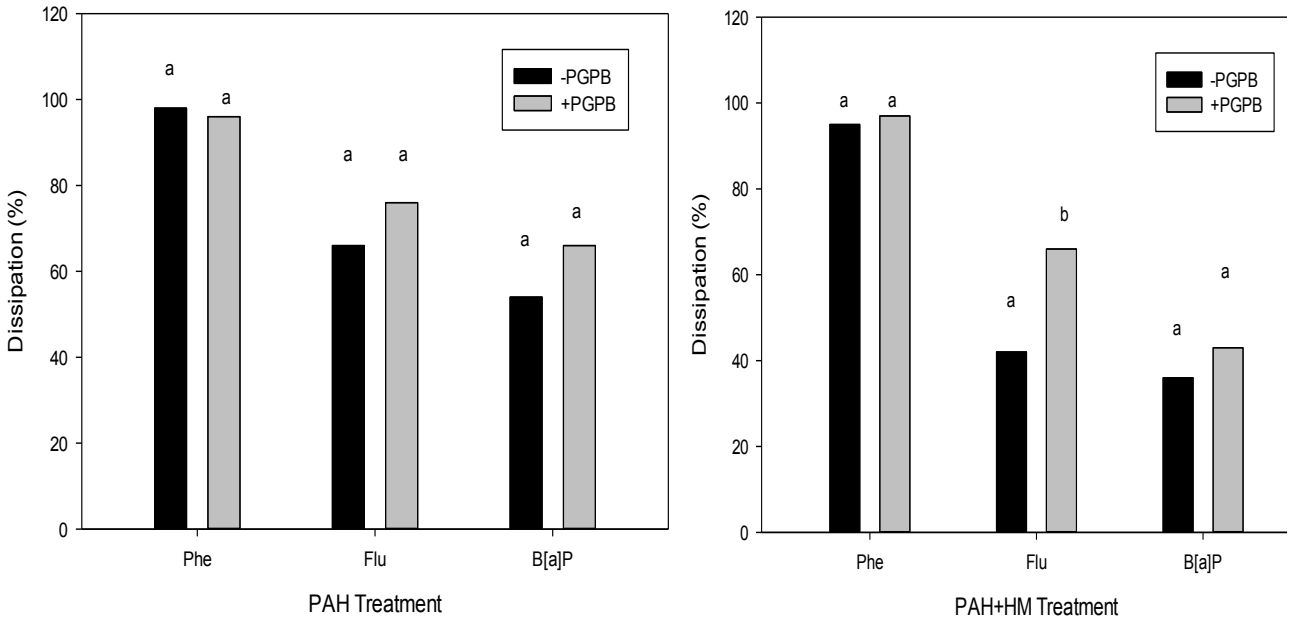


Figure 8.5: Effect of PGPB addition on PAH dissipation from PAH and PAH+HM treatments with *F. arundinacea*. (Bars represent mean dissipation (%) ± SE, n = 3). +PGPB and –PGPB indicate plant grown in treatments with and without PGPB respectively. Bars with different letters indicate significantly different PAH dissipation ($p < 0.05$).

