The Effect of Biotic and Abiotic Factors on Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Bacteria in Soil

Zohreh Khorasanizadeh

A thesis submitted in partial fulfillment of the requirements of the University of Hertfordshire for the degree of Doctor of Philosophy.

2013

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous environmental contaminants with two or more aromatic rings and originating from different emission sources. They are extremely toxic, carcinogenic and mutagenic to human, animals and plants. Consequently, the need to expand economical and practical remediation technologies for PAH contaminated sites is evident. In this study, the effect of biotic and abiotic factors on degradation of PAH was studied. The degradation was studied on the key model PAH (phenanthrene, anthracene, fluoranthene and pyrene) in J. Arthur Bower's top soil. The hypothesis for this study was that roadside soil would contain PAH degrading bacteria; pH would influence the microbial degradation of PAH, chemical oxidation of PAH would be as efficient as microbial breakdown of PAH and mobilising agents, would move PAH throughout soil, potentially making the PAH more available for biodegradation. The greatest degradations were found for the lowest molecular weight PAH, phenanthrene and anthracene; whilst lowest degradation was observed for higher molecular weight PAH, fluoranthene and pyrene.

Twelve bacteria genera were isolated and identified by biochemical and molecular techniques from the roadside soil with the four PAHs as the sole carbon source. However, potentially new PAH biodegrader bacteria species and a novel were found in this study, which was not reported in the literature. The effect of pH between 5.0 and 8.0 at half pH intervals on biodegradation of the four PAHs and on bacterial populations in the soil over 32 days was monitored. The greatest population of bacteria and greatest biodegradation for the four PAHs was found at pH of 7.5. It is likely that the general increase in population was also linked with greater metabolic activities of bacteria at basic pHs which assists pollutant biodegradation. Although there is high pollutant mobility at low pHs, the biodegradation was limited due to reduced microbial activity. High pHs resulted in greater PAH biodegradation suggesting that pH manipulation by liming may be an effective way of stimulating biodegradation of PAH.

The effect of potassium permanganate on oxidation of the four PAHs in the soil was examined. Studies in this thesis, indicated that potassium permanganate had a significant ($p<0.05$) effect on oxidation of the four PAHs at pH 7.5 over 35 days. However in comparison to biodegradation, chemical oxidation has significantly ($p<0.05$) less effect. Finally, the effect of Tween 20 only on translocation and biodegradation of the four PAHs at pH 7.5 over 35 days was examined. Studies indicated that Tween 20 had significantly ($p<0.05$) enhanced translocation of the four PAHs in the sterile soil. Moreover, the greatest biodegradation was found in the soil inoculated with only the roadside soil microorganisms but without Tween 20. This suggested that Tween 20 had a significant ($p<0.05$) inhibitory effect on the roadside soil microorganisms and therefore less microorganism were grown in the soil containing Tween 20. This indicated that Tween 20 was translocated PAH, but inhibited breakdown.

This study indicated microbial biodegradation was the most effective technique for removing of the PAH from contaminated soil, which was cost effective and easier to perform in comparison to the other two techniques.

Microbial biodegradation could be improved by adjusting pH through liming if soil was acid.

Dedicated to the ones I love...

Ahmad and Parivash

Acknowledgement

First and foremost I offer my sincerest gratitude to my supervisor Dr. Avice Hall, who supported me throughout my study with her patience and knowledge. I attribute the level of my degree to her encouragement and effort. I wish to express my warm and sincere thanks to Professor Anwar R Baydoun Head of HHS-RI Research Degrees Programmes and Professor Robert Slater Head of Life and Medical Science department for their kind support and guidance. In my daily work I have been blessed with a friendly and cheerful group of lab-mates. I am grateful to Dr. Arjomand Ghareghani who provided good arguments about the project theory. I especially want to thank my colleague Rakesh for his best cooperation. In the various laboratories I have been aided by Sue, Di and other technicians. I've known them: a friendly smile and a hello every time we met.

I would like to thank my family: my parents Ahmad and Parivash for supporting me spiritually throughout my life. I have no suitable word that can fully describe their everlasting love to me. Without their encouragement and understanding it would have been impossible to finish this work. My special gratitude is due to my brothers Alireza and Hamidreza for their loving support. Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project and my thanks to God for my life through all tests in the past years. You have made my life more bountiful. May your name be exalted and honoured.

Contents Pages

Figures Pages

Tables Pages

Abbreviations

CFU	Colony Forming Units
DOM	Dissolved Organic Matter
HPLC	High Performance Liquid Chromatography Technique
HMW	High Molecular weight
LMW	Low Molecular weight
NA	Nutrient Agar
OM	Organic Matter
PAHs	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
RPM	Revolution Per Minute
SPP	Species
UV	Ultra Violet
WHC	Water-Holding Capacity

Chapter 1

Introduction and Literature Review

1.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs), are a class of toxic xenobiotic aromatic compounds which are generated by incomplete combustion of organic matter, for instance forest fires, volcanoes, oil seeps, petroleum, waste incineration, home heating and combustion engines (Johnsen, 2005). PAH are of environmental concern as they are a significant threat to human health due to their mutagenic and carcinogenic properties (Haritash & Kaushik, 2009; Luo et al., 2009) and are widespread environmental contaminants (Andreoni & Gianfreda, 2007; Mohamed et al., 2012; Muckian et al., 2007). Many PAH are only slightly mutagenic or even nonmutagenic *in vitro*. However, their metabolites or derivatives can be potent mutagens. It is not easy to ascribe observed health effects in epidemiological studies to specific PAH because most exposures are to PAH mixtures. The effects on human health will depend mainly on the extent of exposure (length of time, etc.), the amount one is exposed to (or concentration), the natural toxicity of the PAH and whether exposure occurs via inhalation, ingestion or skin contact. See Table 1.1 for the environmental persistence and toxicity of some of PAH to organisms.

Table 1.1 - Environmental persistence and toxicity of PAH to organisms (ATSDR, 2009; Chouycgai et al, 2007; Ikenaka et al., 2013; Niu, et al., 2009; Oleszczuk, 2006)

Organism affected	PAH	Case study and health effects	Environmental persistence
Human	Phenanthrene Benz(a)anthracene Benzo(a)fluoranthene Benzo(k)fluoranthene Benzo(a)pyrene Indeno(1,2,3-c,d)pyrene	There is no information available from studies on humans to tell what effects can result from being exposed to individual PAH at certain levels. However, breathing PAH and skin contact seem to be associated with lung, skin, and bladder cancers in humans (Niu, et al., 2009). Health effects from chronic or long-term exposure to PAH may include cataracts, kidney and liver damage and jaundice. Repeated contact with skin may induce redness and skin inflammation. Naphthalene, a specific PAH, can cause the breakdown of red blood cells if inhaled or ingested in large amounts (Niu, et al., 2009).	Sewage sludge addition to soils resulted in an increase in the content of PAH in soils. Experimental work showed that during a 42/54-month period, more than half of the individual PAHs introduced into the soil with sewage sludge were degraded (Oleszczuk, 2006). Five metal enriched sewage sludges containing different concentrations of PAH were applied to different plots on field soils at two experimental sites. This resulted in substantial increases in the total PAH soil concentrations in all plots. Since application, losses have occurred, with the high molecular weight PAH being more persistent. (Oleszczuk, 2006).
Animal	Benz(a)anthracene Benzo(b)fluoranthene Benzo(a)pyrene Dibenz(a,h)anthracene Indeno(1,2,3-c,d)pyrene	When pregnant mice ate high doses of benzo(a)pyrene, they experienced reproductive problems. In addition, the offspring of the pregnant mice showed birth defects and a decrease in their body weight, damage to skin, body fluids and the immune system, which help the body fight disease (ATSDR, 2009).	

Organism affected	PAH	Case study and health effects	Environmental persistence
		Animal studies showed that exposing mice to 308 mg.kg^{-1} of PAH (specifically benzo(a)pyrene) in food for 10 days (short term exposure) caused birth defects (ATSDR, 2009). Mice exposed to 923 mg.kg^{-1} of benzo(a)pyrene in food for months developed problems in the liver and blood (ATSDR, 2009). **Animal studies show that certain PAH affect the hematopoietic, immune, reproductive, and neurologic systems and cause developmental effects (ATSDR, 2009).**	
Plant	**Phenanthrene and pyrene**	**The growth of *Zea mays* root was the least sensitive to, but its germination rate was the lowest in the presence of, contaminants, and among the legumes, the growth of *Arachis hypogaea* root was better than others (Chouycgai et al, 2007).** ***Zea mays* and *Arachis hypogaea* were selected to further test their ability to tolerate a mixture of phenanthrene and pyrene in the acidic soil (Chouycgai et al, 2007).** **The presence of both PAH led to a greater decrease in the lengths of shoot and root of *Arachis hypogaea* than phenanthrene or pyrene alone, but the lengths of shoot and root of *Zea mays* were decreased to a similar extent as when phenanthrene or pyrene was present alone. The growth of *Zea mays* root was also better than that of *Arachis hypogaea* root when they were grown in oil contaminated soil (Chouycgai et al, 2007).**	

The four PAHs, which were used in this thesis, are shown below in Figure 1.1 with both names and chemical structures. The four PAHs were chosen due to the low number of rings which cause less toxicity for the user (Bleeker et al., 2002) and a shorter degradation period. Furthermore, they are two by two isomers with a same mollecular weight, but different arrangment of rings (Haritash & Kaushik, 2009).

Phenanthrene **Anthracene** **Fluoranthene** **Pyrene**

Figure 1.1 - PAH chemical structures (Haritash & Kaushik, 2009)

Wong et al. (2001) highlighted concerns about large sites contaminated with pollutant i.e. PAH and their effect on the ecosystem and human health (Balachandran et al. 2012). PAH are considered as a main division of petroleum mixtures. They must be removed from the environment to prevent any possible risk to human health.

The process, by which organic substances are broken down by the enzymes produced by living organisms, is biodegradation. Studies have shown that microbial biodegradation is a major environmental process affecting the fate of PAH in polluted sites. A number of metabolic enzymes have been isolated from microorganisms, which degrade different PAHs (Peng et al., 2008). Haritash and Kaushik (2009) stated that the bacterial enzymes which are responsible for PAH degradation are dioxygenase (a multi component enzyme, which consists of

reductase, ferredoxin and iron-sulfur protein), dehydrogenase and monoxygenase. Fungal enzymes, which are responsible for PAH degradation are monoxygenase and lignolytic enzymes such as lignin peroxidise, manganese peroxidise and laccase. Microbial degradation is an inexpensive and an effective approach to degrade and remove PAH from contaminated soils. In recent years, various microbial species that are effective degraders of hydrocarbons in the natural environment have been identified (Seo et al., 2006a). They have the ability to metabolise various carbon sources such as aliphatic and aromatic compounds. PAH can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae. However, bacteria play a central role in PAH degradation. The driving force for PAH biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs (Haritash & Kaushik, 2009). A possible remedial technology needs microorganisms capable of quick adaptation and efficient use of pollutants of interest in a reasonable period of time (Seo et al., 2009). Microbial community structure has been suggested to be important in PAH biodegradation and is also affected by the presence of PAH (Luo et al., 2009). Leahy and Colwel (1990) reported that different types of biotic and abiotic factors affect ecosystem function. Soil as an ecosystem is affected by abiotic factors such as temperature, availability of nutrients, bioavailability of PAH, moisture content and pH value. The pH has an impact on microbial activity and therefore on biodegradation rate of PAH (Cebron, 2013; Lakshmi et al., 2013).

Chemical oxidation is a rapid and commonly used soil and groundwater remediation technologies, and has proven to be effective for removal of many contaminants such as polycyclic aromatic hydrocarbons (PAHs), petroleum

hydrocarbons and pesticides (Chen et al., 2009). Chemical oxidation has also significant effects on soil properties. Oxidation treatment resulted mainly in breakdown of the soil organic matter component. Hydrogen peroxide (H_2O_2), Fenton's reagent (hydrogen peroxide and ferrous iron), ozone, persulfate ($S_2O_8^{2}$) and permanganate (MnO_4^{-}), are the most commonly used oxidants (Chen et al., 2009 & Silva et al., 2009a).

Leonardi et al. (2008) suggested that applying additives as mobilising agents such as Tween 20 also strongly increased desorption of PAH from soil solid phase to aqueous phase and this influence their degradation. The mass transfer rate of PAH from the solid phase to the aqueous phase is considered as one of the key factors controlling the biodegradation rate. Therefore, the use of mobilising agents as surfactants has been suggested as an appropriate approach to increase microbial degradation of PAH.

In this study, biological and physicochemical methods have been applied to remediate PAH contaminated soils in the environment. The effect of microbial degradation at different pHs, permanganate oxidation and Tween 20 on the rate of PAH degredation in J. Arthur Bower's top soil has been respectively investigated in this study. The J. Arthur Bower's top soil was chosen to ensure constant material during the study. Refer to Table 2.1 in chapter 2 to find out more details about the soil characteristics. However, microbial degradation was found to be the principal method of PAH removal from the environment. Wong et al. (2001) reported that bioremediation by specific microorganisms able to utilise PAH as a sole source of carbon, is a cheap and effectiv method of PAH removal (Lakshmi et al., 2013).

1.2 Different types of chemical pollutants

Chemical pollutants are divided into two main groups: Inorganic such as heavy metals (e.g. zinc, mercury, cadmium, nickel and vanadium etc.), nutrients such as nitrate, nitrite, ammonia and sulfur, and organic pollutants such as pesticides, detergents, petrol, crude oil and polycyclic aromatic hydrocarbons (Bamforth & Singleton, 2005; Hamme et al., 2003; Peng et al., 2008; Straube et al., 2003).

1.2.1 Inorganic pollutants

Inorganic chemical pollutants are naturally found in the environment. The primary inorganic pollutants of concern are metals such as cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn) and inorganic nutrients such as nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3), and phosphate (PO_4^{3-}). These chemicals are often highly toxic to humans and the environment (Defilippis, 1979).

Heavy metal contaminated soils may occur at old landfill sites, old orchards that used insecticides containing arsenic, fungicides containing copper, zinc and iron, fields that are contaminated with waste water or municipal sludge, areas in or around mining waste piles and tailings, industrial areas where chemicals may have been dumped on the ground. Heavy metals may result from synthetic products such as pesticides, paints, and batteries. Heavy metals can enter the cells through ingestion or dermal contact. Microorganisms are affected by binding to cellular ligands such as nucleic acids or proteins. The ligands' structural change, which is caused by metal-ligand binding, leads to loss of normal ligand activity (Defilippis, 1979).

Nutrients are essential to all plant life. They occur naturally in soil, animal waste, plant material, and even the atmosphere or from industries, vehicle exhaust and acid rain. However, an excess of these nutrients can be harmful. Phytoplankton grow rapidly in high amounts of nitrogen and phosphorus, creating dense populations. This reduces the amount of sunlight available to plants. Without light, plants cannot photosynthesize and produce the food they need to survive (Defilippis, 1979).

1.2.2 Organic pollutants

Synthetic organic compounds have been produced by industries for many uses such as plasticizers, lubricants, refrigerants, solvents, pesticides and fuels. Many of these organic compounds are biologically harmful even in very low concentrations. The investigations of Pepper et al., (1996), showed that organic chemicals which are transferred into the soil inhibit or kill the soil organisms. Other chemicals might transfer to water or air from the soil. Therefore it is important to monitor these chemicals in the environment.

Organic pollutants are classified into four main groups (Pepper et al., 1996):

1- Pesticides

2- Aliphatic Hydrocarbons

3- Alicyclic Hydrocarbons

4- Aromatic Hydrocarbons

1.2.2.1 Pesticides

Pesticides are chemicals that include herbicides, insecticides, fungicides, rodenticides and wood preservatives. Pesticides are designed to protect plants, plants products and wood from injurious organisms, and to stop growth of harmful organisms. Pesticides are also used as plant growth regulators, as well as defoliants (used to cause leaves to drop from plants to facilitate harvest) and desiccants, which dry up unwanted plant tops (Crow, 2006).

1.2.2.2 Aliphatic Hydrocarbons

These chemicals have a straight or branched chain structure containing only carbon and hydrogen atoms. Shorter-chain aliphatics have low biodegradation rate as a result of their toxicity to microorganisms. Longer-chain aliphatics are mostly waxy and therefore of lower water solubility which decreases their biodegradation rate (Pepper et al., 1996). Aliphatic Hydrocarbons in the C10-C26 range are the most frequently utilised hydrocarbons in industry. Aliphatic hydrocarbons mostly originate from industrial solvent wastes or the petroleum industry (Venosa & Zhu, 2003).

1.2.2.3 Alicyclic Hydrocarbons

These are a class of compounds containing only carbon and hydrogen atoms joined to form one or more rings and having the properties of both aliphatic and cyclic substances. Alicyclic Hydrocarbons are naturally occurring chemicals

such as crude oil**,** camphor, which is a plant oil; cyclohexyl fatty acids, which are components of microbial lipids; and the paraffin from leaf waxes (Pepper et al., 1996).

1.2.2.4 Aromatic Hydrocarbons

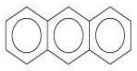
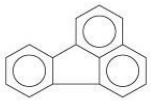
Any organic molecule containing one or more aromatic rings is called an aromatic compound (Seo et al., 2009). Polycyclic aromatic hydrocarbons (PAHs) are allocated in this group (Samanta et al., 2002b).

1.2.2.4a PAH structure and some of their properties

Polycyclic aromatic hydrocarbons are multi-benzene ring chemicals (Straube, et al., 2003) including two or more fused benzene rings (Haritash & Kaushik, 2009; Luo et al., 2009; Peng et al., 2008; Seo et al., 2009). They are nonpolar (Straube, et al., 2003) and hydrophobic (McNally et al., 1998; Yu et al., 2005). Benzene ring arrangements can occur in various structures (Bamforth & Singleton, 2005) such as linear, angular, or cluster shape (Cheung & Kinkle, 2005; Haritash & Kaushik, 2009; Muckian et al., 2007; Peng et al., 2008; Seo et al., 2009). PAH are solid chemicals and are colourless to pale yellow. They have low solubility (Atagana, 2006; Straube et al., 2003; Yu et al., 2005) in water with high melting and boiling points (Pazos et al., 2010). The number of rings and the molecular weight affect the physical and chemical properties of PAH (Seo et al., 2009). The greater the number of fused rings in a PAH chemically stable and hydrophobic is the compound which results in less bioavailability for

the purpose of biodegradation (Kanaly et al., 2000a). By increasing the molecular weight of PAH, aqueous solubility, chemical reactivity and evaporability are decreased (Table 1.2). Therefore, PAH distribution and transportation in the environment will vary. Properties and chemical structures of the four studied PAHs are shown in Table 1.2. (Haritash & Kaushik, 2009; Pazos et al., 2010; Seo et al., 2009; Shafiee, 2006). See Appendix 1-Table 1.1 for properties of other common studied PAH.

Table 1.2 - Properties and chemical structures of the four studied PAHs (modified from Haritash & Kaushik, 2009; Pazos et al., 2010; Seo et al., 2009; Shafiee, 2006)

Chemical	Molecular formula	Chemical structure	Molecular weight ($g.mol^{-1}$)	Water solubility ($mg.dm^{-3}$)	Melting point (◦C)	Boiling point (◦C)
Anthracene	$C_{14}H_{10}$		**178.2**	**1.30**	**218**	**341**
Fluoranthene	$C_{16}H_{10}$		**202.2**	**0.20-0.26**	**111**	**375**
Phenanthrene	$C_{14}H_{10}$		**178.2**	**1.20**	**100**	**340**
Pyrene	$C_{16}H_{10}$		**202.3**	**0.12-0.18**	**156**	**393**

PAH degradation rate depends on the number of benzene rings and the presence or absence of side chains (Haritash & Kaushik, 2009).

1.2.2.4b Sources of PAH

Polycyclic aromatic hydrocarbons occur in sediments (Peng et al., 2008; Yu et al., 2005), groundwater (Muckian et al., 2007), air and soil (Atagana, 2006; Chadhain et al., 2006; Muckian et al., 2007; Peng et al., 2008). PAH originate from two different sources due to incomplete combustion of organic matter: a) natural sources and b) anthropogenic sources (Bamforth & Singleton, 2005; Haritash & Kaushik, 2009; McNally et al., 1998; Yu et al., 2005).

a) Natural sources

PAH generated from natural sources may originate from forest and prairie fires, volcanic eruptions, oil seeps and sediment diagenesis (Bamforth & Singleton, 2005; Chadhain et al., 2006; Haritash & Kaushik, 2009; McNally et al., 1998; Muckian et al., 2007).

b) Anthropogenic sources

PAH generated from human activities such as fossil fuels (coal, diesel and petroleum), wood, garbage, waste incineration, home heating and motor vehicle emissions (Haritash & Kaushik, 2009; Villemin et al., 1994). Wood treatment facilities including preservatives such as creosote (Atagana, 2006; Peng et al.,

2008; Straube et al., 2003) which contains PAH (85 %), phenolic compounds (10 %) and N-, S- and O-heterocyclics (Muckian et al., 2007), fungicides (Straube et al., 2003), tobacco smoke and burnt food are also considered as PAH anthropogenic sources (Cheung & Kinkle, 2005).

1.2.2.4c Persistencey of PAH in the environment

Polycyclic aromatic hydrocarbons are highly resistant molecules that do not break down and can persist in the environment and adsorb onto nonaqueous soil particles (Haritash & Kaushik, 2009; Peng et al., 2008; Silva et al., 2009a; Yu et al., 2005) due to their hydrophobicity (Bamforth & Singleton, 2005; McNally et al., 1998; Mohamed et al., 2012; Silva et al., 2009a; Yu et al., 2005), neutrality and low water solubility which decrease their availability (Atagana, 2006; Bamforth & Singleton, 2005; McNally et al., 1998; Peng et al., 2008; Straube et al., 2003; Villemin et al., 1994; Yu et al., 2005). PAH are stable in soil and are resistant to biological and chemical treatments and are thus more persistent in comparison with other recalcitrant molecules. See Table 1.3 to check half-life of the four PAHs. Biotic and abiotic factors, which affect the persistence of PAH in the environment, are the concentration, molecular structure, water solubility, dispersion and bioavailability of PAH (Seo et al., 2009). For instance, high molecular PAH are more hydrophobic which increases their toxicity and lead to their longer persistence. Soil nutrient availability, pressure, temperature, moisture/water content or humidity, bioavailability and pH are the main environmental factors affecting PAH biodegradation. (Bamforth & Singleton, 2005; Seo et al., 2009).

Table 1.3 - The residence time of the four PAHs in soil (adapted from http://water.epa.gov/polwaste/sediments/cs/upload/Chem-6.pdf; http://www.speclab.com/compound/c3324539.htm)

Chemical	Half-life
Phenanthrene	**16-200 days**
Anthracene	**108-139 days**
Fluoranthene	**5 months-2 years**
Pyrene	**210 days-5.2years**

1.2.2.4d Movement of PAH in the environment

PAHs degrade, translocate and restore in soil and sediment (Table 1.4). Figure 1.2 shows a diagram of the PAH sources and movement in the environment.

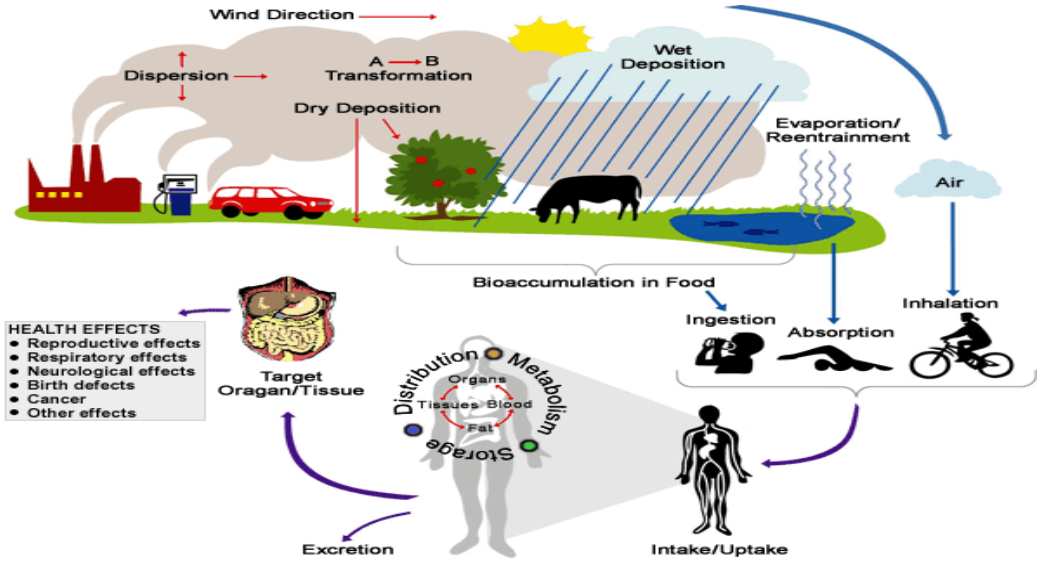


Figure 1.2 - PAH sources and movement in the environment (adapted from Mitch, 2012)

Degradation is the procedure of changing the structure of PAH, such as biological or physicochemical degradation. Translocation is the procedure of

relocating PAH without changing their structure, such as absorption, erosion, leaching and volatilization. Restoration occurs when PAH are transferred from bioavailable pools and stored for long period of time, such as adsorption and diffusion (Pierzynski et al., 2000).

Table 1.4 - Movement of PAH in the environment (adapted from Pierzynski et al., 2000)

Process	Consequence	Factors
Degradation: Altering structure of PAH		
Biological	**Degradation of PAH by microorganisms**	**Nutrient, pressure, temperature, moisture, pH, oxygen, organic matter content, microbial, community present, bioavailability of PAH, structure of PAH, molecular weight of PAH**
Physicochemical	**Conversion of PAH by physicochemical processes**	**Same factors as for biological remediation plus intensity and duration of exposure to sunlight or UV in terms of photo oxidation**
Translocation: Relocating PAH without altering their structure		
Absorption	**Movement of PAH from contaminated soil into plant roots or animal ingestion of the soil, water or vegetation. PAH normally do not transfer into top layer of soil**	**Cell membrane transport, contact time, susceptibility, plant species**
Erosion	**Movement of PAH by water or wind**	**Wind speed, rainfall, size of clay and organic matter particles with adsorbed PAH on them**
Leaching	**Movement of water soluble PAH either laterally or downward through soil**	**Soil water content, soil properties (macropores, texture, clay and organic matter content), rainfall intensity/irrigation**
Volatilization	**Movement of PAH due to evaporation from soil, plant or aquatic ecosystems**	**Intrinsic physiochemical properties of PAH (vapor pressure, solubility, structure), soil properties (moisture, porosity, organic matter and clay), environmental factors (wind speed, temperature, humidity)**
Restoration: Relocating PAH into long-term storage without altering their structure		
Adsorption	**Movement of PAH from bioavailable pools through interaction with soil and sediment**	**Clay and organic matter content, Clay type, moisture**
Diffusion	**Diffusion of PAH into soil micropores where it is unavailable for microbial degradation**	**Hydrophobic nature of micropores and PAH**

1.2.2.4e Environmental effect of PAH on human health

McNally et al. (1998) have suggested that interest has surrounded the occurrence and distribution of polycyclic aromatic hydrocarbons for many decades due to their potentially harmful effects on human health (Figure 1.2). This concern has prompted researchers to find the ways to detoxify or remove them from the environment. PAH enter the body when ingested or via the epidermis. Researches show that the toxicity of PAH can have serious effects on human health (Chadhain et al., 2006; Haritash & Kaushik, 2009). They are carcinogenic and mutagenic (Atagana, 2006; Balachandran et al. 2012; Muckian et al., 2007; Peng et al., 2008; Silva et al., 2009a). PAH have also teratogenic effects on humans (Brown et al., 2003; Luo et al., 2009). Haritash and Kaushik (2009) reported that Benzo(a)pyrene is the most carcinogenic and toxic chemical of petrochemical waste.

The carcinogenic effect of PAH on cells occurs upon oxidation by cytochrome P450 monooxygenase enzyme (Figure 1.3). Epoxides and diol-epoxides are two intermediate products of the reaction by monooxygenase enzyme (Straif et al., 2005).

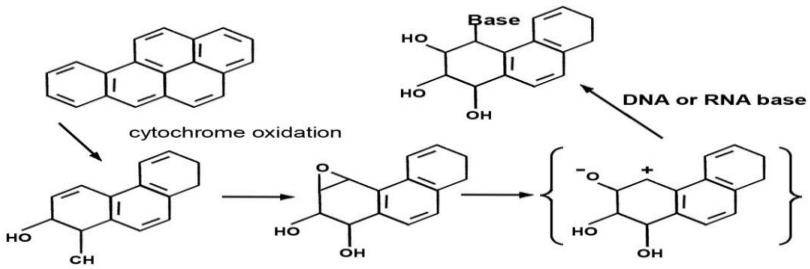


Figure 1.3 - Metabolism of benzo(a)pyrene by cytochrome P450 (Villemin et al., 1994)

The products of the reaction attack DNA to form adduct resulting in mutation. This leads to lung or skin tumours (Straif et al., 2005). They are reported to have impacts on habitats such as marine ecosystems and get into the marine food chain (Straif et al., 2005).

1.3 Remediation

Remediation is defined as the action of providing a remedy, especially prevention or halting damage to the environment (Oxford English Dictionary, 1989). Hence, environmental remediation deals with the removal of contaminants from the environment such as soil, groundwater, sediment, or surface water for the general protection of human health (Oxford English Dictionary, 1989).

1. 3.1 Different remediation methods

Researchers have summarised remediation methods (Appendix 1-Table 1.2) for removing pollutants from contaminated sites. These methods occur either *in situ* or *ex situ* (Hamme et al., 2003; Straube et al., 2003). This study was carried out in laboratory. However the results would be used in the field.

1.3.1.1 *In situ* remediation

In *in situ* remediation methods no excavation is needed and the pollutant remediation is undertaken within the site without excavation or transfer to another place. Land farming (Appendix 1-Table 1.2) in which physical mixing is applied in order to distribute the pollutants over a greater surface area in soil and consequently increase contact between microbes and the pollutant is an *in situ* remediation (Straube et al., 2003). Straube and his colleagues (2003) carried out research on PAH contaminated soil from preservatives. Within their experiment water, ground rice hull (as a bulking agent) and pelletised dried blood (as a nitrogen source) was added and oxygen was provided by tilling the soil. *Pseudomonas aeruginosa* was inoculated as a microbial inoculant. Over a year 86 % of the PAH were removed from the initial concentration of 13,000 $mg.kg^{-1}$ at the start of the year (Straube et al., 2003).

1.3.1.2 *Ex situ* remediation

In *ex situ* remediation excavation is needed which makes it comparatively expensive. *Ex situ* remediation can be on site or off-site. In off-site *ex situ* remediation, the pollutant is transferred to another place. The remediated soil or water may be returned to its origin (Brackney et al., 1997). This method is commonly applied to dissolved contamination via pumping and treatment in above ground bioreactors. Soils are treated above ground via composting (e.g. the addition of straw, compost, manure, etc.). The advantage of *ex situ* approaches is the control over the system (Bamforth & Singleton, 2005; Brackney et al., 1997; Haritash & Kaushik, 2009). This process is good for the

remediation of polluted sites such as soils, sediments or sludges with recalcitrant contaminants for instance polycyclic aromatic hydrocarbons (Robles-Gonzalez et al., 2008).

1.3.2 Bioremediation including biodegradation

Environment-friendly (Haritash & Kaushik, 2009) technology in which microorganisms are utilised to degrade the environmental contaminants into less toxic forms is defined as bioremediation (Andreoni & Gianfreda, 2007; Kazuya, 2001; Lakshmi et al., 2013; Robles-Gonzalez et al., 2008). Bioremediation which is also called bioreclamation and biorestoration, is an approach that has been used to remediate contaminated land and water, and promotes the natural attenuation of the contaminants using the naturally occurring microbial community of the site (Bamforth & Singleton, 2005). It aims to remove PAH compounds from the environment quickly and effectively. The principal process for their removal is biodegradation (Luo et al., 2012; Lakshmi et al., 2013). Bioremediation is a process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state (Mueller et al., 1990). The purpose of bioremediation is to mineralize the organic pollutants into carbon dioxide and water, which are harmless metabolites (Seo et al., 2009). Bioremediation of PAH contaminated soils, sediments and water can be accomplished in a various ways, e.g. *in situ* treatment or *ex situ* methods (Bamforth & Singleton, 2005). Bioremediation provides a technique for cleaning up pollution by enhancing the same biodegradation processes that occur in nature. Bioremediation may be safer and less expensive than alternative

solutions such as landfilling or incineration of the contaminated materials. It also has the advantage of treating the contamination in place so that large quantities of soil, sediment or water do not have to be dug up or pumped out of the ground for treatment (Gillespie & Philp, 2013).

1.3.2.1 Bioremediation strategies

Studies have shown that much research has been done to understand bioremediation technologies for removing pollutants. This method relies on either biostimulation or bioaugmentation (Bamforth & Singleton, 2005; Chadhain et al., 2006; Haritash & Kaushik, 2009; Straube et al., 2003; Yu et al., 2003). "Biostimulation" is an addition of nutrients where microorganisms are available but are limited by lack of nutrients (Bamforth & Singleton, 2005; Chadhain et al., 2006; Silva-Castro, 2013; Straube et al., 2003; Yu et al., 2003). "Bioaugmentation" is an addition of natural or genetically engineered (Haritash & Kaushik, 2009) microorganisms where microorganisms are lacking (Bamforth & Singleton, 2005; Chadhain et al., 2006; Haritash & Kaushik, 2009; Lakshmi et al., 2013; Straube et al., 2003; Yu et al., 2003). In environments such as mangrove sediments biostimulation is highly important as nutrients are often limiting in those areas (Yu et al., 2003).

Furthermore, the naturally occurring degradation process, "Natural attenuation", in which indigenous microorganisms degrade contaminants, has the advantage of avoiding damage to the natural habitats. However due to low populations of the indigenous degrading microorganisms this process may take a long time to complete (Yu et al., 2003). Studies by Atagana, (2006) showed that

the combination of both strategies resulted in 100 % removal of all the PAH with the initial concentration between 100 to 210 $mg.kg^{-1}$ during 10 weeks of incubation.

1.3.2.2 Factors affecting the bioremediation of PAH

The biodegradation of hydrocarbons depends on the nature and amount of the hydrocarbon present, environmental conditions and the activity of the microbial community (Haritash & Kaushik, 2009; Leahy & Colwel, 1990). Many physical and chemical factors (Margesin & Schinner 2001) determine the rate of PAH biodegradability. These environmental factors can be controlled under laboratory conditions whilst in the natural environment they are less controllable (Leahy & Colwel, 1990; Hamme et al., 2003). These environmental factors include: soil nutrients availability, pressure, temperature, moisture/water content or humidity, bioavailability and pH (Andreoni & Gianfreda, 2007; Bamforth & Singleton, 2005; Carter et al., 2010; Lakshmi et al., 2013; Peng et al., 2008; Straube et al., 2003).

1.3.2.2a Nutrient availability

Nutrient sources are categorised as organic (including carbon) and inorganic (mineral) sources. Inorganic sources are divided into two groups: Macronutrients and micronutrients. Macronutrients such as nitrogen, phosphorus, potassium, hydrogen or oxygen are essential for cellular metabolisms in microorganisms and consequently effect on their growth (Bamforth & Singleton, 2005; Lakshmi

et al., 2013; Leahy & Colwel, 1990). Micronutrients such as zinc, manganese, iron, nickel, cobalt, molybdenum, copper, chlorine are required in a very low quantity (Brady & Weil, 1999; Breedveld & Sparrevik, 2000).

Sites contaminated with hydrocarbons such as PAH are high in hydrocarbon concentration and hence inorganic nutrients can rapidly become depleted (Bamforth & Singleton, 2005). The ratios of Carbon/Nitrogen or Carbon/Phosphorus will therefore be high in these sites, which limits microbial biodegradation. This ratio is an important determining factor of biodegradation rates. Adjustments can occur via different mechanisms such as urea-phosphate or ammonium or phosphate salt addition as well as N-P-K fertilisers (Breedveld & Sparrevik, 2000; Carter et al., 2010; Fulthorpe & Wyndham, 1989; Leahy & Colwel, 1990). According to Bamforth and Singleton (2005) a Carbon/Nitrogen/Phosphorus ratio of between 100:15:3 and 120:10:1 leads to optimal microbial growth. However a study has shown that in the soils contaminated with creosote there was no difference in microbial population between Carbon/Nitrogen ratios of 25:1 and 5:1 with the lower level of nutrient, but surprisingly there was no increase in microbial growth with the higher ratio of Carbon/Nitrogen. The level of nutrient required for optimal PAH transformation and hence optimal microbial growth is not yet investigated (Bamforth & Singleton, 2005).

1.3.2.2b Pressure

Deep sea (Margesin & Schinner, 2001), deep ground water, deep sediments and deep oil fields are all under high pressure (Margesin & Schinner, 2001). Studies regarding the effect of pressure on biodegradation of PAH are limited to the deep-sea environment. Investigations have shown samples taken from Atlantic Ocean at 4940 metre depth include microorganisms capable of utilizing PAH. It has been shown that the pressure of 500 atm with the ambient temperature of 20°C significantly increased microbial biodegradation of an in *situ* remediation comparing to the pressure of 1 atm at 20°C (Schwarz et al., 1974). Microorganisms, which require higher pressure than atmospheric to grow, are named barophiles (Piezophiles). Little has been investigated about their ability to degrade hydrocarbons under high pressure (Margesin & Schinner, 2001). It has been reported that microbial degradation of oil, which penetrated to the deep marine environments, is quite slow as high pressure and low temperature prevent microbial activity (Margesin & Schinner, 2001).

1.3.2.2c Temperature

Temperature varies in different seasons of the year. At high temperatures solubility, bioavailability, hydrocarbons distribution and diffusion rate increase, which enhance the biodegradation rate (Leahy & Colwel, 1990; Margesin & Schinner, 2001). Microbial biodegradation increases because of high enzymatic activity at high temperatures (Atlas, 1981). However, high temperature decreases oxygen solubility, which leads to lower aerobic microbial biodegradation rate (Atlas, 1981; Margesin & Schinner, 2001) as well as

affecting the biodegradation of petroleum by changing chemical and physical oil composition (Lakshmi et al., 2013; Leahy & Colwel, 1990). Microorganisms adapted to high temperatures (45˚C to 122˚C), middle temperatures (20˚C to 45˚C) and cold temperatures (20˚C or less) are called 'thermophilic', 'mesophilic' and 'psychrotrophics', respectively. (Klug & Markovetz, 1967). Siron and his colleagues (1995) reported degradation of naphthalene and phenanthrene from crude oil in deep waters at the temperature of 0°C as well as enzymes activity (laccase and manganese peroxidase) of ligninolytic fungi at a temperature between 50°C to 75°C with over 90 % PAH removal.

1.3.2.2d Moisture/water content or humidity

The composition of an organic resource in soil is influenced by several edaphic parameters, including bioavailable moisture (Carter et al., 2010). Soil moisture is expressed in percentage by weight of water in the soil. It is correlated to soil particle size and organic matter (Yuandong et al., 2006). It ranges between 0.0 (complete dryness) to 100 % (complete saturation) in soil, however water content is constant in aquatic ecosystems (Bossert & Bartha, 1984). Normal soil humidity is between 60 to 80 % of its total water capacity (Bossert & Bartha, 1984; Yuandong et al., 2006). In normal fields it varies between 60-80 % of its total capacity. Organisms need over 25-28 % of water-holding capacity. Less than 25 % and over 80 % soil moisture content cause dryness or oxygen depletion, respectively (Yuandong et al., 2006).

1.3.2.2e Bioavailability of PAH

Bioavailability is a dynamic process (Peng et al., 2008) and one of the most important factors in bioremediation, which is determined by the rate of substrate mass transfer into microbial cells (Cebron, 2013; Mueller et al., 1996; Peng et al., 2008). PAH are considered as hydrophobic compounds (Semple et al., 2003) with low water solubility and low accessibility to microorganisms (Miller & Bartha, 1989), which may be described as low bioavailability (Straube et al., 2003). These compounds are resistant to breakdown and they are persistent in the environment due to their high molecular weight and low water solubility (Semple et al., 2003). Furthermore, PAH are very quickly absorbed (Figure 1.4) on the soil matrix (Semple et al., 2003; Haritash & Kaushik, 2009). Those, which have a longer contact with a soil matrix, have greater sorption and consequently the extractability of the contaminant is lower (Hatzinger & Alexander, 1995).

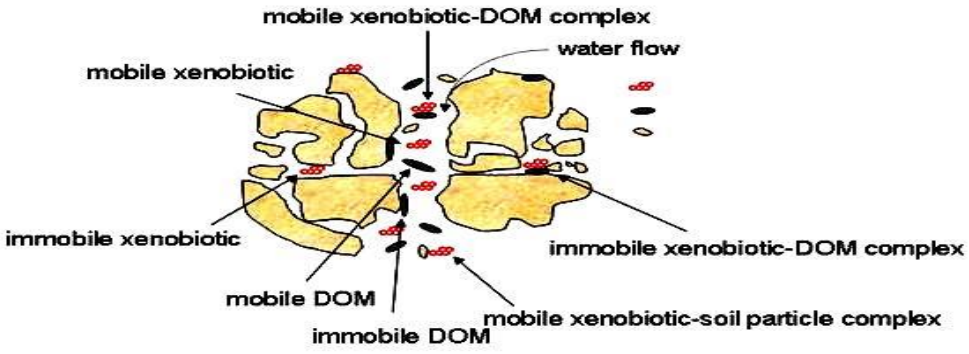


Figure 1.4 - Model of the interactions between xenobiotics and soil matrix. DOM: Dissolved Organic Matter (adapted from Burauel, 2012)

There is high bioavailability of PAH in the soil aqueous phase. However, PAH, which are in interaction with nonaqueous soil phase are less bioavailable

for microorganisms (Andreoni & Gianfreda, 2007). There is low bioavailability in soil nonaqueous phase due to PAH interaction with soil matrix (Figure 1.4). PAH may be released from the soil matrix by using surfactants or detergents as compounds, which contain both a hydrophobic and hydrophilic moiety. They provide a ‘bridge’ between the hydrophobic PAH molecules and hydrophilic microbial cells (Makkar et al., 2003). Emulsan, rhamnolipid, sophorolipids and peptidolipid are biosurfactants (Hamme et al., 2003), which are produced by microorganisms to increase PAH, desorption from the soil particles (Makkar et al., 2003).

1.3.2.2f pH

Soil pH is variable between 2.5 (in mine spoils) to 11.0 (in alkaline deserts and tailings). However pH varies less in aquatic environments (Leahy & Colwell, 1990). Although fungi tolerate acidic conditions for growing, most bacteria and fungi capable of degrading PAH require a neutral pH (Al-Daher et al., 1998; Leahy & Colwell, 1990; Margesin & Schinner, 2001). The group of microorganisms that are metabolically active in environments with low pH values are named "acidophilic microorganisms" and those, which have optimal growth rate at pH above neutrality, are called "alkaliphilic microorganisms". Microbial activity is influenced by extremely low or high pH and hence may result in low hydrocarbon degradation (Leahy & Colwell, 1990). The soil pH may need to be adjusted for biodegradation as not all microorganisms are capable of degrading PAH in extreme acidic or alkaline conditions (Bamforth & Singleton, 2005; Margesin & Schinner, 2001). Bamforth and Singleton (2005)

has reported that 40 % of phenanthrene in a liquid culture at pH 5.5 was degraded by *Burkholderia cocovenenas*. However the degradation at neutral pH in a same conditions was 80 %. Moreover, *Sphingomonas paucimobilis* (strain BA 2) growth was inhibited by the media pH at the value of 5.2 comparing to neutral pH. It is suggested by Bamforth and Singleton (2005) that *Pseudomonas* species are capable of degrading PAH contaminated concrete with a high pH. Although *Pseudomonas fredrikbergen* (DSM 13022) and *Pseudomonas fluorescens* (DSM 6506) were not able to grow at high pH, some *Pseudomonas* species existed in liquid culture contaminated with naphthalene and reduced the pH of soil from 9.0 to 6.5 within 24 hours. This shows some microorganisms tolerate extreme conditions in soil as well as degrading PAH at high pH.

Leahy and Colwell (1990) reported that microbial degradation of naphthalene and octadecane is decreased in sediment environments such as salt marshes with pH 5.0 and it was raised when the pH increased to 6.5 and 8.0. Leaching of demolition wastes such as brick and concrete in gasworks sites enhances soil pH which is not an optimal environmental condition for microorganisms while leaching of coal spoil by oxidation of sulphides will decrease the soil pH (Bamforth & Singleton, 2005). Biodegradation of naphthalene, phenanthrene and anthracene in a soil contaminated with coal spoil at pH 2.0 was evaluated over 28 days. PAH removal was 50 % for naphthalene and between 10 to 20 % for phenanthrene and anthracene (Bamforth & Singleton, 2005). Biodegradation of PAH proceeded well in aquifers with natural pH of 4.5 to 5.0 or in petroleum hydrocarbon contaminated soils from oil spillage with acidic pH of 4 to 6.0. However studies showed anthropogenic activities that had shifted pH from neutral caused lower biodegradation rates (Margesin & Schinner, 2001).

1.3.2.2g Soil microbial community

Environmental pollution caused by the release of a wide range of industrial compounds is now serious (Jain et al., 2005a). Hazardous waste sites occur worldwide resulting in accumulation of xenobiotics in soil and water (Jain et al., 2005a). These environments typically contain a variety of different PAH degrading microorganisms with different metabolic pathways and substrate ranges. Bacteria and fungi are capable of degrading PAH partially or completely. In bioremediation technology metabolic diversity of microorganisms is used to degrade hazardous pollutants (Seo et al., 2009). Like all living creatures, microorganisms need nutrients, carbon, and energy to survive and multiply. Such organisms are capable of breaking down organic contaminants (Figure 1.5) to obtain nutrients and energy, typically degrading them into simple organic compounds, carbon dioxide, water, salts, and other harmless substances (Seo et al., 2009). Refer to Figure 1.6 for the details of the metabolism pathway.

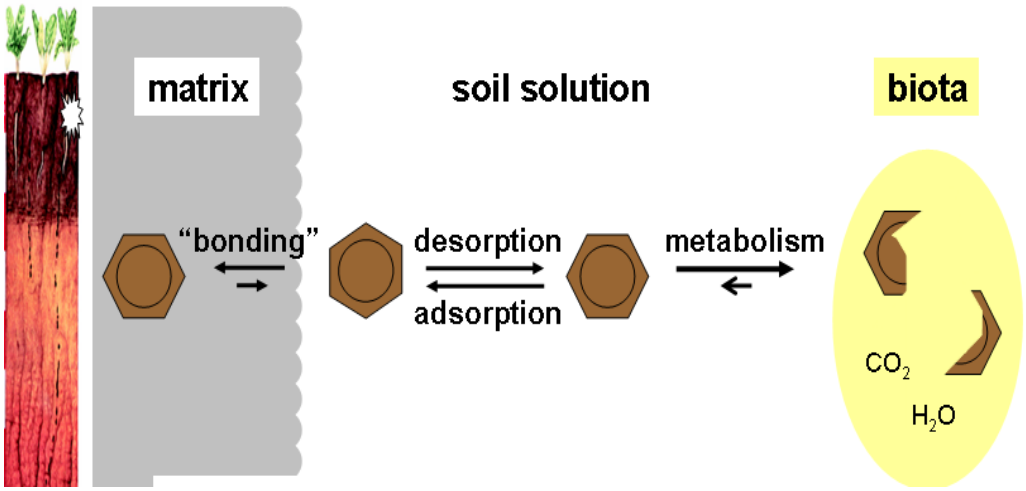


Figure 1.5 - Interaction between microorganisms and PAH in soil solution (adapted from Burauel, 2012)

Haritash and Kaushik (2009) suggested that the slow rate of contaminant desorption from the soil matrix resulted in a slower degradation rate. The presence of heavy metals in soil could also prevent microbial growth and hence limited the metabolism of contaminants under anaerobic conditions (Bamforth & Singleton, 2005). *Sphingomonas paucimobilis* strain EPA 505 (Table 1.5) degraded 5 % of benzo(a)pyrene after 168 hours. Benzo(a)pyrene was also degraded with eleven isolated bacterial strains such as *Pseudomonas, Mycobacterium, Rodococcus, Agrobacterium and Bacillus* spp. (Table 1.5) in refineries or oil, motor oil, wood treatment contaminated sites. The concentration of benzo(k)fluoranthene did not reduce during degradation in freshwater sediments. However the concentration of phenanthrene and fluoranthene remaining was 6 % (Haritash & Kaushik, 2009). Naphthalene and phenanthrene are readily degraded by microorganisms in soil (Peng et al., 2008). Phenanthrene was 100 % degraded by *Pseudomonas aeroginosa* (Table 1.5) in a stream polluted by petroleum refinery after 30 days, whilst 78 % was degraded by isolated bacteria from mangrove sediments. Haritash and Kaushik (2009) isolated *Rhodotorula glutinis* and *Pseudomonas aeruginosa* from a PAH contaminated stream which were able to degrade phenanthrene. Fluoranthene was degraded by *Mycobacterium flavescens* and *Rhodococcus* spp. (Table 1.5) in the sediments of River Grand Calumet and 9-fluorenone-1-1-carboxylic acid was produced as a metabolic product (Haritash & Kaushik, 2009). It is suggested that *Sphingomonas* (Table 1.5) has the ability to degrade pyrene as a high molecular weight PAH (Peng et al., 2008) and 60 % of pyrene with the initial concentration of 0.5 $mg.ml^{-1}$ was degraded by *Mycobacterium* spp. strain KR2

(Table 1.5) after eight days in soil of gaswork plant contaminated with PAH (Haritash & Kaushik, 2009).

Studies showed that a petrochemical waste disposal site contaminated with phenanthrene, anthracene, fluorene, pyrene and acenaphthene contains microorganisms such as *Pseudomonas fluorescens* (Table 1.5) and *Haemophilus* spp. which resulted in PAH degradation of 70 to 100 % over 40 days. Furthermore, 98 % degradation was observed in soil polluted with PAH was caused by microbial activity of the genera *Acenitobacter* and *Klebsiella* after six months (Haritash & Kaushik, 2009). Fungi such as basidiomycetes, white-rot fungi, mitosporic fungi, *Phanerochaete chrysosporium* and *Phanerochaete laevis* are known to be active in the degradation of phenanthrene, anthracene, fluorene and pyrene (Peng et al., 2008). The mycelia of many fungi species have been utilised to increase the extent of PAH biodegradation in soil. The studies of Haritash and Kaushik (2009), also proposed that low molecular weight PAH are degraded by fungi, such as *Aspergillus* spp., *Trichocladium canadense* and *Fusarium oxysporum*. Furthermore, high molecular weights PAH were degraded by *Aspergillus* spp., *Trichocladium canadense*, *Achremonium* spp. and *Verticillium.*

Microbial biodegradation of contaminated sites links to availability as well as physicochemical properties of pollutant (Haritash & Kaushik, 2009). A comprehensive listing of bacteria capable of PAH degradation is shown in Table 1.5 (Hamme, et al. 2003; Haritash & Kaushik, 2009; Juhasz et al., 2000; Seo et al., 2009; Sinha et al., 2000).