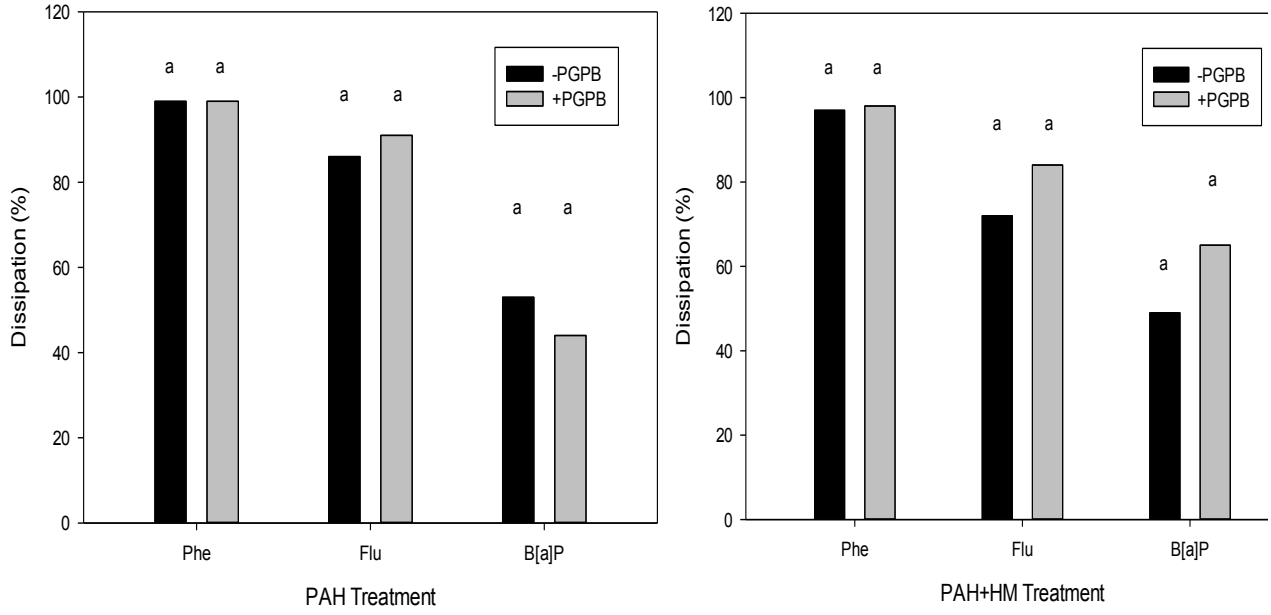


Figure 8.6: Effect of PGPB addition on PAH dissipation from PAH and PAH+HM treatments with mixed plants (*F. arundinacea* + *L. perenne*). (Bars represent mean dissipation (%) ± SE, n = 3). +PGPB and – PGPB indicate plant grown in treatments with and without PGPB respectively. Bars with different letters indicate significantly different PAH dissipations ($p < 0.05$).

8.5 Discussion

8.5.1 *Impact of contaminant type/stress on plant biomass yield*

The effect of PAH and heavy metals (type, range and concentration) on germination, plant biomass yield and difference in plant stress tolerance have been established in a number of studies showing a decrease in plant biomass (Schnoor *et al*., 1995; Lee *et al*., 2008; Wenzel *et al*., 2009; Cheema *et al*., 2010; Van Aken *et al*., 2010), increase in biomass (Reynolds *et al*., 1999; Merkl *et al*., 2004; Gaskin *et al*., 2008) and no growth inhibition (Henner *et al*., 1999).

Decrease in root and shoot biomass yield (Table 8.2) for the different contaminant treatments compared to the control may be attributed to high levels of stress ethylene in response to contaminant toxicity and effect of contaminants on microbial populations with adverse effect on plant-microbe interactions (Glick, 2010; Rajkumar *et al*., 2012). On the other hand, increase in plant biomass despite the presence of contaminants may have been caused by various factors including plant type, plant-microbe interactions, soil microbial diversity and plant root exudate composition which affect contaminant degradation and phytotoxicity. Noteworthy of mention is the fact that the plant tolerance to the contaminant toxicity and growth would depend on the numbers and activities of active degraders and the rate of degradation (Kechavarzi *et al*., 2007 also see discussion section of Chapter 6). Phytotoxicity from PAH in both single and mixed contamination has been discussed previously in Chapter 6.

8.5.1.1 *Effect of Pb on plant biomass yield*

Our finding of decreased biomass yields for *F. arundinacea* shoot, *L. perenne* root and mixed plants root and shoot in the PAH+HM treatment compared with those of the PAH treatment and the control plants is supported by those of previous studies (Singh *et al*., 1997; Brunet *et al*., 2009; Lamhamdi *et al*., 2011; Pinho and Ladeiro 2012). Lamhamdi *et al*., (2013) reported lead treatments at low concentrations (1.5 and 3 mM) affect photosynthetic activity and inhibit growth. Israr *et al.*, (2011) reported a 21% decrease in *Sesbania drummondii* growth following a 10 day treatment with 250 mg L^{-1} of $Pb(NO_3)_2$. Decreased plant biomass may also be attributed to the poor uptake and deficiency of macroelements such as K, P, Ca and Mg, oxidative stress, as a result of lead exposure (Patra *et al*., 2004; Lamhamdi *et al*., 2011; Pinho and Ladeiro, 2012; Rajkumar *et al*., 2012).

The greater adverse effect of lead on root biomass yields compared to those of the shoots agrees with the fact that roots are particularly sensitive to lead during growth and development in the seedling stage (Pinho and Ladeiro, 2012; Wierzbicka and Obidzinska, 1998; Islam *et al*., 2007; Peraltra-Videa *et al*., 2009).

8.5.2 *Impact of PGPB on plant biomass yield for PAH and PAH+HM treatments*

In general, an increased biomass yield was observed for PAH and PAH+HM treatments with PGPB; *Ps. putida* UW4 inoculation compared to those without PGPB inoculation. This is supported by previous studies on PGPB in the presence of organic toxicants (Glick, 2003; Huang *et al*., 2004, 2005; Reed and Glick, 2005; Gurska *et al*., 2009) and metals (Burd *et al*., 1998; Glick, 2003; Reed and Glick, 2005). On the other hand, growth inhibition compared to

the control plants may have resulted from plant responses (stress ethylene production) to contaminant stress in some cases in the PAH and PAH+HM treatments without PGPB inoculation.

Enhanced plant biomass yields following PGPB inoculation may indicate successful colonization and establishment of PGPB as well as their potential impact on plant stress tolerance (Nihorimbere *et al*., 2011). It is noteworthy that growth changes are not typically expected for plants grown with PGPB in the absence of stresses (Glick, 2010). Hence, improved plant growth under contaminant stress, following PGPB inoculation may have been achieved by several mechanisms such as improvement in nutrient acquisition, production of phytohormones and suppression of stress ethylene production (Glick, 2010).

8.5.3 Impacts of PGPB on PAH Dissipation

A significant enhancement PAH dissipation observed for some treatments with *Ps. putida* UW4 addition may be associated with an increase in biomass yield, improved stress tolerance and/or stimulation of specific degraders resulting from successful rhizosphere colonization by *Ps. putida* UW4. Interestingly, the significant effect of PGPB on fluoranthene dissipation (PAH treatment) for *M. sativa* was significant despite the non-significant increase in *M. sativa* biomass yield (Table 8.2). This implies that PGPB may have improved microbial diversity and plant-microbe interaction in the rhizosphere to improve PAH dissipation. However, most studies have reported improved phytoremediation with accelerated plant growth following PGPB application (Huang *et al*., 2004b; Gurska *et al*., 2009).

On the other hand, no enhancement and/or inhibition in phytoremediation despite PGPB addition for some plant treatments, may be attributed to one or more factors; unsuccessful rhizosphere colonisation, survival and/or integration into the soil microbial communities, poor metabolic activity, unsuccessful plant-microbe interaction, decreased inoculum count, limited nutrients, low solubility and bioavailability of contaminants, root exudates profile/composition and environmental factors (Kuiper *et al*., 2004; Tam and Wong, 2008; Lugtenberg and Dekkers, 1999;Raab and Lipson, 2010, Afzal *et al*., 2011; Nihorimbere *et al*., 2011; Khan *et al*., 2013). Also selection of specific bacteria such as degraders and possibly PGPB in the rhizosphere involves complex processes and activities such as changes in soil properties (physicochemical and biological), root structure and root exudation (Hartmann *et al*., 2009).

8.5.4 *Single Plant versus Mixed Plant Treatments*

The mixed plant community of *L. perenne* and *F. arundinacea* is expected to benefit from the advantage of both grasses as well as PGPB addition in the PAH and PAH+HM treatments compared to the single plant experiments. As it is assumed that a combination of different root types and the root exudate profiles will enhance the stimulation and proliferation of metabolically diverse microbial communities with a resultant cumulative benefit from mixed plant communities (Cheema *et al*., 2010) while PGPB enhances plant biomass yield and tolerance (Glick, 2010).

With reference to the biomass result, mixed plant community displayed a greater biomass yield hence would be expected to have a greater PAH dissipation compared to the single plant communities. However, there were cases where the single plants had equal or higher

dissipation than the mixed plant community. This is supported by the findings of Chapter 5 where mixed plant community with *M. sativa* and *L. perenne* displayed lower TPH dissipation in 2% w/w diesel treatment in comparison to plant monoculture. Phillips *et al*. (2006) and Phillips *et al*. (2009) also showed that mixed plant culture with *L. perenne*, *M. sativa* and wheat displayed a lower TPH phytoremediation efficiency in comparison to monoculture.