Table 1.5 - Bacteria capable of degrading aromatic compounds (Hamme, et al. 2003; Haritash & Kaushik, 2009; Juhasz et al., 2000; Seo et al., 2009; Sinha et al., 2009)

Species	Strains	PAH
Achromobacter sp.	NCW	Carbozole
Alcaligenes denitrificans		Fluoranthene
Arthrobacter sp.	F101	Fluorene
Arthrobacter sp.	P1-1	Carbozole, Phenanthrene
Arthrobacter sulphureus	RKJ4	Phenanthrene
Acidovorax delafieldii	P4-1	Phenanthrene
Bacillus cereus	P21	Pyrene
Brevibacterium sp.	HL4	Phenanthrene
Burkholderia sp.	S3702, RP007,2A-12TNFYE-5,	Phenanthrene
Burkholderia sp.	BS3770	Phenanthrene
Burkholderia cepacia	C3	Naphthalene, phenanthrene,
	BU-3	pyrene
Burkholderiacocovenenans		Phenanthrene
Burkholderia xenovorans	LB400	Benzoate, biphenyl
Chryseobacterium sp.	NCY	Carbozole
Cycloclasticus sp.	P1	Pyrene
Desulfomonile tiedjei		Pyrene
Desulfovibrio sp.	G11	Pyrene
Janibacter sp.	YY-1	Dibenzofuran, fluorene, phenanthrene, anthracene, dibenzo-*p*-dioxin
Marinobacter	NCE312	Naphthalene
Mycobacterium sp.		Pyrene, benzo(a)pyrene
Mycobacterium sp.	JS14	Fluoranthene
Mycobacterium sp.	6PY1, KR2, AP1	Pyrene
Mycobacterium sp.	RJGII-135	Pyrene, benzo(a)anthracene, benzo(a)pyrene
Mycobacterium sp.	PYR-1, LB501T	Fluoranthene, pyrene, phenanthrene, anthracene
Mycobacterium sp.	CH1, BG1, BB1, KR20	Phenanthrene, fluorene, fluoranthene, pyrene
Mycobacterium flavescens		Pyrene, fluoranthene
Mycobacterium vanbaalenii	PYR-1	Phenanthrene, pyrene, dimethylbenz(a)anthracene
Mycobacterium sp.	KMS	
Nocardioides aromaticivorans	IC177	Carbozole
Nocardioides sp.	KP7	Phenanthrene

Species	Strains	PAH
***Pasteurella* sp.**	**IFA**	**Fluoranthene**
Polaromonas naphthalenivorans	**CJ2**	**Naphthalene**
***Pseudomonas* sp.**	**C18, PP2, DLC-P11**	**Naphthalene, phenanthrene**
***Pseudomonas* sp.**	**BT1d**	**3-hydroxy-2-formylbenzothiophene**
***Pseudomonas* sp.**	**B4**	**Biphenyl, chlorobiphenyl**
***Pseudomonas* sp.**	**HH69**	**Dibenzofuran**
***Pseudomonas* sp.**	**CA10**	**Carbozole, chlorinated dibenzo-*p*-dioxin**
***Pseudomonas* sp.**	**NCIB 9816-4**	**Fluorene, dibenzofuran**
***Pseudomonas* sp.**	**F274**	**Fluorene**
***Pseudomonas* sp.**	**U2**	**Naphthalene**
Pseudomonas cepacia	**AC1100**	**Fluoranthene**
Pseudomonas paucimobilis		**Phenanthrene**
Pseudomonas vesicularis	**OUS82**	**Fluorene**
Pseudomonas putida	**P16, BS3701, OUS82, BS3750, BS590-P, BS202-P1**	**Naphthalene, phenanthrene**
Pseudomonas putida	**NCIB9816**	**Naphthalene**
Pseudomonas putida	**C18**	**Naphthalene, phenanthrene**
Pseudomonas putida	**CSV86**	**Methyl naphthalene**
Pseudomonas fluorescens	**BS3760**	**Phenanthrene, chrysene, benzo(a)anthracene**
Pseudomonas stutzeri	**AN10**	**Naphthalene**
Pseudomonas stutzeri	**P15**	**Pyrene**
Pseudomonas saccharophilia		**Pyrene**
Pseudomonas aeruginosa		**Phenanthrene**
***Ralstonia* sp.**	**SBUG 290**	**Dibenzofuran**
	U2	**Naphthalene**
***Rhodanobacter* sp.**	**BPC-1**	**Benzo(a)pyrene**
***Rhodococcus* sp.**		**Pyrene, fluoranthene**
***Rhodococcus* sp.**	**WU-K2R**	**Naphthothiophene, benzothiophene**
***Rhodococcus* sp.**	**124**	**Naphthalene**
Rhodococcus erythropolis	**I-19**	**Alkylated dibenzothiophene**
Rhodococcus erythropolis	**D-1**	**Dibenzothiophene**
***Staphylococcus* sp.**	**PN/Y**	**Phenanthrene**
Stenotrophomonas maltophilia	**VUN 10,010**	**Pyrene, fluoranthene, Benzo(a)pyrene**
Stenotrophomonas maltophilia	**VUN 10,003**	**Pyrene, fluoranthene, Benzo(a)anthracene, benzo(a)pyrene, dibenz[*a, h*]anthracene, coronene**

Species	Strains	PAH
Sphingomonas yanoikuyae	**R1**	**Pyrene**
Sphingomonas yanoikuyae	**JAR02**	**Benzo(a)pyrene**
***Sphingomonas* sp.**	**P2, LB126**	**Fluorene, phenanthrene, fluoranthene, anthracene**
***Sphingomonas* sp.**		**Dibenzofuran, carbozole**
Sphingomonas paucimobilis	**EPA505**	**Fluoranthene, naphthalene, anthracene, phenanthrene**
Sphingomonas wittichii	**RW1**	**Chlorinated dibenzo-*p*-dioxin**
Syntrophobacter wolinii		**Fluoranthene**
Syntrophomonas wolfei		**Fluoranthene**
***Terrabacter* sp.**	**DBF63**	**Dibenzofuran, Chlorinated dibenzothophene, chlorinated dibenzo-*p*-dioxin, fluorene**
***Xanthamonas* sp.**		**Pyrene, benzo(a)pyrene, carbozole**

It is important to compare bioremediation in cost and success to physical and chemical treatments of contaminated environments, such as sending to landfill, incineration and soil washing. The applicability of bioremediation varies depending on site conditions. Therefore understanding of factors affecting the bioremediation of site conditions will allow optimising bioremediation and therefore more effective results. In commercial situations when the site is highly contaminated with PAH including those of more than four rings, bioremediation is not carried out as the time taken is not economically viable (Bamforth & Singleton, 2005).

Microorganisms which degrade PAH are distributed in both (a) aerobic (e.g. soil, sediment) and (b) anaerobic (e.g. municipal sewage sludge) environments and microbial metabolism of PAH can be accomplished in both conditions (Bamforth & Singleton, 2005; Haritash & Kaushik, 2009). Bacterial enzymes

which are involved in PAH degradation are dioxygenase (a multi component enzyme, consists of reductase, ferredoxin and iron-sulphur protein), dehydrogenase and monoxygenase (Haritash & Kaushik, 2009). Fungal enzymes, which are responsible for PAH degradation are monoxygenase and lignolytic enzymes such as lignin peroxidise, manganese peroxidise and laccase. PAH degrading enzymes are extracellular and have optimum activity in mesophilic temperatures, whilst their activity decreases at high or low temperatures (Haritash & Kaushik, 2009; Peixoto, 2011).

(a) Aerobic metabolism of PAH

Molecular oxygen is required in biodegradation mechanisms to initiate the enzymatic attack of PAHs' rings (Peng et al., 2008). There are two fundamentally different aerobic metabolism pathways of PAHs by microorganisms. Specific details of bacterial and fungal metabolisms of PAH are discussed and described below (Figure 1.6).

The principle mechanism for aerobic bacterial PAH metabolism is the oxidation of the benzene ring by dioxygenase enzyme resulting in the formation of *cis*-dihydrodiols as the early bioproducts (Bamforth & Singleton, 2005; Peng et al., 2008; Samanta et al., 2002). This enzyme inserts oxygen atoms into two carbon atoms of an aromatic ring (Samanta et al., 2002). The dihydrodiols are dehydrogenated to form a dehydroxylated intermediate either by an *ortho*-cleavage pathway or a *meta*-cleavage pathway (Peng et al., 2008; Samanta et al., 2002), which can further be metabolised via catechols to carbon dioxide and

water (Bamforth & Singleton, 2005; Peng et al., 2008). Dioxygenase oxidises arenes of PAHs' substrates to *cis*-dihydrodiols form (Peng et al., 2008).

Fungal PAH metabolism can occur by two different pathways: Ligninolytic and nonligninolytic (Peng et al., 2008). Ligninolitic fungi utilise lignin catabolism including lignin peroxidase, manganese peroxidase and laccases. These enzymes have the advantage of being located in the fungal cell wall, which enables them to diffuse into soil particles and oxidise PAH with low bioavailability. They also act non-specifically during PAH oxidation (Hamme, 2003; Peng et al., 2008). However nonligninolytic fungi use the cytochrome P450 system that involves the cytochrome P450 monooxygenase enzyme (Haritash & Kaushik, 2009; Peng et al., 2008). Cytochrome P450 monooxygenase enzyme has the ability to insert oxygene into PAH compounds (Peng et al., 2008). However the oxidation mechanism of PAH by fungi is similar in both groups (Peng et al., 2008). In the cytochrome P450 system (Figure 1.6), the aromatic ring is oxidised with cytochrome P450 monooxygenase to produce an arene oxide (Peng et al., 2008; Sutherland et al., 1995). Mammalian metabolism of PAH has the same route. The monooxygenase enzyme inserts only one oxygen atom into the ring to form an arene oxide (Jerina, 1983; Peng et al., 2008). This is then hydrated via an epoxide-hydrolase catalysed reaction to form a *trans*-dihydrodiol (Jerina, 1983). Furthermore, derivatives of phenol may be produced from arene oxides (Mueller et al., 1996). PAH are not toxic for mammals unless they oxidise to epoxides and diol-epoxide via cytochrome P450 monooxygenase enzyme. The final products are however less toxic and more soluble than their parent compounds (Pothuluri et

al., 1992). Aerobic metabolism of PAH is faster, since O_2 is available as an electron acceptor (Peixoto, 2011).

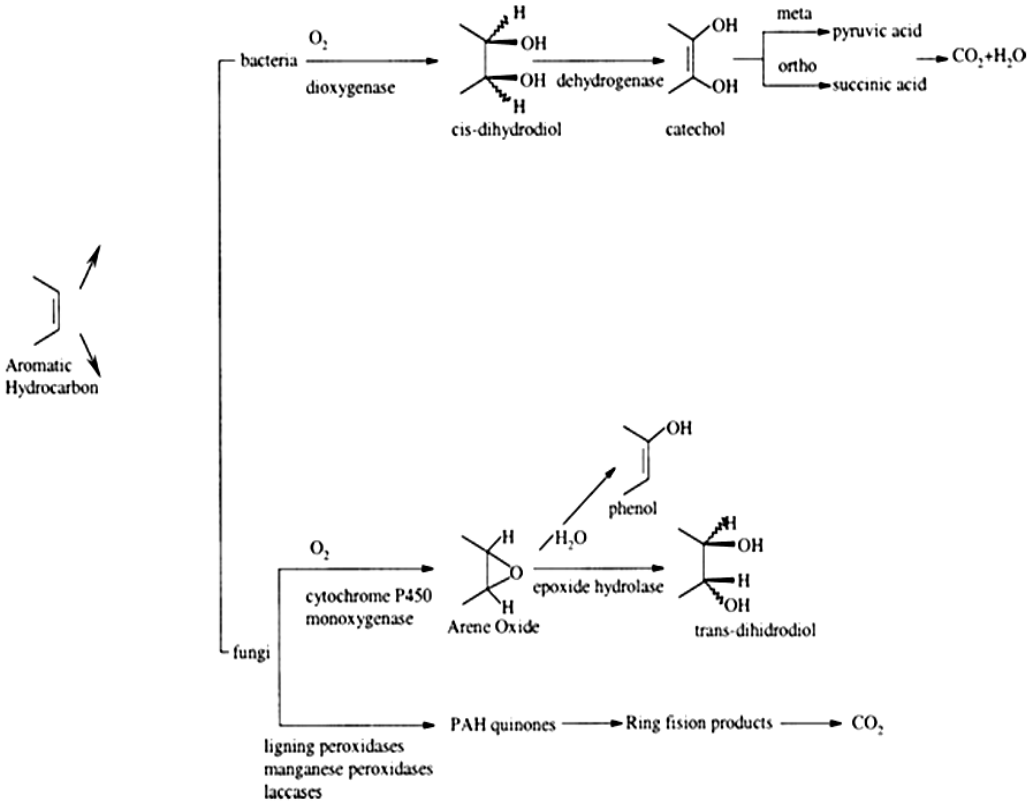


Figure 1.6 - Different PAH metabolism pathways are used by bacteria and fungi (Antizar-Ladislao et al., 2004; Cerniglia, 1992)

(b) Anaerobic metabolism of PAH

PAH can easily be found in anaerobic environments such as aquifers and marine sediments (Anderson & Lovely, 1997; Haritash & Kaushik, 2009). Even aerobic environments such as contaminated soils, sediments and groundwater can develop anaerobic zones (Anderson & Lovely, 1997). Anaerobic zones are created due to available carbon sources stimulating the *in situ* microbial

community, resulting in the depletion of molecular oxygen during aerobic respiration. This molecular oxygen is not replenished at the same rate as its depletion, which results in the formation of anaerobic zones (Bamforth & Singleton, 2005). It was suggested that the degradation rate in anaerobic conditions is slow (Haritash & Kaushik, 2009). Two or three ring PAH are degraded in such conditions. The number of aromatic rings and presence or absence of side chains in PAH molecules effects the rate of degradation (Haritash & Kaushik, 2009). However, the biochemical mechanism has not yet proposed (Bamforth & Singleton, 2005; Haritash & Kaushik, 2009). Anaerobic bacteria such as *Desulfomonile tiedjei*, *Syntrophomonas wolfei*, *Syntrophobacter wolinii* and *Desulfovibrio* spp. strain G11 (Table 1.5) apply the same biodegradation strategy to that of aerobic bacteria (Haritash & Kaushik, 2009). There is a low but an appreciable number of microorganisms capable of degrading PAH in anaerobic conditions (Haritash & Kaushik, 2009). The potential of the microorganisms to degrade PAH in the absence of molecular oxygen has been recognised. In the absence of molecular oxygen, nitrate, ferrous iron and sulphate are used as alternative electron acceptors, which are essential to oxidise these aromatic compounds (Bamforth & Singleton, 2005).

1.4 Chemical oxidation

Chemical oxidation is a rapid and commonly used soil and groundwater remediation technology and has proven to be effective for removal of many contaminants such as polycyclic aromatic hydrocarbons (Chen et al., 2009: Ma et al., 2013). Chemical oxidation also has significant effects on soil properties.

Oxidation treatment results mainly in the breakdown of soil organic matter component. Hydrogen peroxide (H_2O_2), Fenton's reagent (hydrogen peroxide and ferrous iron), ozone, persulfate ($S_2O_8^{2-}$) and permanganate (MnO_4^-), are the most commonly used oxidants (Chen et al., 2009; Doğan et al., 2013; Ma et al., 2013; Silva et al., 2009a). In this thesis specifically the effect of potassium permanganate on the chemical oxidation of PAH in soil at different pHs was investigated.

Potassium permanganate was used to break down inorganic (cyanides, iron and sulfides) and organic (phenol, pesticides and PAH) matter (Silva et al., 2009b). In the presence of permanganate ions, chemical oxidation can occur (Brown et al., 2003). In potassium permanganate oxidation, PAH which are in contact with the soil matrix components are oxidised and their concentration will decrease (Silva et al., 2009b). Permanganate ions quickly oxidise PAH alkene carbon-carbon double bonds (Brown et al., 2003). The chemical oxidation of organic compounds by permanganate ion is shown below. The reaction produces manganese dioxide and carbon dioxide or organic intermediates (Silva et al., 2009b). $R + MnO_4^- \rightarrow MnO_2 + CO_2$, or $ROX + MnO_2$

Brown et al. (2003) indicated that the rate of reduction was significantly variable between specific PAH in soil slurry. The greatest reduction with potassium permanganate (160 mM) was observed for benzo(a)pyrene, pyrene, phenanthrene and anthracene with a reduction of 72.1, 64.2, 56.2 and 53.8 % and minimal reduction in fluoranthene and chrysene at 13.4 and 7.8 %, respectively with the PAH initial concentration of 1.2 $mg.kg^{-1}$ after 30 min. Studies by Ferrarese et al. (2008) showed that the oxidation reactions were frequently rapid

and appear to be completed within few hours. However, in order to assess the total removal efficiency of different reactants including potassium permanganate, the reactions were not quenched and were allowed to continue until the complete consumption of all chemicals before being analysed. The resulting products of chemical oxidation may or may not be more biologically toxic than the original compound (Dabestani & Ivanov, 1999).

1.5 Photo oxidation

Oxidation of molecules caused by the absorption of photons, particularly at wavelengths found in sunlight and ultraviolet light is termed photodegradation. This type of degradation includes the breakup of molecules into smaller fragments by photons or the change of a molecule's shape to make it permanently altered, such as protein denaturation and the addition of other atoms or molecules. Photo degradation is usually an oxidation process. This term is generally used in the oxidation of pollutants by UV-based processes. Photocatalytic oxidation is one of the many developed oxidation processes, relies on the production of $^{\bullet}OH$ by photocatalysts (e.g. titanium dioxide) to prompt oxidative degradation (Woo et al., 2009).

1.6 The efficacy of mobilising agents

The level and rate of biodegradation of contaminated soils is often restricted by PAH solubility, sorption to particles, slow transfer from organic to aqueous phase, and usually low aqueous PAH concentrations unable to maintain

biodegradation (Allan et al., 2007; Giubilei et al., 2009; Yang et al., 2000). Consequently, microbial degradation is reliant on the ability of microorganisms to transfer and degrade contaminants as well as the available concentration of compounds capable of sustaining degradation. In this respect, mobilising agents have been suggested to raise the release and microbial accessibility of PAH in soil (Allan et al., 2007). Mobilising agents are frequently used in treatment technologies to remediate soils, sediments and wastes contaminated with PAH (Yang et al., 2000). Mobilising agents are organic compounds that are amphiphilic, containing both hydrophobic or water insoluble groups such as a hydrocarbon tail and hydrophilic or water soluble groups such as a head. Examples include Tween 20, Tween 80, soybean oil, olive-oil mill waste waters and randomly methylated ß-cyclodextrins (Leonardi et al., 2008). Therefore, mobilising agents are expected to increase desorption rates of PAH from the solid matrix and so improve their solubility in aqueous phase. These agents mobilise polyvalent metal ions, particularly Fe and Al from the soil. Metal ion chelation may disrupt humic-(metal ion)-mineral linkages, re- sulting in mobilization of soil organic matter and accompanying PAH molecules into the aqueous phase; and/or reduce the degree of cross-linking in the soil organic matter phase, which could accelerate PAH diffusion (Yang et al., 2000). Soil organic matter has a more or less flexible structure that allows PAH compound to partition within its inner matrix. Diffusion through the soil organic matter phase probably contributes to the slow desorption of PAH. The results of this study show that PAH compound desorption can be increased in the presence of mobilising agents accompanied by considerable release of organic matter into solution (Yang et al., 2000).

Deschenes et al. (1995) carried out an experiment where soil was inoculated with creosote. In a mobilisation experiment, the soil was treated with sodium dodecyl sulphate (0.005 to 1 w/v), and in a biodegradation experiment the soil was treated with an increased amount of sodium dodecyl sulphate (10,100 and 500 mg.kg $^{-1}$). These studies showed that sodium dodecyl sulphate effected the movement of 3 and 4 benzene ring PAHs and that increasing the amount of sodium dodecyl sulphate effected the movement of 5 and 6 benzene ring PAHs. However, in the biodegradation experiment even though sodium dodecyl sulphate significantly increased the movement of PAHs, but did not affect on their breakdown. Studies suggested that even though sodium dodecyl sulphate improves the mobilisation of PAHs in the soil aqueous phase, but due to destruction of microbial cell membrane it does not affect on PAHs biodegradation (Deschenes et al., 1995). The work of Yang et al. (2000) shows that raising the soil pH up to 8, effects the availability of PAH. It highly effects PAH degradation by deprotonating and charging the acidic groups in soil humic acids, therefore more PAH diffuse into the aqueous phase.

The mass transfer rate of PAH from solid phase to aqueous phase is considered as one of the key factors controlling the biodegradation rate. As a result, the use of mobilising agents as surfactants has been suggested as an appropriate approach to increase microbial degradation of PAH (Leonardi et al., 2008). However, apart from soybean oil, olive-oil and randomly methylated ß-cyclodextrins there has not yet been adequate research to examine the influence of Tween 20 and 80 separately to enhance the mobilisation of PAH contaminated soil.

Rationale

The literature showed broad research on the effect of different biotic and abiotic factors on degradation of PAH in soil (Andreoni & Gianfreda, 2007; Balachandran et al. 2012; Carter et al., 2010; Haritash & Kaushik, 2009; Peng et al., 2008; Straube et al., 2003). The hypothesis for the third chapter was that microorganisms in roadside soil would contain PAH degrading bacteria (Johnsen & Karlson, 2005) and that these can be isolated, identified and used as inoculum through all experiments. Little research was found on the optimum pH for the microbial degradation of the four PAH in the soil. The hypothesis for the fourth chapter was that pH would influence the microbial degradation of PAH in the soil. This study aimed to monitor the rate of degradation at a range of pHs in order to find the optimum pH for PAH degradation in an experimentally prepared soil. Furthermore, some literature examined the effect of potassium permanganate on oxidation of PAH in the absence of microorganisms (Chen et al., 2009; Ferrarese et al. 2008; Silva et al., 2009b). The hypothesis for the fifth chapter was to compare the effect of potassium permanganate oxidation of PAH with microbial degradation. The work reported in this thesis aimed to examine the optimisation of PAH degradation by using potassium permanganate in the presence and absence of microorganisms and the effect of potassium permanganate on bacterial populations in the soil. In addition, there was little research reported on the effect of mobilising agents on degradation of PAH, which had been suggested to raise the release and microbial accessibility of PAH in soil (Allan et al., 2007; Leonardi et al., 2008). The hypothesis for the sixt chapter was to compare the effect of Tween 20 with microbial degradation of

PAH. This thesis examined the effect of Tween 20 only on translocation and microbial degradation of the four PAHs in the soil.

Chapter 2

General Materials and Methods

2.1 Characteristics and preparation of the J. Arthur Bower's top soil for all experiments

A commercially and manufactured available product, J. Arthur Bower's top soil containing 10 % sand, 75 % silt and 15 % clay was used (Table 2.1). The soil contains quality natural English loam blended with organic matter and nutrients. The nutrient analysis (N and P) of soil was carried on using Palintest Kit. Soil organic matter was measured by heating 5 g of soil in foil container at 180 ºC for 48 hours. This soil was selected to provide constant soil characteristics throughout this study. A steel tray was filled with J. Arthur Bower's top soil then covered with aluminum foil and dried in an oven at 90 ^{0}C for two days. Dried soil was sieved through 1 mm metal sieve. Soil was then sterilised by autoclaving on two consecutive days (15 min, 15 psi, 121 ºC). The sterilising process was checked by making a dilution series followed by spread plates and enumerating bacteria colonies. No colonies were found which confirmed the sterilising process.

2.1.1 pH of the soil

The pH of J. Arthur Bower's top soil was confirmed by taking 5.0 g of the soil diluted with 10 cm^3 distilled water in a 50 cm^3 centrifuge tube. The suspension was vortexed for two minutes and left at 20 ^{0}C room temperature for 30 minutes. The pH of the supernatant was measured with pH probe and pH 7.0 was recorded (Kissel et al., 2010).

2.1.2 Percentage water-holding capacity of the soil

100 g of the J. Arthur Bower's top soil was taken and then saturated with Milli-Q water. The soil was filtered, using 25 cm Whatman filter paper (no. 6) in a funnel. The wet filter paper was weighed first and after 15 minutes, the soil and filter paper were weighed again and the weight of a wet filter paper was subtracted. The water-holding capacity was then calculated (Appendix 3.1, 4.1 and 5.1) (Hagood et al., 2012). The soil moisture content for all experiments was then adjusted to 30 % of the water-holding capacity by adding different volumes of Milli-Q water (Appendix 4-Tables 4.2, Appendix 5-Table 5.4 and Appendix 6-Table 6.3).

Table 2.1 - Summary of J. Arthur Bower's soil characteristics (Robertson, 2011)

Content	J. Arthur Bower's top soil
Sand, silt and clay (%)	10, 75 and 15
OM (%)	7.19
pH	7.00
Percentage WHC (%)	41.75 and 49.67 in two different replicates
N (NO_2^-) $mg.dm^{-3}$	0.60
N (NO_3^-) $mg.dm^{-3}$	0.50
N (NH_3) $mg.dm^{-3}$	0.40
P (PO_4^-) $mg.dm^{-3}$	0.10

2.1.3 Preparation of stock solution containing the four PAHs and method of contaminating the soil

The four PAHs used for soil contamination were phenanthrene, anthracene, fluoranthene and pyrene all purchased from Sigma, Steinheim, Germany. The

PAH solutions were prepared by adding 50 mg of each phenanthrene, anthracene, fluoranthene and pyrene to a volumetric flask and then made up to 500 cm^3 with n-hexane HPLC grade (Sigma). This produced a stock solution of four PAHs with a concentration each of 100 $mg.dm^{-3}$. This solution was used to contaminate the soil. The experimental containers for the soil and PAH were mixed manually for 20 minutes to ensure equal distribution of the PAH in the soil. The soil container was weighed and placed under a fume hood for 48 hours to allow n-hexane to evaporate (Sirguey et al., 2008). The container weight was checked frequently until it reached pre-contamination level. The same method but different concentrations and volumes were used during each experiment.

2.1.4 pH adjustment of the soil

The pH of the soil was adjusted in each chapter to monitor the degradation process at varying pH. The natural pH of the J. Arthur Bower's top soil was 7.0. Therefore, different volumes of 1 M hydrogen chloride (HCl) as an acid and 0.1 M sodium carbonate (Na_2CO_3) solution as a base were added to reduce and increase the pH, respectively. This resulted in soils of different pHs (Appendix 4-Table 4.4 and Appendix 5-Table 5.3).

2.2 Characteristics and preparation of the roadside soil

The roadside soil as a source of potential PAH degrading microorganisms was collected from the entrance of the University of Hertfordshire, College Lane campus (AL10 9AB) in a beaker using a metal trowel, air-dried for 48 hours and

sieved through 1 mm metal sterilised sieve. The nutrient analysis (N and P) of soil was carried on using Palintest Kit. Soil organic matter was measured by heating 5 g of soil in foil container at 180 ºC for 48 hours. The HPLC method was applied to check the existence of the PAH in the roadside soil (Appendix 2-Figure 2.1). Different amounts of the roadside soil were added to the experimental containers during each experiment to ensure the presence of microorganisms, which are likely to degrade PAH produced by vehicles' combustion engines (Johnsen & Karlson, 2005). This is the experimental soil used throughout the study. Twenty distinct bacteria genera including *Achromobacter* spp., *Sphingobacterium* spp., *Brevibacterium* spp., *Burkholderiales* spp., *Tetrathiobacter* spp., *Arthrobacter* spp., *Bacillus* spp., *Erwinia* spp., *Pseudomonas* spp., *Rhizobium* spp., *Stenotrophomonas* spp. and *Ochrobactrum* spp. were isolated in this study as the PAH biodegraders in the roadside soil. Refer to chapter 3 (Table 3.4) for methods and results of this identification.

Table 2.2 - Summary of the roadside soil characteristics

Content		**Roadside soil**
Sand, silt and clay (%)		**15, 70, 10**
OM	**(%)**	**9.18**
pH		**8.76**
N (NO_2^-)	**mg.dm^{-3}**	**0.50**
N (NO_3^-)	**mg.dm^{-3}**	**0.09**
N (NH_3)	**mg.dm^{-3}**	**0.70**
P (PO_4^-)	**mg.dm^{-3}**	**0.70**

2.3 Bacterial enumeration after inoculation with the roadside soil during experiment

The total bacteria extracted from the soil were enumerated via dilution series at varying time points to monitor the bacterial populations during degradation or oxidation process. Ringer's solution (Oxoid) was prepared according to the manufacturer's instructions. Universal bottles containing 9 cm^3 of Ringer's solution were prepared and sterilised by autoclaving. 1 g of the soil was taken by sterile spatula and added to 9.0 cm^3 of sterile Ringer's solution. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made up by adding 1 cm^3 of 10^{-n} dilution and add to 9 cm^3 of autoclaved (15 min, 15 psi, 121 ºC) Ringer's solution to make up $10^{-(n+1)}$ dilution. Nutrient agar (Oxoid) was prepared according to manufactures instructions (Appendix 2.3 and Appendix 2-Table 2.2). Spread plates of dilutions 10^{-4} and 10^{-5} were prepared by adding 100 µl of each dilution to the Petri dish and spreading with a sterile glass spreader. Plates were incubated for 48 hours at 24 ºC and all colonies were counted after 48 hours (Asakawa & Hayano, 1995).

2.4 PAH quantification using HPLC

A Dionex P680 High performance liquid chromatography (HPLC) pump fitted with sequential 4 µm particle size silicon columns (C16 and C18) of 35 cm length was used (Appendix 2-Table 2.1). A degassed 90:10 acetonitrile: Milli-Q water mobile phase was utilised (Igwo-Ezikpe et al., 2010; Shafiee et al., 2006). The flow rate was set isocratically at 0.8 cm^3/min. A UV absorbance detector (UVD 170 U) set at 252 nm (Shafiee, 2006) connected to a PC equipped with Chromeleon chromatography software version 6.6 which was used to quantify

the four PAHs. 0.5 g of the soil was removed from experimental containers by sterile spatula and transferred into 2 cm^3 Micro Centrifuge tubes containing carbozole/extraction solution (section 2.6.2). All the samples were capped to prevent evaporation. The standard samples (20 µl of 0.1 ml) were injected into the HPLC machine by using a Terumo I ml syringe. In the chapter 3, 4 and 5 (experiment ii, oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil), percentage remaining (Boonchan et al., 2000; Brown et al., 2002; Ferrarese et al., 2008; Wong, 2001; Zhou et al., 2008) and in the chapter 5 (experiment i, oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate) concentration remaining (Shafiee et al., 2006; Silva et al., 2009a) of the four PAHs in the soil were calculated using the internal standard as a correction factor. The concentration of PAH extracted at time 0 was considered as 100 %. The percentage remaining at all other time points was recorded as a percentage of the PAH recovered at time 0. Thus, the percentage remaining graphs took into account the efficiency of the extraction. The mean values were calculated for all replicates and standard deviation quantified. Table 2.4 turns percentage remaining to $mg.kg^{-1}$ of soil. See Appendix 2.1 and 2.2 for the HPLC analysis method description.

2.4.1 Preparation of carbozole/extraction solution for HPLC

Carbozole solution as a standard with the concentration of 100 $mg.dm^{-3}$ was prepared by adding 20 mg of carbozole (Sigma) to a volumetric flask and made up to 200 cm^3 with acetonitrile (Sigma). 1.5 cm^3 of carbozole/extraction solution

was then added into 0.5 g of prepared soil in 2 cm^3 Micro Centrifuge tubes. Micro Centrifuge tubes were vortexed at 3 rpm using a round table vortex fitted with multi sample holder, which holds a total of 12 samples (Sigma) for 15 minutes and then centrifuged for another 15 minutes. The solid in the Micro Centrifuge tubes were allowed to sediment and the supernatant was transferred into new Micro Centrifuge tubes prior to HPLC analysis. Refer to Table 2.3 for the extraction efficiency of PAH and actual PAH remaining in soil in presence and absence of Tween 20 on day 20 in experiment i (translocation of PAH in the soil) in chapter 6 as an example.

Table 2.3 - The extraction efficiency of PAH

PAH	Initial concentration of PAH exist in soil at T_0 ($mg.kg^{-1}$)	PAH extracted at T_0 ($mg.kg^{-1}$)	Extraction efficiency (%)	Actual PAH remaining in soil treated with Tween 20 at T_{20} ($m.kg^{-1}$)	Actual PAH remaining in soil with no Tween 20 at T_{20} ($mg.kg^{-1}$)
Phenanthrene	**166.66**	**158.39**	**95.03**	**23.18**	**5.10**
Anthracene	**166.66**	**166.44**	**86.31**	**30.68**	**3.49**
Fluoranthene	**166.66**	**137.86**	**82.71**	**20.23**	**4.34**
Pyrene	**166.66**	**128.92**	**77.35**	**16.45**	**3.74**

2.4.2 Preparation of standard solution for the standard curve for HPLC

PAH standard stock solution was prepared. The four PAHs were dissolved in acetonitrile. Varying volumes of stock solution were added along with carbozole dissolved in acetonitrile as the internal standard. The volume was made up to 10 or 20 cm^3 resulting in varying concentrations of PAH based on the potential

highest concentration that could be extracted from the samples. Refer to Table 2.4 for the method of converting percentage remaining (%) of PAH in soil into concentration remaining ($mg.kg^{-1}$) by taking into account the PAH extraction efficiency.

Table 2.4 - Converting percentage remaining (%) of PAH in soil into concentration remaining ($mg.kg^{-1}$) by taking into account the extraction efficiency

Percentage remaining (%) of PAH in soil	Concentration remaining ($mg.kg^{-1}$) of PAH in soil			
	Phenanthrene	Anthracene	Fluoranthene	Pyrene
100	71.40	61.31	59.21	48.12
90	62.26	55.17	53.28	43.30
80	57.12	49.04	47.36	38.49
70	49.28	42.91	41.44	33.68
60	42.84	36.78	35.52	28.87
50	35.7	30.65	29.60	24.06
40	28.56	24.52	23.68	19.24
30	21.42	18.39	17.76	14.43
20	14.28	12.26	11.84	9.62
10	7.14	6.13	5.92	4.81

***Extraction efficiency of phenanthrene, anthracene, fluoranthene and pyrene are 95.03, 86.31, 82.71 and 77.35, respectively.**

2.4.3 Preparation of mobile phase for HPLC

Fresh degassed mobile phase of 90 % acetonitrile and 10 % Milli-Q water was prepared daily to run HPLC samples (Igwo-Ezikpe et al., 2010; Shafiee et al., 2006).

2.5 Preparation of potassium permanganate (0.09 M) solution for the chapter 5

The potassium permanganate was obtained from Fisher Scientific. 5.68 g potassium permanganate was dissolved into 400 cm^3 of sterile deionised water (Brown et al., 2003). See Appendix 5-Table 5.4.

2.6 Preparation of sodium bisulfite (0.09 M) solution for the chapter 5

The sodium bisulfite was obtained from Fisher Scientific. 3.74 g sodium bisulfite was dissolved into 400 cm^3 of sterile deionised water (Brown et al., 2003). See Appendix 5-Table 5.6.

2.7 Preparation of Tween 20 solution for the chapter 6

2.5 % Tween 20 solution was prepared by adding 1.5 cm^3 of Tween 20 into 8.94 cm^3 of distilled water and kept for autoclave (Leonardi et al., 2008). See Appendix 6-Table 6.2.

2.8 Statistical analysis

Graphs were plotted in Microsoft Office Excel 2007. The graphed values are represented as mean with standard deviation. Data analysis was carried out using SPSS Statistic software version 20. One-way ANOVA was used. Post-hoc tests

including LSD (Least Significant Difference) and Tukey's HSD (Honest Significant Difference) were applied to analyse the variance between treated and untreated (control) samples, across different time points and pHs (Appendix 7).

Chapter 3

Selection, Isolation and Identification of PAH Biodegrader Bacteria in the soil used as inoculum

3.1 Introduction

A wide range of bacteria occurs in the soil. A number of bacteria have been found to degrade PAH. Few of them have been used in bioremediation (Lease et al., 2011). The hypothesis for this chapter was that roadside soil would contain PAH degrading bacteria (Johnsen & Karlson, 2005) and that these can be isolated, identified and used through all experiments as inoculum. Therefore, the aims were collecting the roadside soil from an area with heavy traffic, which containing PAH degrading bacteria and isolating and identifying these bacteria. Table 3.1 shows the objectives for this chapter. The selective media containing the four PAHs and the roadside soil was prepared in an attempt to isolate the bacteria from the soil. Isolated microorganisms were identified through biochemical and molecular identification tests.

Table 3.1 - Objectives of chapter 3

1	**Selection of the PAH biodegrader bacteria in the roadside soil**
2	**Isolation of the PAH biodegrader bacteria in the roadside soil**
3	**Identification of the PAH biodegrader bacteria in the roadside soil**

3.2 Materials and methods

See Table 3.2 for the experimental plan.

Table 3. 2 - Experimental layout

- **Capping 15 empty conical flasks with aluminum foil and keeping it for autoclave**
- **Checking weight of flasks**
- **Adding PAH stock solution to first five flasks**
- **Evaporating n-hexane under a fume hood (48 hours)**
- **Checking weight of flasks**
- **Preparing MSM media**
- **Dividing 95 cm^3 of MSM media into the first five flasks**
- **Adding 5 cm^3 of nystatin into each flask**
- **Autoclaving the flasks containing MSM media**
- **Inoculating the first five flasks with roadside soil (5 g) for 14 days**
- **Incubating the flasks (30 °C/24 hours) in a shaker incubator**
- **Taking 5 cm^3 of turbid medium from first five and transferring into new second five flasks containing PAH + MSM media**
- **Taking 5 cm^3 of turbid medium from second five and transferring into new third five flasks containing PAH + MSM media**
- **Transferring the liquid content of each flasks into five centrifuge tubes**
- **Centrifuging the tubes (4,000 rpm/20 min)**
- **Preparing 15 MSM plates solidified with 2% agar, spread from each PAH on surface and kept for n-hexane evaporation**
- **Spreading the supernatant of each centrifuge tubes on each of plates**
- **Incubating MSM plates (20 °C/48 hours)**
- **Identification of colonies on the plates using biochemistry and molecular tests**

Fifteen empty conical flasks were prepared and capped with aluminum foil and autoclaved (15 min, 15 psi, 121 ºC). The stock solution of the four PAHs was prepared with a final concentration of 100 $mg.kg^{-1}$ for the each individual PAH (section 2.2). The conical flasks were weighed before use and then 20 cm^3 of the PAH stock solution was added as sole carbon source into the first five conical flasks (first selective media). The flasks were left under the fume hood for 48 hours to allow n-hexane to evaporate and the weight of flasks was checked to ensure the original weight was achieved.

A minimal salt medium (MSM) was prepared (Appendix 3.1) and autoclaved. The media was then autoclaved and divided into the first five conical flasks (first selective media) each containing 95 cm^3 of the media. 5 cm^3 of nystatin was added as a fungal growth inhibitor (Riccardi et al., 2005). The roadside soil was

collected as described in section 2.3. The soil was kept in an incubator at 30 °C for 24 hours to increase the number of bacteria. 5 g of the soil was inoculated into the each flask. The flasks were incubated in a shaking incubator in the dark at 30 °C for 14 days until they turned turbid. 5 cm^3 of the supernatant was taken from the first five conical flasks and transferred into the second five new autoclaved conical flasks (second selective media). Subsequently, 5 cm^3 of the supernatant was taken from the second set of conical flasks and transferred into the third set of five new autoclaved conical flasks (third selective media). Then the media was transferred into five centrifuge tubes and centrifuged at 4,000 rpm for 20 min. Fifteen MSM plates which had been solidified with 2 % agar were coated with the four PAHs by spreading PAH dissolved in n-hexane using sterile glass spreader and allowing the solvent to evaporate at 20 ^{0}C for an hour (Abd-Elsalam, 2009; Bastiaens, 2000; Lease, 2011). 100 µl of the flasks supernatant was spread on each of the plates. The plates were incubated at 20 °C for 48 hours. The colonies that formed on these plates were selected based on morphological differences by sterilised inoculating needle and transferred onto nutrient agar plates and incubated at 20 °C for 48 hours. The isolates were then identified by biochemical and molecular tests.

3.2.1 Biochemical tests

Five main biochemical tests were performed on isolates, including Gram staining, catalase, oxidase, glucose and O-F test (Cowan & Steel, 2010). See Appendix 3.2 for the method.

3.2.2 Molecular tests

The DNA extraction was performed using bacterial genomic DNA kit (Sigma, GenElute, NA2110-1KT, USA) according to the manufacturer's instructions. Gel electrophoresis (0.8 %) was performed at 100 V for 60 min (Figure 3.3). 16S ribosomal DNA was amplified using the 16S ribosomal DNA universal bacterial primer set (Appendix 6-Table 6.1) 27F, 5`-AGAGTTTGATYMTGGCTCAG-3` and 1492R, 5`-TACGGYTACCTTGTTACGACT-3` , purchased from Invitrogen (Riccardi et al., 2005). PCR was performed on a Hybaid Ltd SPRT001 Issue 2 PCR Sprint machine. Each 50 µl reaction mixture containing 1 µl of each deoxynucleoside triphosphate, 1 µl of each primer, 3 µl $MgCl_2$, 5 µl of 1x reaction buffer (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), 0.2 µl of Taq DNA polymerase (Invitrogen) and 1 µl (12-100 ng) of genomic DNA template (Appendix 3-Table 3.2). Negative controls consisted of an equal volume of nuclease-free water in place of the DNA template. Thermal cycling consisted of an initial denaturation at 95 ^{0}C for 10 min to allow activation of the Taq polymerase, followed by 30 cycles of denaturation at 94 ^{0}C for 30 sec, annealing at 50 ^{0}C for 1 min and extension at 72 ^{0}C for 2 min. A final extension at 72 ^{0}C for 10 min was then performed (Table 3.5). Gel electrophoresis (1.5 %) was performed at 100 V for 60 min to isolate PCR products (Figure 3.4). The isolates were then subjected to a purification step by using PCR clean up kit (Sigma, GenElute, NA21020-1KT, USA) according to the manufacturer's instructions in an attempt to purify the DNA from contaminating nucleic acids or salts. Subsequently, gel electrophoresis (1.5 %) was performed at 100 V for 60 min (Figure 3.5). The absorbance at various wavelengths of 230, 260, 280 and 320 nm was measured

to estimate the purity and concentration of the genomic DNA (Appendix 3-Table 3.3). The samples were then sent to MWG the genomic company (London) for sequencing.

Table 3.3 - Standard PCR cycle conditions

Step	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	**95**	**10**	
Denaturation	**94**	**30 sec**	**30**
Annealing	**50**	**1**	
Extension	**72**	**2**	
Final extension	**72**	**10**	

3.3 Results

Table 3.4 shows PAH degrading microorganisms identified by biochemical methods. *Brevibacterium* spp., *Arthrobacter* spp. and *Bacillus* spp. were found Gram positive, non-spore forming, rod shaped, catalase positive, oxidase, glucose negative and did not react on carbohydrate, whilst only *Bacillus* spp. was spore-forming, *Arthrobacter* spp. either rod or spherical shaped, *Brevibacterium* spp. glucose positive and *Bacillus* spp. was found oxidative positive . *Achromobacter* spp., *Sphingobacterium* spp., *Burkholderiales* spp., *Tetrathiobacter* spp., *Erwinia* spp., *Pseudomonas* spp., *Rhizobium* spp., *Stenotrophomo* spp. and *Ochrobactrum* spp. were found Gram negative, non-spore forming, rod shaped, catalase positive, oxidase positive, glucose negative, whilst only *Tetrathiobacter* spp. found spherical, *Stenotrophomo* spp. oxidase negative, *Tetrathiobacter* spp. and *Rhizobium* spp. glucose positive.

Achromobacter spp., *Sphingobacterium* spp. and *Burkholderiales* spp. did not react on carbohydrate, whilst *Tetrathiobacter* spp., *Pseudomonas* spp. and *Ochrobactrum* spp. were found oxidative. However, *Erwinia* spp. and *Rhizobium* spp. were found fermentative. Figures 3.1 and 3.2 shows Gram staining images of the isolated PAH biodegrader bacteria. Images are labelled according to the cultures' numbers in Table 3.4. Twenty out of forty-five isolates were selected for the further biochemical and molecular tests. Remaining twenty-five had the same colony appearance as those which were select

Table 3.4 - Biochemical tests on the PAH biodegrading bacteria isolated from the selective media (Cowan & Steel, 2010)

Isolate	Gram staining	Endospore	Shape	Catalase	Oxidase	Glucose	O-F test	Genus
1	**-ve**	**-**	**R**	**+**	**+**	**-**	**NA**	***Achromobacter***
2	**-ve**	**-**	**R**	**+**	**+**	**-**	**NA**	***Sphingobacterium***
5	**-ve**	**-**	**R**	**+**	**+**	**-**	**NA**	***Sphingobacterium***
6	**+ve**	**-**	**R**	**+**	**-**	**+**	**NA**	***Brevibacterium***
7	**-ve**	**+**	**R**	**+**	**+**	**-**	**NA**	***Burkholderiales***
10	**-ve**	**-**	**S**	**+**	**+**	**+**	**O**	***Tetrathiobacter***
12	**+ve**	**-**	**RS**	**+**	**-**	**-**	**NA**	***Arthrobacter***
13	**+ve**	**+**	**R**	**+**	**-**	**-**	**O**	***Bacillus***
14	**-ve**	**-**	**R**	**+**	**+**	**-**	**F**	***Erwinia***
17	**+ve**	**-**	**RS**	**+**	**-**	**-**	**NA**	***Arthrobacter***
20	**+ve**	**+**	**R**	**+**	**-**	**-**	**O**	***Bacillus***
21	**+ve**	**+**	**R**	**+**	**-**	**-**	**O**	***Bacillus***
25	**-ve**	**-**	**R**	**+**	**+**	**-**	**O**	***Pseudomonas***
27	**-ve**	**-**	**R**	**+**	**+**	**+**	**F**	***Rhizobium***
28	**-ve**	**-**	**R**	**+**	**-**	**-**	**O**	***Stenotrophomo***
29	**-ve**	**-**	**R**	**+**	**+**	**-**	**O**	***Ochrobactrum***
37	**-ve**	**-**	**R**	**+**	**+**	**+**	**F**	***Rhizobium***
42	**-ve**	**-**	**R**	**+**	**+**	**-**	**O**	***Pseudomonas***
44	**-ve**	**-**	**R**	**+**	**+**	**-**	**O**	***Ochrobactrum***
45	**+ve**	**+**	**R**	**+**	**-**	**-**	**O**	***Bacillus***

+ve = 100-80 % strains positive, -ve = 20-0 % strains positive, R = rod (bacilli), S = sphere (cocci), RS = rod shape during exponential growth and spherical in stationary phase, O = oxidation, F = fermentation, NA = no action on carbohydrate

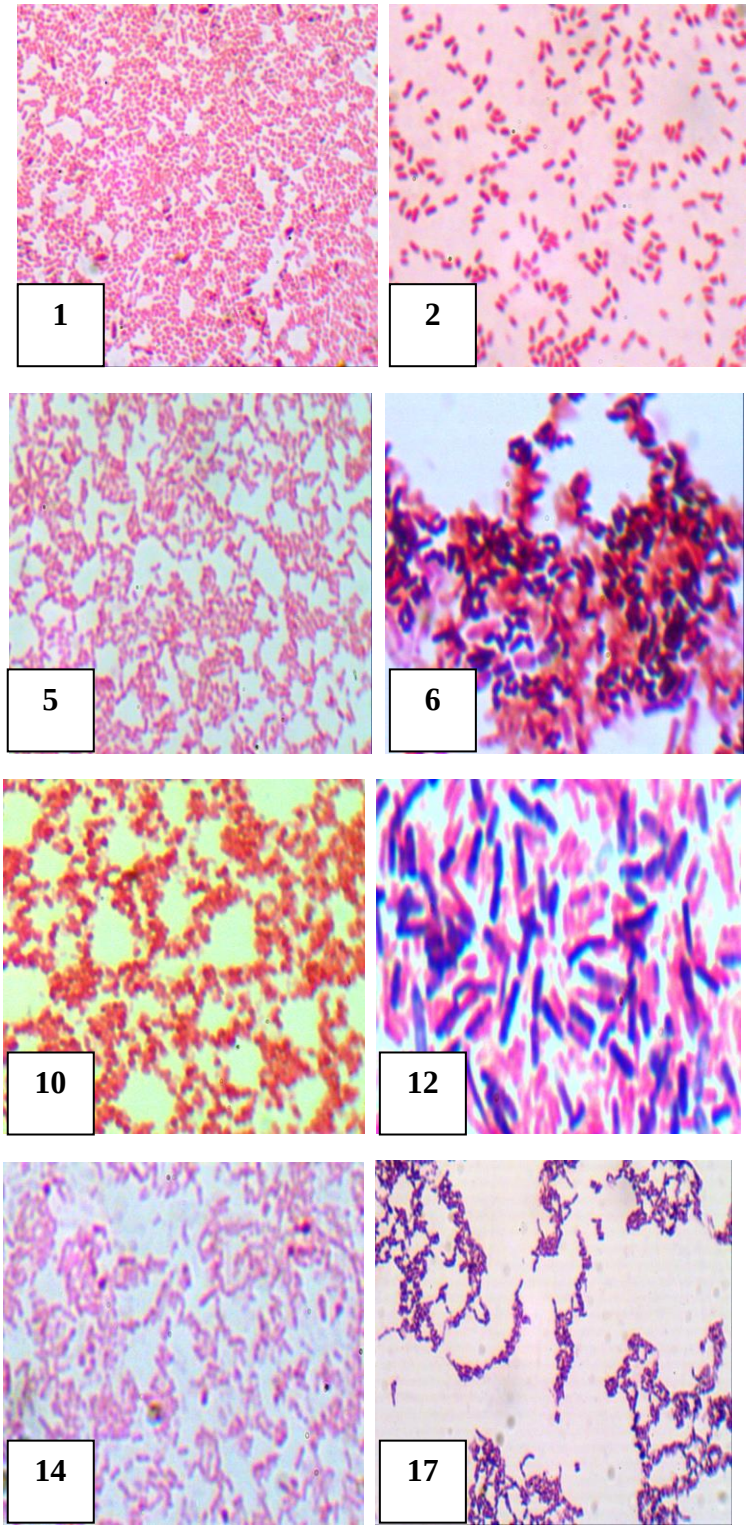


Figure 3.1 - Microscopic images of isolated biodegrader bacteria from the roadside soil; 1-17; Gram stain, magnification X 1,000

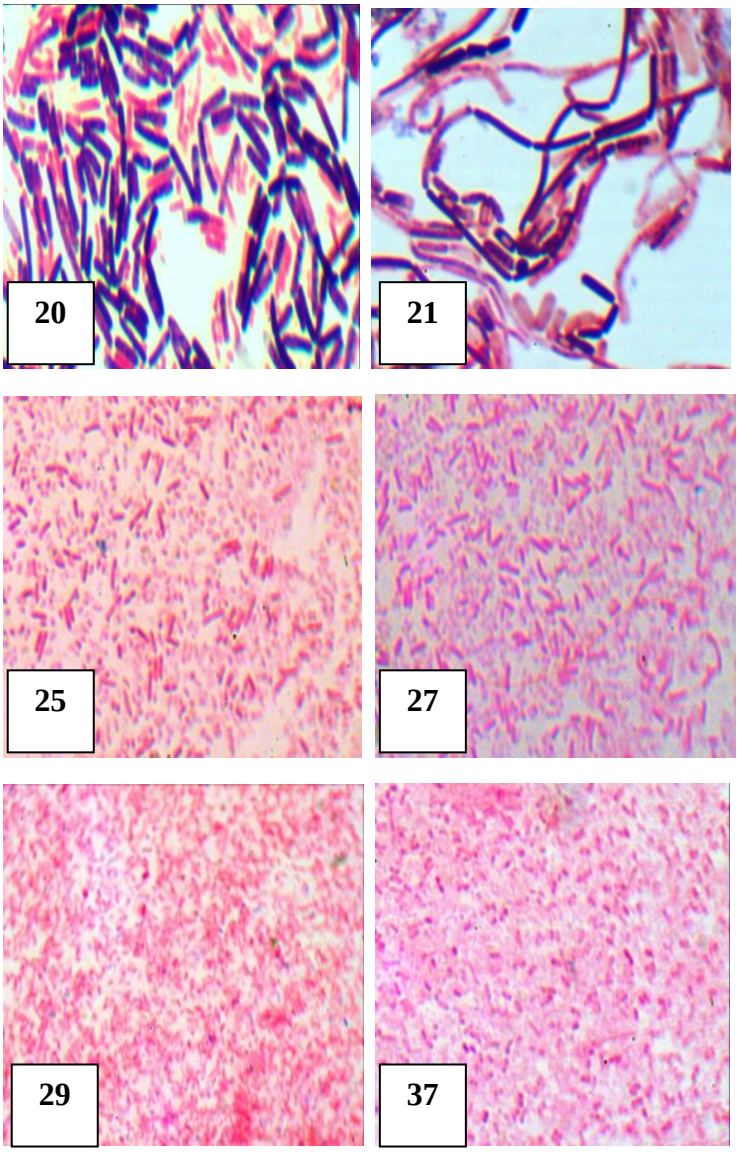


Figure 3.2 - Microscopic images of isolated biodegrader bacteria from the roadside soil; 20-37; Gram stain, magnification X 1,000

All the twenty isolates were subsequently identified by molecular techniques. Figure 3.3 shows genomic DNA extracted from the four PAHs biodegrader bacteria isolated from the roadside soil. The thick bands of high molecular weight indicated the presence of genomic DNA. However, the smear of DNA with low molecular weight showed the presence of sheared genomic DNA in the isolates. Most likely the isolation process had broken up the chromosomes into many pieces. The numbers of the lanes are the numbers of the isolated cultures.

Figure 3.4 shows 16S rDNA gene amplification product of PCR for PAH biodegrader bacteria isolated from the roadside soil. The PCR products (1400 bp) were detected for all the isolates. Comparing Figure 3.4 and 3.5 shows that much of the smears of contaminating nucleic acids (Figure 3.4) were precipitated out and a sharp DNA band of high molecular weight after the purification process has occurred (Figure 3.5).

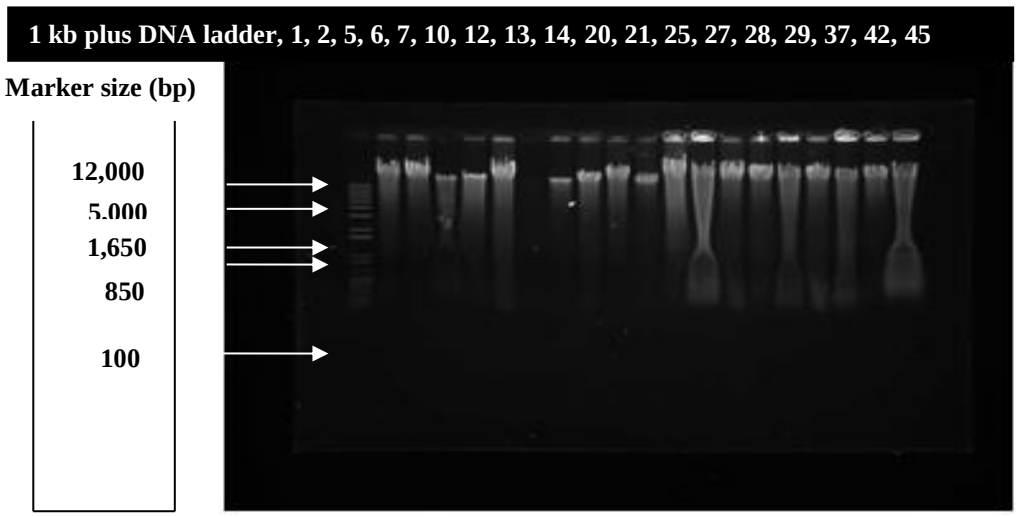


Figure 3.3 - Genomic DNA extracted from the biodegrader bacteria isolated from the roadside soil. Lanes from left to right represent 1 kb plus DNA ladder and the isolates' numbers.

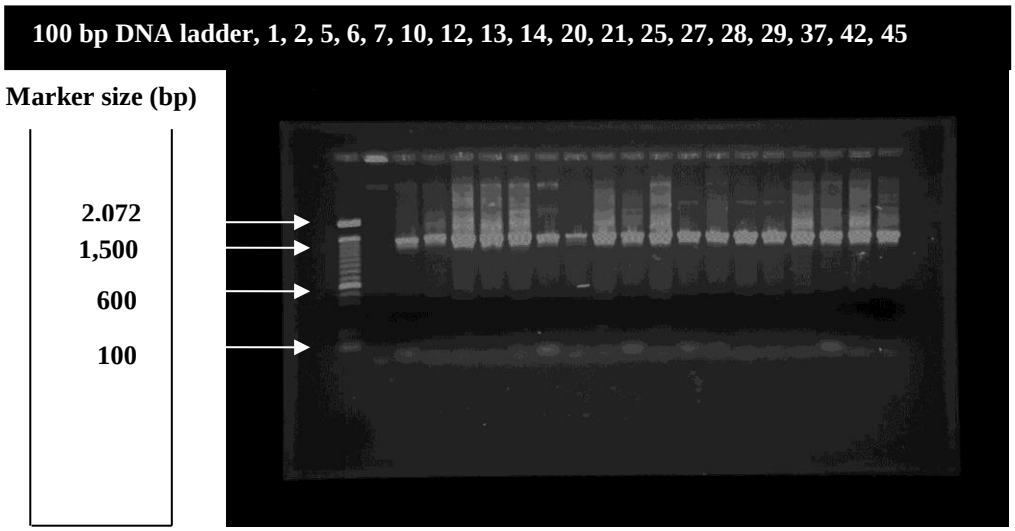


Figure 3.4 - 16S rDNA gene amplification products of PCR (1400 bp) for PAH biodegrader bacteria isolated from the roadside soil. Lanes from left to right represent 100 bp DNA ladder and the isolates' numbers.

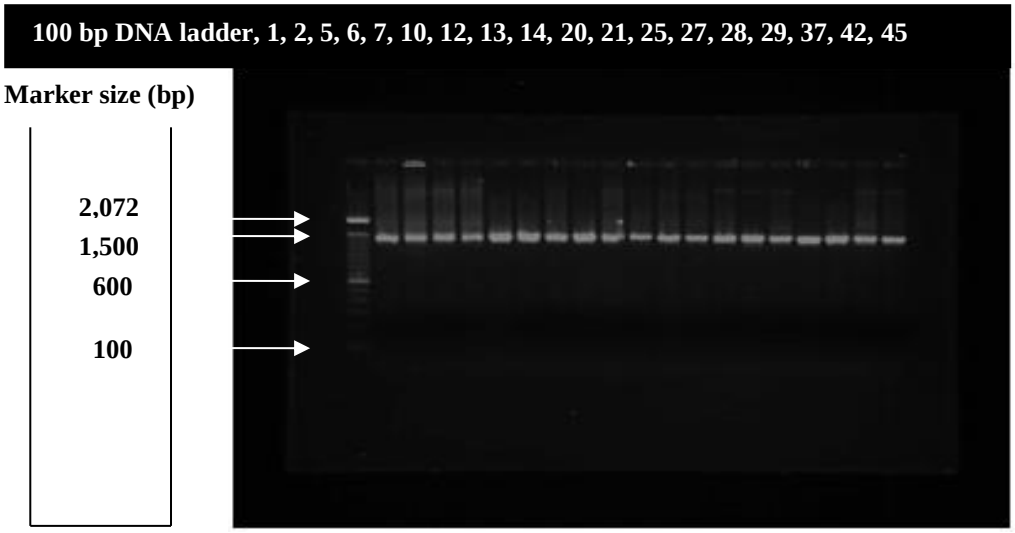


Figure 3.5 - 16S rDNA gene product of PCR (1400 bp) purification for PAH biodegrader bacteria isolated from the roadside soil. Lanes from left to right represent 100 bp DNA ladder and the isolates' numbers.

The successful sequence analysis of *Achromobacter piechaudii* strain TZ4 16S ribosomal RNA gene is shown below as an example and the full sequences of successful sequence analysis are provided in the Appendix 3.1.

TTannatGCaGTcgacgGCAGcAcGGACTTCGGTCTGGTGGCGAGTGGCGAACGGgtgAGTA
ATGTATCGGAACGTGCCTagtAGCGGGGGATAAcTACGCGAAAGCGTAGCTAATACCG
CATACGCCCTACGGGGGAAAGCAGGGGATCGCAAGACCTTGCACTATTAGAGCGGC
CGATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAG
CTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCC
GCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTGGCAGGAAAGAAACGTC
ATGGGCTAATACCCCGTGAAACTGACGGTACCTGCAGAATAAGCACCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGTGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTT
TGGAACTGCATTTTTAACTACCGAGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGC
GTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACACCGATGGCGAagGCAGCCTC
CTGGGATAACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATA
CcCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCTTcngGCCTtnnT
AGCGCancTAACGCGTGAAGTTGACCGCCTGgGGAGTACGGTCGCAAGATTAAnACTC
AaAGGAATTGACggGGACCcgCACAancggTgaaTGATGtggATTaaTTcnaTGcnacnnnnananA
CCTTACcTACCCTtnacaTGTc

Table 3.5 shows the BLAST results for 16S rDNA for the isolates of the roadside soil. The DNA sequences were compared to those in the Genebank database using BLAST analysis. The accession number and the definition of the isolates are described in Table 3.5. These bacterial genera were identified as *Achromobacter* spp., *Sphingobacterium* spp., *Brevibacterium* spp., *Burkholderiale* spp. *s, Tetrathiobacter* spp., *Arthrobacter* spp., *Bacillus* spp., *Erwinia* spp., *Pseudomonas* spp., *Rhizobium* spp., *Stenotrophomonas* spp. and *Ochrobactrum* spp. with identity percentage ranging from 94 % to 100 % except *Ochrobactrum* spp. (88 %). The identification results suggested that most of the

PAH biodegrader bacteria belonged to an aerobic soil bacteria category, and are mostly gram negative.

Table 3.5 - Identification of isolated PAH biodegrader bacteria by sequencing 16S rDNA gene and using BLAST analysis against GenBank database

Isolate and primer used	GenBank accession number	Definition	Max. score	Query coverage (%)	E value	Max. identity (%)
1-27F	GQ92716.1	*Achromobacter piechaudii* strain TZ4 16S ribosomal RNA gene, partial sequence	1664	99	0.0	98
1-1492R	EF550171.1	*Achromobacter piechaudii* strain Shan11 16S ribosomal RNA gene, partial sequence	1890	99	0.0	99
2-27F	FJ816788.1	*Sphingobacterium shayense* strain HS39 16S ribosomal RNA gene, partial sequence	1953	99	0.0	99
2-1492R	FJ816788.1	*Sphingobacterium shayense* strain HS39 16S ribosomal RNA gene, partial sequen	1984	99	0.0	99
5-27F	FJ156081.1	*Sphingobacterium* sp. MOL-1 16S ribosomal RNA gene, partial Sequence	1430	98	0.0	94

Isolate and primer used	GenBank accession number	Definition	Max. score	Query coverage (%)	E value	Max. identity (%)
5-1492R	FJ156081.1	*Sphingobacterium* sp. MOL-1 16S ribosomal RNA gene, partial sequence	326	100	5e-86	96
6-27F	HQ455048.1	*Brevibacterium epidermidis* strain CJ-12 16S ribosomal RNA gene, partial sequence	32.2	100	26	91
6-1492R	Sequencing failure					
7-27F	HE664162.1	*Burkholderiales* sp. B101R-3 partial 16S rRNA gene, strain B101R-3	141	100	7e-31	89
7-1492R	AJXB01000145.1	*Burkholderia thailandensis* MSMB43 Scaffold30_1, whole genome shotgun sequence	75.8	93	1e-10	98
10-27F	HQ845175.1	*Tetrathiobacter kashmirensis* strain AZDF-2 16S ribosomal RNA gene, partial sequence	1814	99	0.0	99
10-1492R	HQ845175.1	*Tetrathiobacter kashmirensis* strain AZDF-2 16S ribosomal RNA gene, partial sequence	1653	100	0.0	98
12-27F	JN662517.1	*Arthrobacter aurescens* 16S ribosomal RNA gene, partial sequence	1428	99	0.0	99

Isolate and primer used	GenBank accession number	Definition	Max. score	Query coverage (%)	E value	Max. identity (%)
12-1492R	JN662517.1	*Arthrobacter aurescens* 16S ribosomal RNA gene, partial sequence	1435	99	0.0	99
13-27F	JQ807860.1	*Bacillus* sp. WYT035 16S ribosomal RNA gene, partial sequence	1406	100	0.0	99
13-1492R	JX406823.1	*Bacillus subtilis* strain b17a 16S ribosomal RNA gene, partial sequence	1563	99	0.0	99
14-1492R	JN695898.1	*Erwinia* sp. E280d 16S ribosomal RNA gene, partial sequence	111	100	3e-22	100
17-27F	FN908795.1	*Arthrobacter nitroguajacolicus* partial 16S rRNA gene, strain SBA86	1810	99	0.0	97
17-1492R	JX293329.1	*Arthrobacter nitroguajacolicus* strain S58 16S ribosomal RNA gene, partial sequence	1421	99	0.0	99
20-27F	JN613469.1	*Bacillus* sp. O-NR1 16S ribosomal RNA gene, partial sequence	1328	100	0.0	99
20-1492R	JN696606.1	*Bacillus* sp. K3-D6L 16S ribosomal RNA gene, partial sequence	1615	100	0.0	99
21-27F	JF496323.1	*Bacillus simplex* strain A1-6 16S ribosomal RNA gene, partial sequence	1369	100	0.0	99

Isolate and primer used	GenBank accession number	Definition	Max. score	Query coverage (%)	E value	Max. identity (%)
21-1492R	JQ693815.1	*Bacillus simplex* strain ARI 16S ribosomal RNA gene, partial sequence	1626	100	0.0	99
25-27F	JQ320089.1	*Pseudomonas* sp. XjGEB-1 16S ribosomal RNA gene, partial sequence	2002	99	0.0	99
25-1497R	JX035946.1	*Pseudomonas* sp. JDG23 16S ribosomal RNA gene, partial sequence	1332	100	0.0	99
27-27F	DQ674859.1	*Rhizobium* sp. CCNWYC119 16S ribosomal RNA gene, partial sequence	1975	99	0.0	99
27-1492R	DQ674859.1	*Rhizobium* sp. CCNWYC119 16S ribosomal RNA gene, partial sequence	1956	99	0.0	99
28-1492R	JX426093.1	*Stenotrophomonas maltophilia* strain A3 16S ribosomal RNA gene, partial sequence	121	100	7e-25	92
29-27F	FJ950614.1	*Ochrobactrum* sp. c279 16S ribosomal RNA gene, partial sequence	56	93	2e-05	88
29-1492R	JX514845.1	*Ochrobactrum* sp. Cr13(2012) 16S ribosomal RNA gene, partial sequence	532	100	4e-148	98
37-27F	AB733647.1	*Rhizobium* sp. L6-8 gene for 16S ribosomal RNA, partial sequence	67.9	77	4e-09	97

Isolate and primer used	GenBank accession number	Definition	Max. score	Query coverage (%)	E value	Max. identity (%)
37-1492R	JN703473.1	*Rhizobium mesoamericanum* strain 5m 16S ribosomal RNA gene, partial sequence	171	95	1e-39	93
42-27F		Sequencing failure				
42-1492R	JQ900536.1	*Pseudomonas aeruginosa* strain B2 16S ribosomal RNA gene, partial sequence	1674	100	0.0	99
44-27F	FJ950646.1	*Ochrobactrum* sp. c268 16S ribosomal RNA gene, partial sequence	44.1	51	0.052	100
44-1492R	JX495605.1	*Ochrobactrum* sp. MS8 16S ribosomal RNA gene, partial sequence	267	96	2e-68	100
45-27F		Sequencing failure				
45-1492R	AB752301.1	*Bacillus flexus* gene for 16S rRNA, partial sequence, strain: RA005	54	100	2e-05	100

***Max. score: Score of high scoring pairs (HSPs) *Query coverage: percent of length coverage for the query *E. Value: The number of hits one can "expect" to see by chance when searching a database of a particular size *Max. identity: Maximal percent identity of the HSP**

3.4 Discussion and conclusion

Isolates of PAH degrading bacteria were identified biochemically and by molecular techniques using PCR amplification and sequencing of 16S rDNA. Sequences were analysed using BLAST (NCBI) and their percentage identity to

known bacterial 16S rDNA sequences in the GeneBank database (NCBI) was compared. Even though a broad range of bacteria have been discovered to be involved in PAH biodegradation (Hamme, et al. 2003; Haritash & Kaushik, 2009; Juhasz et al., 2000; Seo et al., 2009; Sinha et al., 2009), it was observed that PAH degradation in soil is dominated by bacterial strains belonging to a very limited number of taxonomic groups including *Sphingomonas* spp., *Burkholderia* spp., *Pseudomonas* spp. and *Mycobacterium* spp. (Seo et al., 2009). Furthermore, Sinha et al., (2009) showed that twenty-two PAHs degrading bacterial strains isolated from Antarctic soils belonged to the genus *Pseudomonas* spp.. However, it is notable that in this study twenty distinct bacteria genera including *Achromobacter* spp., *Sphingobacterium* spp., *Brevibacterium* spp., *Burkholderiales* spp., *Tetrathiobacter* spp., *Arthrobacter* spp., *Bacillus* spp., *Erwinia* spp., *Pseudomonas* spp., *Rhizobium* spp., *Stenotrophomonas* spp. and *Ochrobactrum* spp. were isolated as the PAH biodegraders (Table 3.5). Many of the PAH biodegrader bacteria were isolated and identified had confirmed the above genera as PAH biodegrader microorganisms (Arulazhagan, 2001; Cubitto, 2004; Goosh, 2005; Juhasz et al., 2000; Teng, 2011; Westerberg, 2000), but potentially new bacteria species including *Burkholderiales thailandensis, Bacillus simplex, Rhizobium mesoamericanum, Sphingobacterium shayense,* and *Tetrathiobacter kashmirensis* and a novel genus of *Erwinia* spp. were found as a PAH biodegraders in this study. This roadside soil was used as a source of PAH degrading bacteria throughout this thesis. Therefore, the hypothesis for this part of thesis was proved.

Chapter 4

The Effect of pH on Bacterial degradation of PAH in Soil

4.1 Introduction

PAH are fused benzene ring compounds and non-polar with low solubility in water and relatively resistant to biodegradation due to their hydrophobicity (Simarro et al., 2011). Microbial biodegradation reduces the toxicity of PAH with a very low environmental impact. Modification of the environmental factors, such as pH may improve the PAH biodegradation process by providing a better growth conditions for microorganisms (Simarro et al., 2011). Little research was found on the optimum pH for the microbial degradation of the four PAHs in the soil. The hypothesis for this chapter was that pH would influence the microbial degradation of PAH in the experimental soil. Therefore, the aim was to monitor the rate of degradation at a range of acidic and basic pHs in the experimental soil. See Table 4.1 for the objectives of chapter 4.

Table 4.1 - Objectives of chapter 4

1	**To monitor microbial degradation of the four PAHs at seven pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0)**
2	**To investigate the effect of pH on biodegradation of the four PAHs (91 mg.kg^{-1}) at seven pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0)**
3	**To investigate bacterial populations in the soil during biodegradation of the four PAHs (91 mg.kg^{-1}) at seven pHs (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0)**

4.2 Materials and methods

See Table 4.2 for the experimental plan.

Table 4.2 - Experimental layout

- **Measuring pH/WHC of J. Arthur Bower's soil**
- **Drying (90 ºC), sieving (2 mm) and sterilising (15 min, 15 psi, 121 ºC) the soil**
- **Filling pots with the prepared and sterilised Arthur Bower's top soil**
- **Contaminating the prepared soil using PAH solution**
- **Evaporating the n-hexane under a fume hood (48 hours)**
- **Checking weights of flasks containing the soil**
- **Adjusting the soil water content to 30 % of the WHC**
- **Adjusting pH of the soil using HCl or Na_2CO_3 for all treatments**
- **Inoculating the soil with roadside soil (0.5 g/1,100g)**
- **Transferring the soil into flasks**
- **Incubating the soil (20 ºC for 32 days)**
- **Sampling for HPLC (every 4 days) and for bacterial enumerating (every week)**
- **Extracting of PAH and enumerating of bacteria from the soil**

Seven plastic pots were filled with 1,100 g of the dried and prepared soil as described in section 2.1.3. Pots were individually contaminated with 1,000 cm^3 of the four PAHs stock solution in n-hexane. Therefore, the final concentration of individual PAH in each pot was 91 $mg.kg^{-1}$ (Appendix 4-Table 4.1). Pots were kept under a fume hood for 48 hours to evaporate n-hexane. The weights were checked to ensure the original weight before contamination was achieved. The soil water content was adjusted to 30 % of the water-holding capacity by adding 138 cm^3 of Mili-Q water (Corrected to the nearest whole number) to 1,100 g of the soil (Appendix 4-Table 4.2). The pH of the soil was adjusted by adding varying volumes of hydrogen chloride and sodium carbonate (section 2.4 and Appendix 4-Table 4.3). Based on this, calculations were performed for larger volumes of experimental soil samples (Appendix 4-Table 4.4). Inoculum of 0.5 g of roadside soil was then added to each pot containing 1,100 g of

prepared, contaminated soil and mixed thoroughly to ensure the presence of PAH biodegrader microorganisms (section 2.3). 200 g of the soil was then transferred into 500 cm^3 conical flasks from each seven pots and covered with sponge bungs and replicated 5 times and incubated in the dark at 20 ºC. Moisture content was monitored every three days and water loss was compensated by addition of sterile Milli-Q water (Appendix 3-Table 3.6). Samples were taken for HPLC analysis and the PAH remaining in the soil samples were extracted from the soil by adding 1.5 cm^3 of acetonitrile containing 100 $mg.dm^{-3}$ carbozole as an internal standard to 0.5 g of soil (section 2.6.1). Micro Centrifuge tubes were vortexed using a round table vortex for 15 minutes and then centrifuged for another 15 minutes. The solid in the Micro Centrifuge tubes were allowed to sediment prior to HPLC analysis. Sampling was carried out every four days for 32 days. The standard solutions of PAH plus carbozole and experimental samples respectively, were injected into the HPLC machine (section 2.6). See Appendix 4-Table 4.5 for preparation of the standard solutions. The mean values were calculated for all the five replicates of samples and standard deviation quantified. See Appendix 4.2 and Appendix 4-Figures 4.1 to 4.2 for the graphs of the HPLC standards and chromatograms. Simultaneously, the culturable bacterial populations were sampled and bacterial CFUs were enumerated at each sample time as described in section 2.5.

4.3 Results

The biodegradation and the effect of pH on the biodegradation of the four PAHs at the seven pHs in the soil were monitored using the HPLC machine.

Figures 4.1 to 4.4 indicate the percentage remaining of the phenanthrene, anthracene, fluoranthene and pyrene, respectively over time in the J. Arthur Bower's top soil at varying pH over 32 days. Statistical analysis (Appendix 7.1) showed that the greatest biodegradation was at pH 7.5 with a significant difference ($p<0.05$) compared to the rest of the pHs.

Figure 4.1 shows that phenanthrene had the greatest biodegradation at pH 7.5. However, anthracene, fluoranthene and pyrene were still present at low levels after 32 days. Figure 4.2 shows percentage remaining of anthracene and its degradation at different pHs over time. Anthracene had a slower biodegradation compared with phenanthrene. The phenanthrene was completely degraded within 32 days; whilst at the same time point anthracene was still remaining in the soil. Figure 4.3 shows percentage remaining of fluoranthene and its degradation at different pHs over time. It was observed that there was a significantly ($p<0.05$) greater biodegradation for fluoranthene at pH 7.5 compared to the rest of the pH (Appendix 7.1). It is evident that the biodegradation of fluoranthene was not completed at pH 6.5 after 32 days. The Figure 4.4 shows percentage remaining of pyrene and biodegradation at different pHs over time. The fastest biodegradation of pyrene was at pH 7.5 and 8.0. It is evident that the biodegradation of pyrene was not completed at pH 6.5 after 32 days.

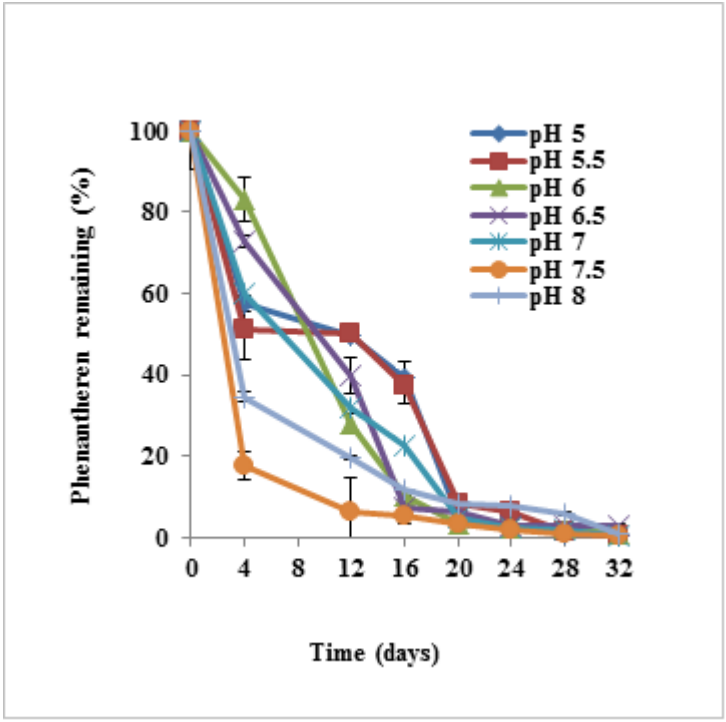


Figure 4.1 - Percentage remaining of phenanthrene over time in the J. Arthur Bower's top soil (n=5 ± SD)

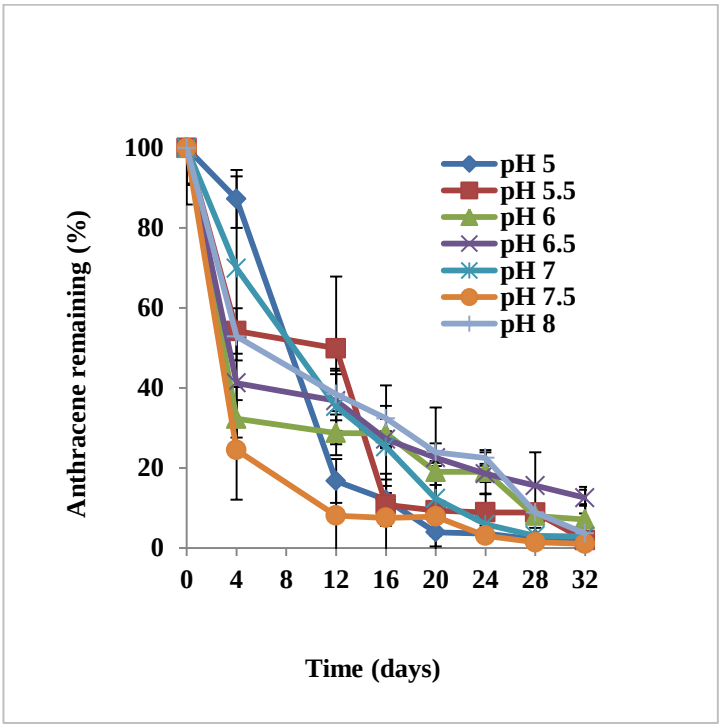


Figure 4.2 - Percentage remaining of anthracene over time in the J. Arthur Bower's top soil (n=5 ± SD)

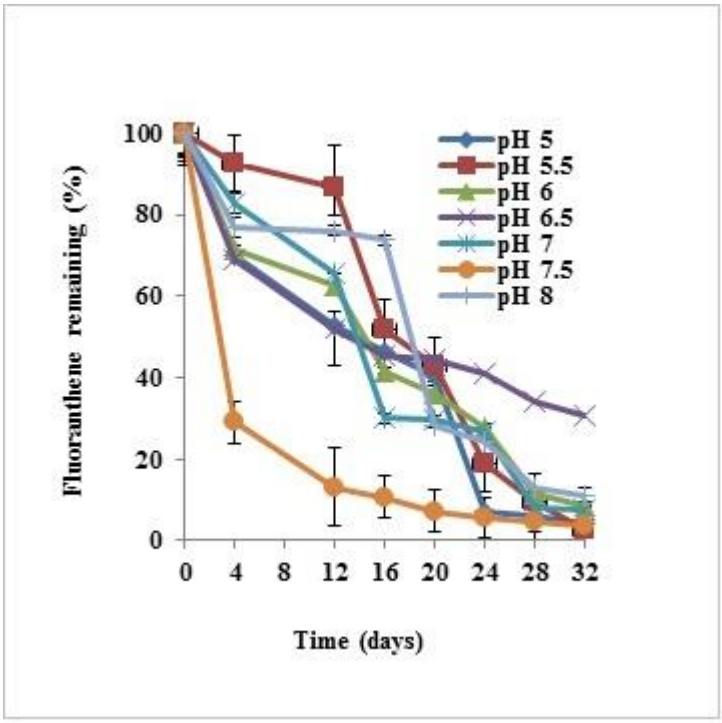


Figure 4.3 - Percentage remaining of fluoranthene over time in the J. Arthur Bower's top soil (n=5 ± SD)

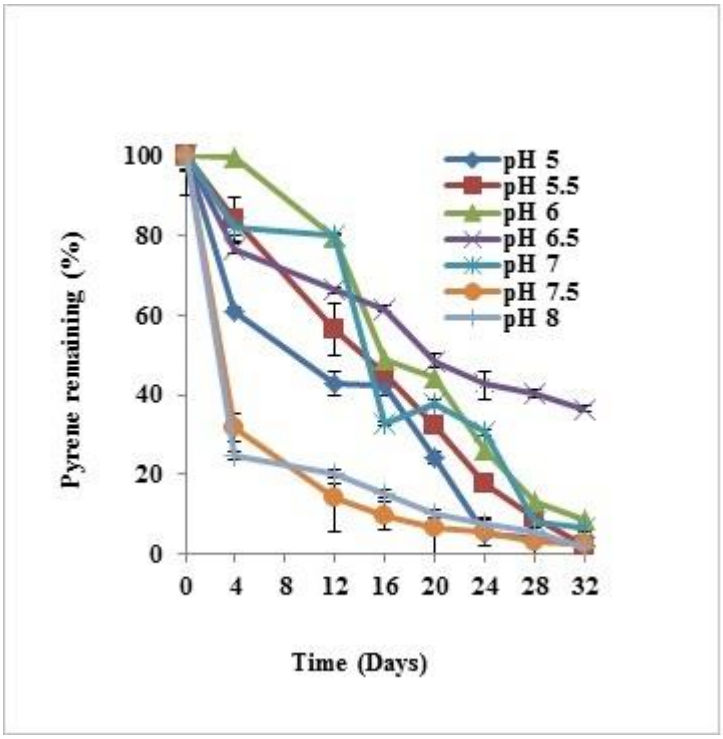


Figure 4.4 - Percentage remaining of pyrene over time in the J. Arthur Bower's top soil (n=5 ± SD)

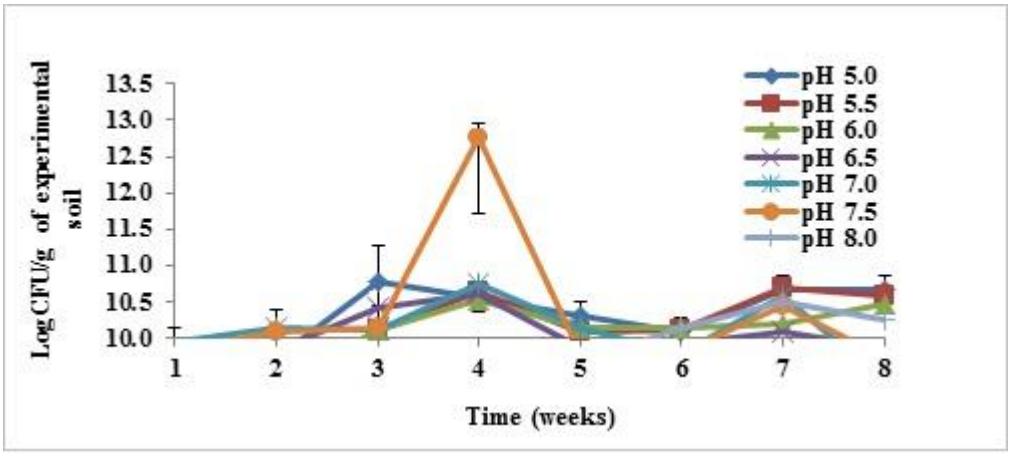


Figure 4.5 - Log total culturable bacteria colony forming units in the J. Arthur Bower's top soil (LogTCFU/g) contaminated with the four PAHs and inoculated with the roadside soil at different pHs against time

Figure 4.5 shows the log total bacterial colony forming units over eight weeks in PAH contaminated J. Arthur Bower's top soil. The greatest bacterial populations were found at pH 7.5. It was observed that bacterial populations had increase in weeks 4 and 7.

4.4 Discussion and conclusion

The greatest degradation was found for the lowest molecular weight PAH, phenanthrene and anthracene; whilst the lowest degradations were observed for higher molecular weight PAH, fluoranthene and pyrene. The results showed that as the number of benzene rings increases in the PAH compounds, the biodegradation decreases (Muckian, et al., 2007; Shafiee, 2006). Biodegradation of PAH and other hydrophobic substrates is believed to be limited by the amounts dissolved in the water phase (Bosma et al., 1997). The lowest molecular weight PAH degrades faster than the higher molecular weight ones due to higher

solubility and greater bioavailability (Pazos et al., 2010). Therefore, the most soluble PAH degrade quicker. Phenanthrene and anthracene are isomers with the same molecular weight. However, anthracene has a linear structure; whilst phenanthrene has a curved structure (Figure 1.1). Fluoranthene and pyrene are isomers. However, degradation of pyrene is slower than fluoranthene. Pyrene was therefore, the most persistent PAH. The linearity of anthracene and pyrene gives a great symmetry to the molecules that consequently leads to less solubility and bioavailability (Table 1.1), which explains the lower degradation (Haritash & Kaushik, 2009; Pazos et al., 2010). This may be due to stronger interactions between more hydrophobic and higher molecular weight PAH molecules and soil particles.

The greatest bacterial populations were found at pH 7.5 (Figure 4.5). The greatest degradation occurred at the same pH. This study showed that acidic pHs (pH 5.0, 5.5, 6.0 and 6.5) resulted in the lower biodegradation compared to neutral or weak alkaline pHs (pH 7.0, 7.5 and 8.0). In general, bacteria are suggested to be more important and involved in the biodegradation of pollutants (Bastiaens et al., 2000; Johnsen et al., 2002; Ho et al., 2000). However, they are more tolerant to neutral and basic pH, therefore, the bacterial populations were expected to be greater at neutral and basic pH. Figure 4.5 shows that bacterial populations are higher at pH 7.5. It is likely that a general increase in population was also linked with greater metabolic activities at basic pHs which assists pollutant degradation. Although there is high pollutant mobility at low pHs, the degradation is expected to be limited due to reduced microbial activity (Chesworth, 2008). This may also be due to the fact that nutrients are commonly more available at pH 7.0. At acidic pH anionic nutrients interact with protons

and are therefore less available. Likewise, at basic pH cationic nutrients have interactions with hydroxyls and are therefore less available. However, at neutral pH these interactions are generally reduced and consequently, nutrients are more available. High pHs resulted in greater PAH biodegradation suggesting that pH manipulation by liming may be an effective way of stimulating biodegradation of PAH (Chesworth, 2008). Therefore, the hypothesis that pH increases biodegradation was shown to be true. Furthermore, the optimum pH for bacterial biodegradation of PAHs was 7.5.

Chapter 5

The Effect of Chemical Oxidation on Degradation of PAH in Soil

5.1 Introduction

Chemical oxidation is a rapid and commonly used soil and groundwater remediation technology and has proven to be effective for removal of many contaminants such as polycyclic aromatic hydrocarbons (Chen et al., 2009; Seol et al., 2003). Fenton's reagent (hydrogen peroxide and ferrous iron), ozone, persulfate ($S_2O_8^{2}$) and permanganate (MnO_4^{-}), are the most commonly used oxidants (Chen et al., 2009; Doğan et al., 2013; Seol et al., 2003; Silva et al., 2009a; Sirguey et al., 2008). The investigations of Ma at al. (2013) showed that potassium permanganate acts as the most effective remediation oxidant compared to others such as hydrogen peroxide, Fenton's reagent, modified Fenton's reagent, activated sodium persulfate.

Potassium permanganate was used in this study as an oxidising agent. Potassium permanganate (Mn^{7+}) reduces to manganese dioxide (MnO_2) and (Mn^{4+}), which precipitates out of solution (Chen et al., 2009). The hypothesis for this chapter was that potassium permanganate oxidation of PAH would be as efficient as microbial breakdown of PAH. Therefore, the main aim of experiment i, oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate was to examine the effect of potassium permanganate on the oxidation of the four PAHs in sterile soil treated with and without potassium permanganate solution at pHs 5.0, 6.0, 7.0 and 8.0 in the J. Arthur Bower's top soil. The main aim of experiment ii, oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil was to compare the effect of potassium permanganate at pH 7.5 on oxidation of the four PAHs in the soil inoculated with the roadside

soil (Treatment A), microbial degradation in the soil inoculated with the roadside soil (Treatment B) and potassium permanganate oxidation in the sterile soil (Treatment C). See Table 5.1 for the objectives of each experiment.

Table 5.1 - Objectives of chapter 5

Oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate	**To study the effect of permanganate (0.09 M) on oxidation of the four PAHs (250 mg.kg^{-1}) in the sterile soil in comparison to degradation of PAH in the sterile soil without permanganate at the four pHs (5.0, 6.0, 7.0, and 8.0)**
	To investigate the effect of pH on permanganate (0.09 M) oxidation of the four PAHs (250 mg.kg^{-1}) in the sterile soil at the four pHs (5.0, 6.0, 7.0 and 8.0)
Oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil	**To monitor the effect of permanganate (0.09 M) on oxidation of the four PAHs (91 mg.kg^{-1}) in the soil inoculated with roadside soil at pH 7.5 (Treatment A)**
	To investigate the biodegradation of the four PAHs (91 mg.kg^{-1}) in the soil inoculated with roadside soil but without permanganate at pH 7.5 (Treatment B)
	To study the effect of permanganate (0.09 M) on oxidation of the four PAHs (91 mg.kg^{-1}) in the sterile soil without inoculation of roadside soil at pH 7.5 (Treatment C)
	To monitor the bacterial growth during permanganate (0.09 M) oxidation of the four PAHs (91 mg.kg^{-1}) at pH 7.5 (Treatment A)
	To examine the bacterial growth during biodegradation of the four PAHs (91 mg.kg^{-1}) in the soil at pH 7.5 (Treatment B)

5.2 Materials and methods

See Table 5.2 for the experimental layout.

Table 5.2 - Experimental layout

- **Measuring pH/WHC of experimental soil**
- **Drying (90 ºC), sieving (2 mm) and sterilising (15 min, 15 psi, 121 ºC) soil**
- **Filling a beaker with the prepared and sterilised Arthur Bower's top soil**
- **Contaminating the prepared soil using PAH solution**
- **Evaporating the n-hexane under a fume hood (48 hours)**
- **Checking weight of a beaker containing the soil**
- **Inoculating the soil with roadside soil**
- **Transferring the soil from beaker into centrifuge tubes**
- **Adjusting the soil water content to 30 % of the WHC**
- **Adjusting pH of the soil using HCl or Na_2CO_3**
- **Adding potassium permanganate solution**
- **Incubating the centrifuge tubes (20 ºC for 144 hours)**
- **Checking moisture content of all the treatments**
- **Adding sodium bisulfite solution to stop the reaction at each time point**
- **Sampling for HPLC (every 48 hours) and for bacterial enumerating (every week)**
- **Extracting of PAH and enumerating of bacteria from the soil**

Experiment i, oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate: The effect of potassium permanganate on oxidation of the four PAHs in the sterile soil in comparison to degradation of PAH in the sterile soil without permanganate at the four pHs (5.0, 6.0, 7.0, and 8.0) was studied. The pH and percentage water-holding capacity of the soil were measured as described in sections 2.1.1 and 2.1.2, respectively. The pH of the soil and water-holding capacity were 7.0 and 41.75 %, respectively (Appendix 5.1). A beaker was filled with 200 g of the prepared soil as described in section 2.1.3. The soil was contaminated with 200 cm^3 of the four PAHs stock solution. Therefore, the final concentration of individual PAH in each beaker was 250 $mg.kg^{-1}$ (Appendix 5-Table 5.2). The sterile soil contaminated with the four

PAHs was left under the fume hood for 48 hours to allow n-hexane to evaporate and the weight of beaker was checked to ensure the original weight was achieved. The sterile soil contaminated with the four PAHs was divided into 32 sterile 50 cm^3centrifuge tubes each containing 5 g of the soil. The pH of the sterile soil was adjusted individually in each tube as described in section 2.4 by adding different volumes of hydrogen chloride and sodium carbonate (Appendix 5-Table 5.3). 0.09 M potassium permanganate solution (Brown et al., 2003) was prepared (Appendix 5-Table 5.4). 10 cm^3 of the prepared potassium permanganate solution was added to each treated sample; whilst 10 cm^3 of sterile deionised water was added to the each control samples. The samples were incubated in a shaking incubator (70 rev/min) at 20 ºC until required for sampling. Moisture content was monitored every three days and water loss was compensated by addition of sterile Milli-Q water. One sample for each pH, with and without permanganate was removed from the incubator at 0, 48, 96 and 144 hours and 10 cm^3 of sodium bisulfite (Appendix 5-Table 5.5) was added to stop the reaction; whilst 10 cm^3 of sterile deionised water was added to the controls and mixed well with a sterile spatula. The samples were centrifuged at 6,000 rpm for five minutes and the supernatant discarded. The soil was transferred into a weighing boat and air-dried in a fume hood for 48 hours. This experiment was replicated four times. PAH remaining in the soil samples were extracted from the soil by adding 1.5 cm^3 of acetonitrile solution containing 200 $mg.dm^{-3}$ of carbozole as an internal standard to 0.5 g of soil in Micro Centrifuge tubes (section 2.6.1). Micro Centrifuge tubes were vortexed using a round table vortex for 15 minutes and then centrifuged for another 15 minutes. The solids in the Micro Centrifuge tubes were allowed to sediment prior to HPLC analysis. The

standard solutions of PAH plus carbozole and experimental samples respectively, were injected into the HPLC machine (section 2.6). See Appendix 4-Table 4.6 for the preparation of standard solutions. The mean values were calculated for all four replicates of samples and standard deviation quantified. See Appendix 5.3 and Appendix 5-Figures 5.1 to 4.7 for the graphs of the HPLC standards and chromatograms.

See Table 5.3 for the experimental layout.

Table 5.3 - Experimental layout

- **Measuring pH/WHC of experimental soil**
- **Drying (90 ºC), sieving (2 mm) and sterilising (15 min, 15 psi, 121 ºC) the soil**
- **Filling a beaker with the prepared and sterilised Arthur Bower's top soil**
- **Contaminating the prepared soil using PAH solution**
- **Evaporating the n-hexane**
- **Checking weight of a beaker containing the soil**
- **Preparing 3 sets of treatments in 3 beakers of sterile prepared soil:**
  - **A – Potassium permanganate inoculated with roadside soil**
  - **B – Inoculated with roadside soil only**
  - **C – Potassium permanganate only**
- **Transferring the soil from beakers into centrifuge tubes**
- **Adjusting the soil pH and water content to 30 % of the WHC**
- **Incubating the centrifuge tubes (20 ºC for 35 days)**
- **Checking moisture content of all the treatments**
- **Adding sodium bisulfite solution to stop the reaction at each time point to treatments A and C**
- **Sampling for HPLC and bacterial enumerating (every week)**
- **Extracting of PAH and enumerating of bacteria from the soil**

Experiment ii, oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil: The effect of potassium permanganate on oxidation of the four PAHs in the soil inoculated with the roadside soil (Treatment A), microbial degradation in the soil inoculated with the roadside soil (Treatment B) and potassium permanganate oxidation only (Treatment C) at pH 7.5 in the J. Arthur Bower's top soil was

compared. The pH and percentage water-holding capacity of the soil were 7.0 and 49.67 %, respectively (Appendix 5.4). A glass beaker was filled with 250 g of the dried and prepared soil as described in section 2.1.3. The soil was contaminated with 227.5 cm^3 of the four PAH stock solution (Appendix 5-Table 5.7). Therefore, the final concentration of individual PAH in a beaker was 91 $mg.kg^{-1}$. The same method, which is detailed in the experiment i, oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate was performed to evaporate the n-hexane. After n-hexane evaporation, 120 g of the soil was placed into two new and sterile beakers (Treatment A and C). 0.5 g of the roadside soil as inoculum (section 2.3) was added only to treatment A to ensure the presence of oil degrading microorganisms for the degradation process. The prepared soils were transferred into 18 sterile 50 cm^3 centrifuge tubes each of which containing 5 g of soil (i.e. six time points plus three replicates equal to 18 tubes). Treatment B was set up with the same method as treatments A and C but without potassium permanganate. The pH of the sterile dried soil was adjusted to 7.5 as described in section 2.4 by adding different volumes of hydrogen chloride and sodium carbonate (Appendix 5.5). pH 7.5 was selected as an appropriated pH for the potassium permanganate oxidation according to previous studies in this thesis. Varying volumes of potassium permanganate solution, sodium bisulfite solution and sterile distilled water were calculated and added into each centrifuge tube individually for the both treatments A and C, respectively to provide the liquid content (Appendix 5.5); whilst the same volume of sterile distilled water was added to the treatment B. All samples were incubated in the dark at 20 ºC. Moisture content was monitored every three days and water loss was

compensated by addition of sterile Milli-Q water. Three samples were taken from each treatment every seven days for 35 days. The weights were checked to ensure the original weight before sampling was achieved. The reaction was stopped at each time point by adding 200 µl of sodium bisulfate to the treatments A and C; whilst 200 µl of sterile distilled water was added to the treatment B, which was not treated with potassium permanganate. Samples were mixed well with a sterile spatula to make a homogenised mixture. This experiment was replicated three times. PAH remaining in the soil samples were extracted from the soil by adding 1.5 cm^3 of acetonitrile solution containing 100 $mg.dm^{-3}$ carbozole as an internal standard to 0.5 g of soil in Micro Centrifuge tubes (section 2.6.1). Micro Centrifuge tubes were vortexed using round table vortex for 15 minutes and then centrifuged for another 15 minutes. The solid in the Micro Centrifuge tubes were allowed to sediment prior to HPLC analysis. The standards and experimental samples were respectively injected into the HPLC machine (section 2.6). See Appendix 5-Table 5.8 for the preparation of standard solutions. The mean values were calculated for all four replicates of samples and standard deviation quantified. See Appendix 5.6 and Appendix 5-Figures 5.8 to 5.16 for the graphs of the HPLC standards and chromatograms. Simultaneously, the bacterial populations were enumerated as described in section 2.5.

5.3 Results

Experiment i, oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate: The effect of permanganate (0.09 M) on the oxidation of the four PAHs (250 $mg.kg^{-1}$) in the sterile soil in comparison to

degradation of untreated control without permanganate in the sterile soil at the four pHs (5.0, 6.0, 7.0, and 8.0) was studied. Moreover, the effect of pH on permanganate (0.09 M) oxidation of the four PAHs (250 $mg.kg^{-1}$) in the sterile soil at the four pHs (5.0, 6.0, 7.0 and 8.0) was investigated.

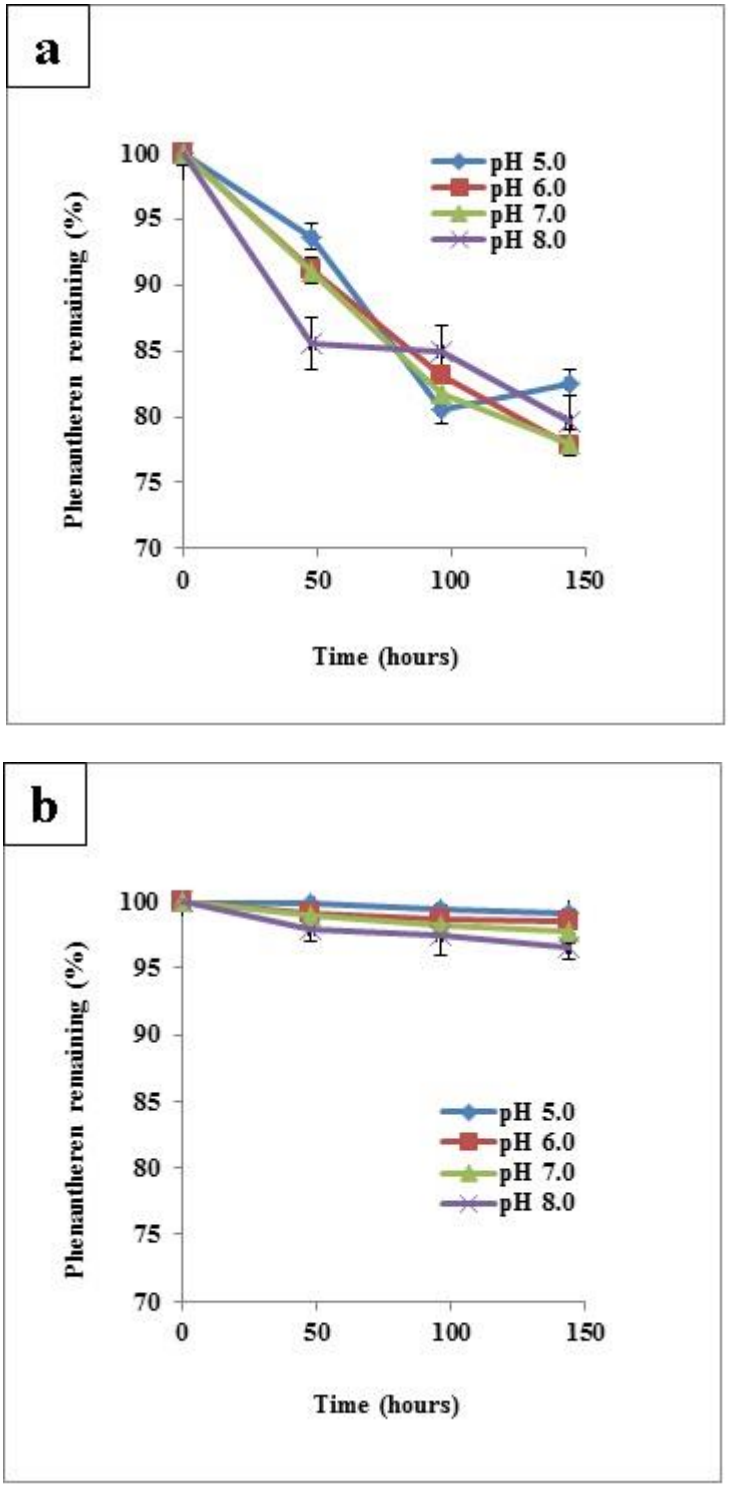


Figure 5.1 - Percentage remaining of phenanthrene at varying pHs over time in the sterile soil. (a) Treated with 0.09 M potassium permanganate solution and (b) Untreated control (n=4 ± SD)

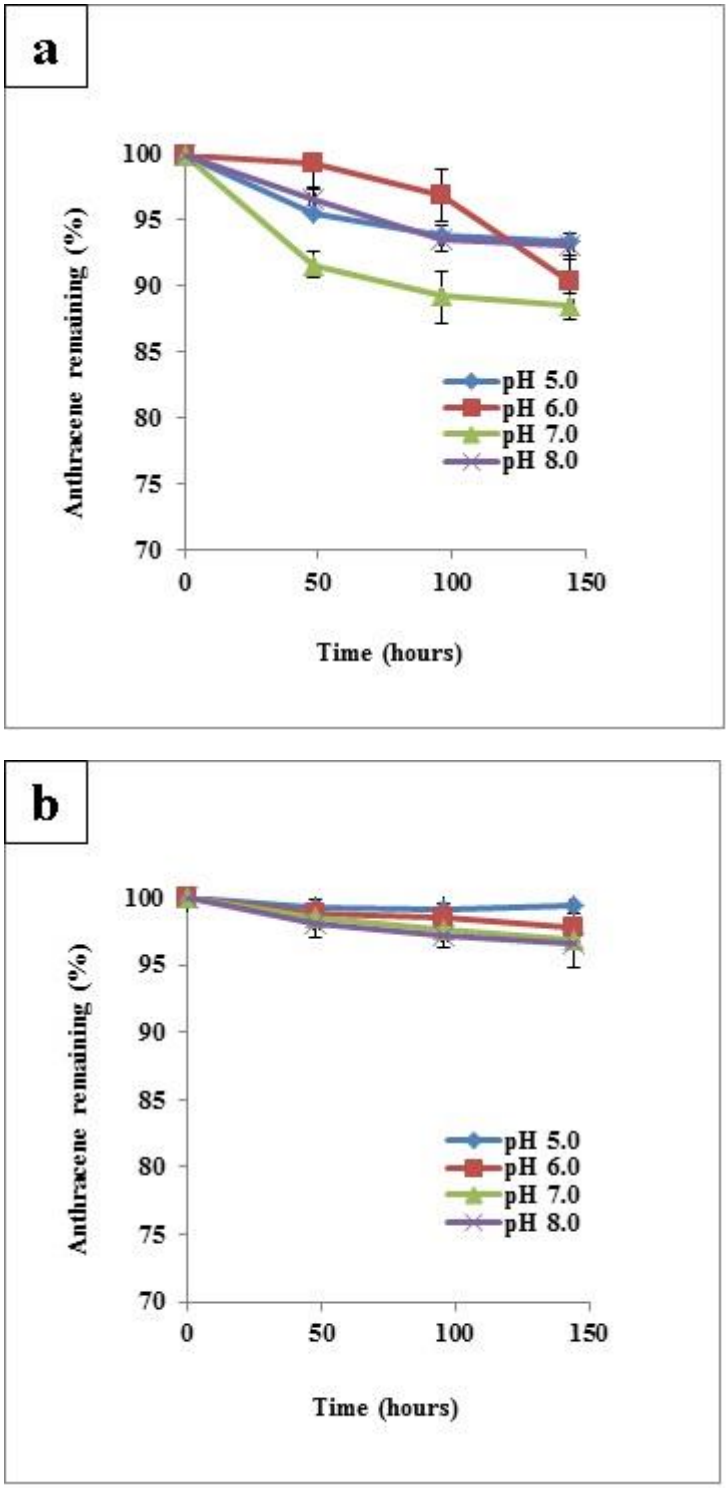


Figure 5.2 - Percentage remaining of anthracene at varying pHs over time in the sterile soil. (a) Treated with 0.09 M potassium permanganate solution and (b) Untreated control (n=4 ± SD)

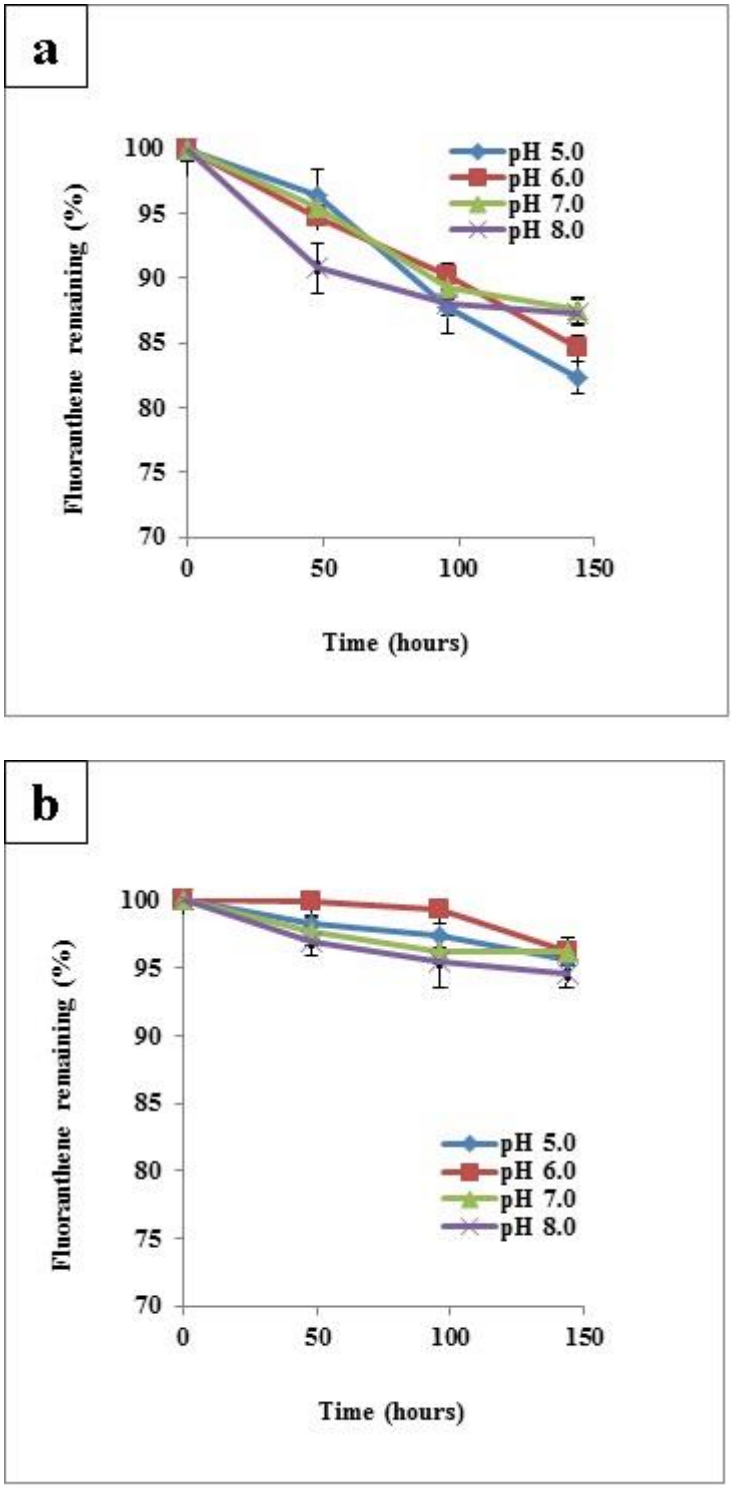


Figure 5.3 - Percentage remaining of fluoranthene at varying pHs over time in the sterile soil. (a) Treated with 0.09 M potassium permanganate solution and (b) Untreated control (n=4 ± SD)

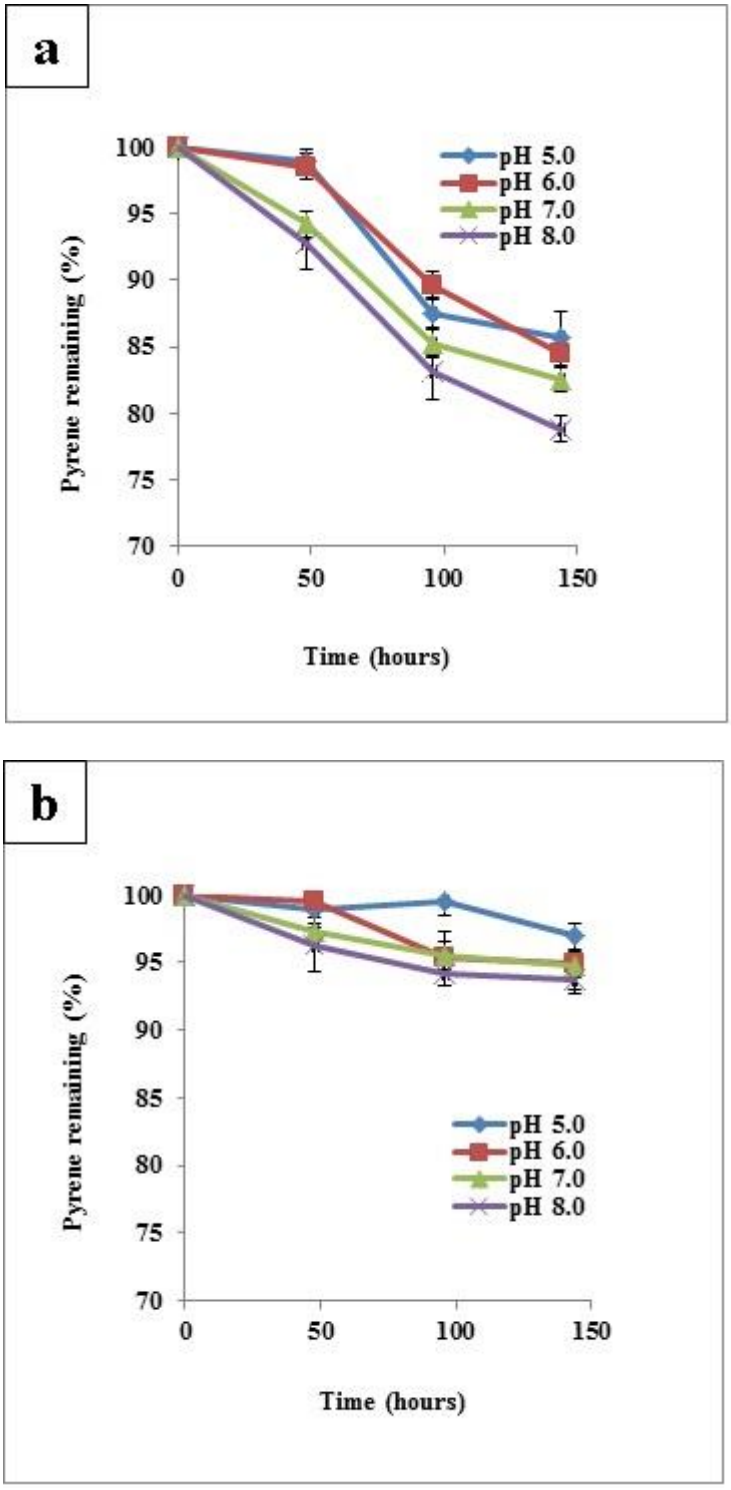


Figure 5.4 - Percentage remaining of pyrene at varying pHs over time in the sterile soil. (a) Treated with 0.09 M potassium permanganate solution and (b) Untreated control (n=4 ± SD)

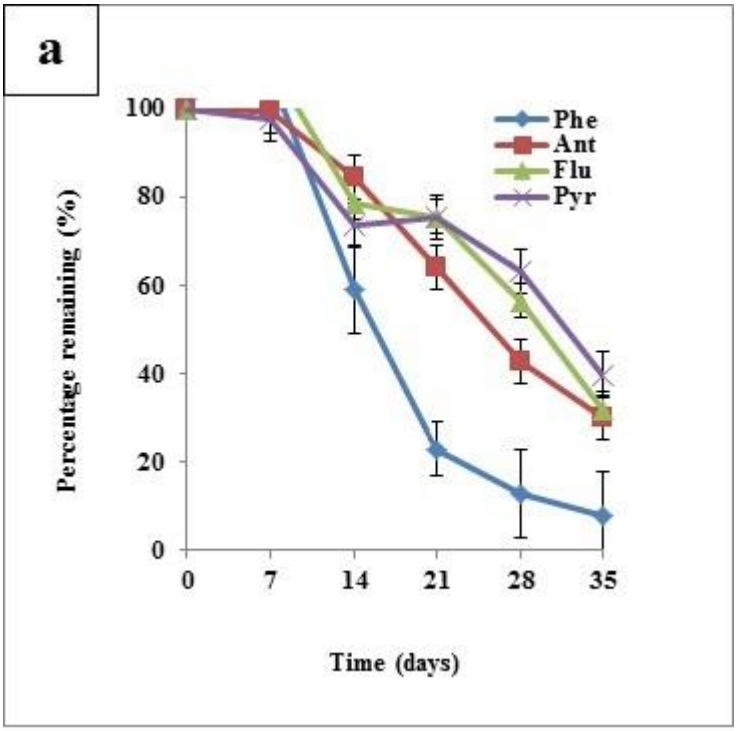
Figures 5.1 to 5.4 indicated the percentage remaining of the four PAHs in the soil at varying pHs over 144 hours. Figure 5.1a shows that potassium permanganate caused some breakdown of phenanthrene; whilst as Figure 5.1b shows very little breakdown of phenanthrene in sterile soil.