In addition, the reasons for the contradicting findings is not understood but may be related to which plant dominated the mixed plant community and how this may have affected root physiology, root colonization, root properties and rhizosphere activities (Cheema *et al*., 2010). Also, as two grasses constitute the mixed plant community, with the same rooting system and mechanism of uptake, there may have been competition for nutrients, water and space in a confined plant pot with an impact on plant growth, biomass yield, microbial diversity and rhizosphere activities (Phillips *et al*., 2009).

8.6 Conclusion

Different contaminant treatments had impact on plant biomass yields. The presence of lead stimulated biomass yields and PAH dissipation of some treatments and inhibited others. The inhibitory effect of lead on biomass yield was greatest for the mixed plant community. The presence of lead increased PAH dissipation in some plant treatments and inhibited others.

PGPB improved biomass yields of some plants in the PAH+PGPB treatment and PAH+HM+PGPB treatment. PGPB also significantly enhanced dissipation of phenanthrene and fluoranthene for *M. sativa*-PAH+PGPB treatment and fluoranthene for *F. arundinacea*-PAH+HM+PGPB treatment ($p<0.05$). PAH dissipation for the mixed plants was not

necessarily higher than those of the single plant in all the treatments and PGPB did not significantly enhance PAH dissipation by the mixed plants. Although PGPB improved plant biomass yield in some cases, this did not essentially have stimulatory effect on PAH dissipation as no enhancement was observed in some treatments.

In addition the impact of PGPB addition on growth inhibition and PAH dissipation during the greenhouse experiments was affected by a number of variables and the complexity of the system. These include number of active PGPB and plant-microbe interaction, contaminant type and plant species. The full exploitation of PGPB in rhizoremediation may depend on plant-microbe selection, establishment and interaction in rhizosphere. To this end, it would worth using PGPB isolated from each plant species (plant-specific PGPB) well-adapted to survival under stressed conditions in contaminated sites. It is likely that field trials over a long period using such plant-specific PGPB/endophytes and indigenous specific degraders would unlock the potential and more realistic remediation outcomes of PEP technology.

CHAPTER 9

SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

9.1 Summary

Phytoremediation is a new technology that exploits plant and microbial activity in the remediation of contaminated land. It involves different mechanisms such as accumulation and degradation, and the contribution of individual mechanisms is determined by the type of contaminant, whether organic or inorganic (Gerhardt *et al*., 2009). Most contaminated sites have a mixture of both organic and inorganic compounds present which has an implication for the selection and the efficiency of remediation techniques (Batty and Dolan, 2013).

The principal phytoremediation technique for organic compounds such as hydrocarbons is phytodegradation which also known as rhizoremediation. Rhizoremediation is based on complex plant-microbe interactions and metabolic activities of the rhizosphere (Alkorta and Garbisu, 2001; James and Strand, 2009). However, rhizoremediation efficiency may be affected by several factors such as contaminant type and concentration, bioavailability, plant choice and nutrient level conditions (Schnoor *et al*., 1995; Semple *et al*., 2003; Vangronsveld *et al*., 2009; Van Aken *et al*., 2010).

Plant growth and biomass yield has also been found to be affected differently by different contaminants hence plant selection is quite important. Consequently on the whole, plant stress tolerance has been recognized to be crucial to phytoremediation success. Studies have recommended the use of mixed plant communities and also the use of plant growth promoting bacteria to improve plant tolerance and enhance phytoremediation efficiency. However, there

is little information in this regard and also conflicting results from different studies (Zhuang *et al*., 2007; Gurska *et al*., 2009; Glick, 2010; De-Bashan *et al*., 2012).).

This thesis was carried out with the overall aim of assessing the phytoremediation potential of selected plants for toxic contaminants in brownfield sites. Contaminants used in this study included diesel fuel, PAHs i.e. phenanthrene, fluoranthrene and benzo[*a*]pyrene and the heavy metal, lead. Selected plant candidates were *Medicago sativa*, *Lolium perenne* and *Festuca arundinacea.* This was achieved by assessing the presence of priority PAHs in two selected brownfield sites in the UK, and understanding the impacts of contaminants on plant growth and soil bacterial community structure, rhizoremediation potential of the selected plant species in contaminant treatments (diesel and PAH) and the impact of PGPB inoculation on plant growth/ stress tolerance and phytoremediation.