Table 5.4 - pH with the greatest and lowest degradation for the four PAHs in 0.09 M potassium permanganate treated samples and untreated controls. * Indicates significant difference (P<0.05).

Chemical	Potassium permanganate treated samples		Untreated controls	
	pH with the greatest degradation	pH with the lowest degradation	pH with the greatest degradation	pH with the lowest degradation
Phenanthrene	**8.0***	**5.0**	**8.0**	**5.0**
Anthracene	**7.0***	**5.0**	**8.0**	**5.0**
Fluoranthene	**8.0***	**5.0**	**8.0**	**6.0**
Pyrene	**8.0***	**5.0**	**8.0**	**5.0**

Experiment ii, oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil: The effect of potassium permanganate on oxidation of the four PAHs in the soil inoculated with the roadside soil (Treatment A), microbial degradation in the soil inoculated with the roadside soil (Treatment B) and potassium permanganate oxidation in the sterile soil (Treatment C) at pH 7.5 were compared. Figures 5.5a, 5.6 and 5.7a indicate the percentage remaining of the four PAHs in the soil at pH 7.5 over 35 days. Moreover, the effect of permanganate (0.09 M) and

biodegradation on bacterial populations during oxidation and degradation of the four PAHs (91 $mg.kg^{-1}$) at pH 7.5 was monitored in Figures 5.5b and 5.6b, respectively.



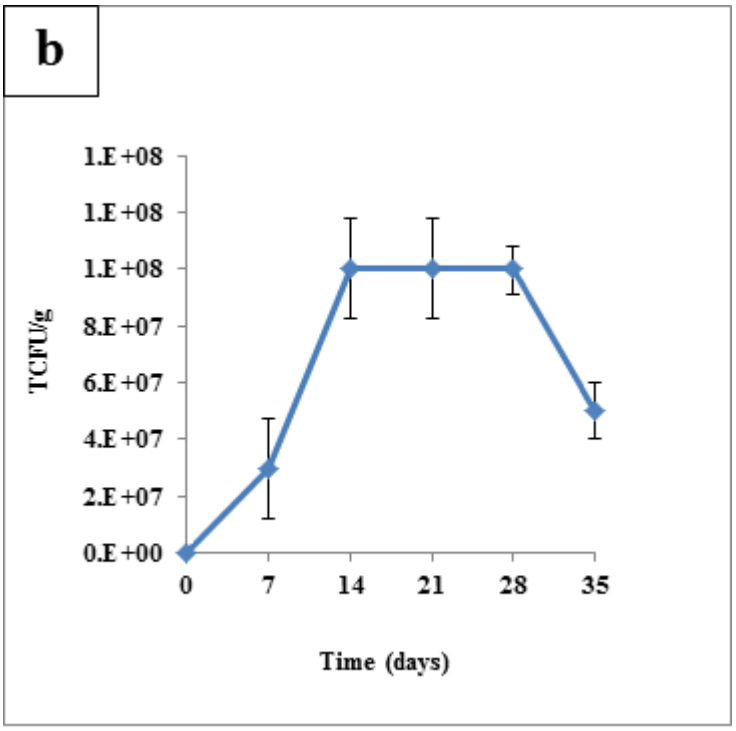


Figure 5.5 - (a) Percentage remaining of the four PAHs over time in the soil inoculated with the roadside soil and treated with potassium permanganate at pH 7.5 against time (n=3 ± SD). (b) Total colony forming units of bacteria in the soil (TCFU/g) inoculated with the roadside soil and treated with potassium permanganate at pH 7.5 against time (n=3 ± SD)

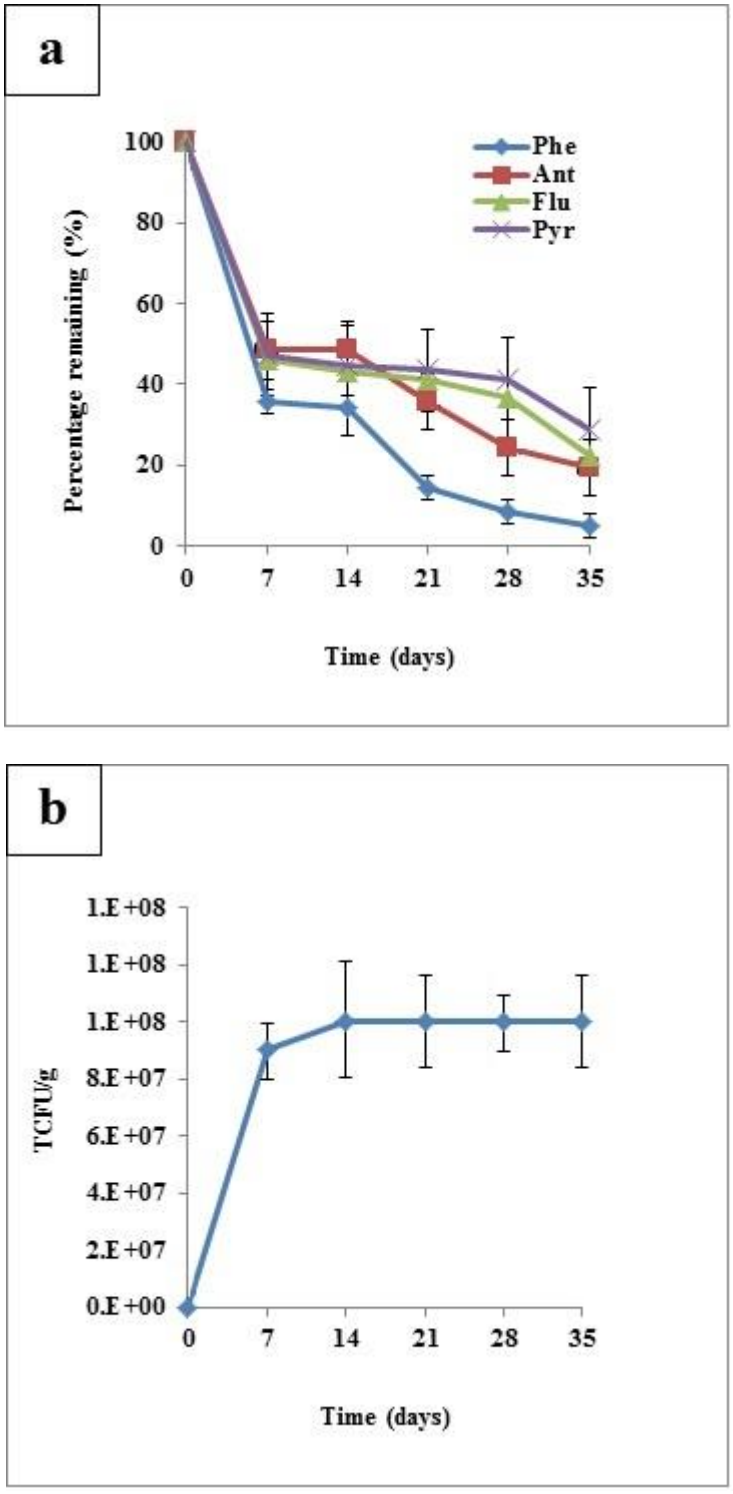


Figure 5.6 - (a) Percentage remaining of the four PAHs over time in the soil inoculated with the roadside soil but without potassium permanganate at pH 7.5 against time (n=3 ± SD). (b) Total colony forming units of bacteria in the soil (TCFU/g) inoculated with the roadside soil but without potassium permanganate at pH 7.5 against time (n=3 ± SD)

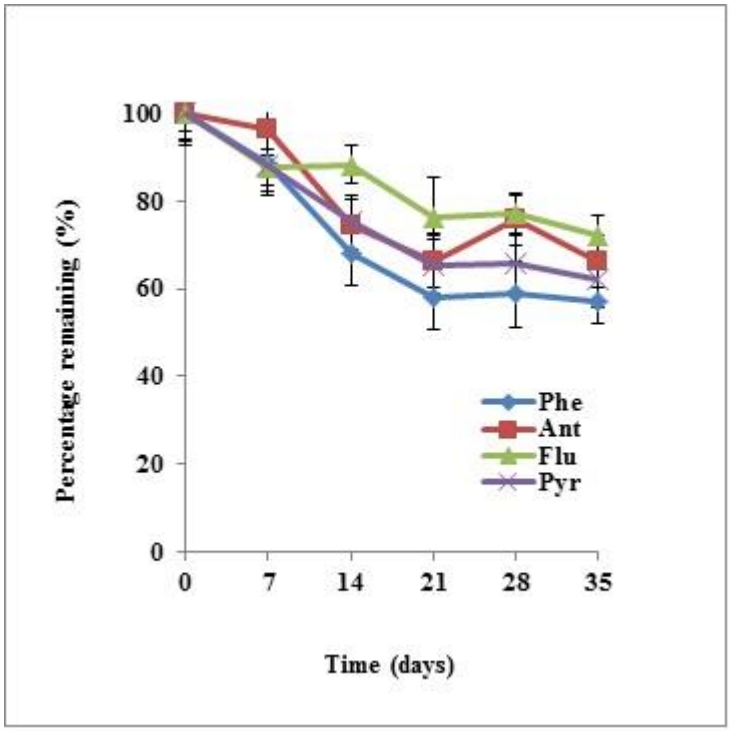


Figure 5.7 - Percentage remaining of the four PAHs over time in the sterile soil without roadside soil inoculation, but treated with potassium permanganate at pH 7.5 against time (n=3 ± SD)

Table 5.5 - Percentage remaining of the four PAHs in the soil on day 35 (n=3 ± SD). * Indicates significant difference (P<0.05) between the three treatments

Chemical	Treatment A: Treated with both roadside soil and potassium permanganate (% remaining)	Treatment B: Treated with roadside soil but no potassium permanganate (% remaining)	Treatment C: Treated with potassium permanganate but no roadside soil (% remaining)
Phenanthrene	7.58*	5.05*	57.26*
Anthracene	30.00*	19.23*	66.20*
Fluoranthene	31.96*	22.24*	72.30*
Pyrene	39.68	28.79*	61.85*

Figure 5.5a, shows that there was a little degradation for the first seven days in presence of potassium permanganate. However, interestingly in Figure 5.6a there was a fast rate of degradation in the first seven days in the absence of potassium permanganate. Figure 5.5a shows that phenanthrene was the most significantly ($p<0.05$) degraded PAH and pyrene was the lowest degraded PAH after 35 days. The figure shows there was a little degradation in the first seven days. However, the degradation of phenanthrene increased between days 7 to 14. The degradation process was continued to day 28. There was a little degradation between days 28 to 35. Statistical analysis (Appendix 7.2) showed that there was a significant difference between the degradation of phenanthrene between treatments A and B on day 7. Interestingly, the degradation of PAH mirrored the bacterial number. Figure 5.5b shows that there was a buildup of bacteria in the first seven days, compared with Figure 5.6b, whereas there was a faster increase in bacterial number. Figure 5.5b shows that bacterial populations had reached up to 3.E+07 on day 7. The bacterial populations reached up to 1.E+08 on day 14. The bacterial populations were constant between days 14 to 28 and it was decreased to 5.E+07 on day 35.

Figure 5.6a shows that phenanthrene was the most significantly ($p<0.05$) degraded PAH and pyrene had the least degradation after 35 days. Figure 5.6a shows that all the four PAHs had a great degradation in the first seven days. However, phenanthrene had a slow degradation between days 7 to 14. The degradation process was continued to day 35. Figure 5.6b shows that bacterial populations had reached up to 9.E+07 on day 7. The bacterial populations reached up to 1.E+08 on day 14. The bacterial populations were constant between days 14 to 28 and it was decreased to 1.E+08 on day 35 (Figure 5.6b).

Figure 5.7 shows that the PAH had a little oxidation in the sterile soil without the roadside soil but treated with potassium permanganate at pH 7.5 after 35 days.

5.4 Discussion and conclusion

Experiment i, oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate: Potassium permanganate oxidation of the four PAHs in the sterile soil at the four pHs were studied. Statistical analysis (Appendix 7.2) showed that the treatment, which contained potassium permanganate had a significantly ($p<0.05$) greatest oxidation compared to controls without potassium permanganate. This indicated that oxidation of the four PAHs in the sterile soil was more effective in the presence of potassium permanganate compared to the sterile soil without potassium permanganate. Moreover, the greatest permanganate oxidation was obtained at higher pHs (7.0 and 8.0); whilst the lowest permanganate oxidation was found at lower pHs (5.0 and 6.0). This indicated that potassium permanganate oxidation has a greater effect on PAH oxidation at higher pHs rather than lower pHs. Investigations indicated that phenanthrene (Figure 5.1a) had the greatest degradation compared to the rest of PAH only in the presence of potassium permanganate.

Experiment ii, oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil: The effect of potassium permanganate on oxidation of the four PAHs in the soil contaminated with the roadside soil (Treatment A), microbial degradation in the soil contaminated with the roadside soil and without potassium permanganate

(Treatment B) and potassium permanganate oxidation in the sterile soil (Treatment C) at pH 7.5 was compared. Moreover, the effect of potassium permanganate (0.09 M) on oxidation and biodegradation of the four PAHs in the soil was compared. Statistical analysis indicated that the treatment B had significantly ($p<0.05$) greatest degradation between the above three treatments on days 7 and 14. This part of the studies showed that the greatest degradation was found in the treatment B, inoculated with only the roadside soil microorganisms and without potassium permanganate (Figure 5.3). This suggested that potassium permanganate oxidation is not as effective as microbial degradation. Silva et al. (2009a) showed that potassium permanganate reduced PAH concentration in contaminated soil. Chemical reactions were studied as a rapid and commonly used soil or groundwater remediation technology (Silva et al., 2009a). Most PAH contaminated sites have a significant number of PAH degrading microorganisms. The bacterial populations are often limited by abiotic factors such as lack of aeration, bioavailability problems, and inadequate nutrients (Straube et al., 2003). Hence, though chemical oxidation was effective for removal of PAH it resulted in breakdown of soil organic matter and inhibited the bacterial populations (Chen et al., 2009; Silva et al., 2009a). Potassium permanganate had either inhibitory effect (direct oxidation of cell material or specific enzyme destruction) on microorganisms or it oxidised soil organic matter (oxidising agent) and therefore there were less microorganisms capable of growing and degrading PAH (Chen et al., 2009).

The greatest degradation was found for the lowest molecular weight PAH phenanthrene and anthracene (Chapter 5). This indicated that the lowest molecular weight PAH degrades faster than the higher molecular weight ones

due to higher solubility and greater bioavailability (Straube et al., 2003). The most degraded PAH was phenanthrene with percentage remaining of 7.58, 5.05 and 57.26 for the treatments A, B and C, respectively after 35 days (Table 5.5). The lowest biodegradation was found for the highest molecular weight PAH fluoranthene and pyrene. This might be related to the number of rings in PAH structure and their molecular weight. This may be due to stronger interactions between more hydrophobic and higher molecular weight PAH molecules and soil particles (Straube et al., 2003). The least degraded PAH was pyrene with the percentage remaining of 39.68, 28.79 and 61.85 for the treatments A, B and C at time 35, respectively (Table 5.5).

Through the *in situ* chemical oxidation, the best system to distribute the oxidants (e.g., potassium permanganate, hydrogen peroxide, and Fenton's reagent) is injecting/withdrawing it into a contaminated area (Seol et al., 2003). Moreover, a successful *in situ* oxidation is highly dependent on the heterogeneous distribution of oxidant. Seol et al. (2003) suggested that the best system for using chemical oxidation *in situ* is to use an injection/withdrawal system in the contaminated area.

Oxidation reduces the PAH in contaminated soils but it may also have an effect on the soil quality. The impact of permanganate and Fenton oxidation on soil quality was investigated. Soil quality is restricted here to the potential for plant growth. Soil samples were collected from an agricultural field (S1) and a former coking plant (S4). Agricultural soil was spiked with phenanthrene and pyrene at two concentrations (S2: 700 mg phenanthrene/kg^{-1}, S3: 700 mg phenanthrene/kg^{-1} and 2100 mg pyrene/kg^{-1}). Soils were treated with both oxidation processes, and analysed for PAH. A plant germination and growth test

was run with rye-grass on treated soils. Results showed that both treatments produced the expected reduction of PAH concentration (from 64% to 97%). Besides, a significant loss of organic C and N, and strong changes in available nutrients were observed. Permanganate treatment increased the specific surface area and the cation exchange capacity in relation to manganese dioxide precipitation. Plant growth was negatively affected by permanganate, related to lower soil permeability and aeration. Both treatments had an effect on soil properties (Sirguey et al., 2008).

The hypothesis for this chapter was that potassium permanganate oxidation of PAH would be as efficient as microbial breakdown of PAH. The results of this chapter disprove the hypothesis as microbial degradation of the PAH is shown to be significantly more effective than chemical oxidation with potassium permanganate.

Chapter 6

The Effect of Mobilising Agents on Degradation of PAH in Soil

6.1 Introduction

The biodegradation of PAH in contaminated soils is often restricted by the solubility of PAH, their sorption onto particles, slow transfer from organic to aqueous phase, and usually low aqueous PAH concentrations unable to maintain biodegradation. Consequently, microbial degradation is reliant on the ability of microorganisms to transfer and degrade contaminants from organic to aqueous phase. In this respect, mobilising agents have been suggested to raise the release of PAH and microbial accessibility in soil (Allan et al., 2007; Giubilei et al., 2009; Yang et al., 2000). Rehmann et al. (2008) suggested an inexpensive process of PAH extraction from soil. In this method, PAHs are concentrated in inert polymer pellets, which can be simply detached from the soil and added to a bioreactor, in which microorganisms degrade a large amount of PAHs.

The hypothesis for this chapter was that Tween 20 (a mobilising agent), would move PAH throughout soil, potentially making the PAH more available for biodegradation. Therefore, the main aim of experiment i, translocation of PAH in the soil was to investigate the effect of Tween 20 on translocation and the main aim of experiment ii, degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil was to monitor the effect of Tween 20 on biodegradation of the four PAHs in the J. Arthur Bower's top soil at pH 7.5. See Table 6.1 for the objectives of each experiment.

Table 6.1 - Objectives of chapter 6

Translocation of PAH in the soil	**To monitor the effect of Tween 20 on translocation of the four PAHs at pH 7.5 in the sterile soil**
Degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil	**To examine the effect of Tween 20 on biodegradation of the four PAHs (91 mg.kg^{-1}) in the soil inoculated with the roadside soil at pH 7.5 (Treatment A)**
	To study the impact of Tween 20 upon bacterial populations in the soil inoculated with roadside soil at pH 7.5 (Treatment A)
	To investigate the biodegradation of the four PAHs (91 mg.kg^{-1}) in the soil inoculated with roadside soil at pH 7.5 (Treatment B)
	To monitor bacterial populations during biodegradation of the four PAHs (91 mg.kg^{-1}) in the soil inoculated with roadside soil and no Tween 20 at pH 7.5 (Treatment B)
	To monitor the degradation of the four PAHs (91 mg.kg^{-1}) in the sterile soil without Tween 20 and roadside soil at pH 7.5 (Treatment C)

6.2 Materials and methods

See Table 6.2 for the experimental layout.

Table 6.2 - Experimental layout

- **Measuring WHC of experimental soil**
- **Drying (90 ºC), sieving (2 mm) and sterilising (15 min, 15 psi, 121 ºC) the soil**
- **Splitting the prepared soil into 2 beakers and contaminating beaker with PAH**
  - **Beaker 1 – Soil contaminated with PAH**
  - **Beaker 2 – Soil only**
- **Evaporating the n-hexane under a fume hood (48 hours)**
- **Checking weight of beaker 1**
- **Splitting beaker 1 into 2 new sterile beakers and adding Tween 20 to one of beakers**
  - **Beaker 1a – Soil contaminated with PAH and treated with Tween 20**
  - **Beaker 1b – Soil contaminated with PAH only**
- **Preparing 2 sets of Petri dishes**
- **Transferring the soil from beakers into Petri dishes**
  - **Petri dish 1: Left side (Soil + PAH + Tween 20), right side (Soil + PAH)**
  - **Petri dish 2: Left side (Soil + PAH), right side (Soil only)**
- **Adjusting the soil water content to 30 % of the WHC**
- **Incubating the Petri dishes (20 ºC for 20 days)**
- **Checking moisture content of all the treatments**
- **Sampling for HPLC (every week)**
- **Extracting of PAH**

Experiment i, translocation of PAH in the soil: The percentage water-holding capacity of the J. Arthur Bower's top soil was measured as described in section 2.1.2. The soil water-holding capacity was 49.67 %. The soil was prepared as described in section 2.1.3. Two beakers, one for the soil to be contaminated with the four PAHs stock solution (section 2.2) and the other one for uncontaminated soil without PAH were prepared each containing 200 g of the autoclaved soil. The soil was contaminated with 200 cm^3 of the PAH stock solution. Therefore, the final concentration of individual PAH in each beaker was 500 $mg.kg^{-1}$ (Appendix 6-Table 6.1). The beaker with soil contaminated with four PAHs was left under the fume hood for 48 hours to allow n-hexane to evaporate and the weight of beaker was checked to ensure the original weight was achieved. Tween 20 solution of 2.5 % was prepared (section 2.9 and Appendix 6-Table 5.2). The required volume of sterile distilled water to adjust the soil moisture

content to 30 % of water-holding capacity was added to the Tween 20 and autoclaved. The autoclaved water plus Tween 20 was added to the PAH contaminated soil (Appendix 6-Table 6.3). Moisture content was monitored every three days and water loss was compensated by addition of sterile Milli-Q water. Two square Petri dishes were used as the soil container. The clear plastic Petri dishes were 120 mm in diameter and purchased from Fisher Scientific. The plates were divided in half with a plastic cover sheet. Afterward, on the line that is offset for 1 cm from the partition line, three points were picked up and soil samples were taken with a sterile spatula. The same method of sampling was applied for the other half of the Petri dish. The cover sheet was then removed from the plates (Figures 6.1 and 6.2). Different sides of each Petri dish were filled with different types of the J. Arthur Bower's top soil containing the four PAHs and Tween 20 (Figures 6.1a), PAH only (Figure 6.2a) or the soil only (Figures 6.1b and 6.2b). Petri dishes were incubated in the dark at 20 ^{0}C with the lids on. 0.5 g of the soil was taken from three wells within 1 cm distance from the barrier in the Petri dishes at each side after each 10 days for 20 days to use for HPLC analysis. This experiment was replicated three times. The four PAHs remaining in the soil samples were extracted from the soil by adding 1.5 cm^{3} of acetonitrile solution containing 500 mg.kg^{-1} carbozole as an internal standard to 0.5 g of soil in Micro Centrifuge tube (section 2.6.1). Micro Centrifuge tubes were vortexed using a round table vortex for 15 minutes and then centrifuged for another 15 minutes. The solid in the Micro Centrifuge tubes were allowed to sediment prior to HPLC analysis. The standard solutions of PAH plus carbozole and experimental samples respectively, were injected into the HPLC machine (section 2.6). See Appendix 6-Table 6.4 for preparation of the standard

solutions. The mean values were calculated for all the three replicates of samples and standard deviation quantified. See Appendix 6.2 and Appendix 6-Figures 6.1 to 6.7 for the graphs of the HPLC standards and chromatograms.

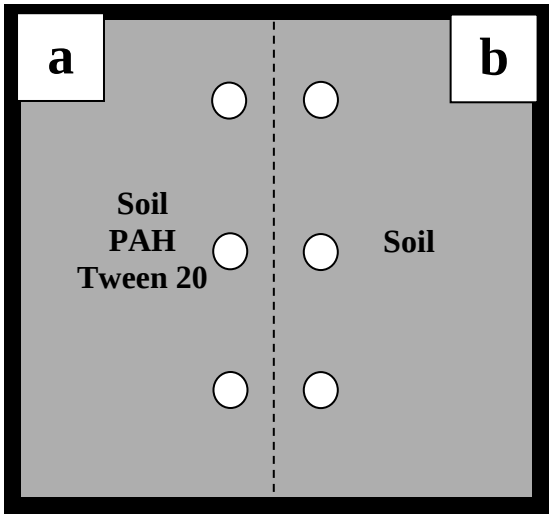


Figure 6.1 - Petri dish layout (a) 60 g of the soil, 500 mg.kg^{-1} of the four PAHs and 1.5 cm^3 of Tween 20 (b) 60 g of the soil only, no PAH and no Tween 20 (n=3 ± SD). White circles represent sampling points.

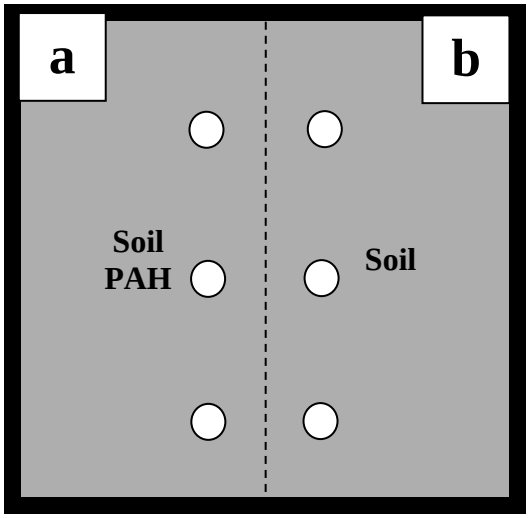


Figure 6.2 - Petri dish layout (a) 60 g of the soil, 500 mg.kg^{-1} of the four PAHs but no Tween 20 (b) 60 g of the soil, no PAH and no Tween 20 (n=3 ± SD). White circles represent sampling points.

See Table 6.3 for the experimental layout.

Table 6.3 - Experimental layout

- **Measuring pH/WHC of experimental soil**
- **Drying (90 ºC), sieving (2 mm) and sterilising (15 min, 15 psi, 121 ºC) the soil**
- **Filling a beaker with the prepared and sterilised Arthur Bower's top soil**
- **Contaminating the prepared soil using PAH solution**
- **Evaporating the n-hexane**
- **Checking weight of a beaker containing the soil**
- **Preparing 3 sets of treatments in a beakers:**
  - **A – Treated with Tween 20 and inoculated with roadside soil**
  - **B – Inoculated with roadside soil only**
  - **C – Sterile soil only**
- **Transferring the soil from beakers into centrifuge tubes**
- **Adjusting the soil pH and water content to 30 % of the WHC**
- **Incubating the centrifuge tubes (20 ºC for 35 days)**
- **Checking moisture content of all the treatments**
- **Sampling for HPLC and bacterial enumerating (every week)**
- **Extracting of PAH and enumerating of bacteria from the soil**

Experiment ii, degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil: The percentage water-holding capacity of the J. Arthur Bower's top soil was 49.67 % (Appendix 6.3). A beaker was filled with 600 g of the prepared soil as described in section 2.1.3. The soil was contaminated with 546 cm^3 of the four PAHs stock solution. Therefore, the final concentration of individual PAH in a beaker was 91 $mg.kg^{-1}$ (Appendix 6-Table 6.5). The same method, which is detailed in experiment i, translocation of PAH in the soil was performed to evaporate the n-hexane. After n-hexane evaporation, 600 g of the four PAHs contaminated soil was split into two new and sterile beakers each containing 420 and 180 g of the soil. The roadside soil was added only to the beaker that containing 420 g of the soil. The soil was then split into two new and sterile beakers each containing 200 g of the soil. The 2.5 % Tween 20 autoclaved solution was prepared by adding the appropriate volume of Tween 20, sterile distilled water and sodium bicarbonate

to provide the required liquid contents and adjusting the pH of the soil at 7.5 at the same time (section 2.4 and Appendix 6-Tables 6.6 and 6.7). The solution was added to one of the beaker containing 200 g of the four PAHs contaminated soil, inoculated with the roadside soil. 18 sterile centrifuge tubes were prepared to transfer soil from beakers, i.e. six time points plus three replicates equal to 18 tubes for each of the three treatments. All samples were incubated in the dark at 20 ºC. Moisture content was monitored every three days and water loss was compensated by addition of sterile Milli-Q water. Three samples were taken from each treatment every seven days for 35 days. The weights were checked to ensure the original weight before sampling was achieved. This experiment was replicated three times. The four PAHs remaining in the soil samples were extracted by adding 1.5 cm^3 of acetonitrile solution containing 100 $mg.dm^{-3}$ carbozole as an internal standard to 0.5 g of soil in Micro Centrifuge tubes (section 2.6.1). Micro Centrifuge tubes were vortexed using a round table vortex for 15 minutes and then centrifuged for another 15 minutes. The solid in the Micro Centrifuge tubes were allowed to sediment prior to HPLC analysis. The standard solutions of PAH plus carbozole and experimental samples were respectively injected into the HPLC machine (section 2.6). See Appendix 6-Table 6.8 for preparation of the standard solutions. The mean values were calculated for all the three replicates of samples and standard deviation quantified. See Appendix 6.4 and Appendix 6-Figures 6.8 to 6.16 for the graphs of the HPLC standards and chromatograms. Simultaneously, the bacterial population was enumerated as described in section 2.5.

6.3 Results

Experiment i, translocation of PAH in the soil: The effect of Tween 20 on translocation of the four PAHs in the sterile J. Arthur Bower's top soil was investigated. Figures 6.3 and 6.4 show the concentration of the four PAHs remaining and transferring in sterile soil at pH 7.5. Comparing Figures 6.3a to 6.3b on day 20 shows the loss of some PAH. In Figure 6.3, graph (a) was sampled from the side of the Petri dish containing Tween 20 and PAH. The level of PAH dropped by day 20. Figure 6.3, graph (b) shows the results from sampling on the side of the Petri dish where the soil was not contaminated with PAH or Tween 20. This shows that the PAH have moved from the contaminated to the uncontaminated soil. In Figure 6.4, graph (a) shows that the levels of the PAH in sterile soil remained high after 20 days; whilst Figure 6.4 graph (b) shows that there was minimal movement of the PAH into the uncontaminated soil. Therefore, the results in Figures 6.3 and 6.4 showed that Tween 20 increased the movement (mobilisation) of PAH.

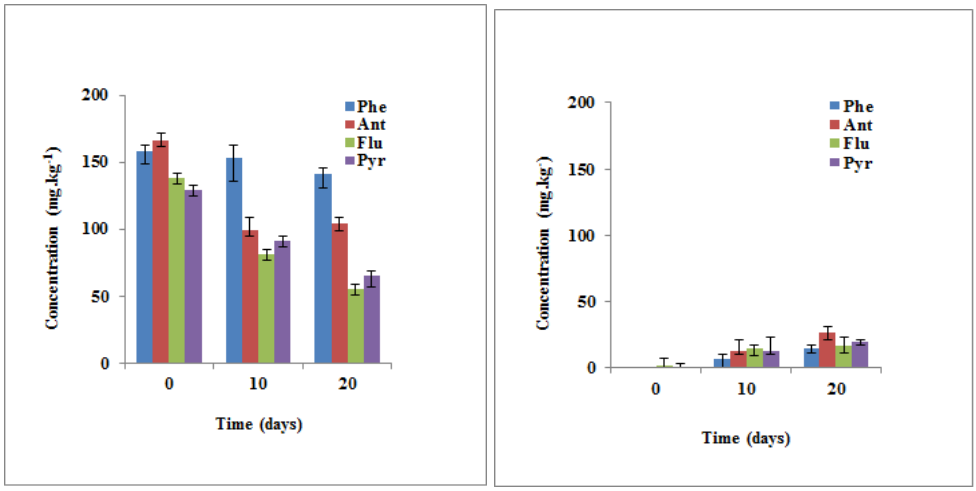


Figure 6.3 - Concentration remaining of the four PAHs over time in the soil. (a) Left side of the Petri dish. Sterile soil treated with PAH and Tween 20 and (b) Right side of the Petri dish. Sterile soil only (n=3 ± SD)

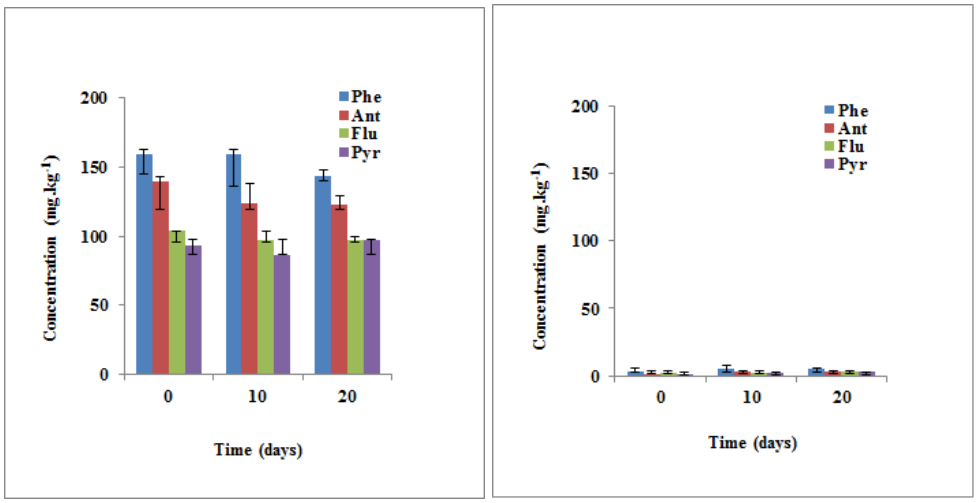
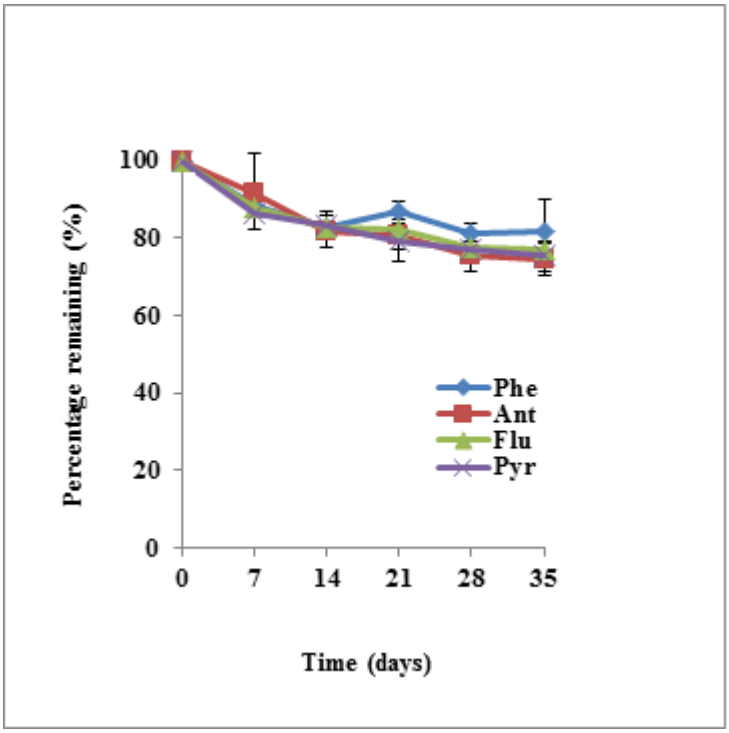


Figure 6.4 - Concentration remaining of the four PAHs over time in the soil. (a) Left side of the Petri dish. Control with PAH only and (b) Right side of the Petri dish. Control with soil only (n=3 ± SD)

Experiment ii, degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil: The effect of Tween 20 on biodegradation of the four PAHs and the impact of Tween 20 upon bacterial populations in the soil inoculated with the roadside soil at pH 7.5 was examined. Moreover, the biodegradation of the four PAHs and the bacterial population

during biodegradation in the soil inoculated with only roadside soil at pH 7.5 was investigated. In addition, the degradation of the four PAHs in the PAH contaminated sterile soil without treatment at pH 7.5 was monitored. Figures indicate the percentage remaining of the four PAHs in the soil at pH 7.5 over 35 days. Figures 6.5a, and 6.5b, shows that there was a little degradation for the first seven days. Figure 6.5a, shows that there was a little degradation in presence of Tween 20 between days 7 and 35. However interestingly in Figure 6.6a there was a fast rate of degradation between days 7 and 35 in the absence of Tween 20. Interestingly, the degradation of PAH mirrored the bacterial number. Figure 6.5b shows that there was a buildup of bacteria in the first 14 days, compared with Figure 6.6b, whereas there was a faster increase in bacterial number. Figure 6.7 shows that there was a little degradation in the sterile soil without roadside soil inoculation and Tween 20.



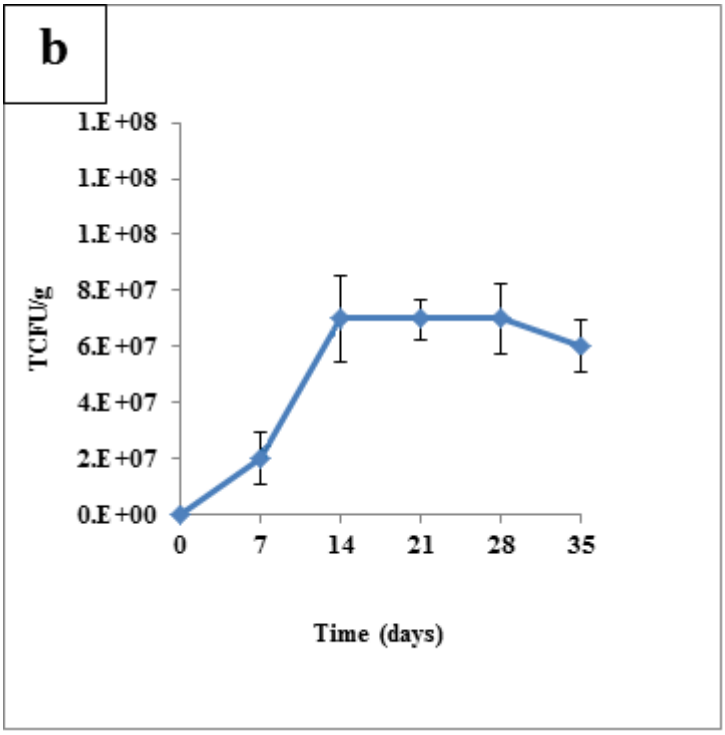


Figure 6.5 - (a) Percentage remaining of the four PAHs over time in the soil inoculated with the roadside soil and treated with Tween 20 at pH 7.5 against time (n=3 ± SD). (b) Total colony forming units of bacteria in the soil (TCFU/g) inoculated with the roadside soil and treated with Tween 20 at pH 7.5 against time (n=3 ± SD)

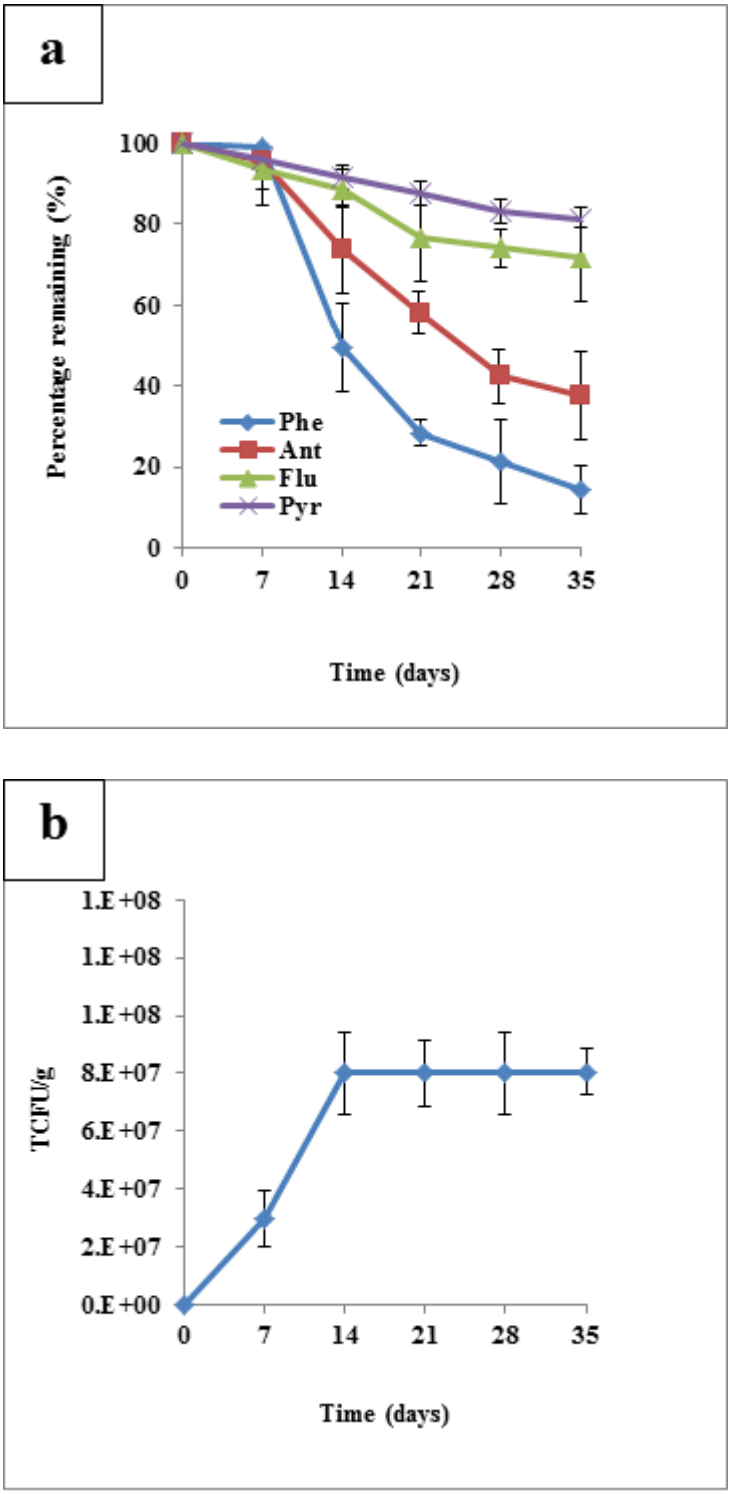


Figure 6.6 - (a) Percentage remaining of the four PAHs over time in the soil inoculated with the roadside soil but without Tween 20 at pH 7.5 against time (n=3 ± SD). (b) Total colony forming units of bacteria in the soil (TCFU/g) inoculated with the roadside soil but without Tween 20 at pH 7.5 against time (n=3 ± SD)

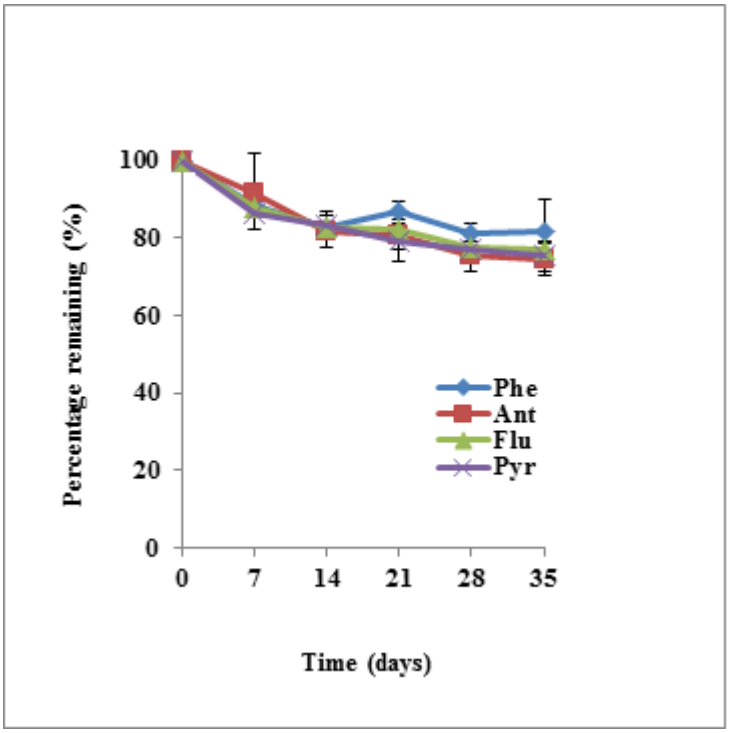


Figure 6.7 - Percentage remaining of the four PAHs over time in the sterile soil without roadside soil inoculation and Tween 20 at pH 7.5 against time (n=3 ± SD)

Table 6.4 - Percentage remaining of the four PAHs in the soil on day 35 (n=3 ± SD). * Indicates significant difference (P<0.05) between the three treatments

Chemical	Inoculated with the roadside soil and Tween 20 (% remaining)	Inoculated with the roadside soil but without Tween 20 (% remaining)	Control without inoculation of the roadside soil and Tween 20 (% remaining)
Phenanthrene	47.80*	14.27*	46.16*
Anthracene	68.40*	37.63*	74.54*
Fluoranthene	89.74*	71.67*	75.30*
Pyrene	91.80*	81.06*	81.90*

6.4 Discussion and conclusion

Experiment i, translocation of PAH in the soil: Effect of Tween 20 on translocation of the four PAHs in the J. Arthur Bower's top soil was investigated. Statistical analysis (Appendix 7.3) showed that there was a significant difference ($p<0.05$) for all the four PAHs between the soil contaminated with the four PAHs and Tween 20 on the left side of the Petri dish (Figure 6.3a) compared to the sterile soil on the right side of the Petri dish (Figure 6.3b) on days 10 and 20. There was also a significant difference ($p<0.05$) between the soil contaminated with the four PAHs on the left side of Petri dish (Figure 6.4a) compared to the sterile soil on the right side of the Petri dish (Figure 6.4b) on days 10 and 20. Moreover, statistical analysis showed that there was a significant difference ($p<0.05$) between sterile soils on the right side of the Petri dish (Figure 6.3a) compared to the sterile soil on the right side of the Petri dish (Figure 6.4b) on day 20. This indicated that Tween 20 had enhanced translocation of the four PAHs into the sterile soil (Figures 6.3 and 6.4).

The greatest translocation was found for the lowest molecular weight PAH including phenanthrene and anthracene (Figures 6.3 and 5.4). The lowest translocation was observed for the highest molecular weight PAH including fluoranthene and pyrene (Figures 6.3 and 5.4). Figure 6.3 and 6.4, show that Tween 20 (a mobilising agent) moved PAH to uncontaminated soil (Yang et al., 2000), thus the first hypothesis of this chapter was proved. Figure 6.3 shows that specific amount of PAH had been translocated from the left side of the Petri dish, however, the same amount were not quantified after 20 days from the right side of the Petri dish. This could be due to the chosen pick up points in the Petri

dish. For a better experimental design it is suggested to sample throughout the Petri dish.

Experiment ii, degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil: The effect of Tween 20 on biodegradation of the four PAHs and the impact of Tween 20 upon bacterial populations in the soil inoculated with the roadside soil at pH 7.5 was examined. Statistical analysis indicated that there was a significant difference ($p<0.05$) between the three treatments among the four PAHs on days 21, 28 and 35. This part of the studies showed that the greatest degradation was found in the treatment B, inoculated only with the roadside soil microorganisms without Tween 20 (Figure 6.6). This suggested that Tween 20 was not as effective as microbial degradation. Tween 20 had either inhibitory effect (surface active agent) on the roadside soil microorganisms and therefore less microorganisms were grown in the soil or it was easier to be used as the carbon source (due to its structure and higher bioavailability) compared to the PAH and therefore less PAH were biodegraded (Gonzalez et al., 2011).

The hypothesis for this chapter was that Tween 20, would move PAH throughout soil, potentially making the PAH more available for biodegradation. The results of this chapter prove the movement of PAH in presence of Tween 20, however, disprove the hypothesis as microbial degradation of the PAH is shown to be significantly more effective in absence of Tween 20.

Chapter 7

General Discussion and Conclusion

7.1 PAH degrader bacteria in roadside soil used as inoculum

The hypothesis for the third chapter was that roadside soil would contain PAH degrading bacteria (Johnsen & Karlson, 2005) and that these could be isolated, identified and the soil used through all experiments as inoculum. The hypothesis was met by collecting the roadside soil from an area with heavy traffic, which was shown to contain PAH degrading bacteria. Many PAH biodegrader bacteria were isolated and identified, and potentially a new PAH biodegrader bacterium and a novel genus with the potential to degrade PAH were found. Bacteria were isolated by shaken selective media using the roadside soil with the four PAHs as the sole carbon source.

Even though a broad range of bacteria have been discovered to be involved in PAH degradation (Hamme, et al. 2003; Haritash & Kaushik, 2009; Juhasz et al., 2000; Seo et al., 2009; Sinha et al., 2009), it has been observed that PAH degradation in soil is dominated by bacterial strains belonging to a very limited number of taxonomic groups including *Sphingomonas* spp., *Burkholderia* spp., *Pseudomonas* spp. and *Mycobacterium* spp. (Seo et al., 2009). However, it is notable that in this study eleven bacteria genera including *Achromobacter* spp., *Sphingobacterium* spp., *Brevibacterium* spp., *Burkholderiales* spp., *Tetrathiobacter* spp., *Arthrobacter* spp., *Bacillus* spp., *Pseudomonas* spp., *Rhizobium* spp., *Stenotrophomonas* spp. and *Ochrobactrum* spp. (Juhasz et al., 2000; Seo et al., 2009; Sinha et al., 2009) were isolated and identified by biochemical and molecular techniques from the roadside soil with the four PAHs as the sole carbon source. Potentially new PAH biodegrader bacterial species, which are not recorded in the literature including *Burkholderiales thailandensis*,

Bacillus simplex, Rhizobium mesoamericanum, Sphingobacterium shayense, Tetrathiobacter kashmirensis and additionally a species from the genus *Erwinia*, which is not recorded as a PAH degrader in the literature was identified. This roadside soil with the PAH degrader bacteria was used as inoculum throughout all experiments.

7.2 The effect of pH on bacterial degradation of PAH in soil

Little previous research was found on the effect of different pHs on biodegradation of PAH and on the bacterial populations during biodegradation. The hypothesis for the fourth chapter was that pH would influence the microbial degradation of PAH in the soil. The hypothesis was met by monitoring the rate of degradation at a range of acidic and basic pHs in the soil, and the results showed that pH 7.5 was the optimum pH. The greatest biodegradation and bacterial populations were found at pH 7.5 (Figure 4.5). In general, bacteria are suggested to be important and involved in the biodegradation of pollutants (Bastiaens et al., 2000; Johnsen et al., 2002; Ho et al., 2000). However, they are more tolerant to neutral or basic pH, therefore, the bacterial populations were expected to be greater at neutral and basic pH.

It is likely that a general increase in bacterial populations (Figure 4.5) was also linked with greater metabolic activities in soils of basic pH, which assists pollutant degradation. Although there is high pollutant mobility at low pHs, the degradation was expected to be limited due to reduced microbial activity (Chesworth, 2008) and this was confirmed in this study. This may also be due to the fact that nutrients are commonly more available at pH 7.0. At acidic pH

anionic nutrients interact with protons and are therefore less available. Likewise, at basic pH cationic nutrients have interactions with hydroxyls and are therefore less available. However, at neutral pH these interactions are generally reduced and consequently, nutrients are more available. Moreover, this study showed that acidic pHs (pH 5.0, 5.5, 6.0 and 6.5) resulted in a lower biodegradation compared to neutral or basic pHs (pH 7.0, 7.5 and 8.0). The greatest biodegradation occurred at pH 7.5 (Figures 4.1 to 4.4). High pHs resulted in a greater PAH biodegradation suggesting that pH manipulation by liming may be an effective way of stimulating biodegradation of PAH (Chesworth, 2008; Lakshmi et al., 2013).

7.3 The effect of chemical oxidation on degradation of PAH in soil

The hypothesis for the fifth chapter was that potassium permanganate oxidation of PAH will be as efficient as microbial breakdown of PAH. The hypothesis was met by examining the effect of potassium permanganate on the oxidation of the four PAHs in sterile soil treated with and without potassium permanganate solution at pHs 5.0, 6.0, 7.0 and 8.0 in the J. Arthur Bower's top soil. The effect of potassium permanganate at pH 7.5 on oxidation of the four PAHs was also compared with degradation in the soil inoculated with the roadside soil (Treatment A), microbial degradation in the soil inoculated with the roadside soil but no potassium permanganate (Treatment B) and potassium permanganate oxidation in the sterile soil (Treatment C). Chapter 5 examined the effect of potassium permanganate on oxidation of the four PAHs in the sterile soil. These studies indicated that oxidation of the four PAHs in the sterile

soil was more effective in the presence of potassium permanganate compared to the sterile soil without potassium permanganate. Moreover, studies showed that potassium permanganate oxidation has a greater effect on PAH oxidation at higher pHs (7.0 and 8.0) rather than lower pHs (5.0 and 6.0). In addition, the effect of potassium permanganate oxidation and biodegradation of the four PAHs in the soil was compared. These results suggested that potassium permanganate oxidation is not as effective as microbial degradation. The bacterial populations in this study suggested that potassium permanganate had an inhibitory effect on the roadside soil microorganisms, and therefore less microorganisms grew in the soil contaminated with potassium permanganate (Chen et al., 2009). The greatest degradation occurred when the bacterial populations were at the highest point at pH 7.5.

Silva et al. (2009b) showed that potassium permanganate reduced PAH concentration in contaminated soil. Chemical oxidation was studied as a rapid and commonly used soil or groundwater remediation technology (Silva et al., 2009b). Most PAH contaminated sites have a significant number of PAH degrader microorganisms. The bacterial population is often limited by abiotic factors such as lack of aeration, bioavailability problems, and inadequate nutrients (Straube et al., 2003). Hence, though chemical oxidation was effective for removal of PAH, it resulted in breakdown of soil organic matter and inhibited the bacterial populations (Chen et al., 2009; Silva et al., 2009b). The work reported here confirmed that bacterial populations were lower in the presence of potassium permanganate thus reduced biodegradation.

7.4 The effect of mobilising agents on degradation of PAH in soil

Finally, there was little previous research reported on the effect of mobilising agents on degradation of PAH. Chapter 6 examined the effect of Tween 20 on translocation and biodegradation of the four PAHs in the soil. The hypothesis for the sixth chapter was that Tween 20 (a mobilising agent), would move PAH throughout soil, potentially making the PAH more available for biodegradation (Allan et al., 2007; Giubilei et al., 2009; Yang et al., 2000). The hypothesis was met by investigating the effect of Tween 20 on translocation and also monitoring the effect of Tween 20 on biodegradation of the four PAHs in the J. Arthur Bower's top soil at pH 7.5. Studies indicated that Tween 20 had enhanced translocation of the four PAHs in the sterile soil. This confirmed the work of Yang et al., 2000. In addition, the effect of Tween 20 on biodegradation of the four PAHs and the impact of Tween 20 upon bacterial populations in the soil treated with the roadside soil at pH 7.5 was examined. These investigations suggested that Tween 20 is not as effective as microbial degradation. The bacterial populations suggested that Tween 20 had an inhibitory effect on the roadside soil microorganisms and therefore less microorganism were grow in the soil containing Tween 20.

7.5 General conclusion

In general, the greatest degradation was found for the lowest molecular weight PAH phenanthrene and anthracene. This indicated that the lowest molecular weight PAH degrades faster than the higher molecular weight ones

due to higher solubility and greater bioavailability This confirmed the work of Straube et al., 2003. The lowest biodegradation was found for the highest molecular weight PAH fluoranthene and pyrene. This might be related to the number of rings in PAH structure and their molecular weight. This may be due to stronger interactions between more hydrophobic and higher molecular weight PAH molecules and soil particles (Straube et al., 2003). It was observed that there was a preferential degradation or oxidation of low molecular weight PAH, based on the fact that micro-organisms prefer to degrade more soluble PAH first (Bosma et al., 1997; Ogram et al., 1985). When more than two sources of carbon are available to microorganisms, they mostly utilize the easier substrate. When the first source is completely declined or considerably destroyed, destruction of others initiates.

The literature showed broad research on the effect of different biotic and abiotic factors on degradation of PAH in soil. Considering that most of the soil in the UK and Europe are acidic (Adamson et al., 1996) and this investigation also suggested that PAH were degraded more rapidly at neutral and basic pH, and PAH mobility was minimised at neutral pH. Changing soil pH could be a suitable method to enhance biodegradation of PAH. This thesis focused upon comparing degradation of PAH using biodegradation, chemical oxidation and biodegradation in presence of mobilising agents. To conclude this thesis, low molecular PAH were degraded faster than high molecular PAH. Very little PAH oxidation was seen in the presence of potassium permanganate and it inhibited the bacterial populations. Tween 20 moved PAH in the soil, but it did not enhance the degradation as it too inhibited bacterial population.

This thesis showed that there are naturally occurring PAH degrading bacteria in roadside soil and this results in efficient biodegradation occurring at pH 7.5. Neither potassium permanganate nor Tween 20 used as a mobilising agent gave as much degradation of PAH as biodegradation alone carried out at pH 7.5.

F u t u r e w o r k

Future Work

- Isolation and identification of mixed PAH biodegrader bacteria were carried out in this thesis. However, isolation, identification and bulking of individual PAH biodegrader bacteria to investigate the most effective genus on degradation of PAH using individual microbial inocula would be a great idea.
- Studies in this thesis showed that high pHs resulted in greater PAH biodegradation suggesting that in future pH manipulation by liming may be an effective way of stimulating biodegradation of PAH.
- Application of genetically engineered microorganisms (GEM)s for bioremediation processes in field scale may show potential for bioremediation of PAH in future.
- Addition of NPK, modifying aeration or moisture content to enhance microbial activity of PAH contaminated soils and enhance microbial degradation can be investigated in future.
- Investigation of metabolic pathways of the bacterial breakdown of PAH can be study in future.
- Degradation of PAH was studied using J. Arthur Bower's top soil to maintain the constant reproducibility throughout the experiments. However, in future carrying out work in several different soil types is required to evaluate the effect of soil type on biodegradation of PAH.

R e f e r e n c e s

References

Abd-Elsalam, H., Hafez, E. E., Hussain, A. A., Ali, A. G. & El-Hanafy, A. A. (2009). Isolation and Identification of Three-Rings Polyaromatic Hydrocarbons (Anthracene and Phenanthrene) Degrading Bacteria: *American-Eurasian J. Agric. & Environ. Sci., 5* (1), 31-38.

Adamson, J. K., Rowland, A. P. & Scott, W. A. (1996). Changes in soil acidity and related variables over 25 years in the north Pennine Uplands, UK. *Soil Use Management*, *12*(2), 55-61.

Al-Daher, R., Balba, M. T. & Al-Awadhi, N. (1998). Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *Journal of Microbiological Methods*, *32*, 155-164.

Allan, I. J., Semple, K. T., Hare, R. & Reid, B. J. (2007). Cyclodextrin Enhanced Biodegradation of Polycyclic Aromatic Hydrocarbons and Phenols in Contaminated Soil Slurries: *Environ. Sci. Technol., 41,* 5498-5504.

Anderson, R. T. & Lovely, D. R. (1997). Ecology and biogeochemistry of *in situ* groundwater remediation: *AdvMicrob Ecol 15*, 289-350.

Andreoni, V. & Gianfreda, L. (2007). Bioremediation and monitoring of aromatic-polluted habitats: *Appl. Microbiol. Biotechnol., 76*, 287–308.

Antizar-Ladislao, B., Lopez-Real, J. & Beck, A. (2004). Bioremediation of Polycyclic Aromatic Hydrocarbon (PAH)-Contaminated Waste Using Composting Approaches: *Critical Reviews in Environmental Science and Technology, 34* (3), 249-289.

Antlar-LadIslao, B., Lopez-Real, J. M, & Beck, A. J. (2004). Bioremediation of Polycyclic Aromatic Hydrocarbon (PAH)-Contaminated Waste Using Composting Approaches: *Critical Reviews in Environmental Science and Technology, 34*, 249-289.

Arulazhagan, P., Vasudevan, N. (2010). Biodegradation of polycyclic aromatic hydrocarbons by a halotolerant bacterial strain *Ochrobactrum* sp. VA1: *Mar. Pollut., 62* (2), 388-94.

Asakawa, S. & Hayano, K. (1995). Populations of methanogenic bacteria in paddy field soil under double cropping conditions (rice-wheat): *biology and fertility of soils, 20* (2), 113-117.

Atagana, H. I. (2006). Biodegradation of polyacyclic aromatic hydrocarbons in contamin-ated soil by biostimulation and bioaugmentation in the presence of copper (II) ions: *World Journal of Microbial Biotechnology, 22* (11), 1145-1153.

Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbons: an environmental perspective: *Microbiol. Rev., 45*, 180-209.Bakken, L. R. & Olsen, R. A. (1987). The relationship between cell size and viability of soil bacteria: *Microbial Ecology, 13,* 103-114.

Balachandran, C., Duraipandiyan, V., Balakrishna, K. & Ignacimuthu, S. (2012). Petroleum and polycyclic aromatic hydrocarbons (PAHs) degradation and naphthalene metabolism in *Streptomyces* sp. (ERI-CPDA-1) isolated from oil contaminated soil: *Bioresource Technolgy, 112*, 83-90.

Balashova, N. V., Kosheleva, I. A., Golovchenko, N. P. & Boronin, A. M. (1999). Phenanthrene metabolism by *Pseudomonas* and *Burkholderia* strains: *Process Biochem., 35*, 291-296.

Bamforth, S. & Singleton, L. (2005). Review: Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. *Journal of Chemical Technology and Biotechnology, 80*, 723-736.

Bastiaens, L., Springael, D., Wattiau, P., Harms, H., deWacher, R., Verachtert, H. & Diels, L. (2000). Isolation of adherent polycyclic aromatic hydrocarbons (PAH)-degrading bacteria using PAH-sorbing carriers: *Applied Environmental Microbiology, 66*, 1834-1843.

Bleeker E. A, Wiegman, S., de voogt, P., Kraak, M., Leslie, H. A., de Hass, E. & Admiraal, W. (2002). Toxicity of azaarenes: *Review Environ Contam Toxicol., 173*, 39-83.

Boldrin, B., Tiehm, A. & Fritzsche, C. (1993). Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp.: *Appl. Environ. Microbiol., 59*, 1927-1930.

Boonchan, S., Britz, M. L. & Stanley, G. A. (1998). Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*: *Biotechnol. Bioeng., 59*, 482-494.

Boonchan, S., Britz, M. L. & Stanley, G. A. (2000). Degradation and Mineralization of High-molecularweight Polycyclic Aromatic Hydrocarbons by Defined Fungal-Bacterial Cocultures: *Appl. Environ. Microbiol., 66*, 1007-1019.

Bosma, T., Middeldrop, P. M., Schraa, G. & Zender, A. B. (1997). Mass transfer limitation of biotransformation: quantifying bioavailability: *Envirnmental Science & Technology, 31*, 248-252.

Bossert, I. & Bartha, R. (1984). The Fate of Petroleum in Soil Ecosystems. In Atlas, R.M. (Ed.), *Petroleum Microbiology.* (pp. 435-473). New York: Macmillan.

Brackney, K. M., Crawford, P. G. & Crawford. R. L. (1997) Ex situ biological petroleum remediation: Landfarm case history. *American Chemical Society, 214*, 21.

Brady, N. & Weil, R. (1999). *The Nature and Property of Soils*. (pp. 343-354). New Jersey: Academic Press.

Breedveld, G. D. & Sparrevik, M. (2000). Nutrient-limited Biodegradation of PAH in Various Soil Strata at a Creosote Contaminated Site: *Biodegradation, 11,* 391.

Brown, G. S., Bartonb, L. L. & Thomsonc, B. M. (2003). Permanganate oxidation of sorbed polycyclic aromatic hydrocarbons: *Waste Management, 23*, 737-740.

Burauel, P. (2012). Bioavailability of aged xenobiotic residues: *Biological Soil Functions.* Retrieved September 21, 2012, from **http://www.fz-juelich.de/ibg/ibg**

Caldini, G., Cenci, G., Manenti, R. & Morozzi, G. (1995). The ability of an environmental isolate of *Pseudomonas fluorescens* to utilize chrysene

and other four-ring polycyclic aromatic hydrocarbons: *Appl. Microbiol. Biotechnol.*, *44*, 225-229.

Carter, D., Yellowlees, D. & Tibbette, M. (2010). Moisture can be the dominant environmental parameter governing cadaver decomposition in soil: *Forensic Science International, 200*, 60-66.

Case Studies in Environmental Medicine (2009), Agency for Toxic Substances and Disease Registry (ATSDR), from http://www.atsdr.cdc.gov/csem/csem.html

Cebron, A., Faure, P., Lorgeoux, C., Ouvrard, S. & Leyval, C. (2013). Experimental increase in availability of a PAH complex organic contamination from an aged contaminated soil: Consequences on biodegradation: *Environmental pollution, 177*, 98-105.

Cerniglia, C.E. (1992). Biodegradation of polycyclic aromatic hydrocarbons: *Biodegradation, 3*, 351-368.

Chadhain, S., Norman, R., Pesce, K., Kukor, J., & Zylstra, G. (2006). Microbial Dioxygenase Gene Population Shifts during PolycyclicAromatic Hydrocarbon Biodegradation: *Applied and Environmental Microbiology, 72* (6), 4078-4087.

Chen, W., Hou, L., Luo, X. & Zhu, L. (2009). Effects of chemical oxidation on sorption and desorption of PAHs in typical Chinese soils: *Environmental pollution, 157*, 1894-1903.

Chapelle, F.H. (2001). *Ground-Water Microbiology and Geochemistry.* (2^{nd} ed.). (pp. 181-182). Printed in the United States of America.

Chesworth, W. (2008). Encyclopedia of soil science. In Chesworth, W. (Ed.), *Soil Chemistry.* (pp. 637-640). The Netherlands: Springer.

Cheung, P. & Kinkle, B. (2005). Changes in Mycobacterium spp. population structure and pyrene mineralization in polycyclic aromatic hydrocarbon-amended soils: *Soil Biology & Biochemistry, 37,* 1929-1937.

Chouychai, W., Thongkukiatkul, A., Upatham, S., Lee, H., Pokethitiyook, P. & Kruatrachue, M. (2007). Phytotoxicity Assay of Crop Plants to Phenanthrene and Pyrene Contaminants in Acidic Soil: *Environmental Toxicology*, *22*, 597-604.

Churchill, S. A., Harper, J. P. & (1999). Churchill, P. F. Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons: *Appl. Environ. Microbiol.*, *65,* 549-552.

Colwell, R. & Walker, J. (1997). Ecological aspects of microbial degradation of petroleum in the marine environment: *Crit. Rev. Microbiol. 5*, 423-445.

Cowan, S. T & Steel, K. J. (2010). Manual for the identification of medical bacteria. (pp. 20-76). Cambridge University: Academic Press.

Crow, J. (2006). The fruit fly's pheromones could be turned against it - Abstinence to control insect numbers: *Chemistry World, 3*, 34-34.

Cubitto, M. A., Morán, A. C., Commendatore, M., Chiarello, M. N., Baldini, M. D., Siñeriz, F. (2004). Effects of *Bacillus subtilis* O9 biosurfactant on the bioremediation of crude oil-polluted soils: Biodegradation, *15* (5), 281-7.

Dabestani, R., & Ivanov, I. N. (1999). A compilation of physical, spectroscopic and photophysical properties of PAHs: *Photochem. Photobiol., 70,* 10-34.

Dean-Ross, D. & Cerniglia, C. E. (1996). Degradation of pyrene by *Mycobacterium flavescens*: *Appl. Microbiol. Biotechnol., 46*, 307-312.

Dean-Ross, D., Moody, J. & Cerniglia, C. E. (2002). Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment: *FEMS Microbiol. Ecol., 41*, 1-7.

Defilippis, L. (1979). Effect of sublethal concentration of mercury and zinc on *chlorella*; V. The counteraction of metal toxicity by selenium and sulfhydryl compounds. *Journal of Plant Physiology, 93,* 63-68.

Denome, S. A., Stanley, D. C., Olson, E. S. & Young, K. D. (1993). Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* Strains: Complete DNA sequence of an upper naphthalene catabolic pathway: *J. Bacteriol., 175*, 6890-6901.

Deschenes, L., Lafrance, P. & Villeneuve, J. P. (1995). The effect of an anionic surfactant on the mobilization and biodegradation of PAHs in a creosote-contaminated soil. Hydrological Sciences, 40 (4), 471-484.

Doğan, E., Accoe, F., Boon, N., Bastiaens, L. & Dejonghe, W. (2013). Impact of Chemical Oxidation on the Heavy Metals and the Microbial Population in Sediments. *Water Air Soil Pollut, 224*, 1386.

Ferrarese, E., Andreottola, G., & Oprea, I.A. (2008). Remediation of PAH-contaminated sediments by chemical oxidation: *Journal of Hazardous Materials, 152* (1), 128-139.

Fewson, C., (1988). Biodegradation of xenobiotic and other persistent compounds: the causes of recalcitrance: *Trends Biotechnol, 6*, 148-153.

Freeman, D. J. & Cattell F. C. R. (1990), Woodburning as a source of atmospheric polycyclic aromatic hydrocarbons: *Environ SciTechnol, 24*, 1581-1585.

Fulthorpe, R., & Wyndham, R. (1989). Survival and Activity of a 3-Chlorobenzoate-Catabolic Genotype in a Natural System: *Applied and Environmental Microbiology, 55*, 1584-1590.

Ghosh, W., Bagchi, A., Mandal, S., Dam, B., Roy, P. (2005). *Tetrathiobacter kashmirensis* gen. nov., sp. nov., a novel mesophilic, neutrophilic, tetrathionate-oxidizing, facultatively chemolithotrophic betaproteobacterium isolated from soil from a temperate orchard in Jammu and Kashmir, India: Int. J. Syst. Evol. Microbiol., *55* (5), 1779-87.

Gillespie, I. M. M. & Philp, J. C. (2013). Bioremediation, an environmental remediation technology for the bioeconomy: *Trend in Biotechnology*, article in press.

Giubilei, M. A., Leonardi, V., Federici, E., Coyino, S., Sasek, V., Novotny, C., Federici, F., D'Annibale, A. & Petruccioli, M. (2009) Effect of mobilizing agents on mycoremediation and impact on the indigenous microbiota: *Journal of Chem Technol Biotechnol, 84*, 836-844.

Gonzalez, N., Simarro, R., Molina, M.C., Bautista, L. F., Delgado, L. & Villa, J. A. (2011). Effect of surfactants on PAH bioderadation by a bacterial

consortium and on the dynamics of the bacterial community during the process: *Bioresource Technology, 102*, 9438-9446.

Grosser, R. J., Warshawsky, D. & Vestal, J. R. (1991). Indigenous and enhanced mineralization of pyrene, benzo(a)pyrene, and carbazole in soils: *Appl. Environ. Microbiol., 57*, 3462-3469.

Guerin, W. F. & Jones, G. E. (1988). Mineralization of phenanthrene by a *Mycobacterium* sp.: *Appl. Environ. Microbiol., 54*, 937-944.

Hagood, M. A., Miller, D. E. & Larsen, E. C. (2012). Water holding capacities of Columbia basin irrigation project soils. Retrieved July 2, 2012, from http://ebookbrowse.com/water-holding-capacities-columbia-basin-irrigation-project-soils-pdf-d196987944

Hamme, V., Singh, A. & Ward, O. (2003). Recent Advances in Petroleum Microbiology: *Microbiology and Molecular Biology Reviews, 67*(4), 503-549.

Haritash, A. K. & Kaushik, C. P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review: *Journal of Hazardous Materials, 169*, 1-15.

Hatzinger, P. & Alexander, M. (1995). Effect of aging on chemicals in soil on their biodegradability and extractability: *Environ Sci Technol, 29*, 537-545.

Herwijnen, R., Sande, B. F., Wielen, F. E. M., Springael, D., Govers, H. A. J. & Parsons, J. R. (2003a). Influence of phenanthrene and fluoranthene on the degradation of fluorene and glucose by *Sphingomonas* sp. strain LB126 in chemostat cultures: *FEMS Microbiol. Ecol., 46*, 105-111.

Herwijnen, R., Springael, D., Slot, P., Govers, H. A. J. & Parsons, J. R. (2003b). Degradation of anthracene by *Mycobacterium* sp. Strain LB501T proceeds via a novel pathway, through *ophthalmic* acid: *Appl. Environ. Microbiol.*, *69*, 186-190.

Herwijnen, R., Wattiau, P., Bastiaens, L., Daal, L., Jonker, L. & Springael, D. (2003c). Elucidation of the metabolic pathway of fluorene and cometabolic pathways of phenanthrene, fluoranthene, anthracene and dibenzothiophene by *Sphingomonas* sp. LB126: *Res. Microbiol., 154*, 199-206.

Ho, Y., Jackson, M., Yang, Y., Mueller, J. G. & Pritchard P. H. (2000). Characterization of fluoranthene- and pyrene-degrading bacteia isolated from PAH-contaminated soils and sediments and comparison of several *Sphingomonas* spp.: *Journal of Industrial Microbiology and Biotechnology, 24*, 100-112.

Huanga, K., Hoaga, G. E., Chhedaa, P., Woodyb, B. A. & Dobbsb, G. M. (2002). Chemical oxidation of trichloroethylene with potassium permanganate in a porous medium: *Advances in Environmental Research*, 217-229.

Igwo-Ezikpe, M. N., Gbenle, O. G., Ilori, M. O., Okpuzor, J. & Osuntoki, A. A. (2010). High Molecular Weight Polycyclic Aromatic Hydrocarbons Biodegradation by Bacteria Isolated from Contaminated Soils in Nigeria: *Research Journal of Environmental Science, 4 (2)*, 127-137.

Ikenaka, Y., Sakamoto, M., Nagata, T., Takahashi, H., Miyabara, Y., Hanazato, T., Ishizuka, M., Isobe, T., Kim, J., Chang, K. (2013). Effects of polycyclic aromatic hydrocarbons (PAHs) on an aquatic ecosystem:

acute toxicity and community-level tocix impact tests of benzo(a)pyrene using lake zooplankton community: *The journal of toxicological sciences, 38* (1), 131-136.

Jain, R., Kapur M., Labana, S., Lai, B., Sarma, P., Bhattacharya, D. & Thakur I. (2005a). Microbial diversity: Application of micro-organisms for the biodegradation of xenobiotics. *Appl. Microbiol. Biotechnol., 76*, 287-308.

Jain, R., Kapur, M., Labana, S., La, B., Sarma, P., Bhattacharya, D. & Thakur, I. (2005b). Microbial diversity: Application of microorganisms for the biodegradation of xenobiotics: *Current Science, 89*(1), 101-110.

Jerina, D. M. (1983). Metabolism of aromatic hydrocarbons by the cytochrome P450 system and epoxide hydrolase: *Drug Metab Dispos 11*, 1-4.

Johnsen, A. & Karlson, U. (2005). PAH degradation capacity of soil microbial communities-Does it depend on PAH exposure? : Microbial ecology, 50, 488-495.

Johnsen, A., Wick, L. & Harms, H. (2005). Principles of microbial PAH-degradation in soil: *Environmental pollution, 133*, 71-84.

Johnsen, A. R., Winding, A., Karlson, U. & Roslev, P. (2002). Linking of microorganisms to phenanthrene metabolism in soil by analysis of 13C-labelled cell-lipids: *Applied Environmental Microbiology, 68*, 6106-6113.

Juhasz, A. L., Stanley, G. A. & Britz, M. L. (2000). Microbial degradation and detoxification of high molecular weight polycyclic aromatic

hydrocarbons by *Stenotrophomonas maltophilia* strain VUN 10,003. *Lett: Appl. Microbiol., 30,* 396-401.

Juhasz, A. L., Stanley, G. A. & Britz, M. L. (2002). Metabolite repression inhibits degradation of benzo(a)pyrene and dibenz[*a,h*]anthracene by *Stenotrophomonas maltophilia* VUN 10,003: *J. Ind. Microbiol. Biotechnol., 28,* 88-96.

Kanaly, R. A., Bartha, R., Watanabe, K., & Harayama, S. (2000a). Rapid Mineralization of Benzo(a)pyrene by a Microbial Consortium Growing on Diesel Fuel: *Applied and Environmental Microbiology, 66*(10), 4205-4211.

Kanaly, R. A., Harayama, S. & Watanabe, K. (2002b). *Rhodanobacter* sp. Strain BPC-1 in a benzo(a)pyrene-mineralizing bacterial consortium: *Appl. Environ. Microbiol., 68*, 5826-5833.

Kang, H., Hwang, S. Y., Kim, Y. M., Kim, E., Kim, Y. S. & Kim, S. K. (2003). Degradation of Phenanthrene and naphthalene by a *Burkholderia* species strain: *Can. J., Microbio., 49*, 139-144.

Kazunga, C. & Aitken, M.D. (2000). Products from the incomplete metabolism of pyrene by polycyclic aromatic hydrocarbon-degrading bacteria: *Appl. Environ. Microbiol., 66*, 1917-1922.

Kazuya, W. (2001). Microorganisms relevant to bioremediation: *Current Opinion in Biotechnology*, 237-241.

Kelley, I., Freeman, J. P., Evans, F. E. & Cerniglia, C. E. (1993). Identification of metabolites from the degradation of fluoranthene by *Mycobacterium* sp. Strain PYR-1: *Appl. Environ. Microbiol., 59*, 800-806.

Khan, A. A., Wang, R. F., Cao, W. W., Doerge, D. R., Wennerstorm, D. & Cerniglia, (2001). Molecular cloning, nucleotide sequence and expression of genes encoding a polycyclic aromatic ring dioxygenase from *Mycobacterium* sp. Strain PYR-1: *Applied Environmental Microbiology, 67*, 3577-3585.

Kim, T. J., Lee, E. Y., Kim, Y. J., Cho, K. S. & Ryu, H. W. (2003). Degradation of polyaromatic hydrocarbons by *Burkholderia cepacia* 2A-12: *World J. Microbiol. Biotechnol., 19*, 411-417.

Kim, Y. H. & Freeman, J. P. (2005). Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbaalenii* PYR-1: *Appl. Microbiol. Biotechnol., 67*, 275-285.

Kissel, D. & Miller, R. O. (2010). Cmparision of Soil pH Methods on Soils of North America: *Nutrient Management & Soil & Plant Analysis, 74* (1), 310-316.

Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Iida, T., Hayashi, H. & Takizawa, N. (1994). Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82: *J. Bacteriol., 176*, 2439-2443.

Klug, M. J. & Markovetz, A. J. (1967). Thermophilic bacteria isolated on n-tetradecane: *Nature (London) 215*, 1082-1083.

Krivobok, S., Kuony, S., Meyer, C., Louwagie, M., Willison, J. C. & Jouanneau, Y. (2003). Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: Evidence for two ringhydroxylating dioxygenase: *J. Bacteriol., 185*, 3828-3841.

Lakshmi, M. B., Perumal, V. & Velan, M. (2013). Bioremediation of phenanthrene by *Mycoplana* sp. MVMB2 isolated from contaminated soil: *Clean Soil Air Water, 41* (1), 86-93. Laurie, A. D. & Lloyd-Jones, G. (1999). Conserved and hybrid meta-cleavage operons from PAHdegrading *Burkholderia* RP007: *Biochem. Biophys. Res. Commun.*, *262*, 308-314.

Leahy, J. & Colwel, R. (1990). Microbial Degradation of Hydrocarbons in the Environment: *Microbiological reviews, 54*, 305-315.

Lease, C. W. M., Bentham, R. H., Gaskin, S. E. & Juhasz, A. L. (2011). Isolation and Identification of Pyrene Mineralising *Mycobacterium* spp. from Contaminated and Uncontaminated Sources: *Applied and Environmental Soil Science, 2011*, 11-16.

Lee, S. E., Seo, J. S., Keum, Y. S., Lee, K. J. & Li, Q. X. (2007). Fluoranthene metabolism and associated proteins in *Mycobacterium* sp. JS14: *Proteomics*, *7*, 2059-2069.

Leonardi, V., Giubilei, M. A., Federici, E., Spaccapelo, R., Sasek, V., Novotny, C., Petruccioli & M., D'Annibale, A. (2008). Mobilizing agents enhance fungal degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria in soil: *Biotechnology and Bioengineering*, *101*(2), 273-285.

Lu, J., Dang, Z., Lu, G., Yang, C., Yi, X. & Guo, C. (2012). Biodegradation Kinetics of Phenanthrene by a Fusant Strain: *Current Microbiology*, *65* (3), 225-230.

Luo, Y. R., Tian, Y., Huang, X., Yan, C. L., Hong, H. S., Lin, G. H. & Zheng, T. L. (2009). Analysis of community structure of a microbial consortium

capable of degrading benzo(a)pyrene by DGGE: *Marine Pollution Bulletin, 58,* 1159-1163.

Ma, W. F., Guo, H., Ye, J. D., Han, D. M. & Ma, X. W. (2013). Removal Efficiency and Distribution Characteristics of PAHs in Coking Plant Contaminated Soils by *In Situ* Chemical Oxidation Remediation: *Advanced Materials Research, 690-693,* 1490-1497.

Mackenzie, A., Ball, A. S. & Virdee, S. R. (2000). Life history patterns. In Hames, B.D. (Ed.): *Instant Notes in Ecology.* (p. 135). Stoodleigh: Bios Scientific Publishers.

Makkar, R. & Rockne, K. J. (2003). Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons: *Environ. Toxicol. Chem., 22,* 2280-2292.

Mallick, S., Chatterjee, S. & Dutta, T. K. (2007). A novel degradation pathway in the assimilation of phenanthrene by *Staphylococcus* sp. strain PN/Y via *meta*-cleavage of 2-hydroxy-1-naphthoic acid: formation of trans-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid: *Microbiology, 153,* 2104-2115.

Margesin, R. & Schinner, F. (2001). Biodegradation and bioremediation of hydrocarbons in extreme environments: *Ap. Microbiol. Biotechnol.,* 59, 650-663.

McNally, J. D., Mihelcic, J. & Lueking, D. (1998). Biodegradation of Three- and Four-Ring Polycyclic Aromatic Hydrocarbons under Aerobic and Denitrifying Conditions: *Environ. Sci. Technol., 32,* 2633-2639.

Miller, R. & Bartha, R. (1989). Evidence of liposome encapsulation for transport limited microbial metabolism of solid alkanes: *App. Environ. Microbiol., 55*, 269-274.

Miller, C. D., Hall, K., Liang, Y. N., Nieman, K., Sorensen, D., Issa, B., Anderson, A. J. & Sims, R. C. (2004). Isolation and characterization of polycyclic aromatic hydrocarbon-degrading Mycobacterium isolates from soil: *Microb. Ecol., 48*, 230-238.

Miller, R. O. & Kissel, D. E. (2010). Comparision of Soil pH Methods on soils of North America: *Soil Science Society of America Journal, 74* (1), 310-316.

Mitch, D. (2012). Risk Assessment: Environmental Managment. Retrieved August 15, 2012, from http://www.in.gov/idem/4144.htm

Mohamed, I. A., Khalil, N. M. & Mohamed, N. A. (2012). Biodegradation of some polycyclic aromatic hydrocarbons by *Aspergillus terreus*: *African Journal of Microbiology Research, 6*(16), 3783-3790.

Moody, J. D., Freeman, J. P., Doerge, D. R. & Cerniglia, C. E. (2001). Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. Strain PYR-1: *Appl. Environ. Microbiol., 67*, 1476-1483.

Moody, J. D., Fu, P. P., Freeman, J. P. & Cerniglia, C. E. (2003). Regio- and stereoselective metabolism of 7,12-dimethylbenz(a)anthracene by *Mycobacterium vanbaalenii* PYR- 1: *Appl. Environ. Microbiol., 69*, 3924-3931.

Muckian, L., Grant, R., Doyle, E. & Clipson, N. (2007). Bacterial community structure in soils contaminated by polycyclic aromatic hydrocarbons: *Chemosphere, 68*, 1535-1541.

Mueller, J. G, Cerniglia, C. E & Pritchard, P. H (1996). Bioremediation of environments contaminated by polycyclic aromatic hydrocarbons. In Crawford Don L. (Ed.), *Bioremediation: Principles and Applications.* (pp. 125-194). Idaho: CambridgeUniversity Press.

Mueller, J. G., Chapman, P. J., Blattmann, B. O. & Pritchard, P. H. (1990). Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*: *Appl. Environ. Microbiol., 56,* 1079-1086.

Niu, Q., Zhang, H., Li, X. & Li, M. (2013). Benzo(a)pyrene-induced neurobehavioral function and neurotransmitter alterations in coke oven workers: *Occup Environ Med, 67*, 444-448.

Ogram, A. V., Jessup, R. E., Ou, L. T. & Rao, P. S. C. (1985). Effect of sorption on biological degradation rates of (2,4-Dichlorophenoxy) acetic acid in soils. *Applied Environmental Microbiology, 49*, 582-587.

Pazos, M., Rosales, E., Alcántara, T., Gómez, J. & Sanromán, M. A. (2010). Decontamination of soils containing PAHs by electroremediation: A review: *Journal of Hazardous Materials, 177*, 1-11.

Peixoto, R. S., Vermelho, A. B. & Rosado, A. S. (2011). Petroleum-Degrading Enzymes: Biodegradation and New Prospects. SAGE-Hindawi Access to Research: *Enzyme Research, 2011* (11), 1-7.

Peng, R., Xiong, A., Xue, Y., Fu, B.X., Gao, F., Zhao, W., Tian, Y. & Yao, Q. (2008). Microbial biodegradation of polyaromatic hydrocarbons: *FEMS Microbiol Rev, 32*, 927-955.

Pepper, I. L, Gerba, C. P & Brusseau, M. L. (1996). *Pollution Science.* (pp. 36-42, 84-88). San diego CA. Academic Press.

Pierzynski, G. M., Sims, J. T. & Vance, G. F. (2000). *Soil and environmental quality*. (2nd ed.), Boca Raton: CRC Press.

Pinyakong, O., Habe, H., Supaka, N., Pinpanichkarn, P., Juntongjin, K. & Yoshida, T. (2009). Identification of novel metabolites in the degradation of phenanthrene by *Sphingomonas* sp. strain P2: *FEMS Microbiol. Lett, 191*, 115-121.

Pinyakong, O., Habe, H., Yoshida, T., Nojiri, H. & Omori, T. (2003). Identification of three novel salicylate 1-hydroxylases involved in the phenanthrene degradation of *Sphingomonas* sp. Strain P2: *Biochem. Biophys. Res. Commun, 301*, 350-357.

Pothuluri, J. V., Heflich, R. H., & Cerniglia, C. E. (1992). Fungal metabolism and detoxification of fluoranthene: *App. Environ. Microbiol., 58*, 937-941.

Prabhu, Y. & Phale, P. S. (2003). Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation: *Appl. Microbiol. Biotechnol., 61*,342-351.

Rahmann, L., Prpich, G. P. & Daugulis, A. J. (2008). Remediation of PAH contaminated soils: Application of a solid–liquid two-phase

partitioning bioreactor: Chemosphere, 73, 798-804.

Ramirez, N., Cutright, T. & Ju, L. K. (2001). Pyrene biodegradation in aqueous solutions and soil slurries by *Mycobacterium* PYR-1 and enriched consortium: *Chemosphere*, *44*, 1079-1086.

Reccardi, C., Papacchini, M., Mansi, A., Ciervo, A., Petrucca, A., Rosa, G. L., Marianelli, C., Muscillo, M., Marcelloni, A. M. And Spicaglia, S. (2005). Characterization of bacterial population coming from a soil contaminated by polycyclic aromatic hydrocarbons (PAHs) able to degrade pyrene in slurry phase: *Annals of Microbiology*, *55* (2), 85-90.

Rehmann, K., Hertkorn, N. & Kettrup, A. A. (2001). Fluoranthene metabolism in *Mycobacterium* sp. Strain KR20: Identity of pathway intermediates during degradation and growth: *Microbiology*, *147*, 2783-2794.

Rehmann, K., Noll, H. P., Steinberg, C. E. W. & Kettrup, A. A. (1998). Pyrene degradation by *Mycobacterium* sp. Strain KR2: *Chemosphere*, *36*, 2977-2992.

Robertson, S. (2011). Direct estimation of organic matter by loss on ignition method, Retrieved December 22, 2012, from http://www.sfu.ca/soils/lab_documents/Estimation_Of_Organic_Matter_By_LOI.pdf

Robles-Gonzalez, I., Fava, F. & Poggi-Varaido, H. (2008). A review on slurry bioreactors for bioremediation of soils and sediments: *BioMed Central*, *7*, 111-117.

Romero, M. C., Cazau, M. C., Giorgieri, S. & Arambarri, A. M. (1998). Phenanthrene degradation by microorganisms isolated from a contaminated stream: *Environ. Pollut.* , *101*, 355-359.

Safinowski, M. & Griebler, C. (2004). Anaerobic degradation of polycyclic aromatic hydrocarbons: *FEMS Microbiology Ecology*, *49*, 27-36.

Samanta, S. K., Chakraborti, A. K. & Jain, R. K. (1999). Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the formation of 1-naphthol: *Appl. Microbiol. Biotechno.l*, *53*, 98-107.

Samanta, S. K., Singh, O. & Jain, R. K (2002). Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation Sudip: *Trends in Biotechnology*, *20*(6), 243-248.

Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W. & Warshawsky, D. (1996). Degradation of pyrene, benz(a)anthracene, and benzo(a)pyrene by *Mycobacterium* sp. Strain RJGII-135, isolated from a former coal gasification site: *Appl. Environ. Microbiol.*, *62*, 13-19.

Schwarz, J. R., Walker, J. D. & Colwell, R. R. (1974). Deep-sea bacteria: growth and utilization of hydrocarbons at ambient and in situ pressure: *Appl. Microbiol. 28*, 982-986.

Semple, K., Morriss, W. & Paton, G.I. (2003). Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis: *Eur. J. Soil Sci.*, *54*, 809-818.

Seo, J. S., Keum, Y. S., Cho, I. K. & Li, Q. X. (2006a). Degradation of dibenzothiophene and carbazole by *Arthrobacter* sp. P1-1: *Int. Biodeter. Biodeg.*, *58*, 36-43.

Seo, J. S., Keum, Y. S., Hu, Y., Lee, S. E. & Li, Q. X. (2006b). Phenanthrene degradation in *Arthrobacter* sp. P1-1: Initial 1,2-, 3,4- and 9,10-dioxygenation, and *meta*- and *ortho*-cleavages of naphthalene- 1,2-

diol after its formation from naphthalene-1,2-dicarboxylic acid and hydroxyl naphthoic acids: *Chemosphere, 65*, 2388-2394.

Seo, J. S, Keum, Y. S. & Li, Q. X. (2009). Bacterial degradation of aromatic compounds: *International Journal of Environmental Research and Public Health, 6*, 278- 309.

Seol, Y., Zhang, H. & Schwartz, F. W. (2003). A Review of *In Situ* Chemical Oxidation and Heterogeneity: *Environmental & Engineering Geoscience, 9*, 37-49.

Sepic, E., Bricelj, M. & Leskovsek, H. (1998). Degradation of fluoranthene by *Pasteurella* sp. IFA and *Mycobacterium* sp. PYR-1: Isolation and identification of metabolites: *J. Appl. Microbiol., 85*, 746-754.

Shafiee, P., Shojaosadati, A. & Charkhabi, A. (2006). Biodegradation of Polycyclic Aromatic Hydrocarbons by Aerobic Mixed Bacterial Culture Isolated from Hydrocarbon Polluted Soils: *J. Chem. Eng., 25*, 73-78.

Silva-Castro, G. A., Rodelas, B., Perucha, C., Laguna, J., Gonzalez-Lopez, J. & Calvo, C. (2013). Bioremediation of diesel-polluted soil using biostimulation as post-treatment after oxidation with Fenton-like reagents: Assays in a pilot plant: *Science of the Total Environment, 445-446*, 347- 355.

Silva, P., Santos, E. C, Menezes, C. R., Faria, A. F., Franciscon, E., Grossman, A. & Durrant, L.R (2009a). Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia: *Bioresource Technology, 100*, 4669-4675.

Silva, P., Silva, V., Neto, B. & Simonnot, M. (2009b). Potassium permanganate oxidation of phenanthrene and pyrene in contaminated soils: *Journal of Hazardous Materials, 168,* 1269-1273.

Simarro, R., Gonzalez, N., Bautista, L.F., Sanz, R. & Molina, M.C. (2011). Optimisation of key Abiotic Factors of PAH (Naphthalene, Phenanthrene and Anthracene) Biodegradation Process by a Bacterial Consortium: *Water Air Soil Pollut, 217,* 365-374.

Simpson, J. A. (1989). *Oxford English Dictionary.* In Simpson, J. A. & Weiner, E. S. C. (Eds.), Oxford: Clarendon Press.

Sinha, S. & Chattopadhyay, P., Pan, I., Chatterjee, S., Chanda, P., Bandyopadhyay, D., Das, K., Sen, S. (2009). *Microbial transformation of xenobiotics for environmental bioremediation. African Journal of Biotechnology, 8* (22), 6016-6027.

Sirguey, C., Silva, P., Schwartz, C. & Simonnot, M. (2008). Impact of chemical oxidation on soil quality: *Chemosphere, 72,* 282-289.

Siron, R., Pelletier, E. & Brochu, H. (1995). Environmental factors influencing the biodegradation of petroleum hydrocarbons in cold seawater: *Arch. Environ. Contam. Toxicol., 28,* 406-416.

Story, S. P., Parker, S. H., Hayasaka, S. S., Riley, M. B. & Kline, E. L. (2001). Convergent and divergent points in catabolic pathways involved in utilization of fluoranthene, naphthalene, anthracene, and phenanthrene by *Sphingomonas paucimobilis* var. EPA505: *J. Ind. Microbiol. Biotechnol., 26,* 369-382.

Straif, K., Baan, R., Grosse, Y., Secretan, B., Ghissassi, FE. & Cogliano, V. (2005). Carcinogenicity of polycyclic aromatic hydrocarbons: *Lancet Oncology 6*(12), 931-932.

Straube, W. L., Nestler, C. C., Hansen, L. D., Ringleberg, D., Pritchard, P.H. & Jones-Meehan, J. (2003). Remediation of Polyaromatic Hydrocarbons (PAHs) through Landfarming with Biostimulation and Bioaugmentation: *Acta. Biotechnol., 23*, 179-196.

Sutherland, J. B., Rafii, F., Khan, A. A. & Cerniglia, C. E. (1995). Mechanisms of polycyclic aromatic hydrocarbon degradation. In Young, LY. & Cerniglia, CE. (Eds.), *Microbial Transformation and Degradation of Toxic Organic Chemicals*. (pp. 269–306). New York: Wiley-Liss.

Teng, Y., Shen, Y., Luo, Y., Sun, X., Sun, M., Fu, D., Li, Z., Christie, P. (2011). Influence of *Rhizobium meliloti* on phytoremediation of polycyclic aromatic hydrocarbons by alfalfa in an aged contaminated soil: J. Hazard. Mater., *186* (2-3), 1271-6.

Uyttebroek, M., Vermeir, S., Wattiau, P., Ryngaert, A. & Springael, D. (2007). Characterization of Cultures Enriched from Acidic Polycyclic Aromatic Hydrocarbon-Contaminated Soil for Growth on Pyrene at Low pH: *Applied and Environmental Microbiology, 73*(10), 3159-3164.

Van, D., Lee, H. & Trevors, J. (1991). Applications of microbial surfactants: *Biotechnol. Adv., 9,* 241-252.

Venosa, A. & Zhu, X. (2003). Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands: *Spill Science & Technology Bulltin, 8*, 163-178.

Vila, J., Lopez, Z., Sabate, J., Minguillon, C., Solanas, A. M. & Grifoll, M. (2001). Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. Strain Ap1: Actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons: *Appl. Environ. Microbiol. , 67*, 5497-5505.

Villemin, D., Cherqaoui, D. & Mesbah, A. (1994). Predicting Carcinogenicity of Polycyclic Aromatic Hydrocarbons from Back-Propagation Neural Network: *J. Chem. Inf. Comput. Sci., 34,* 1288-1293.

Walter, U., Beyer, M., Klein, J. & Rehm, H. J. (1991). Degradation of pyrene by *Rhodococcus* sp. UW1: *Appl. Microbiol. Biotechnol., 34,* 671-676.

Wang, B., Lai, Q., Cui, Z., Tan, T. & Shao, Z. (2008). A pyrene-degrading consortium from deep-sea sediment of the west pacific and its key member *Cycloclasticus* sp. P1: *Environ. Microbiol., 10*, 1948-1963.

Weissenfels, W. D., Beyer, M. & Klein, J. (1990). Degradation of phenanthrene, fluorene, and fluoranthene by pure bacterial cultures: *Appl. Microbiol. Biotechnol., 32*, 479-484.

Westerberg, K., Elväng, A. M., Stackebrandt, E., Jansson, J. K. (2000). *Arthrobacter chlorophenolicus* sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol: Int. J. Syst. Evol. Microbiol., *50* (6), 2083-92.

Wong, J. W. C., Lai, K. M., Wan, C. K., Ma, K. K. & Fang, M. (2001). Isolation and optimization of PAH degradative bacteria from contaminated for PAHs bioremediation: *Water Air Soil Poll.*, *139*, 1-13.

Woo, O. T., Chung, W. K., Wong, K. H., Chow, A. T. & Wong, P. K. (2009). Photocatalytic oxidation of polycyclic aromatic hydrocarbons: Intermediates identification and toxicity testing: *Journal of Hazardous Materials, 168* (2-3), 1192-1199.

Yang, Y., Ratt, D., Smets, B. F., Pignatello, J. J. & Grasso, D. (2000). Mobilization of soil organic matter by complexing agents and implications for polycyclic aromatic hydrocarbon desorption: *Chemosphere, 43*, 1013-1021.

Yamazoe, A., Yagi, O. & Oyaizu, H. (2004). Degradation of polycyclic aromatic hydrocarbons by a newly isolated dibenzofuran-utilizing *Janibacter* sp. Strain YY-1: *Appl. Microbiol. Biotechnol, 65*, 211-218.

Yu, K. S. H., Wong, A. H. Y., Yau, K. W. Y., Wong, Y. S. & Tam, N.F. Y. (2005). Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments: *Mar. Pollut. Bull*., *51*(8-12), 1071-1077.

Yuandong, Z., Shirong, L. & Jiangming, M. (2006). Water-holding capacity of ground covers and soils in alpine and sub-alpine shrubs in western Sichuan, China: *Acta Ecologica Sinica, 26*(9), 2775-2782.

Zhou, H. W., Luan T. G., Zou, F. & Tam, N. F. Y. (2008). Different bacterial groups for biodegradation of three- and four-ring PAHs isolated from

a Hong Kong mangrove sediment: *Journal of Hazardous Materials, 152*, 1179-11.