Presence of PAHs and heavy metals in brownfield sites

Following the analysis of soil samples collected from Saltley and Swansea sites both had PAH and heavy metals (chromium, copper and lead) present in varying concentration. Saltley site had a total PAH concentration of 41.50 mg kg^{-1} with high concentrations of phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene and benzo[*a*]pyrene, total benzo[*a*]pyrene equivalent value of 5.5 mg kg^{-1} and total carcinogenic PAH of 19.43 mg kg^{-1}. Swansea site had total PAH concentration ranging from 5.47 – 84.90 mg kg^{-1} with pyrene, fluoranthene, benzo[*a*]anthracene, chrysene, benzo[*a*]pyrene as the predominant PAHs, total benzo[*a*]pyrene equivalent value of 0.94 – 2.10 mg kg^{-1} and total carcinogenic PAH

concentration of 2.63 – 10.82 mg kg^{-1}. Heavy metal concentration for most samples was above the ambient background concentrations for urban/industrial area. The following conclusions were made:

- The presence and extent of PAH and heavy metal contamination on the sites are attributed to mainly to recent anthropogenic activities on the sites as well as previous activities given the hydrophobicity and persistence of PAH and the effect of ageing on heavy metals.
- The tPAH, cPAH and B[*a*]P-TE values for the PAH and the heavy metal concentration for both sites may indicate the risk to human health depending on the intended land-use. This also determines the need for remediation prior to any redevelopment projects.
- As with most contaminated sites, the presence of mixed contamination with PAH and heavy metals on both sites has an implication for the selection of remediation approaches.

Impact of contaminants on plant growth and biomass yield

The model contaminants used (diesel fuel, PAHs in single and mixed contamination and PAHs and lead) in the different greenhouse experiments had impact on phytotoxicity as reflected by the plant growth and biomass yields. The effect of the contaminants on plant growth and biomass yield was found to be either stimulatory or antagonistic although not significant for some treatments. In general, despite phytotoxic effects from some contaminant treatments, plant biomass yield was generally greater for roots compared to shoots. The findings are highlighted below.

- Diesel-fuel treatments had a negative impact on plant growth and biomass yields. There was an inverse relationship between the plant growth and increasing diesel treatment levels (Figures 5.1, 5.2 and 5.3). There was a decrease in shoot biomass relative to control plants: *M. sativa* (44-73%), *F. arundinacea* (41-64%), *L. perenne* (28-50%) and *M. sativa* + *L. perenne* (13-62%). A decrease in root biomass was observed relative to plant controls: *M. sativa* (81-90%), *Festuca arundinacea* (45-74%), *L. perenne* (27-74%) and *M. sativa* + *L. perenne* (14-86%). The diesel treatment had a significant effect on the root biomass yield of *M. sativa*, *L. perenne*, *F. arundinacea* and *M. sativa* + *L. perenne* and also the shoot biomass yield of *L. perenne*, *F. arundinacea* and *M. sativa* + *L. perenne* ($p<0.05$). The difference in plant growth and biomass production indicate the difference in stress tolerance to diesel-contaminated soils. *L. perenne* was found to be most tolerant while *M. sativa* was least tolerant.
- The single and mixed PAH treatments had stimulatory and inhibitory effects on plant biomass yields relative to the control during the experiment. A biomass yield increase of 80-240% was displayed by *M. sativa* in the single and mixed PAH treatments while *L. perenne* and *F. arundinacea* both displayed increase in biomass yield in some treatment and a decrease in biomass yield in others.

- For the greenhouse experiment with PAH, PAH+HM with/without HM contamination and PGPB inoculation, an increased shoot biomass yield was observed for all plants except *M. sativa* which displayed a 9-14% biomass yield decrease. On the other hand, the decreased root biomass were observed for *M. sativa* in the PAH (9%) and HM (17%) treatments, *L. perenne* for PAH+HM (33%) and HM (4%) treatments, *F.*

arundinacea for PAH (32%) and HM (40%) treatments and mixed plants for HM (13%) treatments (Table 8.2).