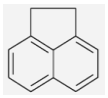
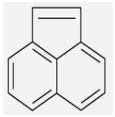
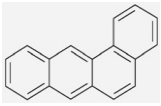
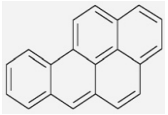
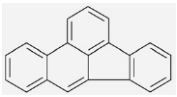
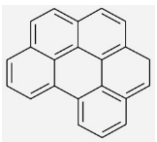
http://water.epa.gov/polwaste/sediments/cs/upload/Chem-6.pdf;

http://www.speclab.com/compound/c3324539.htm

Appendices

Appendix 1

Table 1.1 - Properties and chemical structures of some commonly studied PAHs (modifiedfrom Haritash & Kaushik, 2009; Pazos et al., 2010; Seo et al., 2009; Shafiee, 2006)

Chemical	Molecular formula	Chemical structure	Molecular weight (g/mol)	Water solubility (mg/ dm^{-3})	Melting point (°C)	Boiling point (°C)
Acenaphthene	$C_{12}H_{10}$		**154.2**	**1.93**	**95**	**277**
Acenaphthylene	$C_{12}H_8$		**152.2**	**3.93**	**92-93**	**270**
Benzo(a)anthracene	$C_{18}H_{12}$		**228.2**	**0.01**	**162**	**435**
Benzo(a)pyrene	$C_{20}H_{12}$		**252.3**	**0.002**	**179**	**495**
Benzo(a)fluoranthene	$C_{20}H_{12}$		**252.3**	**0.001 x10^{-7}**	**168.3**	**481**
Benzo(ghi)perylene	$C_{22}H_{12}$		**276.3**	**0.0002**	**273**	**550**

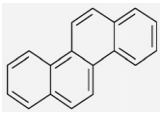
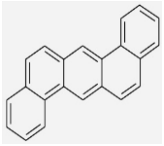
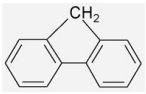
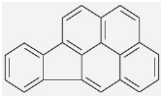
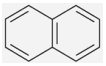
Chemical	Molecular formula	Chemical structure	Molecular weight (g/mol)	Water solubility ($mg.dm^{-3}$)	Melting point (°C)	Boiling point (°C)
Chrysene	$C_{18}H_{12}$		**228.3**	**0.002**	**255**	**448**
Dibenz(a,h)anthracene	$C_{22}H_{14}$		**278.3**	**0.0005×10^{-10}**	**262**	**524**
Fluorene	$C_{13}H_{10}$		**166.2**	**1.68-1.98**	**116**	**295**
Indeno(1,2,3 cd)pyrene	$C_{22}H_{12}$		**276.3**	**0.06**	**163.6**	**530**
Naphthalene	$C_{10}H_8$		**128.1**	**-**	**80.50**	**218**

Table 1.2 - Various remediation processes that alter PAH in the environment (Hamme et al., 2003; Straube et al., 2003)

Remediation	Technology	Comment
Biological: Degradation of PAH by microorganisms **Factors effecting:** • **Nutrient** • **Pressure** • **Temperature** • **Moisture** • **pH** • **Oxygen** **Organic matter content** • **Microbial community present** • **Bioavailability of PAH** • **Structure of PAH** • **Molecular weight of PAH**	**Biostimulation**	**Addition of nutrients/carbon/surfactants to stimulate indigenous microorganisms at contaminated site**
	Bioaugmentation	**Inoculation of specific microorganisms to improve the metabolic capacity of the indigenous microorganisms at contaminated site**
	Bioreactors	**Application of natural and specialized microorganisms in controlled environmental and nutritional conditions – High biodegradation rates – nonhazardous residues – Cost effective – on site**
	Biopiling	**Application of indigenous microorganisms, nutrients and air – Slow degradation rates – year round operation difficult – potential to contaminated ground/surface water**
	Bioventing or volatilisation	**A combination of advective soil venting and biodegradation method for *in situ* treatment of contaminated soil – Most of the LMW hydrocarbons are volatilised**
	Biosparging	**Injection of air into the ground water/saturated zone**
	Composting	**Addition of nutrients/oxygen and moisture in a controlled system – Commonly used for treatment of municipal solid wastes**

	Landfarming	**Addition of nutrients/carbon source, mixing soil to better distribute, injecting oxygen into soil at depth and increasing the chance of contact between microbes and PAH - Slow degradation rates – Year round operation difficult – Potential to contaminated ground/surface water – Inexpensive**
	Phytoremediation	**Application of plants and rhizospheric microorganisms to contaminated site – Most likely cost effective – On site strategy**
	Surfactants	**Enhancing PAH solubility by improving desorption, aqueous mobility and bioavailability – Tween 20/80, cyclodextrins, vegetable oil, etc.**
Physicochemical: Conversion of PAH by physicochemical processes **Factors effecting:** **Same factors as for biological remediation plus intensity and duration of exposure to sunlight or UV in terms of photo oxidation**	**Chemical oxidation**	**Application of chemical oxidants into a contaminated site – The most commonly used oxidants are permanganate, ozone, peroxide and persulphate**
	Incineration	**High temperature treatment – Air pollution risks – expensive control equipment – high cost**
	Photo oxidation	
	Soil washing	**Injection of washing mixture including of water surfactants into the surface to transfer pollutant from soil to liquid phase. The solution is then recovered with an extraction system**
	Soil vapor extraction (SVE)/venting/air stripping	**The pollutant is stripped from soil with a vacuum through pipe or wells. The gaseous product is then treated with catalytic or thermal combustion chambers coupled to activated charcoal filters – Potential to pollutants with high**

		vapor pressure
	Solvent extraction	**Application of solvents and centrifugation or filtration for the separation of pollutant form contaminated site – Hazard of solvent use – High cost**
	Thermal desorbtion	**High/low temperature pollutant removal and recovery method – High cost – nonhazardous residues**

Appendix 2

Table 2.1 - Properties of the HPLC machine used

Properties of the HPLC machine used	
Data Analysis software	**Chromeleon 32® Chromatography Manager 3.2**
Mobile phase/solvent	**Accetonitrile:Milli-Q/de-ionised water (90:10)**
Flow rate	**0.8 cm^3/min**
Column	**C16 (4.6x250 mm) and C18 (150x4.60 mm)**
Detector	**Ultraviolet/Visible detector (UV/VIS D 170 U)**
Run time	**17- 20 minutes**
Temperature	**20 ºC**
Elution	**Isocratic**

Appendix 2.1 HPLC analysis (standard chromatograms)

The peaks appear in the order carbozole, phenanthrene, anthracene, fluoranthene and pyrene, respectively. This reflects the number of rings and molecular weight of each PAH and their relative solubility. The three benzene ringed phenanthrene and anthracene therefore appear earlier than fluoranthene and pyrene. Phenanthrene and anthracene have the same molecular weight and are thus isomers. However, anthracene is a linear molecule and phenanthrene is not. The water solubility for phenanthrene and anthracene is 1.2 and 1.3mg.dm^{-3}, respectively, due to linear structure of anthracene increasing its hydrophobicity and therefore decreasing solubility which leads to it eluting from the HPLC column slower in the acetonitrile:Milli-Q water mobile phase, compared with phenanthrene (Andreoni & Gianfreda, 2007). Fluoranthene and pyrene appear later than phenanthrene and anthracene. They have four rings in their chemical structure. Pyrene has symmetry in its structure. Pyrene is a linear molecule. However, fluoranthene is not (Andreoni & Gianfreda, 2007). They both have a molecular weight of 202.2 g.mol^{-1}. Fluoranthene and pyrene have water solubility of 0.2 and 0.1, respectively. They have lower solubility in extraction solution. Pyrene is more insoluble due to its high hydrophobicity. Phenanthrene and anthracene are the same in molecular weight and number of rings, and so is fluoranthene in respect to pyrene. However, all four PAHs used in this study have different molecular structures (linear or nonlinear), which affects on their hydrophobicity and water solubility. PAH with low molecular weights are more rapidly degraded compared to higher molecular weights and less soluble molecules (Andreoni & Gianfreda, 2007).

Appendix 2.2 HPLC analysis (standard curve)

Standard curve include peak area against PAH concentration. The curve was plotted using the peak area, which obtained from the four PAHs standards at different concentrations. The r^2 (regression coefficient value) and Y value (slope) for all four PAHs was observed. In all chromatograms first, second, third and fourth peaks were anthracene, phenanthrene, fluoranthene and pyrene, respectively. PAH concentrations in samples were calculated against standard curves and the percentage remaining of PAH were calculated using the internal standard as a correction factor. The mean values were calculated for replicates and standard deviation was quantified.

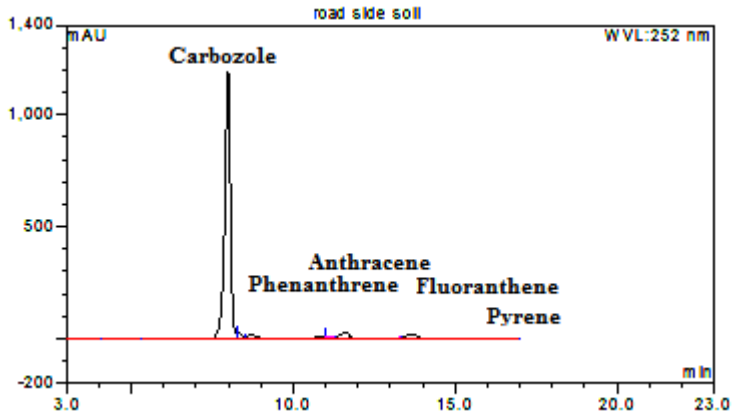


Figure 2.1 - PAH analysis of the roadside soil

Appendix 2.3 Preparation of the nutrient agar culture

Nutrient agar (11.2 g) powder was weighed and added into a 500 cm^3 glass bottle and then distilled water added to make up 400 cm^3 suspension. The bottle was autoclaved (15 min, 15 psi, 121 ºC). After allowing the bottle to get cool 20

mg of mycostatin/nystatin was measured and added to suspension and mixed well. The suspension was then poured into plastic Petri dishes and allowed to solidify.

Table 2.2 - Calculation for preparation of the nutrient agar culture

N.A (g)	Suspension (cm^3)
28	**1,000**
11.3	**400**

Appendix 3

Appendix 3.1 Preparing minimal salt medium

The media containing 8.8 g of $Na_2HPO_4.2H_2O$, 3 g of $KHPO_4$, 1 g of NH_4Cl, 0.5 g of NaCl, 1 cm^3 of 1 M $MgSO_4$, and 2.5 cm^3 of a trace element solution ([per litre] 23 mg of $MnCl_2.2H_2O$, 30 mg of $MnCl_4.H_2O$, 31 mg of H_3BO_3, 36 mg of $CoCl_2.6H_{2O}$, 10 mg of $CuCl_2.2H_2O$, 20 mg of $NiCl_2.6H_2O$, 30 mg of $Na_2MoO_4.2H_2O$, and 50 mg $ZnCl_2$) (pH 7.0) per litre of Milli-Q water (Bastiaens, 2000).

Appendix 3.2 Biochemical tests

a. Gram staining test

The Gram staining test divides bacteria into two major groups: Gram positive and Gram negative. Fresh cultures of isolated bacteria were grown in nutrient broth for 48 hours incubation at 30 ^{0}C. A dried bacterial smear was prepared by applying a drop of sterile water and inoculating the culture on glass slide and heat fixing it. It was then stained with crystal violet for 30 seconds followed by washing with tap water. The slide was then flooded by Lugol's iodine for 30 seconds followed by washing with 95 % alcohol until the washings were virtually colourless. Further, the slides were washed with tap water and followed by carbon fuchsin addition for 10 seconds. The final step was carried out by washing the slide with tap water. The bacteria were observed at X1000 magnification on light microscopy (Meiji EMT model number: 18089), Gram positive bacteria appeared purple while Gram negatives were pink. Simultaneously, the shape, size and presence of spores were assessed.

b. Catalase test

A 3 % hydrogen peroxide (H_2O_2) solution was applied as the catalase reagent. The presence of catalase enzymes can be detected by adding small amount of culture to a slide containing the reagent. An immediate production of gas bubbles was indicative of a positive reaction.

c. Oxidase test

A 1 % tetramethyl p-phenylenediamine aqueous solution stored in the dark in a refrigerator in a glass stoppered bottle covered with alumminium foil was used as the oxidase reagent. To perform the test, two drops of reagent was added on a piece of 7 cm diametre filter paper in a Petri dish. The isolated culture was smeared across the impregnated paper with loop. The reagent was colourless. However, appearance of a dark purple colour within 10 seconds indicated the presence of cytochrome C as an electron transport enzyme resulted into positive reaction test.

d. Glucose metabolism

A 1 % glucose and 15 g peptone per litre of distilled water was used as glucose metabolism reagent. The solution was dispensed in 5 ml universal bottles (4 cm^3 in each) and autoclaved. The bottles were inoculated with isolated bacteria and incubated at 30 ^{0}C for seven days. Acid production from glucose break down was specified by a pink colour.

e. Oxidation or fermentation (O-F) test

An O-F medium containing 2.0 g peptone, NaCl 5.0 g, K_2HPO_4 0.3 g, agarose 3.0 g and 0.2 % aquous solution of bromothymole blue made up to 100 cm^3 in distilled water was heated to 95 ^{0}C and 1 % glucose was then added to the medium. This mix was dispensed to test tubes (9 cm^3 each). The tubes were autoclaved and inoculated in duplicates by stabbing with a straight nichrome wire. A layer of melted sterilised paraffin of 1 cm depth was added to one of the duplicates to create an anaerobic environment. The tubes were incubated at 30 ^{0}C for 14 days. Production of a yellow colour in any tube was an indication of glucose oxidation. If both open and sealed tubes turned yellow it was an indication of fermentation while an unchanged sealed tube and yellow open tube was an indication of oxidation and no change in either tubes destined that bacteria did not metabolise glucose at all.

Table 3.1- Primer details

Name	**27F**	**1492R**
Sequence (5^- to 3^-)	**AGA GTT TGA TYM TGG CTC AG**	**TAC GGY TAC CTT GTT ACG ACT**
Molecular Weight (µg. µmole^{-1})	**6168.5**	**6380.7**
Micromolar Extinction Coeff (OD/µmole)	**222.4**	**218.9**
Number	**E2148B04**	**E2148B05**
Length	**20**	**21**
Scale of Synthesis (n mol)	**25**	**25**
µg per OD	**27.7**	**29.2**
nmoles per OD	**4.5**	**4.6**
Purity	**Desalted**	**Desalted**
Tm (1 M Na^+)	**66**	**68**
Tm (50 mM Na^+)	**45**	**46**
% GC	**45**	**45**
OD's	**4.70**	**6.00**
µg's*	**130.39**	**174.93**
nmoles	**21.2**	**27.4**
Coupling Eff. (%)	**99**	**99**

Table 3.2 - Standard PCR reagents

Reagent	Volumes (µl)
Sterile distilled water	38.8-X
10x reaction buffer	5
25 mM $MgCl_2$	3
10 mM dNTP	1
25 µM primer 1	1
25 µM primer 2	1
Taq Pol (5U/µl)	0.2
DNA template	X

Table 3.3 - Purity and bacterial concentration of genomic DNA; 10 µl of double stranded DNA sample + 40 µl of sterile TE buffer. Absorbance was measured at 230, 260, 280 and 320 nm. Measurement of absorbance and concentration was carried out in a spectrophotometer.

Isolate	A* 260/280 (nm)	A* 260/230 (nm)	A* 230 (nm)	A* 260 (nm)	A* 280 (nm)	A* 320 (nm)	Concentration ($\mu g.cm^{-3}$)
1	1.58	1.27	1.28	1.63	1.03	0.09	408
2	1.78	1.87	0.07	0.14	0.08	0.00	36
5	1.71	1.86	0.08	0.16	0.09	0.01	40
6	1.68	2.16	0.09	0.19	0.11	0.00	50
7	1.77	4.49	0.39	1.77	1.00	0.00	444
10	1.62	1.88	0.09	0.17	0.10	0.01	43
12	1.83	0.78	0.19	0.15	0.08	0.01	38
13	1.85	0.84	0.19	0.16	0.08	0.01	41
14	1.74	3.71	0.53	1.99	1.14	0.03	499
17	1.76	3.78	0.50	1.99	1.13	0.02	500
20	1.79	4.19	0.43	1.80	1.01	0.00	452
21	1.76	2.12	0.08	0.17	0.09	0.00	43
25	1.88	10.5	0.06	0.63	0.33	0.01	159
27	1.89	10.9	0.05	0.64	0.33	0.01	160
28	1.77	4.46	0.39	1.77	1.00	0.00	445
29	1.78	4.43	0.40	1.78	1.00	0.00	447
37	1.77	2.12	0.08	0.17	0.09	0.00	44
42	1.78	4.16	0.43	1.82	1.02	0.00	455
44	1.78	4.14	0.44	1.82	1.02	0.00	456
45	1.78	4.17	0.43	1.80	1.01	0.00	451

A* = Absorbance

Appendix 3.1 16S ribosomal RNA gene, partial sequence of the isolated bacteria followed by the primer used

Isolate 1: *Achromobacter piechaudii* strain TZ4: 27F

>9939257.seq - ID: ZK-1-27F-3 on 2012/7/9-15:26:15 automatically edited with PhredPhrap, start with base no.: 15 Internal Params: Windowsize: 20, Goodqual: 19, Badqual: 10, Minseqlength: 50, nbadelimit: 1

TTannatGCaGTcgacgGCAGcAcGGACTTCGGTCTGGTGGCGAGTGGCGA
ACGGgtgAGTAATGTATCGGAACGTGCCTagtAGCGGGGGATAAcTACG
CGAAAGCGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGG
GATCGCAAGACCTTGCACTATTAGAGCGGCCGATATCGGATTAGCTA
GTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTTTG
AGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGA
TCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCAC
TTTTGGCAGGAAAGAAACGTCATGGGCTAATACCCCGTGAAACTGAC
GGTACCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
TGCGCAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACT
TTGGAACTGCATTTTTAACTACCGAGCTAGAGTGTGTCAGAGGGAGG
TGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGGAGGAACA
CCGATGGCGAagGCAGCCTCCTGGGATAACACTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACcCTGGTAGTCCACGCCC
TAAACGATGTCAACTAGCTGTTGGGGCCTTcngGCCTtnnTAGCGCancT
AACGCGTGAAGTTGACCGCCTGgGGAGTACGGTCGCAAGATTAAnAC
TCAaAGGAATTGACggGGACCcgCACAancggTgaaTGATGtggATTaaTTcna
TGcnacnnnnananACCTTACcTACCCTtnacaTGTc

Isolate 1: *Achromobacter piechaudii* strain Shan11: 1492R

aCgngGTaTCGCcCcCCttgCGGTtAgGCtAACTACTTCTGGTAAAACCCACT
CCCatggtGTGACGGGCGgtgTgtACAAGACCCGGGAACGTATTCACCGCG
ACATGCTGATCCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAG
TTGCAGACTGCGATCCGGACTACGATCGGGTTTCTGGGATTGGCTCC
CCCTCGCGGGTTGGCGACCCTCTGTCCCGACCATTGTATGACGTGTGA
AGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACCTTC
CTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCTTTCGTAGCAAC
TAATGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCAC
GACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCcagTTCTCTTG

CGAGCACTTCCAAATCTCTTCGGAATTCCAGACATGTCAAGGGTAGG
TAAGGTTTTTCGCGTTGCATCGAATTAATCCACATCATCCACCGCTTG
TGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTC
CCCAGGCGGTCAACTTCACGCGTTAGCTGCGCTAcnaAGGCCCGAAGG
CCCCAACAGCTAGTTGACATCGTTTAGGGCGTGGACTACCAGGGTAT
CTAATCCTGTTTGCTCCCCACGCTTTCGTGCATGAGCGTCAGTGTTAT
CCCAGGAGGCTGCCTTCGCCATCGGTGTTCCTCCGCATATCTACGCAT
TTCACTGCTACACGCGGAATTCCACCTCCCTCTGACACACTCTAGCTC
GGTAGTTAAAAATGCAGTTCCAAAGTTAAgcTCTGGGATTTCACATcttT
CTTTCCGAACCGCCTGCGCACGCTTTACgcCCAGTAATTCCgattAACGC
TTGCACCCTACGTATTACcgcnncTGCTGGCACGTantTAGCcGgtgCTTAT
TCTGCAGGTACcgncAGTTTcACGGGGTattagccCAtGACGTTTcTTTCCTG
CcAAAanngcttTACAACC

Isolate 2: *Sphingobacterium shayense* strain HS39: 27F

ctaanatGCaGTcgaCGGgatTTcagTGTAGCTTGCTACGCTGAATGAGAGTGG
CGCACGGGTGCGTAACGCgtgaGCAACctACCcAtatCAGGGGGATAGCCC
GgagAAATCCGGATTAACACCGCATAACATTACCGGatGGCATCATTTG
GTAATCAAATATTTATAGGATATGGATGGGCTCGCgtgaCATTAGCTAG
tTGGAGAGGTAACGGCTCACCAAGGCTACGATGTCTAGGGGCTCTGA
GAGGagaATCCCCCACACTGGTACTGAGACACGgaCCAGACTCCTACG
GGAGGCAGCAGTAAGGAATATTGGTCAATGGAGGCAACTCTGAACC
AGCCATGCCGCGTGCAGGATGACTGCCCTACGGGTTGTAAACTGCTT
TTGTCTAGGAATAACCCTTGGTACgagTACCGAGCTGAATGTACTAGA
AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG
AGGATCCGAGCGTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGG
CGGCCTGTTAAGTCAGGAGTGAAATACGGCAGCTCAACTGTCGCAGT
GCTCTTGATACTGATGGGCTTGAATATCGCTGAAGATGGCGGAATGA
GACAAGTAGCGGTGAAATGCATAGATATGTCTCAGAACACCGATTGC
GAAGGCAGCTGTCTAAACGATTATTGACGCTGATGCACGAAAGCGTG
GGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGAT
GATAACTCGATGTTAGCGATATACtGTTAGCGTCCAAGCGAAAGCGTT
AAGTTATCCACCTGGGGAGTACGCCCGCAAGGGTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGAGGAGTATGTGGTTTAATTCGA
TGATACGCGAGGAACCTTACCCGGGCTTGAAAGTTAGTGAAGGTAGC
AGagaCGCTACCGTCCTTCGGGannCGAAacTAGGTGCTGCATGGCTGTC
GTCAGCTCGTGCCGTGaggtgTTGGGTTAAGTCCcgcAACgnnnncAaCCCnt
ATGTTTAGTTGCCAGcnnAtaATGgtnGGGgacTCTAAaCAg

Isolate 2: *Sphingobacterium shayense* strain HS39: 1492R

acgCTCttgCGGTtacAtGCTTtAGgcacCCCCAACTTtcaTGGCTTGACGGGcG
gtgTGTACAAGGCCCGGGAACGTATTCACCGCGTCATTGCTGATACGC
GATTActAGCGAATCCAACTTCACGGGGTCGAGTTGCAGACCCCGATC
CGAACTGTGAATGGCTTTTAGAGATTAGCATGACATTGCTGTCTAGCT
GCCCGcTGTACCATCCATTGTAGTACGTGTGTAGCCCCGGACGTAAGG
GCCATGATGACTTGACGTCGTCCCCGCCTTCCTCACTGCTTGCGCAGG
CAGTCTGTTTAGAGTCCCCACCATTATGTGCTGGCAACTAAACATAG
GGGTTGCGCTCGTTGCGGgACTTAACCCAACACCTCACGGCACGAGC
TGACGACAGCCATGCAGCACCTAGTTTCGTGTCCCGAAGGACGGTAG
CGTCTCTGCTACCTTCACTAACTTTCAAGCCCGGGTAAGGTTCCTCGC
GTATCATCGAATTAAACCACATACTCCTCCGCTTGTGCGGGCCCCCGT
CAATTCCTTTGAGTTTCACCCTTGCGGGCGTACTCCCCAGGTGGATAA
CTTAACGCTTTCGCTTGGACGCTAACaGTATATCGCTAACATCGAGTT
ATCATCGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATC
CCCACGCTTTCGTGCATCAGCGTCAATAATCGTTTAGACAGCTGCCTT
CGCAATCGGTGTTCTGAGACATATCTATGCATTTCACCGCTACTTGTC
TCATTCCGCCATCTTCAGCGATATTCAAGCCCATCAGTATCAAGAGC
ACTGCGACAGTTGAGCTGCCGTATTTCACTCCTGACTTAACAGGCCG
CCTACGCACCCTTTAnACCCAATAAATCCGGATAACGCTCGGATCCTC
CGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGATCCTTATTCTTCT
AGTACATTCAGCTCGGTAcncGTACCAagGGTTATTCCTAGAcaAAAgCA
GTTTACAACCCgTAGGgCAGTCATCCTgcacgcGGCATGGctggtTCagnntTG
CctCCATTGACCAatATTCCTTActnntg

Isolate 5: *Sphingobacterium* sp. MOL-1: 27F

ctAtgctgAAtgaGAgtgGCGCACgGgngcgtAACGcgtgagCAAcctACCcaTATCA
GGGGGATAGCCCGgagaAAtCCGGAttAACACCGCATAACATTACCggntG
GCATCATttggtAATCAAATATTTATAGGATATGGATGGGctCGCgtgaCAT
TAGctagttggaGagGTAAcGGCTCACCAagGCTACGatgTCTAGGGGctctgaga
GGagAATCCCCCACACtgGtactgAGACACGGACCaGACTCCTACGGgaGG
CAGCAGTAAGGAATATTGGTCAATggaGGCAACTctgAACCAGCCATGC
CGCgtGCAGGATGACTGCCCTACGGGTTGTAAACTGCTTTTGTCTAGG
AATAACCCtTGGTACgtgTACCGAGCTGAATGTACTAGAAGAATAAGG
ATCGGCTAACTCCgtGCCAGCAGCCGCGGTAATACGGAGGATCCGagC
GTTATCCGGATTTATTGGGTTTAAAGGGTGCGTAGGCGGCCTGTTAAg
TCAGGAGTGAAATACGGCAGCTCAACTGTCGCAGtGCTCTTGATACTG
ATGGGCTTGAATATCGCTGAAGATGGCGGAATGAGaCAAGTAGCGGT
GAAATGCATAGATATGTCTCAGAACACCGATTGCGAAGGCAGCTGTC
TAAACGATTATTGACGCTGATGCACGAnAGCGTGgGGATCAAACAGG

ATTAGATACCCTGGTAGTCCACGCCCTAAACGATGATAACTCGATGT
TAGCGATATACTGTTAGCGTCCAAGCGAAAGCGTTAAGTTATCCACC
TGgGGAGTACGCCCGCAAGGGTGAAACTCAnAGGAATTGACGGGggC
CCGCACAAGCGGaGGAGTATGTGgTttaATTCGaTGATACGCgaangaACC
TTACCCGGGCTTGAAAGTTAGTGAAGGTAGCAnAanaCGCTACCGTccT
TCGgGana

Isolate 5: *Sphingobacterium* sp. MOL-1: 1492R

Sequencing failure.

Isolate 6: *Brevibacterium epidermidis* strain CJ-12: 27F

TGcAGTCgnacGcTGAnGCCcg

Isolate 7: *Burkholderiales* sp. B101R-3: 27F

TCggAAaGAaanAtgTnAAATCcCAnancTTAaCTTTggAaCTgCaTTTTTaaCTa
CcnagcTananTGTGtcnnAGGGAGGTGgantTccnCGTGTa

Isolate 7: *Burkholderia thailandensis* MSMB43 Scaffold30_1: 1492R

gGGTTTctGGGattggCtCCCCCTcccGGgttggcgaCCCTctgt

Isolate 10: *Tetrathiobacter kashmirensis* strain AZDF-2: 27F

TtannatGCaGTcgaCGGCAGCGGgnAAGTAGCTTGCtacTTTTGCCGGCGAG
TGGCgaaCGGGTGAgtAATgtaTCGGAACGTGCCCagTAGCGGGGGaTAAC
tACGCGAAAGCgtgGCTAATACCGCATACGCCcTACGGGGGAAAGGGG
GGGATCTTAGGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCt
aGTTGGtGGGgTAAAGGCCTACCAAGGCGACGATCCGtaGCTGGTTTGA
gaGGACGACCAGCCACACTGGGACTGAgaCACGGCCCAGACTCCTACG
GGaGGCAGCAgTGGGGAATTTTGGACAATGGGGGAAACCctGATCCAG
CCATCCCGCGTGTGCGATGAAGGCCTTCGGGTTGTAAAGCACTTTTGT
CAGGGAAGAAAAGGTTTCGGATAATACCCGGAACTGATGACGGTAC
CTGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGC

AGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGG
AACTGCATTTTTAACTACCGAACTAGAGTATGTCAGAGGGGGGTGGA
ATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAACACCGA
TGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCATGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAA
CGATGTCAACTAGCTGTTGgGCCCTTCGGgGCTTAGTAGCGCAGCTAA
CGCGTGAAGTTGACCGCCTGGgGAGTACGGTCGCAAGATTAAnACTC
AAAGGAATTGACGGggACcCGCAcaaGCGGTGGATGATGTGGATTAATT
CGATGCAACGCGAAnAACCTTACCTACCCTTGAcatGTCTGGAATCCTG
AannnaTttaGGAGTGctnnnAAGannaACCggaAcacagGTGCTGCATGGCTGTc
ntc

Isolate 10: *Tetrathiobacter kashmirensis* strain AZDF-2: 1492R

tgtGAcGGGCggtgtgtACAagaCCCGGGaACgtaTTCAcCGCGACATGCTGAT
CCGcgATTActaGCGATTCCGActTCatgCAGGCGagtTgCAGCCTGCAATCC
GgActACGATCGGGTTTATGagATTaGCTCCACCttgCGgntTgggggcCCTCtg
TCCCGACCAttgTATGACgtgTGAAGCCCTACCCATAAGGGCCATGAGG
ACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCAT
TAGAGTGCTCAACTAAATGTAGCAACTAATGACAAGGGTTGCGCTCG
TTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCA
TGCAGCACCTGTGTTCCGGTTCTCTTGCGAGCACTCCTAAATCTCTTC
AGGATTCCAGACATGTCAAGGGTAGGTAAGGTTTTTCGCGTTGCATC
GAATTAATCCACATCATCCACCGCTTGTGCGGGTCCCCGTCAATTCCT
TTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGTCAACTTCACGC
GTTAGCTGCGCTACTAAGCCCCGAAGGGCCCAACAGCTAGTTGACAT
CGTTTAGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAC
GCTTTCGTGCATGAGCGTCAGTATTATCCCAGGGGGCTGCCTTCGcCA
TCGGTGTTCCTCCACATATCTACGCATTTCACTGCTACACGTGGAATT
CCACCCcCCTCTGACATACTCTAGTTCGGTAGTTAAnAATGCAGTTCC
AAGGTTGAGCCCTGggaTTTCACATCTTTCTTTCCGAACCGCCTGCGCA
CGCTTTACGCCCAGTAATTCcGattAACGCTTGCACCCTACGTATTAcnnc
GGCTGCTGGcaCGTanntannncngnncttaTTCTTCAGGTACcnncAtca

Isolate 12: *Arthrobacter aurescens*: 27F

tannatGCaGTcgaCGaTGaTccCaGCTTGCTGGGGgATTAGTGGCGAACGGG
TGagtAACACGTGAgtAACCTGCCCTTGACTCTGGGATAAGCCTGGGAA
ACTGGGTCTAATACCGGATATGACTATCTGACGCATGTCAGGTGGTG
GAAAGCTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTtgTTGGT

GGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGG
GTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA
GTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGC
TAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTAT
CCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTG
CTGTGAAAGACCGGGGCTCAACTCCGGTTCTGCAGTGGGTACGGGCA
GACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAA
ATGCGCAGATATCAGGAGGAACACCGATGGCGAagGCAGGTCTCTGG
GCATTaaCTGACGCtgAGGAGCGAAAGCATGgGGAGCGAACAGGATTA
GATACcCTGGTAGTCCATGCngTAnACGTTGgGCACTnngTGtggGGGaCA
TTCCAcgtTtTCnncGCcnnagCTAACGCATTAnntGCCCcgccctg

Isolate 13: *Bacillus* sp. WYT035: 27F

ggTncTatanatGCaGTCGaGCGaTCGAtGGGaGCTTGCTCCcTGAGATTAGC
GGCGGACGGgtgAGTAACACGTGGGCAACCTGCCTATAAGACTGGGAT
AACTTCGGGAAACCGGAGCTAATACCGGATACGTTCTTTTCTCGCAT
GAGAGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGGCCC
GCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGA
TGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTT
CGGGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCAGAGTAAC
TGCTGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAA
GCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAG
TGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTan
aGATTTGGAGGAACACCAGTGGCGAannCGACTTTCTGGTCTGTAACT
GACACTGAGGCGCGaAAgcGtggGGAGCAAACAGGATTAgATACCCTggt
agTCCAcnccGTAa

Isolate 13: *Bacillus subtilis* strain b17a: 1492R

tgtnncTTagGcgGCtGGCTcCnAtgaAGGTTACCTCACCGACTTCGGGTGTTACAAA
CTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGaACGTATTCACCGC
GGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGC
AGCCTGCAATCCGAACTGAGAATGGCTTTATGGGATTCGCTTACCTTCGCAG
GTTTGCAGcncTTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAA

GGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCA
GTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCG
CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA
TGCACCACCTGTCACTCTGTCCCCCGAAGGGGAAAGCCCTATCTCTAGGGTT
GTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAAC
CACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCC
TTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAA
AGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTT
ACAGACCaGAAAGTCGCctTCgcCACTGGTGTTcCTCCaaATCTCTACGCATTTC
ACCGCtacACTTGgnnntCCACTTTCCTCTTCTGCactCAAGTTCcccagttTCCAAtGA
cnnTCcaCGGTTGAGCCGtgng

Isolate 14: *Erwinia* sp. E280d: 1492R

tGCAacccaCTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAA
CGTATTCACCg

Isolate 17: *Arthrobacter nitroguajacolicus*: 27F

atGCagtcgacgaTGaTCCcaGCTTGCTGGGGgatTAGTGGCGAACGGGTGAG
TAACACGTGAgtAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAACT
GGGTCTAATACCGGATACGACCATCTGGCGCATGTCATGGTGGTGGA
AAGCTTTTGTGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTG
GGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGG
TGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAG
TAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGC
TGTGAAAGACCGGGGCTCAACTCCGGTTCTGCAGTGGGTACGGGCAG
ACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAA
ATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTG
GGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGA
TTAGATACCCTGGTAGTCCATGCCGTAnACGTTGGGCACTAGGTGTGG
gGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCG
CCTGGgGAGTACGGCCGCAAGGCTAAnACTCAnAGGAATTGACGGGG
GCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGA
AGAACCTTACCAAGGCTTGACATGAACCGganAgaCCTGGgAAACAGG

TGCCCcnctTgtggtcngtTTACAGGTGGTGCAtgggtTGtcnnnnanntcnnGtcgnnnn
naTGTTGGGGTTAAGtccCCGCAACGAAGCGCAACCcnncGTTCTAt

Isolate 17: *Arthrobacter nitroguajacolicus* strain S58: 1492R

cagggnttagggCCACCGGCtTcgGgtgttcCAACTTTcgtgAcntnaCGGGCGGTgtG
TACAAGGCCCGGGaAcgtATTCACCGCAGcgttgctgATctGCGAttactagCGA
CTCCGACTTCATGGGGTCGagtTGCAGACCCCAATCCGAActgAGACCG
GCTTTTtGGGAttAGCTCCACCTCACAGTATCGCAACCCTTtgTACCGGC
CATtgtAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGA
CGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCTATGAGT
CCCCGCCATAACGCGCTGGCAACATAGAACGAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGC
ACCACCTGTAAACCGACCGCAAGCGGGGCACCTGTTTCCAGGTCTTT
CCGGTTCATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTA
ATCCGCATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGT
TTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAG
CTACGGCGCGGAAAACGTGGAATGTCCCCCACACCTAgtgCCCAACGT
TTACGGCATggACTACCaggGTATCTAATCCtGTTCGCTCCCCATGCTTT
CGCTCCTCAGCGTCAGTTACAGCCCAGAGACCtgCCtTCGCCATCGgngT
TCCTCCTGATATCTGCGCATTTCACCgctacncnaGGAATTCcnntCTCcccCT
acgg

Isolate 20: *Bacillus* sp. O-NR1: 27F

gctanaCntGCaGTCGaGcgATCgangGGaGCTTGCTCCCTGAGATTAGCGGC
GGAcGGgtGAGTAACACGTGGGCAACCTGCCTATAAGACTGGGATAAC
TTCGGGAAACCGGAGCTAATACCGGATACGTTCTTTTCTCGCATGAG
AGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGGCCCGCG
GCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTTCGG
GTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCAGAGTAACTGC
TGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGC
AGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGcgTAnaGA

TTTGGAGGAACACCAGTGgcgAaggCGACTTTCTGGTCTGTAACTGACA
CTGAGGcgcnnaAGCGTGGGga

Isolate 20: *Bacillus* sp. K3-D6L: 1492R

ggcgGCtgGCTccatgaagGTTACCTCACCGACTTCGGGTGTtacAAACTCTCG
TGGTGTGACGGGCGgtgTGTACAAGGCCCGGGAACGTATTCACCGCGG
CATGCTGATCCGCGATTACTAGCGATTCCGGCTTCAtGCAGGCGAGTT
GCAGCCTGCAATCCGAACTGAGAATGGCTTTATGGGATTCGCTTACC
TTCGCAGGTTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTC
CGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGC
AACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGTCCC
CCGAAGGGGAAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGAC
CTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCG
CTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAAGGGC
GGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACC
AGGGTATCTAATCCTGTTTGCTCCCCACGCTtTCGCGCCTCantGTCagTT
ACAGACcanAnAGTCGCCTTcgCCACTGGTGTTCCTCCAAATCTCTAcgc
ATTTCACCGCTACACTTGgaATTCcaCTTTCCTCTTCTGCACTCAAGTTC
CCCAGTTtCCAATGACcCTCCacGGTTGAGCCGTGggctttnnnaTCAgACTT
AangAACCAcCTG

Isolate 21: *Bacillus simplex* strain A1-6: 27F

gctaTanatGCaGTcgaGCGATCGatgGGAGCTTGCTCCCTGAGATTAGCGGC
GGACGGgtGAGTAACACGTGGGCAACCTGCCTATAAGACTGGGATAA
CTTCGGGAAACCGGAGCTAATACCGGATACGTTCTTTTCTCGCATGA
GAGAAGATGGAAAGACGGTTTACGCTGTCACTTATAGATGGGCCCGC
GGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTTCG
GGTCGTAAAGTTCTGTTGTTAGGGAAGAACAAGTACCAGAGTAACTG
CTGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT
TGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAagnCTGATGTGAAAGCC
CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGC

AGAAGagGAAAGTGGAATTCCAAGTGTAACGGTGAAATGCGTanaGAT
TTGgaggAACACCAGTGGcgAAGGcgACTTTCTGgtcTGTAACTGACACTg
aggcgnnaAagcGTGGGgagCAAACaggatTAGatacCctgg

Isolate 21: *Bacillus simplex* strain ARI: 1492R

cttaGgcggCtGGCTcatgaagGTTACCTCACCGACTTCGGGTGTTACAAACTC
TCgtGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCG
CGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCG
AGTTGCAGCCTGCAATCCGAACTGAGAATGGCTTTATGGGATTCGCT
TACCTTCGCAGGTTTGCAGCCCTTTGTACCATCCATTGTAGCACGTGT
GTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCT
TCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGC
TGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAAC
ATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTG
TCCCCCGAAGGGGAAAGCCCTATCTCTAGGGTTGTCAGAGGATGTCA
AGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGG
CCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAA
GGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGAC
TACCAGGgtATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTG
TCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCanaTC
TCTACGCATTTCACCGCTacACTTGgaaTTCCACTTTCCTCTTCTGCACT
CAAGTTCCcCAGTTTCCAATGACCCTCCAcggTTGAGCcgtGGGcntTcacA
TCanaCTTaanGAAccaCCtg

Isolate 25: *Pseudomonas* sp. XjGEB-1: 27F

GgcTananatGCaGTCGaGCGGatganaggAGCTTGCTCCTGGATTcaGCGGCG
GACGGGtgAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACG
TTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAG
GGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGC
TAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTC
TGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG
ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCA
CTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGA
CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCG
GTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAAC

CTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGT
GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAA
CACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAnACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGC
GCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGT
TAAAACTCAnATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATC
CAATGAACTTTCcAGagaTGGATTGGTGCCTTCGGGaACATTGagacaGGT
GCTGCATGGCTGTCGTCAGCTCGTGTcGTGAGATGTTGgGTTAAGTCCc
GTAACGagcgcaaCCCTTGTCCTTAGTTACCAGCACGTAATGGTGGGcacT
CTAagnng

Isolate 25: *Pseudomonas* sp. JDG23: 1492R

gaGGnTTagaCTAGctaCTTCTgGTGCAACCCactCCCatggtgngACGGGCGgtgt
GtACAAGGCCCGGGaAcgtATTCACCGCGACATTctGATTcGcGAttactaGC
GATTCCGACTTCACGCagtCGAGtTGCAGActgCGATCCGGACTACGATC
GGTTTTATgGGATTAGCTCCACCTCGCGGCtTGGCAACCCTTtgTACCG
ACCATtgTAGCACGTgtgTAGCCCAGGCCGTAAGGGCCAtgATGACTTGA
CGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGT
GCCCACCATTACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTA
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGC
AGCACCTGTCTCAATgctCCCGAAGGCACCAATCCATCTCTGGAAAGT
TCATTGGATGTCAAGGCcTGGTAAGGTTCTTCGCGTTGCTTCGAATTA
AACCACATGCTCCACCGCttgTGCGGGCCCCCGTCAATTCATTTGAGTT
TTAACCTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGcgtTAgctgC
GCCACtaAGAGCTCAAGGCTCCCAACGGCTAgtTGACATcgtTTACGGCgt
ggaCTACCagggtATCTAATCCtgtttgCTCcCCACgCtTTCGCACCTCantgtcaG
TATCantCCAgggTGgTCGcctTCGCcccTGGT

Isolate 27: *Rhizobium* sp. CCNWYC119: 27F

GctaCnnatGCaGTcgaGCGGatganaGGagCTTGCTCCTGGATTCAGCGGCGG
ACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGT
TTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAG
GGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGC
TAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTC
TGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG

ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCA
CTTTAAGTTGGGAGGAAGGGCAGTAAATTAATACTTTGCTGTTTTGA
CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCG
GTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAAC
CTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTATGGTAGAGGGT
GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAA
CACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTG
CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAnACGATGTCAACTAGCCGTTGGGAGCCTTGAGCTCTTAGTGGC
GCAGCTAACGCATTAAGTTGACCGCCTGGgGAGTACGGCCGCAAGGT
TAaaACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATC
CAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGaaCATTGAGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTgAGATgttGGGTTAAGT
CCcGTAACGAGCGCAACcctTGTCCTTantTACCAGcanGTAATGg

Isolate 27: *Rhizobium* sp. CCNWYC119: 1492R

TaCGTCcnccgaGGTTAGACTAGCTACTTCTGGTGCAACCCACTCCCATG
gtgTGACGGGCGgtgTgtACAAGGCCCGGGAACGTATTCACCGCGACATT
CTGATTCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCA
GACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTC
GCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCC
CAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCC
GGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATTACGTGCTGGT
AACTAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAATGCTCC
CGAAGGCACCAATCCATCTCTGGAAAGTTCATTGGATGTCAAGGCCT
GGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCT
TGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC
TCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAGAGCTCA
AGGCTCCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGG
GTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTA
TCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTAC
GCATTTCACCGCTACACAGGAnATTCCACCACCCTCTACCATACTCTA
GCTTGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCAC
ATCCAACTTAACAAAcCACCTACGCGCGCTTTACGCCCAGTAATTCCG
ATTAACGCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTA
GCCGGTGCTTatTCTGTCGGTAACGTcaaAACAGCAAAGTATTAATTTA
CTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCcnnagacCTTCTTCana
nncncGGCnnggCTGGAtCagGCTTTcnnCCATTg

Isolate 28: *Stenotrophomonas maltophilia* strain A3: 1492R

cATGGTGTGACGGGCGGTGTGTAcaagGCCCGGGAACGTATTCACCgnnn
nnntGCTGATCTGCGattacTAGcgat

Isolate 29: *Ochrobactrum* sp. c279: 27F

cCnCACtGgGactGAGAcaCggnccnnActCctacnggagggngca

Isolate 29: *Ochrobactrum* sp. Cr13(2012): 1492R

ctGCCTCCTTGCGGTTAGCAcannnnnccTTCGGGTAAAACCAACTCCCAT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGG
CATGCTGATCCGCGATTACTAGCGATTCCAACTTCATGCACTCGAGTT
GCAGAGTGCAATCCGAACTGAGATGGCTTTTGGAGATTAGCTcgcACT
CGCGTGCTCGCTGCCCACTGTCACCACCATTGTAGCACGTGTGTAGCC
CAGCCCGTAAGGGCCATGAGGACTTGACGTCATCCCCACCTTCCTCTc
ggcttatcaccggc

Isolate 37: *Rhizobium* sp. L6-8: 27F

AaagATTTatCggcaaAgGAtCgGCCCgcgtTGnattnnntanntgga

Isolate 37: *Rhizobium mesoamericanum* strain 5m: 1492R

ctcnnatgGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GCggcatgCTgannngcgATTACTAGCGATTCcancttcatncnctcgagtTgcagnatGcaa
T

Isolate 42: *Pseudomonas aeruginosa* strain B2: 1492R

GCGCCttCGGgtAAAACCAactCCcatggtGtGACGGGCGgtgtgtACAAGGCCC
GGGAACGTATTCACCGCGGCATGCTGATCCGCGATTActAGCGATTCC
AaCTTCAtgCACTcgaGTTGCAGagtGCAATCCGAActgagATGgctTTTGgagat
tAgctCACACtCGCgtgCTCgctGCCCActgtCACCACCATtgtAGCACgtgTGTA
GCCCAGCCCGTAAGGGCCAtGAGGacttgACGTCATCCCCACCTTCCTCT
CGGCtTATCACCGGCAGTCCCCTTAGAGTGCCCAACTAAATGCTGGCA

ACTAAGGGCGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCAGCACCTGTATCCGGTCCAGC
CGAACTGAAAGACACATCTCTGTGTCCGCGACCGGTATGTCAAGGGC
TGGTAAGGTTCTGCGCGTTGCTTCGAATTAAACCACATGCTCCACCGC
TTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTA
CTCCCCAGGCGGAATGTTTAATGCGTTAGCTGCGCCACCGAAGAGTA
AACTCCCCAACGGCTAACATTCATCGTTTACGGcGTGGACTACCAGG
GTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGCGTCAGTA
ATGGTCCAGTGAGCCGCCTTCGCCACTGGTGTTCCTCCGAATATCTAc
gAATTTCACCTCTACACTCGGAATTCCACTCACCTCTACCATACTCAA
GACTTCCAGTATCAnAGGCAGTTCcGGGGTTGAGCCccgGGATTTCACC
CCTGACTTAnAAgtCCGCCtACgTGCGCTTTAcgCCCAGTAnATCnnaanaan
ncTAGCccCCTTCGTATTACCGCGGCTGCTGg

Isolate 44: *Ochrobactrum* sp. c268: 27F

tGcAGTCGaaCGGTCTCttcggnngcngtGngnnnnngtggnt

Isolate 44: *Ochrobactrum* sp. MS8: 1492R

ttctnngTAAAACcaaCTCCCATGGTGTGACGGGCGGTGTGTACAAggcCCG
GGAACGTATTCACCGCggcATGCTGATCCGCGATTACTAGCGATTCcaa
CTTCatgCACTCGAGTTGCAGagtGcAATCCgaaCtgagATGgcttTtg

Isolate 45: *Bacillus flexus*: 1492R

aTGCTGATccgcgATTACTAGCGATtc

Appendix 4

Appendix 4.1 Determination of water-holding capacity in the J. Arthur Bower's top soil

A) Mass of hilgard soil cup + Mass of filter paper + Mass of dried soil = 75.01 g

B) Mass of hilgard soil cup + Mass of filter paper = 65.93 g

C) Mass of dried soil = A-B = 09.08 g

D) Mass of hilgard soil cup + Mass of filter paper + Mass of saturated soil = 81.52 g

E) Mass of hilgard soil cup + Mass of filter paper = 65.93 g

F) Mass of saturated soil= D-E = 15.59 g

G) Mass of water content in saturated soil = F-C = 6.51 g

H) Percentage of water-holding capacity = G/F*100 = 41.75 %

Table 4.1 - The amount, volume and concentration of chemicals used for the soil contamination

Chemical	Chemical added in solution (mg)	n-hexane volume in solution (cm^3)	Chemical concentration in solution ($mg.dm^{-3}$)	Soil (g)	Chemical final concentration in soil ($mg.kg^{-1}$)
Phenanthrene	100	1,000	100	1,100	91
Anthracene	100		100		91
Fluoranthene	100		100		91
Pyrene	100		100		91

Table 4.2 - Adjustment of the soil moisture content to 30 % of the water-holding capacity

Soil (g)	Milli-Q water (cm^3)	Moisture content (%)
100	41.75	100
100	12.52	30
1,100	138	30

Table 4.3 - pH determination of the soil

Soil (g)	Milli-Q water (cm^3)	1 M HCl (µl)	0.1 M Na_2CO_3 (µl)	pH
5.0	9.89	105	-	5.0
5.0	9.90	100	-	5.5
5.0	9.95	52	-	6.0
5.0	9.97	30	-	6.5
5.0	10.00	-	-	7.0
5.0	9.92	-	8	7.5
5.0	9.98	-	15	8.0

Table 4.4 - Adjustment of pH and water content of the soil to 30 % of water-holding capacity

Soil (g)	Milli-Q water (cm^3)	1 M HCl (cm^3)	0.1 M Na_2CO_3 (cm^3)	pH
1,100	115	23.10	-	5.0
1,100	116	22.00	-	5.5
1,100	127	11.44	-	6.0
1,100	132	6.60	-	6.5
1,100	138	-	-	7.0
1,100	136	-	1.76	7.5
1,100	135	-	3.30	8.0

Table 4.5 - Preparation of standard solutions at the concentrations of 1, 10, 20, 30, 40 and 50 $mg.dm^{-3}$

Standard solution concentration ($mg.dm^{-3}$)	PAH standard stock solution with con. of 100 $mg.dm^{-3}$ (µl)	Carbozole stock solution with con. of 100 $mg.dm^{-3}$ (µl)	Acetonitrile (cm^3)
1	200	4,500	Appropriate volume to make the solution up to 20 cm^3
10	2,000	4,500	
20	4,000	4,500	
30	6,000	4,500	
40	8,000	4,500	
50	10,000	4,500	

Table 4.6 - Weights of the conical flasks after each three days to monitor water-holding capacity. The units are based on gram.

Week 1 / 1st					
	Replicate				
pH	A	B	C	D	E
5.0	334	317	326	322	314
5.5	336	330	337	312	318
6.0	339	329	329	358	316
6.5	338	359	322	309	333
7.0	333	310	325	310	332
7.5	315	333	317	321	313
8.0	339	326	312	321	308

Week 1 / 2nd					
	Replicate				
pH	A	B	C	D	E
5.0	325	308	318	314	305
5.5	327	322	327	304	310
6.0	331	320	320	349	307
6.5	328	349	312	301	325
7.0	324	301	315	301	322
7.5	306	323	307	312	304
8.0	329	317	304	321	298

Week 2 / 1st					
	Replicate				
pH	A	B	C	D	E
5.0	316	300	310	307	297
5.5	319	314	318	296	304
6.0	322	312	312	341	299
6.5	321	342	305	293	317
7.0	317	294	308	294	315
7.5	299	316	300	304	297
8.0	322	310	297	314	291

Week 2 / 2nd

	Replicate				
pH	**A**	**B**	**C**	**D**	**E**
5.0	**323**	**306**	**316**	**312**	**306**
5.5	**325**	**321**	**326**	**303**	**309**
6.0	**331**	**319**	**319**	**349**	**308**
6.5	**326**	**349**	**311**	**299**	**325**
7.0	**322**	**301**	**315**	**300**	**322**
7.5	**306**	**323**	**306**	**312**	**304**
8.0	**328**	**317**	**302**	**321**	**299**

Week 3 / 1st

	Replicate				
pH	**A**	**B**	**C**	**D**	**E**
5.0	**323**	**306**	**316**	**312**	**306**
5.5	**325**	**321**	**325**	**302**	**308**
6.0	**330**	**319**	**319**	**348**	**307**
6.5	**326**	**348**	**311**	**299**	**324**
7.0	**322**	**300**	**314**	**299**	**321**
7.5	**305**	**323**	**306**	**311**	**303**
8.0	**327**	**317**	**302**	**320**	**298**

Week 3 / 2nd

	Replicate				
pH	**A**	**B**	**C**	**D**	**E**
5.0	**314**	**297**	**307**	**303**	**297**
5.5	**316**	**312**	**316**	**293**	**300**
6.0	**312**	**310**	**310**	**339**	**298**
6.5	**316**	**340**	**302**	**290**	**316**
7.0	**312**	**291**	**305**	**290**	**312**
7.5	**295**	**313**	**297**	**302**	**295**
8.0	**318**	**307**	**292**	**309**	**290**

Week 4 / 1st

	Replicate				
pH	**A**	**B**	**C**	**D**	**E**
5.0	**314**	**297**	**307**	**303**	**297**
5.5	**317**	**313**	**318**	**294**	**300**
6.0	**322**	**310**	**311**	**340**	**299**
6.5	**318**	**342**	**303**	**241**	**315**
7.0	**314**	**291**	**308**	**241**	**313**
7.5	**297**	**315**	**298**	**303**	**295**
8.0	**318**	**308**	**293**	**309**	**291**

Week 4 / 2nd

	Replicate				
pH	**A**	**B**	**C**	**D**	**E**
5.0	**305**	**288**	**299**	**294**	**289**
5.5	**308**	**308**	**309**	**281**	**291**
6.0	**314**	**302**	**303**	**332**	**290**
6.5	**309**	**333**	**294**	**282**	**307**
7.0	**305**	**283**	**299**	**283**	**305**
7.5	**288**	**306**	**289**	**295**	**286**
8.0	**309**	**299**	**284**	**301**	**282**

Week 5 / 1st

	Replicate				
pH	**A**	**B**	**C**	**D**	**E**
5.0	**305**	**288**	**298**	**294**	**288**
5.5	**307**	**307**	**308**	**282**	**291**
6.0	**312**	**301**	**308**	**332**	**290**
6.5	**308**	**333**	**294**	**282**	**305**
7.0	**304**	**282**	**299**	**282**	**303**
7.5	**288**	**306**	**289**	**294**	**286**
8.0	**309**	**299**	**284**	**300**	**282**

Week 5 / 2nd					
	Replicate				
pH	A	B	C	D	E
5.0	296	280	289	283	282
5.5	299	299	300	273	283
6.0	304	292	293	322	281
6.5	300	324	286	274	297
7.0	296	273	290	274	294
7.5	279	297	280	285	277
8.0	300	290	275	291	273

Appendix 4.2 - HPLC analysis (standard chromatograms)

Standard HPLC chromatograms were obtained of the four PAHs used at six different concentrations of 1, 10, 20, 30, 40 and 50 mg.dm^{-3} dissolved in acetonitrile. Carbozole at a concentration of 22.5 mg.dm^{-3} was also added as an internal standard. The charts show height (mAU) on x axis against retention time (min) on y axis. The wave length used was 252 nm.

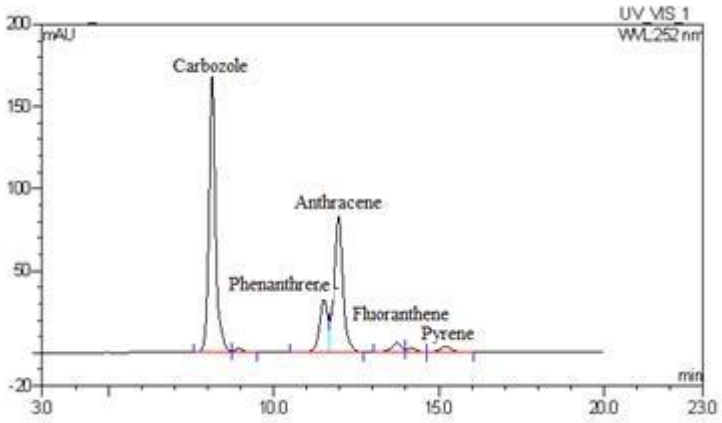


Figure 4.1 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 1 and 22.5 mg.dm^{-3}, respectively

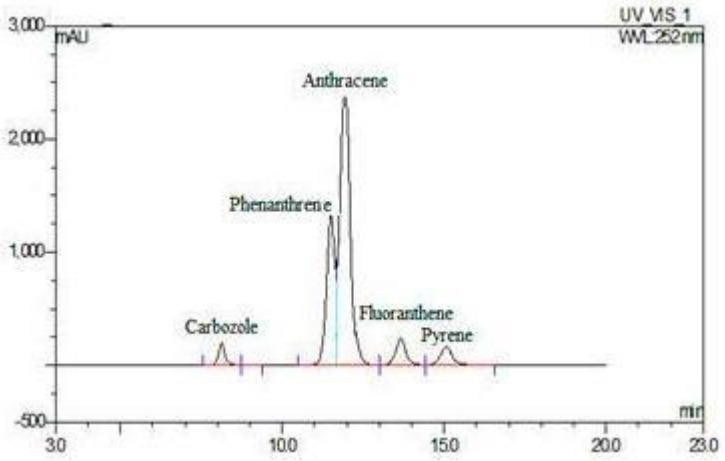


Figure 4.2 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 50 and 22.5 mg.dm^{-3}, respectively

Appendix 4-Figures 4.1 and 4.2 shows that the first peak to elute was carbozole with a retention time of 8.14 minutes. The peak areas for carbozole were 38.63 (Appendix 4-Figure 4.1) and 46.09 (Appendix 4-Figure 4.2) mAU/min at the concentration of 1 and 50 mg.dm^{-3}, respectively. Phenanthrene appeared as the second peak to elute with a retention time of 11.52 minutes with a peak area of 9.35 (Appendix 4-Figure 4.1) and 390.12 (Appendix 4-Figure 4.2) mAU/min at 1 and 50 mg.dm^{-3}, respectively. The third peak was anthracene with a retention time of 11.96 minutes with a peak area of 25.97 (Appendix 4-Figure 4.1) and 919.06 (Appendix 4-Figure 4.2) mAU/min at 1 and 50 mg.dm^{-3}, respectively. In the both Figures phenanthrene and anthracene peaks overlaped before reaching the x axis. Chromeleon software was employed to statistically split the joined peaks of phenanthrene and anthracene. Fluoranthene was the forth peak to elute with a retention time of 13.72 minutes in the both Figures and the peak area of 2.05 (Appendix 4-Figure 4.1) and 87.70 (Appendix 4-Figure 4.2) mAU/min at 1 and 50 mg.dm^{-3}, respectively. The final peak was pyrene with a retention time of 15.21 minutes and the peak area of 1.56 (Appendix 4-

Figure 4.1) and 72.82 (Appendix 4-Figure 4.2) mAU/min at 1 and 50 mg.dm^{-3}, respectively.

Appendix 4.3 HPLC analysis (standard curves)

Data for peak area against the known concentrations of the four PAHs (Appendix 4.2) was used to plot standard curves for the four PAHs. The concentrations used were 1, 10, 20, 30, 40 and 50 mg.dm^{-3}, providing a range above the anticipated maximum concentration of PAH after re-extraction from the soil. The linear regression for each PAH was calculated by the chromeleon software. The r^2 values (regression coefficient value) for all the PAH were above 0.969. The Y value describes gradient of the slope. The r^2 values for phenanthrene, anthracene, fluoranthene and pyrene were 0.98, 0.97, 0.97 and 0.97, respectively. The Y values for phenanthrene, anthracene, fluoranthene and pyrene were 7.66x, 18.63x, 1.40x and 1.40x, respectively.

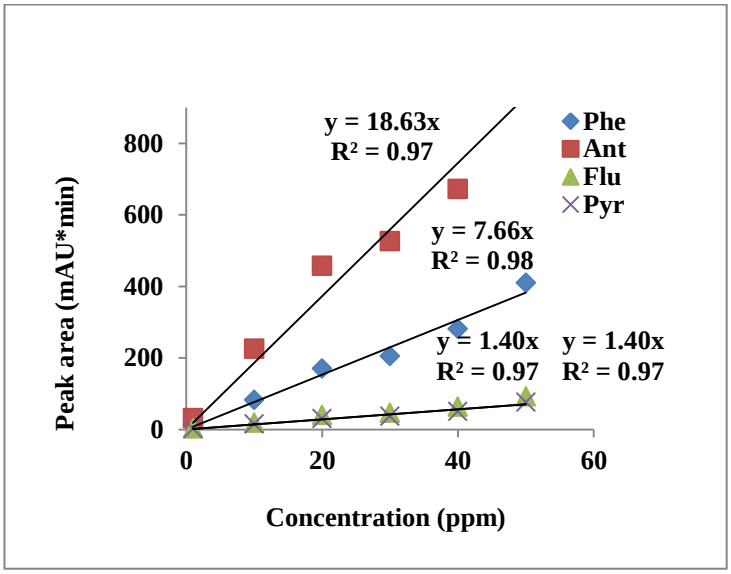


Figure 4.3 - HPLC standard curves for the four PAHs showing peak area against concentration

Appendix 5

Appendix 5.1 - Determination of water-holding capacity in the J. Arthur Bower's top soil

Refer to Appendix 4.1.

Table 5.2 - Experiment i (oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate), the amount, volume and concentration of chemicals used for the soil contamination

Chemical	Chemical added in solution (mg)	n-hexane volume in solution (cm^3)	Chemical concentration in solution ($mg.dm^{-3}$)	Soil (g)	Chemical final concentration in soil ($mg.kg^{-1}$)
Phenanthrene	250		250		250
Anthracene	250		250		250
		1,000		200	
Fluoranthene	250		250		250
Pyrene	250		250		250

Table 5.3 - Experiment i (oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate), pH adjustment of the soil

Soil (g)	0.09 M Potassium permanganate solution (cm^3)	1 M HCl (µl)	0.1 M Na_2CO_3 (µl)	pH
5.0	9.6	400	-	5.0
5.0	9.8	200	-	6.0
5.0	10.0	-	-	7.0
5.0	9.7	-	250	8.0

Table 5.4 - Preparation of the 0.09 M potassium permanganate solution

Potassium permanganate (g)	Final volume of solution diluted with sterile deionised water (cm^3)	Potassium permanganate (M)
158.00	**1,000**	**1.00**
14.22	**1,000**	**0.09**
5.68	**400.00**	**0.09**

5.68 g of potassium permanganate powder was weighed and added into 500 cm^3 Duran glass bottle and then mixed with 400 cm^3 distilled water to make a solution with the concentration of 0.09 M.

Table 5.5 - Preparation of the 0.09 M sodium bisulfite solution

Sodium bisulfite (g)	Final volume of solution diluted with sterile deionised water (cm^3)	Sodium bisulfite (M)
104.06	**1,000**	**1.00**
9.36	**1,000**	**0.09**
3.74	**400.00**	**0.09**

3.74 g of sodium bisulfite powder was weighed and added into 500 cm^3 Duran glass bottle and then mixed with 400 cm^3 distilled water to make a solution with the concentration of 0.09 M.

Table 5.6 - Experiment i (oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate), preparation of standard solutions at the concentrations of 1, 20, 40, 60, 80 and 90 mg.dm^{-3}

Standard solution concentration (mg.dm^{-3})	PAH standard stock solution with con. of 250 mg.dm^{-3} (µl)	Carbozole stock solution with con. of 200 mg.dm^{-3} (µl)	Acetonitrile (cm^3)
1	80	4,500	Appropriate volume to make the solution up to 20 cm^3
20	1,600	4,500	
40	3,200	4,500	
60	4,800	4,500	
80	6,400	4,500	
90	7,200	4,500	

Appendix 5.3 Experiment i (oxidation of PAH at different pHs in sterile soil in the presence or absence of potassium permanganate), HPLC analysis (standard chromatograms and standard curves)

Standard HPLC chromatograms and standard curves were obtained of the four PAHs used at six different concentrations of 1, 20, 40, 60, 80 and 90 mg.dm^{-3}.

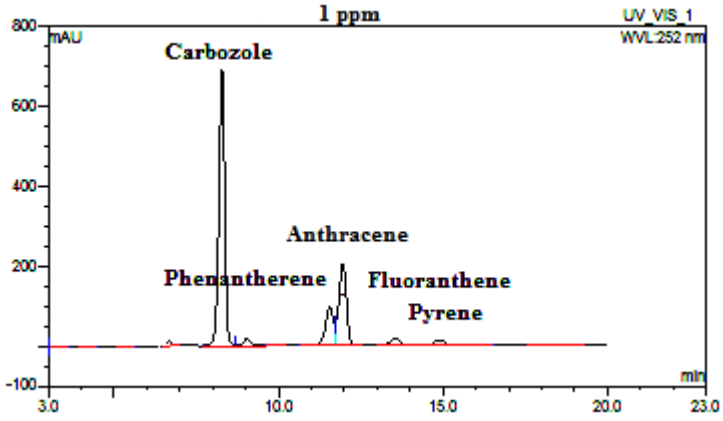


Figure 5.1 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 1 and 45 mg.dm^{-3}, respectively

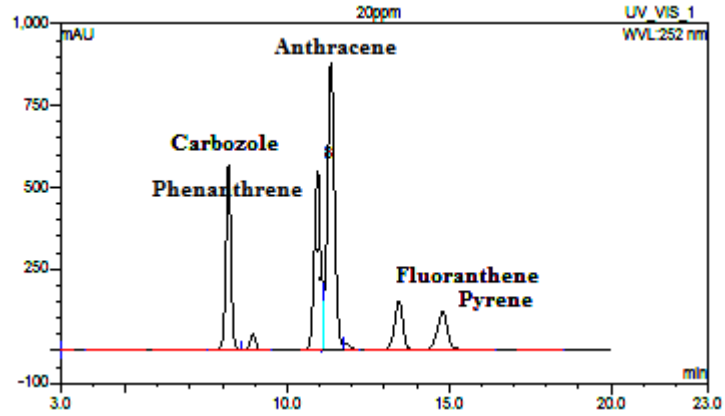


Figure 5.2 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 20 and 45 mg.dm^{-3}, respectively

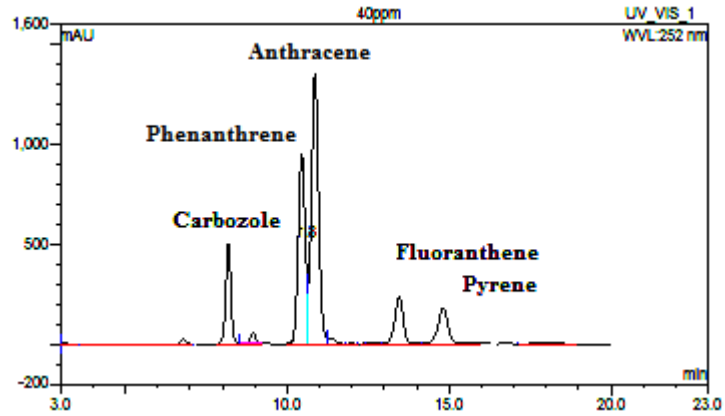


Figure 5.3 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 40 and 45 mg.dm^{-3}, respectively

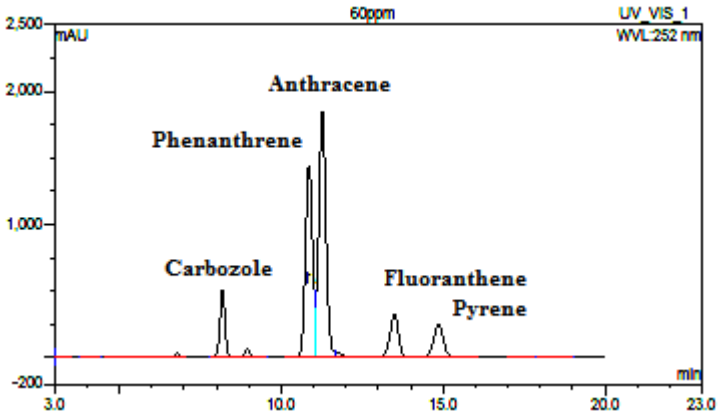


Figure 5.4 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 60 and 45 mg.dm^{-3}, respectively

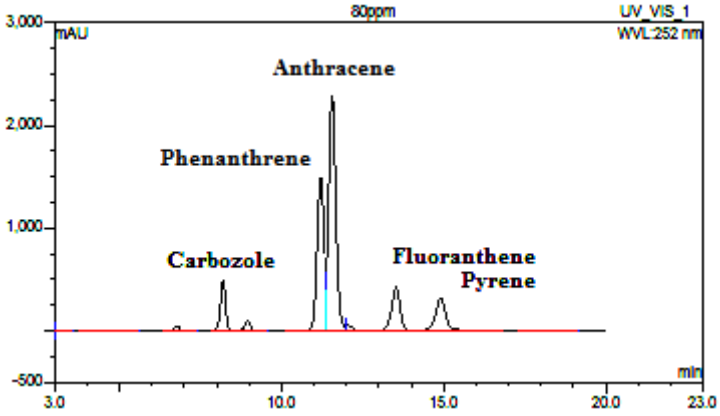


Figure 5.5 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 80 and 45 mg.dm^{-3}, respectively

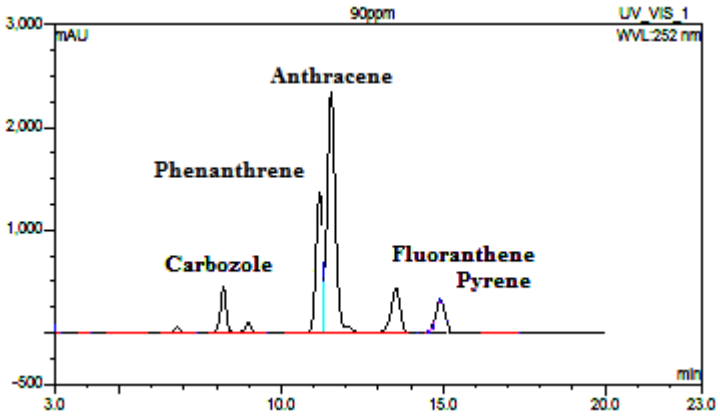


Figure 5.6 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 90 and 45 mg.dm^{-3}, respectively

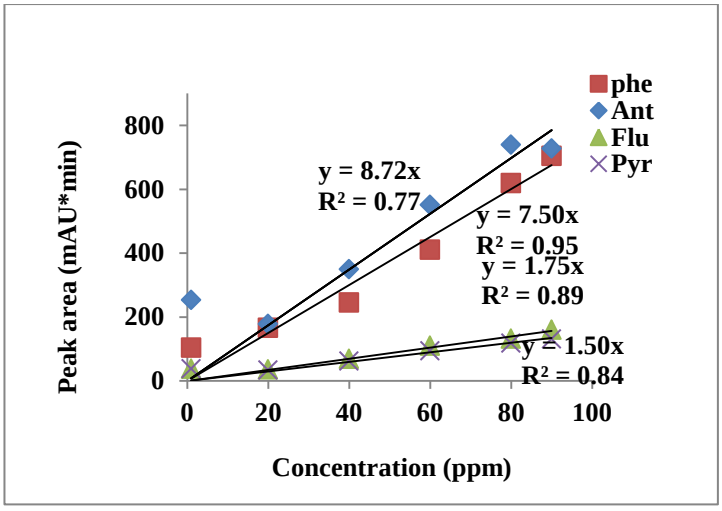


Figure 5.7 - HPLC standard curves for the four PAHs showing peak area against concentration

Appendix 5.4 – Experiment ii (oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil), determination of water-holding capacity in the J. Arthur Bower's top soil

I) Mass of hilgard soil cup + Mass of filter paper + Mass of dried soil= 76.29 g

J) Mass of hilgard soil cup + Mass of filter paper= 67.13 g

K) Mass of dried soil= A-B= 09.16 g

L) Mass of hilgard soil cup + Mass of filter paper + Mass of saturated soil= 85.33 g

M) Mass of hilgard soil cup + Mass of filter paper= 67.13 g

N) Mass of saturated soil= D-E= 18.2 g

O) Mass of water content in saturated soil=F-C= 09.4 g

P) Percentage of water-holding capacity= G/F*100= 49.67 %

Table 5.7 - Experiment ii (oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil), the amount, volume and concentration of chemicals used for soil contamination

Chemical	Chemical added in solution (mg)	n-hexane volume in solution (cm^3)	Chemical concentration in solution ($mg.dm^{-3}$)	Soil (g)	Chemical final concentration in soil ($mg.kg^{-1}$)
Phenanthrene	**50**		**100**		**91**
Anthracene	**50**		**100**		**91**
		500		**250**	
Fluoranthene	**50**		**100**		**91**
Pyrene	**50**		**100**		**91**

Appendix 5.5 Experiment ii (oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil), calculate the volume of liquid which needs to be added into the soil to reach the purposed moisture content to 30 % of water-holding capacity and pH 7.5

745 µl sterile distilled water – (30 µl Na_2CO_3 + 200 µl potassium permanganate)

= 515 µl sterile distilled water

Table 5.8 - Experiment ii (oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil), preparation of standard solution with the concentrations of 1, 5, 10, 15, 20, 25, 30 and 35 mg.dm^{-3}

Standard solution concentration (mg.dm^{-3})	Volume taken from PAH standard stock solution with con. of 100 mg.dm^{-3} (µl)	Volume taken from carbozole stock solution with con. of 100 mg.dm^{-3} (µl)	Volume of acetonitrile (cm^3)
1	200	4,000	Appropriate volume to make the solution up to 20 cm^3
5	1,000	4,000	
10	2,000	4,000	
15	3,000	4,000	
20	4,000	4,000	
25	5,600	4,000	
30	6,000	4,000	
35	7,000	4,000	

Appendix 5.6 Experiment ii (oxidation of PAH at pH 7.5 in sterile soil treated with potassium permanganate in the presence or absence of roadside soil), HPLC analysis (standard chromatograms and standard curves)

Standard HPLC chromatograms and standard curves were obtained of the four PAHs used at eight different concentrations of 1, 5, 10, 15, 20, 25, 30 and 35 mg.dm^{-3}.

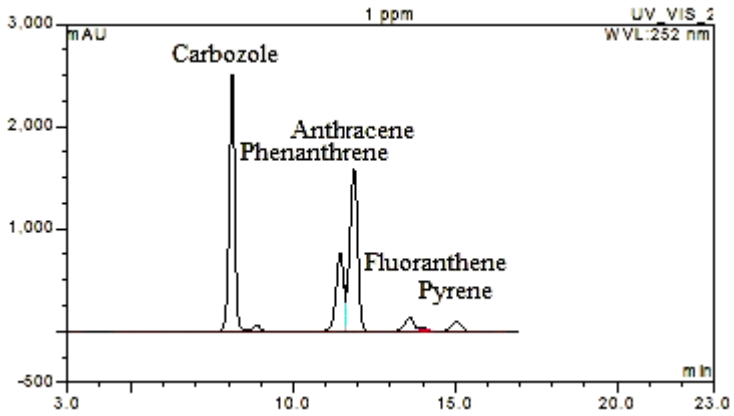


Figure 5.8 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 1 and 20 mg.dm^{-3}, respectively

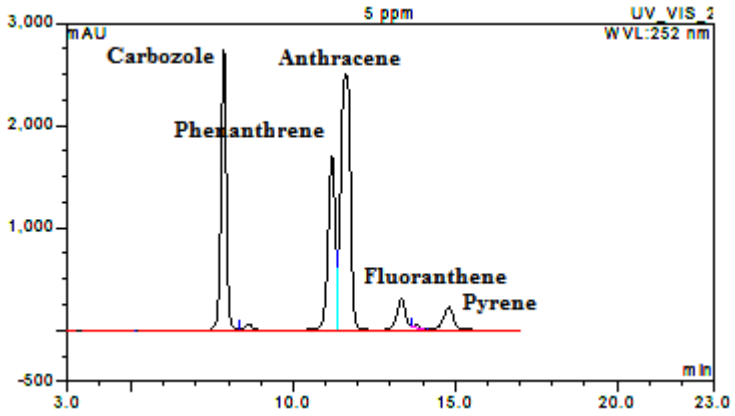


Figure 5.9 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 5 and 20 mg.dm^{-3}, respectively

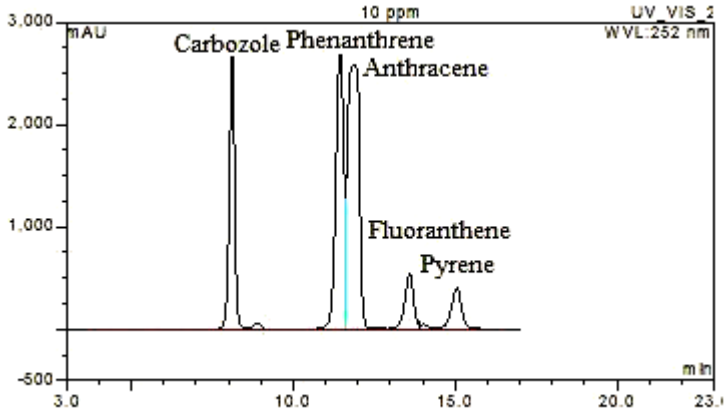


Figure 5.10 - HPLC chromatogram for four PAHs and carbozole at the concentrations of 10 and 20 mg.dm^{-3}, respectively

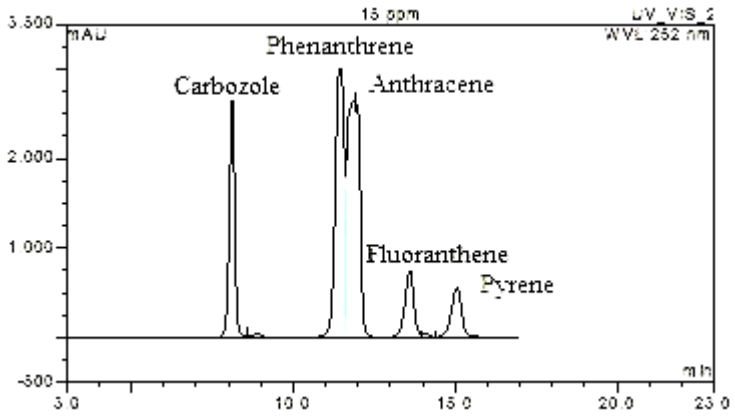


Figure 5.11 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 15 and 20 mg.dm^{-3}, respectively

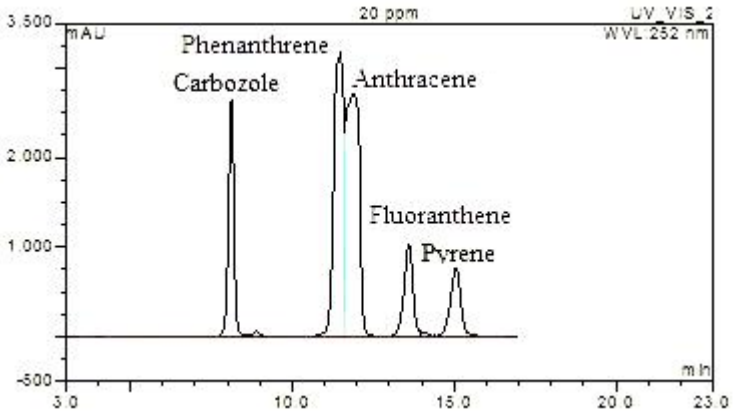


Figure 5.12 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 20 and 20 mg.dm^{-3}, respectively

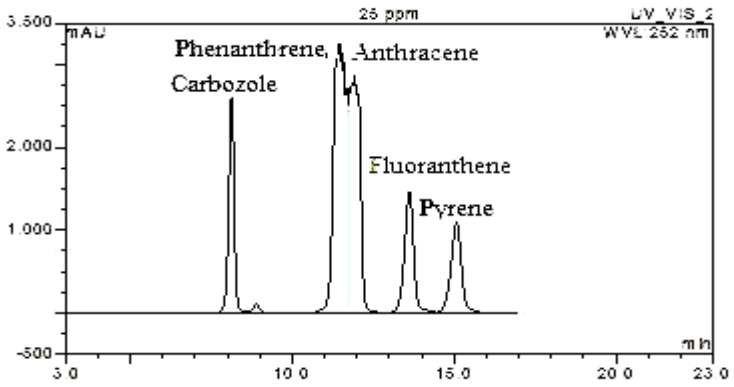


Figure 5.13 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 25 and 20 mg.dm^{-3}, respectively

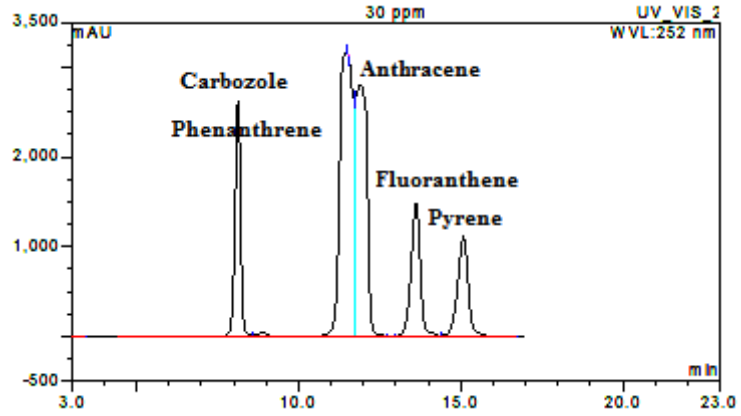


Figure 5.14 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 30 and 20 mg.dm^{-3}, respectively

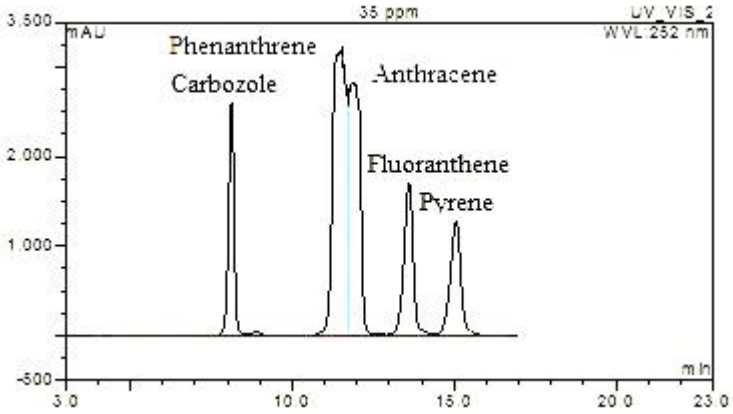


Figure 5.15 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 35 and 20 mg.dm^{-3}, respectively

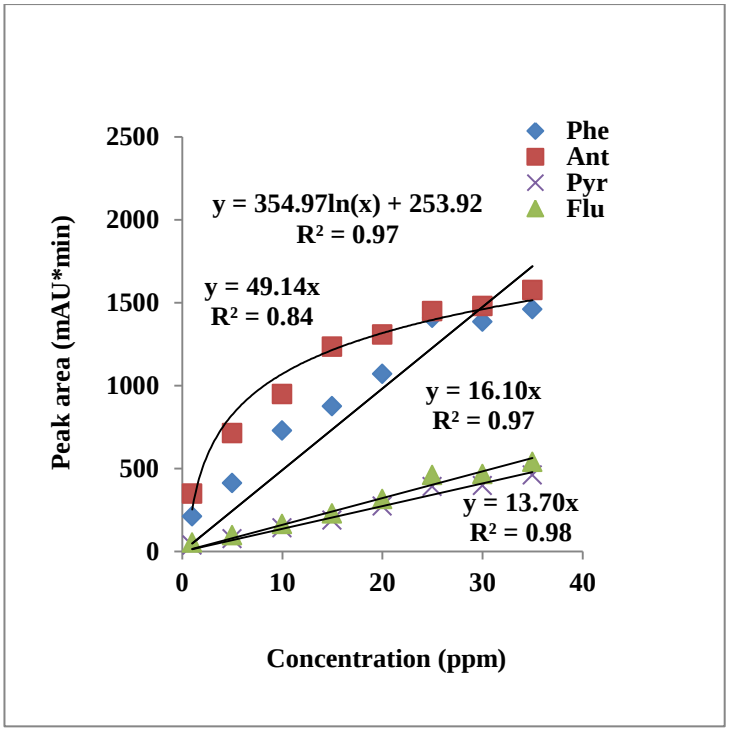


Figure 5.16 - HPLC standard curves for the four PAHs showing peak area against concentration

Appendix 6

Appendix 6.1 - Determination of water-holding capacity in the J. Arthur Bower's top soil

Refer to Appendix 5.2.

Table 6.1 - Experiment i (translocation of PAH in the soil), the amount, volume and concentration of chemicals used for the soil contamination

Chemical	Chemical added in solution (mg)	n-hexane volume in solution (cm^3)	Chemical concentration in solution ($mg.dm^{-3}$)	Soil (g)	Chemical final concentration in soil ($mg.kg^{-1}$)
Phenanthrene	100	200	500	200	500
Anthracene	100		500		500
Fluoranthene	100		500		500
Pyrene	100		500		500

Table 6.2 - Preparation of the 2.5 % Tween 20

Percentage of solution (%)	100 % Tween 20 (cm^3)	Deionised water (cm^3)
2.5	2.5	100
2.5	1.5	60

Table 6.3 - Adjustment of the soil liquid content to 30 % of the water-holding capacity

Soil (g)	Milli-Q water (cm^3)	Moisture content (%)
100	49.67	100
100	14.90	30
60	8.94	30

Individual treatment: 8.94 cm^3 (Milli-Q water) + 1.5 cm^3 (Tween 20) = 10.44 cm^3 x 2 (Two treatments): 10.44 cm^3 x 2 = 20.88 cm^3

Table 6.4 - Experiment i (translocation of PAH in the soil), preparation of standard solutions at the concentrations of 1, 35, 70, 105, 140 and 175 $mg.dm^{-3}$

Standard solution ($mg.dm^{-3}$)	PAH standard stock solution with con. of 100 $mg.dm^{-3}$ (µl)	Carbozole stock solution with con. of 100 $mg.dm^{-3}$ (µl)	Acetonitrile (cm^3)
1	40	3,330	Appropriate volume to make the solution up to 20 cm^3
35	1,400	3,330	
70	2,800	3,330	
105	4,200	3,330	
140	5,600	3,330	
175	7,000	3,330	

Appendix 6.2 Experiment i (translocation of PAH in the soil), HPLC analysis (standard chromatograms and standard curves)

Standard HPLC chromatograms and standard curves were obtained of the four PAHs used at six different concentrations of 1, 35, 70, 105, 140 and 175 mg.dm^{-3}.

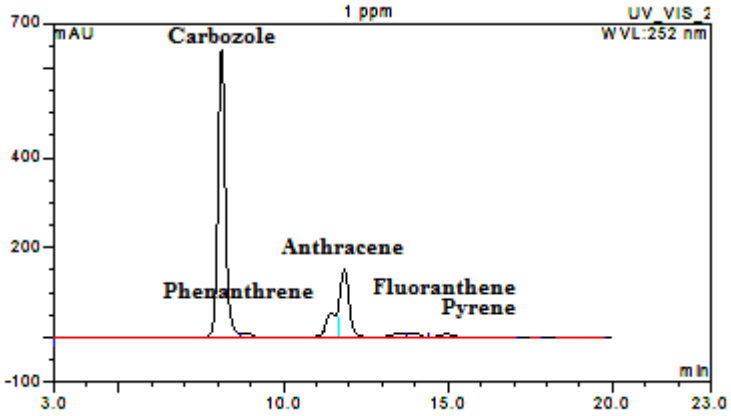


Figure 6.1 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 1 and 83 mg.dm^{-3}, respectively

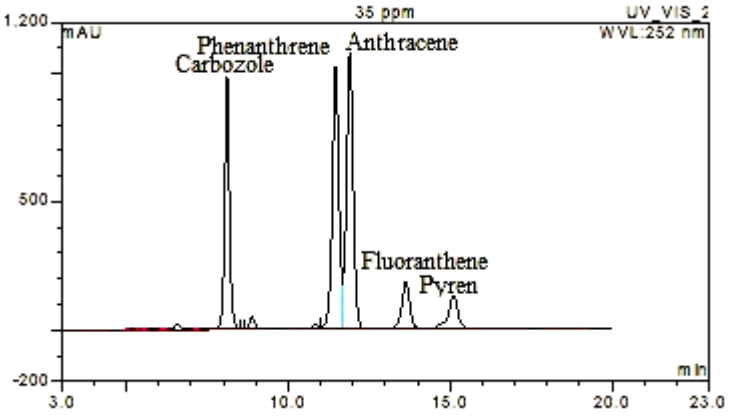


Figure 6.2 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 35 and 83 mg.dm^{-3}, respectively

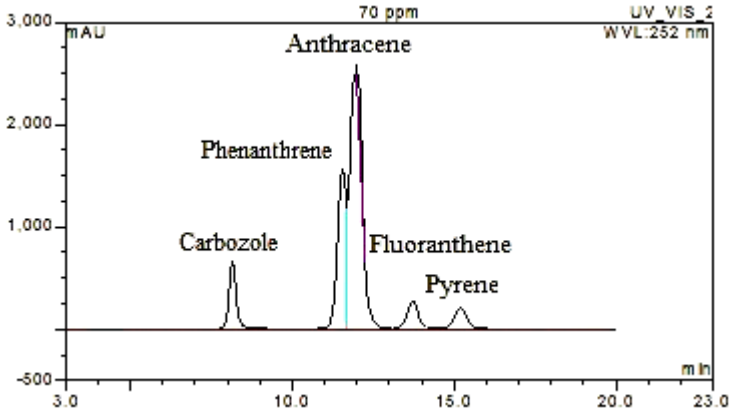


Figure 6.3 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 70 and 83 mg.dm^{-3}, respectively

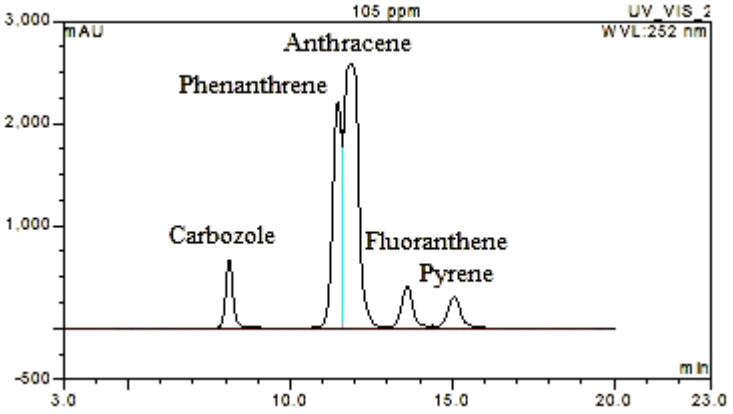


Figure 6.4 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 105 and 83, respectively

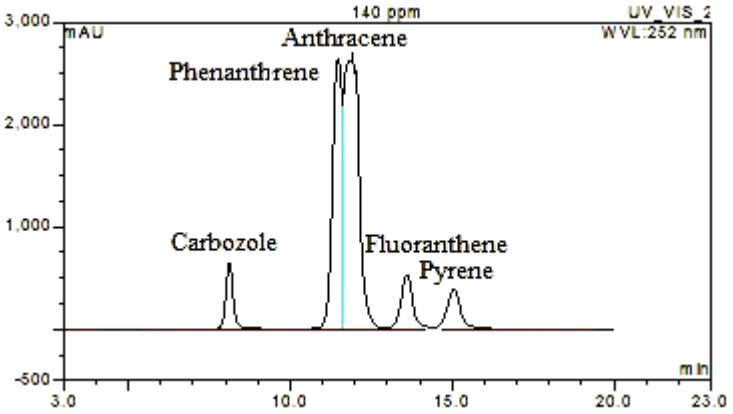


Figure 6.5 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 140 and 83 mg.dm^{-3}, respectively

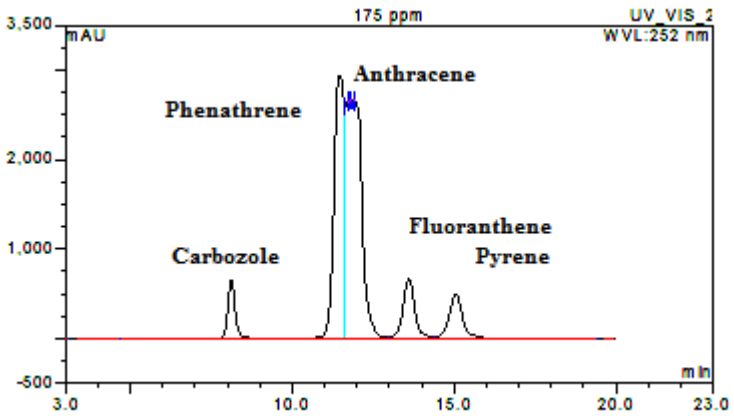


Figure 6.6 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 175 and 83 mg.dm^{-3}, respectively

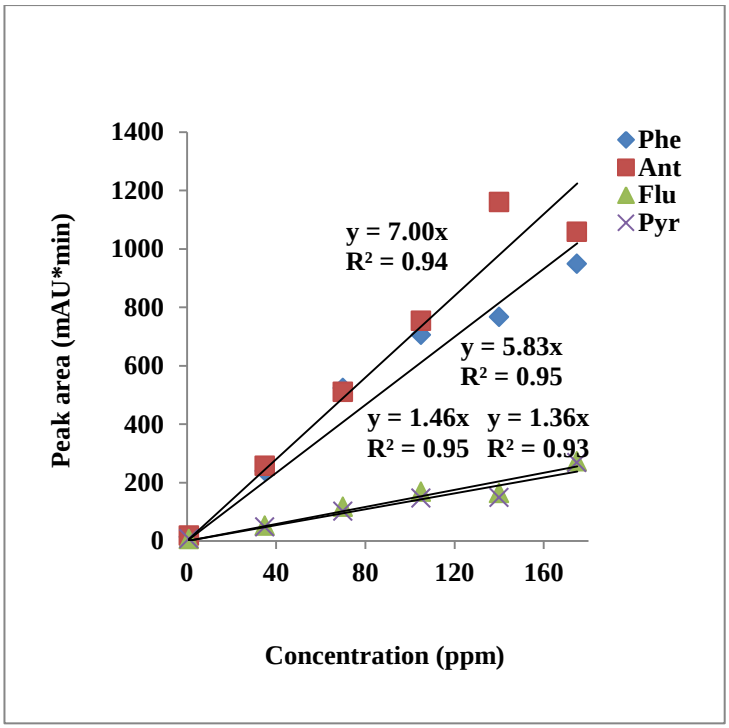


Figure 6.7 - HPLC standard curves for the four PAHs showing peak area against concentration

Appendix 6.3 - Determination of water-holding capacity in the J. Arthur Bower's top soil

Refer to Appendix 4.2.

Table 6.5 - Experiment ii (degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil), the amount, volume and concentration of chemicals used for the soil contamination

Chemical	Chemical added in solution (mg)	n-hexane volume in solution (cm^3)	Chemical concentration in solution ($mg.dm^{-3}$)	Soil (g)	Chemical final concentration in soil ($mg.kg^{-1}$)
Phenanthrene	50	500	100	600	91
Anthracene	50		100		91
Fluoranthene	50		100		91
Pyrene	50		100		91

Table 6.6 - Preparation of the 2.5 % Tween 20

Percentage of solution (%)	100 % Tween 20 (cm^3)	Deionised water (cm^3)
2.5	2.5	100
2.5	0.25	10

0.25 cm^3 x 18 tubes = 4.5 cm^3 Tween 20

Table 6.7 - Adjustment of the soil liquid content to 30 % of the water-holding capacity

Soil (g)	Milli-Q water (cm^3)	Moisture content (%)
100	49.67	100
100	14.90	30
10	1.49	30

Individual treatment: 1.49 cm^3 (Milli-Q water) x 18 tubes = 26.82 cm^3 (Milli-Q water) + 8 µl (Na_2CO_3) x 18 = 144 µl (Na_2CO_3)

Table 6.8 – Experiment ii (degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil), preparation of standard solutions at the concentrations of 1, 5, 10, 15, 20, 25, 30 and 35 $mg.dm^{-3}$

Standard solution ($mg.dm^{-3}$)	PAH standard stock solution with con. of 100 $mg.dm^{-3}$ (µl)	Carbozole stock solution with con. of 100 $mg.dm^{-3}$ (µl)	Acetonitrile (cm^3)
1	200	4,000	Appropriate volume to make the solution up to 20 cm^3
5	1,000	4,000	
10	2,000	4,000	
15	3,000	4,000	
20	4,000	4,000	
25	5,000	4,000	
30	6,000	4,000	
35	7,000	4,000	

Appendix 6.4 Experiment ii (degradation of PAH at pH 7.5 in sterile soil treated with Tween 20 in the presence or absence of roadside soil), HPLC analysis (standard chromatograms and standard curves)

Standard HPLC chromatograms and standard curves were obtained of the four PAHs used at eight different concentrations of 1, 5, 10, 15, 20, 25, 30 and 35 **mg.dm^{-3}**.

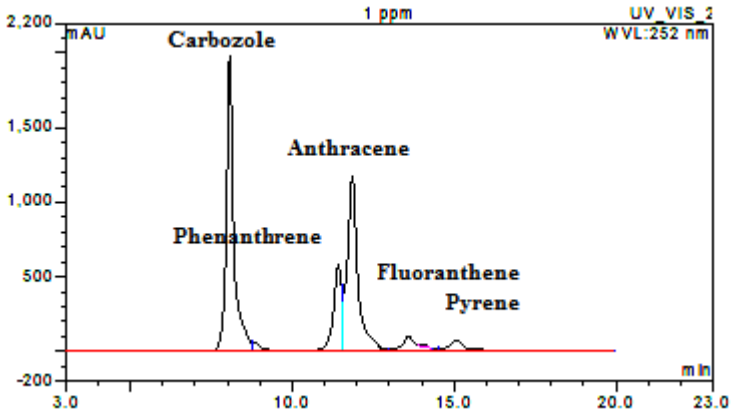


Figure 6.8 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 1 and 20 mg.dm^{-3}, respectively

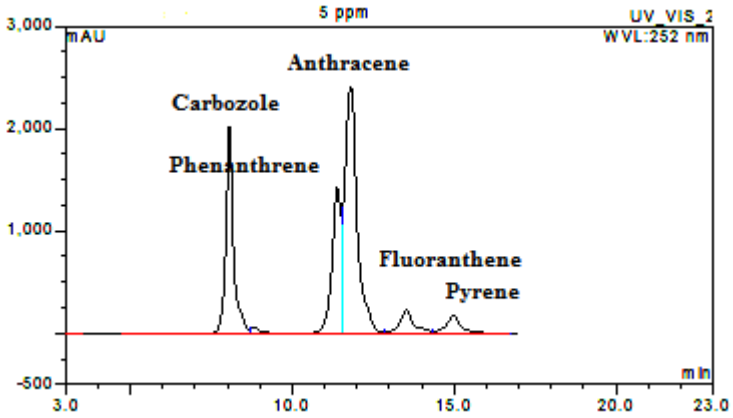


Figure 6.9 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 5 and 20 mg.dm^{-3}, respectively

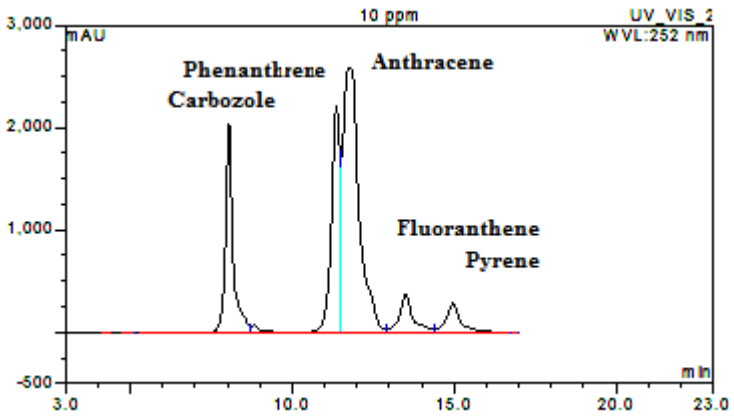


Figure 6.10 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 10 and 20 mg.dm^{-3}, respectively

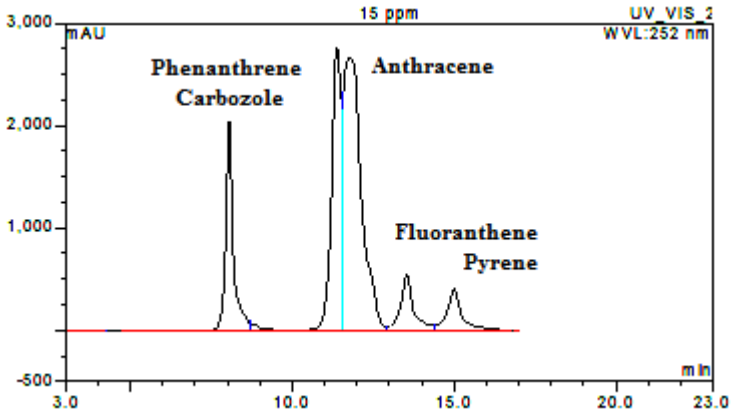


Figure 6.11 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 15 and 20 mg.dm^{-3}, respectively

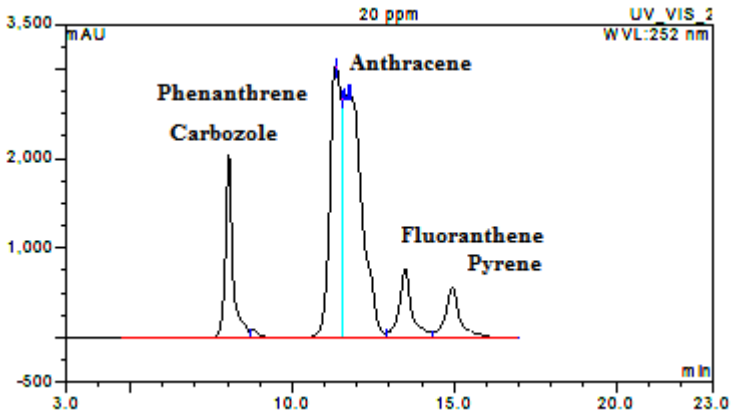


Figure 6.12 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 20 and 20 mg.dm^{-3}, respectively

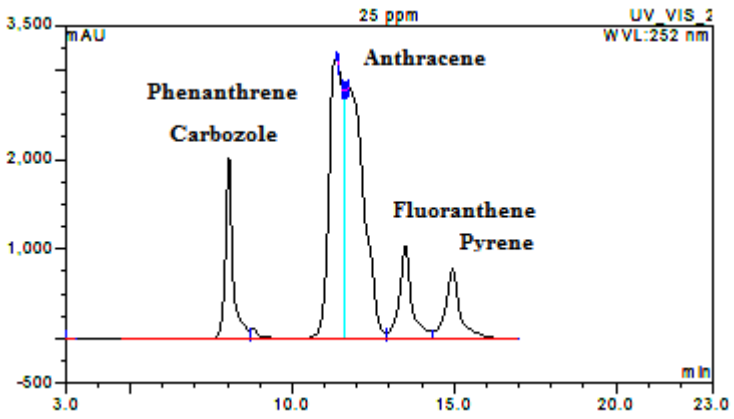


Figure 6.13 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 25 and 20 mg.dm^{-3}, respectively

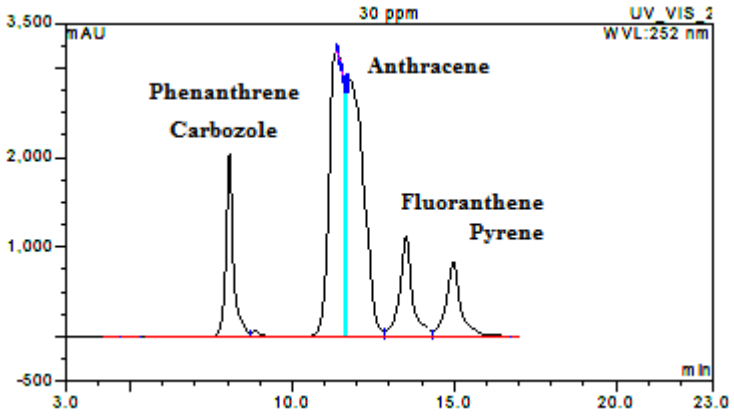


Figure 6.14 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 30 and 20 mg.dm^{-3}, respectively

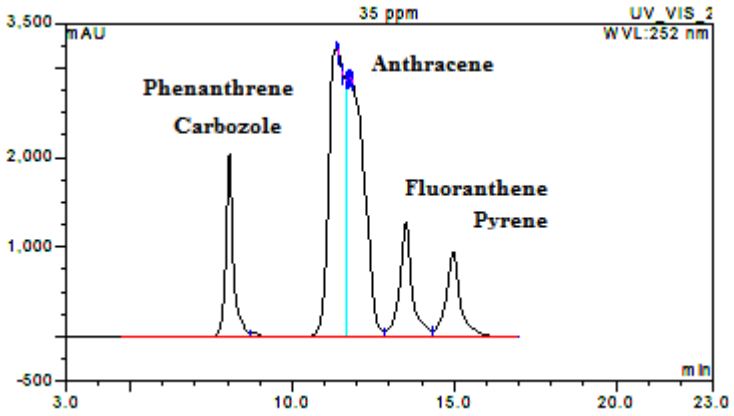


Figure 6.15 - HPLC chromatogram for the four PAHs and carbozole at the concentrations of 35 and 20 mg.dm^{-3}, respectively

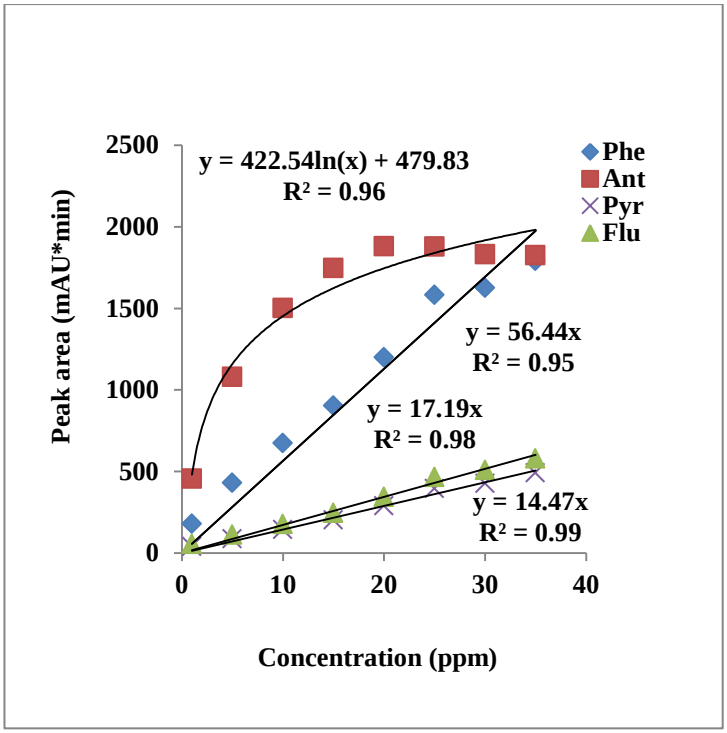


Figure 6.16 - HPLC standard curves for the four PAHs showing peak area against concentration

Appendix 7

Appendix 7.1 Statistical analysis of chapter 4

Oneway - Phenanthrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	86399.98	6.00	14400.00	1.42	0.24
	Within Groups	283645.78	28.00	10130.21		
	Total	370045.77	34.00			
T4	Between Groups	15623.36	6.00	2603.89	1.15	0.05
	Within Groups	63210.08	28.00	2257.50		
	Total	78833.45	34.00			
T12	Between Groups	29032.65	6.00	4838.78	0.56	0.05
	Within Groups	239864.16	28.00	8566.58		
	Total	268896.81	34.00			
T16	Between Groups	704.10	6.00	117.35	2.22	0.06
	Within Groups	1479.70	28.00	52.85		
	Total	2183.80	34.00			
T20	Between Groups	1076.74	6.00	179.46	0.96	0.05
	Within Groups	5241.96	28.00	187.21		
	Total	6318.70	34.00			
T24	Between Groups	14.66	6.00	2.44	1.11	0.05
	Within Groups	61.91	28.00	2.21		
	Total	76.57	34.00			
T32	Between Groups	14.02	6.00	2.34	1.08	0.40
	Within Groups	60.51	28.00	2.16		
	Total	74.54	34.00			

Oneway - Anthracene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	787464.54	6.00	131244.09	1.29	0.30
	Within Groups	2856700.12	28.00	102025.00		
	Total	3644164.66	34.00			
T4	Between Groups	127636.30	6.00	21272.72	1.11	0.06
	Within Groups	538977.55	28.00	19249.20		
	Total	666613.85	34.00			
T12	Between Groups	361894.22	6.00	60315.70	0.52	0.06
	Within Groups	3277791.08	28.00	117063.97		
	Total	3639685.30	34.00			
T16	Between Groups	5383.54	6.00	897.26	1.05	0.41
	Within Groups	23857.82	28.00	852.07		
	Total	29241.36	34.00			
T20	Between Groups	164730.02	6.00	27455.00	1.68	0.16
	Within Groups	457295.56	28.00	16331.98		
	Total	622025.58	34.00			
T24	Between Groups	2890.17	6.00	481.70	0.75	0.62
	Within Groups	17999.97	28.00	642.86		
	Total	20890.14	34.00			
T32	Between Groups	4005.50	6.00	667.58	1.17	0.35
	Within Groups	15911.11	28.00	568.25		
	Total	19916.61	34.00			

Oneway - Fluoranthene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	8147.93	6.00	1357.99	1.37	0.26
	Within Groups	27741.06	28.00	990.75		
	Total	35888.99	34.00			

T4	Between Groups	1383.13	6.00	230.52	1.14	0.05
	Within Groups	5652.92	28.00	201.89		
	Total	7036.05	34.00			
T12	Between Groups	3069.95	6.00	511.66	0.39	0.04
	Within Groups	36992.46	28.00	1321.16		
	Total	40062.41	34.00			
T16	Between Groups	125.34	6.00	20.89	1.00	0.04
	Within Groups	583.41	28.00	20.84		
	Total	708.75	34.00			
T20	Between Groups	7889.72	6.00	1314.95	1.64	0.05
	Within Groups	22454.02	28.00	801.93		
	Total	30343.74	34.00			
T24	Between Groups	165.88	6.00	27.65	0.86	0.54
	Within Groups	899.00	28.00	32.11		
	Total	1064.88	34.00			
T32	Between Groups	275.74	6.00	45.96	1.32	0.28
	Within Groups	977.98	28.00	34.93		
	Total	1253.72	34.00			

Oneway - Pyrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	4238.34	6.00	706.39	1.28	0.30
	Within Groups	15455.97	28.00	552.00		
	Total	19694.31	34.00			
T4	Between Groups	858.81	6.00	143.13	1.22	0.03
	Within Groups	3291.27	28.00	117.55		
	Total	4150.08	34.00			
T12	Between Groups	2212.21	6.00	368.70	0.43	0.05
	Within Groups	23764.53	28.00	848.73		
	Total	25976.74	34.00			

T16	Between Groups	68.48	6.00	11.41	1.07	0.05
	Within Groups	298.75	28.00	10.67		
	Total	367.23	34.00			
T20	Between Groups	5397.46	6.00	899.58	1.67	0.04
	Within Groups	15048.44	28.00	537.44		
	Total	20445.89	34.00			
T24	Between Groups	124.33	6.00	20.72	0.96	0.03
	Within Groups	604.84	28.00	21.60		
	Total	729.18	34.00			
T32	Between Groups	227.14	6.00	37.86	1.43	0.24
	Within Groups	739.44	28.00	26.41		
	Total	966.58	34.00			

Appendix 7.2 Statistical analysis of chapter 5

Oneway - Phenanthrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	78785.46	2	39392.73	6.64	.030
	Within Groups	35551.06	6	5925.17		
	Total	########	8			
T7	Between Groups	11035.33	2	5517.66	330.36	.000
	Within Groups	100.21	6	16.70		
	Total	11135.54	8			
T14	Between Groups	3991.07	2	1995.53	21.75	.002
	Within Groups	550.34	6	91.72		
	Total	4541.42	8			
T21	Between Groups	18222.02	2	9111.01	40.41	.000
	Within Groups	1352.66	6	225.44		
	Total	19574.68	8			
T28	Between Groups	28067.78	2	14033.89	84.81	.000

	Within Groups	992.82	6	165.47		
	Total	29060.61	8			
T35	Between Groups	32393.89	2	16196.94	520.85	.000
	Within Groups	186.58	6	31.09		
	Total	32580.47	8			

Post Hoc Tests - Phenanthrene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + KMnO4	Microbes only	-221.30*	62.84	.02	-414.14	-28.46
			KMnO4 Only	-59.06	62.84	.06	-251.90	133.77
		Microbes only	KMnO4 Only	162.24	62.84	.09	-30.60	355.08
	LSD	Microbes + KMnO4	Microbes only	-221.30*	62.84	.01	-375.09	-67.51
			KMnO4 Only	-59.06	62.84	.05	-212.85	94.725
		Microbes only	KMnO4 Only	162.24*	62.84	.04	8.45	316.02
T7	Tukey HSD	Microbes + KMnO4	Microbes only	72.22*	3.33	.00	61.98	82.45
			KMnO4 Only	-3.96	3.33	.50	-14.20	6.27
		Microbes only	KMnO4 Only	-76.18*	3.33	.00	-86.42	-65.94
	LSD	Microbes + KMnO4	Microbes only	72.22*	3.33	.00	64.05	80.38
			KMnO4 Only	-3.96	3.33	.05	-12.12	4.20
		Microbes only	KMnO4 Only	-76.18*	3.33	.00	-84.34	-68.01
T14	Tukey HSD	Microbes + KMnO4	Microbes only	-21.25	7.81	.07	-45.24	2.74
			KMnO4 Only	-51.33*	7.81	.00	-75.32	-27.33
		Microbes only	KMnO4 Only	-30.07*	7.81	.02	-54.07	-6.08
	LSD	Microbes + KMnO4	Microbes only	-21.25*	7.81	.03	-40.38	-2.11
			KMnO4 Only	-51.33*	7.81	.00	-70.46	-32.19
		Microbes only	KMnO4 Only	-30.07*	7.81	.00	-49.21	-10.94
T21	Tukey HSD	Microbes +	Microbes only	-13.00	12.25	.57	-50.61	24.61

		KMnO4	KMnO4 Only	-101.28*	12.25	.00	-138.90	-63.67
		Microbes only	KMnO4 Only	-88.28*	12.25	.00	-125.89	-50.66
	LSD	Microbes + KMnO4	Microbes only	-13.00	12.25	.33	-43.00	16.99
			KMnO4 Only	-101.28*	12.25	.00	-131.28	-71.28
		Microbes only	KMnO4 Only	-88.28*	12.25	.00	-118.28	-58.28
T28	Tukey HSD	Microbes + KMnO4	Microbes only	-10.17	10.50	.62	-42.39	22.05
			KMnO4 Only	-123.22*	10.50	.00	-155.44	-90.99
		Microbes only	KMnO4 Only	-113.05*	10.50	.00	-145.27	-80.82
	LSD	Microbes + KMnO4	Microbes only	-10.17	10.50	.37	-35.87	15.52
			KMnO4 Only	-123.22*	10.50	.00	-148.92	-97.52
		Microbes only	KMnO4 Only	-113.05*	10.50	.00	-138.75	-87.35
T35	Tukey HSD	Microbes + KMnO4	Microbes only	-5.44	4.55	.49	-19.41	8.53
			KMnO4 Only	-129.90*	4.55	.00	-143.87	-115.92
		Microbes only	KMnO4 Only	-124.46*	4.55	.00	-138.43	-110.48
	LSD	Microbes + KMnO4	Microbes only	-5.44	4.55	.27	-16.58	5.70
			KMnO4 Only	-129.90*	4.55	.00	-141.04	-118.75
		Microbes only	KMnO4 Only	-124.46*	4.55	.00	-135.60	-113.31

. The mean difference is significant at the 0.05 level.