- Consequently, plant type was found to be important in plant survival and stress tolerance in contaminated soil. Generally, the grasses; *L. perenne* and *F. arundinacea* had greater biomass yields than the legume; *M. sativa* in all the experiments. Although *M. sativa* is said to be more tolerant to soil toxicity than grasses (Hall *et al*, 2011), *M. sativa* displayed better tolerance in the PAH treatments compared to the diesel treatments. The biomass yields and tolerance of the grasses may be attributed to the extensive fibrous rooting systems, fast growth and dissipation rates of different contaminants as supported by the findings of previous studies (Kuiper *et al*., 2004; Olson *et al*., 2007; Gerhardt *et al*., 2009; Gurska *et al*., 2009; Vangronsveld *et al*., 2009).

Phytoremediation potential of selected plants

Although the highest contaminant dissipation was displayed by *L. perenne,* the contribution of plants to contaminant dissipation (diesel fuel and PAH) was generally low. The dissipation from vegetated treatments was found to be either comparable or slightly greater than that of the unvegetated controls in the different experiments as discussed in chapters 5 and 6. This signifies the importance of abiotic processes (especially volatilization) and microbial degradation in contaminant dissipation (Kaimi *et al*, 2006; Sun *et al*., 2010; Smith *et al*., 2011). This was clearly observed in the greenhouse experiment with single and mixed PAH treatments which included abiotic controls to assess the relative contributions of plants, soil

microbes and abiotic processes to PAH dissipation. The main outcomes of the phytoremediation potential following the experiments are:

- Although it is difficult to maintain abiotic controls, the major contribution of microbial degradation and volatilization to contaminant dissipation in comparison to phytoremediation cannot be ignored. While volatilization and microbial degradation were most important for phenanthrene dissipation due to its low molecular weight, microbial degradation was more important with some contribution by plants to fluoranthene and benzo[*a*]pyrene dissipation (Table 6.6). Microbial degradation is attributed to the selective proliferation of metabolically diverse microbes in contaminated sites but may be enhanced in the presence of plants by the rhizosphere effect (Robertson *et al*., 2010; Santos *et al*., 2011).
- Interestingly, the presence of plants inhibited PAH dissipation in some cases (Single PAH treatment: *M. sativa*- and *L. perenne*-benzo[*a*]pyrene and *M. sativa*-fluoranthene; and mixed PAH treatment; *M. sativa*-and *L. perenne*- phenanthrene and *M. sativa*-and *L. perenne*- benzo[*a*]pyrene) (Tables 6.7 and 6.9).This is attributed to the inhibition of PAH degraders (Liste and Alexander, 2000) and catabolite repression by root extracts (Louvel *et al*., 2011).
- As there was comparable dissipation for vegetated and unvegetated soils, the importance of mixed plant community compared to the single plant community was not apparent. The impact of mixed plants in the rhizoremediation of hydrocarbon-contaminated soil may vary with site properties. Hence plant selection and the full exploitation of the benefits of mixed plant communities may rely on the proper understanding of the individual site variables and conditions.

Impact of PGPB inoculation on plant growth and PAH dissipation

The impact of PGPB inoculation on plant growth and PAH dissipation for the experiment on PAH and PAH+HM contaminated soil is summarised below:

- PGPB inoculation did not significantly enhance plant biomass yields for all plants in the PAH treatment ($p>0.05$) but displayed a significant impact on those of mixed plants in the PAH+HM treatment ($p<0.05$). PGPB addition significantly enhanced mixed plants shoot biomass yield in the PAH+HM+PGPB treatment such that biomass yields were not significantly different from those of the control despite the presence of the contaminants. Also the shoot biomass for the PAH+HM treatment was significantly different to those of the control. Factors such as inoculum size (small microbial biomass), decrease in PGPB population after inoculation, survival and successful establishment of PGPB, plant-microbe interactions and bioavailability of contaminants (Zhuang *et al*., 2007; Tam and Wong 2008; Hartmann *et al*., 2009; Nihorimbere *et al*., 2011) likely influenced our findings. Consequently, a variation in the number of metabolically active PGPB population required to suppress plant growth inhibition resulting from the above-mentioned factors may not be excluded.
- PGPB inoculation significantly enhanced phenanthrene and fluoranthene dissipation for *M. sativa*-PAH treatment and fluoranthene for *F. arundinacea*-PAH+HM ($p<0.05$). This indicates that PGPB may enhance PAH dissipation without significantly enhancing plant biomass yields.