Oneway - Anthracene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	225744.54	2.00	112872.27	4.96	0.05
	Within Groups	136643.51	6.00	22773.92		
	Total	362388.06	8.00			
T7	Between Groups	37278.74	2.00	18639.37	20.87	0.00
	Within Groups	5359.85	6.00	893.31		
	Total	42638.59	8.00			
T14	Between Groups	5241.90	2.00	2620.95	4.96	0.05

	Within Groups	3170.47	6.00	528.41		
	Total	8412.37	8.00			
T21	Between Groups	17281.02	2.00	8640.51	6.90	0.03
	Within Groups	7516.64	6.00	1252.77		
	Total	24797.66	8.00			
T28	Between Groups	109985.77	2.00	54992.88	40.61	0.00
	Within Groups	8125.84	6.00	1354.31		
	Total	118111.61	8.00			
T35	Between Groups	107119.51	2.00	53559.76	48.63	0.00
	Within Groups	6607.58	6.00	1101.26		
	Total	113727.09	8.00			

Post Hoc Tests - Anthracene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + KMnO4	Microbes only	-382.22*	123.22	0.05	-760.29	-4.16
			KMnO4 Only	-133.67	123.22	0.56	-511.74	244.39
		Microbes only	KMnO4 Only	248.55	123.22	0.19	-129.51	626.62
	LSD	Microbes + KMnO4	Microbes only	-382.22*	123.22	0.02	-683.73	-80.72
			KMnO4 Only	-133.67	123.22	0.05	-435.18	167.83
		Microbes only	KMnO4 Only	248.55	123.22	0.09	-52.95	550.06
T7	Tukey HSD	Microbes + KMnO4	Microbes only	34.50	24.40	0.07	-40.38	109.38
			KMnO4 Only	-115.96*	24.40	0.01	-190.84	-41.09
		Microbes only	KMnO4 Only	-150.46*	24.40	0.00	-225.34	-75.59
	LSD	Microbes + KMnO4	Microbes only	34.50	24.40	0.05	-25.21	94.21
			KMnO4 Only	-115.96*	24.40	0.00	-175.68	-56.25
		Microbes only	KMnO4 Only	-150.46*	24.40	0.00	-210.18	-90.75
T14	Tukey HSD	Microbes + KMnO4	Microbes only	-14.31	18.77	0.74	-71.90	43.28
			KMnO4 Only	-56.83	18.77	0.05	-114.42	0.76

		Microbes only	KMnO4 Only	-42.52	18.77	0.14	-100.11	15.07
	LSD	Microbes + KMnO4	Microbes only	-14.31	18.77	0.05	-60.23	31.62
			KMnO4 Only	-56.82*	18.77	0.02	-102.75	-10.90
		Microbes only	KMnO4 Only	-42.52	18.77	0.06	-88.45	3.41
T21	Tukey HSD	Microbes + KMnO4	Microbes only	-15.81	28.90	0.85	-104.49	72.86
			KMnO4 Only	-99.84*	28.90	0.03	-188.52	-11.17
		Microbes only	KMnO4 Only	-84.03	28.90	0.06	-172.71	4.64
	LSD	Microbes + KMnO4	Microbes only	-15.81	28.90	0.60	-86.53	54.90
			KMnO4 Only	-99.84*	28.90	0.01	-170.56	-29.13
		Microbes only	KMnO4 Only	-84.03*	28.90	0.03	-154.75	-13.32
T28	Tukey HSD	Microbes + KMnO4	Microbes only	-15.32	30.05	0.87	-107.51	76.87
			KMnO4 Only	-241.79*	30.05	0.00	-333.98	-149.60
		Microbes only	KMnO4 Only	-226.47*	30.05	0.00	-318.66	-134.28
	LSD	Microbes + KMnO4	Microbes only	-15.32	30.05	0.63	-88.84	58.20
			KMnO4 Only	-241.79*	30.05	0.00	-315.31	-168.27
		Microbes only	KMnO4 Only	-226.47*	30.05	0.00	-299.99	-152.95
T35	Tukey HSD	Microbes + KMnO4	Microbes only	-27.27	27.10	0.60	-110.41	55.87
			KMnO4 Only	-243.85*	27.10	0.00	-326.99	-160.72
		Microbes only	KMnO4 Only	-216.58*	27.10	0.00	-299.72	-133.45
	LSD	Microbes + KMnO4	Microbes only	-27.27	27.10	0.35	-93.57	39.03
			KMnO4 Only	-243.85*	27.10	0.00	-310.16	-177.56
		Microbes only	KMnO4 Only	-216.58*	27.10	0.00	-282.89	-150.29

*. The mean difference is significant at the 0.05 level.

Oneway - Fluoranthene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	2661.48	2.00	1330.74	53.65	0.00

	Within Groups	148.82	6.00	24.80		
	Total	2810.31	8.00			
T7	Between Groups	258.79	2.00	129.40	40.74	0.00
	Within Groups	19.06	6.00	3.18		
	Total	277.85	8.00			
T14	Between Groups	137.64	2.00	68.82	13.98	0.01
	Within Groups	29.53	6.00	4.92		
	Total	167.17	8.00			
T21	Between Groups	28.39	2.00	14.20	1.45	0.31
	Within Groups	58.74	6.00	9.79		
	Total	87.14	8.00			
T28	Between Groups	250.77	2.00	125.38	10.23	0.01
	Within Groups	73.55	6.00	12.26		
	Total	324.32	8.00			
T35	Between Groups	845.64	2.00	422.82	18.93	0.00
	Within Groups	134.01	6.00	22.34		
	Total	979.65	8.00			

Post Hoc Tests - Fluoranthene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + KMnO4	Microbes only	-38.20*	4.07	0.00	-50.68	-25.73
			KMnO4 Only	-3.74	4.07	0.08	-16.22	8.73
		Microbes only	KMnO4 Only	34.46*	4.07	0.00	21.99	46.94
	LSD	Microbes + KMnO4	Microbes only	-38.20*	4.07	0.00	-48.16	-28.26
			KMnO4 Only	-3.74	4.07	0.05	-13.69	6.21
		Microbes only	KMnO4 Only	34.46*	4.07	0.00	24.51	44.41
T7	Tukey HSD	Microbes + KMnO4	Microbes only	13.10*	1.46	0.00	8.64	17.56
			KMnO4 Only	7.38*	1.46	0.01	2.92	11.84
		Microbes only	KMnO4 Only	-5.72*	1.46	0.02	-10.18	-1.26

	LSD	Microbes + KMnO4	Microbes only	13.10*	1.46	0.00	9.54	16.66
			KMnO4 Only	7.38*	1.46	0.00	3.82	10.94
		Microbes only	KMnO4 Only	-5.72*	1.46	0.01	-9.28	-2.16
T14	Tukey HSD	Microbes + KMnO4	Microbes only	0.36	1.81	0.68	-5.20	5.92
			KMnO4 Only	-8.11*	1.81	0.01	-13.67	-2.55
		Microbes only	KMnO4 Only	-8.47*	1.81	0.01	-14.03	-2.91
	LSD	Microbes + KMnO4	Microbes only	0.36	1.81	0.05	-4.07	4.79
			KMnO4 Only	-8.11*	1.81	0.00	-12.54	-3.68
		Microbes only	KMnO4 Only	-8.47*	1.81	0.00	-12.90	-4.04
T21	Tukey HSD	Microbes + KMnO4	Microbes only	0.77	2.55	0.95	-7.07	8.61
			KMnO4 Only	-3.32	2.55	0.44	-11.16	4.52
		Microbes only	KMnO4 Only	-4.09	2.55	0.32	-11.93	3.75
	LSD	Microbes + KMnO4	Microbes only	0.77	2.55	0.77	-5.48	7.02
			KMnO4 Only	-3.32	2.55	0.24	-9.57	2.93
		Microbes only	KMnO4 Only	-4.09	2.55	0.16	-10.34	2.16
T28	Tukey HSD	Microbes + KMnO4	Microbes only	-4.39	2.86	0.34	-13.16	4.38
			KMnO4 Only	-12.72*	2.86	0.01	-21.50	-3.96
		Microbes only	KMnO4 Only	-8.34	2.86	0.06	-17.11	0.43
	LSD	Microbes + KMnO4	Microbes only	-4.39	2.86	0.18	-11.38	2.61
			KMnO4 Only	-12.72*	2.86	0.00	-19.72	-5.73
		Microbes only	KMnO4 Only	-8.34*	2.86	0.03	-15.34	-1.34
T35	Tukey HSD	Microbes + KMnO4	Microbes only	-3.85	3.86	0.60	-15.69	7.99
			KMnO4 Only	-22.21*	3.86	0.00	-34.06	-10.38
		Microbes only	KMnO4 Only	-18.36*	3.86	0.01	-30.20	-6.52
	LSD	Microbes + KMnO4	Microbes only	-3.85	3.86	0.36	-13.30	5.59
			KMnO4 Only	-22.21*	3.86	0.00	-31.66	-12.77
		Microbes only	KMnO4 Only	-18.36*	3.86	0.00	-27.81	-8.92

*. The mean difference is significant at the 0.05 level.

Oneway - Pyrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	905.31	2.00	452.66	2.83	0.04
	Within Groups	960.15	6.00	160.02		
	Total	1865.46	8.00			
T7	Between Groups	199.49	2.00	99.74	8.56	0.02
	Within Groups	69.91	6.00	11.65		
	Total	269.39	8.00			
T14	Between Groups	91.93	2.00	45.97	12.39	0.01
	Within Groups	22.26	6.00	3.71		
	Total	114.19	8.00			
T21	Between Groups	11.67	2.00	5.84	0.88	0.46
	Within Groups	39.69	6.00	6.62		
	Total	51.36	8.00			
T28	Between Groups	61.31	2.00	30.65	5.07	0.05
	Within Groups	36.27	6.00	6.05		
	Total	97.58	8.00			
T35	Between Groups	315.89	2.00	157.94	8.79	0.02
	Within Groups	107.77	6.00	17.96		
	Total	423.66	8.00			

Post Hoc Tests - Pyrene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + KMnO4	Microbes only	-23.66	10.33	0.13	-55.35	8.03
			KMnO4 Only	-6.09	10.33	0.08	-37.78	25.60
		Microbes only	KMnO4 Only	17.57	10.33	0.28	-14.12	49.26
	LSD	Microbes + KMnO4	Microbes only	-23.66	10.33	0.06	-48.93	1.62
			KMnO4 Only	-6.09	10.33	0.05	-31.36	19.18

		Microbes only	KMnO4 Only	17.57	10.33	0.05	-7.71	42.84
T7	Tukey HSD	Microbes + KMnO4	Microbes only	8.10	2.79	0.06	-0.45	16.65
			KMnO4 Only	-3.06	2.79	0.09	-11.61	5.49
		Microbes only	KMnO4 Only	-11.16*	2.79	0.02	-19.71	-2.61
	LSD	Microbes + KMnO4	Microbes only	8.09*	2.79	0.03	1.28	14.92
			KMnO4 Only	-3.06	2.79	0.05	-9.88	3.76
		Microbes only	KMnO4 Only	-11.16*	2.79	0.01	-17.98	-4.34
T14	Tukey HSD	Microbes + KMnO4	Microbes only	0.17	1.57	0.09	-4.66	5.00
			KMnO4 Only	-6.69*	1.57	0.01	-11.52	-1.87
		Microbes only	KMnO4 Only	-6.86*	1.57	0.01	-11.69	-2.04
	LSD	Microbes + KMnO4	Microbes only	0.17	1.57	0.05	-3.68	4.02
			KMnO4 Only	-6.69*	1.57	0.01	-10.54	-2.85
		Microbes only	KMnO4 Only	-6.86*	1.57	0.00	-10.71	-3.02
T21	Tukey HSD	Microbes + KMnO4	Microbes only	1.67	2.10	0.72	-4.77	8.11
			KMnO4 Only	-1.10	2.10	0.86	-7.54	5.34
		Microbes only	KMnO4 Only	-2.77	2.10	0.44	-9.21	3.67
	LSD	Microbes + KMnO4	Microbes only	1.67	2.10	0.46	-3.47	6.81
			KMnO4 Only	-1.10	2.10	0.62	-6.24	4.04
		Microbes only	KMnO4 Only	-2.77	2.10	0.24	-7.91	2.37
T28	Tukey HSD	Microbes + KMnO4	Microbes only	-2.01	2.01	0.60	-8.17	4.15
			KMnO4 Only	-6.26*	2.01	0.05	-12.42	-0.10
		Microbes only	KMnO4 Only	-4.25	2.01	0.17	-10.41	1.91
	LSD	Microbes + KMnO4	Microbes only	-2.01	2.01	0.36	-6.92	2.91
			KMnO4 Only	-6.26*	2.01	0.02	-11.17	-1.35
		Microbes only	KMnO4 Only	-4.25	2.01	0.08	-9.17	0.66
T35	Tukey HSD	Microbes + KMnO4	Microbes only	-3.03	3.46	0.67	-13.65	7.58
			KMnO4 Only	-13.80*	3.46	0.02	-24.42	-3.19
		Microbes only	KMnO4 Only	-10.77*	3.46	0.05	-21.39	-0.16

	LSD	Microbes + KMnO4	Microbes only	-3.03	3.46	0.41	-11.50	5.43
			KMnO4 Only	-13.80*	3.46	0.01	-22.27	-5.34
		Microbes only	KMnO4 Only	-10.77*	3.46	0.02	-19.24	-2.31

*. The mean difference is significant at the 0.05 level.

Appendix 7.3 Statistical analysis of chapter 6

Oneway - Phenanthrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	2742225.01	3.00	914075.00	36.39	0.00
	Within Groups	200930.20	8.00	25116.27		
	Total	2943155.21	11.00			
T10	Between Groups	1857612.91	3.00	619204.30	58.35	0.00
	Within Groups	84893.80	8.00	10611.73		
	Total	1942506.72	11.00			
T20	Between Groups	1195604.41	3.00	398534.80	91.15	0.00
	Within Groups	34980.06	8.00	4372.51		
	Total	1230584.47	11.00			
PAHs	Between Groups	0.00	3.00	0.00		
	Within Groups	0.00	8.00	0.00		
	Total	0.00	11.00			

Post Hoc Tests - Phenanthrene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	331.77	129.40	0.12	-82.61	746.15
			Soil Only - Right	1112.08*	129.40	0.00	697.70	1526.47

			Soil Only (Control) - Right	1072.53*	129.40	0.00	658.15	1486.91
		PAHs Only (Control) - Left	Soil Only - Right	780.31*	129.40	0.00	365.93	1194.70
			Soil Only (Control) - Right	740.76*	129.40	0.00	326.38	1155.14
		Soil Only - Right	Soil Only (Control) - Right	-39.55	129.40	0.99	-453.94	374.83
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	331.77*	129.40	0.03	33.37	630.17
			Soil Only - Right	1112.08*	129.40	0.00	813.69	1410.48
			Soil Only (Control) - Right	1072.53*	129.40	0.00	774.13	1370.93
		PAHs Only (Control) - Left	Soil Only - Right	780.31*	129.40	0.00	481.92	1078.71
			Soil Only (Control) - Right	740.76*	129.40	0.00	442.36	1039.16
		Soil Only - Right	Soil Only (Control) - Right	-39.55	129.40	0.77	-337.95	258.84
T10	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-268.72	84.11	0.05	-538.07	0.63
			Soil Only - Right	625.45*	84.11	0.00	356.10	894.80
			Soil Only (Control) - Right	633.00*	84.11	0.00	363.65	902.35
		PAHs Only (Control) - Left	Soil Only - Right	894.17*	84.11	0.00	624.83	1163.53
			Soil Only (Control) - Right	901.72*	84.11	0.00	632.38	1171.08
		Soil Only - Right	Soil Only (Control) - Right	7.55	84.11	1.00	-261.80	276.90
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-268.72*	84.11	0.01	-462.68	-74.77
			Soil Only - Right	625.45*	84.11	0.00	431.50	819.41
			Soil Only (Control) - Right	633.00*	84.11	0.00	439.05	826.96

		PAHs Only (Control) - Left	Soil Only - Right	894.17*	84.11	0.00	700.22	1088.13
			Soil Only (Control) - Right	901.72*	84.11	0.00	707.77	1095.68
		Soil Only - Right	Soil Only (Control) - Right	7.55	84.11	0.93	-186.41	201.51
T20	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-754.43*	53.99	0.00	-927.33	-581.53
			Soil Only - Right	-77.62	53.99	0.51	-250.51	95.28
			Soil Only (Control) - Right	-8.74	53.99	1.00	-181.64	164.16
		PAHs Only (Control) - Left	Soil Only - Right	676.81*	53.99	0.00	503.92	849.71
			Soil Only (Control) - Right	745.69*	53.99	0.00	572.79	918.59
		Soil Only - Right	Soil Only (Control) - Right	68.88	53.99	0.10	-104.02	241.77
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-754.43*	53.99	0.00	-878.93	-629.93
			Soil Only - Right	-77.62	53.99	0.19	-202.12	46.89
			Soil Only (Control) - Right	-8.74	53.99	0.88	-133.24	115.76
		PAHs Only (Control) - Left	Soil Only - Right	676.81*	53.99	0.00	552.31	801.32
			Soil Only (Control) - Right	745.69*	53.99	0.00	621.19	870.19
		Soil Only - Right	Soil Only (Control) - Right	68.88	53.99	0.04	-55.63	193.38

Oneway - Anthracene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	2969747.33	3.00	989915.78	68.42	0.00

	Within Groups	115749.16	8.00	14468.65		
	Total	3085496.49	11.00			
T10	Between Groups	1890537.18	3.00	630179.06	16.01	0.00
	Within Groups	314795.28	8.00	39349.41		
	Total	2205332.46	11.00			
T20	Between Groups	1996661.35	3.00	665553.78	83.95	0.00
	Within Groups	63420.22	8.00	7927.53		
	Total	2060081.57	11.00			
PAHs	Between Groups	0.00	3.00	0.00		
	Within Groups	0.00	8.00	0.00		
	Total	0.00	11.00			

Post Hoc Tests - Anthracene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-375.86*	98.21	0.02	-690.38	-61.35
			Soil Only - Right	774.61*	98.21	0.00	460.10	1089.12
			Soil Only (Control) - Right	767.07*	98.21	0.00	452.56	1081.59
		PAHs Only (Control) - Left	Soil Only - Right	1150.47*	98.21	0.00	835.96	1464.99
			Soil Only (Control) - Right	1142.94*	98.21	0.00	828.43	1457.45
		Soil Only - Right	Soil Only (Control) - Right	-7.54	98.21	1.00	-322.05	306.98
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-375.86*	98.21	0.01	-602.35	-149.39
			Soil Only - Right	774.61*	98.21	0.00	548.13	1001.09
			Soil Only (Control) - Right	767.07*	98.21	0.00	540.59	993.55

		PAHs Only (Control) - Left	Soil Only - Right	1150.47*	98.21	0.00	924.00	1376.96
			Soil Only (Control) - Right	1142.94*	98.21	0.00	916.46	1369.42
		Soil Only - Right	Soil Only (Control) - Right	-7.54	98.21	0.94	-234.02	218.94
T10	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-623.49*	161.97	0.02	-1142.17	-104.82
			Soil Only - Right	310.86	161.97	0.29	-207.82	829.53
			Soil Only (Control) - Right	382.11	161.97	0.16	-136.57	900.78
		PAHs Only (Control) - Left	Soil Only - Right	934.35*	161.97	0.00	415.68	1453.03
			Soil Only (Control) - Right	1005.60*	161.97	0.00	486.93	1524.28
		Soil Only - Right	Soil Only (Control) - Right	71.25	161.97	0.97	-447.42	589.92
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-623.49*	161.97	0.00	-996.99	-250.00
			Soil Only - Right	310.86	161.97	0.09	-62.64	684.35
			Soil Only (Control) - Right	382.10*	161.97	0.05	8.61	755.60
		PAHs Only (Control) - Left	Soil Only - Right	934.35*	161.97	0.00	560.86	1307.85
			Soil Only (Control) - Right	1005.60*	161.97	0.00	632.11	1379.10
		Soil Only - Right	PAHs + Tween 20 - Left	-310.86	161.97	0.09	-684.35	62.64
			PAHs Only (Control) - Left	-934.35*	161.97	0.00	-1307.85	-560.86
			Soil Only (Control) - Right	71.25	161.97	0.67	-302.24	444.74
		Soil Only (Control) - Right	Soil Only - Right	-71.25	161.97	0.67	-444.74	302.24
T20	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-978.35*	72.70	0.00	-1211.16	-745.55

			Soil Only - Right	-158.32	72.70	0.21	-391.12	74.49
			Soil Only (Control) - Right	10.96	72.70	1.00	-221.85	243.77
		PAHs Only (Control) - Left	Soil Only - Right	820.03*	72.70	0.00	587.23	1052.84
			Soil Only (Control) - Right	989.31*	72.70	0.00	756.51	1222.12
		Soil Only - Right	Soil Only (Control) - Right	169.28	72.70	0.17	-63.53	402.08
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-978.35*	72.70	0.00	-1146.00	-810.71
			Soil Only - Right	-158.32	72.70	0.06	-325.96	9.33
			Soil Only (Control) - Right	10.96	72.70	0.88	-156.68	178.60
		PAHs Only (Control) - Left	Soil Only - Right	820.03*	72.70	0.00	652.39	987.68
			Soil Only (Control) - Right	989.31*	72.70	0.00	821.67	1156.96
		Soil Only - Right	Soil Only (Control) - Right	169.27*	72.70	0.05	1.63	336.92

*. The mean difference is significant at the 0.05 level.

Oneway - Fluoranthene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	44851.35	3.00	14950.45	10.67	0.00
	Within Groups	11212.50	8.00	1401.56		
	Total	56063.85	11.00			
T10	Between Groups	36662.09	3.00	12220.70	64.62	0.00
	Within Groups	1512.82	8.00	189.10		
	Total	38174.92	11.00			
T20	Between Groups	61227.84	3.00	20409.28	80.11	0.00
	Within Groups	2038.09	8.00	254.76		

	Total	63265.93	11.00			
PAHs	Between Groups	0.00	3.00	0.00		
	Within Groups	0.00	8.00	0.00		
	Total	0.00	11.00			

Post Hoc Tests - Fluoranthene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-53.81	30.57	0.36	-151.69	44.08
			Soil Only - Right	82.05	30.57	0.10	-15.83	179.94
			Soil Only (Control) - Right	95.74	30.57	0.06	-2.15	193.62
		PAHs Only (Control) - Left	Soil Only - Right	135.86*	30.57	0.01	37.97	233.75
			Soil Only (Control) - Right	149.54*	30.57	0.01	51.66	247.43
		Soil Only - Right	Soil Only (Control) - Right	13.68	30.57	0.97	-84.20	111.57
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-53.81	30.57	0.12	-124.30	16.68
			Soil Only - Right	82.05*	30.57	0.03	11.56	152.54
			Soil Only (Control) - Right	95.73*	30.57	0.01	25.25	166.23
		PAHs Only (Control) - Left	Soil Only - Right	135.86*	30.57	0.00	65.37	206.35
			Soil Only (Control) - Right	149.54*	30.57	0.00	79.05	220.03
		Soil Only - Right	Soil Only (Control) - Right	13.68	30.57	0.67	-56.81	84.17
T10	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-14.79	11.23	0.58	-50.75	21.16
			Soil Only - Right	98.99*	11.23	0.00	63.03	134.95

			Soil Only (Control) - Right	106.09*	11.23	0.00	70.13	142.05
		PAHs Only (Control) - Left	Soil Only - Right	113.78*	11.23	0.00	77.83	149.74
			Soil Only (Control) - Right	120.88*	11.23	0.00	84.93	156.84
		Soil Only - Right	Soil Only (Control) - Right	7.10	11.23	0.92	-28.86	43.06
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-14.79	11.23	0.22	-40.69	11.10
			Soil Only - Right	98.99*	11.23	0.00	73.10	124.88
			Soil Only (Control) - Right	106.09*	11.23	0.00	80.20	131.98
		PAHs Only (Control) - Left	Soil Only - Right	113.78*	11.23	0.00	87.89	139.68
			Soil Only (Control) - Right	120.88*	11.23	0.00	94.99	146.78
		Soil Only - Right	Soil Only (Control) - Right	7.10	11.23	0.54	-18.79	32.99
T20	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-175.79*	13.03	0.00	-217.52	-134.06
			Soil Only - Right	-19.19	13.03	0.49	-60.92	22.54
			Soil Only (Control) - Right	-15.85	13.03	0.63	-57.58	25.88
		PAHs Only (Control) - Left	Soil Only - Right	156.60*	13.03	0.00	114.87	198.33
			Soil Only (Control) - Right	159.94*	13.03	0.00	118.21	201.67
		Soil Only - Right	Soil Only (Control) - Right	3.34	13.03	0.06	-38.39	45.07
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-175.79*	13.03	0.00	-205.84	-145.74
			Soil Only - Right	-19.19	13.03	0.18	-49.24	10.86

		Soil Only (Control) - Right	-15.85	13.03	0.26	-45.90	14.20
	PAHs Only (Control) - Left	Soil Only - Right	156.60*	13.03	0.00	126.55	186.65
		Soil Only (Control) - Right	159.94*	13.03	0.00	129.89	189.99
	Soil Only - Right	Soil Only (Control) - Right	3.34	13.03	0.05	-26.71	33.39

*. The mean difference is significant at the 0.05 level.

Oneway - Pyrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	44926.63	3.00	14975.54	97.94	0.00
	Within Groups	1223.20	8.00	152.90		
	Total	46149.82	11.00			
T10	Between Groups	31815.56	3.00	10605.19	89.14	0.00
	Within Groups	951.80	8.00	118.97		
	Total	32767.36	11.00			
T20	Between Groups	37072.86	3.00	12357.62	49.21	0.00
	Within Groups	2008.80	8.00	251.10		
	Total	39081.65	11.00			

Post Hoc Tests - Pyrene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-1.25	10.10	1.00	-33.58	31.08
			Soil Only - Right	115.54*	10.10	0.00	83.21	147.87
			Soil Only (Control) - Right	127.37*	10.10	0.00	95.05	159.71

		PAHs Only (Control) - Left	Soil Only - Right	116.79*	10.10	0.00	84.46	149.12
			Soil Only (Control) - Right	128.62*	10.10	0.00	96.30	160.96
		Soil Only - Right	Soil Only (Control) - Right	11.83	10.10	0.66	-20.50	44.16
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-1.25	10.10	0.90	-24.53	22.03
			Soil Only - Right	115.54*	10.10	0.00	92.26	138.83
			Soil Only (Control) - Right	127.37*	10.10	0.00	104.09	150.66
		PAHs Only (Control) - Left	Soil Only - Right	116.79*	10.10	0.00	93.51	140.08
			Soil Only (Control) - Right	128.62*	10.10	0.00	105.34	151.91
		Soil Only - Right	Soil Only (Control) - Right	11.83	10.10	0.27	-11.45	35.12
T10	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-4.59	8.91	0.95	-33.11	23.93
			Soil Only - Right	99.78*	8.91	0.00	71.26	128.30
			Soil Only (Control) - Right	101.47*	8.91	0.00	72.96	130.00
		PAHs Only (Control) - Left	Soil Only - Right	104.37*	8.91	0.00	75.85	132.89
			Soil Only (Control) - Right	106.06*	8.91	0.00	77.55	134.59
		Soil Only - Right	Soil Only (Control) - Right	1.70	8.91	1.00	-26.82	30.22
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-4.59	8.91	0.62	-25.13	15.95
			Soil Only - Right	99.78*	8.91	0.00	79.24	120.32
			Soil Only (Control) - Right	101.47*	8.91	0.00	80.94	122.01
		PAHs Only (Control) - Left	Soil Only - Right	104.37*	8.91	0.00	83.83	124.91
			Soil Only (Control) - Right	106.06*	8.91	0.00	85.53	126.60

		Soil Only - Right	Soil Only (Control) - Right	1.70	8.91	0.85	-18.84	22.23
T20	Tukey HSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-136.02*	12.94	0.00	-177.46	-94.59
			Soil Only - Right	-7.83	12.94	0.93	-49.27	33.60
			Soil Only (Control) - Right	-17.57	12.94	0.56	-59.01	23.86
		PAHs Only (Control) - Left	Soil Only - Right	128.19*	12.94	0.00	86.76	169.62
			Soil Only (Control) - Right	118.45*	12.94	0.00	77.02	159.88
		Soil Only - Right	Soil Only (Control) - Right	-9.74	12.94	0.08	-51.17	31.69
	LSD	PAHs + Tween 20 - Left	PAHs Only (Control) - Left	-136.02*	12.94	0.00	-165.86	-106.19
			Soil Only - Right	-7.83	12.94	0.56	-37.67	22.00
			Soil Only (Control) - Right	-17.57	12.94	0.21	-47.41	12.26
		PAHs Only (Control) - Left	Soil Only - Right	128.19*	12.94	0.00	98.35	158.03
			Soil Only (Control) - Right	118.45*	12.94	0.00	88.61	148.29
		Soil Only - Right	Soil Only (Control) - Right	-9.74	12.94	0.05	-39.58	20.10

*. The mean difference is significant at the 0.05 level.

Oneway - Phenanthrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	1653.69	2.00	826.85	2.30	0.18
	Within Groups	2153.25	6.00	358.88		
	Total	3806.94	8.00			
T7	Between Groups	1261.46	2.00	630.73	0.24	0.79

	Within Groups	15692.36	6.00	2615.39		
	Total	16953.82	8.00			
T14	Between Groups	8541.43	2.00	4270.71	3.19	0.11
	Within Groups	8037.58	6.00	1339.60		
	Total	16579.01	8.00			
T21	Between Groups	33513.20	2.00	16756.60	41.24	0.00
	Within Groups	2438.12	6.00	406.35		
	Total	35951.32	8.00			
T28	Between Groups	38825.20	2.00	19412.60	78.16	0.00
	Within Groups	1490.26	6.00	248.38		
	Total	40315.46	8.00			
T35	Between Groups	33854.90	2.00	16927.45	12.72	0.01
	Within Groups	7985.14	6.00	1330.86		
	Total	41840.04	8.00			

Post Hoc Tests - Phenanthrene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + Tween	Microbes only	32.85	15.47	0.16	-14.61	80.31
			PAHs only (Control)	12.22	15.47	0.72	-35.24	59.68
		Microbes only	PAHs only (Control)	-20.63	15.47	0.43	-68.09	26.83
	LSD	Microbes + Tween	Microbes only	32.85	15.47	0.08	-5.00	70.69
			PAHs only (Control)	12.22	15.47	0.46	-25.63	50.07
		Microbes only	PAHs only (Control)	-20.63	15.47	0.23	-58.47	17.22
T7	Tukey HSD	Microbes + Tween	Microbes only	-0.25	41.76	1.00	-128.37	127.87
			PAHs only (Control)	-25.24	41.76	0.82	-153.36	102.88
		Microbes only	PAHs only (Control)	-24.99	41.76	0.83	-153.11	103.13
	LSD	Microbes + Tween	Microbes only	-0.25	41.76	1.00	-102.43	101.92
			PAHs only (Control)	-25.24	41.76	0.57	-127.41	76.93

		Microbes only	PAHs only (Control)	-24.99	41.76	0.57	-127.16	77.19
T14	Tukey HSD	Microbes + Tween	Microbes only	57.53	29.88	0.21	-34.17	149.22
			PAHs only (Control)	-13.53	29.88	0.90	-105.22	78.16
		Microbes only	PAHs only (Control)	-71.06	29.88	0.12	-162.75	20.64
	LSD	Microbes + Tween	Microbes only	57.53	29.88	0.10	-15.60	130.65
			PAHs only (Control)	-13.53	29.88	0.67	-86.65	59.59
		Microbes only	PAHs only (Control)	-71.06	29.88	0.05	-144.18	2.07
T21	Tukey HSD	Microbes + Tween	Microbes only	112.36*	16.46	0.00	61.87	162.87
			PAHs only (Control)	-29.18	16.46	0.26	-79.68	21.32
		Microbes only	PAHs only (Control)	-141.54*	16.46	0.00	-192.05	-91.05
	LSD	Microbes + Tween	Microbes only	112.36*	16.46	0.00	72.09	152.64
			PAHs only (Control)	-29.18	16.46	0.13	-69.45	11.09
		Microbes only	PAHs only (Control)	-141.54*	16.46	0.00	-181.82	-101.27
T28	Tukey HSD	Microbes + Tween	Microbes only	110.18*	12.87	0.00	70.70	149.67
			PAHs only (Control)	-46.43*	12.87	0.03	-85.92	-6.95
		Microbes only	PAHs only (Control)	-156.61*	12.87	0.00	-196.10	-117.13
	LSD	Microbes + Tween	Microbes only	110.18333*	12.87	0.00	78.70	141.67
			PAHs only (Control)	-46.43333*	12.87	0.01	-77.92	-14.95
		Microbes only	PAHs only (Control)	-156.61*	12.87	0.00	-188.10	-125.13
T35	Tukey HSD	Microbes + Tween	Microbes only	79.80	29.79	0.08	-11.59	171.20
			PAHs only (Control)	-70.33	29.79	0.12	-161.72	21.06
		Microbes only	PAHs only (Control)	-150.13*	29.79	0.01	-241.53	-58.74
	LSD	Microbes + Tween	Microbes only	79.80*	29.79	0.04	6.92	152.69
			PAHs only (Control)	-70.33	29.79	0.06	-143.22	2.56
		Microbes only	PAHs only (Control)	-150.13*	29.79	0.00	-223.02	-77.25

*. The mean difference is significant at the 0.05 level.

Onway - Anthracene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	14301.84	2.00	7150.92	3.50	0.10
	Within Groups	12246.38	6.00	2041.06		
	Total	26548.22	8.00			
T7	Between Groups	19623.16	2.00	9811.58	1.62	0.27
	Within Groups	36303.91	6.00	6050.65		
	Total	55927.07	8.00			
T14	Between Groups	21390.55	2.00	10695.28	1.46	0.30
	Within Groups	43888.88	6.00	7314.81		
	Total	65279.43	8.00			
T21	Between Groups	29246.90	2.00	14623.45	22.90	0.00
	Within Groups	3830.81	6.00	638.47		
	Total	33077.71	8.00			
T28	Between Groups	55464.81	2.00	27732.41	21.26	0.00
	Within Groups	7825.08	6.00	1304.18		
	Total	63289.89	8.00			
T35	Between Groups	64245.83	2.00	32122.92	24.29	0.00
	Within Groups	7934.31	6.00	1322.38		
	Total	72180.14	8.00			

Post Hoc Tests - Anthracene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + Tween	Microbes only	41.44	36.89	0.54	-71.74	154.62
			PAHs only (Control)	-55.85	36.89	0.35	-169.03	57.33
		Microbes only	PAHs only (Control)	-97.29	36.89	0.09	-210.47	15.89
	LSD	Microbes + Tween	Microbes only	41.44	36.89	0.30	-48.82	131.70
			PAHs only (Control)	-55.85	36.89	0.18	-146.11	34.41
		Microbes only	PAHs only	-97.29*	36.89	0.04	-187.55	-7.03

			(Control)					
T7	Tukey HSD	Microbes + Tween	Microbes only	-90.62	63.51	0.39	-285.49	104.25
			PAHs only (Control)	15.13	63.51	0.97	-179.75	210.00
		Microbes only	PAHs only (Control)	105.75	63.51	0.29	-89.13	300.62
	LSD	Microbes + Tween	Microbes only	-90.62	63.51	0.20	-246.03	64.79
			PAHs only (Control)	15.13	63.51	0.82	-140.28	170.53
		Microbes only	PAHs only (Control)	105.75	63.51	0.15	-49.66	261.15
T14	Tukey HSD	Microbes + Tween	Microbes only	21.03	69.83	0.95	-193.23	235.29
			PAHs only (Control)	-91.29	69.83	0.44	-305.55	122.98
		Microbes only	PAHs only (Control)	-112.32	69.83	0.31	-326.58	101.95
	LSD	Microbes + Tween	Microbes only	21.03	69.83	0.77	-149.84	191.90
			PAHs only (Control)	-91.29	69.83	0.24	-262.16	79.59
		Microbes only	PAHs only (Control)	-112.32	69.83	0.16	-283.19	58.56
T21	Tukey HSD	Microbes + Tween	Microbes only	113.82*	20.63	0.00	50.52	177.13
			PAHs only (Control)	-13.13	20.63	0.81	-76.43	50.17
		Microbes only	PAHs only (Control)	-126.95*	20.63	0.00	-190.26	-63.65
	LSD	Microbes + Tween	Microbes only	113.82*	20.63	0.00	63.34	164.31
			PAHs only (Control)	-13.13	20.63	0.05	-63.61	37.35
		Microbes only	PAHs only (Control)	-126.95*	20.63	0.00	-177.44	-76.47
T28	Tukey HSD	Microbes + Tween	Microbes only	162.64*	29.49	0.00	72.17	253.12
			PAHs only (Control)	-7.51	29.49	0.97	-97.99	82.96
		Microbes only	PAHs only (Control)	-170.16*	29.49	0.00	-260.63	-79.69
	LSD	Microbes + Tween	Microbes only	162.64*	29.49	0.00	90.50	234.80
			PAHs only (Control)	-7.51	29.49	0.01	-79.66	64.64

		Microbes only	PAHs only (Control)	-170.16*	29.49	0.00	-242.31	-98.01
T35	Tukey HSD	Microbes + Tween	Microbes only	168.63*	29.69	0.00	77.53	259.74
			PAHs only (Control)	-19.58	29.69	0.05	-110.68	71.53
		Microbes only	PAHs only (Control)	-188.21*	29.69	0.00	-279.32	-97.11
	LSD	Microbes + Tween	Microbes only	168.63*	29.69	0.00	95.98	241.29
			PAHs only (Control)	-19.58	29.69	0.03	-92.23	53.08
		Microbes only	PAHs only (Control)	-188.21*	29.69	0.00	-260.87	-115.56

*. The mean difference is significant at the 0.05 level.

Oneway - Fluoranthene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	523.42	2.00	261.71	60.87	0.00
	Within Groups	25.80	6.00	4.30		
	Total	549.21	8.00			
T7	Between Groups	339.86	2.00	169.93	1.59	0.28
	Within Groups	639.29	6.00	106.55		
	Total	979.15	8.00			
T14	Between Groups	885.84	2.00	442.92	3.11	0.12
	Within Groups	853.56	6.00	142.26		
	Total	1739.39	8.00			
T21	Between Groups	181.93	2.00	90.97	10.19	0.01
	Within Groups	53.59	6.00	8.93		
	Total	235.52	8.00			
T28	Between Groups	224.81	2.00	112.41	5.37	0.05
	Within Groups	125.57	6.00	20.93		
	Total	350.38	8.00			
T35	Between Groups	155.41	2.00	77.70	32.52	0.00
	Within Groups	14.34	6.00	2.39		
	Total	169.75	8.00			

Post Hoc Tests - Fluoranthene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + Tween	Microbes only	-6.52*	1.69	0.02	-11.71	-1.33
			PAHs only (Control)	-18.42*	1.69	0.00	-23.61	-13.23
		Microbes only	PAHs only (Control)	-11.90*	1.69	0.00	-17.09	-6.71
	LSD	Microbes + Tween	Microbes only	-6.52*	1.69	0.01	-10.66	-2.38
			PAHs only (Control)	-18.42*	1.69	0.00	-22.56	-14.28
		Microbes only	PAHs only (Control)	-11.90*	1.69	0.00	-16.04	-7.76
T7	Tukey HSD	Microbes + Tween	Microbes only	-14.89	8.43	0.26	-40.75	10.97
			PAHs only (Control)	-9.34	8.43	0.54	-35.20	16.52
		Microbes only	PAHs only (Control)	5.56	8.43	0.79	-20.30	31.42
	LSD	Microbes + Tween	Microbes only	-14.89	8.43	0.13	-35.52	5.73
			PAHs only (Control)	-9.34	8.43	0.31	-29.96	11.29
		Microbes only	PAHs only (Control)	5.56	8.43	0.53	-15.07	26.18
T14	Tukey HSD	Microbes + Tween	Microbes only	-13.65	9.74	0.40	-43.53	16.23
			PAHs only (Control)	-24.24	9.74	0.10	-54.12	5.64
		Microbes only	PAHs only (Control)	-10.58	9.74	0.56	-40.46	19.30
	LSD	Microbes + Tween	Microbes only	-13.65	9.74	0.21	-37.48	10.18
			PAHs only (Control)	-24.23*	9.74	0.05	-48.07	-0.41
		Microbes only	PAHs only (Control)	-10.58	9.74	0.32	-34.41	13.25
T21	Tukey HSD	Microbes + Tween	Microbes only	0.16	2.44	0.05	-7.33	7.65
			PAHs only (Control)	-9.45*	2.44	0.02	-16.94	-1.97
		Microbes only	PAHs only (Control)	-9.61*	2.44	0.02	-17.10	-2.13
	LSD	Microbes + Tween	Microbes only	0.16	2.44	0.05	-5.81	6.13
			PAHs only (Control)	-9.45*	2.44	0.01	-15.43	-3.49
		Microbes only	PAHs only (Control)	-9.61*	2.44	0.01	-15.59	-3.65

T28	Tukey HSD	Microbes + Tween	Microbes only	1.74	3.74	0.89	-9.72	13.20
			PAHs only (Control)	-9.63	3.74	0.09	-21.09	1.83
		Microbes only	PAHs only (Control)	-11.36	3.74	0.05	-22.82	0.10
	LSD	Microbes + Tween	Microbes only	1.74	3.74	0.05	-7.40	10.88
			PAHs only (Control)	-9.62*	3.74	0.04	-18.77	-0.49
		Microbes only	PAHs only (Control)	-11.36*	3.74	0.02	-20.50	-2.22
T35	Tukey HSD	Microbes + Tween	Microbes only	0.00	1.26	0.08	-3.88	3.87
			PAHs only (Control)	-8.81*	1.26	0.00	-12.69	-4.94
		Microbes only	PAHs only (Control)	-8.81*	1.26	0.00	-12.69	-4.94
	LSD	Microbes + Tween	Microbes only	0.00	1.26	0.05	-3.09	3.09
			PAHs only (Control)	-8.81*	1.26	0.00	-11.91	-5.73
		Microbes only	PAHs only (Control)	-8.81*	1.26	0.00	-11.90	-5.72

*. The mean difference is significant at the 0.05 level.

Oneway - Pyrene

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
T0	Between Groups	429.61	2	214.806	31.06	0.00
	Within Groups	41.48	6	6.914		
	Total	471.09	8			
T7	Between Groups	8.71	2	4.358	.047	0.95
	Within Groups	558.89	6	93.149		
	Total	567.60	8			
T14	Between Groups	809.14	2	404.571	4.73	0.05
	Within Groups	512.67	6	85.446		
	Total	1321.81	8			
T21	Between Groups	103.03	2	51.515	12.90	0.00
	Within Groups	23.94	6	3.992		
	Total	126.97	8			
T28	Between Groups	150.12	2	75.061	6.72	0.03

	Within Groups	66.98	6	11.164		
	Total	217.10	8			
T35	Between Groups	89.97	2	44.988	18.48	0.00
	Within Groups	14.60	6	2.434		
	Total	104.57	8			

Post Hoc Tests - Pyrene

Multiple Comparisons

Dependent Variable				Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
T0	Tukey HSD	Microbes + Tween	Microbes only	-5.49	2.14	0.09	-12.08	1.09
			PAHs only (Control)	-16.61	2.14	0.00	-23.19	-10.02
		Microbes only	PAHs only (Control)	-11.11*	2.14	0.00	-17.70	-4.52
	LSD	Microbes + Tween	Microbes only	-5.49*	2.14	0.04	-10.75	-0.24
			PAHs only (Control)	-16.61*	2.14	0.00	-21.86	-11.35
		Microbes only	PAHs only (Control)	-11.11*	2.14	0.00	-16.36	-5.85
T7	Tukey HSD	Microbes + Tween	Microbes only	-1.85	7.88	0.97	-26.02	22.32
			PAHs only (Control)	0.41	7.88	0.99	-23.76	24.59
		Microbes only	PAHs only (Control)	2.26	7.88	0.95	-21.91	26.44
	LSD	Microbes + Tween	Microbes only	-1.85	7.88	0.82	-21.13	17.43
			PAHs only (Control)	0.41	7.88	0.96	-18.86	19.69
		Microbes only	PAHs only (Control)	2.26	7.88	0.78	-17.01	21.54
T14	Tukey HSD	Microbes + Tween	Microbes only	-5.80	7.54	0.73	-28.95	17.35
			PAHs only (Control)	-22.37	7.54	0.05	-45.53	0.78
		Microbes only	PAHs only (Control)	-16.57	7.54	0.15	-39.73	6.58
	LSD	Microbes + Tween	Microbes only	-5.80	7.54	0.47	-24.26	12.66
			PAHs only (Control)	-22.37	7.54	0.02	-40.84	-3.90
		Microbes only	PAHs only (Control)	-16.57	7.54	0.07	-35.04	1.89
T21	Tukey HSD	Microbes + Tween	Microbes only	-2.38	1.63	0.37	-7.39	2.61

			PAHs only (Control)	-8.06	1.63	0.00	-13.07	-3.06
		Microbes only	PAHs only (Control)	-5.68	1.63	0.03	-10.68	-0.67
	LSD	Microbes + Tween	Microbes only	-2.38	1.63	0.19	-6.37	1.60
			PAHs only (Control)	-8.06	1.63	0.00	-12.05	-4.07
		Microbes only	PAHs only (Control)	-5.68	1.63	0.01	-9.67	-1.68
T28	Tukey HSD	Microbes + Tween	Microbes only	-3.20	2.72	0.05	-11.57	5.16
			PAHs only (Control)	-9.81	2.78	0.02	-18.18	-1.43
		Microbes only	PAHs only (Control)	-6.60	2.72	0.11	-14.97	1.76
	LSD	Microbes + Tween	Microbes only	-3.20	2.72	0.02	-9.88	3.46
			PAHs only (Control)	-9.81	2.72	0.01	-16.48	-3.13
		Microbes only	PAHs only (Control)	-6.60	2.72	0.05	-13.27	0.07
T35	Tukey HSD	Microbes + Tween	Microbes only	-2.41	1.27	0.22	-6.32	1.49
			PAHs only (Control)	-7.58	1.27	0.00	-11.48	-3.67
		Microbes only	PAHs only (Control)	-5.16*	1.27	0.01	-9.07	-1.25
	LSD	Microbes + Tween	Microbes only	-2.41	1.27	0.10	-5.53	0.70
			PAHs only (Control)	-7.58	1.27	0.00	-10.69	-4.46
		Microbes only	PAHs only (Control)	-5.16*	1.27	0.00	-8.28	-2.05

*. The mean difference is significant at the 0.05 level.