Impact of PAH treatment and plant type on bacterial community structure

Contaminant treatments, presence of vegetation, and the plant type had a significant impact on the bacterial community structure. The impact of the contaminant may be attributed to contaminant toxicity to plant and microbes whereas phytotoxicity and rhizosphere effect may account for the impact of plants on the soil bacterial community.

The rhizosphere bacterial community of *M. sativa* with a higher relative abundance of TRF 133 bp was significantly different from that of *F. arundinacea* which displayed a higher relative abundance of TRF 135 bp. This may be attributed to the fact that root exudate composition and quantities vary with plant species with impact on soil microbial community and diversity (Diogo *et al*., 2010; Hall *et al*., 2011; Wang *et al*., 2011).

A decrease in the average number of TRFs at the end of the experiment indicated a decrease in soil bacterial diversity. The highest mean TRFs were observed for *M. sativa* in the phenanthrene treatment and *F. arundinacea* for the benzo[*a*]pyrene and PAH mix treatments. Furthermore, the bacterial community abundance and diversity may account for the low enhancement and inhibition of PAH dissipation by plants in Chapter 6.

9.2 Conclusion

The presence and concentrations of the PAHs and heavy metals on the Saltley and Swansea brownfield sites pose health risks hence the need for remediation prior to the proposed redevelopment projects. Contaminant concentration and plant stress tolerance remain important factors to be considered for selecting suitable plant candidates as contaminant stress has an impact on plant growth and microbial diversity. This may influence the outcome of rhizoremediation. Although, PGPB inoculation was employed to suppress plant growth

inhibition and enhance contaminant degradation, a number of factors may influence the impact of PGPB. An understanding of the contaminated sites variables, plant stress tolerance, plant-microbe interaction and rhizosphere activities would allow the exploitation of phytoremediation as sustainable remediation technology.

9.3 Research gaps and future perspectives

- Continuous screening for highly tolerant plants with good biomass yields is important in overcoming or reducing the impact of stress during phytoremediation. The use of salt tolerant plants (halophytes) has been recently proposed for heavy metal contaminated sites and may also be employed on co-contaminated sites.
- Future research should focus on understanding the impacts of contaminants on plant growth, root exudation and the intricate interactions in the rhizosphere which affect selective proliferation of metabolic diverse microbial communities and PAH dissipation. It would also be useful to better understand how root exudate compositions may affect the selection of carbon sources in single and mixed plant communities or aid co-metabolism during phytoremediation.
- An understanding of plant-microbe interaction and plant-microbe-contaminant interaction, response to contaminant stress with indigenous plants and microbes on contaminated sites would be useful.
- Studies to understand the root exudate compositions that stimulate or suppress proliferation of contaminant degraders which may determine the impact of plants in rhizoremediation are important. This may promote the application of a rhizosphere metabolomics-driven approach involving the use of root exudates to increase

microbial biomass to enhance degradation in the rhizosphere; a strategy reported by Narasimhan *et al*., (2003).

- Further studies would be required to provide insight on PGPB colonization and survival, minimal effective population of innoculum, plant-microbe association, plant stress response and effect of PGPB inoculation on root exudation, soil microbial population and diversity especially degraders.
- Other recommendations include further studies on the use of a multi-process phytoremediation system with endophytic PGPB from individual plant and bioaugmentation with indigenous contaminant degraders (Huang *et al*., 2004a; Glick, 2010; De-Bashan *et al*., 2012, Khan *et al*., 2013). As the understanding of plant-microbe-contaminant interaction improves, the full exploitation of PGPB technology for efficient degradation during in phytoremediation may depend on regulations that support use of genetically engineered bacteria after assuring the scientific community and the public of their safety with research findings in the future (Glick, 2010).

The exploitation of phytoremediation as a cost-effective green technology would require monitoring and specific tinkering to understand the activities and interactions in rhizosphere which are quite complex.

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